# Dendritic Cells in HLA-B27 Transgenic Rat Model of Spondyloarthropathy

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# Abstract

The Spondyloarthritides (SpAs) are a group of related inflammatory disorders that share common clinical features and a genetic predisposing factor, the MHC class I gene HLA-B27. Rats transgenic for the human HLA-B27 and $\beta_2$ microglobulin genes (B27-TG rats) develop a multisystem inflammatory disease, offering a model for studying SpA. Several lines of evidence from both humans and rats suggest that intestinal inflammation and the commensal microbiota in the gut are important in disease development. The intestine is the site of entry for vast amounts of antigens, and the intestinal immune system has evolved to be tolerant towards harmless non-self antigens, but still retain the ability to mount protective immune responses against invading pathogens. Dendritic cells (DCs) are professional antigen presenting cells residing in the intestinal tissue at sites of antigen entry, and continuously migrate from the intestine into the mesenteric lymph nodes, via lymph, where they interact with naïve T cells. Hence, DCs have important role in maintaining the balance between tolerance and immunity in the intestine.

The aim of this thesis was to investigate the roles of migrating intestinal lymph DCs (L-DCs) in disease development in the B27-TG rat. These DCs were collected by thoracic duct cannulation and their phenotype and functions were investigated. In addition, mesenteric lymph node (MLN) and bone marrow derived DCs (BMDCs) were compared between B27-TG and non-TG animals.

B27-TG animals lack one of the three subsets of L-DCs, the MHCII$^{hi}$ CD103$^{+}$ CD172a$^{lo}$ cells, in the pseudo-afferent lymph, and the remaining L-DCs appear more activated. The proportion of this subset was also reduced in the MLNs of B27-TG animals. The CD172a$^{lo}$ DC subset has been implicated in the induction and maintenance of intestinal tolerance, and thus lack of this subset could lead to breakdown in tolerance and to systemic disease. In addition, BMDCs cultured with mouse Flt3L survived less well than non-TG BMDCs, and this defect could be reversed by addition of the apoptosis inhibitor Q-VD into the cultures. This indicated there could be a defect in the development of B27-TG DCs from their bone marrow precursors. Furthermore, B27-TG BMDCs stimulated significantly less proliferation from naïve CD4$^{+}$ T cells than their non-TG counterparts in a mixed leukocyte reaction. In spite of this, more IL-17F and less IFN-γ were detected in the B27-TG BMDC-T cell co-cultures compared to non-TG DCs, indicating skewing of the immune response from Th1 to Th17. Together, these results reveal previously unrecognised defects in HLA-B27$^{+}$ DCs both *in vivo* and *in vitro*, which could have a role on SpA disease pathogenesis. This opens up new exciting avenues of research for the future.

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*"If we knew what it was we were doing, it would not be called research, would it?"*

*– Albert Einstein*

# Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature: …… Printed name: Lotta Utriainen…….

# List of abbreviations

| | |
|---|---|
| **A** | |
| Ab | Antibody |
| Ag | Antigen |
| ALDH | Aldehyde dehydrogenase |
| ANOVA | Analysis of Variance |
| APC | Antigen presenting cell |
| AS | Ankylosing Spondylitis |
| ASAS | Assessment of Spondyloarthritis International Society |
| **B** | |
| Bio | Biotin |
| BiP | Binding immunoglobulin protein |
| BM | Bone Marrow |
| BMDC | Bone marrow derived dendritic cell |
| bp | Base pair |
| **C** | |
| CCL | CC-chemokine ligand |
| CCR | CC-chemokine receptor |
| CD | Cluster of differentiation |
| cDC | Conventional dendritic cell |
| cDNA | Complementary DNA |
| CDP | Common DC progenitor |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CHO | Chinese hamster ovary |
| CHOP | CCAAT/enhancer binding protein (C/EBP), epsilon |
| CIA | Collagen induced arthritis |
| CLN | Cutaneous lymph node |
| CLP | Common lymphoid progenitor |
| CMP | Common myeloid progenitor |
| cpm | Counts per minute |
| CT | Cholera Toxin |
| CTL | Cytotoxic T lymphocyte |
| CXCR | CXC-chemokine receptor |
| Cys | Cysteine |
| **D** | |
| DAPI | 4',6-diamidino-2-phenylindole |
| DC | Dendritic cell |
| DNA | Deoxyribonucleic acid |
| **E** | |
| EAE | Experimental autoimmune encephalomyelitis |
| EDTA | ethylenediaminetetraacetic acid |
| ER | Endoplasmic reticulum |
| ERAD | ER-associated degradation |
| ERAP | ER aminopeptidase |
| **F** | |
| FACS | Fluorescence activated cell sorting |
| FAE | Follicle associated epithelium |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| Flt3 | Fms-like tyrosine kinase |
| Flt3L | Fms-like tyrosine kinase ligand |
| FoxP3 | Forkhead box protein P3 |
| FSC | Forward scatter |
| **G** | |
| GALT | Gut-associated lymphoid tissue |
| GM-CSF | Granulocyte macrophage colony-stimulating factor |
| GWAS | Genome wide association study |

**H**

| | |
|---|---|
| h | Human |
| hi | High |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |

**I**

| | |
|---|---|
| i.v. | Intravenous |
| IBD | Inflammatory bowel disease |
| IDO | Indoleamine 2,3-dioxygenase |
| IEC | Intestinal epithelial cell |
| IEL | Intraepithelial lymphocyte |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| ILF | Isolated lymphoid follicle |
| iNOS | Inducible nitric oxide |
| int | Intermediate |
| ITAM | Immunoreceptor tyrosine-based activation motif |
| ITIM | Immunoreceptor tyrosine-based inhibition motif |

**J**

| | |
|---|---|
| Jak | Janus kinase |

**K**

| | |
|---|---|
| KIR | Killer-cell immunoglobulin-like receptor |

**L**

| | |
|---|---|
| L-DC | Lymph derived dendritic cell |
| LC | Langerhans cell |
| LIN | Lineage markers |
| LN | Lymph node |
| lo | Low |
| LP | Lamina propria |
| LPS | Lipopolysaccharide |

**M**

| | |
|---|---|
| m | Mouse |
| M-CSF | Macrophage colony stimulating factor |
| mAb | Monoclonal antibody |
| MACS | Magnetically activated cell sorter |
| MAdCAM-1 | Mucosal vascular addressin cell adhesion molecule 1 |
| MDP | Monocyte-DC progenitor |
| MHC | Major histocompatibility complex |
| MLN | Mesenteric lymph node |
| MLNX | Mesenteric lymphadenectomy |
| MLR | Mixed leukocyte reaction |
| mRNA | Messenger RNA |

**N**

| | |
|---|---|
| NK | Natural killer |
| Non-TG | Non-transgenic |

**O**

| | |
|---|---|
| OVA | Ovalbumin |
| OX | Oxford |

**P**

| | |
|---|---|
| PAMP | Pathogen associated molecular pattern |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| pDC | Plasmacytoid dendritic cell |
| PE | Phycoerythrin |
| PerCP | Peridinin-chlorophyll-protein |
| PP | Peyer’s patch |
| PRR | Pattern recognition receptor |
| PsSpA | Psoriatic Spondyloarthritis |

**Q**

| | |
|---|---|
| QPCR | Quantitative PCR |

**R**

| | |
|---|---|
| R848 | Resiquimod |
| RA | Rheumatoid arthritis |
| Ra | Retinoic acid |
| RALDH | Retinaldehyde dehydrogenase |
| RBC | Red blood cell |
| ReA | Reactive arthritis |
| RNA | Ribonucleic acid |
| RPMI | Roswell Park Memorial Institute-1640 medium |

**S**

| | |
|---|---|
| s.c. | Sub cutaneous |
| SA | Streptavidin |
| SD | Standard deviation |
| SED | Sub-epithelial dome |
| SEM | Standard error of the mean |
| SpA | Spondyloarthritis |
| SpA-IBD | Spondyloarthritis associated with inflammatory bowel disease |
| SSC | Side scatter |
| STAT | Signal transducers and activators of transcription |

**T**

| | |
|---|---|
| TBP | Tata-box binding protein |
| TG | Transgenic |
| TGF | Transforming growth factor |
| Th | T helper cell |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| Treg | T regulatory cell |
| TSLP | Thymic stromal lymphopoietin |

**U**

| | |
|---|---|
| UPR | Unfolded protein response |
| uSpA | Undifferentiated spondyloarthritis |

# 1. Introduction

## 1.1 Spondyloarthritides

The Spondyloarthritides (SpAs) are a group of inter-related but heterogeneous inflammatory disorders that share common genetic predisposing factors. These diseases also share some clinical features, however each disease in the group also has its own distinct features. Historically, it was suggested by Moll et al in 1974 that this group of diseases indeed form their own group of related disorders separate from rheumatoid arthritis, and they were originally named as seronegative spondarthrides (1). Today, the term spondyloarthritis, or spondyloarthritides in plural, is preferred. The SpA group includes psoriatic SpA (PsSpA), reactive arthritis (ReA), arthritis related to inflammatory bowel disease (SpA-IBD), a subgroup of juvenile idiopathic arthritis, undifferentiated SpA (uSpA) and ankylosing spondylitis (AS), which is the most common and most studied member of the group (Figure 1.1). Common clinical features, described in the original SpA publication (1) and expanded more recently (2-4), include sacroiliitis with inflammatory back pain, peripheral arthropathy, enthesitis, extra-articular or extra-spinal involvement, including that of the eye, heart, lung, and skin, and the absence of rheumatoid factor and subcutaneous nodules. In addition, all the SpA diseases share a common genetic factor, the major histocompatibility (MHC) class I gene HLA-B27 (5). The prevalence rate for the combined SpA diseases in the general population is estimated to be roughly the same as for RA, ranging from 0.3 % to 1.9 % depending on the population studied and the methods used (6, 7).

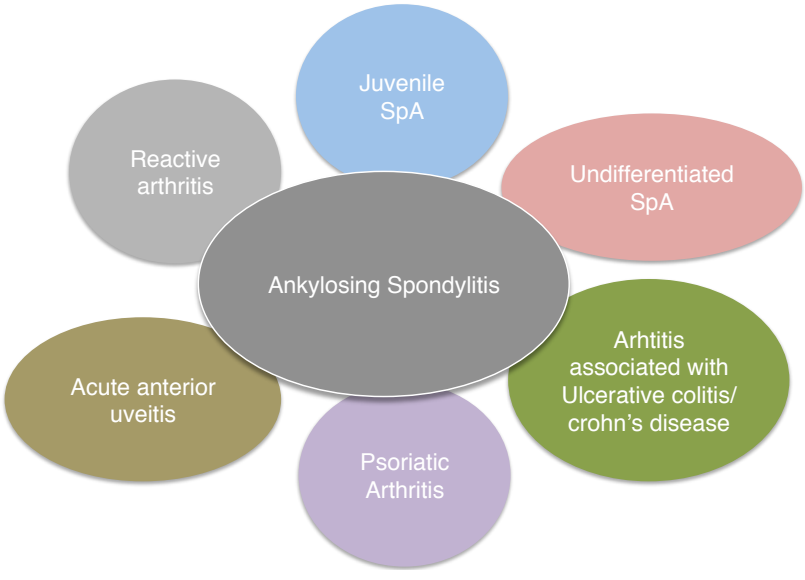


**Figure 1.1. Diseases belonging to the SpA group.** Modified From the ASAS slide collection, http://www.asas-group.org, open access.

SpA patients can be divided into two groups based on their symptoms: 1) patients presenting with axial SpA, characteristic of AS, or 2) patients presenting with peripheral symptoms only. Thus, two different diagnostic classification criteria have been developed recently by the Assessment of Spondyloarthritis International Society (ASAS) to be able to identify patients presenting with or without axial symptoms as having SpA (8). For axial SpA, the inclusion criteria are back pain for at least three months and a young age (<45), combined with either sacroiliitis or HLA-B27 positivity combined with other classical SpA features (Figure 1.2.). Peripheral SpA inclusion criteria are arthritis, enthesitis and dactylitis, combined with one or two additional SpA features (Figure 1.2.). These two criteria can be combined by dictating that all patients presenting with back pain (with or without peripheral features) follow the assessment for axial SpA, whereas for patients manifesting with only peripheral symptoms, the ASAS criteria for peripheral SpA are followed.

As the diseases belonging to the SpA family vary greatly in their symptoms and all of them have some specific characteristics not necessarily present in the other members of the SpA family, it is not possible to describe all of them in great detail within the scope of this thesis. Thus, I will mostly concentrate on ankylosing spondylitis, as it is the archetypal disease of the family and it is also the most studied. Information about the other members of the SpA family will be given when relevant, and can also be found in several other publications (2-4, 9-11).

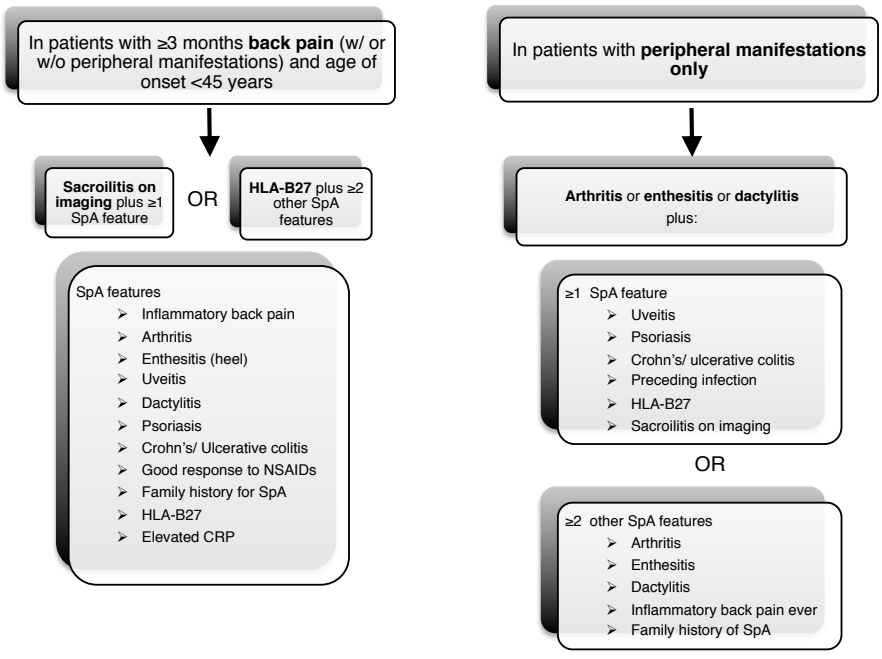


**Figure 1.2. SpA assessment**

Combined use of the Assessment of SpondyloArhritis Society (ASAS) criteria for axial and peripheral SpA in the entire SpA population. Modified from (8).

### 1.1.1 Epidemiology of AS

The most common diagnosis for patients presenting with axial symptoms only is AS, and its prevalence among adults has been estimated to be between 0.2 and 0.9 % according to studies carried out in Berlin and France (6, 7). Ankylosing spondylitis generally affects younger people, as 80 % of AS patients present with their first symptoms before the age of 30 and less than 5 % older than 45 years (12). Unlike many other autoimmune diseases, AS affects men more often than women with the ratio of about 2-3:1. Overall the prevalence of AS in Europe is estimated to be 0.1 - 1.4 % and the incidence of the disease is between 0.5-14 people per 100,000 people per year (13).

### 1.1.2 Pathogenesis and genetics of SpA

In spite of vigorous research, the exact genetic and environmental triggers of AS, or other SpAs, are not known. In 1973 two groups reported independent findings of a strong association between the MHC class I molecule HLA-B27 and AS (14, 15). However, after three decades of research the functional role of HLA-B27 in SpA disease pathogenesis is still obscure. Through twin studies it has been established that AS has a strong genetic component; monozygotic twins have concordance rate of 63 % compared with a dizygotic concordance rate of 12.5 % (16). Overall, it has been estimated that genetic factors account for over 90 % of AS disease susceptibility with the rest being attributed to random, but widespread, environmental factors (16). It is now commonly accepted that the overall contribution of *HLA-B27* to the AS susceptibility is 30-40 %; the rest are attributed to other genes within and outside the MHC locus such as IL-23 receptor (*IL-23R*) and endoplasmic reticulum aminopeptidase (*ERAP1*), which have been proposed to contribute 9 % and 26 %, respectively, to disease susceptibility (Table 1.1) (10). Even though other genes have been linked to AS, HLA-B27 remains by far the most strongly associated with about 90-95 % of AS patients being positive for the HLA-B27 gene (14, 17). Other SpA diseases also show a strong association with HLA-B27 as about 70 % of Caucasian reactive arthritis patients are positive for HLA-B27, as well as 60-70 % of psoriatic spondylitis patients and 50-60 % of patients presenting with SpA-IBD (10).

#### *HLA-B27*

*HLA-B27* is a class I MHC gene expressed by all the nucleated cells in the body. It is a relatively common subtype of MHC class I, with on average 7-8 % of Caucasians expressing it (18). Despite being very closely linked to the development of SpA, as

discussed above, fewer than 5 % of HLA-B27$^+$ individuals in the general population develop AS or other SpAs (7, 19). However, there is a strong familiar heritability as first degree relatives of AS patients have 5-16 times greater chance of developing AS than HLA-B27$^+$ individuals in the general population (20). The low percentage of HLA-B27$^+$ people developing SpA suggests there are other factors, genetic or environmental, affecting the risk of developing the disease. Indeed, as mentioned HLA-B27 only accounts for about 30-40 % of disease susceptibility, with other genes inside and outside the MHC locus together with environmental factors accounting for the rest. Thus, for the disease to develop, a certain genetic background may be required as well as environmental factors to trigger it. Also, at present over 80 subtypes of HLA-B27 have been defined (21), some of which confer disease susceptibility more strongly than others (22). Some are so rare it has not been possible to link them to AS or other SpAs.

The B*2705 allele is thought to be the parent molecule for all the other subtypes, and together with B*2704 and B*2702 subtypes it accounts for almost all HLA-B27-positive north European Caucasians (23). These three alleles are also the two most strongly disease associated alleles (23). Several other HLA-B27 subtypes have also been associated with AS, but more weakly than B*2705 and B*2704. These other subtypes are believed to have developed from B*2705 through population migration, as different ethnic populations carry different mixture of HLA-B27 subtypes (16, 24, 25). B*2706 and B*2709 have been reported not be associated with AS, and even though some cases of AS have been reported on people with this background, it is thought that these alleles are at most very weakly associated (26-28). The variability in the mixture of HLA-B27 subtypes seen in different populations might be the result of evolution and in response to environmental stresses. This variability may have been maintained through natural selection by local infectious diseases, as has been shown to be the case to other HLA antigens (29). Indeed, HLA-B27 has been shown to confer protection towards viral infections such as Epstein-Barr virus and herpes simplex virus type 2 (29). Furthermore, HIV progression has been shown to be slower in HLA-B27$^+$ individuals (29).

As mentioned above, no definite role for HLA-B27 in SpA disease pathogenesis has been defined. However, several hypotheses try to address this issue: (a) The *Arthritogenic peptide theory* hypothesises that HLA-B27 presents a distinct peptide repertoire, where some of the peptides might be molecular mimics of self-antigens, which would cause immune reaction against self; (b) *HLA-B27 misfolds* and contributes to inflammation by activating a series of intracellular events known as the unfolded protein response; and (c)

*HLA-B27 forms a homodimer* on the cell surface, and this serves as an activating ligand for natural killer cells. All these theories are discussed in detail later in this chapter.

### *Association with genes other than HLA-B27*

Traditionally, genetic studies of diseases have been hypothesis led; that is, a suspect gene or genes are selected, and it is then investigated whether they are differentially expressed between healthy controls and patients. In recent years genetic studies have changed as the technology and knowledge of how to carry out studies investigating the whole genome have expanded. These genome wide association studies (GWAS) are carried out without any underlying hypothesis, using the whole genome of affected individuals and healthy controls. Following initial analysis, differentially expressed genes are identified and subsequently verified by more traditional methods of Q-PCR, for example. Use of these GWAS studies to study AS patients have led to the identification of several additional genes linked to the disease (Table 1.1). Of these, *ERAP1* and *IL-23R* have been studied most, and their association has also been verified in other independent studies (30). ERAP1 is an intracellular molecule expressed in the endoplasmic reticulum (ER), and is ubiquitously expressed throughout the body. The role of ERAP1 in AS pathogenesis is unclear, but it is thought it could be involved through one or both of its two different functions. First, ERAP1, together with ERAP2, trims peptides destined for loading onto MHC I molecules to a correct length (31), and it could be that this function is defective in AS patients, leading to disruption in antigen presentation on MHC class I molecules. Supporting this theory, ERAP1$^{-/-}$ mice have been shown to be more prone to infection with the intracellular parasite *Toxoplasma gondii* due to defective presentation of parasite antigen on MHC class I molecules (32). The second role proposed for ERAP1 is to cleave proinflammatory cytokine receptors, such as IL-1R2, TNFR1 and IL-6Rα, from the cell surface and thus create soluble receptors with potential to bind their respective ligands and attenuate the effect of these cytokines (33-35). However, these data have been obtained using *in vitro* cell lines and no *in vivo* data are available to support this latter function of ERAP1.

Another gene found to be associated with AS in the GWAS study was *IL-23R*. Unlike ERAP1, which has not been found to be associated with other diseases, single-nucleotide polymorphisms (SNPs) in the gene encoding IL-23R have also been linked to IBD (36), psoriasis (37) and more recently to psoriatic arthritis another disease of the SpA family (38). IL-23R is a heterodimeric receptor for the cytokine IL-23. It is composed of IL-

12Rβ1 chain, a component of IL-12 receptor complex, and a specific IL-23R chain (39). The IL-12Rβ1 receptor binds to janus kinase (Jak) family member Tyk2, whereas IL-23R associates with Jak2 (39). Signalling through the receptor complex leads to phosphorylation of Jaks, and they in turn phosphorylate tyrosine residues in the intracellular portions of the receptor subunits. STATs (signal transducers and activators of transcription) in turn bind to these phosphorylated tyrosine residues (39). In humans IL-23R is expressed on activated/memory T cells, natural killer (NK) cells, and at low levels on activated macrophages and dendritic cells (DCs) (40). In mice it is expressed by activated macrophages, bone marrow derived DCs and activated T cells (40). These activated T cells expressing IL-23R were found to be a novel $CD4^+$ helper T cell subset, named Th17, which produce the cytokine IL-17 (41, 42). Th17 cells have raised considerable research interest lately as they have been indicated to have a pathological role in many animal models of autoimmune diseases, such as collagen induced arthritis and experimental autoimmune encephalomyelitis (43, 44), as well as in many human diseases such as RA, IBD and psoriasis (45). These cells are discussed in detail later in this chapter.

One *IL-23R* SNP, R381Q, has been found to confer protection from several inflammatory diseases, and in a recent publication it was shown that memory $CD4^+$ and $CD8^+$ T cells from healthy people carrying this SNP mutation showed decreased IL-23-dependent IL-17 and IL-22 production relative to individuals with WT IL-23R (46). In addition, this phenomenon was associated with a lower percentage of circulating Th17 cells. These data indicate, perhaps, that non-protective mutations could work in the opposite manner, increasing the numbers of circulating Th17 cells. There is, however, no direct evidence for a role for *IL-23R* mutations in SpA pathogenesis. Indeed, it is not currently known whether the disease-associated variants of *IL-23R* cause gain or loss function. However, several genes associated with IL-23R signalling have been linked to AS, thus indicating that IL-23R can indeed have a role in SpA pathogenesis. These genes include: 1) *IL-12B*, which encodes the 40-kd (p40) chain, a component of both IL-12 and IL-23; in the latter, it is paired with a 19-kd protein (p19); 2) caspase recruitment domain 9 (*CARD-9*), which encodes a major signaling intermediate in the pathway whereby fungal products induce IL-23 production from DCs; and 3) *STAT-3* (24). STAT3 is a key transcription factor in IL23R signaling, and loss-of-function mutations of *STAT3* cause failure of development of Th17 lymphocytes, resulting in Job's (hyper-IgE) syndrome, which is associated with severe, recurrent fungal and bacterial infections (47).

**Table 1.1. Genes linked to development of AS, and the relationship of these genes to psoriasis and IBD, using GWAS studies (modified form (5)).**

| Gene | Function | Associated with | | |
|---|---|---|---|---|
| | | Ankylosing spondylitis | Psoriasis | Inflammatory bowel disease |
| **HLA-B** | Ag presentation | Yes | -- | -- |
| **ERAP1** | Aminopeptidase | Yes | Probable | -- |
| **IL23R** | Cytokine receptor | Yes | Yes | Yes |
| **TNFRSF1A** | Cytokine receptor | Probable | -- | Yes |
| **TRADD** | Signalling | Probable | -- | -- |
| **TNFSF15** | Inflammatory cytokine | Probable | -- | Yes |
| **IL1A** | Inflammatory cytokine | Probable | -- | -- |
| **IL1R2** | Cytokine receptor | Probable | -- | -- |
| **CARD9** | Innate immune defence | Probable | -- | -- |
| **ANTRX2** | Vascular morphogenesis | Probable | -- | -- |

### *1.1.3 Role for intestinal microbiota in SpA pathogenesis?*

The role of intestinal inflammation and microbiota in SpA pathogenesis has long been under debate. It is reported that 25-70 % of SpA patients display signs of intestinal inflammation, and moreover, 6-13 % of these patients eventually develop overt IBD. Furthermore, 50 % of HLA-B27$^+$ IBD patients develop AS, compared with 1-10 % of HLA-B27$^+$ individuals in the general population (48). Also, as mentioned above, both IBD and AS have additional shared genetic factors, such as IL23R polymorphisms, further suggesting that these two diseases might have shared pathogenic mechanisms. Thus, I will briefly discuss evidence linking intestinal inflammation, and especially the intestinal microbiota, to the development of SpA.

In several studies a role for the intestinal microbiota in AS development has been suggested, but no concrete evidence have been found. In ReA, a subgroup of SpA, peripheral joint inflammation in genetically predisposed individuals has been shown to be triggered by particular bacterial gut infections, such as *Salmonella typhimurium*, *Campylobacter jejuni*, *Yersinia enterocolitica* and *Shigella*, and antigens from these bacteria have also been found in the joints of these patients (49). About 20 % of these ReA patients will eventually develop AS, providing a link for the role of gut inflammation in SpA. In addition, there is some evidence to support the role of intestinal bacteria *Klebsiella*

in AS (reviewed in (50)), although in some cases these observations have been controversial; they have proved difficult to replicate, and in addition the studies carried out have been relatively small. Nonetheless, higher titers of antibodies against these bacteria were found in the serum of AS patients compared to RA patients or healthy controls (50). Additionally, elevated levels of antibodies against *K. pneumoniae* were associated with a higher degree of gut inflammation in Finnish patients with axial AS (51); and in a study from 1980, cross-reactivity between antisera against a specific isolate of *Klebsiella* and HLA-B27 itself was also reported (52). To support the role for bacterial infections in AS pathogenesis, it has also been noted that HLA-B27 shares an unusually high degree of sequence similarity with proteins derived from gram-negative bacteria (53, 54), possibly lending support for the hypothesis of arthritogenic peptide presentation described above.

### *1.1.4 Treatment of SpA*

There is no cure for SpA, but several treatments can be used to alleviate the symptoms (reviewed in (55). Conventional anti-rheumatic drugs, such as sulfasalazine, methotrexate and leflunomide have no effect on the axial or enthesopathic features of SpA, but have some effects on peripheral arthritis and other extra-articular features such as psoriasis, uveitis and IBD. The most commonly used treatment of SpA is anti-tumour necrosis factor (TNF)-α therapy. It has been shown to reduce general symptoms such as fatigue, and improve the overall function and quality of life. However, TNF-α therapy appears to halt joint destruction, but has no effect on the formation of new bone regularly observed in SpA. Anti-TNF-α therapy is not effective for all patients, and thus several therapies are either under trial or being developed. For example, B cell therapy with rituximab, shown to be effective in RA, was shown not to have a similar efficacy in SpA as in RA. T cell targeted therapies, such as efalizumab (anti-CD11a/LFA-1), alefacept (anti-LFA3) and abatacept (CTLA-4-Ig), have not been successful in the treatment of SpAs. Based on the newly suggested role for Th17 cells in SpA pathogenesis, antibodies against IL-6 (Th17 differentiation) and IL-17 (effector cytokine) are currently being trialled.

## 1.2 Dendritic cells

DCs have several roles in immune regulation in several locations in the body. As professional antigen presenting cells (APCs), they are responsible for the induction of antigen specific immune responses by T cells. However, they also have important roles in induction and maintenance of peripheral tolerance. Because DCs have such a big role in

the immune system, it is possible they could be involved in the pathogenesis of SpA. Thus, in this section I take a closer look at DCs and their interaction with other immune cells, and their role in immune regulation in general terms. As has been mentioned above, the intestinal immune system seems to also have an important role in the pathogenesis of SpA, and hence the intestinal immune system and in particular intestinal DCs are discussed in greater detail in their own section.

### *1.2.1 What is a dendritic cell?*

DCs are highly specialised cells of the immune system present in most tissues and organs of the body, where they act as phagocytic cells continuously sampling their environment. The first descriptions of DCs were published in 1973 by Ralph Steinman's group, when they found cells with a unique morphology in murine lymph nodes (LNs) and Peyer's patches (PP) (56), and a few years later in mouse spleens (57). These cells were named dendritic cells due to the visible "dendrites" these cells possess. DCs can also sample antigen, either self- or foreign, from the tissues where they reside, and migrate out of the tissues via lymphatic vessels and into organised lymphoid tissues, where they act as APCs capable of activating cells of the adaptive immune system. In fact, DCs were also found to be unique in their ability to induce naïve T cell proliferation in allogeneic mixed leukocyte reactions (MLRs) (58). Thus, whilst DCs can act as a link between the innate and adaptive immune systems, they also have an additional role in the induction and maintenance of peripheral tolerance. All of these functions of DCs will be discussed in more detail in the sections that follow.

### *1.2.2 DC classification*

Not only are DCs unique regarding their function and phenotype, many would also say they are a uniquely heterogeneous group of cells – reflecting their many diverse functions and physical locations within the body. This heterogeneity poses many challenges to researchers, the first of which is classification. Traditionally, DCs have been referred to as either myeloid DCs or plasmacytoid DCs (pDCs), as it was thought that all DCs are derived from myeloid precursors. However, as discussed below, DCs can be derived from both myeloid and lymphoid bone marrow precursors, another unique DC feature, thus making the traditional classification inaccurate. Using a more modern approach DCs are first divided into either conventional (cDCs) or non-conventional DCs. Conventional DCs arise from pre-DC precursors and have classical DC functions, and can be further divided into migrating and resident DCs. Migrating DCs reside in peripheral tissues, such as skin

and intestine, and upon acquiring antigen they are able to migrate to draining LNs, where they interact with T cells. Resident DCs, on the other hand, reside in the lymphoid organs such as spleen, thymus and LNs, and lack migratory function. Non-conventional DCs include pDCs, which, although derived from the same common myeloid progenitors (CDPs) as cDCs, are distinguished from cDCs by their unique ability to secrete large amounts of IFN upon activation. Also it has been recently shown that DCs can be derived *in vivo* and *in vitro* from monocytes under specific conditions both in steady state and under inflammatory conditions; these DC subsets are also classified as non-conventional DCs. Subsets of DCs belonging to these various groups are discussed later on this section.

### *1.2.3 Cytokines in DC development: evidence from in vitro and in vivo studies*

Because DCs are such a heterogeneous group of cells, details of their complex developmental pathways have only recently started to emerge. In addition, as is common, most of what is known about DC development at the moment is derived from studies carried out in the mouse, and in some cases in humans. In the rat, not much information is available, and thus at this point we can only assume that DC development is similar in mice and rats. However, what is known is that all DCs, like other leukocytes, are differentiated from hematopoietic stem cells in the bone marrow (BM), which requires the integration of environmental signals surrounding the cell. These signals include for example cytokines or growth factors, which can act either locally in the immediate microenvironment, or they can act distantly by traveling in the bloodstream or lymph vessels from the site where they were produced. Here, cytokines and growth factors most commonly associated with DC development, granulocyte-macrophage colony stimulating factor (GM-CSF), fms-like tyrosine kinase 3 ligand (Flt3L) and macrophage-colony stimulating factor (M-CSF), are discussed.

DCs cultured from bone marrow (BMDCs) are often used in experiments due to the rarity and difficulty of obtaining DCs *in vivo* or *ex vivo*. Two main cytokines used to generate DCs *in vitro* from BM cells, are GM-CSF and Flt3L. GM-CSF was the first cytokine described to be capable of supporting DC differentiation *in vitro* (59). Addition of this cytokine, with or without the cytokine IL-4, to murine monocyte or bone marrow cultures leads to generation of high numbers of BMDCs, which were thought to be the *in vitro* equivalent of steady state cDCs. However, these cells are both functionally and morphologically different from cDCs isolated *ex vivo* (60); they are larger, contain more granules and also produce higher amounts of TNF-α and nitric oxide (iNOS) after

stimulation than cDC (61). Similar cells were also identified in rat BMDC cultures supplemented with IL-4 and GM-CSF (62). *In vivo*, cells with a similar phenotype were first discovered in spleens of mice 1-2 days after infection with *Listeria monocytogenes;* these were named TNF-α and iNOS producing DC (Tip-DC) (61). Furthermore, GM-CSF-deficient mice or mice lacking the GM-CSF receptor have only a very slight decrease in splenic and LN cDCs compared to wild type mice (63). Another factor factoring against the role for GM-CSF in steady state DC development is the fact that GM-CSF levels are undetectable in the blood in the absence of inflammation (64), and thus it is unlikely it could drive differentiation of monocytes under non-inflammatory conditions. These data derived from both *in vitro* and *in vivo* animal studies have led researchers to consider these GM-CSF-dependent cells likely to be monocyte derived cells resembling the inflammatory *in vivo* tip-DCs.

In contrast to BMDCs grown with GM-CSF, rat or mouse BM cells but not monocyte cultures, supplemented with Ftl3L give rise to both pDCs and cDCs resembling steady state DC populations (60, 65, 66). In addition, Flt3L or Flt3 receptor knock out mice have severely reduced DC numbers in spleen, LN and thymus (67, 68). *In vivo,* injection of Flt3L to mice or humans leads to massive expansion of cDCs, pDCs and myeloid cells, but not B or T lymphocytes (69-71). Unlike GM-CSF, Flt3L is also readily detected in the serum even in the absence of inflammation (72).

M-CSF null mice, or mice deficient in M-CSF receptor have severe problems in monocyte development in early life and lack some macrophage subsets (73). Total DC numbers are reduced in these animals, however they also present with reduced spleen size, which could explain the lower DC numbers observed. In addition, splenic DC subsets are present in their expected number and density, thus suggesting that M-CSF is not needed for steady-state DC development from DC precursors, nor from monocyte precursors.

Taken together, these data indicate that Flt3L is essential for the development of cDCs and pDCs under steady state conditions, whereas GM-CSF and M-CSF are not.

### *1.2.4 DC ontogeny and life cycle*

The development of DCs from bone marrow precursors is an active field of study, and much is still not known. Here I seek to give a brief summary of what is known about DC development and ontogeny thus far, with the intentional omission of precursors prior to

lymphoid and myeloid precursors. DC development has also been recently discussed in several reviews (73-75).

Two distinct cell lineages can be recognised in the bone marrow: myeloid and lymphoid lineages. Common myeloid progenitor (CMP) cells gives rise to monocytes, macrophages, granulocytes, megakaryotes and erythrocytes, whereas B, T and natural killer (NK) cells differentiate from the common lymphoid progenitor (CLP). DCs are unique, as it has been shown that they can be derived from both myeloid and lymphoid lineages. However, about 90 % of DCs in all compartments, except for thymus, are derived from the myeloid precursors. A very simplified picture of DC development in the mouse, which probably also takes place in rats, is provided in Figure 1.3. CMPs give rise to monocyte-DC progenitors (MDP), which have the potential to differentiate into monocytes, or into the common DC progenitor cell (CDP). This is dependent on the signals provided: it is thought that presence of growth factor M-CSF instructs MDP to produce monocytes, whereas Flt3L drives differentiation of CDPs. Consequently, all cells following the DC differentiation pathway express the receptor for Flt3L, Flt3, whereas its expression is downregulated on cells following the monocyte differentiation pathway.

CDPs in turn can give rise to both pDCs and a cell type called pre-DC, which is the immediate precursor for cDCs. Monocytes, pDCs and pre-DCs leave the BM and travel vial blood to peripheral tissues, where pre-DCs differentiate into tissue resident DCs, and monocytes into macrophages. In addition to giving rise to resident splenic and LN DCs, it has also been shown that pre-DCs are precursors for all $CD103^{+}$ DCs in non-lymphoid tissues such as skin, lung, kidney and intestine, and also for some $CD103^{-}$ DCs in those organs. As mentioned above, monocytes can also give rise to DCs under some circumstances. This is thought to occur under inflammatory conditions, when monocytes are exposed to the inflammatory cytokine GM-CSF. However, in rats it has been shown that $CD43^{hi}$ $CCR2^{lo}$ $CX3CR1^{hi}$ monocytes, corresponding to mouse $Ly6C^{lo}$ monocytes, can give rise to some DC subsets even in steady-state (76).

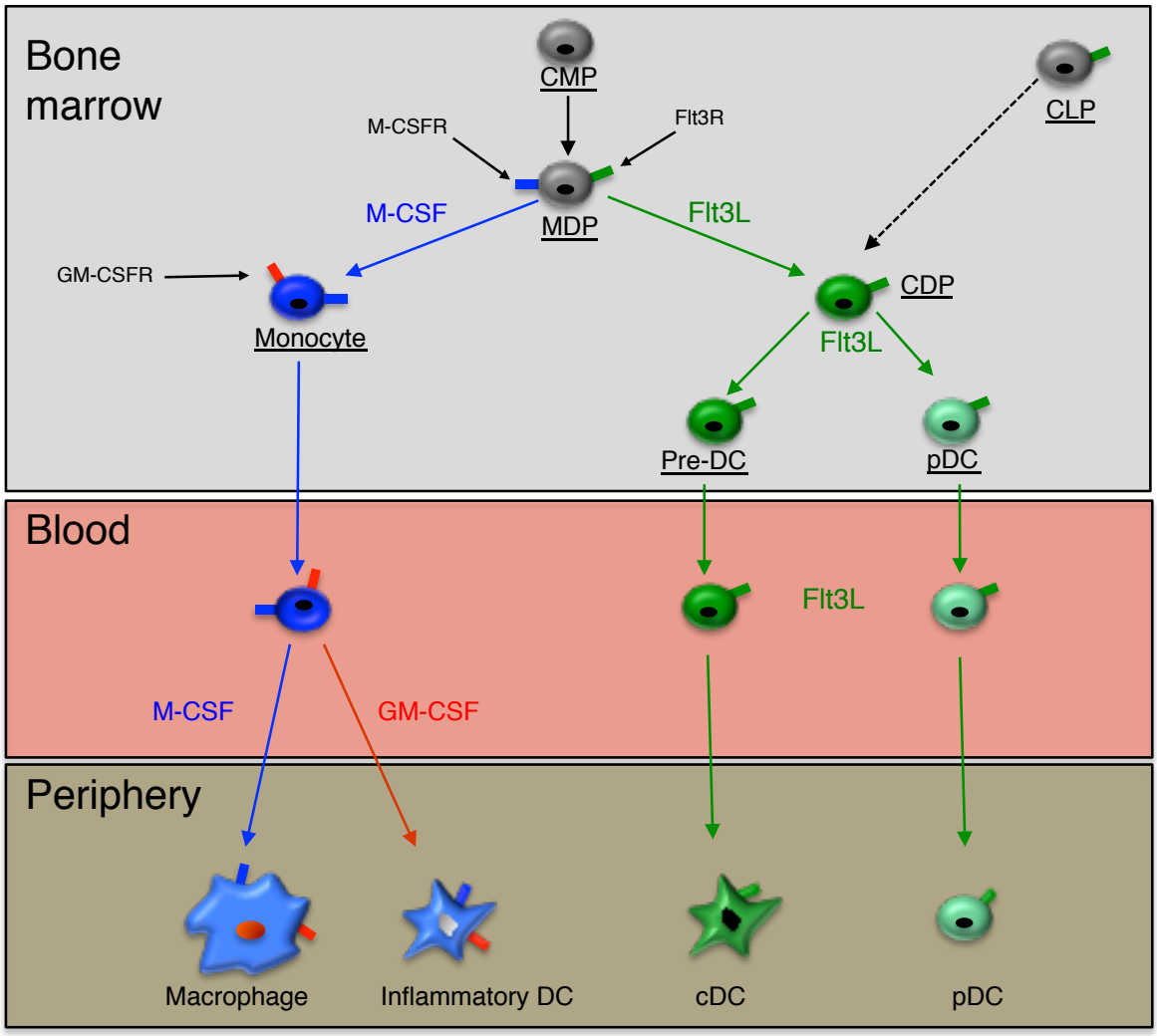


**Figure 1.3. Development of DCs from BM precursors – a simplified view**

In the BM, common myeloid precursors (CMP) give rise to macrophage-DC precursors (MDP). DC potential is retained in all CMPs and common lymphoid progenitors (CLPs), and is restricted to progenitors expressing Flt3 receptor (green receptor on cells). MDPs in turn give rise to both monocytes and common DC precursors (CDP). In addition, CLPs are thought to be able to give rise to CDPs as well.

Monocytes leave the bone marrow (BM) and under influence of M-CSF give rise to macrophages in the peripheral tissues (blue receptor denotes M-CSF receptor). However, it is also believed that monocytes can be induced to become inflammatory DCs under inflammatory conditions under the influence of GM-CSF (denoted by red arrows, and red GM-CSF receptor).

CDP in the BM give rise to both pDCs and to classical DC precursors (Pre-DCs). pDCs exit the bone marrow, circulate in the blood and can enter the peripheral tissues. Pre-DCs also exit the bone marrow, but it is not certain whether they circulate in blood, or immediately home to peripheral tissues where they give rise to the various cDC subsets under the guidance of the local microenvironment. It is believed that Flt3L directs the development of DCs from MDP downwards. The dotted lines demonstrate additional developmental pathways that may lead to development of cDCs.

### 1.2.5 Subsets of dendritic cells

As noted above, DCs can be classified into several groups and subgroups. However, almost all the groups contain several subsets of DCs. In this section I will introduce some of these DC subsets belonging to these groups; most of the information comes from studies with mice, but I will also incorporate elements of what is known about rat and human DCs.

#### *Conventional DCs*

Conventional DCs are present in most tissues of the body, including internal organs, lymphoid tissues and mucosal surfaces. However, as cDCs are relatively scarce in peripheral tissues and difficult to isolate, most research has focused on cDCs present in lymphoid tissues such as spleen and LNs. Mouse splenic DCs were originally divided into two subsets according to the expression of the surface CD8αα homodimer (77); $CD8\alpha\alpha^+$ and $CD8\alpha\alpha^-$ (referred to as $CD8^+$ and $CD8^-$, respectively). $CD8^+$ cells are also DEC $205^+$ $CD11b^{lo}$ $CD172a^{lo}$, whereas $CD8^-$ cells are DEC $205^-$ $CD11b^+$ and $CD172a^+$. Furthermore, it was found that splenic $CD8^-$ DCs could be divided into two additional populations according to expression of CD4: $CD8^-$ $CD4^+$ and $CD8^-$ $CD4^-$ (DN, double negative). $CD8^+$ splenic DCs are all negative for CD4 (78). These same DC subsets can also be found in mouse LNs (79). In addition to displaying different surface markers, in the steady state these subsets of DCs are also found in different areas within the lymphoid organs: $CD8^+$ DCs are mainly found in the T cell areas, whereas $CD8^-$ DCs reside in the marginal zones (80). However, upon stimulation $CD8^-$ cells are able to migrate into the T cell areas (81).

$CD8^+$ and $CD8^-$ cells also differ in their functions, although the $CD8^-$ DCs are not as well characterised as $CD8^+$ DCs. DN DCs have been shown to be poor stimulators of $CD8^+$ T cells *in vitro* and instead they have been shown to prime OVA-specific OTII $CD4^+$ T cells, which secrete IL-10 and suppress the immune response via a transforming growth factor (TGF)-β dependent mechanism (82). However, when DN DCs were stimulated with Toll-like receptor (TLR) 9 agonist CpG, they were converted from tolerogenic DCs into immunogenic DCs and induced development of $CD4^+$ Th1/Th17 T cells, and were able to stimulate efficient OVA specific $CD8^+$ T cell responses (83). $CD8^-$ $CD4^+$ DCs were able to prime $CD8^+$ T cells as well as $CD8^+$ DCs (83). This could mean that under steady-state conditions DN DCs have a role in tolerance induction, but upon TLR stimulation they revert to an immunogenic phenotype. $CD8^+$ DCs, on the other hand, have a key role in viral immunity and in inducing immunity against intracellular pathogens especially in the

spleen and LNs. Splenic $CD8^+$ DCs are more efficient at phagocytic uptake of apoptotic cells (84), and more importantly they have been shown to be much more efficient at cross-presenting both soluble and cell-bound antigens on MHC class I compared with other splenic DC subsets (85, 86). As a consequence, it has been observed that *in vivo* $CD8^+$ DCs are better at activating $CD8^+$ T cells, whereas $CD8^-$ DCs are more efficient at activating $CD4^+$ T cells. In addition, upon activation with TLR ligands and when stimulated with either CD40 or with inflammatory cytokines, $CD8^+$ DCs produce high levels of bioactive IL-12p70 (87, 88). IL-12 production, together with presentation of exogenous antigens on MHC class I make $CD8^+$ DCs capable of driving development of both Th1 T cells and cytotoxic T-cell (CTL) responses from $CD8^+$ T cells. $CD8^+$ DCs have also been shown to produce anti-inflammatory cytokine TGF-β in the steady state (89), suggesting a role for this DCs subset in tolerance as well and immunity. Indeed, it has been indicated that splenic $CD8^+$ DCs are more efficient than their $CD8^-$ counterparts at inducing forkhead box P3 $(FoxP3)^+$ regulatory T cells from $FoxP3^-$ precursors without the presence of supplemental TGF-β, both *in vitro* and *in vivo* (89). Furthermore, $CD8^+$ DCs have been shown to participate in the induction of peripheral self-tolerance by capturing self-antigens and presenting them to naïve $CD4^+$ and $CD8^+$ T cells using the cross-presentation pathway (90).

Human DCs are much less well characterised than murine DCs due to the limited availability of human lymphoid tissues for study, and due to rarity of these cells. Unlike mouse DCs, human DCs can be found in the blood, and much work has been conducted to find out if the human blood derived DCs could be compared to the mouse $CD8^+$ and $CD8^-$ subsets. Human blood DCs comprise about 1 % of all peripheral blood mononuclear cells (PBMCs), and they can be characterised as negative for lineage markers (CD3, 14, 15, 19, 20, 56) and some are positive for MHC class II (HLA-DR) (91), although the usefulness of this marker is under debate. Human DCs, like mouse DCs, can be divided into pDCs and conventional DCs; pDCs are defined as $CD11c^-$ $CD123^+$ $CD304^+$, whereas cDCs are $CD11c^+$ $CD123^-$. Additionally, cDCs can be divided into $CD141^+$ (BDCA-3), $CD16^+$ and $CD1c^+$ (BDCA-1) subsets (92). Given that human DCs do not express CD8α, correlation of mouse and human DC subsets has been difficult. Recently, however, progress has been made in understanding of the relationship of DCs from these two species. First of all, genome-wide expression profiling revealed similarities in the genetic signatures of human $CD141^+$ DCs and mouse $CD8^+$ DCs, and with human $CD1c^+$ and mouse $CD8^-$ DCs (93). In addition, the human $CD141^+$ subset shares some phenotypic similarities with the mouse $CD8^+$ subset, such as expression of TLR3 (94, 95), c-type lectin 9A (CLEC9A) (96),

nectin-like molecule 2 (NECL2) (97) and the chemokine receptor XCR1 (98, 99). Recently, functional studies form several groups have revealed more similarities between these two DC subsets by demonstrating that, like murine $CD8^+$ DCs, human $CD141^+$ DCs show more efficient cross-presentation capabilities than other DC subsets (98-101). Furthermore, the $CD141^+$ DC subset has also been shown to expand *in vivo* after Flt3L injection (97), as do the mouse $CD8^+$ DCs (discussed above). In addition to blood, human $CD141^+$ DCs have also been found in the spleen, bone marrow, tonsils and LNs, suggesting a similar distribution pattern to mouse $CD8^+$ DCs (97, 100, 102).

Similar to human DCs, rat DCs do not express CD8α, and the subsets are not very well defined. Rat peripheral tissue DCs have been identified in the intestine, lungs and liver. However, most of these studies have carried out using histological sections and different subsets have not been identified. However, in spleen and LNs, three subsets of DCs have been identified; two cDC subsets identified as $CD103^{lo}$ $CD11b^+$ $CD4^+$ $CD172a^+$ ($CD4^+$) and $CD103^{hi}$ $CD11b^+$ $CD4^-$ $CD172a^-$ ($CD4^-$), and pDCs identified as $CD103^-$ $CD11b^-$ $CD4^+$ $CD172^+$ (103). $CD4^+$ DCs have been shown to have better spontaneous survival in culture compared to $CD4^-$ DCs, but survival of both cell types was enhanced by stimulation with TLR agonists (103). In addition, $CD4^+$ DCs exhibited spontaneous maturation after overnight culture, as measured by CD86 expression, whereas $CD4^-$ DCs required TLR stimulation for their maturation. However, $CD4^-$ DCs were shown to produce more IL-12p40 after TLR stimulation compared to $CD4^+$ DCs. Regarding their capacity to induce proliferation of T cells, both DC subsets were equally capable of driving $CD4^+$ T cell proliferation, but whereas $CD4^-$ DCs induced Th1 type T cells, $CD4^+$ DCs drove non-polarized T cell differentiation. $CD4^+$ DCs were also shown to be better stimulators of $CD8^+$ T cells compared to $CD4^-$ DCs.

In addition to blood derived DC subsets described above, LNs also contain DCs that have arrived via afferent lymph from peripheral tissues. The phenotype of these cells depends on the tissue the LN drains, and thus DCs found in the LNs draining the skin are different from those found in the mesenteric lymph nodes (MLNs) that drain the intestine. Most information concerning migrating DCs has been derived from studies involving murine DCs migrating from the skin. In the steady state, five subsets of DCs can be identified in the skin dermis and epidermis, and they differ in their expression of CD207 (langerin), CD11b and CD103 (104). Epidermal DCs called Langerhans cells (LC) are all $CD207^{hi}$, whereas dermal DC populations express less CD207 and also include cells negative for this marker (104). All five populations have also been suggested to migrate from the skin to

the skin draining cutaneous lymph node (CLN) even in the absence of inflammation (105). In humans, corresponding LC populations of $CD207^+$ $CD11b^+$ DCs can be identified in the skin, but understandably less in known about the migratory behaviour of these cells (105). As was mentioned above, DCs also migrate from the intestine to the MLN. In rats, these migrating intestinal lymph DCs (L-DCs) consist of three subsets that share some phenotypical characteristics with the $CD4^+$ and $CD4^-$ DCs found in the spleen and described above, and have also shown to exhibit different functions. These DCs, together with the recently described murine L-DCs, are discussed in section 1.3.

### *1.2.6 Migration of dendritic cells*

As mentioned above, immature DCs reside at the sites of pathogen entry, where they sample and collect antigen. Subsequently, they migrate out of the tissues and into draining LNs through the lymphatic system (reviewed in (106)). This migration occurs both in steady state and under inflammatory conditions, however it is increased under inflammatory conditions compared to steady state. To detect invading pathogens or other foreign antigens, DCs carry pathogen recognition receptors (PRRs) on their surface, which recognise conserved pathogen associated molecular patterns (PAMPs) shared by large groups of potentially pathogenic microorganisms. Best known among the PRRs are TLRs, which have evolved to recognise a large variety of both viral and bacterially derived antigens. Innate recognition of pathogens by TLRs and other PRRs has recently been reviewed (107), and will not be discussed here further. Upon recognition of pathogenic antigens, DCs start to express more MHC molecules on their surface, as well as upregulating their expression of co-stimulatory molecules, leading to maturation. Activated DCs migrate out of the tissue, and into the draining LNs via lymph in a CCR7 dependent manner. Lymphatic vessels drain all peripheral tissues, and the lymph flow is unidirectional from the periphery towards the heart. Eventually, lymphatic vessels from the abdominal viscera join the thoracic duct, from which lymph and its contents return to the circulation. Given that very few DCs are detected in the efferent lymph leaving the LNs, it is thought that once DCs have arrived in the LNs, the vast majority of DCs do not travel any further but die there. This contrasts with lymphocytes, which after interacting with DCs leave the LNs and return to the circulation.

Direct investigation of migrating DCs involves either cannulation of afferent lymphatics in bigger animals, or on rats and mice a two-stage surgical method is used. This requires removal of draining LNs prior to cannulation and collection of the thoracic duct pseudo-

afferent lymph, as described in materials and methods. Using these techniques, migrating DCs have been detected in the skin draining lymph of cows (108), sheep (109) and pigs (110, 111) , as well as in the intestinal lymph of rats, mice (112, 113) and pigs (114). From these studies, it has been observed that migration of dendritic cells from the periphery to the LNs is continuous and occurs even in the absence of inflammatory stimulation. It has also been shown to be independent of intestinal flora, as demonstrated by cannulation of germ-free rats where DC migration can still be observed (115). Once in the LNs, DCs present antigen to lymphocytes and this interaction leads to either activation or tolerance of the cells with which they interact (116). DCs migrating in the steady state can carry self-antigens or antigens derived from commensal bacteria in the case of intestine, and presentation of these antigens to T cells without inflammatory stimulus leads to induction or maintenance of peripheral tolerance. The role of intestinal DCs in inflammation and tolerance will be discussed in detail later in this chapter.

As investigation of migrating DCs involves surgical procedures, it could be that the surgery induces a low level of inflammation that drives migration of DCs. However, studies conducted in cattle, where lymph can be collected for several weeks, indicate that the surgical procedure does not affect the number or phenotype of the DCs as their characteristics remain stable for duration of the lymph collection (117). In addition, DC migration from the periphery to LN can be enhanced in the cannulated animals by proinflammatory stimuli, such as skin contact sensitization (118), i.v. administration of TLR4 agonist LPS (119) or oral administration of a TRL7/8 agonist R848 (120). This increased DC migration results in peripheral tissues becoming almost completely devoid of DCs, and this effect is dependent on TNF$\alpha$.

Migration of DCs from the periphery into the LNs is regulated by chemokine receptor CCR7. DCs in peripheral tissues exist in an immature state, and upon encountering inflammatory signals they rapidly mature. This maturation is also linked to upregulation of CCR7 and downregulation of CCR6 (121). The crucial role for CCR7 in DC migration was revealed when mice deficient in CCR7 were investigated, and it was noted that there was a defect in DC migration into LNs (122), and furthermore, CCR7 deficient DCs failed to migrate when transferred into wild type recipients (123).

### 1.2.7 DC interactions with immune cells

Interactions between DCs and other cells of the immune system are crucial for the induction of effective immune reactions, as well as for development of peripheral tolerance. It is therefore important to understand the biology of the DC : T cell interactions in order to gain greater understanding of the role DCs can have in disease pathogenesis. In this section, interactions of DCs with both $CD4^+$ and $CD8^+$ T cells are briefly reviewed.

#### *$CD4^+$ T cells*

$CD4^+$ T cells have critical roles during adaptive immune responses, including helping B cells to produce antibodies, recruitment and activation of $CD8^+$ T cells, as well as attracting other effector cells such as macrophages and neutrophils. These diverse functions are mirrored in the heterogeneity of the $CD4^+$ effector T cell populations, which all have a variety of functions. Four main lineages currently recognised are Th1, Th2, Th17 and regulatory T cells, and these populations differ in their cytokine production and function (124). Recently, however, it has been suggested there is much more plasticity in the $CD4^+$ T cell populations than previously thought, with cells of one subset being able to acquire the cytokine profile of another subset, at least *in vitro,* and to some extent *in vivo* (125, 126). For the purpose of this brief review, the traditional lineage commitment models are discussed, but this picture may change in the future as new data emerge. As all the $CD4^+$ T cell subsets share the same early activation events, these are first discussed, followed by a brief introduction to each of the different subsets.

As the development of activated effector cells under non-inflammatory conditions could harm the host, their differentiation is tightly regulated. Thus, $CD4^+$ T cells need at least 3 different types of signal for full differentiation into effector cells (127). The first interaction, or signal 1, is provided by DCs expressing exogenously derived peptides on their MHCII molecules, which are recognised by the T cell receptor complex of a particular T cell clone. This interaction leads to activation of the naïve, non-committed $CD4^+$ T cell, but does not lead to proliferation or commitment to a specific lineage. The same DC, from whom the T cell acquired signal 1, also delivers signal 2. This involves ligation of co-stimulatory molecules such as CD80 and CD86 on the mature DC with activating CD28 or inhibitory CTLA-4 on the T cell, leading to activation of specific signalling pathways on the T cell. The mechanisms behind the balancing act between activating and inhibiting signals delivered by the above mentioned two receptors have been recently elegantly described (128). In addition to these interactions, ligation of CD40 or 4-

1BB on DC by CD40 ligand or 4-1BBL, respectively, on T cells leads to bi-directional signalling, where ligation of these molecules leads to activating signals being directed to both DC and T cell (129). Signal 1 and 2 together lead to production of the cytokine IL-2 by the T cell, which is needed for the survival and proliferation, or clonal expansion, of the $CD4^+$ T cells. T cells that receive either only signal 1, without signal 2, or cells that only receive co-stimulatory signal 2, are not activated but instead enter a state of anergy (130). These anergic cells continue to circulate, but do not subsequently respond to specific antigen even when it is presented to them by an APC expressing co-stimulatory molecules. These events provide a mechanism to maintain peripheral self-tolerance, required because not all self-reactive T cells are deleted in the thymus during development. Thus, DCs as APCs have an important role in the shaping of early immune response.

Mediators, such as chemokines or cytokines, secreted by DCs provide signal 3, and this directs the differentiation of $CD4^+$ T cells into different lineages (124). There are currently four recognised $CD4^+$ T cell lineages: Th1, Th2, Th17 and Tregs. Th1 and Th2 cells were the first ones discovered in 1986 by Mosmann and Coffman, who demonstrated the existence of two $CD4^+$ T cell types, Th1 and Th2, which differed in their cytokine secretion profiles (131, 132). Th1 cells produce IFN-γ, which is important for macrophage activation and clearance of intracellular pathogens, such as viruses and intracellular bacteria. In addition, Th1 cells can also activate naïve $CD8^+$ T cells to become cytotoxic T lymphocytes (CTLs). In contrast, Th2 cells are involved in allergic responses and produce IL-4, IL-5, IL-10 and IL-13, which have key roles in IgE production, eosinophil and basophil recruitment and clearance of extracellular pathogens, such as helminths. These two cell types also require different cytokines for their differentiation; Th1 cells require cytokines IL-12 and IFN-γ, which are derived from DCs or other innate cells, whereas Th2 cells need IL-4, whose initial source is not entirely clear. As the response develops, IFN-γ produced by Th1 cells, or IL-4 produced by Th2 cells create a positive feedback loop, further driving the differentiation of these effector cells. The development of one or the other effector subset is driven by the DCs, which can be activated by different microbial stimuli to drive differentiation of the most suitable effector cell type.

It has been observed that Th1 cytokine IFN-γ expression in target tissues correlates with clinical signs in both experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), and treatment with polyclonal antibodies against IL-12 in both of these disease models works as a therapy (133, 134). Thus, for a long time Th1 cells were thought to be the main T cell subset driving the pathology in several animal models of T

cell mediated autoimmune diseases. However, this view was challenged when data emerged showing that mice deficient for several molecules in the Th1 differentiation pathway, such as IFN-γ, IL-12p35 or IL-12 receptor β2 were not protected from EAE and CIA but rather were more susceptible (135-137). These observations led researchers to believe there could be another cell type independent of Th1 that is responsible for the induction of disease pathogenesis in these models. IL-12 is a dimer formed by two chains: p40 and p35, and in 2000 a novel cytokine chain, p19, was discovered that shared the p40 chain with IL-12 (138). This novel cytokine was named IL-23, and it is predominantly produced by activated dendritic cells and macrophages, especially in the gut (139). Cua and colleagues discovered that it was IL-23 rather than IL-12 that was responsible for induction of EAE by comparing IL-23p19 deficient mice to IL-12p35 deficient mice (140). Furthermore, in a follow-up publication they also showed that IL-23 expands a specific subset of IL-17A (referred to as IL-17) producing $CD4^+$ T cells in EAE that are capable of transferring disease into naïve WT recipients (43). Since these original observations, a wealth of data has emerged linking these IL-17 producing T cells, named Th17, to pathogenesis in several experimental animal models, as well as human autoimmune diseases such as RA, psoriasis and IBD (for review see (141)). In addition, it has been observed that AS patients have increased number of circulating Th17 cells as well as increased IL-17 levels in the serum (142, 143). Furthermore, Th17 cells have been indicated to have a pathological role in the animal model for SpA, where rats transgenic for the human HLA-B27 gene have more Th17 cells in mesenteric and popliteal lymph nodes as well as in arthritic joints, than non-transgenic animals (144). This SpA animal model is discussed in detail in a later section of this chapter.

Differentiation of Th17 cells from naïve mouse T cells requires cytokines TGF-β and IL-6, produced by a variety of cell types such as DCs, and these cytokines in turn upregulate expression of IL-23R on the surface of these T cells (145, 146). The precise function of IL-23 on Th17 cells is still elusive but it is thought that it is required for proliferation and survival of differentiated Th17 cells, as shown by the observation that IL-23p19 deficient mice have limited number of Th17 cells (43), and that treatment of mice with IL-23p19 neutralising antibody inhibits development of EAE (147). Initial reports about human Th17 cells suggested that, in contrast to mice, it was not possible to create human Th17 cells using TGF-β and IL-6. Instead, it was suggested that a combination of IL-1β, IL-6 and/or IL-23 was needed for the differentiation of IL-17 producing cells from $CD4^+$ peripheral blood T cells (148, 149). However, it was noticed that the culture medium used for the *in vitro* experiments contained TGF-β. Thus, when TGF-β free medium was used together

with naïve T cells derived from cord blood, it was indeed shown that TGF-β is also required for the differentiation of human Th17 cells (150, 151).

Th17 cells produce a variety of cytokines in addition to their signature cytokine IL-17A. These include other members of the IL-17 family, such as IL-17F, IL-21 and IL-22, as well as TNF-α. These cytokines produced by Th17 cells have been proposed to have a role in protection against extracellular bacteria, protozoa and fungi, and in addition they have been shown to be key mediators of chronic inflammation in several experimental animal models of diseases such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis (reviewed in (152)). In addition, as discussed earlier, IL-23R polymorphisms have been linked to several inflammatory disorders. Interestingly, Th17 cells have also been shown to produce GM-CSF, which is critical for their pro-inflammatory functions (153, 154). For example, GM-CSF was shown to induce IL-23 production by APCs. Like the other T helper subsets, development of Th17 cells is tightly regulated, and it has been reported Th1 and Th2 cytokines IFN-γ and IL-4 both inhibit IL-17 production as well as the differentiation of Th17 cells (41, 42).

There is also a reciprocal relationship between Th17 cells and regulatory T cells, another $CD4^+$ T cell type. There are two types of regulatory T cells: Natural regulatory T cells (nTregs) develop in the thymus from $CD4^+$ T cells as a separate population, whereas inducible Tregs (iTregs) can be generated from naïve $CD4^+$ T cells in the periphery (155). There is considerable overlap between these two populations, and as of yet no reliable surface markers have been found to distinguish iTregs from nTregs. Despite differences in the origins of these cells, most Tregs secrete anti-inflammatory IL-10 and/or TGF-β, which can inhibit T cell responses, and thus these cells are important in tolerance and control of inflammatory responses. Similarly to Th17 cells, differentiation of iTregs from naïve precursors requires TGF-β (156). This induces the expression of the Treg specific transcription factor FoxP3 on these cells, which is required for the induction and maintenance of iTregs in the peripheral immune compartment. However, in the presence of IL-6, the Treg specific developmental pathway is stopped and instead these T cells develop into Th17 cells as previously discussed. Many cell types produce TGF-β, and it has broad inhibitory effects on the whole immune system. Thus under non-inflammatory conditions it could be that generation of iTregs is favoured, whereas under inflammatory conditions where IL-6 production is induced, inflammatory Th17 cells would be produced. In addition, retinoic acid, a vitamin A metabolite, can drive generation of Tregs while inhibiting the differentiation of Th17 cells. Retinoic acid is primarily produced by

intestinal $CD103^+$ DCs, and it is thought to act directly on naïve T cells by enhancing TGF-β signalling while inhibiting IL-6 signalling (157, 158). This provides further evidence for the crucial role DCs in general, and specifically in the intestine, play in the initiation and regulation of various T cell responses.

### *CD8*

In contrast to $CD4^+$ T cells, $CD8^+$ T cells show less heterogeneity in their differentiation. Current knowledge suggests that upon activation, all $CD8^+$ T cells become CTLs, which migrate to their instructed peripheral target sites and kill their target cells. This role is particularly important in defence against intracellular pathogens, like viruses and intracellular bacteria. For example, virus-infected cells can express viral proteins derived from virus replicating inside them on their MHCI, which the CTLs recognise. As uncontrolled activity of CTLs could be destructive, their activation is very tightly regulated and naïve CD8+ T cells need more co-stimulatory activity to drive the development of effector CTLs compared to $CD4^+$ T cell differentiation. There are two ways in which this can be achieved, with or without $CD4^+$ T cells help. In inflammatory conditions, DCs are activated by binding of PAMPs derived from viruses and bacteria to their TLRs, leading to maturation (107). Mature DCs express all necessary co-stimulatory molecules and can directly stimulate CD8 T cells to synthesize IL-2, which drives proliferation and differentiation of CD8 CTLs. However, this type of CD8 T cell stimulation only occurs in some specific settings, and most often the DC alone is not able to induce CD8 T cell differentiation. The extra help required is provided by $CD4^+$ effector T cells recognising their cognate antigen on the surface of the same DC, which leads to upregulation of co-stimulatory molecules on the DC (159). Especially important for this interaction is the CD40 on DCs that interacts with CD40L on T cells, leading to upregulation of CD80 and CD86 molecules on the DC (160). These activated DCs are then able to stimulate CD8 T cell differentiation. This mechanism of action is referred to as licensing of DCs by the CD4 effector T cells. After differentiation, effector CTLs proliferate and dramatically increase in numbers. This phase is referred to as the effector phase, which leads to pathogen clearance. This activity is followed by the contraction phase, where most effector CTLs die and leave behind only a small population of long-lived memory cells. These memory cells are maintained at stable levels for several years, so that in case of pathogen re-exposure, they can mount a rapid recall response. CD4 T cell help for CTL differentiation seems to play a critically important role in the formation of these long lived memory CTLs. Memory CTL cells derived from CD4 competent mice were able to mount an effective immune

response when transferred into CD4 deficient hosts, whereas memory CTL from CD4 deficient mice transferred into CD4 competent mice did not (161, 162).

## 1.3 The Intestinal Immune system

The intestinal immune system is the largest and most complex of all the immune compartments in the body. It is the site of entry for vast amounts of antigens, including harmless antigen from food alongside harmful pathogenic bacteria. In addition, the intestinal system is a home to a large colony of commensal bacteria, which, whilst harmless, are still seen as foreign by the immune system. Thus, the intestinal immune system has evolved to ignore, or be tolerant, towards harmless non-self antigens, but still retain the ability to mount protective immune responses against invading pathogens. A break in tolerance can result in hypersensitivity reactions against either food antigens or commensal bacteria, as demonstrated by coeliac disease and Crohn's disease. As has been discussed in the above paragraphs, DCs as professional APCs have a central role in inducing immune responses throughout the body, so it not surprising that they have an important role in maintaining the balance between tolerance and immunity in the intestine.

Lymphoid tissues in the intestine are referred to as gut-associated lymphoid tissue (GALT) and it consists of both non-organised and organised lymphoid tissue sites. The non-organised tissues contain aggregations of effector lymphocytes and are scattered throughout the epithelium and lamina propria (LP). In adult humans the single layer of epithelial cells, which make up the epithelium lining the lumen of the gut, covers an area of about 200-400 $m^2$. It absorbs nutrients and fluids, but it also acts as a barrier between the environment and the inside of the body, and as such it forms the largest entry site for pathogens. Thus, the epithelium contains immune cells acting as the first line of defence against invading pathogens. These cells are antigen-experienced T lymphocytes called intraepithelial lymphocytes (IEL), and it has been demonstrated using parabiotic mice that they do not seem to recirculate (163). Many IELs express the molecule CD103 (integrin $\alpha_e$) that binds E-cadherin on the epithelial cells and is important for their localisation (164, 165). The various types of IELs have different functions but as a whole they have been indicated to: condition and repair the epithelial barrier; control the growth and turnover of the epithelium; contain cytoplasmic granules for cytotoxic activity; and secrete various inflammatory cytokines such as IFN-γ, IL-2, IL-4 and IL-17 (166).

DCs can be found in the LP, which unlike the epithelium contains several different populations in addition to DCs such as macrophages, $CD4^+$ and $CD8^+$ T cells, plasma cells and eosinophils (167). In addition to the lamina propria, DCs can be found in the organised lymphoid tissues, sites of induction of immune reactions (168). Intestinal organised lymphoid tissues include MLNs, Peyer's patches (PP) and isolated lymphoid follicles. MLNs, like other LNs of the body, are highly organised structures allowing circulating naïve T cells to interact with their cognate antigen on DCs, which have arrived from the periphery (169). This interaction can lead to either tolerance or induction of immunity, and this will be discussed in more detail below. PPs, like MLNs, are organised into separate B and T cell areas, and they are primarily responsible for the induction of T cell dependent IgA responses (167). DCs are found in the submucosa along the length of the small intestine, and they are separated from the gut lumen by a single layer of specialised epithelial cells called follicle-associated epithelium (FAE). Under the FAE layer an area called sub-epithelial dome (SED) can be found, which also further separates the B and T cell areas from the lumen. DCs can be found both in the SED and around the B and T cells areas.

The intestine is an immunologically active organ, and due to its constant exposure to numerous antigens it is in a constant state of low-grade inflammation. However, this rarely results in overt inflammation and disease because of sophisticated homeostatic mechanisms, in which DCs play important roles.

### *1.3.1 DCs of the intestinal system*

Like in other parts of the body, several intestinal subsets of $CD11c^{hi}$ DCs can be identified in the intestine, including both cDCs and plasmacytoid DCs. In the mouse, PPs contain $CD8^-$ $CD11b^+$ DCs that are found mainly in the SED (170), and preferentially secrete IL-10 and induce Th2 type responses (171). In contrast, $CD8^+$ $CD11b^{lo}$ DCs are mainly located in the T cell rich areas of the PP, whereas a third subset of double negative $CD8^-$ $CD11b^-$ DCs can be found in both areas (170). Both of the latter mentioned subsets have been reported to produce mainly IL-12 and drive the differentiation of IFN-γ producing T cells (171). Uniquely, DCs in PP express a chemokine receptor CCR6, which has been shown to be required for their recruitment of DCs into the FAE upon entry of pathogenic invading *Salmonella typhimurium* (172). In addition, upon activation PP DCs also start to express chemokine receptor CCR7 enabling them to migrate to the interfollicular T cell areas (170).

The same three subsets found in the PP can also be detected in small intestinal LP of mice, however in the LP the majority are of the $CD8^-$ $CD11b^+$ subset (173). In addition, most LP DCs express CD103, and these $CD103^+$ DCs consist of both $CD8^-CD11b^+$ and $CD8^+$ $CD11b^-$ subsets, which are functionally different (174). $CD103^+$ DCs are the only subset shown to be able to imprint gut homing molecules CCR9 and $\alpha_4\beta_7$ on T and B cells, and to induce FoxP3 expression on naïve T cells (175). These functions are dependent on retinoic acid, and the role of $CD103^+$ DCs in tolerance induction will be discussed below. It was reported that another DC population present in the LP was able to extend dendrites through the epithelial layer, and thus directly sample the antigen content within the intestinal lumen (170). These cells were later reported to express high levels of the chemokine receptor CX3CR1 (176, 177). However, it was recently shown that $CX3CR1^{hi}$ cells do not migrate in the lymph to the MLNs, a hallmark DC function, and are poor stimulators of T cells (112). In addition they were found to express the macrophage marker F4/80 but not CD103 (178). Thus, these $CX3CR1^{hi}$ cells are now generally accepted to be macrophages rather than DCs. Intriguingly, another heterogeneous population of $MHCII^+$ $CD11c^+$ cells expressing intermediate levels of CX3CR1 ($CX3CR1^{int}$ $CD103^-$) were found in the LP, and these appeared distinct from the $CX3CR1^{hi}$ cells (112, 179). In addition to these cDC populations, LP in mice also includes a population of $MHCII^{lo}$ $B220^+$ pDCs (173).

Human intestinal DCs are poorly characterised. In the MLN, a population of $CD103^+$ DCs have been recognised, which share similar functions, such as induction of CCR9 expression on T cells, with the equivalent mouse $CD103^+$ DCs (180). In the colonic lamina propria, $MHCII\ (HLA\text{-}DR)^+$ $CD11c^+$ $LIN^-$ (CD3, 14, 16, 19, 20, 26) DCs have been recognised, and they were shown to make up less than 1 % of all colonic LP mononuclear cells. These DCs were shown to migrate out of colonic biopsies in culture, be able to stimulate proliferation of T cells, and upregulate expression of co-stimulatory molecules upon TLR stimulation (181, 182). More recently, a $CD1c^+$ subset of $MHCII^+$ $CD11c^+$ $LIN^-$ DCs was recognised in the small and large intestinal LP, and this population also included some $CD103^+$ cells (183).

The DC populations in the LNs of mice have been discussed above, and the same subsets can also be found in the MLN. These are: $CD8^-$ $CD4^+$ $CD11b^+$, $CD8^+$ $CD4^-$ $CD11b^-$, $CD8^-$ $CD4^-$ $CD11b^+$ and an additional $CD8^-$ $CD4^-$ $CD11b^-$ subset. MLN also contain DCs expressing CD103, and these DCs include both $CD8^+$ and $CD8^-$ populations. $CD103^+$ DCs have unique functions, such as induction of gut homing molecules on T cells and induction of $FoxP3^+$ T regulatory cells, as has been discussed elsewhere. The number of $CD103^+$

DCs is reduced in the MLNs of CCR7 deficient mice (174), leading to idea that most $CD103^+$ cells found in the MLN have migrated there via lymph, whereas $CD103^-$ DCs are blood derived. CD103 seems to be a conserved marker between species, as $CD103^+$ DCs can also be found in the MLNs of rats and humans (180).

Until recently it has not been possible to directly examine the phenotype of murine DCs arriving to the MLN from the intestine. However, we have adopted the cannulation techniques, successfully used to study rat migrating DC populations, for use with mice. Using this technique it was determined in our laboratory that the majority of mouse lymph $MHCII^+$ $CD11C^+$ L-DCs express CD103, with two distinct populations observed: $CD8^+$ $CD11b^-$ and $CD8^-$ $CD11b^+$ subsets, which can also be detected in the LP (Dr Vuk Cerovic, manuscript in preparation). Surprisingly, some $CD103^-$ $CD8^-$ cells were also observed within the $MHCII^+CD11c^+$ gate, with the majority also expressing CD11b. These cells have been shown to be able to prime both naïve $CD4^+$ and $CD8^+$ T cells, and to have normal DC characteristics. Thus, this new information challenges the view that $CD103^-$ DCs observed in the MLNs would have all arrived there via blood. Further investigation into the functional differences between these subsets is on going.

Using the cannulation technique in the rat, three subsets of $MHCII^+$ $CD103^+$ DCs can be identified in the lymph. These subsets differ in their expression of CD11b and CD172a: $CD172a^{hi}$ $CD11b^{lo}$ (referred to as $CD172a^{hi}$), $CD172a^{int}$ $CD11b^{hi}$ ($CD172a^{int}$) and $CD172a^{lo}$ $CD11b^{int}$ ($CD172a^{lo}$). It is difficult to directly compare these subsets to the mouse ones, as rat DCs do not express CD8, and no good rat-specific anti-CD11c antibodies have been developed. In addition, CD4 is co-expressed with CD172a in the rat whereas in the mouse the three L-DC subsets all express only very low levels of CD4, and CD172a is co-expressed with CD11b (Dr Vuk Cerovic, manuscript in preparation). It is possible that in the rat there are also some $CD103^-$ L-DCs (personal observations), but these have not been further investigated.

In the steady state, DCs carry self-antigens and antigen derived from commensal bacteria or food proteins. Upon arrival in the LNs they present these to naïve T cells, in the absence of co-stimulatory signals, and induce immunological tolerance towards the antigens they carry. Paradoxically, $CD172a^{hi}$, $CD172a^{int}$ and $CD172a^{lo}$ L-DC subsets in the rats have been shown to induce strong proliferation and IFN-γ production from naïve $CD4^+$ T cells in MLR reactions in steady state (177), suggesting that at least in *in vitro* even steady state DCs are capable of T cell activation. There were some differences between the three L-DC

subsets, as the CD172a$^{hi}$ and CD172a$^{int}$ populations were shown to promote stronger proliferative responses than the CD172a$^{lo}$ population (177), suggesting that the CD172a$^{lo}$ population might be less immunogenic. In addition, the CD172a$^{lo}$ subset was shown to contain cytoplasmic apoptotic DNA and cytokeratins restricted to epithelial cells (115). These CD172a$^{lo}$ DCs were also shown to be present in PPs, MLNs and LP, but not in other tissues. Together, these data indicate that this subset is carrying antigen from apoptotic epithelial cells from the intestine into the MLN (115). The cytoplasmic inclusions were also observed in the absence of normal microbiota in gnotobiotic rats, thus suggesting that the CD172a$^{lo}$ subset might be involved in the induction and maintenance of tolerance to intestinal antigens. In spite of these differences observed between steady-state CD172a$^{hi}$/CD172$^{int}$ and CD172a$^{lo}$ subsets, all three L-DC subsets induced comparable proportions of CD25$^{+}$ FoxP3$^{+}$ Treg cells to divide in the absence of inflammatory stimuli (184).

Under inflammatory conditions, the number of DCs migrating from the periphery to LNs increase, and they also upregulate their expression of co-stimulatory molecules. Arrival of these DCs in the LN results in T cell proliferation and activation. When TLR7/8 agonist R848 was orally fed to rats, it was observed that the the migration of all three subsets increased 30-50 fold (120). However, the DC subsets responded differently to *in vivo* R848 stimulation, as only the CD172a$^{hi}$ and CD172$^{int}$ subsets upregulated expression of CD25 (185). Furthermore, *in vitro* studies demonstrated that CD172a$^{hi}$ and CD172a$^{int}$, but not CD172a$^{lo}$ cells, were shown to express TLR8 receptor and respond to R848 stimulation (185). Differences between CD172a$^{hi}$ and the CD172a$^{int}$ subsets have also been observed. In response to *in vitro* R848 stimulation the CD172a$^{hi}$ L-DCs included a higher proportion of CD25$^{+}$ cells than the CD172$^{int}$ cells, and CD172a$^{hi}$ subset was also the only subset secreting IL-6 and IL-12p40 *ex vivo* (185). In addition, oral admininistration of another antigen, heat labile *E. coli* enterotoxin, was shown to promote CD25 expression on the CD172a$^{hi}$ subset as well as increasing the ability of these DCs to induce naïve T cell proliferation in MLRs (186).

Some intestinal cell populations have been described to be hyporesponsive to stimulation with TLR4 ligand LPS (187). When rats were given LPS i.v., the number of migrating L-DCs did increase, but the DCs did not show signs of activation, as they failed to upregulate CD80 and CD86 (188). Furthermore, L-DCs were shown to be hyporesponsive to TLR4 ligand LPS *in vitro* (188). This function was later shown to be restricted to TLR4, as L-DCs responded to stimulation with TLR2 and TLR9 ligands Pam3Cys and CpG *in vitro,* as

shown by upregulation of CD25, co-stimulatory molecules CD80 and CD86, as well as an increase in the production of cytokines IL-6 and IL-12p40 by all three subsets of DCs (189). However, it was observed that the CD172a$^{lo}$ subset was the only one increasing production of TNF-α after stimulation with both TLR ligands (189). The above experiments do demonstrate that the CD172a$^{lo}$ subset does behave differently from the other two L-DC subsets, however, when these experiments were carried out no reagents were available to assess whether CD172a$^{lo}$ L-DCs induced a truly tolerogenic phenotype on the T cells. However it should not be forgotten that real differences were noted in the ability of CD172a$^{lo}$ L-DCs to induce proliferation of naïve T cells compared to the other two subsets.

### *1.3.2 Intestinal DC antigen uptake and migration*

Several theories have been proposed to describe how the intestinal DCs acquire antigen. The best understood method is via specialised enterocytes called microfold (M) cells situated in the FAE of PPs (167). FAE itself differs from the normal epithelial layer covering the intestine as it has lower levels of digestive enzymes and a less pronounced brush border. M cells, on the other hand, completely lack microvilli as well as the thick layer of mucus otherwise covering the surfaces of the intestinal lumen. These features are designed to allow easier access for antigens through the M cells. DCs are present both in the epithelium directly associated with M cells and in the dome regions directly below. In addition, LP DCs can acquire antigen through the epithelial layer in a yet unknown mechanism. It is, for example, possible that the LP CX3CRI$^{hi}$ cells, that extend dendrites into the lumen to sample antigen (177), could transfer antigen to the DCs in the LP.

DCs carrying antigen migrate either to local T and B cell areas in the PP, or to the MLN via afferent lymphatics. It is not yet clear if DCs from PPs migrate to the MLNs, or if they just act locally. In the MLN, DCs interact with T cells and induce either activation or tolerance, mechanisms of which will be discussed in the next section. The vast majority of DCs die in the MLN as only a few can be detected in the efferent lymph leaving the LNs. The migration of DCs from the intestine to the MLN is constitutive; in steady state, every day about $8x10^5$ DCs, from the total pool of about 7-24 million intestinal DCs, migrate out (186). DCs in the lymph have upregulated their expression of MHCII, but not the expression of costimulatory molecules, and thus migrating DCs are described as semi-mature (190).

### *1.3.3 Immunity vs. tolerance*

Induction of protective immune responses in the gut is important for host survival, and as has been discussed DCs are closely involved in this. DCs have been indicated to be involved in the induction of immune reactions against a variety of pathogens, including bacteria, viruses and parasites (reviewed in (191). $CD103^+$ intestinal DCs have been proposed to have many unique features, and they are important in both induction of immune responses as well as in tolerance. They were first found to be uniquely capable of inducing gut homing molecules $\alpha_4\beta_7$ and CCR9 on the T and B cells that they interact with in the MLN, as well as inducing FoxP3 expression on naïve T cells (174, 192-194). Activated effector lymphocytes expressing CCR9 and $\alpha_4\beta_7$ exit the MLN to the blood, where they circulate and eventually home back to the intestinal sites, as directed by CCR9 and $\alpha_4\beta_7$ ligands. $\alpha_4\beta_7$ ligand mucosal addressin cell-adhesion molecule 1 (MAdCAM1) is expressed by the vasculature of mucosal tissues, whereas small intestinal epithelial cells express the CCR9 ligand CCL25. These functions of the $CD103^+$ mucosal DCs are dependent on the vitamin A metabolite retinoic acid, which can be produced by $CD103^+$ DCs that express the necessary retinaldehyde dehydrogenase enzymes needed for the conversion. Retinoic acid, together with TGF-β has also been implicated in antibody class-switching from IgM to IgA by B cells (195). IgA antibodies are the dominant antibody class produced in the intestine, and have an important role in the protection against harmful pathogens.

Despite being very effective at inducing immunity towards a variety of pathogens under inflammatory conditions, in the steady state intestinal DCs induce tolerance towards self- or food antigens as well as towards commensal microbiota. The best-studied model of tolerance is oral tolerance. In oral tolerance, antigen delivered via the oral route induces systemic unresponsiveness, revealed when animals are re-challenged in peripheral tissues with the same antigen. The mechanisms behind this phenomenon are not completely understood, but it is known that T cells, commensal bacteria and DCs all play a role. DCs have been shown to induce peripheral tolerance using two mechanisms: induction of T cell anergy/deletion, and through generation of Tregs. Recently, it was shown that constitutive ablation of DCs in mice led to spontaneous fatal autoimmunity, demonstrating the importance of DCs in maintaining immunological tolerance (196). Furthermore, expansion of DCs using Flt3L led to increased oral tolerance after feeding of soluble proteins (197). Many factors can contribute to the induction of tolerogenic properties on DCs: the

maturation stage of the DC, subsets, nature of the microbial stimuli and environmental cues from the local microenvironment. These will be briefly discussed below.

As has been discussed earlier in this chapter, immature DCs promote tolerogenic responses, whereas mature DCs promote immunogenic responses. Under steady state conditions, DCs predominantly adopt an immature phenotype, with the expression of some MHCII, but only low levels of costimulatory molecules. Often these immature DCs carry self-antigens, and interaction of these DCs with T cells can lead to anergy or deletion of self-reactive T cells or induction of Tregs *in vivo* (198-201). Maturation of DCs leads to upregulation of MHCII and costimulatory molecule expression and induce T cell activation and differentiation.

The site of tolerance induction can play a role as well. Antigen can gain access to DCs through M cells in the PPs, or the antigen can be transported by DCs to the MLN via lymph, where it is presented to the local T cells. PPs were originally considered to be the main site for tolerance induction, but this view has been challenged by more recent studies observing induction of oral tolerance in PP deficient intestines (202-204). Thus, the MLN is now considered the main site for oral tolerance induction. Supporting this, it was recently observed that tolerance could not be induced in mice from which MLNs were removed (205). In addition, in the same study it was found that oral tolerance could not be induced in CCR7 deficient mice, where the migration of DCs from the intestine to the MLNs is impaired.

As described above, mucosal $CD103^+$ DCs have many unique features and can be found both in the LP and the MLNs of mice, rats, and humans. One of their functions is to convert dietary vitamin A into retinoic acid in a two-step process. This process involves enzymatic oxidation of retinol by retinaldehyde dehydrogenase (ALDH), with the major isoform expressed by DCs being retinaldehyde dehydrogenase 2, encoded by the *Aldh1a2* gene (206). Compared to DCs from many other tissues, as well as to colonic $CD103^+$ DCs, murine small intestinal LP and MLN $CD103^+$ DCs express higher level of *Aldh1a2* mRNA, display increased ALDH activity and induce enhanced retinoic acid signalling in responding T cells (206). Under steady state conditions, retinoic acid expressing $CD103^+$ DCs promote Treg conversion from naïve T cells, together with TGF-β (discussed earlier) (157, 193, 207). Interestingly, retinoic acid has also shown to be able to inhibit the TGF-β -dependent generation of inflammatory Th17 cells, even in the presence of IL-6 (158, 208).

In addition to its effects on T cells, retinoic acid promotes generation of IgA producing B cells (195).

Another molecule produced by LP $CD103^+$ DCs is indoleamine 2,3 dioxygenase (IDO), which catalyses amino acid tryptophan from the environment, leading to generation of toxic metabolites. These can inhibit generation of effector T cells and induce Treg cells, and inhibition of IDO *in vivo* results in defective oral tolerance (209). It has been shown recently that although the generation of gut homing Tregs take place in the local MLN, and involves antigen presentation by DCs, full maturation of these cells does not take place in the MLN, but requires Tregs to migrate into the intestinal mucosa. In the gut mucosa Tregs undergo a second round of expansion, which is directed by IL-10 producing $CX3CR1^+$ macrophages, suggesting an intriguing cooperation in the maintenance of tolerance by different mucosal cell types (210). The functions of $CD103^+$ DCs seems to be conserved between species, as human $CD103^+$ DCs show similar characteristics to their mouse counterparts (180).

How do intestinal $CD103^+$ DCs acquire their tolerogenic phenotype? It is now believed these DCs are not generated from specialised precursors, but that the local microenvironment in the intestine directs these cells to become tolerogenic. These factors can involve intestinal commensal bacteria and interaction with intestinal epithelial cells (IEC) as well as other leukocytes (reviewed in (175, 206). IECs can produce several immunomodulatory factors, such as TGF-β, retinoic acid, thymic stromal lymphopoietin (TSLP), and GM-CSF, and the production of these molecules can be enhanced by interactions between IEC and commensal bacteria. Supporting this, addition of exogenous retinoic acid, GM-CSF + IL-4 or supernatant from cultured IECs can program inflammatory BMDCs or monocyte derived DCs to become tolerogenic (211, 212). IEC supernatant can promote generation of $CD103^+$ DCs from human $CD103^-$ cells, and these conditioned $CD103^+$ DCs inhibit development of Th1 and Th17 cells (212, 213). TSLP was found to be one of the molecules in the human IEC supernatants responsible for the generation of tolerogenic DCs. Intriguingly, some commensal bacteria seem to favour induction of Tregs, while other suppress Tregs and induce inflammatory Th17 cells. For example, colonization of germ-free mice with human commensal *Bacteroides fragilis* induces development of $FoxP3^+$ Tregs, and polysaccharide A (PSA) derived from *B. fragilis* mediates the conversion of $CD4^+$ T cells into $FoxP3^+$ Tregs that produce IL-10 (214). It was also noted that both induction of Tregs and IL-10 production were dependent on TLR2 signalling. Some helminths can also promote differentiation of regulatory T cells

(215). Whereas some commensals promote induction of Tregs, colonization of the small intestine of mice with segmented filamentous bacteria leads to appearance of $CD4^+$ Th17 cells producing IL-17 and IL-22 in the lamina propria (216). Furthermore, it has been shown that DNA from intestinal flora can signal through TLR9, promoting effector T cell induction while suppressing Tregs (215, 217). These results indicate that commensal bacteria exert complex effects on DCs, which shape the immune response by inducing generation of effector cells or regulatory T cells depending on the signals DCs receive from the environment.

All in all, intestinal dendritic cells need to make constant decisions about whether to induce inflammation or tolerance in response to particular antigens. Several unique features make the intestinal immune system geared towards generation of tolerogenic responses, and a breakdown in this tolerance can lead to overt inflammatory reactions or autoimmunity. In fact, it has been shown both in humans and mice that DCs can have protective or immunogenic roles in several diseases, including inflammatory bowel disease and celiac disease (168) and rheumatoid arthritis (218).

## 1.4 B27-TG rats

When the strong genetic link between HLA-B27 and AS was discovered in 1973, researchers were curious to learn whether HLA-B27 alone was responsible for AS disease pathogenesis. When the technology to create transgenic mammals became available in the mid 1980's, mice transgenic for the human HLA-B27 and human β2 microglobulin (β2m) genes were among the first ones generated. However, despite expressing a functional HLA-B27 gene, no evidence of spontaneous inflammatory disease was reported in these mice (219-221), although it was observed that the transgenic mice were more susceptible to *Yersinia enterocolitica* infection (222). As several classical models of chronic arthritis, such as adjuvant arthritis, occur in rats but not in mice, it was thought that rats would provide a better model for the HLA-B27 induced disease as well.

### *1.4.1 Disease phenotype in B27-TG rats*

With the idea that rats might provide a better model for HLA-B27-dependent disease, in 1989 Joel Taurog generated rats transgenic for the human HLA-B27 and human β2m genes (B27-TG rats) (223). These transgenic animals were created by randomly inserting both HLA-B27 and β2m DNA fragments into the rat genome, and assessing which of the

founders had integrated both genes in the genome and also expressed them as proteins on the cell surface. Several lines on the LEW background and one line on the F344 background fulfilled both requirements, and these lines differed in their copy number of the incorporated genes (Table 1.2). It was then observed that some, but not all, lines of these transgenic animals developed a spontaneous inflammatory disease. The development of the symptoms seemed to correlate with the gene copy number: the more copies of the gene the animals had, the more likely they were to develop disease symptoms. In addition, it was observed that the level of HLA-B27 mRNA and HLA-B27 surface expression seemed to increase as the rats got older and displayed more disease symptoms. It has also been noted that HLA-B27$^+$ AS patients express higher level of HLA-B27 on the surface of their PBMCs than HLA-B27$^+$ healthy controls, suggesting that the level of HLA-B27 expression correlates with disease symptoms (224).

**Table 1.2. HLA transgenic rat lines**

| Line | HLA gene | HLA copy number | β2m copy number | Original strain | Other strains | Disease |
|---|---|---|---|---|---|---|
| **21-4H** | B*2705 | 150 | 90 | LEW | F344, DA, SD | Yes |
| **21-3** | B*2705 | 20 | 15 | LEW | | In homozygotes |
| **25-6** | B*2705 | 7 | 7 | LEW | | No |
| **21-4L** | B*2705 | 6 | 6 | LEW | PVG | No |
| **33-3** | B*2705 | 55 | 66 | F344 | LEW, PVG | Yes |
| **195-2** | B*2705 | 9 | 17 | F344 | F344 rnu/rnu | No |
| **120-4** | B*0702 | 26 | 5 | LEW | | No |

The disease in these transgenic animals has shown little variation since they were developed, and the main symptoms are (adapted from (225)):

1. Inflammatory gastrointestinal disease, in most cases affecting the colon and cecum; stomach and small intestine are affected with a variable distribution and frequency
2. Inflammatory peripheral arthritis, mostly in the tibiotalar and tarsal joints
3. Inflammatory and fibrotic spinal lesions (rare, and not investigated in detail)
4. Variety of skin lesions, such as psoriasiform dermatitis, folliculitis, alopecia and nail dystrophy
5. Orchitis and epididymitis

6. Carditis (has not been investigated in detail)

Not all symptoms mentioned above are present in all the transgenic lines used for research, and not all have been followed up since the original description of the rats. For example, spinal lesions and carditis are not well characterised in this model. The two main lines of transgenic rats, 33-3 (F344) and 21-4H (LEW), both present with intestinal inflammation, arthritis, nail and skin symptoms and genital lesions (226). Intestinal inflammation is the first symptom to occur, with the onset in both female and male rats of the 33-3 line around age of 5 weeks (226). In males, this is followed by development of skin and nail lesions, arthritis and genital lesions, with gradual increase in the percentage of animals affected from age 10 weeks. By 36 weeks, all the animals are affected by intestinal symptoms, about 80 % with skin and nail lesions and arthritis and at least 60 % by genital lesions. In female animals, the disease course is slightly different with later onset of arthritis and fewer skin- and nail-lesions. By 36 weeks, all the females are also affected by intestinal inflammation, about 60 % with arthritis, but only about 20 % present with skin and nail lesions.

As can be seen, many of the spontaneously-arising HLA-B27-dependent disease characteristics in the rats represent symptoms observed across the whole range of HLA-B27-linked SpA diseases in humans, such as skin symptoms present in psoriatic SpA, and gastrointestinal disease observed especially in AS and IBD-SpA. Thus, these transgenic animals offer a good model for human SpA.

### *1.4.2 Factors affecting disease development in B27-TG animals*

Development of spontaneous disease in the B27-TG animals suggests that the HLA-B27 and β2m genes alone are capable of driving the development of SpA. But, as it was observed that a very high copy number of HLA-B27 and β2m genes were needed for the disease to develop in the animals, it was suggested that the disease was caused by the insertion of human transgene in the rats, irrespective of the gene. Thus, rats transgenic for another human MHC class I molecule, HLA-B7, which is not associated with any human disease, were created and named line 120-4 (Table 1.2). These animals also have a high copy number of the human transgene, but they do not show any signs of spontaneous disease development, which is why these animals today serve as useful controls in experiments where B27-TG animals are used. In humans HLA-B27 is responsible for only about 30-40 % of disease susceptibility, with other genes and environmental factors

contributing the rest, whereas in rats HLA-B27 seems to be able to drive the development of spontaneous inflammatory disease on its own. However, the disease in transgenic rats is also strain dependent. The 33-3 line, originally on the F344 background, shows normal disease development when backcrossed to both PVG and LEW backgrounds (225, 227), but when the F344 B27-TG rats are backcrossed to the DA strain, the animals exhibit a strikingly reduced disease phenotype (225). In addition, it was also noticed that the surface expression of the two transgenes was lower on the disease resistant DA.33-3 rats than on the susceptible F344.33-3 animals. This difference in the susceptibility of different rats strains for SpA phenotype also hints that, as in humans, additional genetic factors are required for SpA disease development. However, it has not been possible to identify the factors on rats of the DA strain that confer protection from the disease.

Environmental factors also play a role in development of disease in the B27-TG animals. This was made especially clear by Joel Taurog's group, when they encountered an extreme change in the disease phenotype in one of their barrier rooms (225). In this instance, the 33-3 line on an F344 background developed an accelerated disease, with rats at weaning presenting with symptoms normally present at about 3 months of age, including severe diarrhoea. DA.33-3 animals housed in the adjacent rack had been completely symptom free until then, but eventually they also developed severe bowel inflammation and diarrhoea. This observation suggests that whatever factors offer protection in the DA background, they can be overcome by some, in that case unknown, environmental factors.

As discussed before, there has been much interest in the role of intestinal factors in the pathogenesis of SpA in humans. Whereas the above-mentioned isolated observation does not provide direct evidence for the role of intestinal factors in disease development, other studies performed using these animals provide more reliable and consistent observations. Perhaps the most convincing evidence linking the intestinal microbiota to disease development on the B27-TG animals was acquired when B27-TG animals on 33-3 and 21-4H backgrounds were re-derived in a germ-free environment. Surprisingly, no gut or joint inflammation was observed in either of the strains used (228). However, skin and male genital lesions were still present and not affected by the germ-free environment, suggesting that these two symptoms could be a direct consequence of transgene expression, whereas the development of joint and intestinal inflammation is dependent on additional environmental factors. Supporting this, upon transferring the animals from germ-free environment into a specific pathogen-free environment, they developed the complete disease phenotype (228). Furthermore, it was subsequently shown that it was possible to

induce the disease phenotype in germ-free animals by giving them a specific cocktail of bacteria including the anaerobic species *Bacteroides vulgatus* (229). This same group elaborated on their earlier observations and showed that reconstitution with this species alone, but not with *E. coli*, was sufficient to induce colitis in germ-free B27-TG animals (230). Regarding the intestinal microbiota in B27-TG animals, it has also been observed that the cecal content of *E. coli* and *Enterococcus* rise in parallel with disease activity (231). It was mentioned earlier that there has been much controversy regarding the role of *Klebsiella* bacteria in AS pathogenesis. This bacterium does not appear to be relevant for the disease in rats, as no evidence for the presence of these bacteria was found when cecal bacteria from B27-TG rats were studied (231).

### *1.4.3 Immunological considerations*

The immunological mechanisms driving disease in the B27-TG animals have been of great interest to researchers, and much has been learned since the model was developed. Here, the main points are briefly discussed.

After the generation of the B27-TG animal model, researchers were interested to discover which cells were responsible for disease induction in the animals. One of the first experiments to try to elucidate this was performed by Joel Taurog's group, where either LN or bone marrow BM cells were transferred from disease prone 21-4H and 33-3 lines to lethally irradiated non-transgenic recipients (232). All the recipients of B27-TG BM cells, but not LN cells, developed disease symptoms. In addition, transfer of non-transgenic BM cells to lethally irradiated B27-TG 33-3 animals with established disease resulted in disappearance of skin lesions and diarrhoea. In the same publication, it was also observed that there was an increased presence of $B27^{bright}$ $CD4^{dim}$ $OX42^{+}$ (CD11b) cells with monocyte-macrophage morphology in the peripheral blood of the animals that developed disease symptoms. The percentage of these cells also increased with disease progression. Thus, the authors concluded that BM derived hematopoietic precursors are required for disease development, and that these cells could be dendritic cells, or cells of the monocyte lineage.

HLA-B27 is an MHC class I molecule involved in antigen presentation to $CD8^{+}$ T cells. Thus, despite the findings above indicating that BM-derived non-lymphoid cells are needed for disease development, it is likely that other cell types are involved as well. Following the above observations, Taurog's group were interested to discover whether T

cells are required for the development of disease in these animals (233). They performed a series of elegant experiments involving the use of B27-TG 33-3 rats crossed with nude animals, which lack T cells. First, it was observed that these nude B27 transgenic animals remained healthy, suggesting that T cells are indeed required for the disease progression. Supporting this, transfer of BM from nude 33-3 animals into non-transgenic recipients did induce disease in the recipients. By transferring either total LN cells or LN cells enriched for either $CD4^+$ T cells or $CD8^+$ T cells into nude 33-3 recipients, it was surprisingly observed that $CD4^+$ cells induced a more pronounced disease in the recipients than $CD8^+$ cells. However, in both cases the induced disease was mild, and thus this observation has been followed up more recently using two different approaches. First, disease prone rats from 21-4H and 33-3 lines underwent thymectomy, and subsequent *in vivo* treatment with anti-CD8α (OX-8) antibody to deplete $CD8\alpha\beta^+$ T cells (234). These animals developed a disease comparable to control animals, suggesting that $CD8^+$ T cells are not required for the disease pathogenesis. However, long-term treatment of B27-TG animals with OX8 antibody led to a less severe arthritis compared with non-treated animals. There are other CD8α expressing cells in the rat that are not thymically derived, such as NK cells and monocytes, and thus it could be that depletion of these cells is responsible for the effects seen using the long-term treatment. To address this question more specifically, recently a new model was developed where rats with a mutation in the CD8α chain were backcrossed to LEW.33-3 transgenic line (235). This led to generation of transgenic animals with no surface expression of either CD8α or CD8β. Supporting the earlier observations, no difference in either the onset of disease or the phenotype was seen when comparing CD8 deficient $CD8\alpha^{-/-}$ animals to $CD8\alpha^{-/+}$ littermates with normal CD8 surface expression. From the above observations it can be concluded that normal MHC class I antigen presentation to $CD8^+$ T cells does not seem play a significant role in HLA-B27 driven disease development. And as it was noted in one of the experiments that $CD4^+$ T cells derived from disease prone animals were sufficient to induce disease in nude 33-3 animals, and that hematopoietic BM derived cells were needed, it has been suggested that interactions between dendritic cells and monocytes and $CD4^+$ T cells have a central role in the development of inflammatory disease in the B27-TG rat model.

DCs are APCs that are uniquely capable of activating naïve $CD4^+$ T cells, a cell type, as noted above, that was able to transfer disease to non-TG recipients. Recently, there have been several publications suggesting a role for DCs in the SpA animal model. When B27-TG animals were backcrossed to nude rats, creating 33-3 B27-TG rats that lack T and B cells, it was observed that these animals did not develop spontaneous disease. When non-

TG MLN cells were transferred to these nude 33-3 B27-TG animals, the donor non-TG cells were capable of inducing colitis in the recipient animals (236). This suggests that an additional HLA-B27 expressing cell type present in the recipient animals is needed for the disease induction on nude 33-3 animals, together with lymphocytes. As this cell type needs to be capable of activating and instructing the immune response, DCs were implicated. Furthermore, several additional observations support the role for DCs in SpA pathogenesis in the animal model:

- Splenic dendritic cells from B27-TG animals exhibit reduced capability to stimulate allogeneic T cells responses (237).
- Reduced and altered formation of T cell-DC conjugates has been observed when using B27-TG DCs (237, 238).
- Deficient cytoskeletal dynamics on B27-TG DCs leads to reduced DC mobility and impacts the formation of immunological synapse with T cells; and reduced expression of surface MHC class II, which in turn leads to increased apoptosis of B27-TG splenic DCs (239).
- Splenic DCs from B27-TG rats have been shown to induce more Th17 T cells when compared to non-TG DCs (144).

Thus, several defects in splenic and LN DC behaviour have been identified in the B27-TG animal model. However, given the mounting evidence for the role of intestinal inflammation in the pathogenesis of both human SpA and B27-TG animal model, it is surprising that only a few studies have been conducted using DCs from more physiologically relevant sites, such as the MLN or the intestine. This is likely to be due, at least in part, to the lack of reliable DC markers in the rat. Therefore, several issues regarding DC function in SpA still need further research and clarification.

### *1.4.4 HLA-B27 driven mechanisms of disease*

As was mentioned earlier in this chapter, the association between HLA-B27 and SpA is well established. However, the functional role this molecule has in disease induction remains unclear. Several theories have been proposed to explain the pathogenic role of HLA-B27, including presentation of arthritogenic peptides, aberrant folding of surface heavy chains, and HLA-B27 misfolding. These are described briefly below.

### *1.4.5 Presentation of arthritogenic peptides*

Presentation of arthritogenic peptides by HLA-B27 is sometimes referred to as the molecular mimicry theory. It is thought that HLA-B27 may present peptides arising from degradation of endogenous proteins to $CD8^+$ T cells, and the sequence similarity between microbial and self-antigens might lead to break in tolerance and lead to autoimmunity. In support of this theory, HLA-B27 was shown to present a peptide derived from its own molecule, which was shown to be highly homologous to a peptide derived from *Chlamydia trachomatis* (240). This peptide is a natural ligand to HLA-B27 subtypes strongly associated with SpA, namely B*2705, B*2704 and B*2702, but not to weakly associated B*2706 and B*2709 (240). Furthermore, peptides derived from cartilage or bone-related proteins were shown to be possible ligands to HLA-B27, and a high number of the peptides binding HLA-B27 shared a homology to bacterial sequences (241). It has also been shown that self-peptide derived from vasoactive intestinal peptide 1 (VIPR1) show homology to an Epstein-Barr derived peptide when bound in a non-conventional way to B*2705. B*2709 can only bind this peptide in a conventional way, and when presented in this conformation, VIPR1 peptide does not share the sequence similarity with Epstein-Barr virus. Thus, the authors hypothesise that individuals with B*2705 allele could have circulating self-reactive T cells that have escaped the central tolerance in the thymus, which can induce cross-reactivity to self-antigens (242, 243). This theory is also supported by the fact that reactive arthritis, a subtype of SpA, is triggered either by genitourinary infections with *Chlamydia trachomatis* or by gram-negative enterobacteria such as *Shigella*, *Salmonella*, *Yersinia* or *Campylobacter* species (244), and thus HLA-B27 presenting peptides from these pathogens could lead to induction of immunity towards self-antigens and thus to autoimmunity. Furthermore, 10-20% of $HLA\text{-}B27^+$ patients with reactive arthritis develop AS in the space of 10-20 years (245).

In favour of this theory, a single amino acid substitution from aspartate in the disease conferring allele B*2705 to histidine in B*2709 leads to loss of association with AS (246). This mutation is in the antigen-binding region of the molecule, which suggests that the disease-conferring alleles might bind unique set of peptides, supporting the theory that B*2705 binds a unique set of peptides that can induce disease development. However, some cases of SpA have been identified in individuals carrying the B*2709 allele, thus undermining the credibility of this particular piece of evidence. In addition, none of the above mentioned pathogens have been noted as being causative agents in SpA. Also, studies from B27-TG rats indicate that $CD8^+$ T cells are not needed for the induction of

disease, whereas $CD4^+$ T cells are (see section 1.4.3), further suggesting that HLA-B27 might not have a traditional antigen presenting role in the disease pathogenesis of SpA.

Thus, the absence of autoantibodies and scarce evidence for HLA-B27 restricted T cell responses could raise the questions whether SpA is a genuine autoimmune disease driven by B and T cells. Other theories discussed below suggest that HLA-B27 could have a role in triggering innate immune responses rather than in antigen presentation, thereby arguing for a more autoinflammatory rather than autoimmune origin for the disease.

### *1.4.6 HLA-B27 misfolding in the ER*

It has been noted that the HLA-B27 molecule has some unusual features outside of its role in presenting antigens. Notably, HLA-B27 has been shown to have a tendency to misfold inside cells, in the endoplasmic reticulum (ER). The ER is a sub-cellular organelle, where proteins are assembled and correctly folded before transit to the cell surface or to other organelles. This process is dependent on chaperones and other molecules that assist in protein folding and is under strict quality control, which limits accumulation of protein aggregates by ensuring fidelity of transcription and by selectively degrading unfolded or misfolded proteins (247). Altered ER homeostasis may lead to accumulation of protein aggregates that can be detrimental to cell survival, and lead to an ER "stress response" or "unfolded protein response" (UPR) (247). The UPR can trigger three kinds of protective cellular responses: (i) up-regulation of ER chaperones, e.g. BiP which assist to refold the proteins; (ii) downregulation of protein translation and (iii) degradation of misfolded proteins by the proteasome in a process called ER associated degradation (ERAD) (247). If these three protective measures fail to resolve the ER stress, it may lead to NF-κB activation and upregulation of host defence mechanisms or, if stress is prolonged, to apoptosis or autophagy (247).

HLA-B27 is an unusual molecule in that it has been shown to undergo slower protein folding and greater cytosolic accumulation than other MHC class I molecules, even in the presence of a normal supply of peptide and β2m (248). The unpaired $Cys^{67}$ residues in the peptide-binding region of the molecule appear to allow HLA-B27 heavy chains to dimerise when they are reassembled in the absence of β2m (249). Supporting this, in the B27-TG rats, the B27 molecule has shown prolonged binding to the BiP chaperone suggesting that misfolding is taking place (250). In contrast, the HLA-B7 allele, which is not linked to disease, did not show any evidence of misfolding when expressed in rat cells (251).

Further evidence for HLA-B27 misfolding comes from work with the transgenic rats where it was noted that macrophages activated with IFNγ showed evidence of UPR whereas there was no evidence of UPR in macrophages derived from non-transgenic rats or from B7-TG rats (250). In this same study, it was found that tissue from the gastrointestinal tract of B27-TG rats showed signs of UPR, unlike other tissues such as spleen and thymus where this effect was not seen. More recently, it was also shown that BM macrophages from B27-TG rats produced more IL-23p19 than macrophages from non-TG animals when stimulated with LPS (252). In the same study it was found that IL-23p19 and IL-17 mRNA were increased in the colon of B27-TG rats compared to non-TG animals, and most of these transcripts were localised in the LP of the rats. LP cells were divided into lymphocytes and APCs (DCs and macrophages) according to CD11b and FSC/SSC characteristics. It was found that there was an increase in the expression of BiP in the $CD11b^+$ APCs from B27-TG animals, and these cells also showed increased expression of IL-23p19 compared to other cell populations observed. The increase in the IL-17 expression was noticed mostly in the $CD4^+$ $TCR\alpha\beta^+$ lymphocytes. The authors name these $CD11b^+$ cells macrophages. However, some of these cells could be LP DCs, populations of which also express CD11b. This would fit with other data from B27-TG rats showing defects in the DC populations, discussed above. In concordance with rats, human monocyte derived macrophages from AS patients were also noted to produce increased amounts IL-23 compared to cells from healthy individuals (253). However, no increase was noted in expression of ER stress molecules CHOP or BiP.

The propensity of HLA-B27 molecules to misfold and activate UPR may lead activation of proinflammatory NF-κB pathways and thus to antigen-independent activation of NK cells, T cells and macrophages via cytokines, such as IFN-γ and IL-12, secreted from the stressed cells. For example, DCs would be ideally placed to drive these inflammatory responses. Not only can they activate T cells, it has also been noted that DCs can interact with and activate NK cells. However, there is little or no evidence of UPR or ER stress in human SpA cells, so it is still unclear how important this mechanism is for the human disease development.

### *1.4.7 Aberrant folding of surface heavy chains*

In 1999 Allen, RL et al showed that HLA-B27 molecules can form homodimeric or heterotrimeric structures on cell surfaces (249). The HLA-B27 molecule normally consists of β2-microglobulin (β2m), which together with α3 domain supports peptide binding to

the groove formed by α1 and α2 domains. HLA-B27 has an unusual feature of having an unpaired cysteine residue ($Cys^{67}$) in its α1 domain. In their publication Bird, LA et al demonstrated that HLA-B27 was able to form heavy chain homodimers on the cell surface that were dependent on $Cys^{67}$ but free of β2m. These disulfide-bridged homodimeric complexes did not seem to derive from the ER but seemed to originate from cell surface heavy chain/β2m/peptide complexes that have lost β2m, and is was proposed they were formed either on the surface or in the endoplasmic vesicles during transport to the surface (254). However, these observations were carried out using HLA-B27 transfected B cell lines *in vitro*, and it is possible that on other cells, like DCs, some of the surface complexes are formed intracellularly in the ER and then circulated to the cell surface. Indeed, complexes staining with HC10 antibody, that recognises B27 dimers, are found both in the surface and within the cell in B27-TG rat splenocytes and DCs (255). HC10 staining has also revealed surface homodimers to be present in other cell types both from AS patients and from HLA-B27 transgenic rats (255, 256).

As it has been shown that $CD8^+$ T cells are not necessary for disease induction, at least in HLA-B27 transgenic rats, it raises a question about which cells are driving disease pathogenesis. Hill Gaston showed that $CD4^+$ T cells are capable of directly recognising forms of mutated HLA-B27 (257), and the authors conclude that these abnormal forms could arise as a result of infection and thus $CD4^+$ T cells might play a pathogenic role in spondyloarthropathies. Furthermore, MHC class I restricted $CD4^+$ T cell lines have been isolated both from humans (258) and mice (259) supporting this argument. The mechanism for this interaction is not fully understood but the CD4 co-receptor is thought to play a role.

In addition to $CD4^+$ T cells, NK cells are also capable of binding to HLA-B27 molecules. NK cells have both inhibitory and activatory receptors on their surface, and these belong to the family of killer cell immunoglobulin-like receptors (KIRs) (260). Inhibitory KIRs (KIR2/3DL) have long cytoplasmic tails, and incorporate two immunoreceptor tyrosine inhibitory motifs (ITIMs). Stimulatory KIRs (KIR2/3DS) have shorter cytoplasmic tails and they interact with immunotyrosine activation motifs (ITAMs). NK cell activation occurs as a result of the overall sum of the inhibitory and activating signals the cell receives. Some populations of T cells, mostly a small population of CD8 memory effector cells, also express KIRs (261). $KIR^+$ CD4 T cells are scarce on healthy individuals, but are expanded in circumstances where chronic T cell activation occurs, such as in some autoimmune diseases (262, 263).

Kollnberger, S et al showed that NK cell receptor KIR3DL2 is capable of binding to HLA-B27 homodimers independent of the bound peptide sequence (264). Co-culture of KIR3DL2 NK cells with HLA-B27 transfected cells expressing B27 dimers leads to inhibition of IFN-γ production by NK cells, whereas co-culture with HLA-B7 transfected cells did not affect IFN-γ production (264). Interestingly, KIR3DL2 expressing CD4 T cells are expanded in the peripheral blood of patients with HLA-B27 associated SpA, and have been shown to account for almost 30 % of memory effector CD4 T cells in these patients (265). Ligation of KIR3DL2 with B27 dimers was shown to inhibit activation induced cell death of KIR3DL2 expressing leukocytes from patients, and thus binding of B27 dimers to KIRs could promote survival of proinflammatory NK and T cell population in SpA patients (237). Intriguingly, RA patients also have an expanded population of KIR expressing T cells, but no preferential expansion of $KIR3DL2^+$ is seen (265, 266). More recently, it was also noted that co-culture of $KIR3DL2^+$ $CD4^+$ T cells with a cell line expressing B27 homodimers induced survival, proliferation and IL-17 production from the T cells (267). Furthermore, IL-17 producing $KIR3DL2^+$ $CD4^+$ T cells were expanded in the blood and synovial fluid of SpA patients. $KIR3DL2^+$ $CD4^+$ T cells from SpA patients also produced more IL-17 ex-vivo than $KIR3DL2^+$ T cells from healthy controls. These observations are interesting as they again enforce the observations about possible importance of Th17 cells in the pathogenesis of SpA, and offer an additional explanation for the role of HLA-B27 in SpA pathogenesis.

## 1.5 Hypothesis and aims

DCs act between innate and adaptive arms of the immune system, and thus have a crucial role as orchestrators of the immune response. Nowhere is this role more pronounced than within the intestinal immune compartment, where DCs have to be capable of directing protective immune responses against pathogens, but must also maintain tolerance to harmless antigens. Defects in the maintenance of peripheral tolerance for commensal bacteria or food antigens can lead to disease, as demonstrated in coeliac disease and Crohn's disease. Intestinal inflammation has also been linked to several HLA-B27-linked human SpA diseases. The most convincing evidence for a link between intestinal inflammation and systemic disease comes from the HLA-B27 transgenic rat model of disease, where animals reared in germ-free conditions fail to develop the disease.

Taking into account the central role of DCs in intestinal immune homeostasis, and the mounting evidence for the role of intestinal inflammation in systemic disease, I sought to

investigate the role of intestinal DCs in the pathogenesis of B27-TG rat model of SpA. DCs continuously migrate from peripheral intestinal sites to the MLNs, and these DCs can be collected by thoracic duct cannulation. Due to lack of reliable DC markers in the rat, investigation of DCs from B27-TG animals had mostly been limited to LNs, BMDCs and splenic DCs. Using our unique surgical method, I was, for the first time, able to investigate DCs migrating from the intestines to the MLNs in this animal model. I hypothesised that there would be a defect in the migrating intestinal DCs, leading to development of systemic inflammatory disease in these animals.

In the first results chapter (Chapter 3), I describe the disease in the transgenic animals we used; this is important because the animals were backcrossed to the PVG strain to enable collection of L-DCs. The expression of HLA-B27 in the animals is also characterised in this chapter. Chapter 4 contains data describing the migrating intestinal lymph DCs from B27-TG, B7-TG and non-TG animals. These findings are extended in chapter 5 by studying similar DC populations in the animals' MLNs. In chapter 6 I describe data, obtained by studying the functions of BM-derived DCs from B27-TG and non-TG rats, and argue that the DC defect in the B27-TG rats is systemic, and may contribute to pathology in these animals. In the final chapter I describe two possible models that will try to explain the events that could lead to development of inflammatory disease in both B27-TG animals and the human SpA.

# 2. Materials and methods

## 2.1 Animals

Rats transgenic for human HLA-B27 and $\beta_2$ microglobulin genes (B27-TG) on the F344 background (F344/NTac-Tg(HLA-B2705, β2M)33-3Trg) were purchased from Taconic Inc. (Hudson, NY) and bred under licence. Subsequently, these animals were backcrossed to a PVG strain (PVG/OlaHsd; Harlan, UK), and animals used for *in vivo* experiments (Chapter 4) are from generations F3-F5, whereas animals used for *in vitro* experiments (Chapter 5-6) are F7 and up. Hemizygous B27-TG animals were crossed with non-transgenic PVG rats, and all offspring were screened for the presence of HLA-B27 gene using a FITC conjugated anti-human HLA-B27 antibody. Non-transgenic age-matched littermates were used as controls, as well as rats transgenic for human HLA-B7 gene (B7-TG), which were kindly provided by Maxime Breban (Université René Descartes, Paris, France). B7-TG animals were also backcrossed to a PVG strain as above. At the time of *in vivo* experiments B7-TG animals were F2-F3 progeny, and for *in vitro* data they were F3-F4. DA rats used for T cell proliferation experiments were purchased from Harlan, UK. All rats were used at the age of 8-24 weeks, except for mesenteric lymphadenectomy (MLNX), where rats were 5-6 weeks old.

Animals were bred and maintained under specific pathogen-free conditions at the Central Research Facility of University of Glasgow. All procedures were performed under licenses obtained from the UK Home Office.

## 2.2 Surgical procedures

These have previously been described in detail (268), but a brief description is given below.

Male rats of 5-6 weeks of age were used for MLNX. Animals were shaved and treated with subcutaneous analgesics Carprofen (Rimadyl; Pfizer) and Buprenorphine (Vetergesic; Reckitt Benckiser Healthcare); both diluted 1:10, and dose rates 0.1 ml/100 g and 0.15 ml/100 g, respectively). Anaesthesia was induced by inhalation of isoflurane (Abbot Laboratories Ltd) and a small midline incision (about 2 cm) was made in the skin and along the linea alba. Intestines were externalised through this incision, and the chain of mesenteric lymph nodes was removed, along with any isolated lymph nodes visible near

the cecum. The intestines were replaced, and the wound was closed using a continuous stitch for the muscle layers, and metallic clips for the skin. Animals were placed in a recovery cage for a few hours, after which they were moved to normal cages.

A polyurethane cannula (3fr, Harvard Apparatus, UK) was prepared in advance for the second part of the surgery. The cannula was first rinsed with acetone using a 5ml syringe and 23G needle, followed by 10-minute incubation with 5 % (v/v) 3-aminopropyltriethoxysilane. After 10 minutes, the cannula was again rinsed, first with acetone and then with PBS, and finally the cannula was filled with Heparin (Wockhardt UK) and left at 4ºC overnight.

A minimum of 5 weeks after MLNX, when the lymphatics had re-anastomosed, animals were subject to a second round of surgery. The animals underwent laparotomy under inhalation anaesthesia, and the prepared cannula was inserted into the thoracic duct. The cannula was externalised between the shoulder blades of the rats, and inserted through a metal tube attached to a Covance harness (Harvard Apparatus). Animals wore this harness for the entire duration of the lymph collection. For the first 24h after surgery, animals were placed in a Bollman restraining cage and received i.v. saline. After 24h, they were removed to a normal cage for further 24 or 48 hours of lymph collection.

## 2.3 Histology

For histochemical analysis, tissues were collected from animals under terminal anaesthesia and immediately fixed in neutral buffered formalin. Formalin-fixed tissues were processed by James Reilly and Shauna Kerr (University of Glasgow; Institute of Infection, Immunity and Inflammation) using a Shandon Citadel 1000 tissue processor (ThermoFisher) and embedded in paraffin wax using a Shandon Histocentre 3 (ThermoFisher) for subsequent sectioning using a Shandon Finesse 325 (ThermoFisher). Sections were cut at 5 μm thickness and floated on distilled water at 40°C in a Tissue Floatation Bath (ThermoFisher) to stretch and remove any wrinkles/folds from the section. Sections were then adhered to Polysine microscope slides (VWR).

Paraffin-embedded sections were incubated at 60°C on a Hotplate (ThermoFisher) for at least 35 minutes to soften the paraffin wax. They were then incubated in xylene (ThermoFisher) for 10 minutes to remove the wax, followed by 5-minute immersions in 100 %, 90 % and 70 % ethanol, and finally a 5-minute immersion in running tap water to

hydrate the tissue. Slides were then stained as follows: immersion in haemotoxylin (Sigma) for 2 minutes; immersion in running tap water until water runs clear; two short immersions in 1 % acid/alcohol (ThermoFisher); rinse in running tap water; immersion in Scotts Tap Water Substitute (CellPath, UK) for 30 seconds; rinse in cold running tap water; immersion in eosin (Sigma) for 2 minutes; rinse in running tap water; immersion in 70% ethanol for 30 seconds; immersion in 90 % ethanol for 1 minute; immersion in 100 % ethanol for 3 minutes x 2; immersion in xylene for 3 minutes first, and then left in for 30 minutes. Slides were then allowed to air-dry before glass coverslips were mounted onto the sections using DPX mountant (VWR) and air-dried for at least an hour. Images were obtained using a light microscope (Olympus BX11) with an x10 objective and were analysed and archived using cell^B software (Olympus, Southend-on-Sea, UK).

## 2.4 Cell culture reagents

Where indicated, cells were cultured in complete media consisting of: RPMI 1640 medium supplemented with 5 % FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol (all Invitrogen, UK).

Medium was supplemented with one or several of the following, as described in the text: 10 % supernatant from mFlt3L producing CHO cell line prepared in house (see section 2.5), or 100 ng/ml recombinant human Flt3L (Hatfield, UK); recombinant rat GM-CSF (100 ng /ml) or Pan Caspase OPH Inhibitor Q-VD (0.01-10 μM, as indicated; both from R&D systems), Thapsigargin (0.4 - 400 nM, Sigma) or Salubrinal (1-15 μg/ml; Calbiochem).

## 2.5 Generation of Flt3L culture supernatant

Mouse Flt3L transfected Chinese hamster ovary (CHO) cells were grown in flasks in ProCHO$_4$ media (Lonza) supplemented with 1x ProHT (Lonza), 2 mM L-glutamin, 100 U/ml penicillin and 100 μg/ml streptomycin (all Invitrogen) until confluence was achieved. Supernatant was harvested from the flasks, and filtered through a 0.2 μM filter (Millipore). The filtered supernatant was aliquoted and placed at -20°C until use.

## 2.6 Isolation of peripheral blood cells for HLA-B27 genotyping by FACS

To determine whether the rats carried the HLA-B27 gene, offspring were tested at weaning (about 4 weeks of age) by FACS. Animals were anaesthetised using inhalation anaesthetic, and their tail veins were nicked using a scalpel blade. About 200 µl of blood was collected into 1.5 ml tubes containing 5 µl of heparin (5000 U/ml), and mixed thoroughly. 100 µl of blood was transferred into 15 ml tubes, and the RBCs were lysed using ammonium chloride solution (Stem Cell Technologies) at a ratio of 1:10 v/v. Cells were washed twice using complete media or FACS buffer, and resuspended in 200 µl of FACS buffer for staining. Antibody specificities and concentrations can be found in Table 2.1.

## 2.7 Isolation of thoracic duct leukocytes

Thoracic duct leukocytes were collected from animals on ice in PBS supplemented with 10 mM EDTA and 20 U/ml heparin. They were passed through a 40 µm cell strainer (BD Biosciences), and RBCs were lysed with Red Blood Cell Lysing Buffer Hybri-Max (Sigma) for 3 minutes at room temperature and washed with complete media. Cells were either directly stained and analysed by flow cytometry, or enriched for L-DCs using anti-CD103 MACS beads (Miltenyi Biotec) (see section 2.9). On occasion, cells were stained and further purified by FACS sorting using BD Aria cell sorter.

## 2.8 Isolation of MLN and spleen cells

MLNs and spleens were collected from rats in serum free RPMI, cut into small pieces and digested using DNase (50 µg/ml) and liberase (0.4 Wunsch units/ml; both Roche) for 45 min in a shaking incubator set at 37°C. After this, cells were passed through a 40 µm cell strainer, washed and stained with antibodies and analysed by flow cytometry. On occasion, MLN cells were first enriched for CD103 using magnetic beads, and then further stained.

## 2.9 Enrichment of cell populations using magnetic beads

Dendritic cells were enriched from thoracic duct lymph prior to FACS analysis by using magnetic beads specific for CD103 (Miltenyi Biotec) according to manufacturer's protocol, with minor modifications. Briefly, single cells suspensions were spun down at 380 g x 5 min, supernatant was aspirated and the cell pellet was resuspended in 80 µl of

buffer per $1x10^7$ total cells. Due to the fragile nature of the DC subsets being enriched, complete media supplemented with 2 mM EDTA was used as buffer throughout the process. 10 µl of MicroBeads per $1x10^7$ total cells was then added, mixed thoroughly and incubated at 4°C for 15 minutes. Cells were washed with excess buffer, spun down 380 g x 5 min and resuspended in buffer 500 µl /$10^8$ cells. Depending on the number of cells, either MS or LS columns (Miltenyi Biotec) were prepared by placing them on the magnetic MACS separator and washing them with the buffer using amounts indicated in the protocol. Cells were then applied onto the column, and it was washed three times with buffer, using volumes appropriate for the column size. After the unlabelled cells were washed away from the column, it was removed from the magnet and placed on top of a suitable collection tube. The appropriate amount of buffer was added on top of the column, and a plunger was used to flush out the magnetically labelled cells.

## 2.10 Labelling of antibodies

Monoclonal antibodies specific for rat CD103 and CD172a were conjugated to Alexa Fluor 488 and Alexa Fluor 647, respectively, using protein-labelling kits from Invitrogen, UK according to manufacturer's instructions. Briefly, 50 µl of 1 M sodium bicarbonate was added to 0.5 ml of 2 mg/ml protein solution to raise the pH of the solution for optimum labelling conditions. This solution was transferred to a vial of reactive dye, and stirred at room temperature for 1h. Meanwhile, the purification column was assembled and prepared by pipetting the purification resin into the column. Reaction mixture was carefully added on top of the resin, and allowed to enter the column. Elution buffer was carefully added to the top, and the reaction mixture separated into two separate coloured bands as it moved through the column. The faster moving band contains the labelled antibody, and it was collected from the bottom of the column. The second, slower moving band contains the unincorporated dye, which was discarded.

## 2.11 Flow cytometry

Cell surface staining was performed in PBS supplemented with 2 mM EDTA and 2 % FBS (FACS buffer) for 30 minutes on ice. Where a biotin-conjugated antibody was used, cells were washed with FACS buffer, and further stained with a streptavidin-fluorochrome conjugate for 15 min on ice, and washed again before analysis.

To determine proportions of live and dead cells, a combination of Annexin-V and DAPI (4',6-diamidino-2-phenylindole) was used in addition to surface staining. Cells were stained with cell surface antibodies as above, washed with PBS and resuspended in an appropriate volume of 1x Annexin-V binding buffer (BD). 4 µl of Annexin-V antibody was added to each stain, and left for 15 minutes at room temperature. 50 ng/ml DAPI was added to the samples for 2-4 minutes before acquisition.

For full list of monoclonal antibodies used see Table 2.1. Antibodies marked as 'In house' were provided by Neil Barclay, Cellular Immunology Unit, University of Oxford and have been conjugated to their fluorochromes or biotin in the laboratory either by others or by me. Lineage markers consist of: OX-12, OX-22, OX-33 and TCR α/β. All samples were acquired using either MACS Quant analyser (Miltenyi Biotec) or BD LSRII, or sorted and analysed by BD FACS Aria. All data analysis was carried out using FlowJo software (Tree Star, versions 8.8.6 and 9.3.1).

## 2.12 Cell sorting

Cells were prepared for sorting as above. When sorting fragile DC subsets, cells were kept at all times in complete media with 2 mM EDTA added, on ice. Cells were kept in complete media for the entire duration of the sort, and they were also sorted into media. Other cell types were sorted in FACS buffer.

## 2.13 Cytospins

To assess the morphological features of the three different L-DC populations in lymph, cytospins were performed. Migrating DCs from lymph were enriched for CD103 by using magnetic beads, and the MHCII$^{+}$ CD103$^{+}$ DCs were sorted into three subsets: CD11b$^{lo}$ CD172a$^{hi}$, CD11b$^{hi}$ CD172a$^{int}$ and CD11b$^{lo}$ CD172a$^{lo}$. Cells were then spun down and resuspended in 200 µl of complete medium. Slides were prepared with a filter card and a cuvette in a metal holder, and the 200 µl of cell suspension was loaded into the cuvette. Cells were spun for 6 minutes at 450 rpm to transfer them onto microscopic slides, allowed to air dry, and fixed in acetone for 10 minutes at -20°C. Slides were stained with Rapid Romanowsky (RA Lamb) observed under light microscope and analysed and archived using cell^B software (Olympus, UK).

**Table 2.1 List of monoclonal antibodies**

| Specificity | Clone | Fluorochrome/Bio | Supplier | Dilution (v/v) |
|---|---|---|---|---|
| **CD3** | G4.18 | PE | BD | 1:400 |
| **CD4** | OX-38 | FITC | BD | 1:400 |
| | OX-35 | PE/APC/Pe-Cy5 | BD | 1:200 |
| | | Biotin | In house * | 1:200 |
| | W3/25 | Biotin | In house * | 1:200 |
| **CD5** | OX-19 | Biotin/FITC | In house * | 1:200 |
| **CD8a** | OX-8 | PerCP | BD/Biolegend | 1:400 |
| **CD8b** | 341 | FITC | BD | 1:400 |
| **CD11b** | OX-42 | PE | BD | 1:400 |
| **CD25** | OX-39 | PE | BD | 1:400 |
| **CD45R** | HIS24 | FITC/PE | BD | 1:200 |
| **CD45RA** | OX-33 | Biotin | Biolegend | 1:200 |
| **CD45RC** | OX-22 | FITC | BD | 1:200 |
| | | Biotin | Biolegend | 1:200 |
| **CD80** | B7-1 | PE | BD | 1:400 |
| **CD86** | B7-2 | PE | AbD Serotec | 1:50 |
| **CD103** | OX-62 | A488 | In house * | 1:400 |
| **CD172a** | OX-41 | A647 | In house * | 1:400 |
| **Annexin-V** | | PE | BD | 4ul/stain |
| **OX-40L** | ATM-2 | PE | Biolegend | 1:200 |
| **HLA-B27** | HLA-ABC-m3 | FITC | AbD Serotec | 1:200 |
| **HLA-B7** | BB7.1 | FITC | AbD Serotec | 1:200 |
| **MHC class II** | OX-6 | PerCP | BD | 1:400/600 |
| **TCR α/β** | R73 | FITC/ Biotin | In house * | 1:200 |
| **Igκ light chain** | OX-12 | Biotin | In house * | 1:200 |
| **CD68** | ED-1 | Biotin | In house * | 1:200 |
| **DAPI** | | | Molecular Probes | 1:10,000 |
| **Streptavidin** | | FITC/ APC/ APC-Cy7/ V450 | BD | 1:200-400 |
| **Ms $IgG_1$, κ**† | MOPC-21 | PE | BD | 1:400 |

* See section 2.10 for details

† Isotype control for CD25 and CD80

## 2.14 Generation of bone marrow derived DCs (BMDCs)

Hind legs of rats were removed and placed in complete media until processed. Bones were separated from tissue and snapped using bone snips below the hip joint and above the knee, for femur, and below the knee and above the ankle for tibia. Bone marrow was flushed out from femurs and tibias using a 20 ml syringe and a 23G needle. Cells were passed through a 40 μm filter to make a single cell suspension, spun down, followed by a red blood cell lysis. After washing, cells were counted and resuspended at a concentration of $2x10^6$ cells/ml. These were plated on flat-bottom 6-well plates with 5 ml/well, and cultured for 7 days in the presence of either 10 % mouse Flt3L supernatant, or with 100 ng/ml recombinant human Flt3L. On occasion, cell cultures were also supplemented with other reagents either at D0 or at another time point, depending on the experiment. After 7 days, cells were harvested from the plates by removing the semi-adherent layer containing dendritic cells with a pipette, and the wells were washed with complete media.

## 2.15 Activation of BMDC in vitro

For some experiments BMDC were activated with 100 ng/ml *Salmonella enterica, serovar: typhimurium* LPS (Invivogen) by adding it to the BMDC cultures overnight. Cells were then collected and analysed as before, and compared to cells grown without LPS.

## 2.16 In vitro T cell proliferation assay

To assess the ability of DCs to stimulate proliferative responses from $CD4^+$ T cells, mixed lymphocyte reactions (MLR) were performed, as previously described (184). Briefly, L-DCs were enriched for CD103 with magnetic beads, and MHC $II^+$ $CD103^+$ lymph DCs were sorted from PVG ($RT1^c$) B27-TG animals or from non-TG littermates. For BMDCs, live (Annexin-$V^-$ $DAPI^-$) $CD103^+$ DCs were flow sorted on day 7 of BMDC cultures. Both L-DCs and BMDCs were cultured, at different densities in triplicate, with $1x10^5$ T cells from DA ($RT1^{av1}$) rats: depending on the experiment, these came from either a digest of MLN cells without an enrichment for T cells; or from flow sorted naïve-phenotype ($CD45RC^{hi}$ $CD25^-$) $CD4^+$ T cells from MLN. Cells were plated out on 96 well plates for five days at 37°C and 5 % $CO_2$ in complete medium.

Two different methods were used to assess T cell proliferation. In the first one, 1 μCi/well tritiated thymidine ($^3$H-TdR) (West of Scotland Radionucleotide Dispensary) was added to the wells for the final twelve hours of culture, and cellular DNA was harvested onto filter

mats (Wallac, UK) before addition of scintillation fluid and analysis with Betaplate scintillation counter (Wallac) using the Microbeta programme.

In the second method T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) before plating: T cells were resuspended in 1 ml of complete media at $2x10^7$ cells/ml, and 5 μM CFSE was added and the cells were incubated at room temperature with gentle shaking for 3 minutes. Cells were then washed with complete media, counted again, and finally plated out as indicated above. After 5 days, proliferating T cells were identified by flow cytometry by CD4 and CFSE staining.

## 2.17 Luminex

Supernatants from BMDC and L-DC MLRs were collected on day 5, and frozen at -20ºC until use. IFN-γ, IL-17F, IL-6, IL-10, IL-2 and TNF-α levels were measured from the supernatants by Luminex assay (Biolegend), according to manufacturers instructions. Briefly, 25 μl of neat MLR supernatant was added into wells of a 96-well filter plate containing 25 μl of assay buffer. 25 μl of premixed beads were added into each well, and the plate was incubated with agitation overnight on a plate shaker at 4ºC. Following day, the plate was washed twice with wash buffer, and 25 μl of detection antibody was added to each well, and the plate was incubate with agitation on a plate shaker for 2 hours at room temperature. After 2h, 25μl of Streptavidin-Phycoerythrin was added into each well and incubated with agitation on a plate shaker for 30 minutes at room temperature. The plate was then washed twice, and 150 μl of sheath fluid was added into each well, and the plate was read with Bio-Rad Bio-Plex 200 system Luminex plate reader.

## 2.18 Isolation of RNA from single cell suspensions

Cells were isolated from tissues and washed with PBS. Samples were spun down at 400 g, supernatant was removed completely and each sample was resuspended in 350-600 μl of denaturing RLT Lysis Buffer (Qiagen), and either stored at -80°C or processed immediately. RNA was isolated following the Qiagen RNeasy Mini Kit instruction manual. This included the optional on-column DNase digestion step using the Qiagen RNase-Free DNase Set. Briefly, samples with added RLT buffer were homogenised using the QIAshredder spin columns, and 1 volume of ethanol was added to the samples to ensure efficient binding of RNA to the spin column membrane. Up to 700 μl of sample was placed on the spin column containing a silica-based column that binds the RNA.

Tubes were spun 15 s at 10,000 rpm, washed once with buffer RW1 and 80 µl of DNase I incubation mix was added directly to the spin membrane. This was incubated for 15 min at room temperature and the spin column membrane was washed additional two times with buffer RW1, and twice with buffer RPE. Spin column was then placed to a fresh collection tube and 40 µl of RNase free water was added on the membrane, columns were spun down and the RNA concentration of each sample was determined using a ThermoFisher Nanodrop 1000' spectrophotometer. Samples were either processed immediately for cDNA synthesis, or stored at -20°C until use.

## 2.19 Synthesis of cDNA from RNA

Complementary DNA (cDNA) was reverse-transcribed from RNA using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, UK) according to manufacturer's instructions. Briefly, equal amounts (50-100 ng, depending on experiment) of starting RNA for each sample were made up to 10 µl with diethylpyrocarbonate (DEPC) treated water and 1 µl of oligo(dT) primer (0.5 µg/ul) and 1 µl of dNTP mix (10 mM). Samples were then incubated at 65°C for 5 minutes to remove any secondary structures in the RNA and then placed on ice for 5 minutes to allow primers to anneal to the RNA. To each sample, a 9 µl mix of the following components was added: 2 µl of 10X RT Buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT and 1 µl of RNaseOUT™ Recombinant RNase Inhibitor. Samples were incubated at 42°C for 2 minutes to initiate the reaction, after which 1 µl (50 units) of SuperScript™ II Reverse Transcriptase was added, except to the no RT control samples, to which 1 µl nuclease free water was added. Samples were then incubated at 42°C for 50 minutes to extend the products and 15 minutes at 70°C to terminate the reaction. Reactions were stored at either 4°C or -20°C until they were used for QPCR.

## 2.20 Flt3 primer design

Primers for Flt3 receptor were designed by using primer-BLAST (Primer3 and BLAST) program to find primers for *Rattus norvegicus* fms-related tyrosine kinase 3 (Flt3) mRNA sequence (Accession: NM_001100822). Parameters for the search were set as:

- PCR product size: 90-200 bp
- Primer $T_m$: 57-62°C, with 60°C being optimum and maximum $T_m$ difference between primers 3°C

- Primer length between 18-23 bp
- Primer GC content 40-65%
- Max self complementarity: 2
- Max 3' end complementarity: 1

Once primers were suggested by the program, the following points were taken into account when selecting the primers: No more than three G or C bases in last 5 at 3' end of each primer, and stretches of 4 or more G or C bases were avoided. Using these parameters, 6 primer pairs were selected and ordered. These primers were tested using BMDC from non-TG rats and the SYBR green protocol (see section 2.21), with melt curves run at the end to determine the quality of the primers. All but two of the primers tested functioned well, and finally Flt3 1 primer pair was selected for use in experiments (Table 2.2).

## 2.21 Measuring relative mRNA expression by quantitative polymerase chain reaction (QPCR)

Synthesised cDNA was used to determine the expression of Flt3 receptor on BMDCs at the level of mRNA. TATA box binding protein (TBP) was used as a housekeeping gene. All information about primers is shown on Table 2.2.

Agilent Brilliant SYBR® Green QPCR Master Mix was used for the QPCR reaction. Briefly, equal amounts of 1:5 diluted cDNA were added to a 96-well MicroAmp® Fast Optical Reaction Plate (Applied Biosystems) in triplicate per biological sample, and the final volume per reaction was adjusted to 25 µl by adding a mixture of the following components: Nuclease free water, 12.5 µl of 2x Master Mix, forward and reverse primers at final concentration of 100 nM, and 0.375 µl of 1:50 diluted reference dye (300 nM final concentration). Plates were covered with an optical adhesive cover (Applied Biosystems) and were spun at 450 g for 1 min.

Plates were loaded into a 7500 Fast Real-Time PCR System machine (Applied Biosystems) and incubated at 95°C for 10 minutes followed by 40 cycles of incubation at 95°C for 15 seconds, then 60°C for 1 minute. A melt curve was run at the end of each experiment to determine if the PCR reaction was successful.

**Table 2.2 Primer sequences and other details**

| Gene | Primer (5'-3') | $T_m$ (°C) | GC content (%) | Amplicon length (bp) |
|---|---|---|---|---|
| Flt3 1 | Forward: GGCTGCCGCTGCTTGTTGTTT | 59.33 | 57.14 | 143 |
| | Reverse: TGGAACCATGACGTGCGACGA | 58.8 | 57.14 | |
| TBP | Forward: TCCTGCCACACCAGCCTCTGA | 59.49 | 61.90 | 150 |
| | Reverse: ACTGCAGCAAACCGCTTGGGA | 59.51 | 57.14 | |

## 2.22 Statistical analysis

Data were analysed using GraphPad Prism software (San Diego, CA) by using either a Student's unpaired two-tailed t test, one-way Anova or two-way Anova with Bonferroni post tests, as described on figure legends. Probability values of $p<0.05$ were considered statistically significant.

# 3. HLA-B27 transgenic rats

## 3.1 Introduction

Rats transgenic for human HLA-B27 and human β2m have been used for research since their development in the 1990's (223) and, as discussed in detail in the introduction, they provide a good model for the human SpA diseases. Mostly these rats have been used in their original F344 background, but in some cases they have been backcrossed into LEW or PVG strains. The B27-TG strain 33-3, which is used in this study, has been successfully backcrossed into both LEW and PVG, with few reported changes in the disease course. For the purpose of studies conducted in this thesis, rats of the 33-3 strain on the F344 background were purchased, and subsequently backcrossed onto PVG. This was necessary in order to be able to carry out the surgical procedures used to collect lymph DCs by thoracic duct cannulation (see introduction and Ch4). In rats of the F344 and LEW strain, the thoracic duct is anatomically far less accessible than it is on the PVG animals, considerably lowering the success rate of the surgery. In addition, the thoracic duct of F344 and LEW animals is crisscrossed with numerous blood vessels that would inadvertently be damaged during the surgery leading to contamination of the collected lymph with blood, compromising the analysis. But as B27-TG PVG animals are not as commonly used, it was necessary to assess the expression of HLA-B27 molecule across tissues used in these studies, as well as to observe the disease symptoms in the animals. Rats transgenic for a non-disease-associated human HLA-B7 molecule were likewise backcrossed from F344 to PVG in order to maintain the usefulness of these animals as controls. However, as these rats do not develop disease, no detailed description of these animals on PVG background is provided.

## 3.2 Results

For the backcrossing, most often B27-TG females were bred with WT PVG males, as male B27-TG rats have been reported to develop epidymitis and orchitis by 3 months of age and thus become sterile. However, we found that it was possible to use the young B27-TG males for breeding, although they did stop reproducing as they aged. The presence of epidymitis and/or orchitis on our male B27-TG rats was not verified in these studies. The infertility of B27-TG males is also the reason why even the commercially available B27-TG F344 animals are bred as hemizygotes. The HLA-B7 animals are bred as homozygotes,

but as they were backcrossed onto PVG, this generated hemizygous animals. Thus, all the offspring from both B7-TG and B27-TG animals need to be screened for the presence of the corresponding HLA transgene. This was done by sampling blood from the tail vein of about 4-week old, weaned animals under anaesthesia, and staining the peripheral blood mononuclear cells (PBMCs) with anti-HLA-B27 or anti-HLA-B7 antibody. The presence or absence of the transgene was verified by FACS. As there were going to be many animals to screen, the HLA-B27 antibody was first titrated to see if it could be used at a lower concentration than the recommended 1:25. It was noted that it was still possible to reliably identify the transgenic animals even when the antibody was used at 1:100 or 1:200, and thus for the rest of the experiments and screening one of these concentrations was used (Figure 3.1A). When 4-week old B27-TG rats were screened for the presence of HLA-B27, it was observed that about 50 % of all PBMCs were positive for the transgene, whereas non-TG animals only showed background level of staining (Figure 3.1B). About half of the offspring from all the litters are HLA-B27$^+$, as would be expected. Curiously, when blood was taken from 3-week old B27-TG rats, only about 10 % of PBMCs showed surface expression of HLA-B27 (Figure 3.2A). However, when these same rats were screened again at 5 weeks, they showed increased surface expression of HLA-B27, similar to that observed at 4 weeks (data not shown). Screening of 8-week old rats revealed that about 62 % PBMCs were HLA-B27$^+$ (Figure 3.2B), whereas at 21 weeks over 90 % of PBMCs had this phenotype (Figure 3.2C). Figure 3.2D shows staining of PBMCs from B27-TG animals using the HLA-B7 antibody. There is no specific staining at all, proving that this antibody does not cross react with HLA-B27.

As with B27-TG rats, the offspring of B7-TG rats were also screened for the presence of the HLA transgene by FACS. When peripheral blood from the original homozygous B7-TG animals, aged 12 weeks, was stained with the HLA-B7 antibody, as expected a vast majority of PBMCs were positive for the HLA-B7 transgene (Figure 3.3A). When 4-week-old B7-TG animals were screened, as with B27-TG animals, about 50 % of PBMCs were positive for HLA-B7 (Figure 3.3B), and there was only a very slight increase in the percentage of HLA-B7$^+$ cells in 8-week old animals (Figure 3.3C). It was also observed that the HLA-B27 antibody cross-reacts very strongly with HLA-B7, as the staining of PBMCs from B7-TG animals with HLA-B27 antibody resulted in almost identical staining pattern than staining with the HLA-B7 antibody (Figure 3.3D, E).

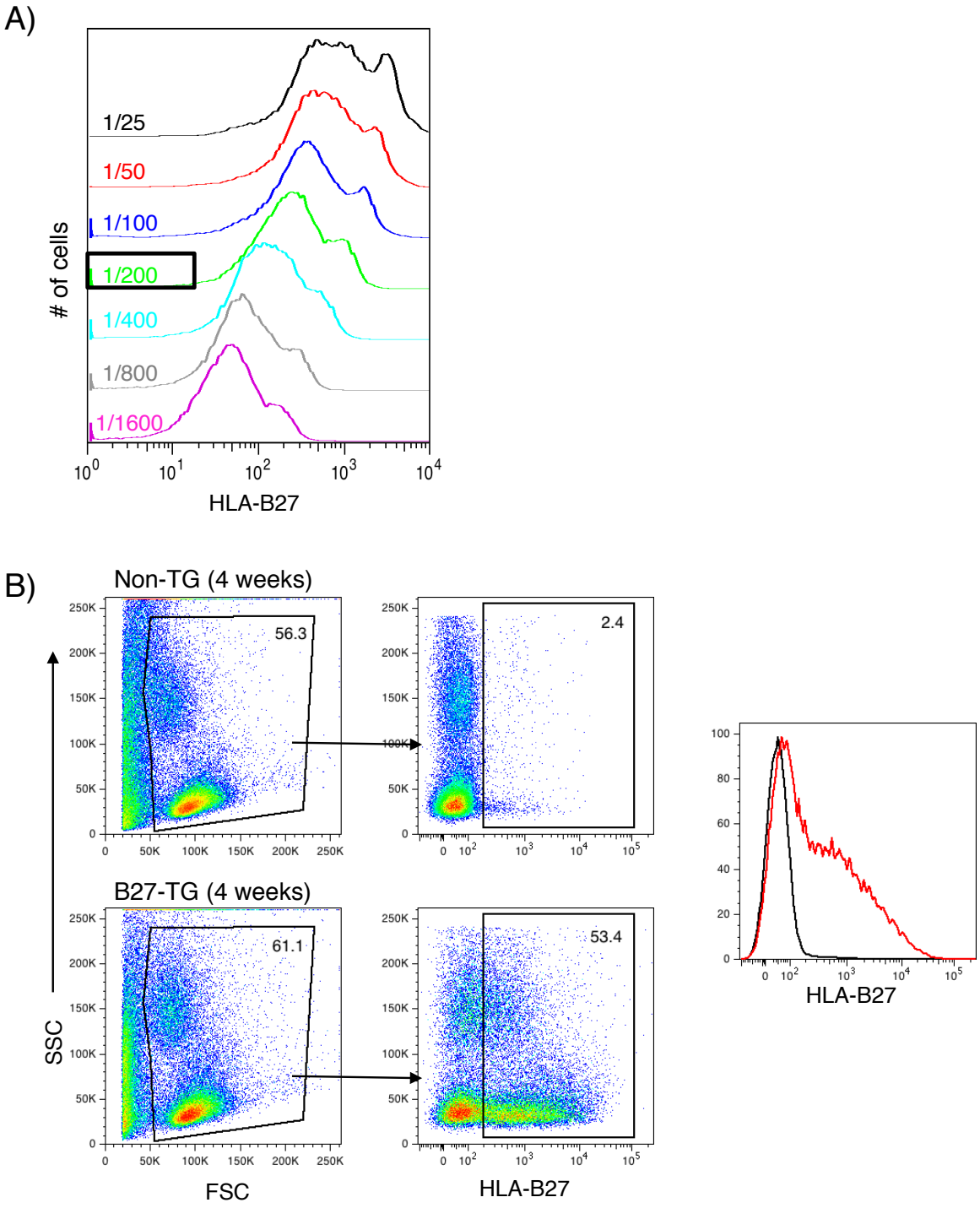


**Figure 3.1 Screening of hemizygous HLA-B27 animals**

(A) The HLA-B27 antibody was titrated to find the ideal concentration, using peripheral blood from 8-week old B27-TG animals. (B) Representative results showing screening results from peripheral blood of 4-week old hemizygous littermates demonstrating levels of binding of HLA-B27-specific antibody for B27-TG and non-TG animals.

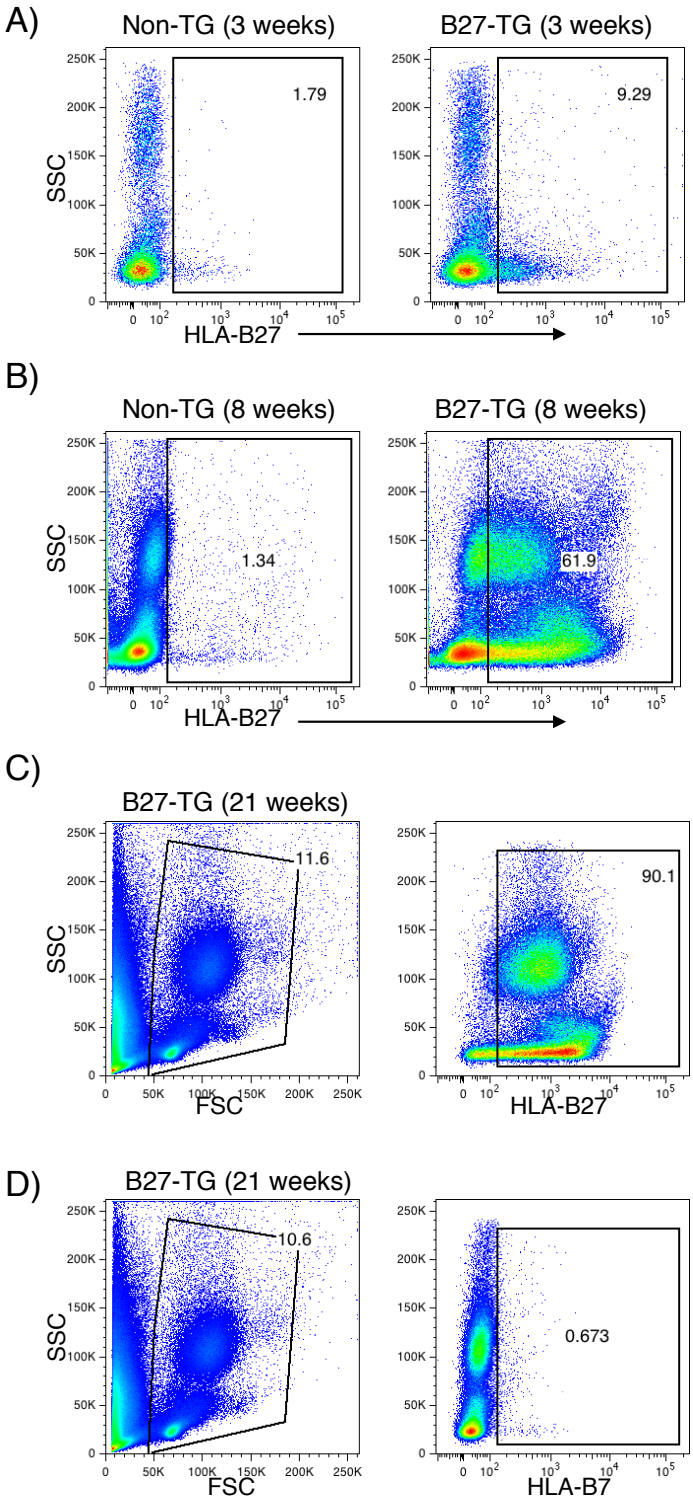


**Figure 3.2 HLA-B27 expression by B27-TG peripheral blood cells**

Peripheral blood was collected from hemizygous non-TG and B27-TG animals, RBCs were lysed and remaining cells were stained with HLA-B27 antibody and cell surface expression was assessed by FACS. Animals at ages of (A) 3 weeks, (B) 8 weeks and (C) 21 weeks were used. (D) Peripheral blood cells from 21-week old B27-TG animals were stained with HLA-B7 antibody.

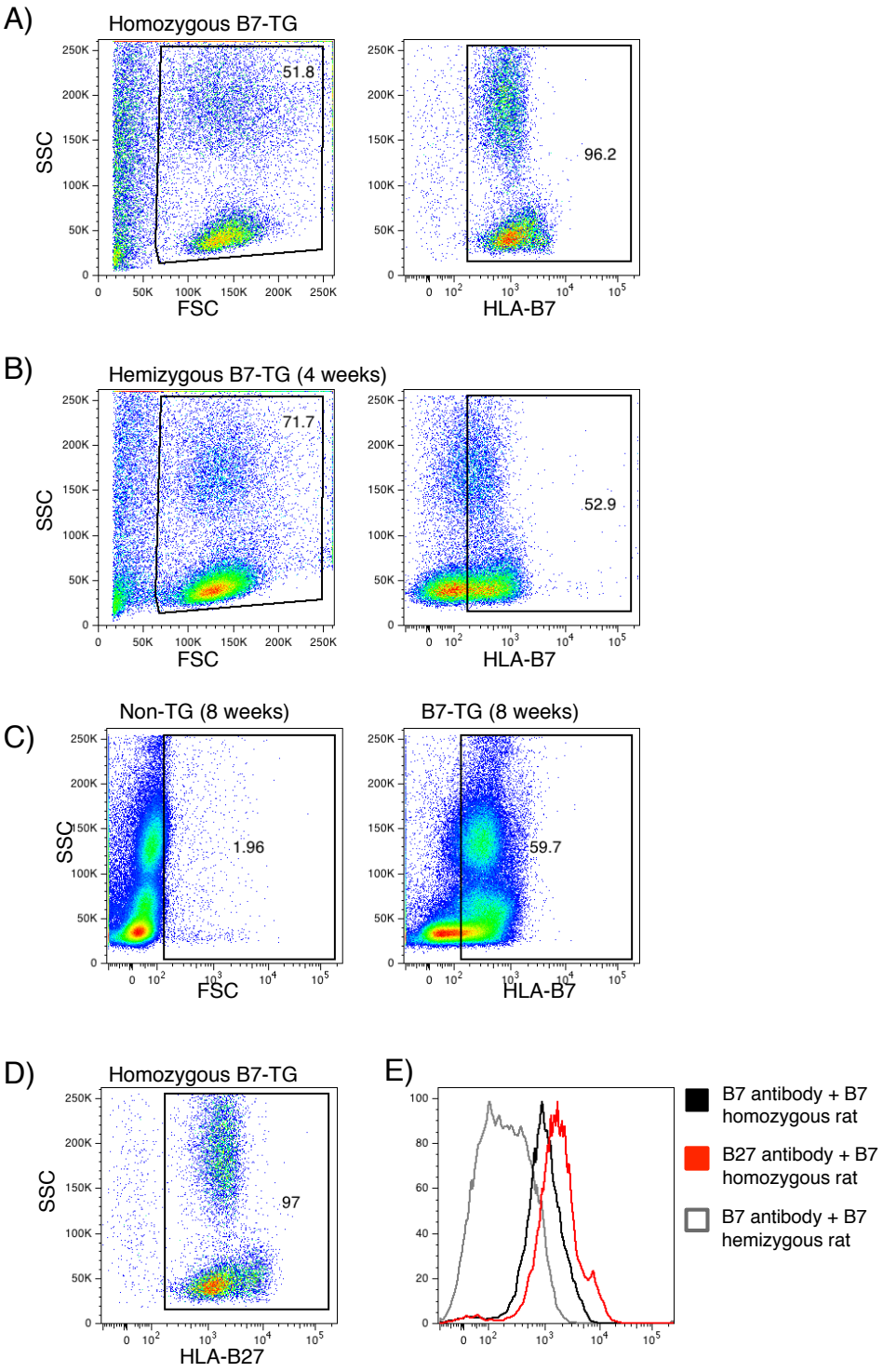


**Figure 3.3 Expression of HLA-B7 by B7-TG peripheral blood cells**

Peripheral blood was collected from B7-TG animals, RBCs were lysed and cells were stained with HLA-B7 antibody. Blood cells were collected from: (A) homozygous B7-TG animals on F344 background at age of 14-weeks, (B) 4-week old hemizygous B7-TG rats backcrossed into PVG for 4 generations, (C) 8-week old non-TG and hemizygous B7-TG animals on PVG background. (D) Blood cells from homozygous 14-week old B7-TG animals were stained with HLA-B27 antibody, and in (E) histogram overlay of results from B7-TG results from A, B and D are shown.

It was also decided to investigate whether cells in the various tissues that will be used in the following chapters express HLA-B27. Cells collected by cannulation from non-TG and B27-TG animals were stained with the HLA-B27 antibody, and it was observed that almost 98 % of lymph cells were HLA-B27$^+$ compared to only background level of staining in the non-TG animals (Figure 3.4). Unfortunately no other stains were included together with HLA-B27, so it was not possible to ascertain if all the DCs were HLA-B27$^+$. However, as will be seen in the next figure, all MLN MHCII$^+$ cells are very strongly positive for HLA-B27 on 6-week old animals, and thus all lymph DCs are also likely to be HLA-B27$^+$. MLNs were collected from 6-week old animals and digested with DNase and liberase, and the cells were stained with antibodies specific for HLA-B27, MHCII and lineage markers. HLA-B27 expression on all MLN cells was first investigated, and it was noted that over 70 % of all MLN cells were positive for HLA-B27 (Figure 3.5A). DCs in the rat MLNs can be difficult to identify, as will be discussed in more detail in Chapter 5, but they can be recognised as LIN$^{-/lo}$ and MHCII$^+$ cells (Figure 3.5B). When expression of HLA-B27 on these cells was investigated, it was noted that over 97 % of LIN$^{-/lo}$ MHCII$^+$ cells were also HLA-B27$^+$ (Figure 3.5B), indicating that all DCs in the MLN are also HLA-B27$^+$. In fact, the cells expressing highest level of MHCII also expressed highest level of HLA-B27. When expression of HLA-B27 was evaluated on total MLN cells from 21-week old animals, it was noted that at this time most of the MLN cells were HLA-B27$^+$ (Figure 3.5C), compared to 70 % in animals of 6 weeks of age. The FCS/SSC characteristics of the cells that appeared not to express HLA-B27 suggested that they are lymphocytes, or other small cells (Figure 3.5C).

As discussed in the introduction, the B27-TG animals present with variety of symptoms, such as psoriasiform skin and nail lesions, nail hyperplasia and intestinal inflammation. Observation of B27-TG animals, backcrossed into PVG, revealed that the animals on this background presented with similar symptoms as has been reported for the original F344 strain. Animals experienced hair loss, starting from the periorbital area and spreading towards the ears, then down towards the forepaws (Figure 3.6A). These symptoms did not appear until 12-16 weeks of age, and did not occur in all animals. Extensive hair loss shown in 3.6B was observed on a 24-week old rat, and hair loss at this scale was hardly ever observed on animals under 16 weeks of age. Another prominent feature of B27-TG animals are nail deformations. Figure 3.6C features a commonly observed nail dystrophy on a 24-week old B27-TG animal. None of these symptoms were observed in either non-TG or B7-TG animals at any age.

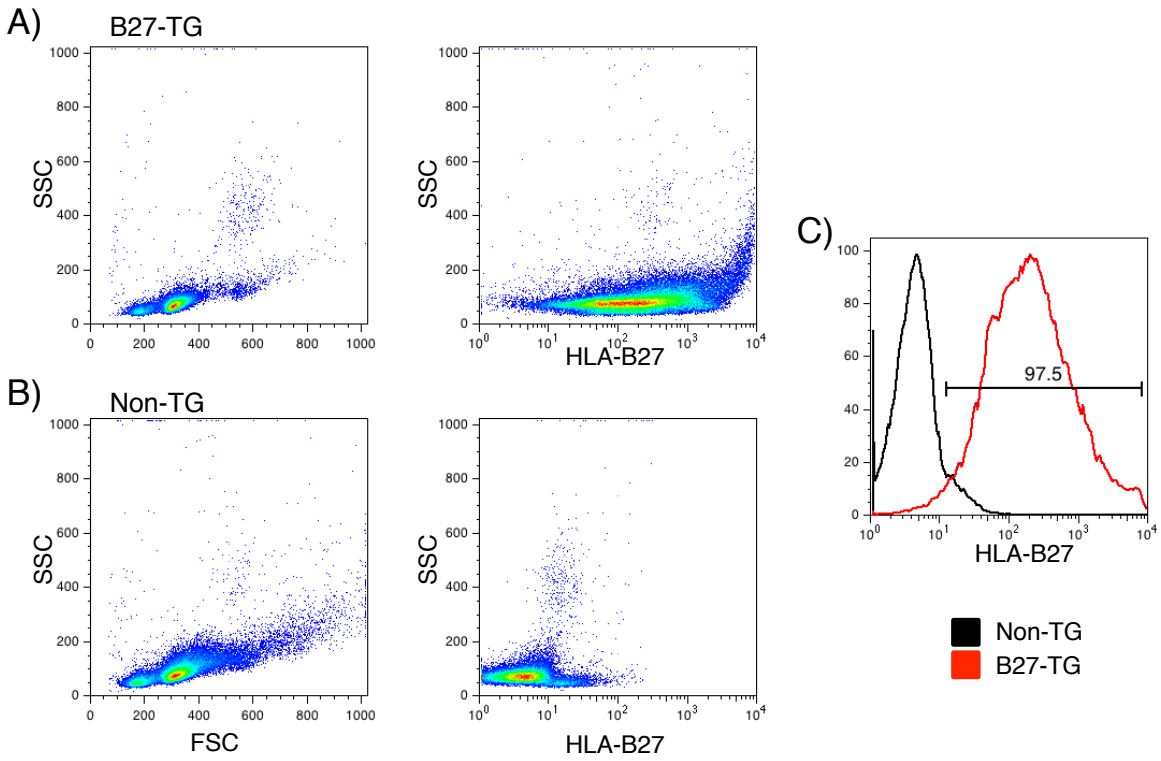


**Figure 3.4 Expression of HLA-B27 on thoracic duct lymph cells from B27-TG and non-TG animals**

Cells from thoracic duct lymph were collected from cannulated non-TG and B27-TG animals, RBC were lysed and remaining cells were stained with HLA-B27 antibody. Expression of HLA-B27 on total lymph cells from (A) B27-TG and (B) non-TG animals was analysed by FACS. (C) shows histogram overlays comparing HLA-B27 expression on non-TG and B27-TG lymph cells

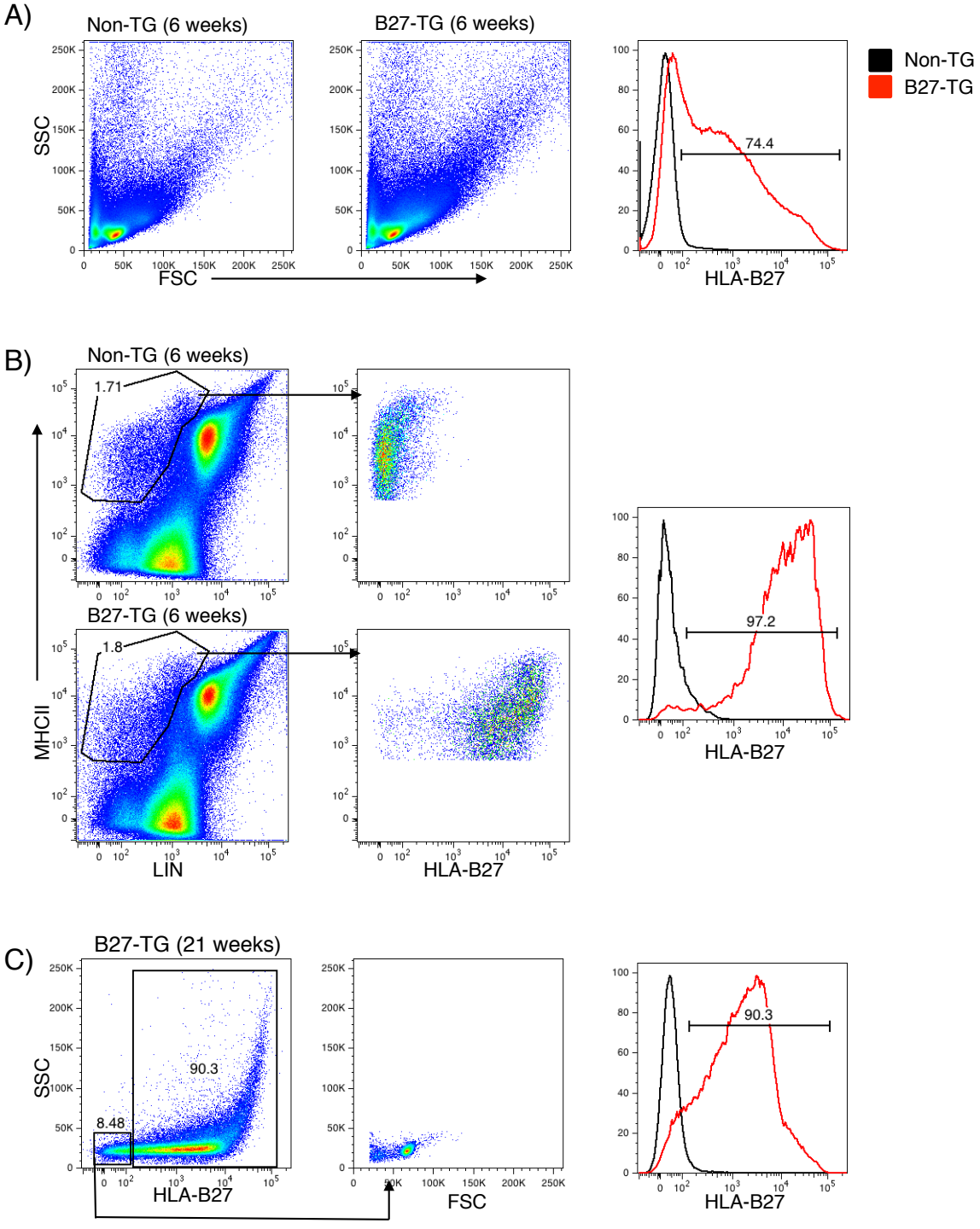


**Figure 3.5 Expression of HLA-B27 on B27-TG MLN cells**

MLNs were collected from B27-TG animals of different ages, and digested with DNase and liberase. (A) Total MLN cells from 6-week old B27-TG animals were stained with HLA-B27 antibody and surface expression was assessed by FACS. (B) Expression of HLA-B27 on non-TG and B27-TG MLN DCs from 6-week old animals was assessed by staining the cells with LIN markers (CD45RC, CD45RA, TCRαβ, Igκ light chain), MHCII and HLA-B27. (C) Total MLN cells from 21-week old B27-TG animals were stained with ant-HLA-B27 antibody and assessed for surface expression. FSC/SSC characteristics of HLA-B27$^-$ cells were also analysed, as shown in the middle panel.

Inflammation of the small and large intestine is another reported feature of the B27-TG animals. Thus, it was investigated whether our animals would present with these symptoms as well. Small intestinal inflammation was evaluated on animals of 9 (F10 generation), 15 (F5 generation) and 52 (F3 generation) weeks of age. When looking at the ileum (Figure 3.7A-C), it was noted that 9-week old rats displayed a slightly changed architecture, as the villi appeared to be flatter, and the LP seemed more cellular when compared to non-TG animal. At 15-weeks the ileum has lost its normal architecture; villi were fused and flattened, and cellular infiltrates were visible in the LP. No visible changes are seen in the jejunum of 9-week old animals (Figure 3.7D-F), however again at 15 weeks increased cellularity and shorter and thicker villi were observed. No inflammatory changes were observed in the duodenum of either 9-week or 15-week old B27-TG animals (Figure 3.7G-I). Small intestinal sections from jejunum of 52-week old F3 generation rats revealed signs of slight inflammation with flattened, fused villi and some inflammatory cell infiltrates present in the LP (Figure 3.8). Unfortunately, no sections are available from other parts of the small or large intestine from these animals. Histology of ascending colon from 9-week old animals showed no signs of inflammation with normal colonic architecture (Figure 3.9B). In contrast, histology of ascending colon from 15-week old animals show signs of crypt hyperplasia and presence of inflammatory cell infiltrate throughout the lamina propria (Figure 3.9D).

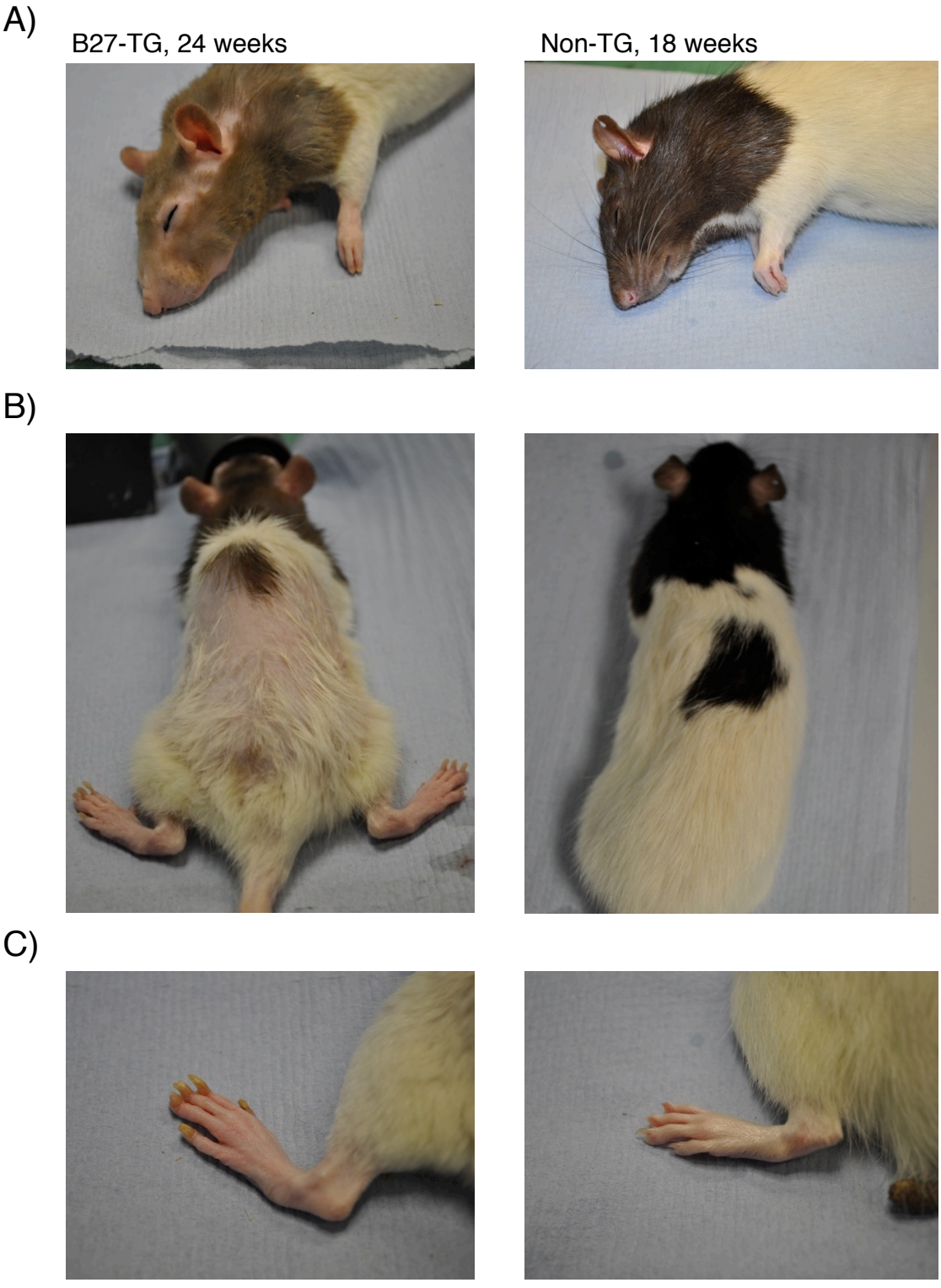


**Figure 3.6 peripheral disease symptoms on B27-TG animals**

(A) Typical pattern of hair loss on a 24-week old B27-TG animal; starting from the periorbital area in the head and spreading down to forepaws. (B) Alopecia and thinning of hair on the back of a B27-TG animal. (C) Nail dystrophy on the hindpaw of B27-TG animals. Animals used here are from F7 generation of backcrossing.

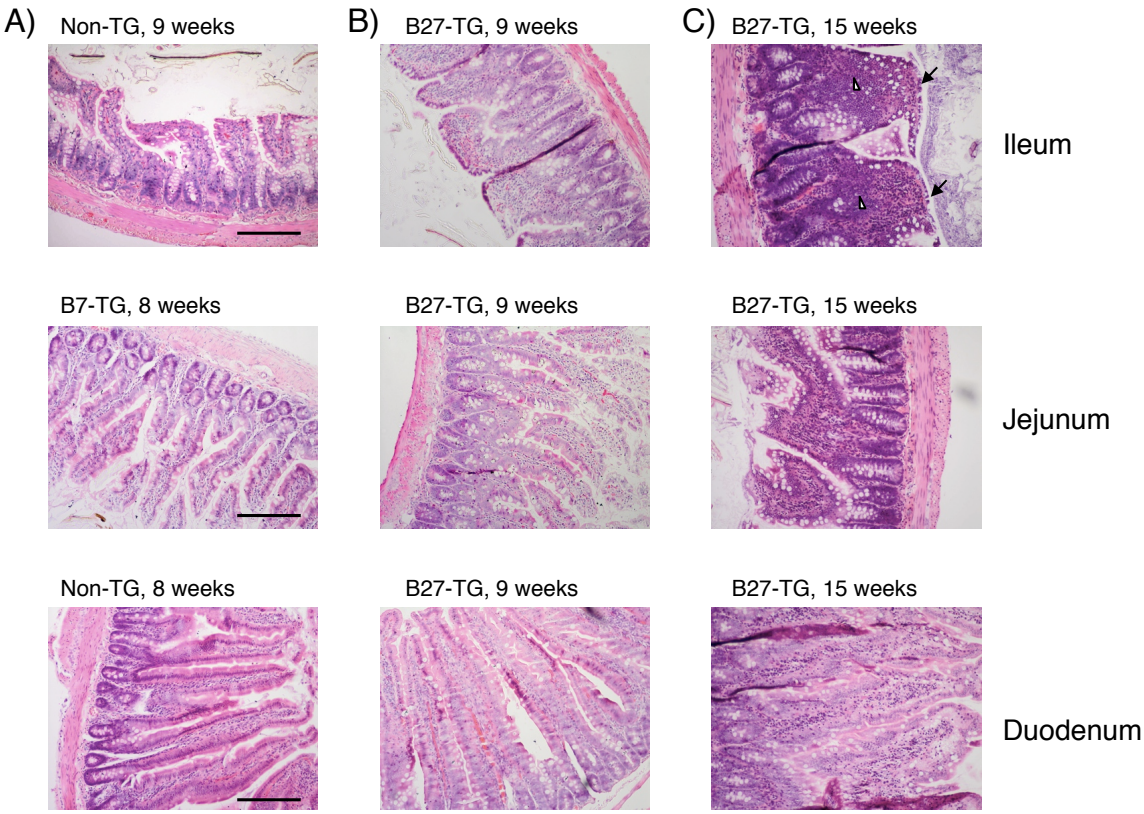


**Figure 3.7 Histology of small intestinal sections**

Paraffin embedded sections from ileum, jejunum and duodenum of non-TG, B7-TG and B27-TG animals were stained with H&E. (A) Sections from 8-9 week old non-TG and B7-TG animals showed no signs of intestinal inflammation, (B) and neither did sections from 9-week old B27-TG animals. (C) Sections of ileum and jejunum from 15-week old animals showed signs of abnormal villi (arrows) and inflammatory cell infiltrates (arrowheads). No inflammation was observed in the duodenum. Magnification x10 and scale bars 50 µm, n=1.

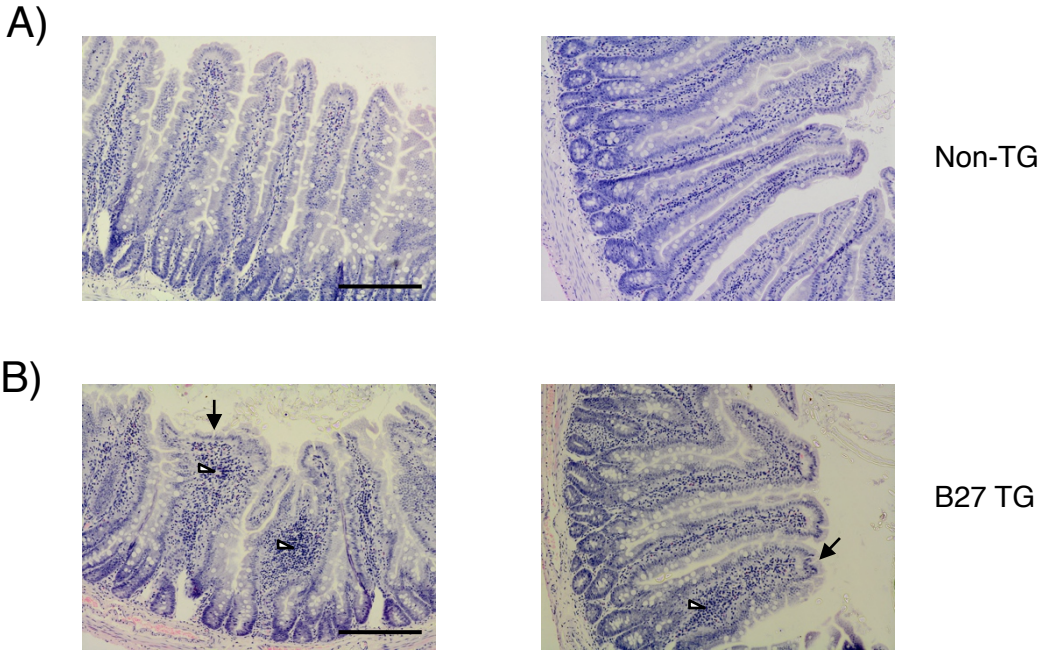


**Figure 3.8 Small intestinal histology of 52-week old animals.**

Paraffin embedded sections from 52-week old rat jejunums were stained with H&E. (A) non-TG animals showed no signs of inflammation, whereas B27-TG animals presented with inflammatory cell infiltrates (arrowheads) and flattened and fused villi (arrows). Magnification x10, scale bars µm, n=1.

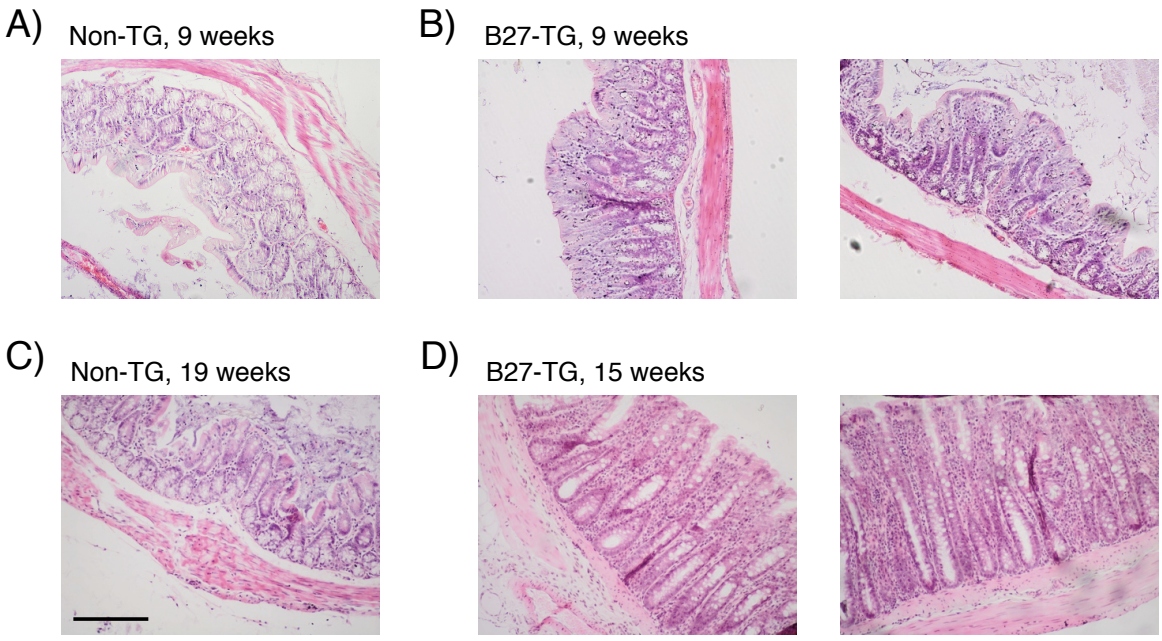


**Figure 3.9 B27-TG animals show signs of colonic inflammation**

Paraffin embedded sections from 9-15 week old non-TG and B27-TG animals were cut and stained for H&E. Colon sections from 9-week (A) and 19-week old (C) non-TG animals show normal colon structure and histology. (B) Colon sections from 9-week old B27-TG animals show normal structure, whereas in (D) 15-week old B27-TG animals show elongated crypts, and inflammatory cell infiltrate can be seen in the LP. (A, B) F10 generation, (C, D) F5 generation. Magnification x10, scale bars 50 µm, n=2.

## 3.3 Discussion

In this chapter the expression of HLA-B27 in various tissues, as well as the disease phenotype on the backcrossed B27-TG animals were investigated. It was first noticed that the surface expression of HLA-B27 increased in an age-dependent manner, from 10 % of HLA-B27$^+$ PBMCs at 3-week of age, to over 90 % on rats of 21-weeks of age. This age-dependent increase of HLA-B27 surface expression has been reported before for splenic cells on the 33-3 strain on F344 background, and in the same publication it was also reported that the level of surface expression correlated with the disease status (226). No such dramatic age-dependent increase of HLA-B7 molecule was noted when HLA-B7 expression on PBMCs from 4-week and 8-week old B7-TG rats was compared. It was also noted that the HLA-B27 antibody also bound to HLA-B7 molecules as well, if not better, than the HLA-B7 antibody. This cross-reactivity in the HLA-B27 antibody does not pose a problem to us, as the rats only express one or the other HLA transgene, but for human work carried in our laboratory it does have an effect and was important to see. In contrast, the HLA-B7 antibody was specific for HLA-B7 molecule only. The age-dependent increase of surface HLA-B27 expression was not only limited to PBMCs, as it was also observed that HLA-B27 expression by MLN cells increased from about 70 % on 6-week old animals to over 90 % on 21-week old animals. However, it was noted that most MLN DCs were already expressing high levels of HLA-B27 at 6 weeks, suggesting that the expression of HLA-B27 is upregulated on APCs early in life. Over 97 % of lymph cells from 10-week old B27-TG rats were HLA-B27$^+$, as would be predicted when looking at the expression patterns elsewhere in the body. Unfortunately, no DC stain was included in the stain together with HLA-B27, and so it cannot be said if the DCs in the lymph express higher levels of HLA-B27 than other lymph cells.

The reason for this curious effect of increasing HLA-B27 surface expression is not known. B27-TG animals were originally made by DNA microinjection of both HLA-B27 and β2m genes into fertilized rat eggs, and inserting these eggs into pseudo-pregnant females. The insertion of the genes is random, both in number and in location, as can be seen from the original publication from 1990 where several founders with different numbers of integrated HLA-B27 and β2m genes were identified (223). Both genes were inserted with flanking sequences, but it is not known for sure whether the promoters of these genes were contained within these sequences, and it is not known where in the chromosome the transgenes were inserted. Thus, it could be that the transgenes are under a promoter for some other genes, which are only upregulated as the animals get older. Weaning of the rats

could potentially have an effect, as at this time the animals start eating more solid food and rapidly develop their intestinal microflora. However, the 3-week old rats used for the experiments here were already weaned at the time of screening and still expressed only low levels of HLA-B27.

B27-TG rats have been reported to present with a variety of psoriasiform skin and nail lesions, such as hyperkeratosis of digits and nail dystrophy, alopecia, edema, psoriasiform hyperplasia and flaking of the skin on the tail or on the body, as well as arthritis and intestinal inflammation. In our colony the most common symptoms were alopecia of the face and back skin and hyperkeratosis and dystrophy of nails. The hair loss starts at the periorbital area, and spreads from there and eventually the animals exhibit thinning hair all over. This phenomenon has also been reported for the same 33-3 B27-TG rat line on the original F344 background (269). In addition, the 33-3 F344 line has been previously backcrossed into PVG, and disease symptoms were reported to be the same as in the original F344 background (227)[1]. In our colony, hair loss and nail dystrophies were rarely seen on animals younger than 16 weeks, and not all the older animals showed the symptoms either. This is not surprising, as the original publication detailing the development of inflammatory disease on 33-3 F344 strain reported that by 12 weeks, most animals present with diarrhoea, but less than 10 % of animals have nail or skin lesions or arthritis (226). Considering most animals were sacrificed before they reached the age of 12 weeks, it not so surprising most of the other symptoms were not frequently observed in our experiments. We did not detect arthritis in our animals, but this could be because the onset of arthritis is quite late: it has been reported that on the original F344 background at 15 weeks only less than 20 % of males and less than 10 % of females show signs of arthritis. On the PVG background, the onset of arthritis is even later (Joel Taurog, personal communication). We have however observed some reddening and swelling of the feet on some of the older animals, but no overt inflammation of the joints has been detected.

Intestinal inflammation is another common symptom observed in the B27-TG animals. In our colony it was observed that most of the rats developed diarrhoea by age of 16-weeks, indicating ongoing inflammation in the intestine. Histological sections from the small intestine of 9-week old animals did not appear inflamed, which was to be expected, as the

[1] Note: This paper was retracted in 1999 as the presented CTL data turned out to be fabricated. However, in the letter of retraction main author J. Taurog assured that all the data concerning genetics and phenotype of the 33-3 rats on PVG background remained accurate. This was also confirmed by personal communication with J. Taurog.

animals on the PVG background are unlikely to develop signs of intestinal inflammation before age of 14-weeks (Joel Taurog, personal communication). In contrast, by 15 weeks inflammatory infiltrates were visible in the LP of the ileum and jejunum, and the villi were flattened. Sections from jejunum of 52 old week showed signs of inflammatory cell infiltrates as well as fused and flattened villi. The inflammation in these old animals does not seem to be severe, but most signs of intestinal inflammation in these animals are detected in the ileum and the colon. Unfortunately no sections of these are available from these older animals. Sections from the ascending colon of 9-week old animals did not show signs of inflammation, but as expected 15-week old animals showed signs of colonic inflammation. Inflammatory lesions in the stomach have also been reported for B27-TG animals, but it was not investigated if these were present in our animals. Together these results suggest that B27-TG animals on the PVG background do exhibit symptoms similar to those seen on the original F344 background and are thus considered suitable model to use in our studies.

# 4. *In vivo* phenotype and function of migrating intestinal DCs in B27-TG rats

## 4.1 Introduction

The intestinal system is constantly exposed to vast amounts of foreign antigens – most of these are harmless, such as food proteins and commensal bacteria, but some are potentially harmful pathogens. Dendritic cells (DCs) carry antigens from the intestine to the mesenteric lymph nodes (MLNs) where they are responsible for activating naïve T cells. DCs are not only involved in induction of active immune reactions, but as was discussed in the introduction DCs also play an important role in maintaining balance between immunity and tolerance in the intestine (116). Since DCs and intestinal inflammation have both been suggested to have a role in the disease pathogenesis in both human SpA as well as in the animal model of SpA (See introduction), it was decided to investigate whether intestinal DCs could have a role in the pathogenesis of the SpA animal model. The animal model makes use of rats transgenic for human HLA-B27 gene together with human $\beta_2$-microglobulin (B27-TG rats), and these animals develop a spontaneous disease that shares many symptoms with SpA patients, such as peripheral arthritis (223). HLA-B27 is an MHC class I gene, and while it is closely linked to SpA diseases its role in the disease pathogenesis is unclear (14). Data regarding DCs in the B27-TG animal model has mainly concentrated on functions of either splenic or BMDCs, as intestinal DCs are not as easy to acquire. However, in our laboratory we can make use of a sophisticated surgical method involving cannulation of the thoracic duct of the rats (270), which allows *in vivo* investigation of DCs migrating in the lymph from the intestine towards MLNs. As inflammation in B27-TG animals and in AS patients often affects the intestine (223, 228, 239), *we hypothesised that the DCs migrating from the intestine may play an important role in initiating the pathogenic inflammatory responses associated with SpA*.

As DCs are very sensitive to changes in their environment, it is difficult to obtain them directly from the intestinal tissues as the harsh treatment needed to extract the cells from the tissues is likely to alter their phenotype, and function. Thus, to test our hypothesis, well-established surgical methods of mesenteric lymphadenectomy (MLNX) and thoracic duct cannulation were used to collect the DCs that migrate from the intestine to the MLNs in the afferent lymph (for details of this method, see Materials and Methods and (268)). This method allows the collection of DCs with minimum *in vitro* manipulation, and hence

the cells are able to retain their *in vivo* phenotype. These DCs are phenotypically MHCII$^{hi}$ CD103$^{+}$, and they have been found to divide into three subsets (113, 271): CD11b$^{lo}$ CD172a$^{hi}$, CD11b$^{hi}$ CD172a$^{int}$ and CD11b$^{int}$ CD172a$^{lo}$. In this chapter, for the first time, the phenotype and function of these migrating intestinal DCs, or lymph-derived DCs (L-DCs), from the B27-TG animals are compared to L-DCs derived from non-TG littermates, or non-disease-prone rats transgenic for the human HLA-B7 gene and b2m genes (B7-TG).

## 4.2 Results

### *4.2.1 B27-TG animals have fewer L-DCs in the lymph*

As lymph cells from B27-TG animals were found to express HLA-B27 on their surface (CH3), it was next determined if the B27-TG and the control non-TG and B7-TG animals had the same number and proportion of L-DCs in their lymph. Initially, the total yield of lymph-derived cells from B27-TG rats was compared to that of non-TG animals to make sure there was no inherent problem with the general migration of cells from tissues draining to the thoracic duct. In humans, and presumably in rats as well, these tissues include most of the body, except for right arm, right side of the chest, neck and head and lower left lobe of the lung. It was also assessed whether i.v. saline received by the animals for the first 24h after surgery had an effect on the number of cells in the lymph. To answer these questions, lymph was collected from individual animals at three different time points after cannulation: 0-24h, 24-48h and 48-72h. The volume of lymph was calculated for the first two time points, and as no difference was observed between non-TG and B27-TG animals, their results were pooled for the two time points (Figure 4.1A). As expected, lymph flow was significantly higher on the 0-24h collection than on the 24-48h one, reflecting the i.v. fluid provided for the animals for the first 24h after surgery. To determine if the increased lymph flow affected the number of migrating cells and if there were any differences in the number of cells between non-TG and B27-TG animals, the total number of lymph cells for non-TG, B7-TG and B27-TG animals were calculated separately for each of the three time points (Figure 4.1B). No differences in the cell numbers were observed between any of the time points, indicating that cell migration in the lymph is stable for the period of observation and is not affected by extra fluid given to the animals. There was a trend towards a slight increase in the number of B27-TG cells compared to control animals, however this was not statistically significant.

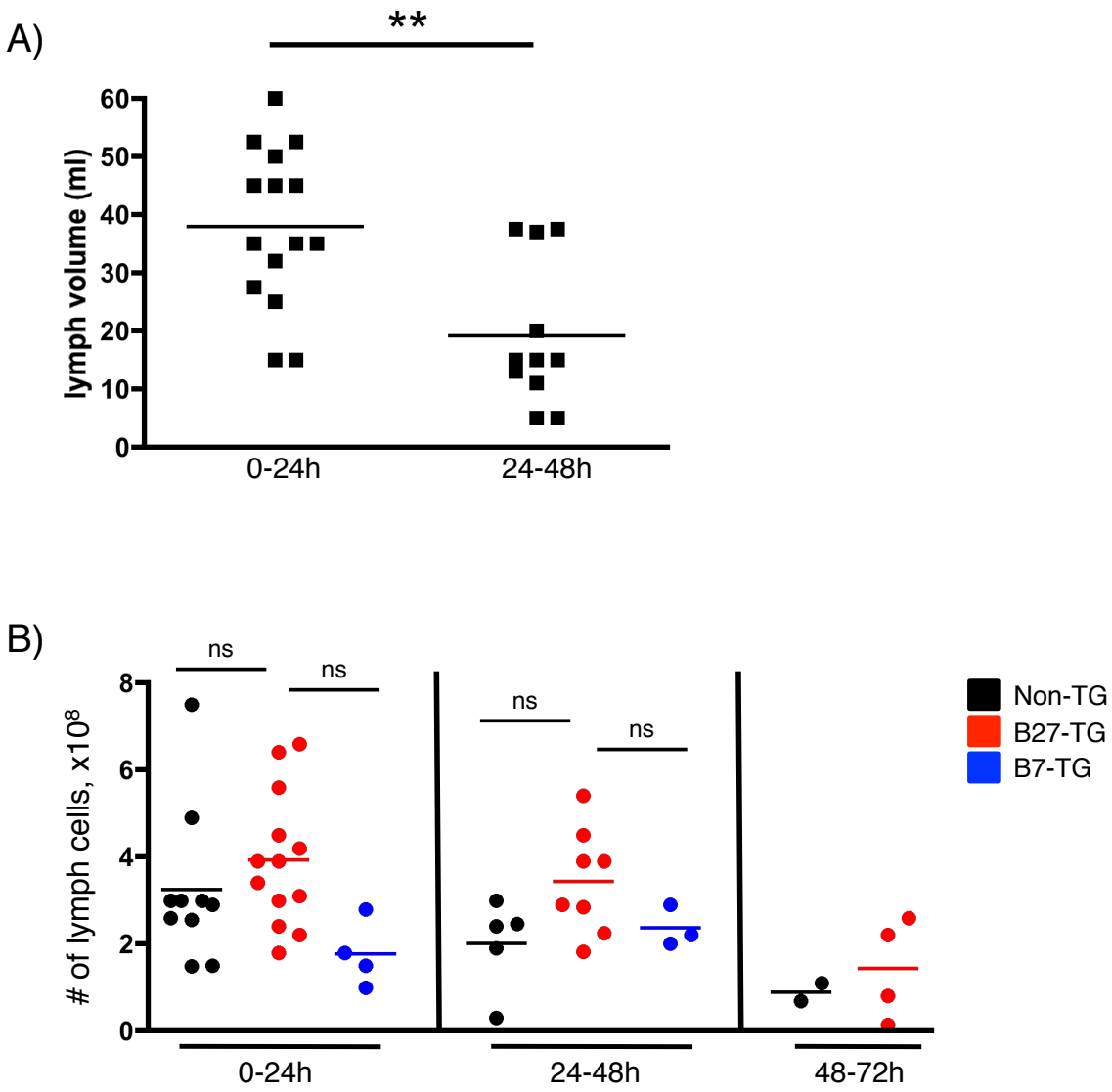


**Figure 4.1. No difference in the total number of cells in the lymph between non-TG and B27-TG animals.**

Lymph was collected from non-TG, B27-TG and B7-TG rats at three different time points, and volume of lymph at 0-24h and 24-48h are shown in (A), with results for each timepoint pooled from non-TG and B27-TG animals. (B) The total number of cells in non-TG, B27-TG and B7-TG lymph was calculated using a haemocytometer. Each dot represents an individual animal, and data are from 3-8 individual experiments. Horizontal line is the mean of all the values within that time point. Data was analysed by Student's T-test (A), or 2-way Anova (B), **p<0.01.

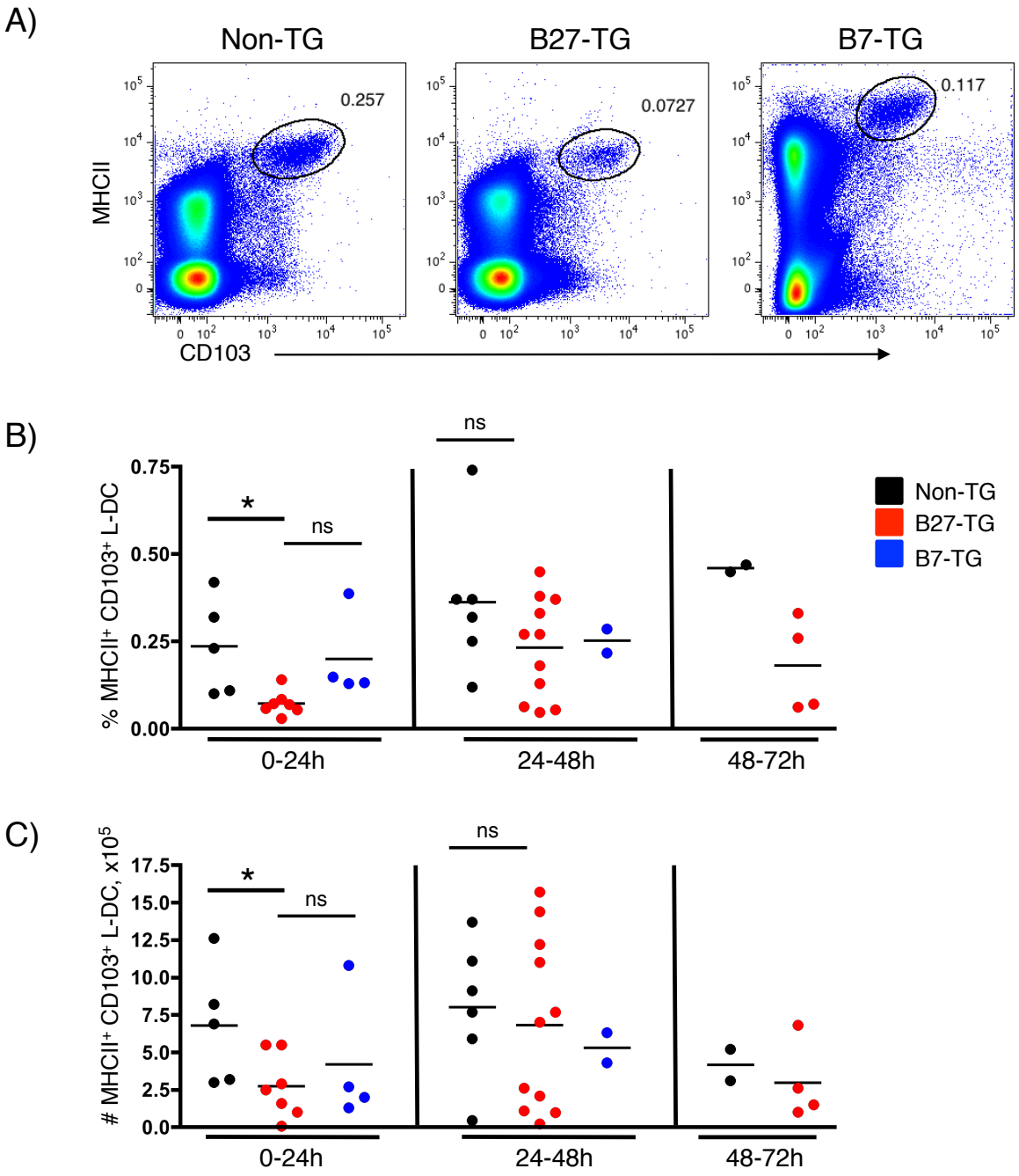


**Figure 4.2 B27-TG rats have fewer MHCII$^+$ CD103$^+$ L-DCs than control animals**

Lymph was collected from individual animals in three 24-hour periods following thoracic duct cannulation: 0-24h, 24-48h and 48-72h. Cells were stained for DC markers CD103 and MHCII on each collection period, and the number and percentage of DCs was determined by FACS. (A) Representative FACS plots from 0-24h collection showing the percentage of CD103$^+$ MHCII$^+$ DCs in B27-TG animals, and control Non-TG and B7-TG lymph. Data from 5-6 (Non-TG and B27-TG) or 1 (B7-TG) independent experiments showing percentage (B) and number (C) of DCs in lymph. Each dot represents an individual animal. Horizontal lines show mean values, and data were analysed by two-tailed Student's T-test. *p<0.05.

As there were no differences in the total number of migrating lymph cells between B27-TG and control animals, it was next determined if the proportion and number of lymph derived DCs (L-DCs) in B27-TG rats was comparable to non-TG animals. Again, lymph was collected from individual animals for three 24h periods after cannulation, but this time the cells were stained with antibodies specific for MHC class II (MHCII) and CD103, markers for L-DCs in the rat. The percentage and absolute number of the double positive $MHCII^+$ $CD103^+$ cells was determined separately for each collection period (Figure 4.2). Figure 4.2A shows representative FACS plots for B27-TG, non-TG and B7-TG animals from 0-24h collection, with gating for $MHCII^+$ $CD103^+$ cells. When data was collated from 5-6 (B27-TG, non-TG) or 1 (B7-TG) individual experiments, it was noted that B27-TG animals had significantly fewer $MHCII^+$ $CD103^+$ L-DCs at the 24h collection compared to non-TG animals, both proportionally (mean percentage of 0.226% for non-TG vs. 0.072% for B27-TG) (Figure 4.2B) and in absolute numbers (mean: $6.72x10^5$ vs. $2.73x10^5$) (Figure 4.2C). At 24-48h more variation in both DC percentage and number was observed, and no statistical significances were observed between non-TG and B27-TG derived L-DCs. Very few data points were available for 48-72h, and thus no firm conclusions could be drawn. Importantly, no differences between non-TG and B7-TG animals were observed.

### *4.2.2 A population of L-DCs from B27-TG animals express more CD25*

As it was established that there were only very slight differences in the L-DC number and proportion between non-TG and B27-TG animals, it was next determined if there were any differences in the activation status of L-DCs. It was hypothesised that L-DCs from B27-TG animals would be more activated, as these animals might already present with intestinal inflammation at this time. However, this has not been investigated by histology. If the hypothesis was proved to be right, these DCs could cause enhanced immune reactions in the MLN, or present self-antigens in an inflammatory setting, and thus drive the disease development in the rats. To assess this, expression of classical costimulatory molecules CD80 and CD86 on L-DCs was determined together with CD25, or IL-2Rα, which is the most sensitive marker of activation on rat DCs (272). Rats were cannulated and the expression of these three molecules on $MHCII^+$ $CD103^+$ L-DCs at 0-24h (Figure 4.3), 24-48h (Figure 4.4) and 48-72h (Figure 4.5) were determined. In the first 24h-collection, it was noticed that about 50 % of B27-TG $MHCII^+$ $CD103^+$ L-DCs expressed CD25, compared to 26.2 % of non-TG L-DCs, as can be seen in the representative dot plot and histogram overlay in Figure 4.3A. When data from at least three independent

experiments were pooled, there was a small but significant increase in the proportion of MHCII$^+$ CD103$^+$ CD25$^+$ cells on B27-TG lymph compared to non-TG (Figure 4.3B).

There were no significant differences in CD80 and CD86 expression between non-TG and B27-TG L-DCs, although when looking at the dot plot (Figure 4.3A), there appeared to be slightly more CD80$^+$ cells in the B27-TG lymph. The trend towards increased CD25 expression continued when data from 24-48h was collated (Figure 4.4), but was not statistically significant at this time point. Again, it was noticed that there seemed to be more CD80$^+$ cells within the B27-TG L-DCs (Figure 4.4A), but as before, it was not statistically significant (Figure 4.4B). Results from the final 48-72h collection resemble those of 0-24h and 48-72h, with slight increase in CD25 expression, and more CD80$^+$ cells on B27-TG MHCII$^+$ CD103$^+$ L-DCs (Figure 4.5A, B). Unfortunately, for non-TG there was only one experiment with one animal for the 48-72h data point, so no conclusions can be drawn. Expression of OX-40L on L-DCs at the 0-24h time point was also examined, as this is an important costimulatory molecule involved in T cell activation (Figure 4.6). Neither non-TG nor B27-TG animals expressed OX-40L at a high level; in fact, there was only a very slight shift when compared to the isotype, and this shift was identical in both non-TG and B27-TG L-DCs.

It has been reported previously that MHC class II expression on CD103$^+$ splenic DCs is lower on the B27-TG than on non-TG animals (239). Thus, it was investigated whether this was also true for MHCII$^+$ CD103$^+$ L-DCs (Figure 4.7). It was noted that in most experiments L-DCs from B27-TG animals did indeed have a slightly lower MHCII expression, as shown on Figure 4.7A, where representative results from one representative experiment are shown. When the geometric means for MHCII on CD103$^+$ cells were calculated and data from 4 independent experiments combined, there was a trend towards B27-TG L-DCs expressing less MHCII, but the difference was not significant (Figure 4.7B).

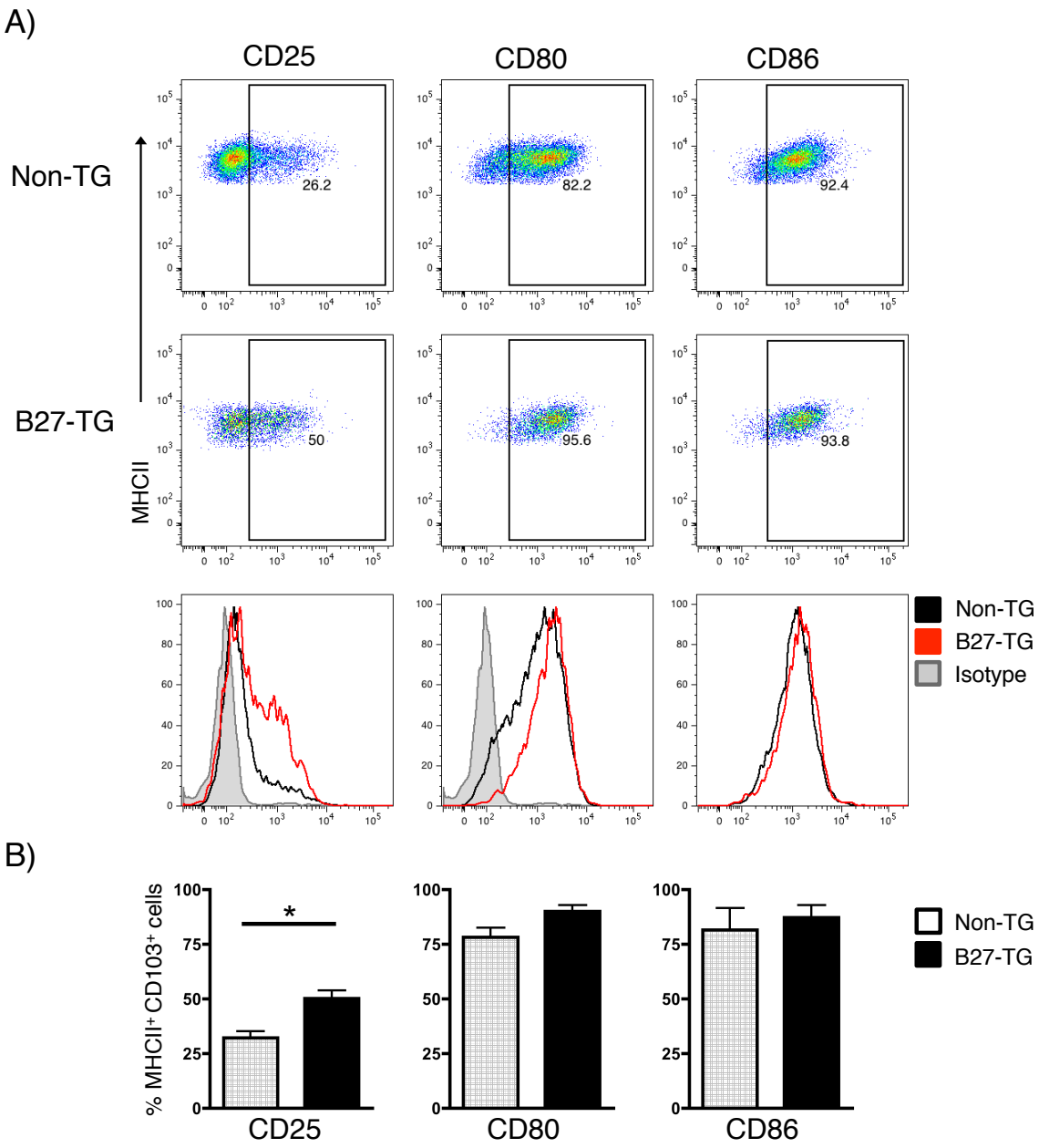


**Figure 4.3 A proportion of B27-TG L-DCs express significantly more CD25 than non-TG control DCs.**

L-DC were collected by thoracic duct cannulation for the first 24 hours (0-24h) from B27-TG and non-TG animals. The expression level of various costimulatory molecules on $CD103^+$ $MHCII^+$ DCs was determined by FACS. (A) Two upper panels show representative FACS plots of CD25, CD80 and CD86 expression on $CD103^+$ $MHCII^+$ DCs from non-TG and B27-TG animals. Bottom panels show histogram overlays from figures above. (B) Pooled data from at least 3 independent experiments (non-TG, n=3-4; B27-TG, n=4-6). Error bars represent SEM, and data were analysed by two-tailed Student's T-test. *$p<0.05$.

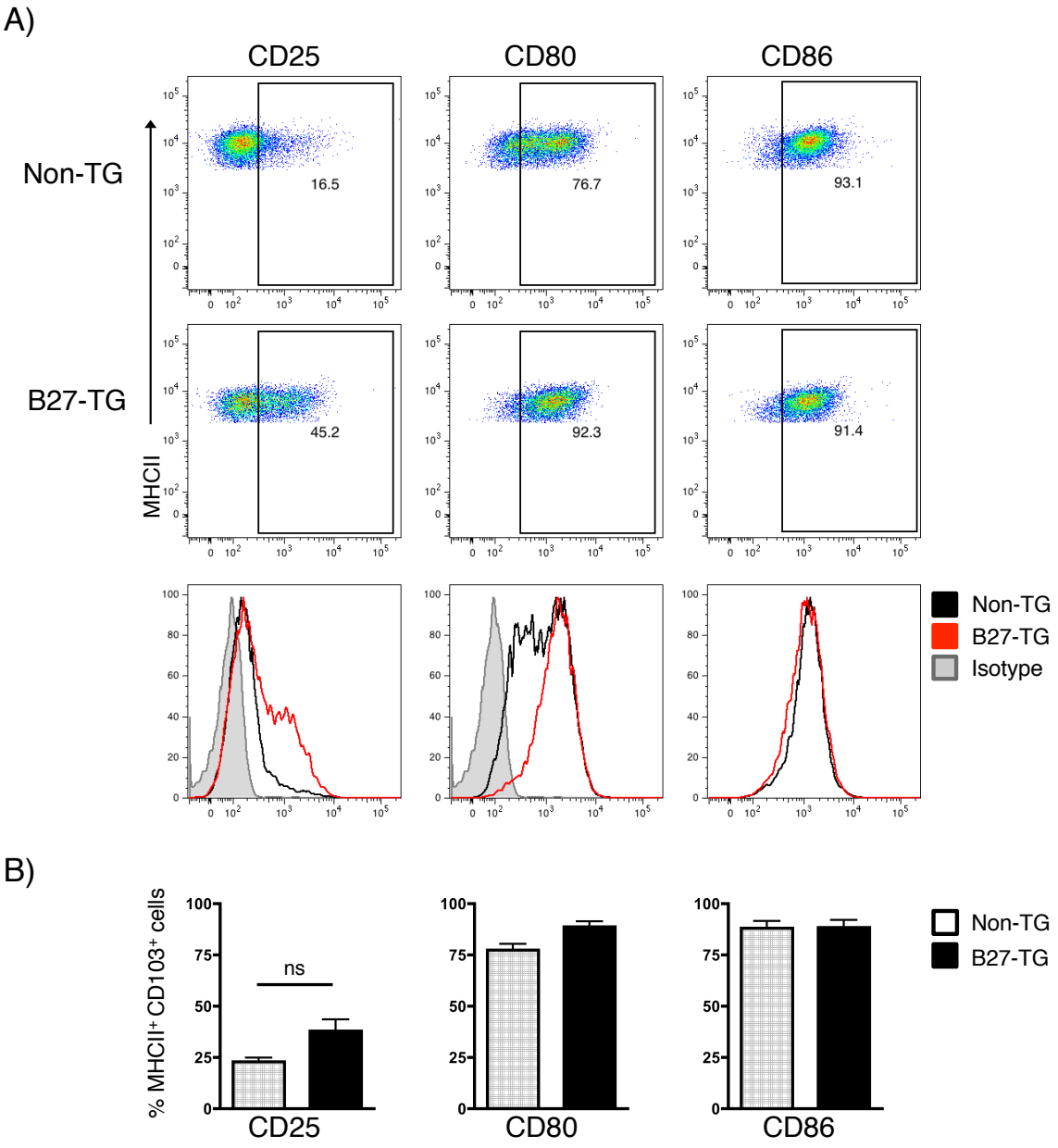


**Figure 4.4 CD103$^+$ MHCII$^+$ DCs from B27-TG animals express more CD25 also at 24-48h**

L-DC were collected by thoracic duct cannulation for the second 24 hours (24-48h) from B27-TG and non-TG animals. The expression level of various costimulatory molecules on CD103$^+$ MHCII$^+$ DCs was determined by FACS. (A) Two upper panels showing representative FACS plots of CD25, CD80 and CD86 expression on CD103$^+$ MHCII$^+$ DCs. Lower panels show histogram overlays from figures above. (B) Pooled data from at least 3 independent experiments (non-TG, n=3-5; B27-TG, n=4-8). Data analysed by Student's T-test. Error bars represent SEM.

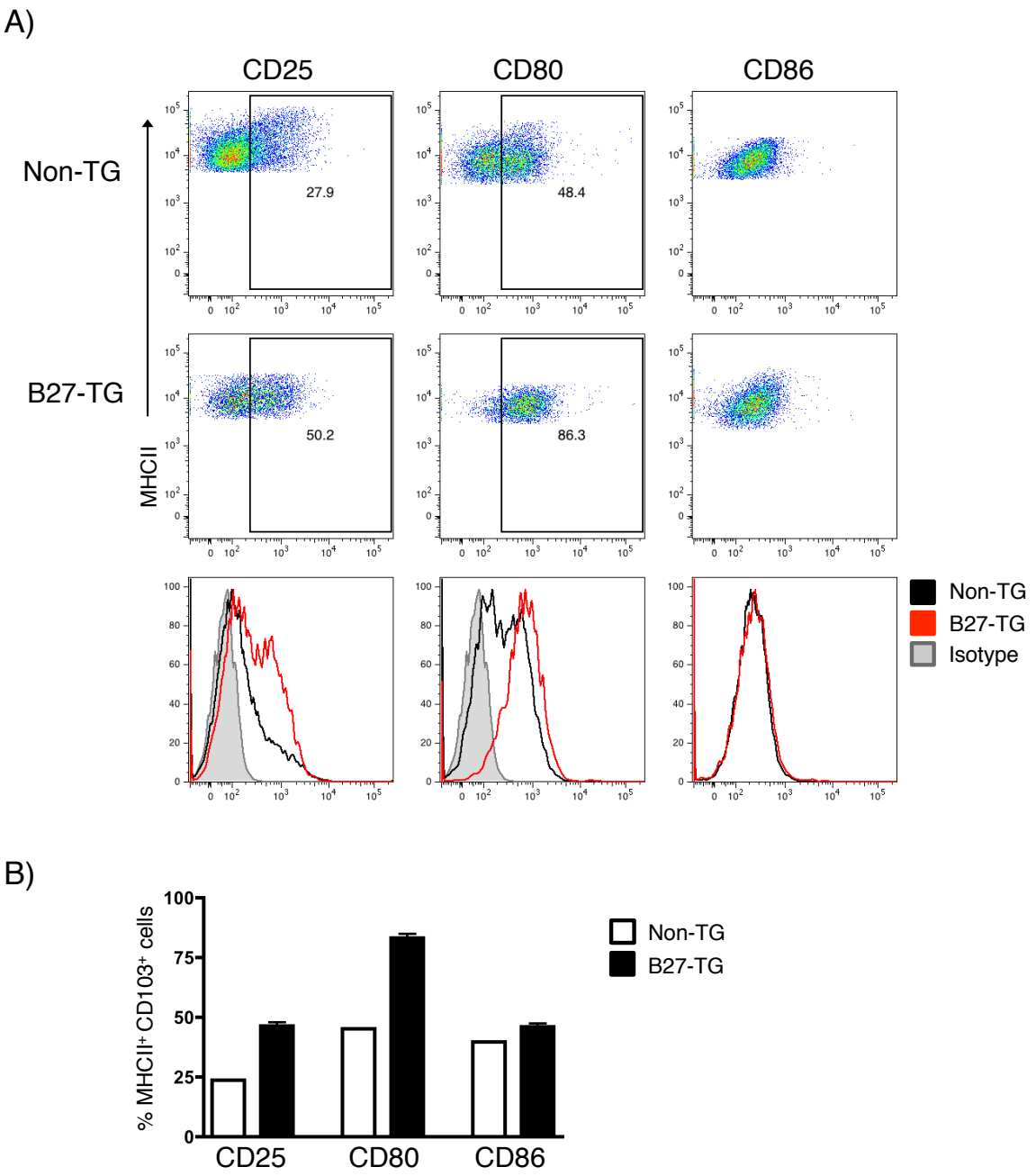


**Figure 4.5. DCs from B27-TG animals continue to express more CD25 at 48-72h than control non-TG DCs**

L-DC were collected by thoracic duct cannulation at 48-72h time point from B27-TG and non-TG animals. The expression level of various costimulatory molecules on $CD103^+$ $MHCII^+$ DCs was determined by FACS. (A) Two upper panels showing representative FACS plots of CD25, CD80 and CD86 expression on $CD103^+$ $MHCII^+$ DCs. Lower panels show histograms from figures above. (B) Collated data from two experiments for B27-TG and one experiment for non-TG (non-TG, n=1; B27-TG, n=2). Error bars are SEM.

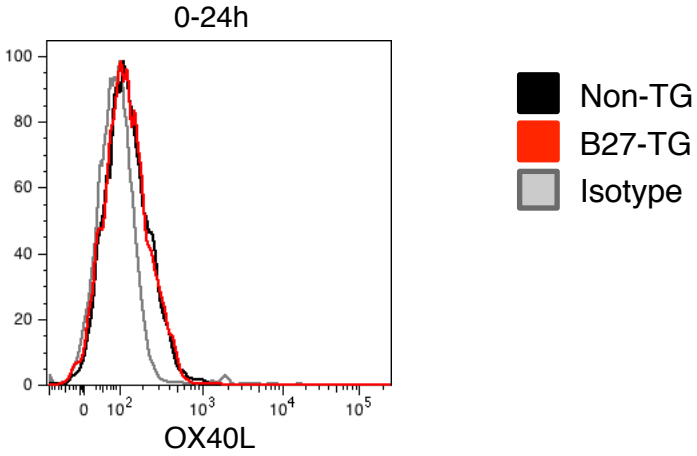


**Figure 4.6. B27-TG and non-TG L-DCs express similar levels of OX40L**

L-DCs were collected by thoracic duct cannulation and cells were stained for MHCII, CD103 and OX40L, and expression level of OX40L on $MHCII^+CD103^+$ DCs was determined by FACS at 0-24h. n=1.

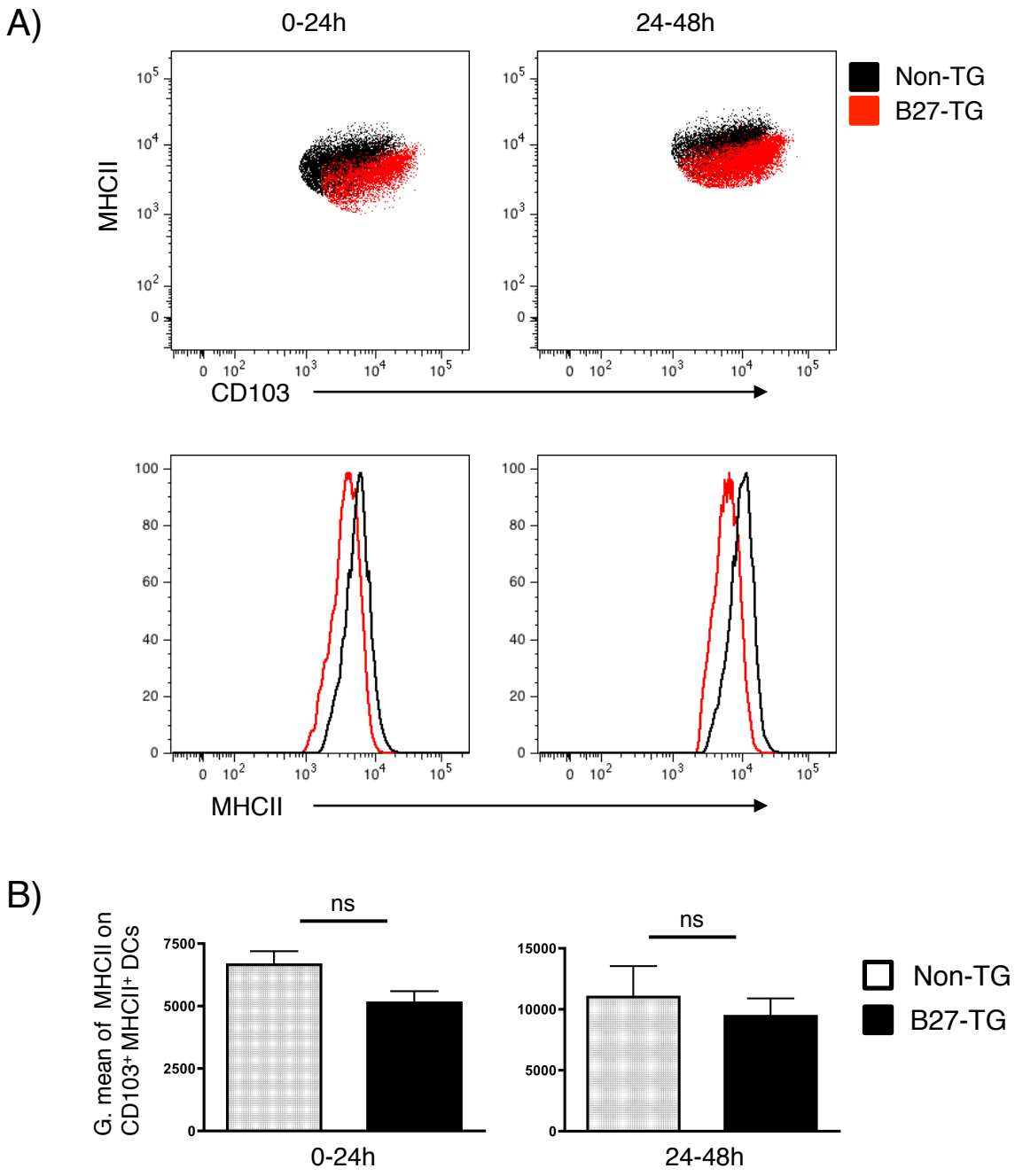


**Figure 4.7. Comparison of MHCII expression level on MHCII$^+$ CD103$^+$ L-DCs from non-TG and B27-TG animals**

L-DCs were collected by thoracic duct cannulation from the lymph, stained with MHCII and CD103 antibodies, and geometric mean for MHCII on MHCII$^+$ CD103$^+$ DCs from non-TG and B27-TG animals was determined. (A) Representative dot plots (upper panels) and histograms of MHCII expression on non-TG and B27-TG L-DC at 0-24h and 24-48h. (C) Pooled data + SEM from at least 5 independent experiments (non-TG, n=4-5; B27-TG, n=5-8). Data were analysed by Student's T-test.

### 4.2.3 L-DCs from B27-TG animals are not better at stimulating allogeneic T cell proliferation

As it was observed that a population of L-DCs from B27-TG animals express more CD25, and might thus be in a more activated state, the ability of DCs to drive T cell proliferation was assessed using mixed leukocyte reactions (MLRs). Lymph cells were enriched for CD103 using magnetic beads, and $MHCII^+$ $CD103^+$ L-DCs from B27-TG or non-TG animals were flow sorted as shown in Figure 4.8A. These DCs were plated out in triplicate in 2-fold dilutions, from 20,000 to 1,250 DCs/well, with $1x10^5$ MLN cells from DA rats, and were cultured at 37ºC for 5 days. Thymidine was added to the wells for the final 12h of culture, and the level of T cell proliferation was assessed by thymidine incorporation in each well. The experiment was repeated 5 times; non-TG and B27-TG cells were similarly capable at supporting allogeneic T cell proliferation (Figure 4.8B).

T cell proliferation was also assessed using CFSE, as using this method it is possible to determine the proliferation of a specific cell type in the cultures. Thus, MLN cells from DA rats were incubated with CFSE before plating them out with sorted L-DCs, as above, and the plates were cultured at 37ºC for 5 days. Cells were then harvested from the plates and stained with a CD4-specific antibody to assess proliferation of $CD4^+$ T cells. A representative FACS plot showing CFSE staining on all acquired cells with 12,500 DCs is shown in Figure 4.9A, with a gate showing the percentage of all $CD4^+$ cells. As can be seen, both non-TG and B27-TG samples appear to have similar proportion of $CD4^+$ cells (53-59%). When CFSE staining was assessed on the $CD4^+$ cells (Figure 4.9B), it was noticed that non-TG L-DC induced more T cell proliferation than B27-TG L-DCs (Figure 4.9C). However, as seen with the other proliferation experiments (Figure 4.8) the experiment should be repeated several times before any conclusions could be drawn.

Concentrations of various cytokines at day 5 of MLR from experiment shown in Figure 4.9 were also measured by Luminex (Figure 4.10). IFN-γ and IL-17F were present at highest concentrations in the cell culture supernatants, and both were higher in the cultures with non-TG L-DCs. IL-10 and IL-2 were also present, and IL-12 was also higher when 12,500 non-TG L-DCs were used, whereas no obvious differences between non-TG and B27-TG were noted with 6,500 L-DCs. Concentrations of IL-6 and TNFα were low in both culture conditions, however slightly more of both were observed in the cultures with non-TG L-DCs.

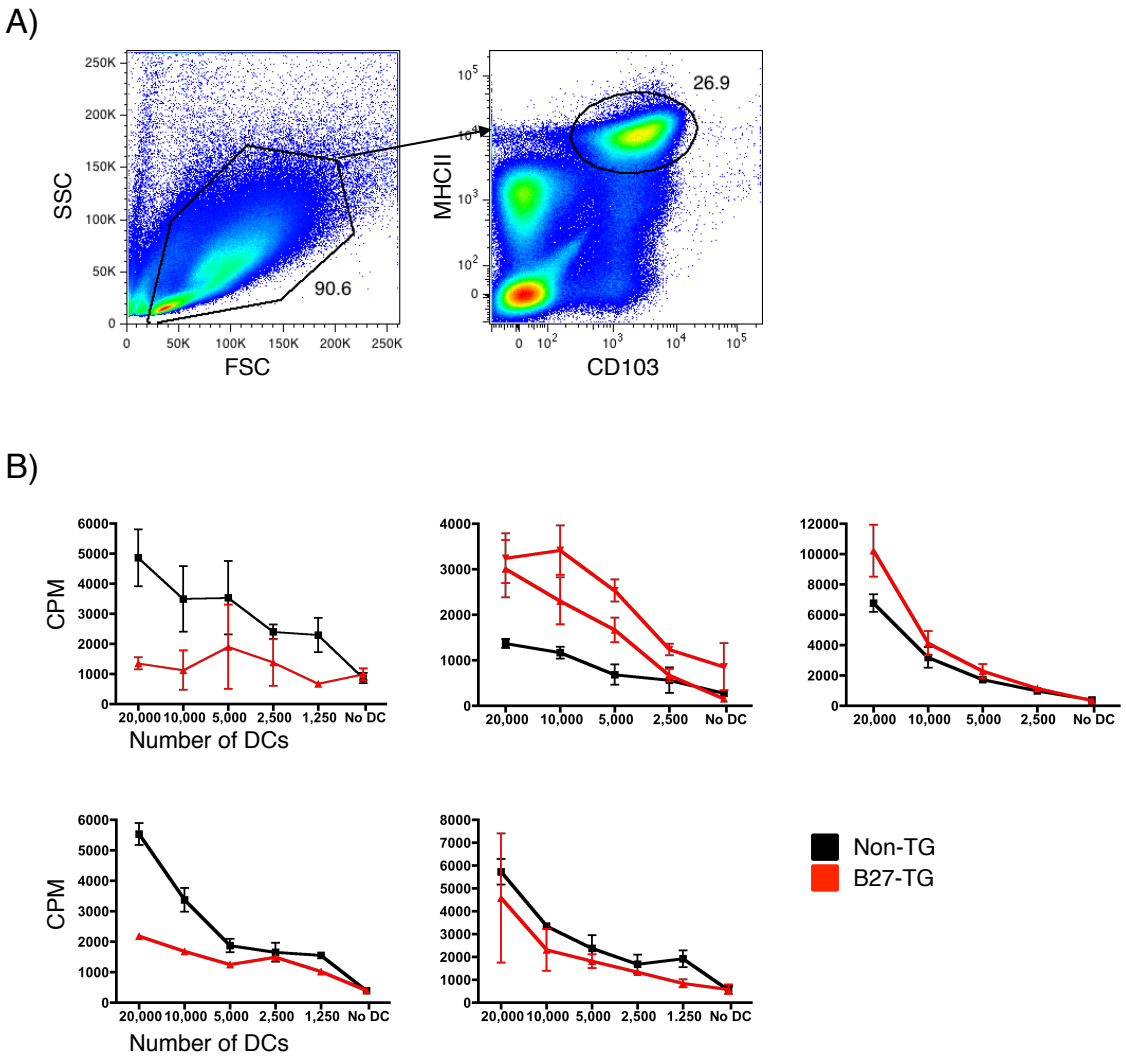


**Figure 4.8. Mixed lymphocyte reactions revealed no consistent differences in the ability of L-DCs from non-TG or B27-TG animals to induce T cell proliferation**

Thoracic duct lymph was collected from non-TG and B27-TG animals in order to acquire L-DCs. Lymph cells were enriched for CD103 with magnetic beads, and stained with CD103 and MHCII antibodies, and $CD103^+$ $MHCII^+$ DCs were flow-sorted. Single cell suspension was made from MLNs of Dark Agouti (DA) rat, and $1x10^5$ MLN cells were incubated in triplicate on 96-well plates for 5 days with 0-20,000 flow-sorted $CD103^+$ $MHCII^+$ DCs from either non-TG or B27-TG thoracic duct lymph. Tritiated thymidine was added to the wells for the final 12h of the 5d incubation to assess cell proliferation. (A) Gating strategy for sorting $MHCII^+$ $CD103^+$ DCs from thoracic duct lymph. (B) 5 independent MLR experiments, error bars mean±SD.

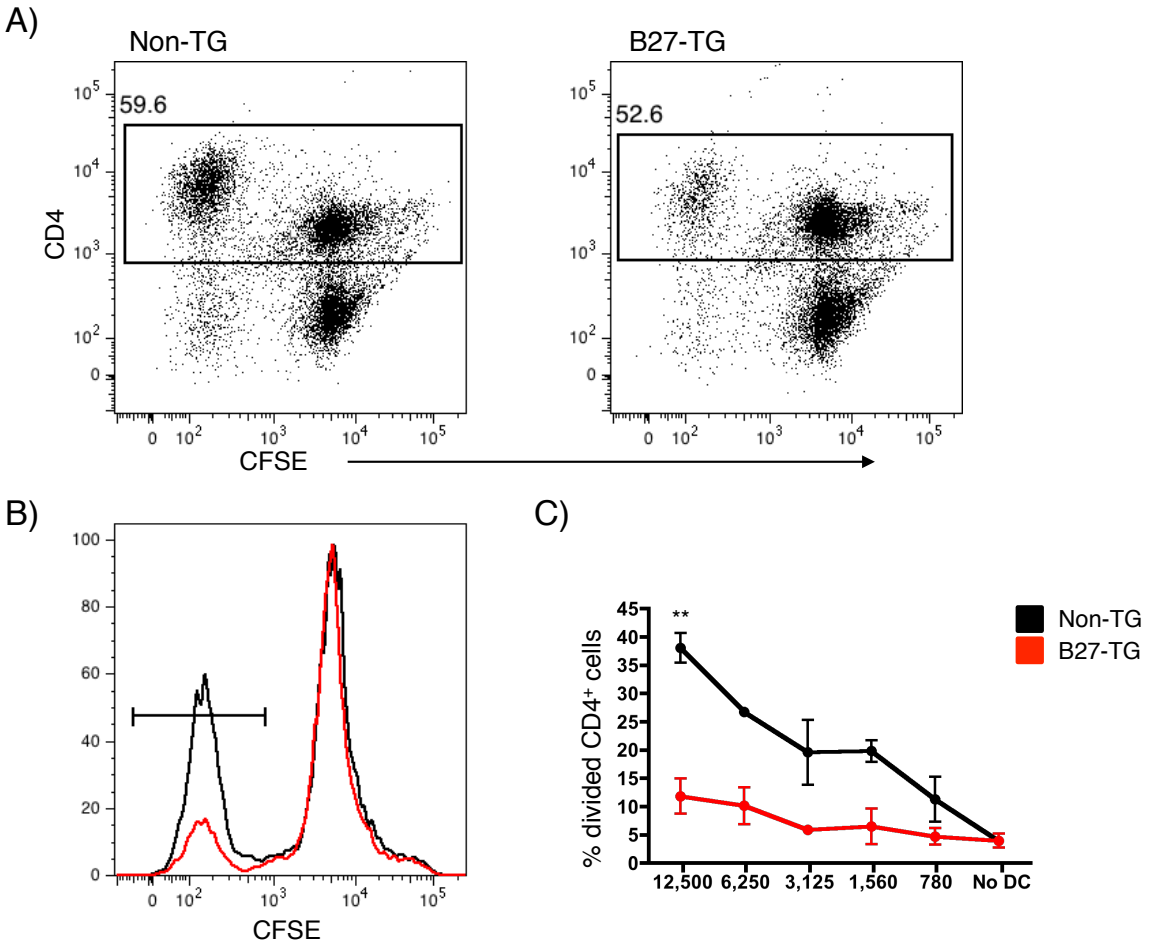


**Figure 4.9. CFSE based MLR showed non-TG L-DCs to be more effective at inducing CD4+ T cell proliferation than B27-TG L-DC**

L-DC were sorted from cannulated non-TG and B27-TG animals as before, and dilutions of sorted MHCII$^{+}$ CD103$^{+}$ DCs were incubated in triplicate for 5d with 1x10$^{5}$ CFSE labelled MLN cells from DA rats. After 5d, cells were harvested from the well and stained with CD4 antibody. Proliferation of CD4$^{+}$ cells was assessed by FACS. (A) a representative CD4 and CFSE staining of cells incubated with 12,500 DCs, showing the percentage of CD4$^{+}$ cells in both non-TG and B27-TG preparations. (B) Percentage of proliferated CD4 T cells was assessed using a gate shown in the histogram overlay showing CFSE staining intensity in both non-TG and B27-TG samples from (A), and the results of the MLR can be seen in (C). **p<0.01 by two-way Anova. Error bars mean±SD.

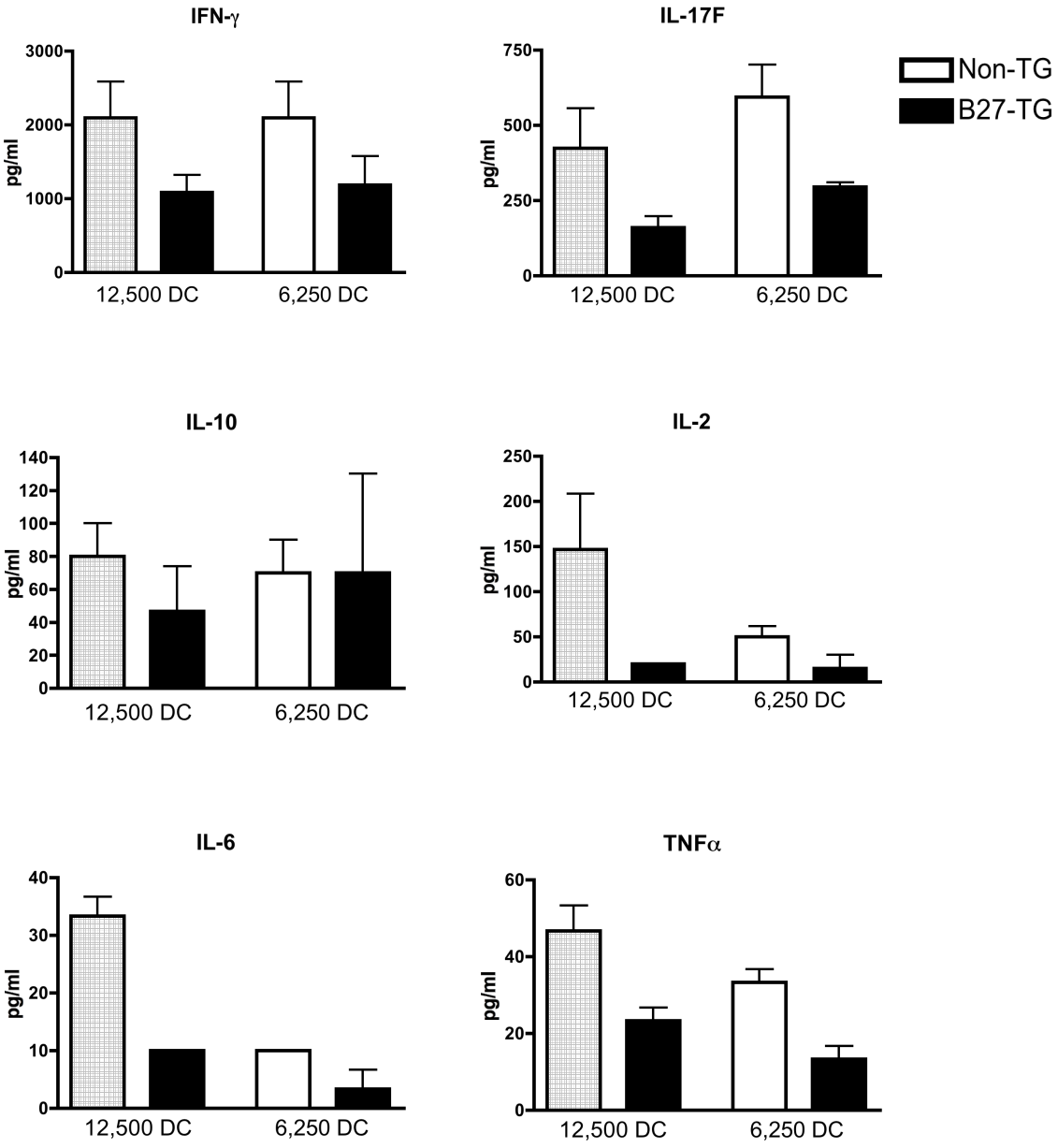


**Figure 4.10 Luminex results from MLR**

Supernatants at day 5 of MLR were collected from experiment shown in Figure 4.9, and the concentrations of various cytokines were measured by Luminex. Results from one experiment with 3 technical replicates. Error bars are SEM.

### 4.2.4 B27-TG rats lack a subset of migrating intestinal DCs

Rat MHCII$^{+}$ CD103$^{+}$ lymph DCs can be divided into three well-characterised populations according to their expression of CD11b and CD172a (113). These three populations are: CD172a$^{hi}$ CD11b$^{-}$ (CD172a$^{hi}$) CD172a$^{int}$ CD11b$^{+}$ (CD172a$^{int}$) and CD172a$^{lo}$ CD11b$^{int}$ (CD172a$^{lo}$). The appearance of these three populations in non-TG rats was assessed by sorting the three subsets (Figure 4.11A), followed by a cytospin and staining of the subsets with H&E (Figure 4.11B).

CD172a$^{hi}$ cells were mainly large, mononuclear cells with a large amount of cytoplasm. CD172a$^{int}$ cells resembled CD172a$^{hi}$ cells, but were in general slightly smaller, had less cytoplasm, and a more regular shaped nucleus. In contrast, CD172a$^{lo}$ cells appeared different from the other two subsets: it was possible to see cells resembling activated DCs, many displaying dendrites on their surface. Cells like this were not seen in preparations from the other two subsets. Some of the cells in the CD172a$^{hi}$ preparation resembled cells seen in CD172a$^{int}$ sample, but these might be a contamination from that population that has occurred during cell sorting.

Next it was investigated whether all the three MHCII$^{+}$ CD103$^{+}$ L-DC subsets were present in the lymph of B27-TG animals with same proportions as in control non-TG and B7-TG rats. B27-TG, non-TG and B7-TG animals were cannulated, and the lymph cells were stained with antibodies for MHCII, CD103, CD11b and CD172a, and analysed by FACS. As expected, all three subsets could be found in the lymph of non-TG and B7-TG animals (Figure 4.12A, B), with similar proportions. However, when the lymph cells from B27-TG animals were analysed, unexpectedly it was noted while in non-TG animals the CD172a$^{lo}$ subset comprised about 40 % of all MHCII$^{+}$ CD103$^{+}$ L-DCs, in B27-TG animals this population only comprised less than 10 % (Figure 4.12C). This result is highly reproducible, as can be seen when data from 6 independent experiments with 2-3 animals/group was analysed (Figure 4.13). Figure 4.13A shows the percentage of each subset from MHCII$^{+}$ CD103$^{+}$ L-DCs, and B27-TG animals had a significantly smaller proportion of CD172a$^{lo}$ when compared to non-TG and B7-TG animals: on average, B27-TG MHCII$^{+}$ CD103$^{+}$ DCs contained 5 % CD172a$^{lo}$ cells, whereas non-TG and B7-TG animals had on average 28 % and 20 %, respectively. In contrast, B27-TG animals had significantly higher proportion of CD172a$^{int}$ DCs when compared to non-TG (39 % vs. 27 %), and also seemed to have slightly higher proportion of CD172a$^{hi}$ cells, but this was not significant. When the absolute numbers for each DC subset out of MHCII$^{+}$ CD103$^{+}$

population were calculated, B27-TG animals again had significantly fewer CD172a$^{lo}$ L-DCs compared to non-TG animals (Figure 4.13B). On average, B27-TG animals had 1.9 x $10^4$ MHCII$^+$ CD103$^+$ CD172a$^{lo}$ lymph DCs compared to 2.1 x $10^5$ cells in non-TG.

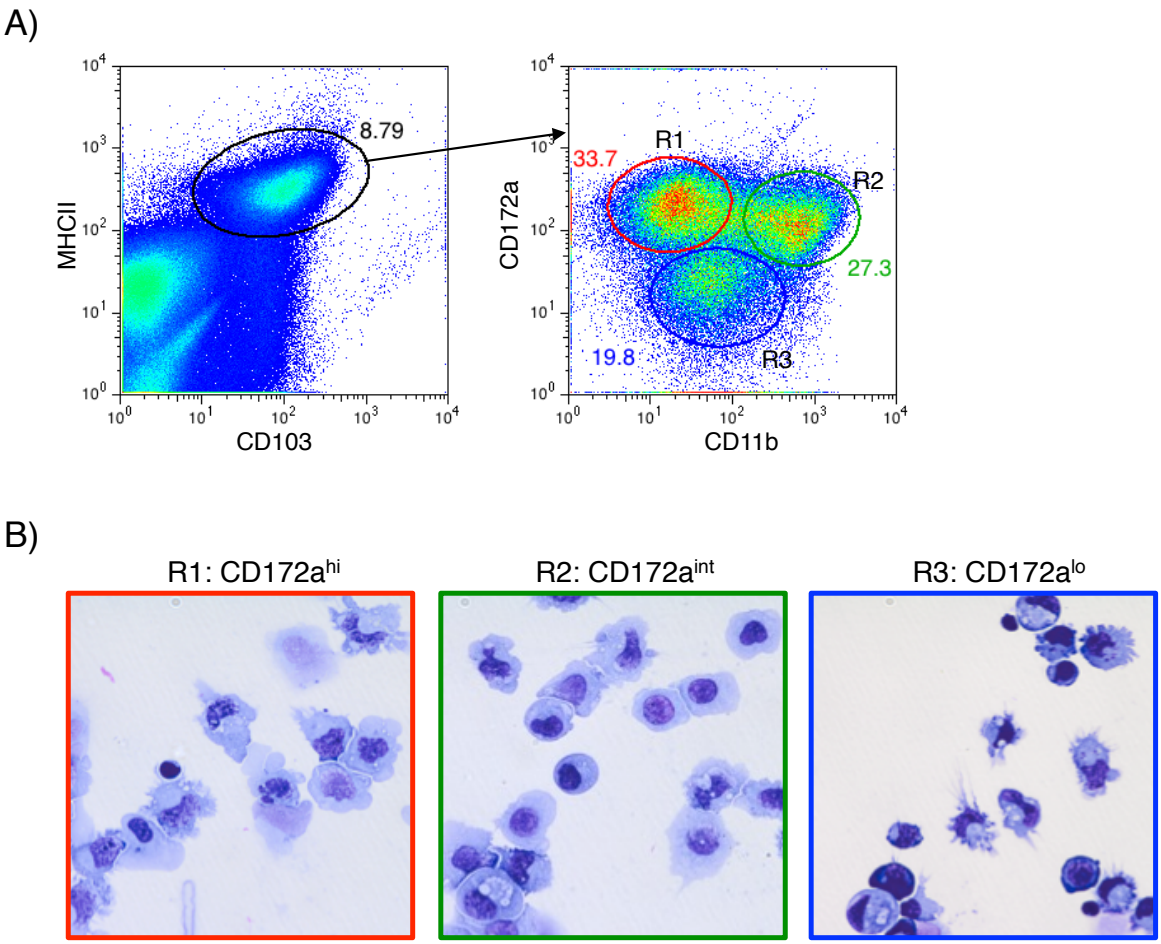


**Figure 4.11 H&E staining of sorted L-DC subsets.**

Cells collected at 0-24h by thoracic duct cannulation were enriched for CD103 using magnetic beads, and stained with CD103, MHCII, CD11b and CD172a antibodies. (A) Three subsets of MHCII$^+$ CD103$^+$ L-DCs were flow-sorted as shown, and cytospins were performed for these three populations. (B) H&E staining of the slides from cytospins. Pictures were taken using x40 magnification and a light microscope.

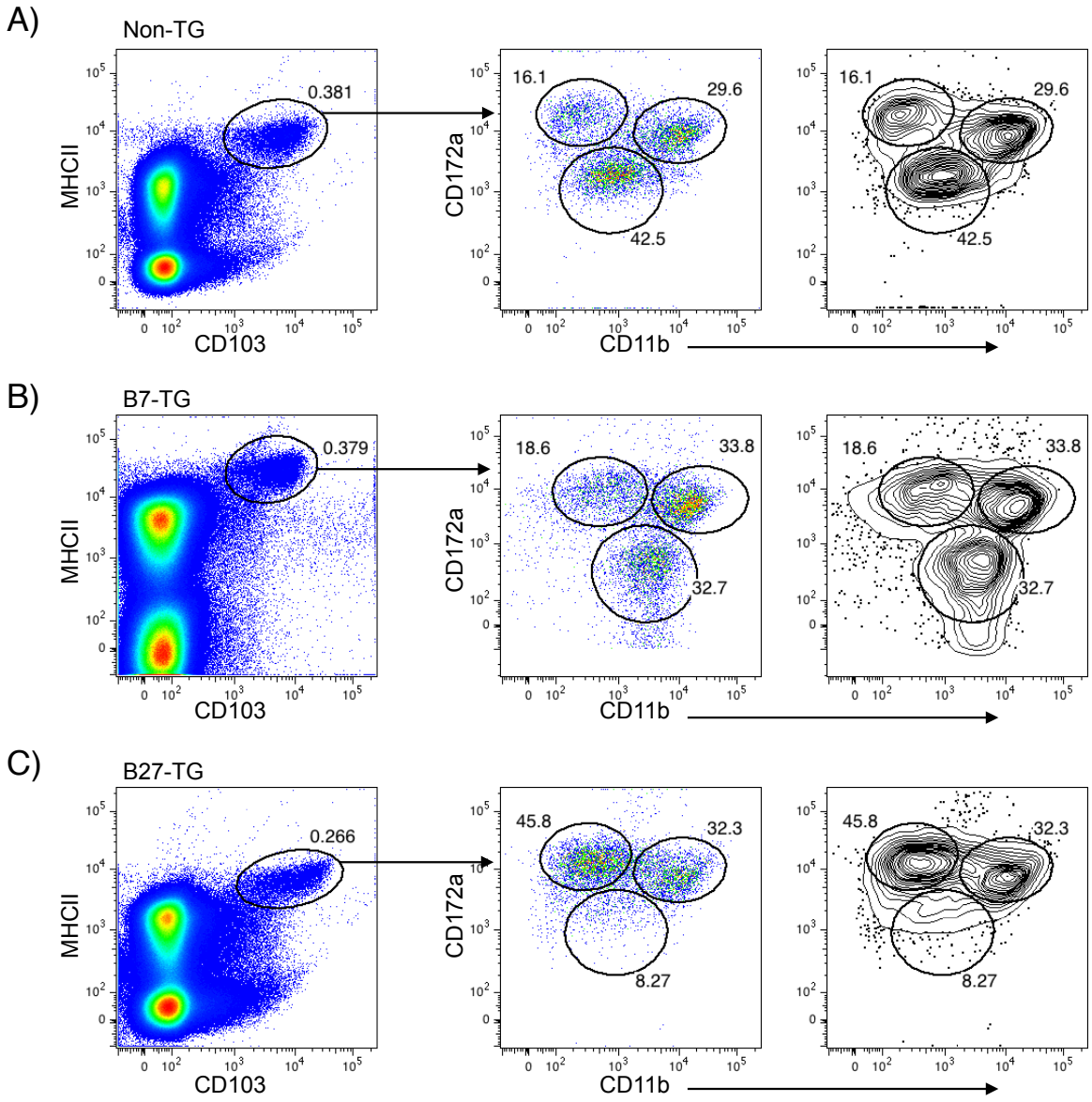


**Figure 4.12. B27-TG rats lack the CD172a$^{lo}$ subset of L-DCs.**

Cells were collected at 0-24h by thoracic duct cannulation, and stained with antibodies for CD103, MHCII, CD11b and CD172a to identify migrating DC subsets. (A) Three CD103$^{+}$ MHCII$^{+}$ subsets of L-DC can be identified in non-TG rat lymph: CD11b$^{lo}$CD172a$^{hi}$, CD11b$^{hi}$CD172a$^{int}$ and CD11b$^{int}$CD172a$^{lo}$. (B) Only two L-DC subsets can be found in B27-TG rat lymph. In control B7-TG rats all three subsets can be found (C). Data are summarised in the next figure.

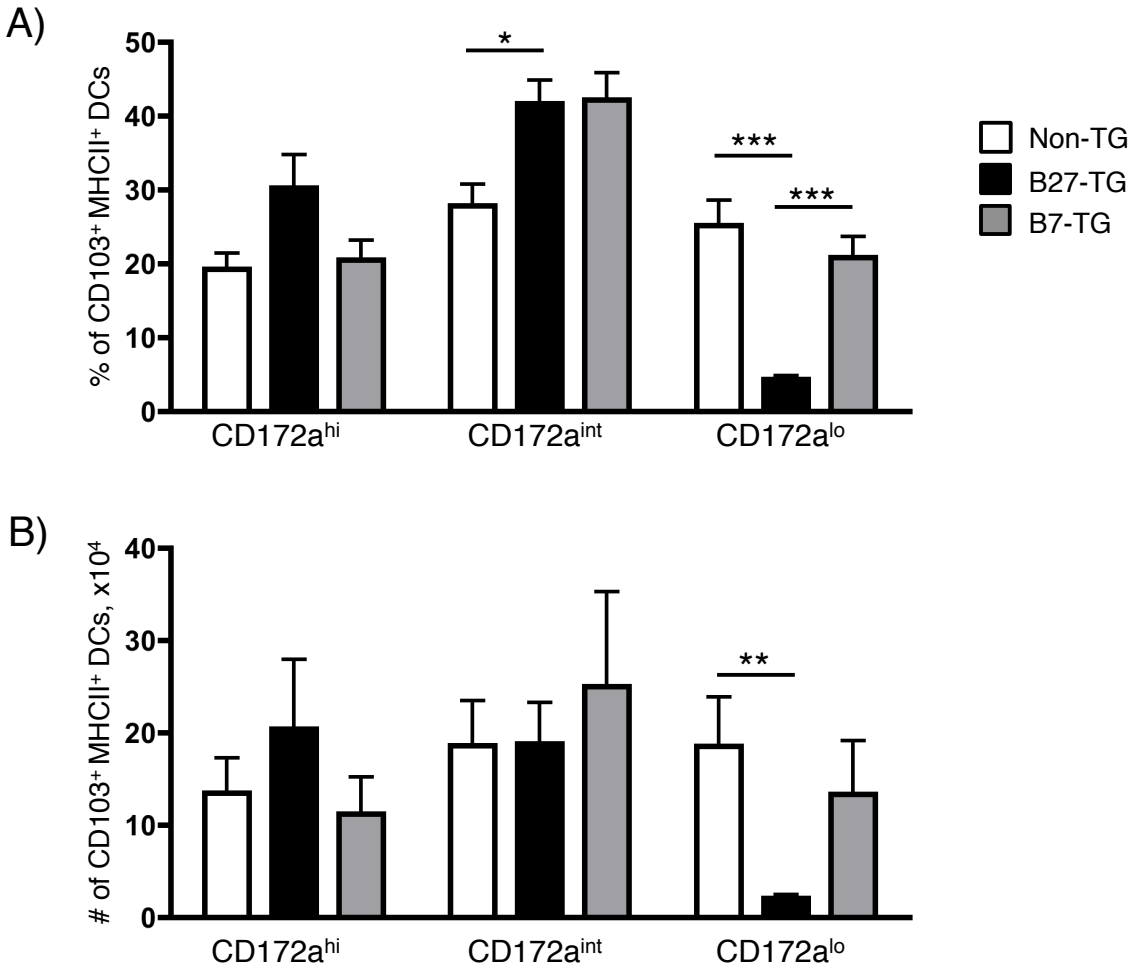


**Figure 4.13. B27-TG rats lack the CD172a$^{lo}$ L-DC subset**

Data from 6 (for non-TG and B27-TG) or 1 (for B7-TG) independent cannulation experiments were compiled for 0-24h timepoint, and the percentage and absolute number of DCs in each subset was calculated. (A) Percentage of each subset from total MHCII$^+$ CD103$^+$ DCs, and of each subset from total CD103$^+$MHCII$^+$ DCs. Error bars are SEM, and data were analysed by 1-way Anova, *p<0.05, **p<0.01, ***p<0.001. Total number of animals used: 4 (B7-TG), 7 (non-TG) and 12 (B27-TG).

### 4.2.5 Expression of CD80 and CD25 on L-DC subsets

As it was noticed earlier that there were small differences in the expression of CD25, as well as CD80, on MHCII$^{+}$ CD103$^{+}$ DCs between B27-TG and control non-TG animals (Figures 4.3 - 5), it was next determined if these differences could traced back to a specific L-DC subset. As before, rats were cannulated, and lymph cells were stained with MHCII, CD103, CD11b, CD172a, and either CD25 or CD80. Non-TG CD80 expression was similar between the CD172a$^{hi}$ and CD172a$^{int}$ DC subsets, but the CD172a$^{lo}$ subset expressed a slightly lower level of CD80 both at 0-24h (Figure 4.14A) and 24-48h (Figure 4.14B). This finding could explain the difference in CD80 expression that was observed when total MHCII$^{+}$ CD103$^{+}$ DCs were used (Figures 4.4 - 6), as B27-TG animals lack this CD172a$^{lo}$ subset that on non-TG animals expresses less CD80. It was then investigated if the increase in CD25 expression noted earlier on B27-TG derived cells could be attributed to one subset upregulating the expression of CD25, or if both subsets upregulated it equally. Figure 4.15 is a representative of one experiment out of two, and as can be seen on B27-TG derived cells, it is the CD172a$^{hi}$ subset that expresses more CD25. In contrast, all three non-TG L-DC subsets express CD25 on an equally low level.

### 4.2.6 Are B27-TG L-DCs more susceptible to apoptosis?

As it was noted that the CD172a$^{lo}$ subset was missing from the B27-TG animals, it was hypothesised that this could be because these cells are more susceptible to apoptosis. There is some evidence supporting this hypothesis, as it has been reported that the correlating B27-TG CD103$^{+}$ population in the spleen is more susceptible to apoptosis (239), and in addition it has been reported that this population survived less well than the CD172a$^{hi}$ population when cultured with GM-CSF (273). Thus, non-TG and B27-TG animals were cannulated and lymph cells stained with L-DC markers CD103 and MHCII, as well as Annexin-V and DAPI to assess if more cells in the B27-TG animals were undergoing apoptosis. First, the proportion of live (Annexin-V$^{-}$ DAPI$^{-}$ ) cells in the total lymph cell population were compared between non-TG (Figure 4.16A) and B27-TG animals (Figure 4.16B). No obvious differences were noticed, as the proportion of live cells was similar in the lymph of both non-TG and B27-TG animals. Next it was postulated that maybe the difference in viability of L-DCs would be too small to be seen when looking at the total lymph cell population, so the gating strategy was changed. This time, cells were first gated on MHCII$^{+}$ CD103$^{+}$ cells, and Annexin-V and DAPI expression on these cells was assessed on non-TG (Figure 4.16C) and B27-TG (Figure 4.16D) animals. There were no

differences in the number of live, apoptotic (Annexin-V+) or dead (DAPI+) MHCII$^{+}$ CD103$^{+}$ cells between non-TG and B27-TG L-DCs.

Since there were no differences in the viability of non-TG and B27-TG MHCII$^{+}$ CD103$^{+}$ DCs, and as it is not possible to directly investigate the CD172a$^{lo}$ population from B27-TG animals, it was decided to examine whether the CD172a$^{lo}$ population in the non-TG animals would be more susceptible to apoptosis. As was mentioned earlier, in culture CD172$^{lo}$ cells survive less well than the CD172a$^{hi}$ cells, and thus it was thought that maybe it was an inbuilt character of these cells. Thus, four non-TG rats were cannulated and their 0-24h-collection lymph was pooled together. Lymph cells were enriched for CD103 using magnetic beads, stained for CD103 and MHCII and double positive L-DCs were flow sorted. The sort resulted in 6x10$^{5}$ L-DCs, and these were divided into two wells in a 96-well plate (3x10$^{5}$ L-DCs/well) and were incubated overnight in complete media. The following day, cells were harvested from the wells and re-stained for CD103, MHCII, CD11b, CD172a and DAPI. Results for both replicates are shown on Figure 4.17. Cells were first gated on live (DAPI$^{-}$) MHCII$^{+}$ cells (Figure 4.17A, top panels), and the expression of CD103 on these cells was then analysed (Figure 4.17A, bottom panels). Firstly, it was noticed that majority (>90%) of the L-DCs had died when cultured overnight. In addition, the homogenous MHCII$^{+}$ CD103$^{+}$ population that had been sorted (see Figure 4.11 for an example of sorted cells), had divided into two separate populations: MHCII$^{hi}$ CD103$^{+}$ (R1) and MHCII$^{int}$ CD103$^{lo}$ (R2). As we wanted to find out whether the CD172a$^{lo}$ expressing cells were more prone to apoptosis, the expression of CD172a on these two populations was assessed (Figure 4.17C). Surprisingly, R1 and R2 populations also differed in their expression of CD172a: R1 cells were CD172a$^{hi}$, whereas R2 cells expressed only intermediate level of CD172a. When comparing the expression level of CD172a of all the MHCII$^{+}$ (R1+R2) cultured cells to that of freshly analysed MHCII$^{+}$ CD103$^{+}$ L-DCs from non-TG animals, it was noticed that actually the CD172a expression pattern on these two preparations was quite similar (Figure 4.17D). CD172a staining on B27-TG MHCII$^{+}$ CD103$^{+}$ was clearly different (Figure 4.17D). Thus, these results do not indicate a reduction in the non-TG CD172a$^{lo}$ population after over-night culture.

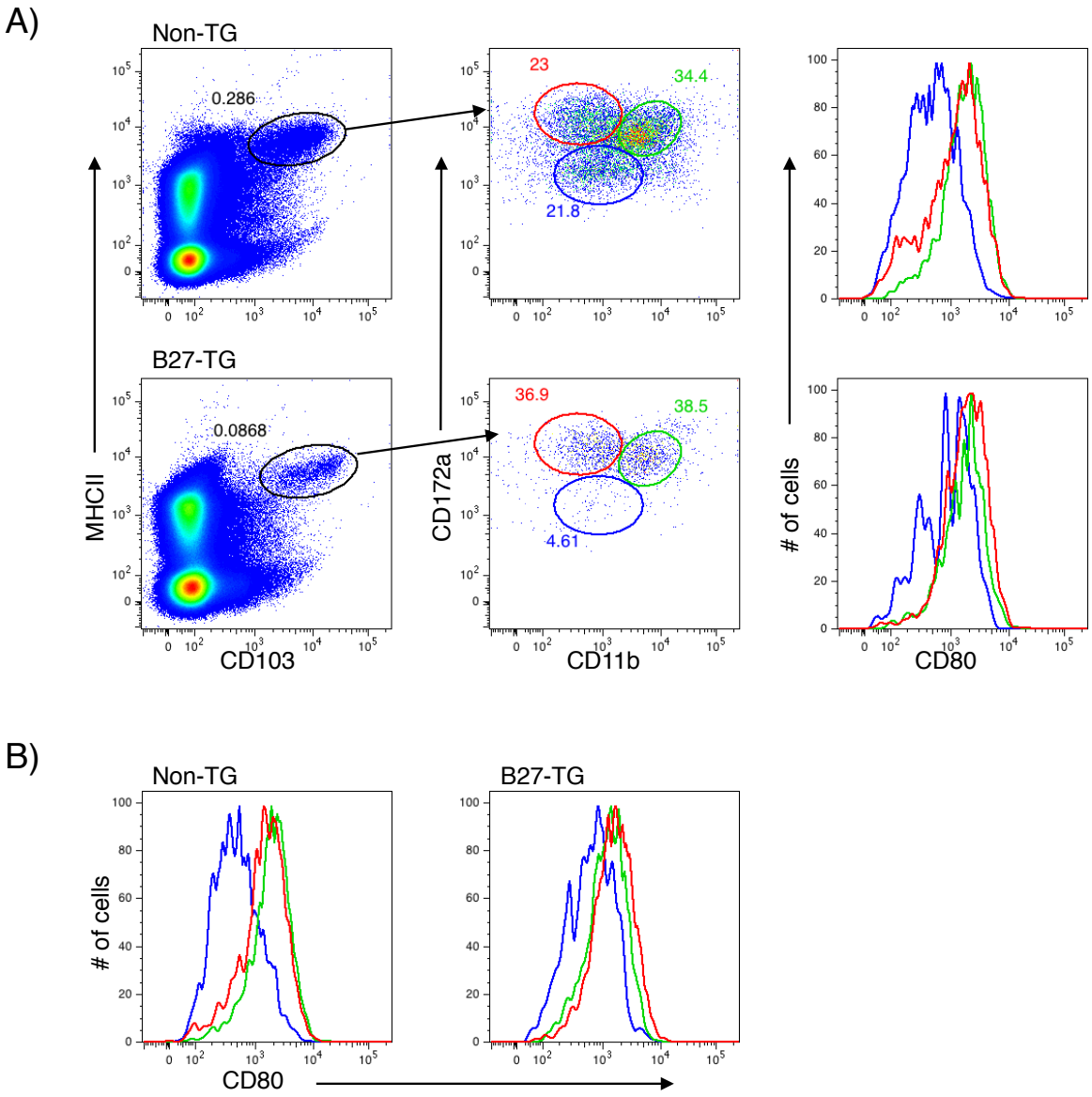


**Figure 4.14. Expression of CD80 by L-DC subsets**

Lymph cells were collected by thoracic duct cannulation as before, and the cells were stained for CD103, MHCII, CD11b, CD172a and CD80, and the expression level of CD80 in the different L-DC subsets was determined by FACS. (A) Lymph was collected for 0-24h, and CD80 expression was determined on the three DC subsets in both Non-TG (upper panels) and B27-TG animals (lower panels). (B) CD80 expression in the subsets on the 24-48h collection. Gates show the frequency of parent, and the gate colours on the dot plots match those on the histograms, n=1.

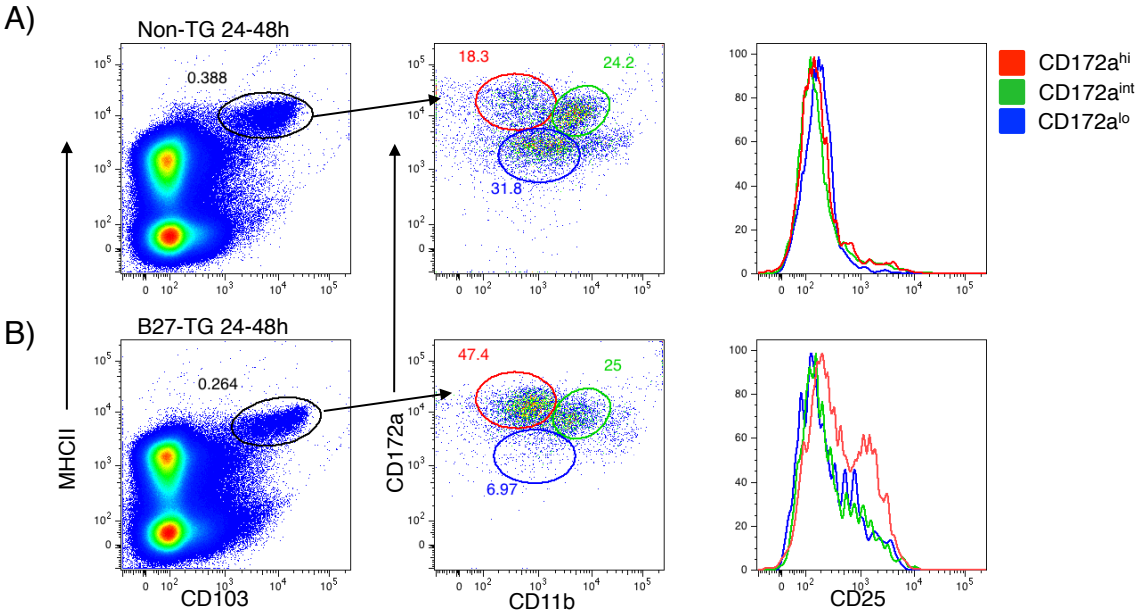


**Figure 4.15. CD172a$^{hi}$ L-DC subset from B27-TG animals might be the population expressing CD25 more highly.**

Lymph was collected from 24-48h, and the cells stained for CD103, MHCII, CD11b, CD172a and CD25, and the expression of CD25 on the three different L-DC subsets from non-TG (A) and B27-TG (B) animals was determined by FACS. Gates show the frequency of the parent and the gate colours on the dot plots match those on the histograms. This is a representative data from 2 independent experiments.

Since there were no differences in the viability of non-TG and B27-TG MHCII$^{+}$ CD103$^{+}$ DCs, and as it is not possible to directly investigate the CD172a$^{lo}$ population from B27-TG animals, it was decided to examine whether the CD172a$^{lo}$ population in the non-TG animals would be more susceptible to apoptosis. As was mentioned earlier, in culture CD172$^{lo}$ cells survive less well than the CD172a$^{hi}$ cells, and thus it was thought that maybe it was an inbuilt character of these cells. Thus, four non-TG rats were cannulated and their 0-24h-collection lymph was pooled together. Lymph cells were enriched for CD103 using magnetic beads, stained for CD103 and MHCII and double positive L-DCs were flow sorted. The sort resulted in 6x10$^{5}$ L-DCs, and these were divided into two wells in a 96-well plate (3x10$^{5}$ L-DCs/well) and were incubated overnight in complete media. The following day, cells were harvested from the wells and re-stained for CD103, MHCII, CD11b, CD172a and DAPI. Results for both replicates are shown on Figure 4.17. Cells were first gated on live (DAPI$^{-}$) MHCII$^{+}$ cells (Figure 4.17A, top panels), and the expression of CD103 on these cells was then analysed (Figure 4.17A, bottom panels). Firstly, it was noticed that majority (>90%) of the L-DCs had died when cultured overnight. In addition, the homogenous MHCII$^{+}$ CD103$^{+}$ population that had been sorted (see Figure 4.11 for an example of sorted cells), had divided into two separate populations: MHCII$^{hi}$ CD103$^{+}$ (R1) and MHCII$^{int}$ CD103$^{lo}$ (R2). As we wanted to find out whether the CD172a$^{lo}$ expressing cells were more prone to apoptosis, the expression of CD172a on these two populations was assessed (Figure 4.17C). Surprisingly, R1 and R2 populations also differed in their expression of CD172a: R1 cells were CD172a$^{hi}$, whereas R2 cells expressed only intermediate level of CD172a. When comparing the expression level of CD172a of all the MHCII$^{+}$ (R1+R2) cultured cells to that of freshly analysed MHCII$^{+}$ CD103$^{+}$ L-DCs from non-TG animals, it was noticed that actually the CD172a expression pattern on these two preparations was quite similar (Figure 4.17D). CD172a staining on B27-TG MHCII$^{+}$ CD103$^{+}$ was clearly different (Figure 4.17D). Thus, these results do not indicate a reduction in the non-TG CD172a$^{lo}$ population after over-night culture.

Since non-TG cells were not expected to be more prone to apoptosis, it was decided to repeat the experiment above, but this time, induce apoptosis on the non-TG cells. To induce apoptosis, a compound called thapsigargin was used. Thapsigargin binds to endoplasmic reticulum $Ca^{2+}$ ATPases, inhibiting the function of $Ca^{2+}$ pumps, and thus raises the cytosolic $Ca^{2+}$ level, causing ER stress and apoptosis (274). B27-TG cells have been reported to undergo more ER stress compared to non-TG cells (250-252). The 24-48h-collection of lymph from the same cannulation as in Figure 4.17 was used for the experiment.

As before, $6x10^5$ $MHCII^+$ $CD103^+$ DCs were sorted, but this time they were divided into three wells of a 96-well plate, $2x10^5$ cells/well. Two different protocols were used to induce apoptosis with thapsigargin; either 4 nM thapsigargin was incubated with the cells overnight (Figure 4.18B), or 400 nM thapsigargin was added to the culture for the final 3h of the incubation (Figure 4.18C). One well was left untreated (Figure 4.18A). The higher concentration of thapsigargin was selected as it was recommended by Dr Darren Asquith (personal communication) in the laboratory, whereas the low concentration was selected empirically by culturing BMDCs with various concentrations, and selecting the concentration that caused similar level of reduction in cell viability as was seen with 7-day culture of B27-TG BMDCs without thapsigargin (see Chapter 5 for BMDC analysis). The cells were analysed as before, by first gating on live $MHCII^+$ cells, followed by CD103. As in Figure 4.17, two distinct populations were again found based on MHCII and CD103 expression (Figure 4.18A-C, bottom panels). There were no differences in the proportions of these two populations between the groups. When the expression of CD172a on the two populations was compared between the treatments, no differences were noticed either in the R1 or R2 populations. It should also be noted that as there was no difference in the proportion of live cells between the groups (Figure 4.18A-C, top panels), it is possible that thapsigargin failed to induce apoptosis in these cells.

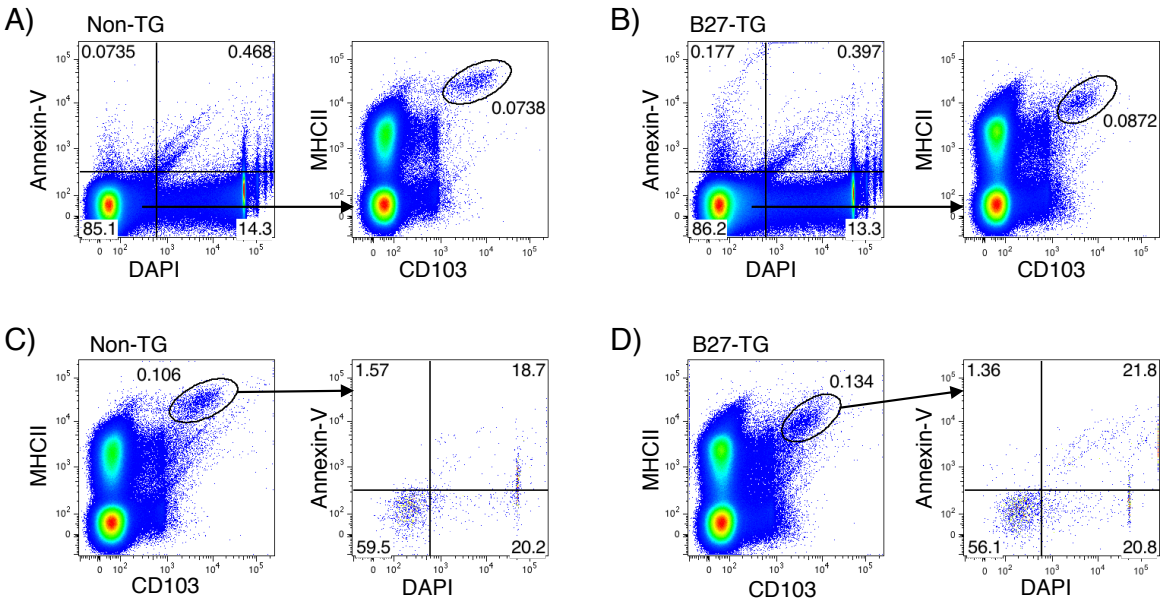


**Figure 4.16. No difference in the viability of lymph derived cells from non-TG and B27-TG animals**

Thoracic duct lymph was collected from non-TG and B27-TG animals, and stained with DAPI, Annexin-V, MHCII and CD103. Data were analysed in two different ways: In (A) and (B) cell viability of all lymph cells was determined by gating first on Annexin-V$^-$ DAPI$^-$ cells, and the expression of MHCII and CD103 was determined on these live cells. Viability of L-DCs was assessed by first selecting MHCII$^+$ CD103$^+$ cells, followed by gate for Annexin-V and DAPI (C, D). Numbers on plots refer to frequency of the parent. n=1.

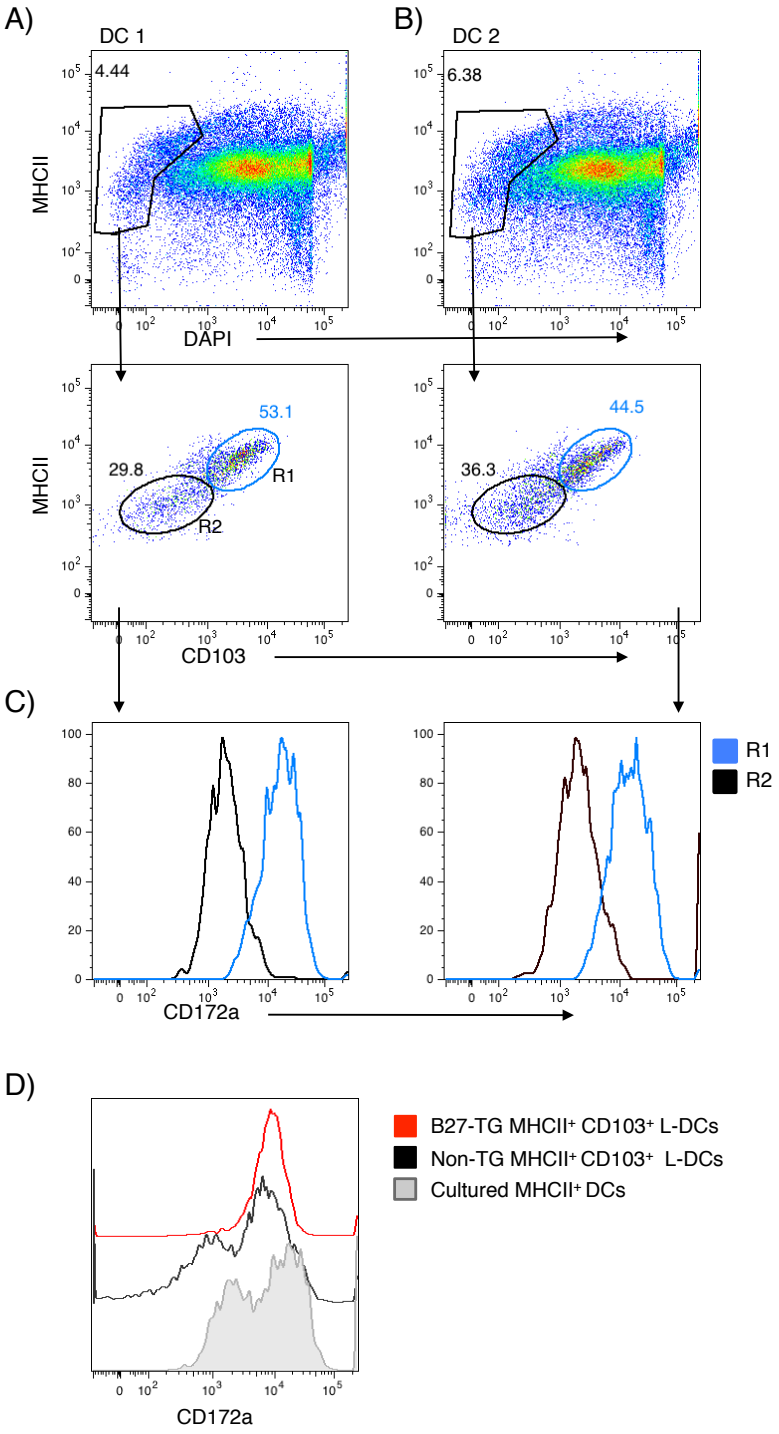


**Figure 4.17. In vitro culture of flow sorted L-DCs**

Lymph was collected from 4 non-TG rats at 0-24h and pooled together. Cells were then enriched for CD103 with magnetic beads and stained for MHCII and CD103. Double positive cells were sorted, and placed into 2 wells of a 96-well plate 200,000 cells/well in complete media for 12h at 37°C. After 12h, cells were harvested, and restained for DAPI, CD103, MHCII and CD172a. (A, B) Cells from two replicates (DC 1 and DC 2) were gated on live ($DAPI^-$) $MHCII^+$ cells. These were further divided into $CD103^{hi}$ (R1) and $CD103^{lo}$ (R2) populations. (C) CD172a expression on R1 and R2 populations from both replicates was determined by FACS, and shown as histogram overlays. (D) Expression of CD172a on all $MHCII^+$ cultured DCs (R1+R2) compared to expression on freshly isolated and analysed $MHCII^+$ $CD103^+$ DCs from non-TG and B27-TG animals. Numbers in figures refer to frequency of the parent.

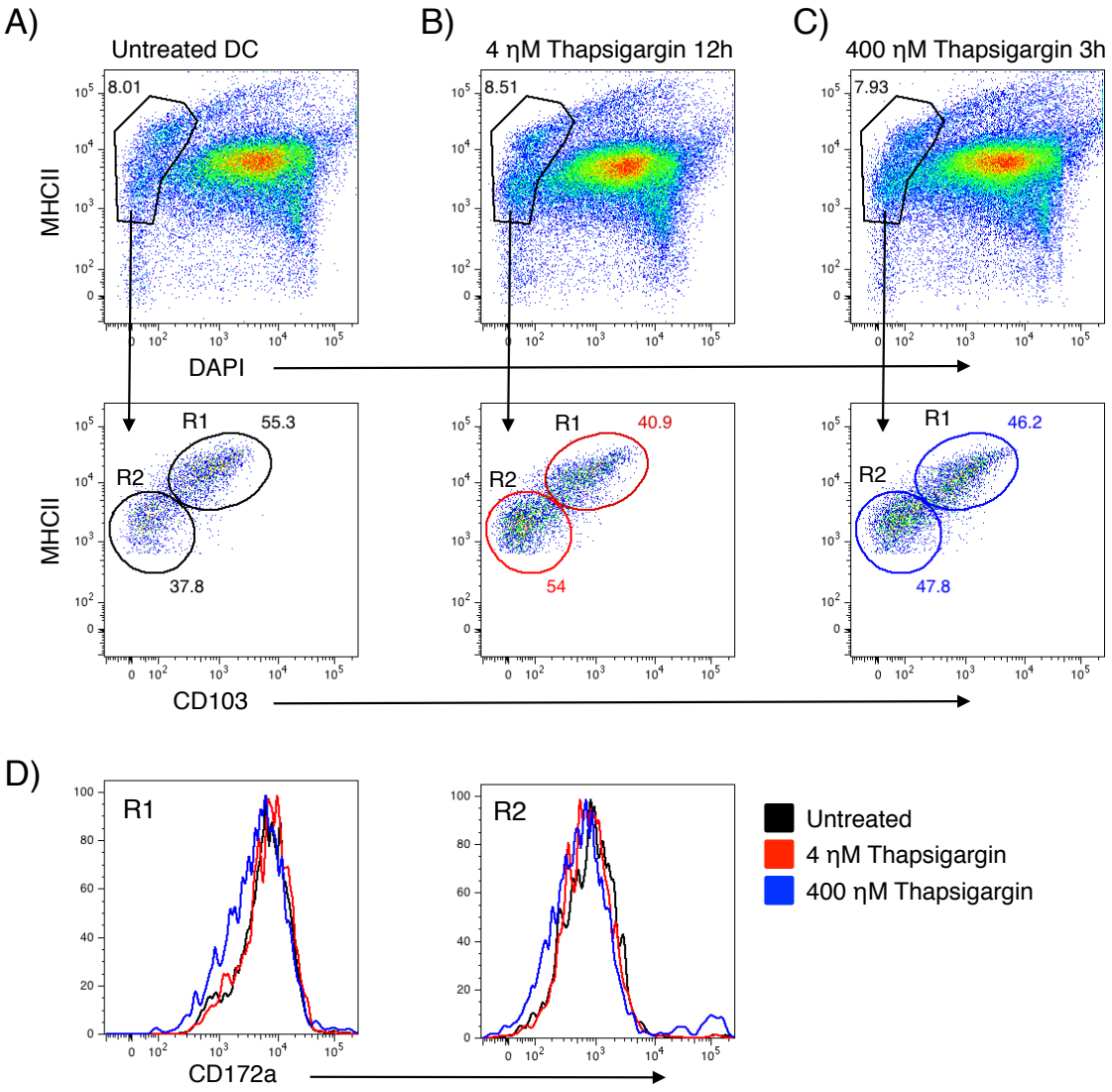


**Figure 4.18. In vitro culture of flow-sorted L-DCs with Thapsigargin**

24-48h lymph collection from 4 Non-TG rats was pooled together. Cells were enriched for CD103 with magnetic beads and stained for MHCII and CD103. Double positive DCs were sorted, and placed into 3 wells of a 96-well plate 200,000 cells/well in complete media +/- Thapsigargin for 12h at 37°C. After 12h, DCs were harvested and restained for DAPI, CD103, MHCII and CD172a. (A, B, C) Live MHCII$^{+}$ Dapi$^{-}$ cells were selected, and the expression of CD103 on these cells was determined in three different conditions: DCs cultured in complete media only (A), DCs treated with 4 ηM Thapsigargin for 12h (B) and DCs treated with 400 ηM Thapsigargin for the final 3h of the 12h culture. (C) Histogram overlays depicting expression of CD172a on MHCII$^{+}$ CD103$^{+}$ (R1) and MHCII$^{+}$ CD103$^{-}$ (R2) cells in all three conditions shown above. Numbers shown in figures are frequency of the parent.

## 4.3 Discussion

B27-TG animals have been used extensively to investigate the pathogenesis of SpA. Other investigators have demonstrated roles for both $CD4^+$ T lymphocytes (233, 234) and DCs (238, 239) in the disease. However, the mechanism by which DCs may influence the development of inflammation has been unclear. As both patients and the B27-TG animals present with intestinal symptoms, it was hypothesised that DCs that migrate from the intestine to the MLN would have a role in the disease pathogenesis. Collection of migrating intestinal DCs by thoracic duct cannulation of mesenteric lymphadenectomised rats is an established method used to study the phenotype and properties of these cells (268). Here, this technique was used, for the first time, to investigate the properties L-DCs in B27-TG rats.

It was first determined if the total number of cells in the lymph of non-TG and B27-TG animals would be the same. No differences were expected and indeed it was found that there was no defect in the general migration of cells as the total cell numbers collected from the non-TG and B27-TG animals were similar. Furthermore, it was established that the cell number/24h remained constant throughout the three-day collection of the lymph, despite the animals receiving i.v. saline for the first 24h after cannulation. As shown, this leads to a significantly larger volume of lymph on 0-24h-collection, compared to 24-48h.

At the time of surgery the rats are a minimum of 10 weeks old, and some may present with signs of intestinal inflammation at this time point. Inflammation in the intestine leads to activation of DCs situated at the site of inflammation. DC activation in turn leads to increased migration of these cells out of the intestine and into the MLNs via lymph. Indeed, it has been shown in rats and mice that during acute inflammation or TLR stimulation the number of migrating DCs from the intestine increases dramatically (185). Thus, it was hypothesised that B27-TG animals could have increased number of DCs in the lymph due to inflammation in the intestine. Migrating DCs in the rat are characterised as $MHCII^+$ $CD103^+$, and they have been shown to be able to stimulate proliferation and differentiation from naïve allogeneic $CD4^+$ T lymphocytes (184), a DC hallmark. Surprisingly, instead of increased DC numbers, it was found that the B27-TG L-DCs were significantly decreased both in proportion and in absolute numbers compared to non-TG animals at 0-24h timepoint. This observation could be caused by B27-TG animals having less DCs in the intestine in general, maybe due to chronic inflammation causing long-term increased DC migration out of the intestine. However, no difference in DC numbers

between B27-TG and B27-TG animals at other time points were observed, suggesting that this is not the case. Ideally, the number of DCs in the intestine would be determined by comparing the DC numbers in the intestinal tissues of non-TG and B27-TG animals to see if same numbers are present. This could be achieved by microscopy, as the necessary physical and enzymatic disruption of tissues necessary for FACS analysis would be likely to alter the functions and phenotype of these cells and thus make it difficult to identify them. Also, the animals could be treated orally with TLR7/8 ligand R848, which has been shown to lead to an increased migration of L-DCs from the small intestine, and to an almost complete depletion of DCs within lamina propria (120). This would allow a comparison of DC numbers present in the small intestine of non-TG and B27-TG animals. Also, as it was later noticed that B27-TG animals lack one of the three migrating DC subsets. This could lead to decreased total number of DCs if the other two subsets do not compensate for the loss.

It was next decided to determine if L-DCs from B27-TG and non-TG animals would express activation and costimulatory molecules similarly. It has been reported before that $MHCII^+$ $CD103^+$ DCs from non-TG animals express only low levels of CD80, CD86 and CD25 (189), thus being semi-mature cells. However, in these experiments L-DCs were found to express intermediate levels of CD80 and CD86, whereas the expression on CD25 was low, as reported. The higher level of CD80 and CD86 observed here compared to previously published results could be explained by slight differences in the protocols used, such as antibody concentration or clone, or length of staining with the antibody. When comparing the expression of CD86 between the different time points, it was noticed that CD86 was expressed at a lower level at 48-72h time point compared to the other two time points. It is not known why this would be the case, but there were only two replicates for this time point, so more repeats would be required to see if the phenomenon is real. There was no difference, however, in the expression of CD86 between non-TG and B27-TG animals in any of the time points. When analysing the CD80 expression, more $MHCII^+$ $CD103^+$ cells from B27-TG animals compared to non-TG expressed CD80 at all the time points, but this was not statistically significant. In addition, there were more $CD25^+$ cells in the B27-TG lymph compared to non-TG in all the time points, but it only reached significance at the 0-24h time point. Interestingly, the pattern of CD25 expression on B27-TG animals resembles that observed on non-TG animals after stimulation with various TLR2 and TLR3 ligands (189). CD25 has been reported to be a very sensitive marker of activation on rat DCs, and it is upregulated very quickly when the cells are exposed to GM-CSF *in vitro* (272). GM-CSF, on the other hand, is normally present *in vivo* at very

low levels, but its production it rapidly upregulated under inflammatory conditions. Thus, it could be that in the B27-TG rat small intestine more GM-CSF or some other inflammatory cytokines are present, due to inflammation, and the B27-TG L-DCs would be exposed to these factors before migrating to the MLN, leading the DCs to be more activated. In addition, increased numbers of Th17 cells have been reported in the colonic LP of B27-TG animals (252), and splenic DCs from B27-TG animals have been noted to preferentially induce Th17 cells (144) and GM-CSF is one of the Th17 effector cytokines (275). Thus, it could be that inflammation and defective DCs lead to induction of Th17 cells, which then in turn activates DCs to upregulate CD25 expression and migrate to the MLN.

In addition to markers mentioned above, expression of OX-40L was also analysed, as this has been shown to be an important molecule driving Th2 cell proliferation and maturation by intestinal DCs (276). Difference in the expression level of this molecule between non-TG and B27-TG animals could give a hint about functional differences the DCs could have. However, the expression level for OX-40L was found to be low, and there were no differences between non-TG and B27-TG animals. It is possible, though, that this marker is only upregulated once the cells reach MLN and are exposed to the microenvironment there, and thus it would be interesting to see if OX-40L was upregulated if the DCs were stimulated *in vitro,* as indicated above. It has also been reported that splenic DCs from B27-TG animals express lower level of MHCII on their surface (239), and it was analysed if this was the case for the B27-TG $MHCII^+$ $CD103^+$ L-DCs. When analysing the results from individual experiments, it was often observed that MHCII was lower on B27-TG animals, however when data from several experiments were collated, the difference in MHCII expression between non-TG and B27-TG L-DCs was not statistically significant. There was always some variation in the intensity of the MHCII staining between individual experiments, probably due to slight differences in cell numbers used for each staining. Also, it is in general difficult to assess the level of MHCII expression on DCs as they express so much of it on their surface, and thus careful measurements must be made to find out the concentration of antibody that saturates the cells.

As it was observed that a proportion of B27-TG DCs had a more activated phenotype with more $CD25^+$ cells, it was hypothesis that B27-TG L-DCs would be better able to stimulate naïve $CD4^+$ T cell proliferation. When measuring proliferation using total MLN cells derived from DA animals and a thymidine-based assay, there were differences within the individual experiments, but when comparing five individual experiments, B27-TG cells

were better as often as the non-TG ones. Importantly though, both non-TG and B27-TG L-DC were able to induce cell proliferation, implicating that the B27-TG DCs are functioning normally. When using a CFSE cell proliferation assay, and specifically investigating the proliferation of $CD4^+$ cells, non-TG cells seemed to be better in this one experiment. However, this experiment has only been done once, so should be repeated to obtain consistent results. The MLR done with sorted naïve $CD4^+$ T cells is much more specific, and also one can be sure that the same number of naïve cells is added for each for each condition. On the other hand, when using total MLN cells, it is not sure how many naïve cells are actually used. Thus, variations in the number of naïve T cells in the MLN could have an effect on the reproducibility of the MLRs observed on the thymidine based assays. It could also be that non-TG and B27-TG L-DCs actually are comparable in their ability induce T cell proliferation, but there could be a difference in the outcome of the T cell activation. For example, it has been shown that B27-TG splenic DCs induce more Th17 T cells compared to non-TG DCs (144), which induce more IFN-γ and thus Th1 type responses. Thus, luminex was performed to measure cytokine production by the T cells when activated in a MLR. Supernatants from the MLR with sorted naïve $CD4^+$ T cells were used. Contrary to our prediction, non-TG DCs induced more IL-17F as well as IFN-γ than B27-TG DCs, whereas better IL-6 and IL-2 induction from non-TG L-DCs was expected based on the poor proliferation by B27-TG DCs. If true, these results indicate that intestinal migrating DCs behave differently from their splenic counterparts, and this merits some more investigation. However, as this is a result from just one MLR experiment, which itself has only been performed once, it is not sure how reliable these results are. In addition, in the light of experiments from other groups that have reported increased IL-23 production in the B27-TG animals, it would also be important to see if the L-DCs themselves differ in their production of cytokines after stimulation.

It is well established that there are three $MHCII^+$ $CD103^+$ L-DC subsets present in the rat lymph: $CD172a^{hi}$ $CD11b^{lo}$, $CD172^{int}$ $CD11b^{hi}$ and $CD172a^{lo}$ $CD11b^{int}$ . These subsets seem to differ somewhat morphologically. $CD172a^{hi}$ and $CD172a^{int}$ populations consist of mononuclear cells, with $CD172a^{int}$ cells being slightly smaller in size than $CD172a^{hi}$ cells. $CD172a^{lo}$ cells, however, are smaller, and have more of a textbook DC appearance with visible dendrites.

It was next established whether all three L-DC subsets were present at similar frequencies in the lymph of control B7-TG and non-TG animals, as well as B27-TG animals. Surprisingly, lymph from B27-TG animals lacks the $CD172a^{lo}$ DC subset. This

phenomenon has never been reported before, as in other models of inflammation DCs have been shown to maintain similar relative frequencies (185, 188). The CD172a$^{lo}$ subset was significantly reduced both proportionally and in absolute numbers. The lack of this subset could explain the reduction in the absolute number of B27-TG MHCII$^{+}$ CD103$^{+}$ DCs observed at 0-24h timepoint before, however it does not explain why there was no difference at 24-48h timepoint. We then hypothesised that the differences in CD80, CD86 and CD25 expression seen when comparing total MHCII$^{+}$ CD103$^{+}$ L-DCs could be explained by the DC subsets being present in different proportions in non-TG and B27-TG animals. Indeed, when the CD80 expression on each of the subsets was analysed, it was noticed that the CD172a$^{lo}$ subset in non-TG animals seemed to express lowest level of CD80. As a result, B27-TG animals, which lack this subset, would appear to have more CD80$^{+}$ L-DCs. In contrast, when the expression of CD25 was analysed in all three subsets, it was noticed that it was the CD172a$^{hi}$ subset in B27-TG animals that contained most CD25$^{+}$ cells. Hence, B27-TG L-DCs appear more activated by having an increased expression of CD25 on the CD172a$^{hi}$ subset.

In this context, it is then somewhat curious that B27-TG L-DCs were less effective at stimulating CD4$^{+}$ T cell proliferation when a more refined method of MLR with CFSE was used. It would, of course, be good to sort all three subsets separately and perform an MLR, and analyse if the subsets differ in their abilities. It is very difficult, however, to obtain enough L-DCs to perform this kind of functional analyses and it would require pooling of the lymph from several animals. Even if we did not observe the expected differences between B27-TG and non-TG DCs in the MLRs, it does not rule out a DC defect. Indeed, preliminary data from our laboratory (Drs Dawn Firmin and Aude Aumeunier, personal communication) suggest that T cells derived from non-TG and B27-TG MLNs do express different levels of homing markers, such as chemokine receptors CCR4 and CCR6 on them. In addition, our data and data from Maxime Breban's group (144) do indicate there is an increase in the number of activated CD4$^{+}$ CD25$^{+}$ T cells in the MLNs of B27-TG animals when compared to non-TG or B7-TG animals. Furthermore, other experiments by our group do indicate that B27-TG T cells sorted from the MLN, and transferred back to non-TG animals home preferentially in the small intestine, whereas this is not seen when non-TG cells are transferred to non-TG animals (preliminary data, Dr Dawn Firmin). Thus, it does seem DCs from B27-TG and non-TG animals do indeed induce different responses from T cells *in vivo*, even if there are no obvious differences in the ability to induce proliferation of CD4$^{+}$ T cells.

The nature of the subset that the B27-TG rats lack might also have a significant role on the induction or perpetuation of the disease in these animals. Previous work has revealed that the three subsets differ from one another in several ways. Only CD172a$^{hi}$ and CD172a$^{int}$ populations have been shown to upregulate CD25 expression after feeding with TLR7/8 agonist R848, they both express TLR8, and secrete cytokines in response to R848 *in vitro* (120). Furthermore, CD172a$^{hi}$ population has been shown to be the first subset migrating from the intestine to the MLN in response to feeding of TLR agonists (185), as well as having largest proportion of CD25$^{+}$ cells. The CD172a$^{lo}$ subset has been shown to have some unique properties compared to the other two subsets. For example, it has been shown to be less effective at inducing T cell proliferation than the other two, but instead it induces IL-10 production (184, 273). In addition, previous work has indicated that CD172a$^{lo}$ subset both in rats and in pigs contains cytoplasmic apoptotic DNA inclusions, and in rats also epithelial cell-restricted cytokeratins and non-specific esterase-positive inclusions (111, 115). These features have been shown to be present in DCs derived from germ-free animals as well, suggesting that these cells constitutively endocytose and transport apoptotic intestinal epithelial to the MLNs, even in the absence of bacterial stimuli (115). Thus, it has been suggested that the CD172a$^{lo}$ cells might be involved in the induction and maintenance peripheral self-tolerance to intestinal antigens. Mouse lymph also contains subsets of DCs, most notably CD8$^{+}$ CD103$^{+}$ CD11b$^{lo}$ (referred as CD8$^{+}$), CD11b$^{+}$ CD103$^{+}$ CD8$^{lo}$ (CD11b$^{+}$) and CD8$^{lo}$ CD11b$^{lo}$ CD103$^{-}$ (CD103$^{-}$) (113). Of these, CD8$^{+}$ cells have been suggested to resemble the CD172a$^{lo}$ subset, as they also pick up and present antigen from apoptotic cells. As the B27-TG animals lack CD172a$^{lo}$ population, they could have a deficiency in either maintenance or induction of self-tolerance. Taking into account that this CD172a$^{lo}$ subset has been noted to produce more anti-inflammatory IL-10, it could be that a presence of two highly active L-DC populations might lead to presentation of self-antigens in an inflammatory context, and to activation of self-reactive T cells. As mentioned above, preliminary data from our laboratory indicates that there might also be differences in the induction of homing molecules on T cells by DCs, and thus these auto-reactive T cells could end up homing to other places outside the intestine as well explaining the systemic disease seen both in humans and in the animal model.

Some might argue that the CD172a$^{lo}$ population is just a precursor for the CD172a$^{hi}$ and/or CD172a$^{int}$ subsets, and under inflammatory conditions CD172a$^{lo}$ DCs upregulate their CD172a expression, and become the more inflammatory-like CD172a$^{hi}$ DCs. This does not, however, seem to be the case, as previous elegant BrdU-labelling experiments show that the CD172a$^{lo}$ population is not a precursor to the CD172a$^{hi}$ DCs (76). In addition,

almost all the species investigated seem to have both CD172a$^{+/hi}$ and CD172a$^{lo/-}$ subsets, and they do seem to have separate ontogeny and functions. One possible experiment to investigate this problem further would be to isolate CD172a$^{lo}$ L-DCs from non-TG animals, and then adoptively transfer these cells to the B27-TG rats, and monitor the disease progression. This experiment is technically very difficult to accomplish, however, as the number of L-DCs needed for the transfer is very high, and the CD172a$^{lo}$ subset only comprises maximum of 30 % of all non-TG L-DCs, which in turn comprise 0.2 % of all lymph cells. Thus, a very large number of animals would be needed to perform a single transfer. It might be more feasible to transfer total MHCII$^{+}$ CD103$^{+}$ non-TG lymph DCs to irradiated B27-TG recipients, and see if all three subsets could be recovered from the recipients. If not, it implies there could an active and very specific unknown mechanism in place leading to depletion of CD172a$^{lo}$ L-DCs in B27-TG animals. And if all subsets were found, it could be that there is an inherent and maybe a genetic problem in the development of CD172a$^{lo}$ DCs in these animals.

It has been reported that a population of CD4$^{-}$ CD103$^{+}$ DCs in the spleen, which correspond to the CD172a$^{lo}$ DC subset in lymph, are more prone to apoptosis (239). Furthermore, as was mentioned before, it has been shown that the CD172a$^{lo}$ cells survive less well when cultured *in vitro* compared to CD172a$^{+}$ subsets (272). In addition, it has been previously shown the HLA-B27$^{+}$ molecule is prone to misfolding in the cells, leading to activation of unfolded protein response, and possibly to apoptosis (250). It was thus hypothesised that the L-DC CD172a$^{lo}$ subset might be more prone to die by apoptosis, and that is why it is missing in the B27-TG rats. In order to find out if this was the case, lymph cells from both non-TG and B27-TG animals were stained with Annexin-V and DAPI to analyse the proportions of apoptotic, dead and live cells. It was observed that, there was no difference in the proportion of live cells between non-TG and B27-TG animals either when looking at total live cells, or when looking at the Annexin-V and DAPI stain on MHCII$^{+}$ CD103$^{+}$ cells.

Since it is not possible to directly analyse properties of the missing CD172a$^{lo}$ subset from the B27-TG animals, it was decided to see if CD172a$^{lo}$ cells from non-TG animals were more prone to apoptosis when cultured in complete media overnight. It was noticed that most of the sorted DCs died after overnight culture, and in addition two MHCII$^{+}$ populations could be seen: MHCII$^{hi}$ CD103$^{hi}$ CD172a$^{hi}$ and MHCII$^{int}$ CD103$^{lo}$ CD172a$^{int}$. This already indicates that at least two CD172a expressing populations are present, and when expression of CD172a on all the cultured cells (MHCII$^{hi}$ + MHCII$^{int}$), was compared

to that of freshly analysed L-DCs, no difference in the staining pattern was observed. This indicates that no specific loss of one subset over another has taken place.

As the non-TG DCs are not expected to be more prone to spontaneous apoptosis, it was then decided to use thapsigargin to induce apoptosis in these cells, and see if this would make a difference to the CD172a$^{lo}$ population. Two different protocols were used: low concentration (4 nM) of thapsigargin for the duration of culture (12h) or high concentration (400 nM) for the last three hours of cell culture. Again, we observed two MHCII$^{+}$ populations. When the level of CD172a on these two populations was compared between the treatments, no differences were observed. More importantly, no differences in the percentage of DAPI$^{-}$ cells were observed between the treatments, which might indicate that thapsigargin had failed to induce apoptosis on these cells using this protocol. It should also be noted that on both experiments mentioned above, with or without thapsigargin, extremely few cells survived the overnight culture (less than 10%). This could be a result of the treatment the cells have to go thorough before they are even plated out: RBC lysis, many washes and spins, enrichment with magnetic beads, and cell sorting. Any, and all of these could stress the cells sufficiently enough to cause the low viability observed when cultured overnight. In addition, L-DCs are on their way to the MLN, where they would not live very long even in *in vivo*. But in previous experiments, where L-DCs were sorted to use them in MLR, they were perfectly capable of supporting T cell proliferation. This could indicate that L-DCs need other cells, such as T cells, with them in the culture to provide survival signals. Thus, this experiment could be repeated by maybe co-culturing the DCs together with T cells. However, using non-TG DCs to try and predict what might be happening with B27-TG DCs is never going to give a reliable answer, however it might point us to the right direction and open up new avenues of research.

In conclusion, it was observed that B27-TG animals lack the CD172a$^{lo}$ subset of migrating DCs, and in addition a population of DCs that are left appear to be more activated. This however did not translate to B27-TG L-DCs being more stimulatory in an allogeneic MLR. Cytokine production by T cells in the MLR was surprising, as contrary to expectations B27-TG DCs induced less IL-17F. However, these experiments should be repeated to confirm these observations. CD172a$^{lo}$ cells have been indicated to carry self-antigens from the periphery to the lymph nodes in steady state conditions, and thus the lack of these DCs in the B27-TG animals could lead to defect in the peripheral tolerance induction and thus to increased number of auto-reactive T cells in the circulation. Most of the previously published results indicating various defects or differences in B27-TG DC function and

phenotype have been obtained using splenic DCs, suggesting that DC defects in B27-TG animals are found in several locations, suggesting systemic defects. Thus, in the following chapter it was decided to investigate DCs found in the MLNs, as this is where DCs would normally interact with T cells, leading to either induction of immunity or to tolerance. DC development is dependent on Flt3L, as discussed in Introduction, and it could be that the lack of CD172a$^{lo}$ subset could be caused by a problem in the development of DCs from their precursors. Hence, in addition to MLN DCs, also DCs derived from their BM precursors and grown under Flt3L are investigated in the following chapter.

# 5. Mesenteric lymph node dendritic cells

## 5.1 Introduction

In the previous chapter it was shown that B27-TG animals lack one of the three subsets of migrating intestinal DCs, the CD11b$^{lo}$ CD172a$^{lo}$ cells. While this is an interesting observation, it raises many questions: Is the loss of this L-DC subset a cause or a symptom of the disease? How can a defect in intestinal L-DCs lead to disease in other peripheral tissues? Recent findings indicating defective functions of splenic B27-TG DCs, such as a failure to form stable DC-T cells complexes (237), as well as an observation that splenic DCs induce more Th17 cells than their non-TG counterparts (144), suggest that aberrant B27-TG DC functions are not limited to the intestinal compartment. Most of the work investigating the roles of DCs in the B27-TG animal model has involved the use of splenic DCs. There are several reasons for this: First, splenic DCs are well characterised and are easy to acquire. Second, it is possible to obtain large enough numbers of them to perform functional analyses. However, as there are links between intestinal inflammation and SpA in both humans and rats (reviewed in (49) and see Introduction), DCs isolated from mesenteric lymph nodes (MLNs) would provide more physiologically relevant information.

L-DCs carry antigen from the intestine to the MLN, where they can activate, or tolerise, naïve T cells. As these important events are taking place in the MLNs, and since MLNs are the destination for the L-DCs observed in the previous chapter, we were interested in analysing the phenotype of DCs in the MLNs of non-TG and B27-TG animals. In particular, *we hypothesised that the CD172a$^{lo}$ population that is missing in the lymph of B27-TG animals would also be missing from the MLN of these rats*, and this could have consequences on the activation of T cells. Identification of migrating DCs in the MLNs would provide the means to analyse DCs from younger animals, and address one of the questions above - whether the loss of the subset is a cause or a symptom of disease in the animals. The MLNX-cannulation process requires at least six weeks, and can only begin once animals reach approximately four weeks of age, which means that all the rats analysed using this approach are a minimum of 10 weeks old. At this stage, the animals already have established disease, and thus it is possible that inflammatory disease in these animals causes the loss of the subset by some unknown mechanism.

As was shown in the previous chapter, it is relatively easy to identify L-DCs in rat lymph, as they form a homogenous population of $MHCII^+$ $CD103^+$ cells. This is because there is no other cell type with similar characteristics present in the lymph draining from the intestine. However, identifying DCs in the peripheral tissues or secondary lymphoid organs, such as spleen or MLN, can be difficult as many other cell types are present that share a similar phenotype to DCs. In the following pages an analysis of MLN DCs derived from 10-16 week old animals is provided. Unfortunately, it was not possible to include analysis of MLN DCs from younger animals in this thesis. This could have provided some insight into whether the decrease in numbers of a specific subset of DCs, noticed in the lymph, precedes the onset of symptoms in these animals, or whether this loss is a consequence of disease.

## 5.2 Results

### *5.2.1 Increased cellularity of B27-TG MLNs*

First, it was evaluated whether there were differences in the general viability or number of cells from non-TG, B27-TG and B7-TG MLNs. MLNs were collected from non-TG and B27-TG animals, digested with DNase and liberase, passed through a cell strainer and stained with Annexin-V and DAPI to assess cell viability (Figure 5.1A). No difference in the overall viability of MLN cells was observed in this experiment. However, when MLNs were collected from B27-TG, B7-TG and non-TG, passed through a cell strainer and counted, B27-TG rats had significantly more total MLN cells compared to both non-TG and B7-TG animals (Figure 5.1B).

### *5.2.2 Identification of L-DCs in MLN preparations*

In MLNs, dendritic cells are tightly embedded in the tissue, and thus in order to extract and analyse them, the MLNs need to be enzymatically digested. To do this, MLNs collected from non-TG and B27-TG animals were digested using DNase and liberase, as indicated in Materials and Methods. Due to the lack of an effective CD11c-specific antibody in rats, which together with high MHCII expression is commonly used to identify murine DC populations, it is difficult to unambiguously identify DC populations with certainty in tissues other than lymph. Unlike lymph, MLN contains a mixture of resident DCs, macrophages and migrating DCs, which can share similar phenotypes. Furthermore, as shown in the previous chapter, L-DCs only comprise about 0.4 % of all lymph cells, and as such their proportion of all MLN cells is very small.

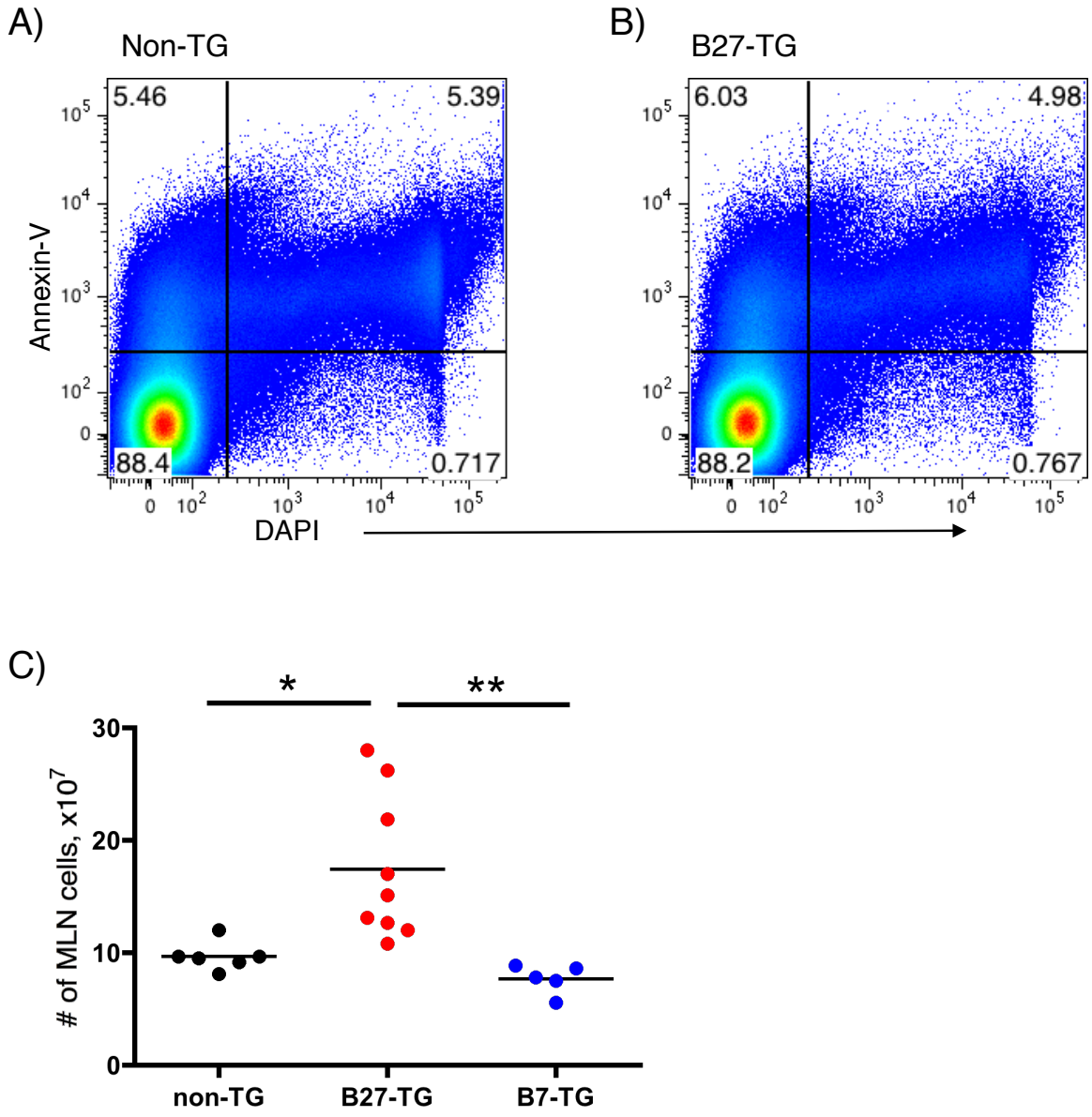


**Figure 5.1 B27-TG animals have more MLN cells**

(A)+(B) MLN cells from B27-TG and non-TG rats were collected in serum free medium, and digested with liberase and DNase. Cells in single cell suspension were stained with Annexin-V and DAPI, and the cell viability was assessed by FACS. Total MLN cell viability in a non-TG rat (A) and a B27-TG rat (B) are shown. Numbers refer to percentages of total cells. (C) MLNs were collected from non-TG, B27-TG, and B7-TG animals, passed through 40 µM cell strainer and counted. Each dot represents an individual animal. Results in (C) are from 7 individual experiments. Data was analysed by 1-way Anova. *p<0.05, **p<0.01.

As shown in Chapter 4, CD103, together with MHCII, is a very useful marker in identifying migrating L-DCs. Thus, it was first decided to enrich the MLN cells for CD103 in order to make it easier to identify this small migrating DC population. Single cell suspensions from non-TG MLNs were enriched for CD103 using magnetic beads, and the $CD103^+$ fraction was stained with LIN markers (Igκ chain, CD45RA, CD45RC and TCR α/β), DAPI, CD103, MHCII, CD11b and CD172a. Analysis of the LIN versus MHCII plot (Figure 5.2A) revealed two $MHCII^+$ $LIN^-$ populations: $MHCII^+$ (R1) and $MHCII^{hi}$ (R2). It was decided to analyse these populations separately, as resident DCs and macrophages in the MLN express less MHCII than the arriving migratory DCs. Thus, it was hypothesised that the $MHCII^{hi}$ population would include the migratory L-DCs. Indeed, it was first noticed that the $MHCII^{hi}$ cells were both bigger and more granular than the $MHCII^+$ cells (Figure 5.2B), with $MHCII^{hi}$ cells more closely resembling L-DCs from Chapter 4 in regards to FSC and SSC characteristics. Our hypothesis was further supported by the observation that $MHCII^{hi}$ cells were $CD103^+$, whereas the $MHCII^+$ cells expressed lower levels of CD103 (Figure 5.2C). CD11b and CD172a distinguish the three L-DC subsets in the lymph, and thus $MHCII^+$ and $MHCII^{hi}$ populations were analysed for their expression of these markers. It was found that whereas $MHCII^+$ cells were predominantly $CD11b^+$ $CD172a^+$, the $MHCII^{hi}$ cells formed a more heterogeneous population (Figure 5.2D, E). Thus, it is likely that $MHCII^{hi}$ cells contain the migratory DC population arriving from the lymph, whereas $MHCII^+$ cells might consist of MLN resident DCs.

### *5.2.3 Reduced proportion of $MHCII^+$ $CD103^+$ $CD172a^{lo}$ cells in B27-TG MLN*

Because it was possible that the enrichment with CD103-specific magnetic beads might make it more difficult to distinguish the $CD103^+$ and $CD103^-$ populations from each other or otherwise affect the results, it was decided to identify the DCs without the use of pre-enrichment. As before, MLN cells were collected from 10-16 week old F10 generation non-TG and B27-TG animals, digested with DNase and liberase and stained without pre-enrichment with antibodies specific for LIN, MHCII, CD103, CD11b and CD172a. As can be seen in Figure 5.3, it was not possible with certainty to separate the $LIN^-$ $MHCII^+$ cells into two populations as was done with the CD103 enriched sample before. Thus, all $LIN^-$ $MHCII^+$ cells were included in the analysis. However, as was shown with the CD103 enriched sample, the $MHCII^{hi}$ and $MHCII^+$ populations could be distinguished, first, according to their FSC and SSC characteristic, and secondly, according to their CD103 expression. Hence, the $FSC^{lo}$ $SSC^{lo}$ cells were gated out of the analysis (Figure 5.3, top right), followed by selection for $CD103^+$ cells.

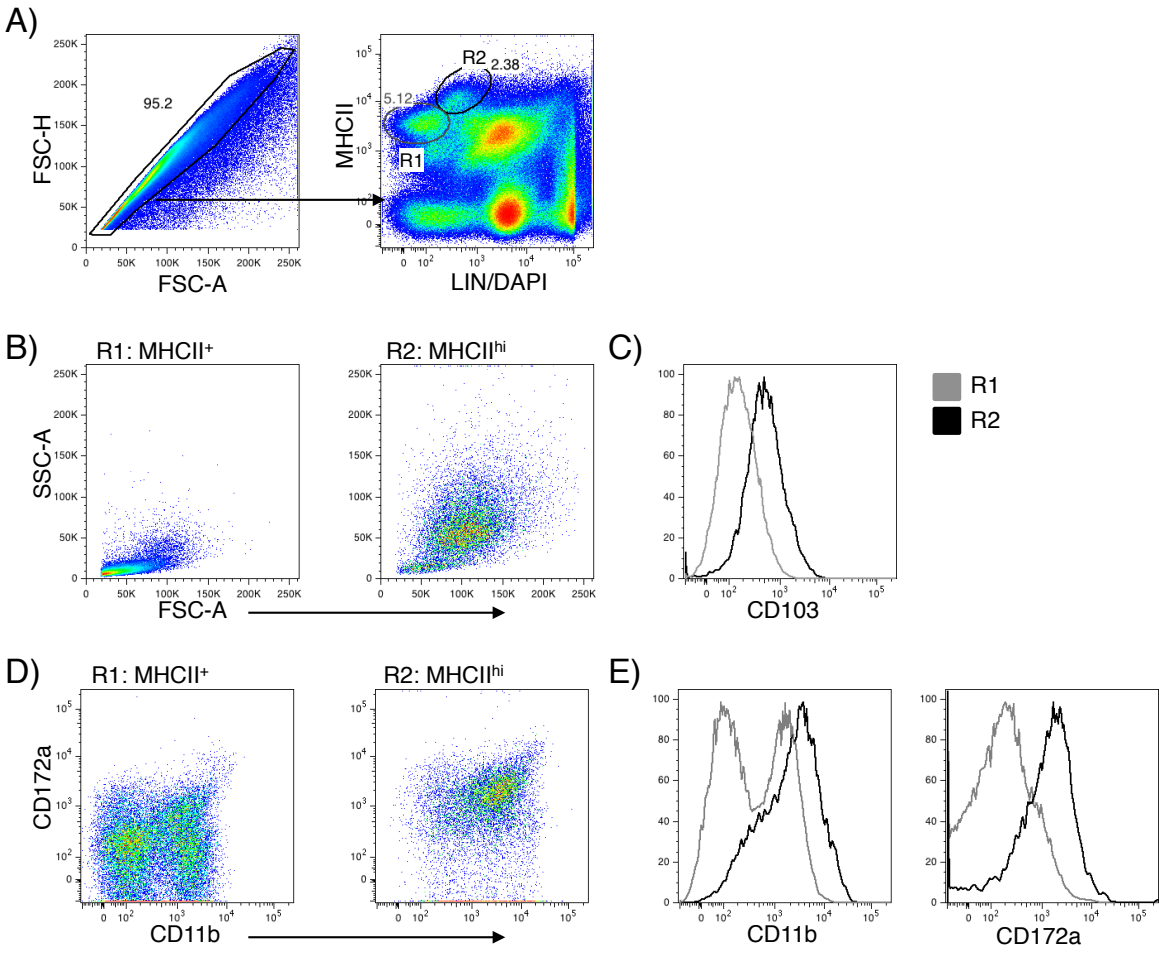


**Figure 5.2 Analysis of MLN DCs – enrichment for CD103 followed by FACS analysis**

MLNs were collected from a non-TG rat, and enriched for CD103 with magnetic beads. Cells were then stained with antibodies for LIN markers (CD45RA, CD45RC, TCRα/β, Igκ chain), MHCII, CD103, CD11b and CD172a and analysed by FACS. (A) Two MHCII$^{+}$ populations were analysed separately: MHCII$^{+}$ (R1) and MHCII$^{hi}$ (R2). FSC/SSC phenotype (B) and the expression level of CD103 (C) were determined for the two populations. (D) Expression of CD11b and CD172a on MHCII$^{+}$ and MHCII$^{hi}$ populations shown as a dot plot and (E) as a histogram overlay. This experiment has been only been performed once.

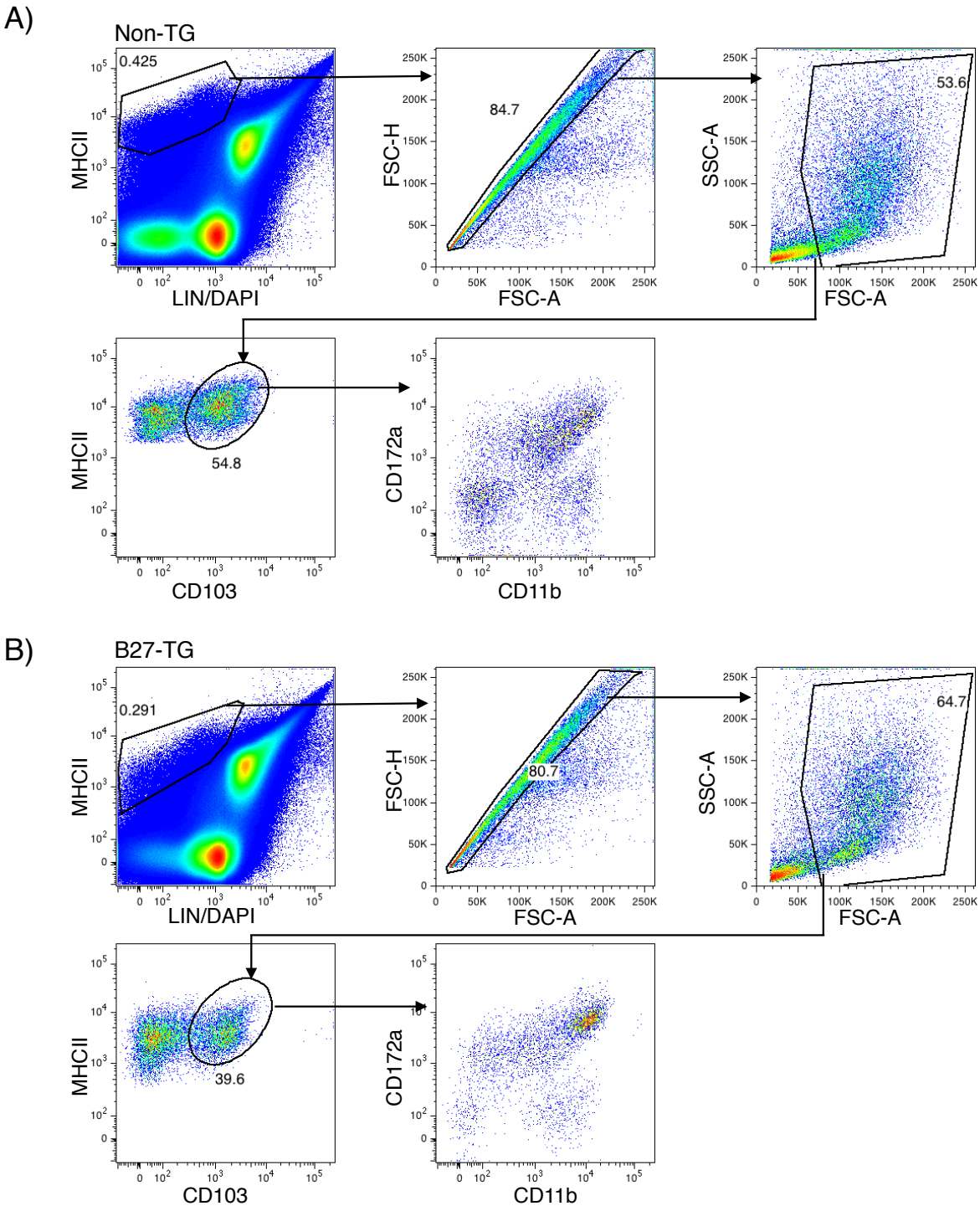


**Figure 5.3 Analysis of MLN DCs – without CD103 enrichment**

MLN cells were collected from both non-TG and B27-TG rats, digested with liberase and DNase, and stained with LIN markers (CD45RA, CD45RC, TCRα/β, Igκ chain), DAPI, MHCII, CD103, CD11b and CD172a. (A) Gating strategy for non-TG MLN cells. (B) Gating for B27-TG MLN cells.

Comparison of MHCII$^+$CD103$^+$ cells from non-TG and B27-TG animals revealed that B27-TG animals had significantly fewer MHCII$^+$ CD103$^+$ cells proportionally (mean: B27-TG 37 % vs. non-TG 52 %) (Figure 5.4B). However, when numbers of MHCII$^+$ CD103$^+$ cells were calculated, the difference did not reach statistical significance (Figure 5.4C).

Even though it was not possible to identify the three L-DC populations in the MLN according to CD11b and CD172a expression as in lymph, it was possible to examine CD172a$^{hi}$ and CD172a$^{lo}$ cells within the CD103$^+$ population (Figure 5.5). Indeed, it was observed that B27-TG animals had fewer CD172a$^{lo}$ cells (Figure 5.5A, B) and comparison of data from four independent experiments revealed that the B27-TG animals had significantly fewer MHCII$^+$ CD103$^+$ CD172$^{lo}$ DCs proportionally than non-TG animals (means: 23 % vs. 37 %) (Figure 5.5C). However, again there was no significant difference in the absolute number of MHCII$^+$ CD103$^+$ CD172a$^{lo}$ cells between non-TG and B27-TG animals (Figure 5.5D). B7-TG MLNs showed a slightly different pattern of staining when compared to non-TG MLNs, with more CD172a$^{lo}$ cells (Figure 5.5B).

In the previous chapter it was shown that B27-TG L-DCs in the lymph express a slightly lower level of MHCII than non-TG cells, and in addition it has been reported that splenic CD103$^+$ DCs from B27-TG animals express less MHCII than non-TG cells (239). Thus, the MHCII expression level on non-TG and B27-TG MHCII$^+$ CD103$^+$ DCs was determined. It was noticed that B27-TG cells had significantly lower geometric mean values for MHCII than non-TG DCs (Figure 5.6C).

When the phenotype MHCII$^+$ CD103$^-$ populations from non-TG and B27-TG animals were analysed, MHCII$^+$ CD103$^-$ cells differed from CD103$^+$ DCs in their CD11b and CD172a expression (Figure 5.7A, B). In addition, no difference was observed in the CD172a expression between B27-TG and control B7-TG and non-TG animals (Figure 5.7C). However a slight decrease of MHCII expression on B27-TG DCs was observed (Figure 5.6A, Figure 5.7D).

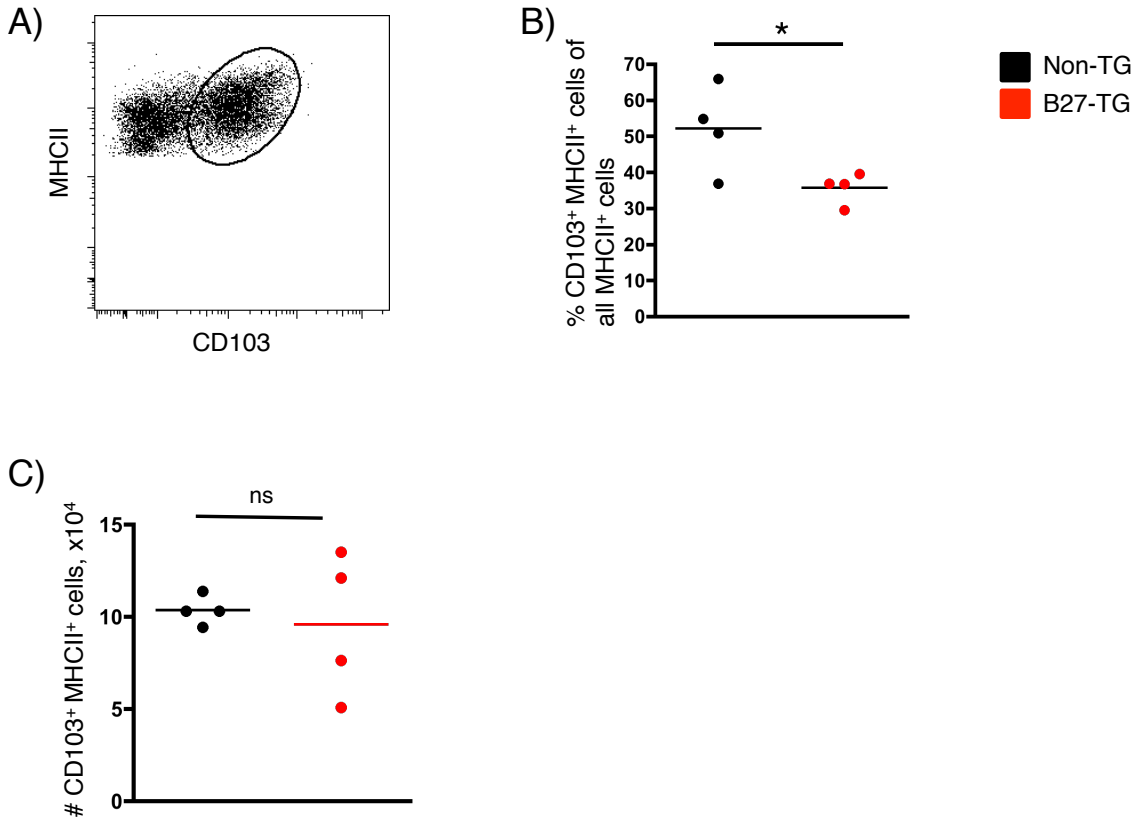


**Figure 5.4 B27-TG rats have proportionally fewer MHCII$^+$ CD103$^+$ DCs than non-TG rats**

Percentage and number of LIN$^-$ (CD45RA, CD45RC, TCRα/β, Igκ chain), MHCII$^+$ CD103$^+$ DCs in MLNs of non-TG and B27-TG rats was assessed by FACS. (A) MHCII$^+$ cells from MLN, gate shows CD103$^+$ cells. (B) Percentage of MHCII$^+$ CD103$^+$ cells (gate in A) out of all MHCII$^+$ cells. (C) Total number of MHCII$^+$ CD103$^+$ DCs from the MLN. Horizontal bars represent mean values and data were analysed by unpaired Student's T-test. *$p<0.05$

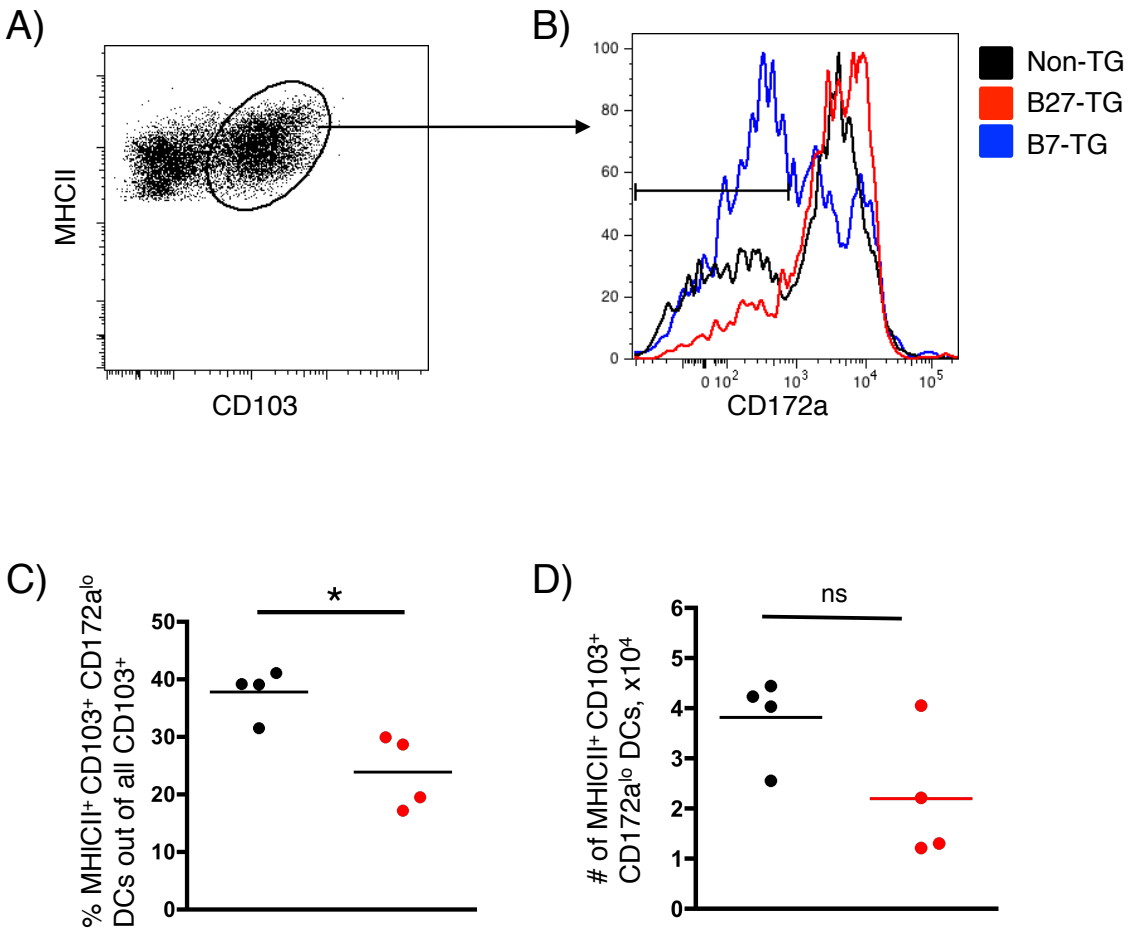


**Figure 5.5 B27-TG rats have a lower proportion of MHCII$^+$ CD103$^+$ CD172a$^{lo}$ MLN DCs than non-TG animals**

MLN cells were collected, digested and stained as before, and the percentage and number of LIN$^-$ (CD45RA, CD45RC, TCRα/β, Igκ chain) MHCII$^+$ CD103$^+$ CD172a$^+$ MLN DCs was assessed in B27-TG, B7-TG and non-TG animals. (A) gate used to identify MHCII$^+$ CD103$^+$ DCs. (B) a representative histogram overlay of CD172a expression on MHCII$^+$ CD103$^+$ DCs (gate in (A)) from non-TG, B27-TG and B7-TG rats. (C) Percentage of CD172a$^+$ cells within MHCII$^+$ CD103$^+$ DCs. (D) Number of MHCII$^+$ CD103$^+$ CD172a$^+$ cells from the total MLN cells. Horizontal bars represent mean values, and data were analysed by unpaired Student's T-test, *p<0.05. B7-TG staining, n=1.

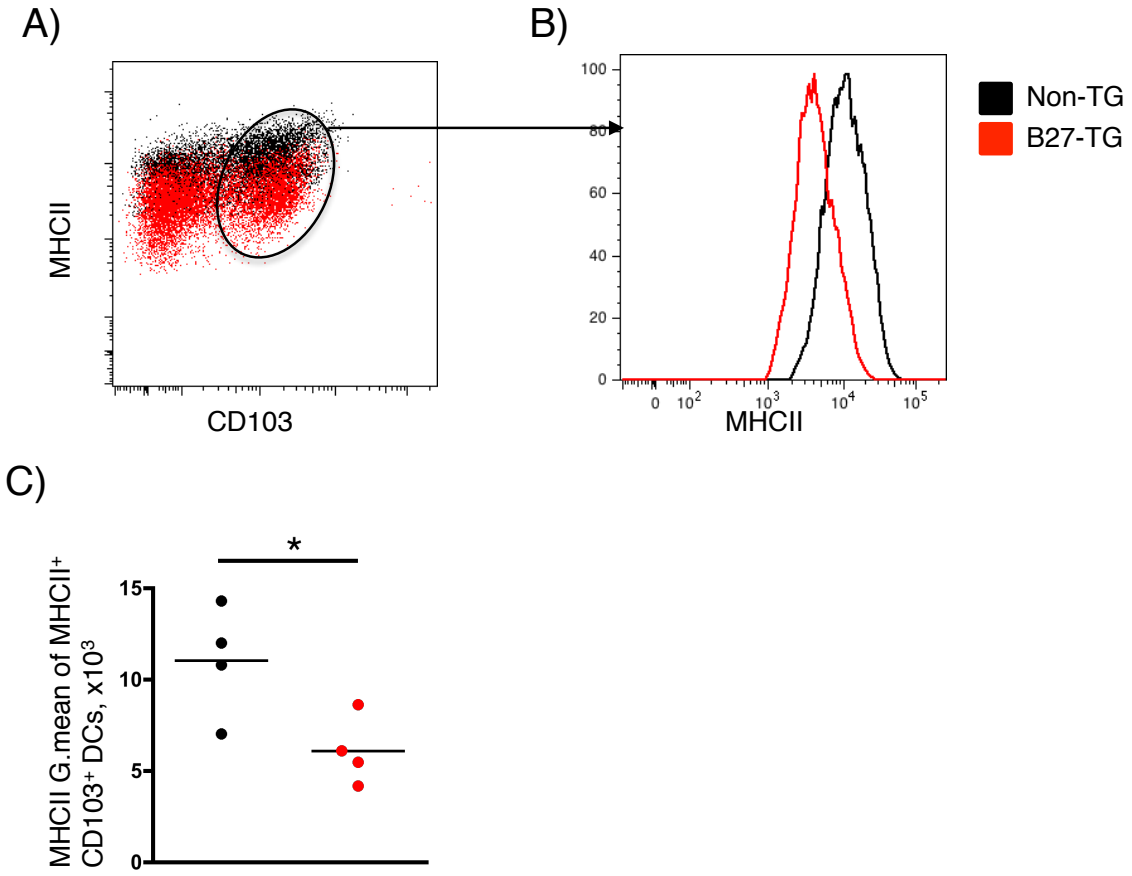


**Figure 5.6 MHCII$^+$ CD103$^+$ MLN DCs from B27-TG rats express lower level of MHCII than DCs from non-TG rats**

MLNs were collected, digested and stained as before, and the expression of MHCII on LIN$^-$ (CD45RA, CD45RC, TCRα/β, Igκ chain) CD103$^+$ DCs was determined. (A) A dot plot overlay for MHCII$^+$ cells from non-TG and B27-TG rats. (B) Histogram overlay showing expression of MHCII on CD103$^+$ cells gated on (A), and the geometric mean is enumerated in (C). Horizontal bars represent mean values, and data were analysed by unpaired Student's T-test. *p<0.05

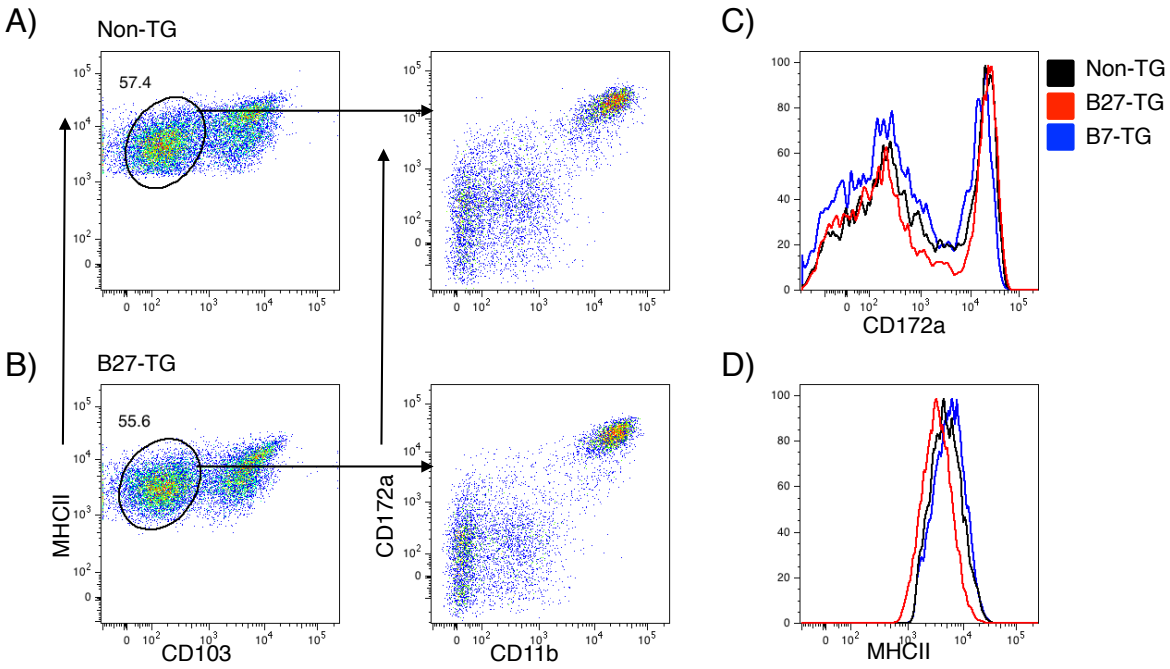


**Figure 5.7 Expression of CD172a and MHCII on MHCII$^+$ CD103$^-$ MLN cells**

MLNs from non-TG, B7-TG and B27-TG animals were collected, digested and stained as before, and the expression of MHCII and CD172a on LIN$^-$ (CD45RA, CD45RC, TCRα/β, Igκ chain) MHCII$^+$ CD103$^-$ cells were determined by FACS. CD172a and CD11b expression on MHCII$^+$ CD103$^-$ cells from non-TG MLN (A) and B27-TG MLN (B), with a histogram overlay of CD172a expression on non-TG, B27-TG and B7-TG rats (C). (D) Expression of MHCII on MHCII$^+$ CD103$^-$ cells from B27-TG MLN and from B7-TG and non-TG control rats. Results are representative of 4 experiments.

## 5.3 Discussion

Most of the evidence indicating a role for DCs in disease pathogenesis of B27-TG animals has been obtained using either splenic or BM-derived DCs. However, there is mounting evidence for the role of intestinal cells in both the human disease (277), as well as in the transgenic rat model. Furthermore, in the previous chapter it was shown that the B27-TG animals lack one subset of migrating DCs, the $CD172^{lo}$ expressing subset. As discussed before, this subset has been implicated in transport of self- or commensal bacterial antigens from the intestine into the MLN (115), thus contributing to the induction and maintenance of peripheral tolerance. In view of this, it was decided to investigate whether it would be possible to identify L-DCs in the MLNs. This would allow a comparison of non-TG and B27-TG migrating DCs that have arrived in the MLN, and been exposed to the local microenvironment. No conclusive differences in the ability of migrating L-DCs from non-TG and B27-TG animals to induce T cell proliferation were observed in Chapter 4, but it is possible that once the cells arrive in the MLN, they change their phenotype and/or function. Unfortunately due to time constraints and difficulties with our animal colony, it was not possible to analyse the functions of L-DCs from the MLNs.

To begin with, it was observed that B27-TG MLNs had significantly more total cells than non-TG or B7-TG MLNs, and there was no difference in the viability of MLN cells after digestion, as show by Annexin-V and DAPI staining. The increased number of cells could be caused by inflammation in the B27-TG animals. However, in the previous chapter it was observed that there was no difference in the total number of cells migrating in the lymph between B27-TG and non-TG animals, meaning that the increase seen in the MLN is more likely to be caused by cells arriving via blood or local proliferation of resident MLN cells. This could mean there is an increased number of circulating cells in the blood, or that the cells that arrive in the MLN spend a longer time in there, leading to accumulation of cells. It should be investigated whether there is a difference in the number of cells present in the blood, and if other lymph nodes in the B27-TG animals also have increased numbers of cells present. The possible cell type responsible for the increase of MLN cells number will be discussed in the next paragraph.

We were then interested in finding out if the number of DCs would be altered in the MLNs of B27-TG animals. As it was noticed in the previous chapter that there were fewer L-DCs present in B27-TG lymph compared to non-TG at 0-24h collection, even though there was no obvious difference between B27-TG and B7-TG animals, it was hypothesised there

could be differences in the number of DCs in the MLNs as well. Analysis of L-DCs in the MLNs of rats can be difficult due to lack of specific markers for DCs, as MHCII is expressed by several other cell types, such as macrophages, and CD103 is not exclusive to migrating DCs as some resident DC populations in the MLN also express it. Nonetheless, by pre-enriching the MLN cell populations for CD103 prior to staining, two $LIN^-$ MHCII-expressing populations were identified, $MHCII^+$ and $MHCII^{hi}$. $MHCII^{hi}$ cells expressed more CD103 and had the same FSC/SSC characteristics as L-DCs in the previous chapter, thus suggesting they could include the migrating DC populations. Curiously, the $MHCII^{hi}$ population was also slightly more positive for the lineage markers, maybe indicating that these cells express low levels of one or several of the markers used. Alternatively, as the samples were not Fc blocked prior to staining, it could be that DCs express more Fc receptors on their surface, leading to non-specific binding of antibodies and false positive results. In addition, these DCs could be more autofluorescent, thus again falsely appearing to be positive for these markers. When CD103 enrichment was not used, the separation between the two MHCII expressing $LIN^-$ populations was not as clear. However, to avoid excluding any DC populations from the analysis, it was decided not to use the pre-enrichment, and to include all $LIN^-$ $MHCII^+$ cells in the analysis, and then separate them according to CD103 expression. This way, it was noted that B27-TG MLNs had a lower proportion, but not absolute number, of $MHCII^+$ $CD103^+$ cells, in accordance to the data seen in pervious chapter about lymph DCs. Thus, the increased total number of MLN cells does not reflect on the number of DCs in the MLN, but must be an effect of increase in other cell populations. Recent data from our laboratory indicates these cells are not T cells, as similar numbers have been detected in both non-TG and B27-TG MLNs (Dr Aude Aumeunier, unpublished observation). Other studies in our laboratory have indicated increased numbers of cells staining positive for rat granulocyte marker RP-1 in the spleens of B27-TG animals (Pamela Wright, personal communication), and thus it could be these cells are responsible for the increased cell numbers in the MLN as well.

Whether or not CD103 enrichment was used, in the MLNs it was not possible to clearly identify the three subsets of migrating lymph DCs seen *in vivo* in the lymph. However, the proportions of $MHCII^+$ $CD103^+$ $CD172a^{hi}$ and $CD172a^{lo}$ DCs could be compared between non-TG, B7-TG and B27-TG animals, and it was observed that B27-TG animals had a significantly decreased proportion of $CD172a^{lo}$ cells compared to non-TG $CD103^+$ cells. This decrease was much less marked than the one observed in the lymph. This could be because the $CD103^+$ population examined here is unlikely to be formed purely from migrating L-DCs. In fact, some rat splenic $CD103^+$ DCs are also $CD172a^-$ (278), and as

spleen does not have lymphatic drainage, these cells must be blood derived. It is thus likely that similar cells would also be present in the MLNs. In addition, murine MLN-resident DCs consist of both CD172a$^{+}$ and CD172a$^{-}$ populations (279). There was no significant reduction in the number of CD172a$^{lo}$ cells, again most likely reflecting the increased total number of cells in the B27-TG MLN.

The failure to clearly identify the three migrating populations seen in lymph could be caused by many reasons. For example, DCs are very sensitive to signals from their microenvironment and could change their phenotype as they enter MLNs from lymph. Also, it has to be taken into account that the MLNs are subjected to harsh physical and enzymatic disruption prior to staining, and it is not known what effect this treatment could have on DCs. However, mouse lymph also contains several populations of DCs (Dr Vuk Cerovic, University of Glasgow, personal communication), and these DCs can also be identified in the MLNs after digestion, making it unlikely that rat DCs would react to digestion very differently. More likely, the lack of suitable markers in the rat for MLN DCs makes it difficult to identify the specific, small subsets without them being contaminated by other cells with similar characteristics. The lack of a reliable anti-CD11c antibody in the rat is particularly important in this context.

Similarly to the reduced MHCII expression observed by B27-TG CD103$^{+}$ cells in the lymph, B27-TG MLN CD103$^{+}$ DCs expressed lower levels of MHCII. As discussed in the previous chapter, the reason for this HLA-B27-dependent reduction of class II molecule expression is not known, but in a recent publication it was shown that *ex vivo* splenic DCs from B27-TG animals show reduced expression of cathepsin S enzyme (239). Cathepsin S is a thiol protease in the ER critical for the cleavage of the invariant chain that occupies the peptide-binding cleft of immature MHCII molecules (280, 281). These complexes are then directed to the endocytic pathway, where the invariant chain is cleaved, allowing peptide binding and formation of functional complexes. In addition, ER stress is thought to cause retention of MHCII-Ag complexes in the ER, and thus result in decreased surface expression (282). The functional effect of reduced MHCII on DCs and other immune cells is not clear, however it could lead to decreased antigen presentation to CD4$^{+}$ T cells. In addition, results presented in the following chapter clearly show that *in vitro* B27-TG BMDCs are less efficient at inducing proliferation of naïve CD4$^{+}$ T cells in MLRs, even though the same phenomenon was not conclusively detected using *in vivo* L-DCs in Chapter 4. Unfortunately it was not possible to include functional data concerning MLN DC populations in this study. There were no differences in the expression of CD103,

CD172a or MHCII on MHCII$^+$ CD103$^-$ cells from non-TG, B7-TG or B27-TG animals, further indicating that the differences between non-TG and B27-TG DCs lie within the CD103$^+$ population.

These results suggest that the of B27-TG CD172a$^{lo}$ subset missing in lymph is also proportionally reduced in the MLN of these animals, even though the number of CD172a$^{lo}$ MLN cells was not significantly different between non-TG and B27-TG animals. As no difference in the T cell stimulatory capacity between non-TG and B27-TG L-DCs were noticed in the previous chapter, it would be important to analyse the function of these cells once they have arrived in the MLN. A further intriguing possibility is that they induce different CD4$^+$ T cell populations, as indicated in a recent publication, where it was observed that splenic B27-TG DCs induce more Th17 cells than non-TG cells (144). In addition, it has been shown both in mice and in humans that the CD103$^+$ cells in the MLN and small intestinal lamina propria are uniquely capable at inducing gut homing receptors $\alpha_4\beta_7$ and CCR9 on T cells, and they also have an important role in maintenance of peripheral tolerance. These functions are dependent on conversion of vitamin A to retinoic acid by these DCs (see introduction). CD103$^+$ DCs express enzyme retinaldehyde dehydrogenase, which is required for this action. Another function attributed to CD103$^+$ DCs and retinoic acid is induction of FoxP3$^+$ regulatory T cells. Therefore, it would be interesting to investigate whether CD103$^+$ DCs from non-TG and B27-TG animals are both capable of converting retinol to retinoic acid at similar rates, giving an indication whether there is defect on either induction of homing markers on T cells, or in induction and maintenance of peripheral tolerance by B27-TG DCs. Defect in induction of homing markers is potentially interesting, as a defect in the imprinting of gut specific homing markers would generate populations of activated T cells that would not home to the site of infection, but to somewhere else in the body. This could lead to non-specific systemic inflammation as seen in SpA. Expression of homing markers by T cells is a subject of active research in our laboratory. MHCII$^+$ CD103$^+$ DCs could also be sorted from non-TG and B27-TG MLNs, and co-cultured with naïve CD4$^+$ MLN T cells. The phenotype and cytokine production by these T cells could then be analysed and compared between non-TG and B27-TG animals.

These studies have shown it is possible to identify DCs in rat MLNs, but much remains still unclear. A more detailed phenotype of these cells should be gathered to make it possible to use these cells for detailed studies. The precise functions of different MLN DC populations are still also unclear, and much work remains for the future to assess the role

these DCs have in the disease observed in the B27-TG animals. As results seen here are derived from animals of 10-16 weeks of age, it is still unclear whether the changes in DC subsets are a cause or a symptom of the inflammatory disease. However, preliminary data from 6-week old non-TG and B27-TG MLNs suggests that the proportion of CD172a$^{lo}$ cells is already reduced in the MLNs of young B27-TG animals compared to non-TG rats (data not shown), in parallel with data shown here. As the rats do not show signs of inflammatory disease at this time, it is feasible to assume that the DC defect precedes the onset of disease symptoms. More work is still required to address this in more detail, and functional data from MLN DCs at different ages should be investigated in the future.

# 6. Bone marrow derived dendritic cells

## 6.1 Introduction

As both lymph and MLN DCs represent cells already differentiated and influenced by the factors in their environment, it was also decided to investigate whether there were any differences between non-TG and B27-TG DCs grown from bone marrow precursors (bone marrow dendritic cells, BMDCs). Due to difficulty in obtaining DCs *in vivo,* BMDCs are widely used in *in vitro* experiments. Despite widespread use of BMDCs in experiments involving B27-TG animals, there have not been any reports indicating defects in the development of BMDCs from these animals. However, most investigators who have used BMDCs have used the originally developed method of growing rat BMDCs, with GM-CSF and IL-4 (283). But, as discussed in detail in the introduction, it has been reported that DCs grown with GM-CSF and IL-4 do not resemble steady state DCs, but have a more inflammatory DC phenotype (60). In contrast, the L-DCs and MLN DCs in which we have identified defects represent steady state populations. Thus, for the purpose of the experiments in this chapter, BM cells were cultured with Flt3L instead, which has been shown to induce cells that resemble steady state DCs (60, 66). In view of previous reports indicating several defects, or differences, in B27-TG DCs compared to non-TG DCs, *it was hypothesised that there would be a defect in the development of B27-TG steady state DCs form their bone marrow precursors*, and this Flt3L culture system would allow us to investigate this in more detail.

## 6.2 Results

### *6.2.1 BMDCs from B27-TG animals are less viable than non-TG BMDCs when grown with mouse Flt3L*

As has been shown earlier, differences in non-TG and B27-TG DCs could not only be found in lymph (chapter 4), but the data presented in the previous chapter also suggests that there might be differences in MLN DCs. Thus, it was decided to investigate whether there was a systemic or developmental problem associated with the observed DC defects. Flt3L is essential for DC development from their precursors and thus it was investigated whether DCs grown from BM precursors with mouse (m) Flt3L would be different between non-TG and B27-TG animals. BM cells were collected from non-TG and B27-TG animals as indicated in Materials and Methods, and grown for seven days in complete

media supplemented with 10 % Flt3L supernatant derived from mouse Flt3L producing CHO cell line (referred to as mFlt3L). 10 % mFlt3L supernatant was selected after various concentrations, from 5-25 %, were tested for their capacity to generate BMDCs. 5 % supernatant was not sufficient for generating BMDCs, and there was no difference in the cell numbers when either 10 or 25 % was used (data not shown). Hence, 10 % supernatant was used for all the subsequent experiments. When grown under these conditions, it was noticed that BMDCs derived from the B27-TG animals were less viable than non-TG cells, as shown with Annexin-V and DAPI staining (Figure 6.1). In addition, the FSC and SSC characteristics of the non-TG and B27-TG cells were different, with B27-TG cells having more small, $FSC^{lo}$ cells (Figure 6.1). As it was possible that this phenotype seen on day seven could be caused by, for example, overcrowding or lack of growth factors, it was decided to investigate the viability of BMDCs on day four. Surprisingly, there was already a marked difference in the proportion of live Annexin-$V^-$ $DAPI^-$ cells between non-TG and B27-TG animals at this early timepoint (Figure 6.2). As no defects in the viability of BMDCs grown with GM-CSF and IL-4 have been reported, it was decided to investigate whether the reduced viability observed was Flt3L dependent. Thus, BM cells from B27-TG and non-TG animals were grown with 10 % mFlt3L supernatant supplemented with 1 ng/ml recombinant rat GM-CSF. Surprisingly, we observed that the viability of B27-TG BMDCs under these conditions was the same, if not better, than that of non-TG BMDCs (Figure 6.3).

When data from at least three individual experiments were combined, it was observed that there were indeed a significantly lower proportion of live cells present in B27-TG BMDCs than in non-TG BMDCs, when grown with mFlt3L (Figure 6.4A). In contrast, when GM-CSF was added to the cultures, significantly more B27-TG BMDCs were Annexin-$V^-$ $DAPI^-$, compared to B27-TG BMDCs grown with Flt3L only (Figure 6.4A). In addition, there was no difference in the cell viability between non-TG and B27-TG BMDCs when the two growth factors were both used. The number of Annexin-$V^-$ $DAPI^-$ cells was calculated per harvested well, and it showed a similar trend to the proportion of cells, however it did not reach statistical significance (Figure 6.4B). The percentage and number of live $CD103^+$ cells were also calculated, as these cells are phenotypically closest to *in vivo* lymph DCs. There were no obvious differences in the proportion of $CD103^+$ cells between non-TG and B27-TG animals (Figure 6.4C), but there was a decrease in the absolute number of B27-TG $CD103^+$ with Flt3L alone, which increased to the level of non-TG mFlt3L only when GM-CSF was added to the cultures (Figure 6.4D). The proportions

of apoptotic (Annexin-V$^+$ DAPI$^-$) and dead (Annexin-V$^+$ DAPI$^+$, and DAPI$^+$) cells were also compared between non-TG and B27-TG animals and the two different conditions.

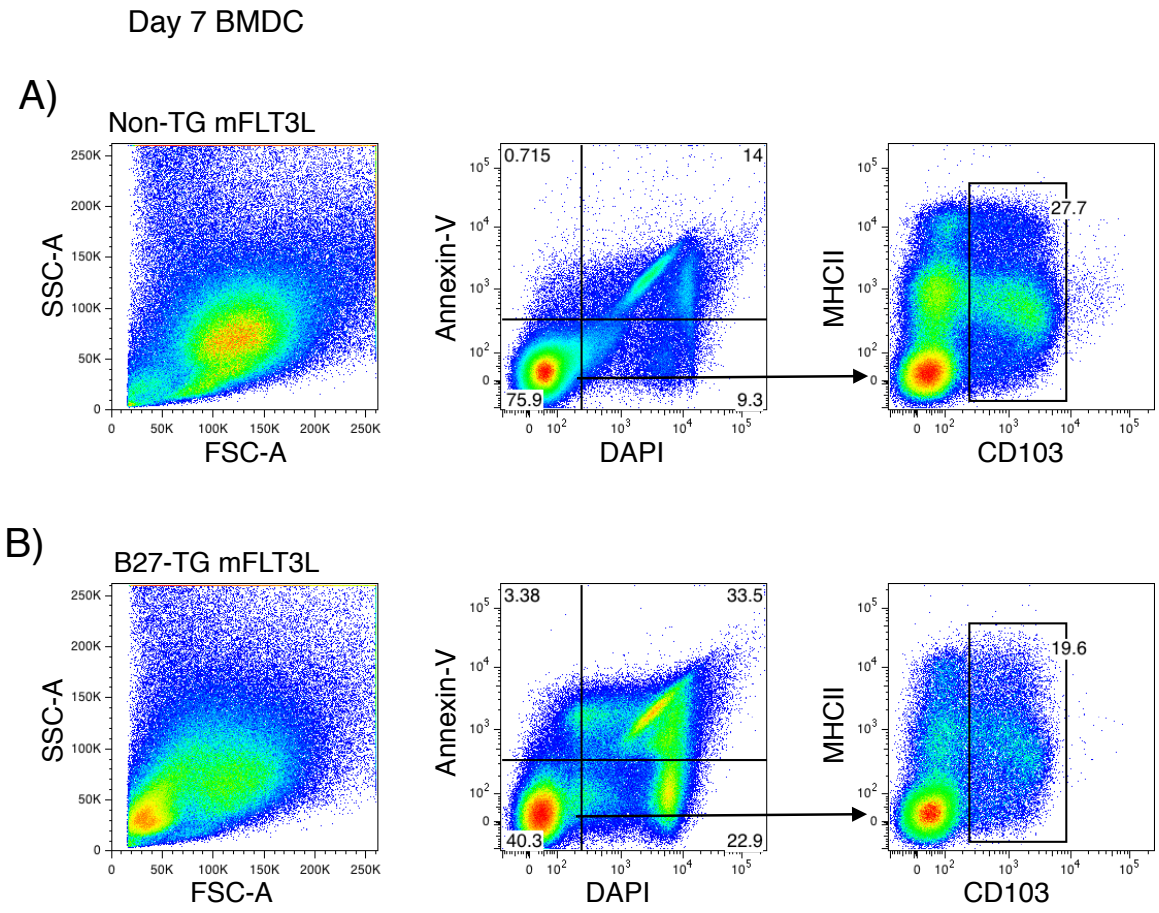


**Figure 6.1. Viability of B27-TG and non-TG BMDCs on day 7 of culture supplemented with mFlt3L**

BM cells were harvested from non-TG and B27-TG animals, and cultured for 7 days with 10 % mFlt3L. On day 7, the cells were harvested and stained with DAPI and antibodies for Annexin-V, MHCII and CD103. The proportions of live (Annexin-V$^-$ DAPI$^-$) cells as well as the proportion of CD103$^+$ within the live cell gate were determined for both non-TG (A) and B27-TG (B). (A) is a representative of 10 and (B) 8 biological replicates.

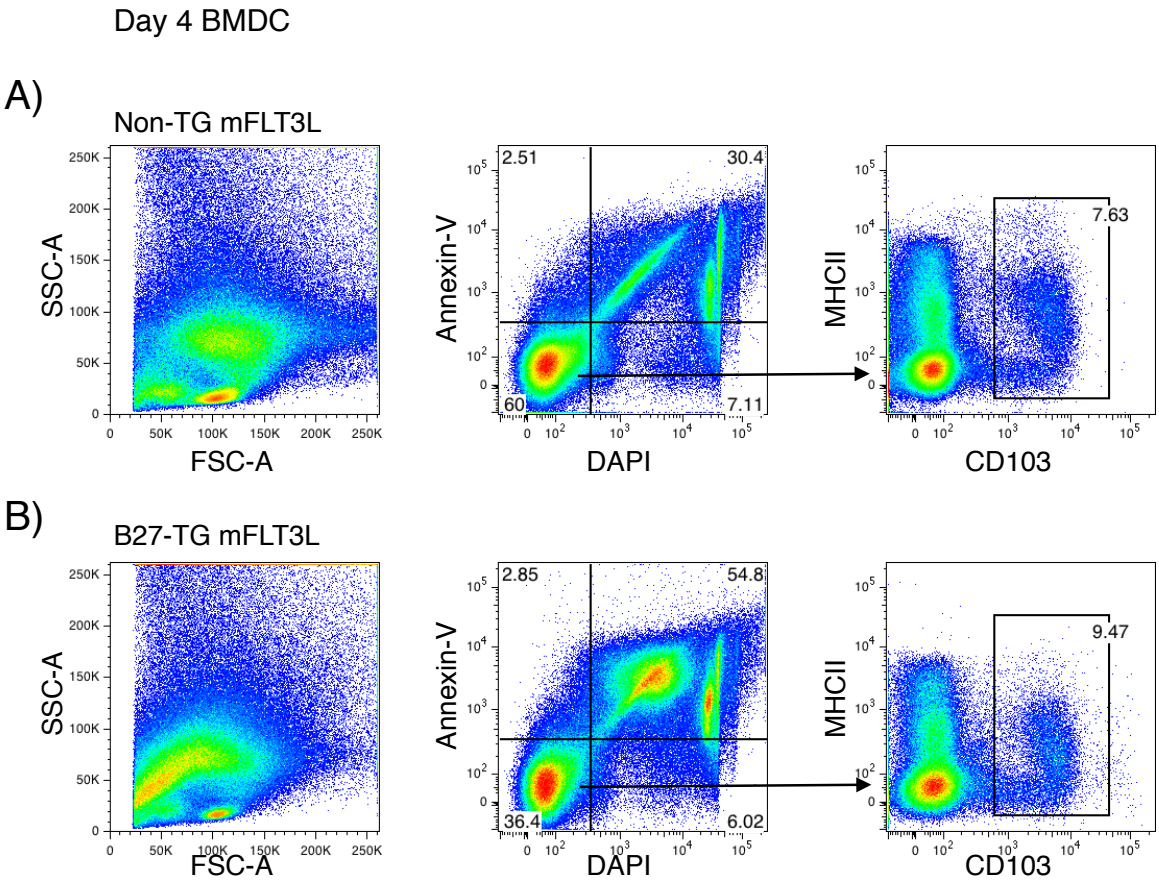


**Figure 6.2. Viability of B27-TG and non-TG BMDCs on day 4 of culture supplemented with mFlt3L**

BM cells were harvested from non-TG and B27-TG animals, and cultured for 4 days with 10 % mFlt3L. On day 4, the cells were harvested and stained with DAPI and antibodies for Annexin-V, MHCII and CD103. The proportions of live (Annexin-V$^-$ DAPI$^-$) cells as well as the proportion of CD103$^+$ within the live cell gate were determined for both non-TG (A) and B27-TG (B). n=1.

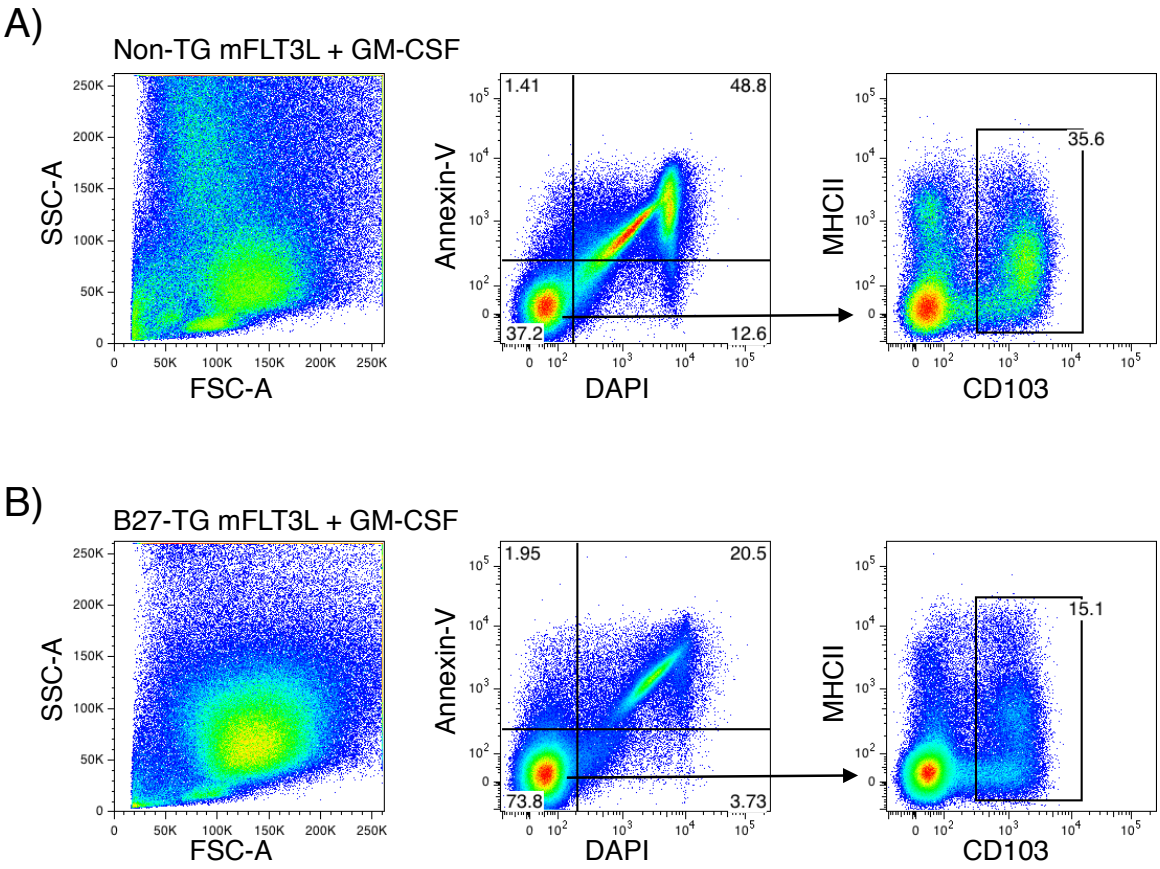


**Figure 6.3. Viability of B27-TG and non-TG BM cells grown with both mFlt3L and GM-CSF**

BM cells were collected from non-TG and B27-TG animals, and cultured for 7 days in the presence of both 10 % mFlt3L supernatant and 1 ng/ml recombinant rat GM-CSF. On day 7 the cells were harvested, and the viability of the cells was assessed by staining with Annexin-V and DAPI (A, B). Percentage of $CD103^{+}$ cells was also assessed. Figure is representative of 3 independent experiments.

Both non-TG and B27-TG BMDCs had a very low proportion, less than 5 %, apoptotic cells when cultured with both GM-CSF and mFlt3L, and there were no differences between non-TG and B27-TG BMDCs (Figure 6.4F). Not surprisingly, B27-TG BMDCs had more Annexin-$V^+$ $DAPI^+$ and $DAPI^+$ cells than non-TG animals, and these proportions were decreased in the B27-TG BMDC cultures when GM-CSF was used in addition to mFlt3L (Figure 6.4E, G). Results from B7-TG BMDCs do not deviate significantly from those observed from non-TG animals. If anything, they look like there are more live cells and less Annexin-$V^+$ cells than was observed on non-TG BMDC cultures.

### *6.2.2 Phenotype of BMDCs cultured with mFlt3L*

The expression of surface markers on B27-TG, B7-TG, and non-TG BMDCs cultured for 7 days with 10 % mFlt3L were assessed (Figure 6.5). The only observed differences between non-TG and B27-TG BMDCs were in the expression of MHCII and CD172a – all the other markers were expressed at similar levels. As expected, only a very small proportion of $CD68^+$ macrophages/granulocytes were found, and CD4 and CD8α were expressed by less than 5 % of BMDCs.

To assess the presence of DC populations similar to *in vivo* DCs, non-TG and B27-TG BMDCs grown with 10 % mFlt3L were stained with Annexin-V, DAPI, MHCII, CD103, CD11b and CD172a. Three populations of live (Annexin-$V^-$ $DAPI^-$) cells were found in both non-TG and B27-TG BMDC preparations (Figure 6.6A, C): $MHCII^-$ $CD103^-$ (R1), $MHCII^+$ $CD103^-$ (R2) and $MHCII^{lo}$ $CD103^+$ (R3). Expression of CD11b and CD172a were determined in all three populations (Figure 6.6B, D), but no obvious differences were observed between non-TG and B27-TG BMDCs in regards to those markers in any of the three populations. The $MHCII^+$ $CD103^+$ R3 population, that most closely resembles lymph DCs phenotypically, expressed uniformly high level of CD172a and CD11b, whereas the R1 and R2 populations were more homogeneous (Figure 6.6B, D).

The ability to upregulate expression of MHCII and costimulatory molecules in response to TLR stimulation is an important function for immature DCs. Thus, it was decided to investigate whether non-TG and B27-TG BMDCs differ in their response to TLR4 stimulation with LPS. When non-TG and B27-TG BMDCs grown for 7 days with mFlt3L were treated with 100 ng/ml LPS overnight, both non-TG and B27-TG $CD103^+$ (R3) cells strongly upregulated expression of MHCII, creating uniformly $MHCII^{hi}$ $CD103^+$ cells (Figure 6.7A, C).

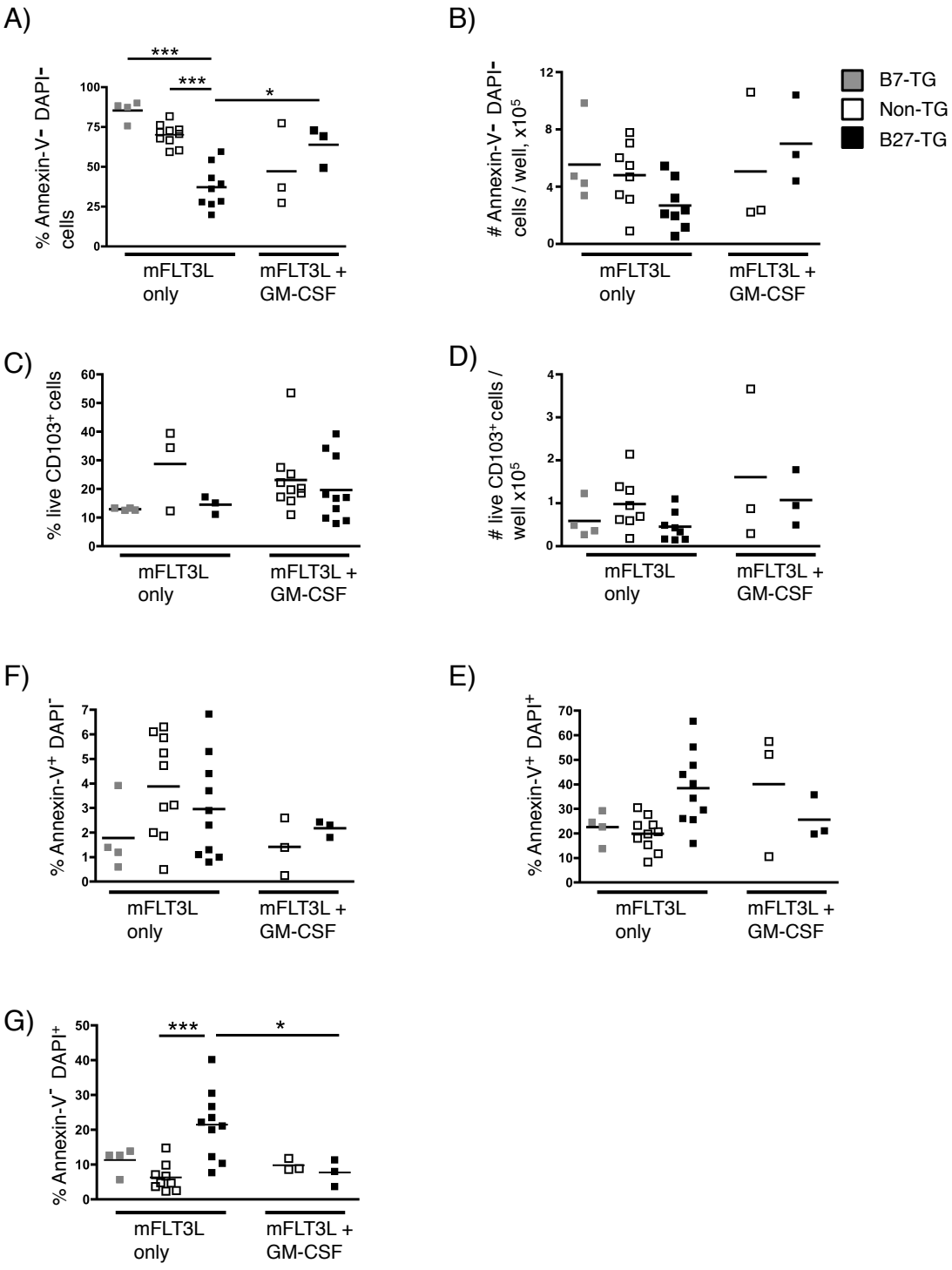


**Figure 6.4. Combined results for mFlt3L cultured BMDCs**

Combined results are shown for day 7 non-TG and B27-TG BMDC cultures supplemented with either only 10 % mFlt3L supernatant, or with both mFlt3L and 1 ng/ml recombinant rat GM-CSF. (A) Proportion of live (Annexin-V$^-$ DAPI$^-$) cells out of all BMDCs, and (B) the number of live cells/well of culture. Percentage (C) and number/well (D) of live CD103$^+$ cells were also determined. (F) shows the proportion of apoptotic Annexin-V$^+$ DAPI$^-$ cells, whereas dead Annexin-V$^+$ DAPI$^+$ and DAPI$^+$ Annexin-V$^-$ are shown in (E) and (G), respectively. “mFlt3L only” shows results from 8-10 and “mFlt3L+GM-CSF” from 3 individual experiments with one animal used in each. Data were analysed by a 1-way Anova, *p<0.05, ***p<0.001..

No obvious differences were noticed in the expression of CD11b and CD172a in any of the three above-mentioned populations (R1-R3) between non-TG and B27-TG BMDCs (Figure 6.7B, D). As addition of GM-CSF into BMDC cultures together with mFlt3L was shown earlier to improve viability of B27-TG BMDCs, it was decided to investigate if this would have an effect on LPS stimulated cells. The number and percentage of total non-TG and B27-TG BMDCs, either grown with mFlt3L only or with both mFlt3L and GM-CSF, were compared after overnight LPS stimulation. A significantly lower proportion of live Annexin-V$^-$ DAPI$^-$ cells were found in mFlt3L only B27-TG BMDC cultures compared to non-TG BMDCs (Figure 6.8A). However, an opposite pattern was observed when the proportion of live B27-TG and non-TG BMDCs cultured with both mFlt3L and GM-CSF were compared (Figure 6.8A). There were no significant differences in the number of live cells but, as with percentage, B27-TG animals had more viable cells compared to non-TG after LPS stimulation when both mFlt3L and GM-CSF were used (Figure 6.8B).

As the MHCII$^+$ CD103$^+$ population is phenotypically closest to *in vivo* L-DCs in the steady state, the number and percentage of CD103$^+$ cells in LPS treated non-TG and B27-TG BMDC cultures were compared. There was no difference in the proportions of either non-TG or B27-TG CD103$^+$ cells when GM-CSF was added to the cultures (Figure 6.8C). In addition, BMDCs from B27-TG animals contained only a slightly lower proportion of CD103$^+$ cells compared to non-TG BMDCs under both culture conditions (Figure 6.8C). Calculation of cell numbers revealed that as with percentages, mFlt3L cultured B27-TG BMDCs had fewer CD103$^+$ cells compared to non-TG BMDCs under the same conditions (Figure 6.8D). Interestingly, mFlt3L+GM-CSF cultured BMDCs from non-TG and B27-TG animals differed slightly. The absolute number of CD103$^+$ cells stayed the same in B27-TG BMDC cultures, whereas the number of non-TG CD103$^+$ BMDCs was strongly reduced compared to mFlt3L only cultures (Figure 6.8D). In addition, the expression of MHCII, CD11b, CD172a and CD25 on BMDCs grown with only mFlt3L were compared between non-TG and B27-TG, and with and without LPS stimulation (Figure 6.8E). In this experiment B27-TG cells express slightly lower level of MHCII both with and without LPS, however this phenomenon is not consistent between individual experiments (data not shown). CD11b and CD172a were downregulated slightly on the B27-TG BMDCs after LPS stimulation, but as the experiment has only been done once, no conclusions can be drawn from these observations. In addition, both non-TG and B27-TG BMDCs expressed only low levels of the activation marker CD25, and its expression was not upregulated after LPS stimulation.

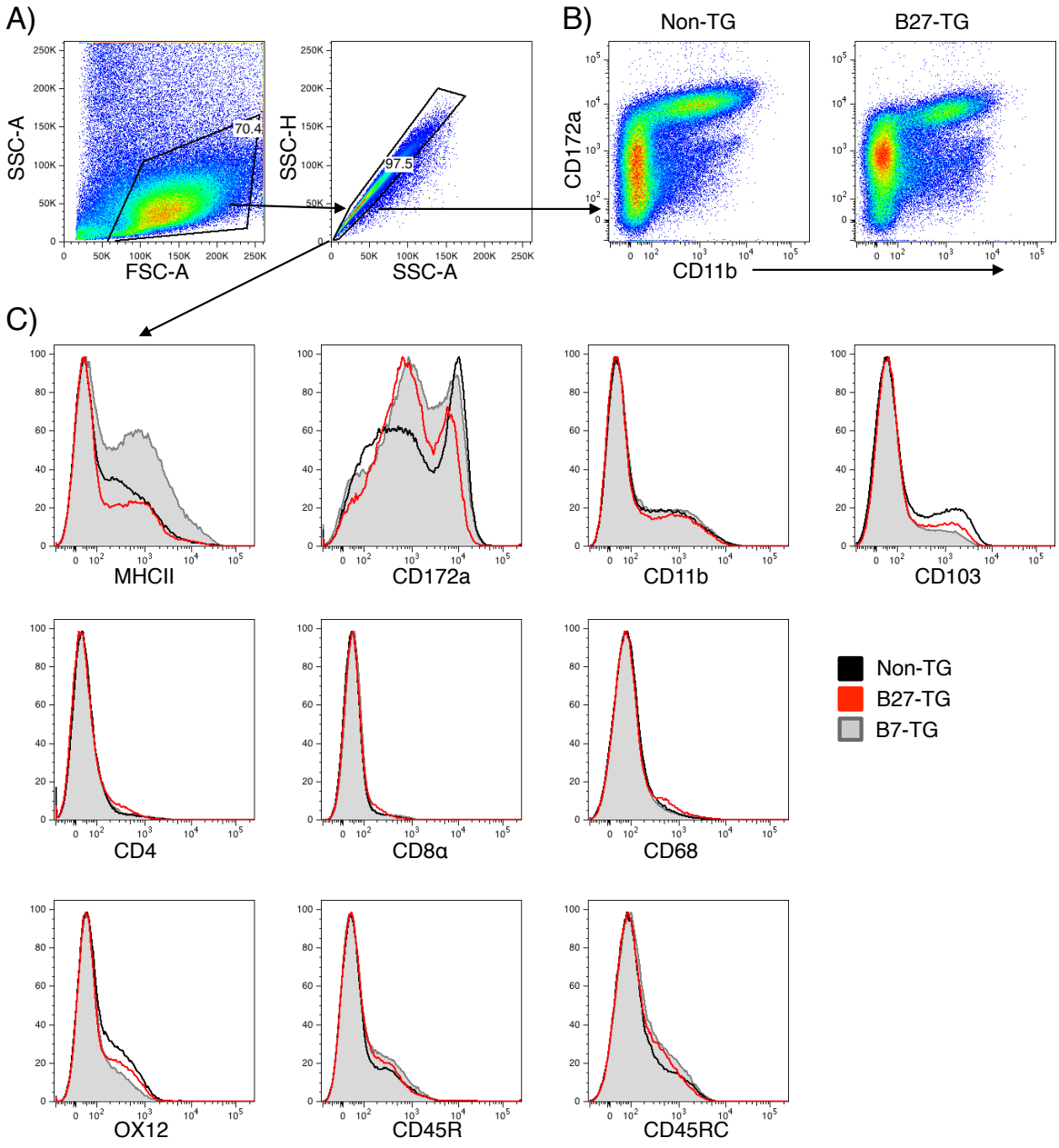


**Figure 6.5 Phenotype of mFlt3L cultured BMDCs**

BM cells from non-TG, B27-TG and B7-TG animals were incubated with 10 % mFlt3L for 7 days to obtain BMDCs. On day 7, semi-adherent cells were harvested and stained with various markers as indicated in the figure. This experiment has been performed once, n=1.

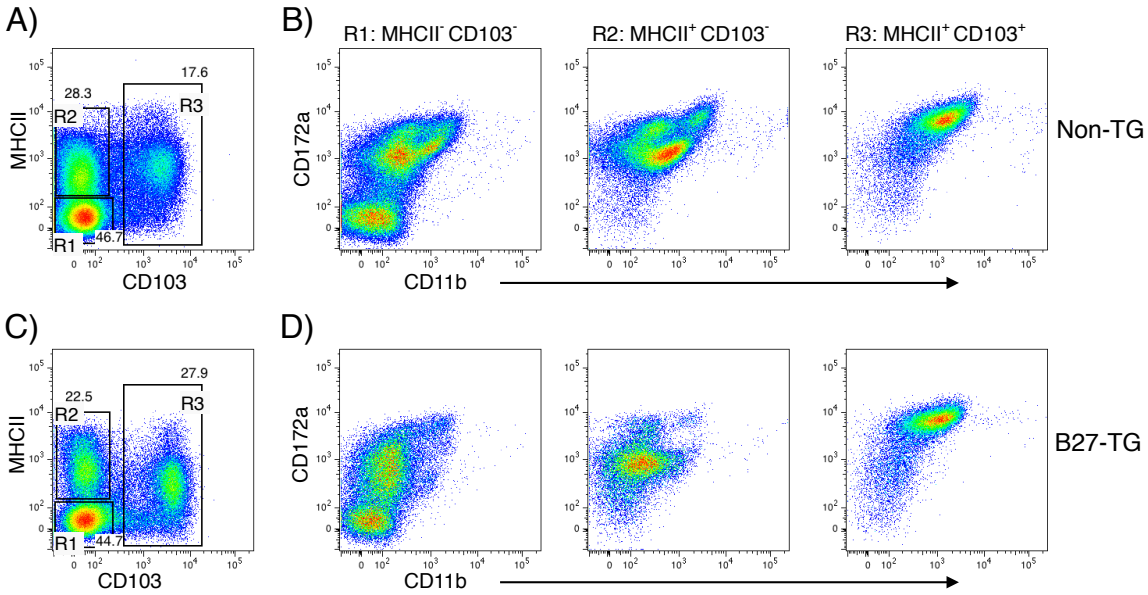


**Figure 6.6. Phenotype of non-TG and B27-TG BMDCs generated with mFlt3L**

Non-TG and B27-TG BMDCs were grown as before with 10 % mFlt3L cell culture supernatant for 7 days. On day 7 cells were harvested and stained with Annexin-V, DAPI, MHCII, CD103, CD11b and CD172a. Cells were were pre-gated on live (Annexin-V$^-$ DAPI$^-$) cells, and analysed for the expression of MHCII and CD103. Three populations were identified in both non-TG (A) and B27-TG BMDCs (C): MHCII$^-$ CD103$^-$ (R1), MHCII$^+$ CD103$^-$ (R2) and MHCII$^{lo/int}$ CD103$^+$ (R3). Each of these subsets were further analysed for their expression of CD11b and CD172a (B, D). N=1.

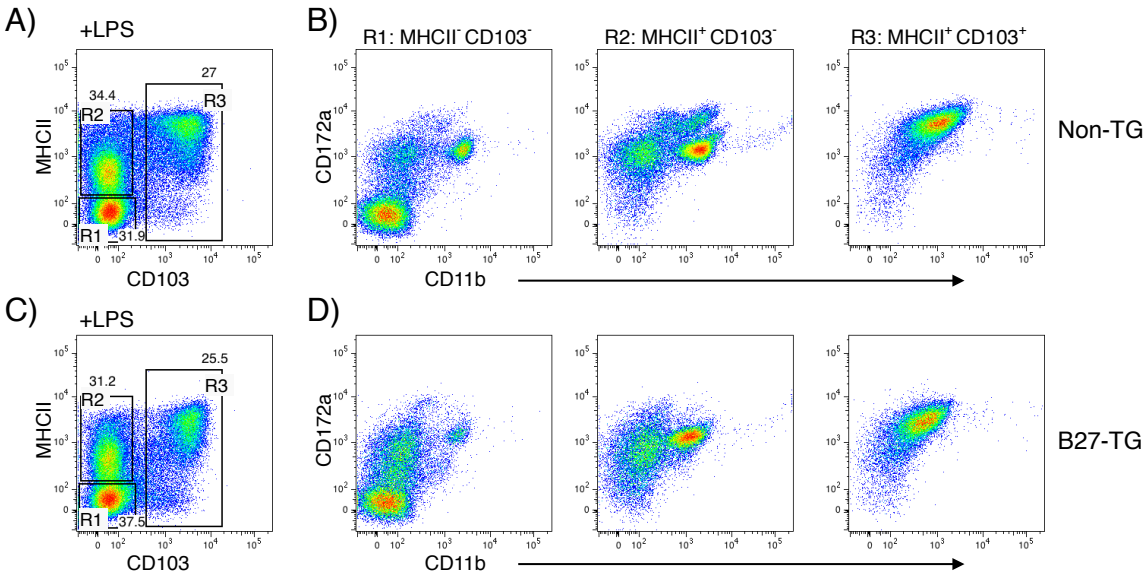


**Figure 6.7. Phenotype of mFlt3L derived BMDCs activated with LPS.**

Non-TG and B27-TG BMDCs were grown with 10 % mFlt3L for 6 days and treated with LPS overnight. On day 7 cells were harvested, and stained with Annexin-V, DAPI, MHCII, CD103, CD11b and CD172a. Three subsets were identified on both non-TG (A) and B27-TG (C) live BMDCs: MHCII$^{-}$ CD103$^{-}$ (R1), MHCII$^{+}$ CD103$^{-}$ (R2), and MHCII$^{+}$ CD103$^{+}$ (R3). All three subsets were analysed for the expression of CD11b and CD172a (B, D). (A, C) are representatives of 3 individual experiments with one non-TG and B27-TG animal used in each, (B, D) n=1.

### 6.2.3 Caspase inhibitor Q-VD can rescue B27-TG BMDCs

In the previous figures it was shown that B27-TG BMDCs cultured with 10 % mFlt3L contain a large proportion of Annexin-$V^+$ cells, indicating they are dying by apoptosis. Thus, it was investigated whether this loss of viability was caspase-dependent by inhibiting caspase activation in the BM cultures, and observing if this would rescue the cells. As it was not known which caspase pathway would be activated, pan-caspase inhibitor Q-VD-oph (Q-VD) was selected because it has been reported to inhibit all three major caspase pathways, caspase 9/3, caspase 8/3 and caspase 12, and in addition it is not toxic to the cells even when used at very high concentrations (284). To investigate the effects of Q-VD on BMDC cultures, non-TG and B27-TG BM cells were treated at day zero with various concentrations of Q-VD, and the viability of the cells was determined by Annexin-V and DAPI staining at day seven of culture. When treated with a small concentration of Q-VD (0.01 μM), about 60 % of non-TG BMDCs were live Annexin-$V^-$ $DAPI^-$ on day 7 (Figure 6.9A, top panels), compared to only about 13 % of B27-TG BMDCs (Figure 6.9A, bottom panels). However, when the concentration of Q-VD was raised to 10 μM, 60-70 % of both non-TG and B27-TG BMDCs were viable, and in addition both non-TG and B27-TG BMDCs had similar FSC/SSC characteristics (Figure 6.9B). When data from 2-5 individual experiments were pooled, it was noticed that when supplemented with 10 μM Q-VD, B27-TG BMDCs survived as well as the non-TG BMDCs. In fact, there was a significant increase in the proportion of viable B27-TG BMDCs between non-treated cells and cells treated with 10 μM Q-VD, with mean percentage of 27 % viable cells with no Q-VD, and 61 % with 10 μM Q-VD (Figure 6.9C). There was no significant difference in the absolute number of Annexin-$V^-$ $DAPI^-$ cells, however the number of B27-TG live cells did increase in a Q-VD concentration dependent manner (Figure 6.9D).

In addition to Q-VD, it was also investigated if the elF2α inhibitor salubrinal would have an effect on the survival of B27-TG BMDCs. elF2α is a key signalling molecule involved in the ER stress response, and has been reported to specifically inhibit apoptosis driven by cell stress and UPR (285), and this process is different from the caspase driven apoptosis Q-VD inhibits. As splenic cells and BM derived macrophages in rats have been shown to undergo the UPR more than non-TG cells (250, 251, 286), it was thought that use of elF2α inhibitor might be a more specific way to inhibit apoptosis on B27-TG cells. As with Q-VD, non-TG and B27-TG BM cells were treated at day zero with various concentrations of salubrinal.

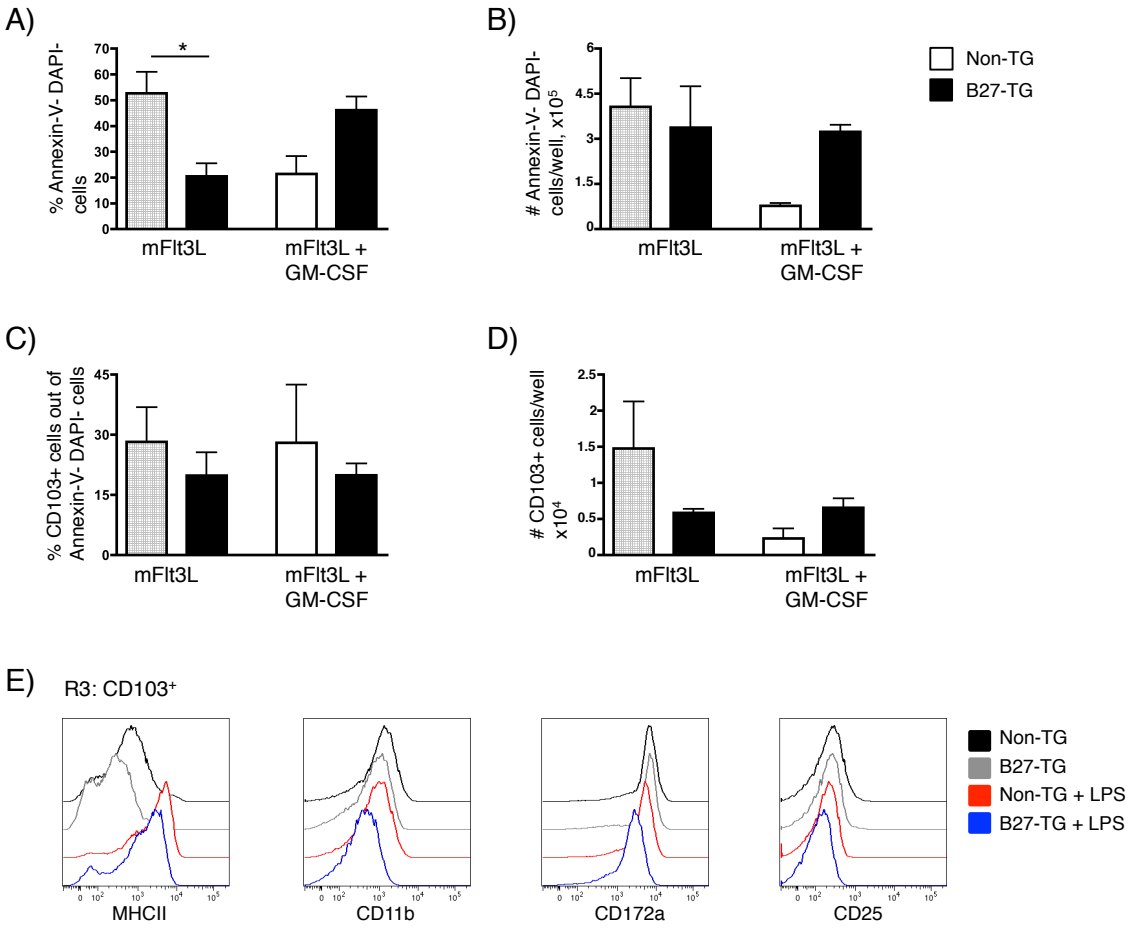


**Figure 6.8. Effect of LPS on non-TG and B27-TG BMDCs**

BM cells were grown with either 10 % mFlt3L only, or with 10 % mFlt3L + 1 ng/ml GM-CSF for 6 days, and treated with 100 ng/ml LPS. On day 7 BMDCs were harvested and stained with Annexin-V, DAPI, MHCII and CD103. The percentage and number of viable (Annexin-V$^-$ DAPI$^-$) cells were calculated (A. B), as well as the percentage and number of live CD103$^+$ BMDCs (C, D). (E) Comparison of MHCII, CD11b, CD172a and CD25 expression levels on non-TG and B27-TG CD103$^+$ BMDCs cultured with mFlt3L before and after (+LPS) LPS treatment. mFlt3L, n=4; mFlt3L+GM-CSF, n=2-4; (C) has been performed once, n=1. Error bars are SEM, and data were analysed by a Student's T test, *p<0.05.

No effect was seen in the proportion (Figure 6.9E) or in the number (Figure 6.9F) of Annexin-V$^-$ DAPI$^-$ B27-TG BMDCs in any of the concentrations investigated. Salubrinal had no effect on the viability of non-TG BMDCs (data not shown).

### *6.2.4 B27-TG BMDCs are not as efficient as non-TG cells at inducing CD4$^+$ T cell proliferation*

It was now established that B27-TG BMDCs were less viable than non-TG cells, and that addition of pan-caspase inhibitor Q-VD to the BM cultures inhibited the cell death. No obvious differences were noted in the phenotype of non-TG and B27-TG BMDCs either with or without LPS stimulation. The function of non-TG and B27-TG BMDCs was investigated next. Thus, the capacity of non-TG and B27-TG BMDCs to induce T cell proliferation was assessed by way of allogeneic mixed leukocyte reaction (MLR).

To obtain BMDCs, BM cells from PVG-strain (RT1c) were grown in the presence of 10 % Flt3L for 7 days, as before, and stained for Annexin-V and DAPI. Live Annexin-V$^-$ DAPI$^-$ cells were flow sorted, and plated out at various concentrations with 1x10$^5$ CFSE labelled MLN cells from a DA-strain (RT1$^{av1}$) rat. These co-cultures were incubated for 5 days at 37°C, and on day five cells were stained with CD4 antibody and the percentage of proliferated (CFSE$^{lo}$) CD4$^+$ cells was determined by FACS (Figure 6.10). Surprisingly, even when 1x10$^4$ BMDCs were used, only about 10% of CD4$^+$ cells had proliferated in response to the B27-TG DCs, compared to over 40 % with the same number of non-TG BMDCs (Figure 6.10A, B). Although no obvious differences were noticed in the FSC/SSC characteristics of non-TG and B27-TG cells from the co-cultures, it was proposed that B27-TG BMDCs might not survive sufficiently long in the MLR co-culture. To find out if this was the case, the DC-T cell co-cultures were supplemented on day zero of the MLR with 10 μM Q-VD, which was shown earlier to effectively rescue B27-TG BMDCs. However, the Q-VD treated B27-TG BMDCs did not induce more proliferation from CD4$^+$ cells compared to non-treated cells (Figure 6.10C). Importantly, neither Q-VD nor its vehicle (DMSO) had an inhibitory effect on CD4 cell proliferation in the non-TG cultures (Figure 6.10C).

We then tested whether it was too late to treat the BMDCs with Q-VD at the time of MLR set-up, even though live cells were sorted prior to culturing them. As was shown earlier in this chapter, the phenotype of B27-TG BMDCs is already different on day four of BMDC culture (Figure 6.2), indicating an inherent problem in the generation of Flt3L dependent cells.

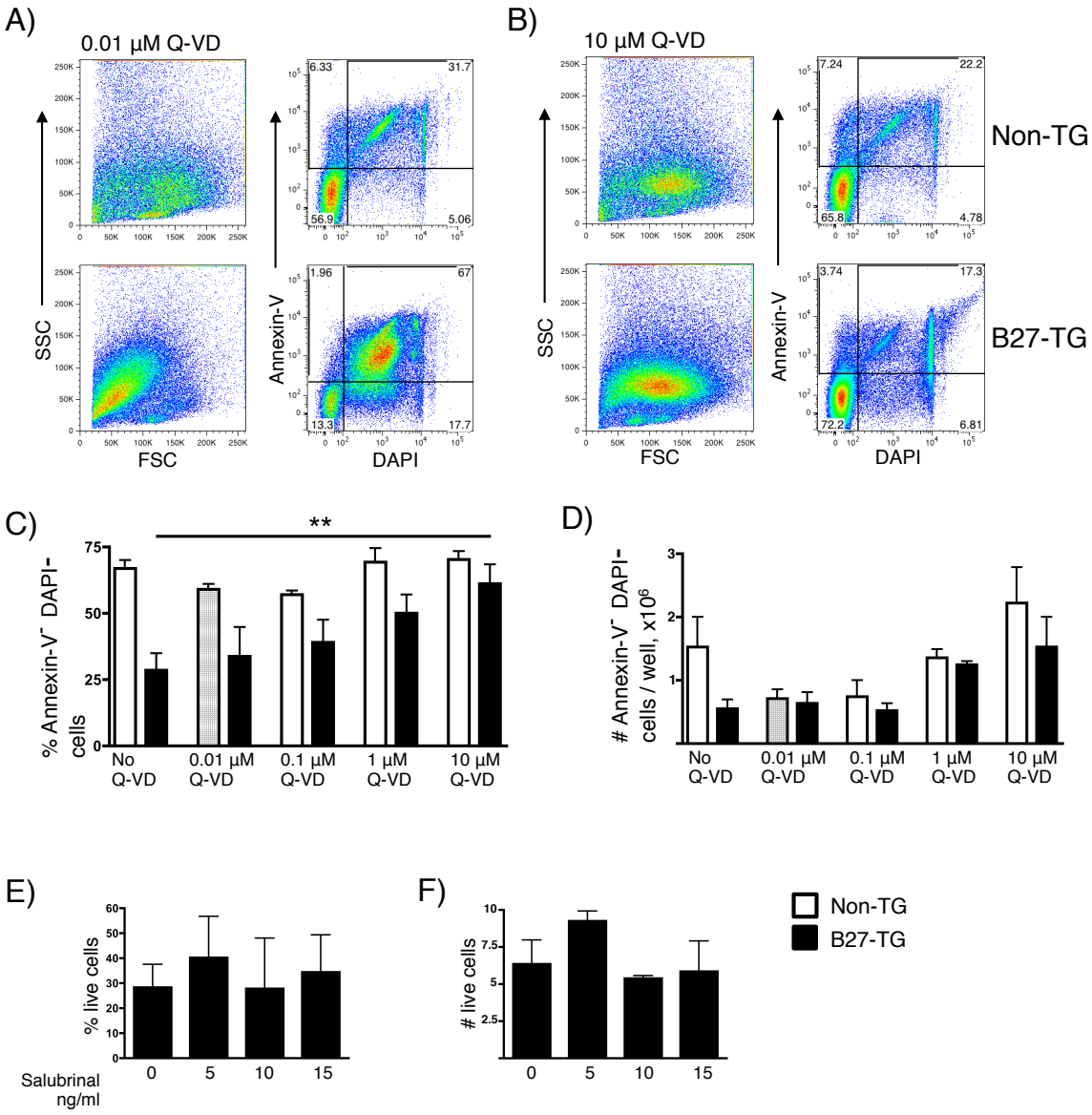


**Figure 6.9 Caspase inhibitor Q-VD rescues B27-TG BMDCs**

BM cells were harvested from non-TG and B27-TG animals, and cultured for 7 days in the presence of 10 % mFlt3L supernatant. In addition, some wells were further supplemented on day 0 with various concentrations of Q-VD (0.01-10 µM). On day 7, cells were harvested and stained with Annexin-V and DAPI, and the viability of the cells was determined by FACS. Representative dot plots for cells treated with either 0.01 µM Q-VD (A) or 10 µM Q-CD (B), with non-TG on top and B27-TG on bottom rows. (C, D) Summary of results, with (C) showing percentage of live Annexin$^-$V$^-$ DAPI$^-$ cells, and (D) the absolute number. Results are from 2 (non-TG 0.01-1µM Q-VD), 3 (B27-TG 0.01-1µM Q-VD, non-TG 10 µM) or 5 (no Q-VD, B27-TG 10 µM) individual experiments. (E, F) BM cells were treated on day 0 with various concentrations of elf2α-inhibitor Salubrinal, and cultured for 7 days in the presence of 10 % mFlt3L. On day 7 cells were harvested and stained with Annexin-V and DAPI, and the cell viability was assessed by FACS. (E) percentage and (F) absolute number of live (Annexin-V$^-$ DAPI$^-$) cells. Results from 2-3 individual experiments with 1-2 animals in each. Error bars are SEM, and data were analysed by 1-way Anova, **p<0.01.

Thus, non-TG and B27-TG BMDCs were grown in the presence of either 10 % mFlt3L alone, or with both mFlt3L and 10 μM Q-VD for 7 days. On day 7, live BMDCs were flow sorted as before, and plated out at various concentrations. On this occasion, naïve-phenotype $CD4^+$ $CD25^-$ $CD45RC^+$ T cells were sorted from MLNs of DA rats, stained with CFSE and $1x10^5$ of these cells were cultured with the DCs. In addition, some wells with the Q-VD treated B27-TG BMDCs were further treated with 10 μM Q-VD. After 5 days of co-culture, cells were stained with CD4, and again the percentage of proliferating $CD4^+$ T cells was assessed by FACS. Using this system, B27-TG BMDCs that had not received Q-VD (media only) were again significantly worse than the non-TG BMDCs at inducing proliferation from $CD4^+$ naïve T cells (Figure 6.11A, B). Furthermore, even the additional Q-VD added to the DC-T cell cultures at the time of MLR set up (Q-VD+Q-VD) was not sufficient to induce more $CD4^+$ T cell proliferation from cultures with B27-TG BMDCs (Figure 6.11C).

As was shown earlier, BMDCs could be divided into three populations: $CD103^+$ $MHCII^+$ (R3), $CD103^-$ $MHCII^+$ (R2) and $CD103^-$ $MHCII^-$ (R1) (Figure 6.12A). For the MLR experiments above, total live BMDCs had been used. However, it was next decided to investigate if these three populations would differ in their ability to induce naïve T cell proliferation. It was hypothesised that the $CD103^+$ DCs would be the best, as they were the only population to strongly upregulate their MHCII expression upon LPS stimulation (Figure 6.7). MLRs were set up as before with $1x10^5$ flow sorted CFSE labelled $CD4^+$ $CD25^-$ $CD45RC^+$ naïve T cells. In Figure 6.12B, live BMDCs were flow sorted into two populations: i) R3 and ii) R1+R2 combined. After 5 days of co-culture $CD103^+$ DCs induced a robust proliferation, whereas $CD103^-$ DCs failed to induce T cell proliferation. The experiment was then repeated, but this time $CD103^+$ (R3) and $CD103^-$ $MHCII^+$ (R2) DCs were used (Figure 6.12C). Again, $CD103^+$ DCs induced more proliferation than $CD103^-$ $MHCII^+$ DCs, but the difference was less pronounced than before when all $CD103^-$ cells were used.

To assess whether there would be a difference in the T cell populations non-TG and B27-TG DCs induce, the concentration of cytokines in the BMDC MLR co-culture supernatants were also measured. The supernatants from MLRs performed with flow sorted $CD103^+$ DCs with sorted naïve T cells were used. The cytokines were measured in triplicate from individual experiments, and representative results are shown for one experiment. The results are shown in Figure 6.13. Consistently, B27-TG BMDC co-culture wells contained significantly more IL-17F than non-TG co-cultures.

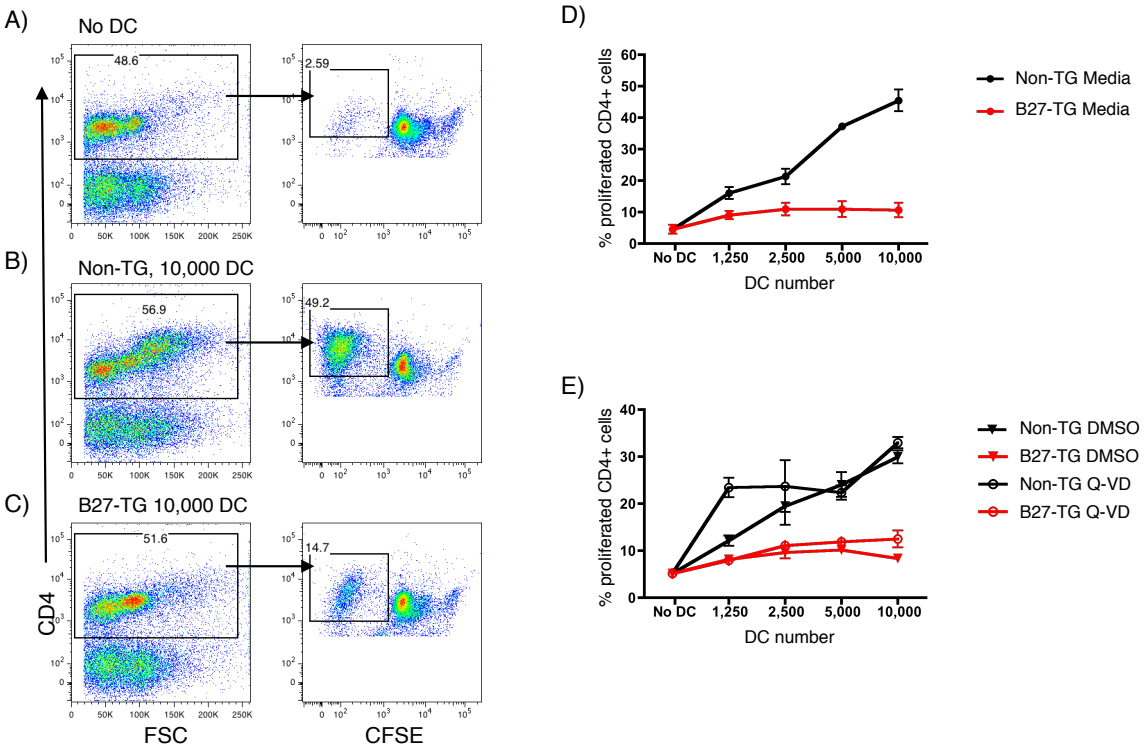


**Figure 6.10 B27-TG BMDCs are less capable at inducing T cell proliferation than non-TG BMDCs**

BM was harvested from both non-TG and B27-TG animals, and cultured for 7 days in the presence of 10 % mFlt3L supernatant. On day 7, BMDCs were harvested, stained with Annexin-V and DAPI, and the live (Annexin-V$^-$ DAPI$^-$) cells were flow sorted and plated out in triplicate at various concentrations, as shown. MLN cells from DA rats were labelled with CFSE, and 1x10$^5$ labelled cells were added to the cultures with BMDCs. After 5 days of co-culture, the cells were harvested and stained with anti-CD4 antibody, and the proliferation of CD4 cells was assessed by FACS (A-C) shows an example of proliferation with no DC control and 10,000 DCs. (D) CD4$^+$ T cells proliferation shown in graph format. Using another approach, day 7 BMDCs were co-cultured with CFSE labelled MLN cells, as above, but this time either DMSO (vehicle) or 10 µM Q-VD was added to the co-cultures for the 5-day incubation. On day 5, cells were harvested from the plate, and stained as above, and the proliferation of CD4$^+$ cells was again assessed by FACS (E). All MLR reactions were performed in triplicate. (A-D) are a representative of 3 individual experiments (n=3), whereas C has only been performed once (n=1). Error bars are SD.

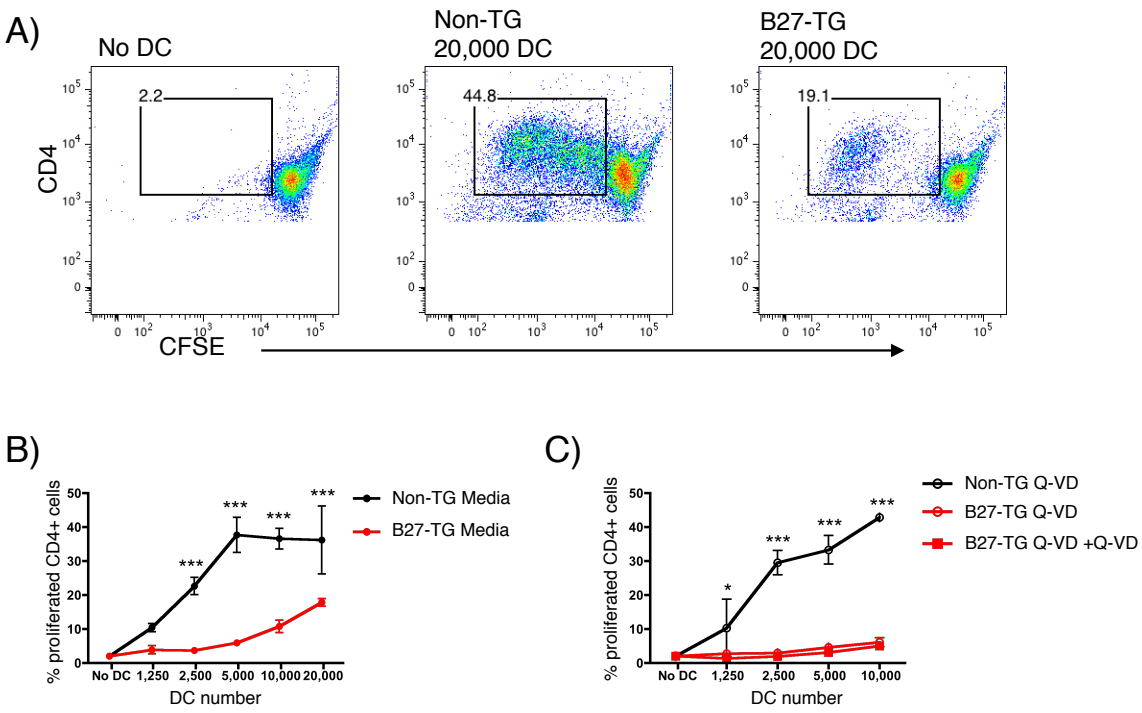


**Figure 6.11 B27-TG BMDCs are less able to induce proliferation from naïve T cells compared to non-TG BMDCs**

As before, BM cells from non-TG and B27-TG animals were cultured for 7 days in the presence of 10 % mFlt3L supernatant, and additionally some wells of the B27-TG cells were supplemented with 10 µM Q-VD. On day 7, BMDCs were harvested, stained with Annexin-V and DAPI, and the live (Annexin-V$^-$ DAPI$^-$) cells were flow sorted and plated out in triplicate at various concentrations, as indicated. Naïve CD4$^+$ CD25$^-$ CD45RC$^{hi}$ MLN T cells from DA rats were also flow-sorted, labelled with CFSE and 1x10$^5$ labelled cells were added to the wells with BMDCs. Some wells of the B27-TG BMDCs grown with Q-VD were further supplemented with 10 µM Q-VD for the duration of the co-culture. After 5 days, cells were harvested and the proliferation of CD4$^+$ T cells was assessed by FACS. (A) and (B) show the results without Q-VD treatment, and (C) shows results with BMDCs either treated with Q-VD for 7 days (Q-VD) or 7+5 days (Q-VD+Q-VD). (A) and (B) are results from 3 experiments, (C) has only been performed once. Error bars are mean +/$^-$ SD, and the data was analysed by a 2-way Anova, *p<0.05, ***p<0.001.

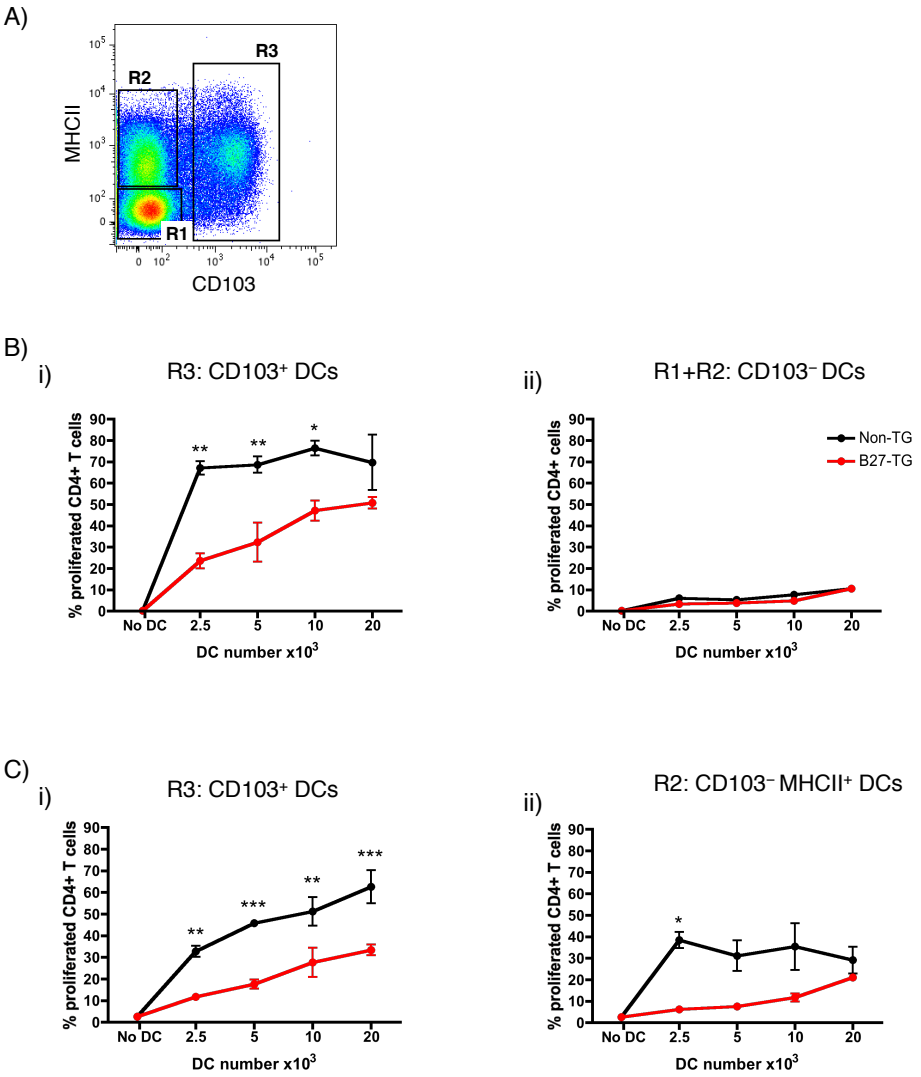


**Figure 6.12 CD103$^-$ DCs are not as efficient at inducing proliferation of naïve CD4$^+$ T cells**

Annexin-V$^-$ DAPI$^-$ BMDCs grown with mFlt3L for 7 days were sorted into two different populations in two separate experiments according to MHCII and CD103, either R1+R2 and R3, or R2+R3 as show in (A). 2-fold dilutions of DCs in triplicate were incubated for 5 days with 1x10$^5$ sorted, CFSE stained naïve CD4$^+$ T cells from DA rats. (B) is experiment one, where R3 (CD103$^+$) (i) and R1+R2 (all CD103$^-$) (ii) BMDCs were sorted, as shown in (A), and the proliferation of T cells was assessed on day 5 by FACS. (C) is experiment 2, where R3 population (i) and R2 (CD103$^-$ MHCII$^+$) (ii) BMDCs were sorted, and T cell proliferation was assessed as before. Data were analysed by 2-way Anova, *p<0.05, **p<0.01, ***p<0.001. Experiments were performed in triplicate; (B, C), n=1.

The IL-17F concentration was also dependent on the number of B27-TG BMDCs used. In contrast, more IFN-γ was found in the non-TG co-cultures, especially when $1x10^4$ or $5x10^3$ DCs were used There was some variation in the concentration of the cytokines between experiments, however the trend was always similar to that indicated in the figure. Non-TG BMDCs induced more of IL-6, IL-2 and TNFα compared to B27-TG DCs, however there was no difference in the IL-10 level. No IL-12p70, IL-4, IL-1 or IL-5 were detected.

### *6.2.5 B27-TG BMDCs grown with hFlt3L are more viable than cells grown with mFlt3L*

There have been no reports to indicate that rat BM cells would not respond properly to mouse derived Flt3L, and indeed results shown earlier in this chapter with non-TG BMDC developing normally when grown with mFlt3L supports this. Nonetheless, as many investigators have used recombinant human Flt3L (hFlt3L) instead of mFlt3L cell culture supernatant, the effect of hFlt3L on BMDCs was investigated.

The concentration of hFlt3L used in literature for culturing either murine or rat BMDCs ranges from 100-200 ng/ml (60, 66). It was decided to use 100 ng/ml as the initial concentration for culturing BMDCs. Non-TG and B27-TG BM cells were and cultured for 7 days in the presence of 100 ng/ml hFlt3L. As before, cells were harvested on day seven, and stained for Annexin-V, DAPI, MHCII and CD103. When the viability of the cells was assessed by FACS, the difference between non-TG and B27-TG BMDCs was no longer apparent (Figure 6.14). First, FSC and SSC characteristics of the BMDCs looked similar in both non-TG (Figure 6.14, top panels) and B27-TG cultures (Figure 6.14, bottom panels). Second, viability of the cells was similar, as measured by Annexin-V and DAPI staining. In addition, there seemed to be a slight decrease in the proportion of B27-TG $CD103^+$ cells in the B27-TG cultures compared to the non-TG.

Since it had been noted earlier that addition of GM-CSF to mFlt3L BMDC cultures had a positive effect on the proportion of live cells, it was decided to see how the addition of GM-CSF would affect the hFlt3L BMDC cultures. Thus, non-TG and B27-TG BM cells were cultured for seven days in the presence of 100 ng/ml hFlt3L and 1 ng/ml GM-CSF, and the viability was assessed by FACS (Figure 6.15). It was noticed that under these conditions that the viability of both non-TG and B27-TG BMDCs seemed to be lower than with 100 ng/ml hFlt3L alone. However, there were no differences between non-TG and B27-TG BMDCs in terms of Annexin-V and DAPI staining, nor in the proportion of $CD103^+$ cells, or in the FSC and SSC characteristics.

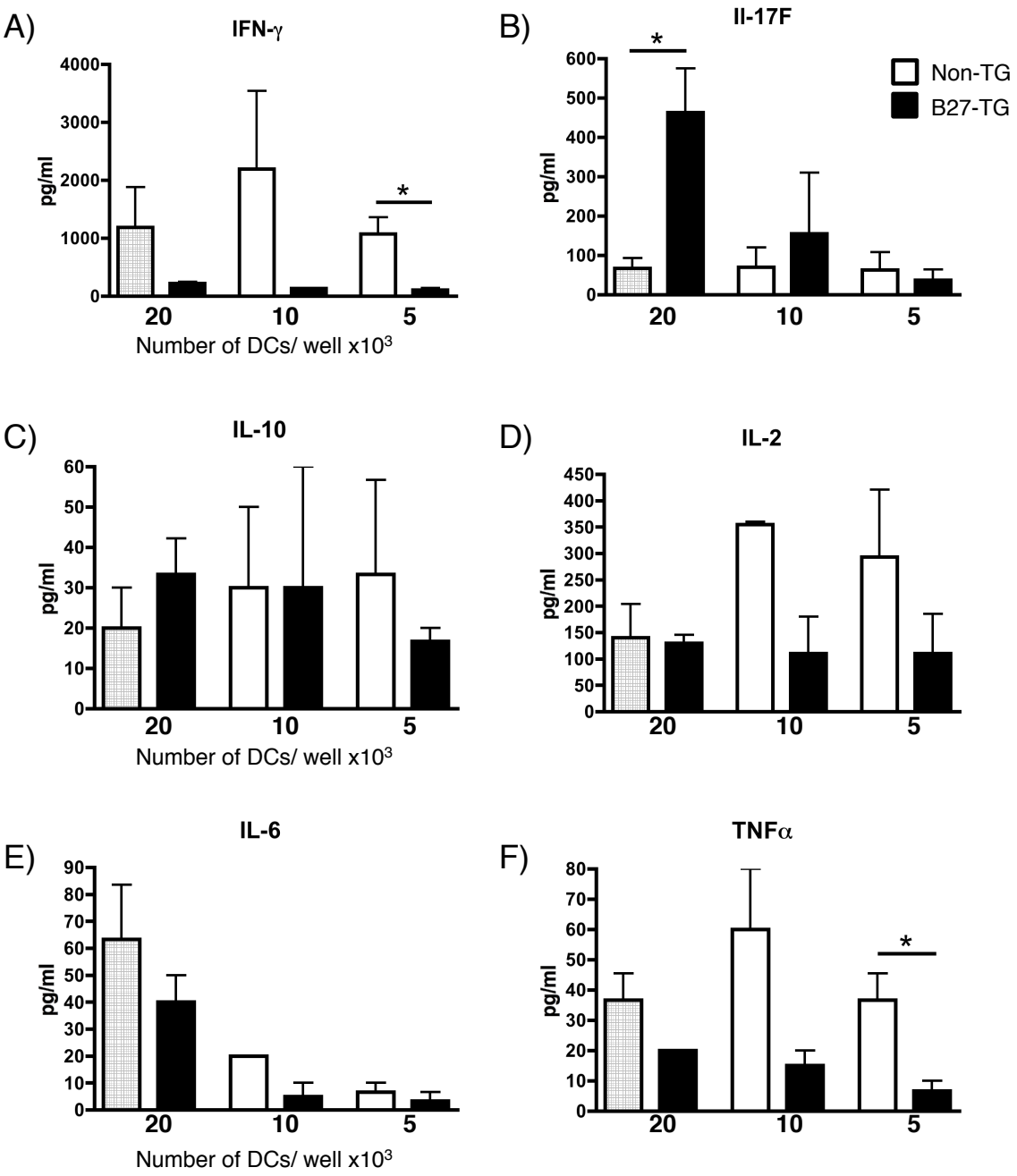


**Figure 6.13. B27-TG BMDCs induce more IL-17 and less IFN-γ than non-TG BMDCs**

$CD103^+$ mFlt3L BMDCs from non-TG and B27-TG animals and naïve $CD4^+$ T cells from DA rats were flow sorted, as described before, and co-cultured for 5 days. On day 5, the cell culture supernatants were collected, and frozen at -20ºC until use. Luminex was performed in triplicate (20 and $5x10^3$ DCs) or in duplicate ($10x10^3$ DCs), and results here are a representative of 3 (20 and 5 $x10^3$ DCs) or 2 (10 x $10^3$ DCs) biological replicates. Data were analysed by Student's T-test, *$p<0.05$.

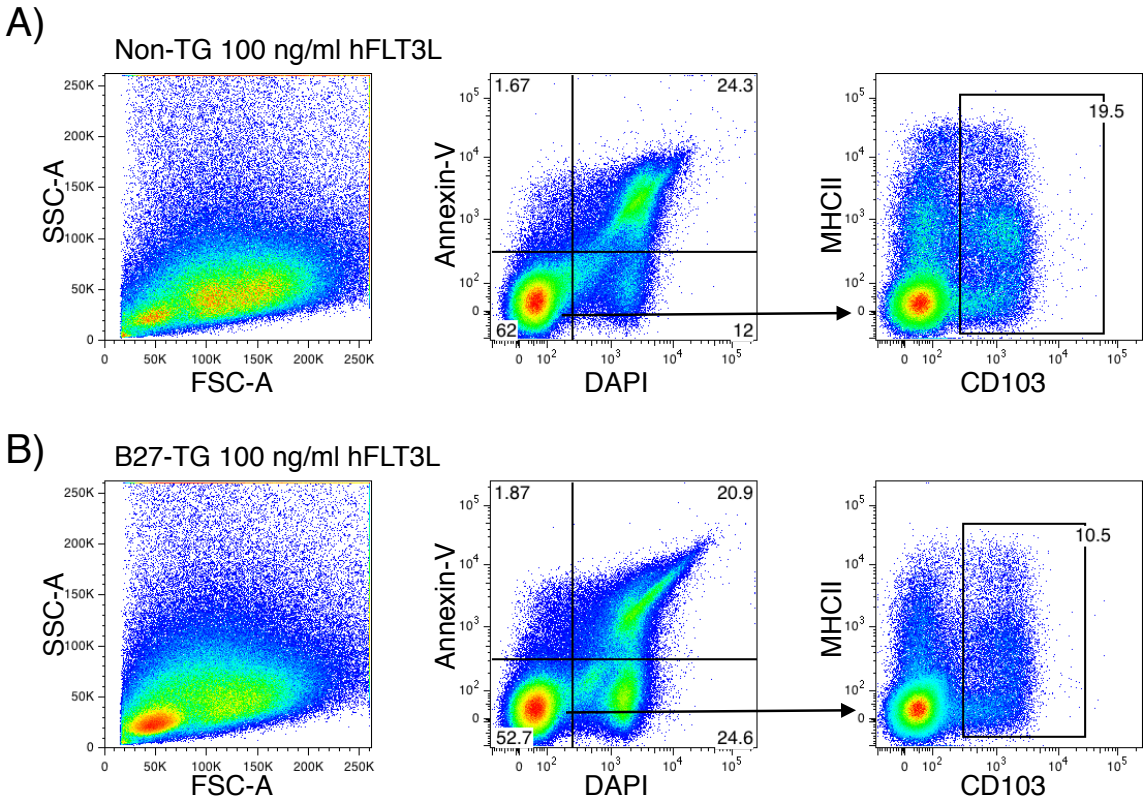


**Figure 6.14 Viability of non-TG and B27-TG BMDCs cultured with 100 ng/ml hFlt3L**

BM cells were harvested from non-TG and B27-TG animals, and cultured for 7 days in the presence of 100 ng/ml recombinant human Flt3L. On day 7 cells were harvested, and stained with Annexin-V, DAPI, MHCII and CD103 and analysed by flow cytometry. The percentages of live (Annexin-V$^-$ DAPI$^-$) and CD103$^+$ cells were assessed for both non-TG (A) and B27-TG (B) BMDCs. Frequencies in the figure relate to frequency of the parent. Figure is a representative of 7 individual experiments with 1-3 animals/experiment.

As the viability of B27-TG BMDCs was improved with 100 ng/ml hFlt3L compared to mFlt3L, it was decided to use a higher concentration of Flt3L to see if this would have an additional effect on the BMDCs. When BM cells were cultured in the presence of 10 ug/ml hFlt3L for 7 days, and analysed as above, it was noticed that the higher concentration of hFlt3L did not further increase the proportion of live B27-TG BMDCs, nor were there any differences in the FSC and SSC characteristics (Figure 6.16).

When data from several individual experiments and the three conditions were combined and compared, no significant differences were observed either in the proportion or absolute number of live Annexin-$V^-$ $DAPI^-$ cells between non-TG and B27-TG BMDCs (Figure 6.17A, B). It was observed, however, that on average 48 % of B27-TG hFlt3L derived BMDCs were live (Annexin-$V^-$ $DAPI^-$), compared to 34 % with mFlt3L (Figure 6.17 and Figure 6.4). When comparing the proportion and number of $CD103^+$ BMDCs, more were present in both non-TG and B27-TG BMDC hFlt3L cultures than when either GM-CSF was added to the cultures, or when a higher concentration of hFlt3L was used (Figure 6.17C, D). Again, as with mFlt3L, there was a relatively low proportion of cells undergoing apoptosis (Annexin-$V^+$ $DAPI^-$) in both non-TG and B27-TG preparations (Figure 6.17E). There was a slight increase in the percentage of apoptotic cells when GM-CSF was added to the culture, but this was not significant. Furthermore, the proportion of late apoptotic/dead (Annexin-$V^+$ $DAPI^+$) cells was not different between non-TG and B27-TG BMDCs, between any of the three conditions (Figure 6.17F). As with mFlt3L+GM-CSF grown BMDCs, also with hFlt3L+GM-CSF the proportion of $DAPI^+$ Annexin-$V^-$ cells was lower, but this time the difference was not significant (Figure 6.17G).

### *6.2.6 Phenotype of hFlt3L derived BMDCs*

It was also investigated if hFlt3L grown BMDCs would share a similar phenotype to that seen when mFlt3L was used. As before, non-TG, B27-TG and B7-TG BMDCs on day 7 were stained with various surface markers (Figure 6.18). The staining pattern was identical to that seen on mFlt3L derived BMDCs, with only a small proportion of $CD68^+$ cells, and even smaller proportion of $CD4^+$ or $CD8\alpha^+$ cells. I also determined whether BMDCs cultured with hFlt3L would respond similarly to LPS stimulation as cells grown with mFlt3L. As before, BMDCs were treated with LPS on day 6, and on day 7 the cells were harvested and stained with DAPI and antibodies to Annexin-V, MHCII and CD103.

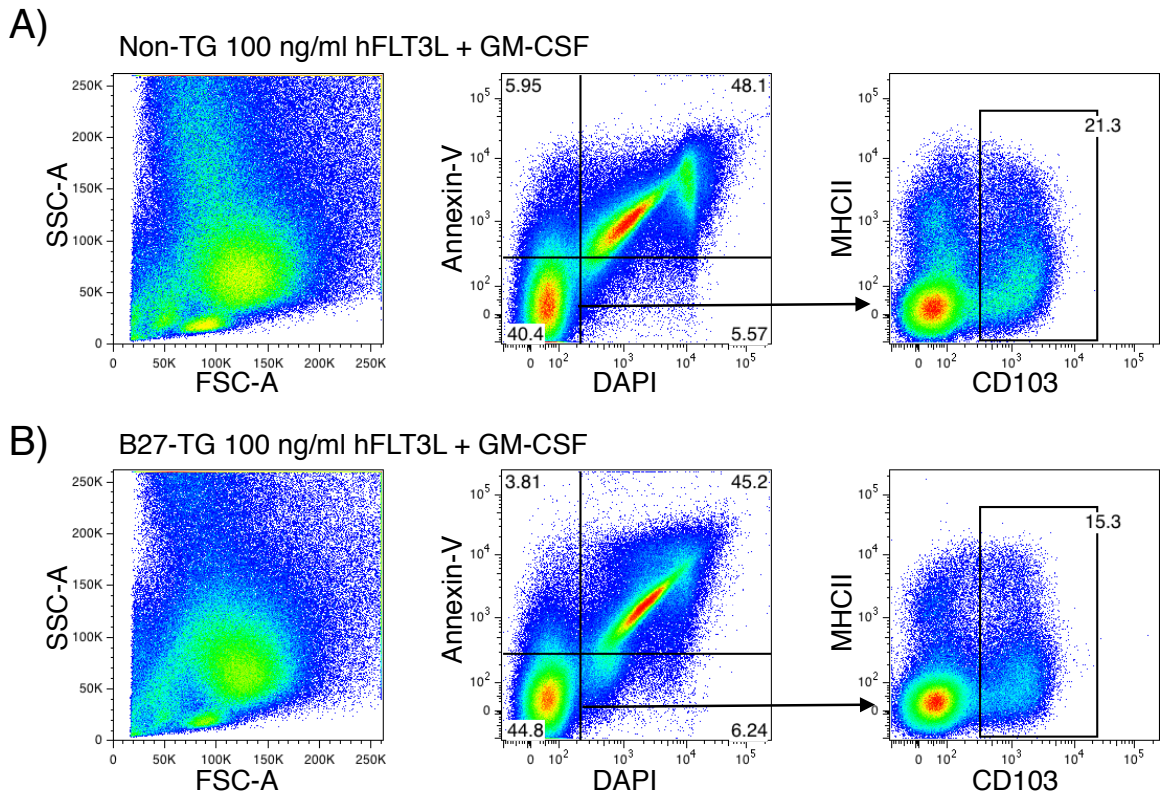


**Figure 6.15. Viability of non-TG and B27-TG BMDCs grown with both 100 ng/ml hFlt3L and GM-CSF**

BM cells were harvested from non-TG and B27-TG animals, and cultured for 7 days in the presence of both 100 ng/ml recombinant human Flt3L and 1ng/ml recombinant rat GM-CSF. On day 7 cells were harvested, and stained with Annexin-V, DAPI, MHCII and CD103 and analysed by flow cytometry. The percentages of live (Annexin-V$^-$ DAPI$^-$) and CD103$^+$ cells were assessed for both non-TG (A) and B27-TG (B) BMDCs. Frequencies in the figure relate to frequency of the parent. Figure is a representative of 3 individual experiments.

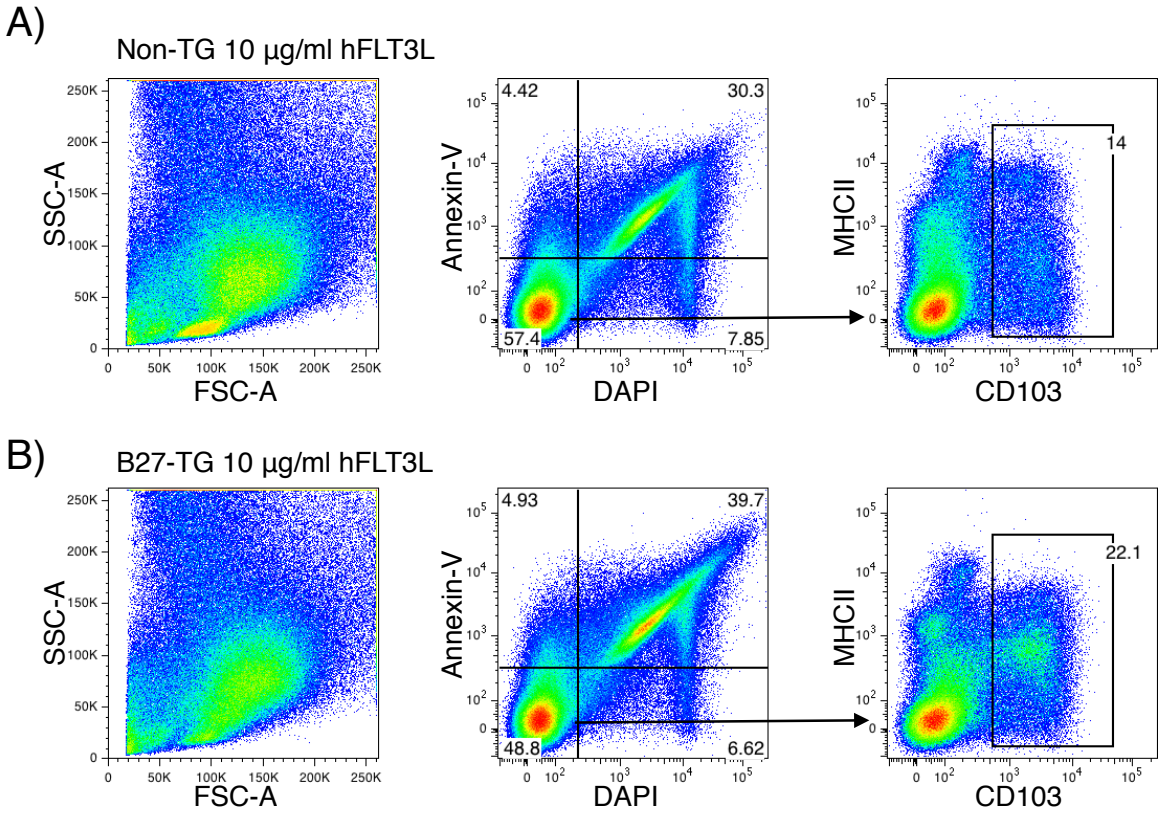


**Figure 6.16 Non-TG and B27-TG BMDC viability with 10 µg/ml hFlt3L**

BM cells were harvested from non-TG and B27-TG animals, and cultured for 7 days in the presence of 10 µg/ml recombinant human Flt3L. On day 7 cells were harvested, and stained with Annexin-V, DAPI, MHCII and CD103 and analysed by flow cytometry. The percentages of live (Annexin-V$^-$ DAPI$^-$) and CD103$^+$ cells were assessed for both non-TG (A) and B27-TG (B) BMDCs. Frequencies in the figure relate to frequency of the parent. Figure is a representative of 3 individual experiments.

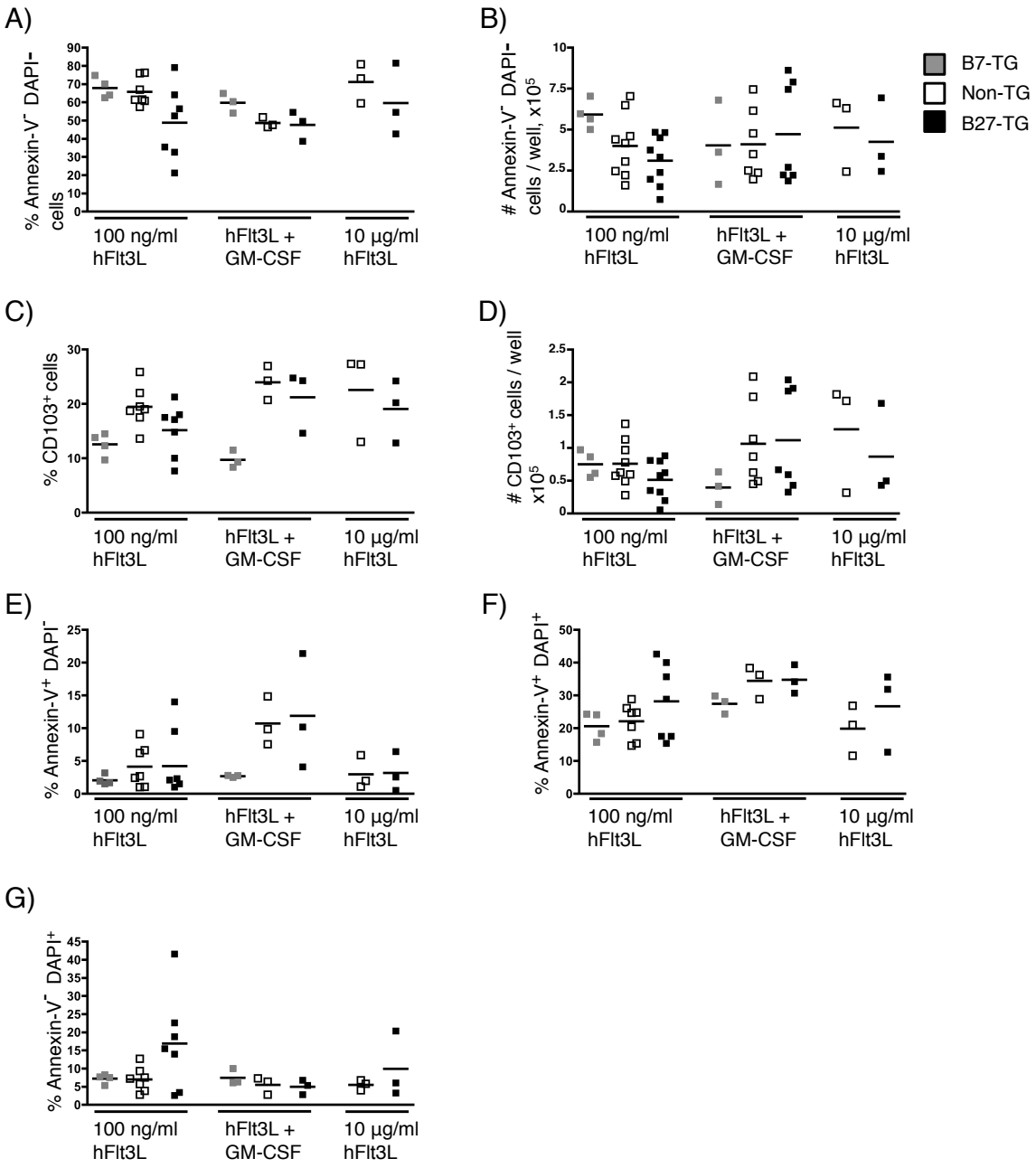


**Figure 6.17. Combined results for hFlt3L cultured BMDCs**

Combined results are shown for non-TG and B27-TG BMDC cultures supplemented with 100 ng/ml hFlt3L, 100 ng/ml hFlt3L+ 1 ng/ml GM-CSF, or with 10 µg/ml hFlt3L. (A, B) proportion and number/well of live (Annexin-$V^-$ $DAPI^-$) cells out of all BMDCs. Percentage (C) and number/well (D) of live $CD103^+$ cells were also determined. Proportion of apoptotic Annexin-$V^+$ $DAPI^-$ cells are shown in (F), whereas dead Annexin-$V^+$ $DAPI^+$ and $DAPI^+$ Annexin-$V^-$ are shown in (E) and (G), respectively. Results from 3-7 individual experiments, and data were analysed by 1-way Anova, *p<0.05, ***p<0.001. Error bars mean+SEM.

As with mFlt3L derived BMDCs, $CD103^+$ cells showed the strongest upregulation of MHCII after LPS stimulation in hFlt3L cultures (Figure 6.19). No differences were noted between non-TG and B27-TG BMDCs.

Since B27-TG derived BMDCs were shown to be deficient when grown with mFlt3L, it was tested whether non-TG and B27-TG BM cells express similar levels of the Flt3 receptor. In the absence of suitable antibody reagents, expression of Flt3 receptor transcript was measured by RT-qPCR. Freshly isolated BMDCs were lysed with RBC lysis buffer to deplete red blood cells, and RNA was isolated from the cells. RNA was reverse transcribed into cDNA, and the amount of Flt3 receptor RNA was assessed, during the PCR reaction, by SYBR green incorporation. The experiment was repeated twice, and no significant difference was observed in the amount of Flt3 receptor RNA present in B27-TG and non-TG freshly isolated BM cells (Figure 6.20A). In addition, it was investigated whether cells grown with either 10 % mFlt3L supernatant or with 100 ng/ml hFlt3L would express different amount of Flt3 receptor after 7 days of culture. Not surprisingly, it was found that both B27-TG and non-TG BMDCs grown with Flt3L had more Flt3 receptor when compared to freshly isolated BM cells (Figure 6.20B, C). Furthermore, both non-TG and B27-TG BMDCs grown with hFlt3L expressed about 20-fold more Flt3 receptor than cells grown with mFlt3L (Figure 6.20B, C).

## 6.3 Discussion

As DC development is dependent on the growth factor Flt3L, and defects in both intestinal and in splenic B27-TG DCs have been observed by others (144, 237, 239, 287) and by us, it is possible that there is an inherent developmental problem with DCs in B27-TG animals. Thus, we investigated whether the development of DCs from bone marrow with Flt3L would be the same between non-TG and B27-TG animals.

When BMDCs were grown for 7 days with 10 % mFlt3L supernatant, a significantly reduced viability of B27-TG BMDCs compared to either B7-TG or non-TG BMDCs was seen. This defect was already present on day 4 of the culture, indicating that lack of cytokines or nutrients in the medium was unlikely to be the cause for this loss of viability. This observed reduced viability of B27-TG BMDCs has not been reported before, but as described in the introduction, there are no previous reports of Flt3L being used to culture BMDCs from B27-TG rats.

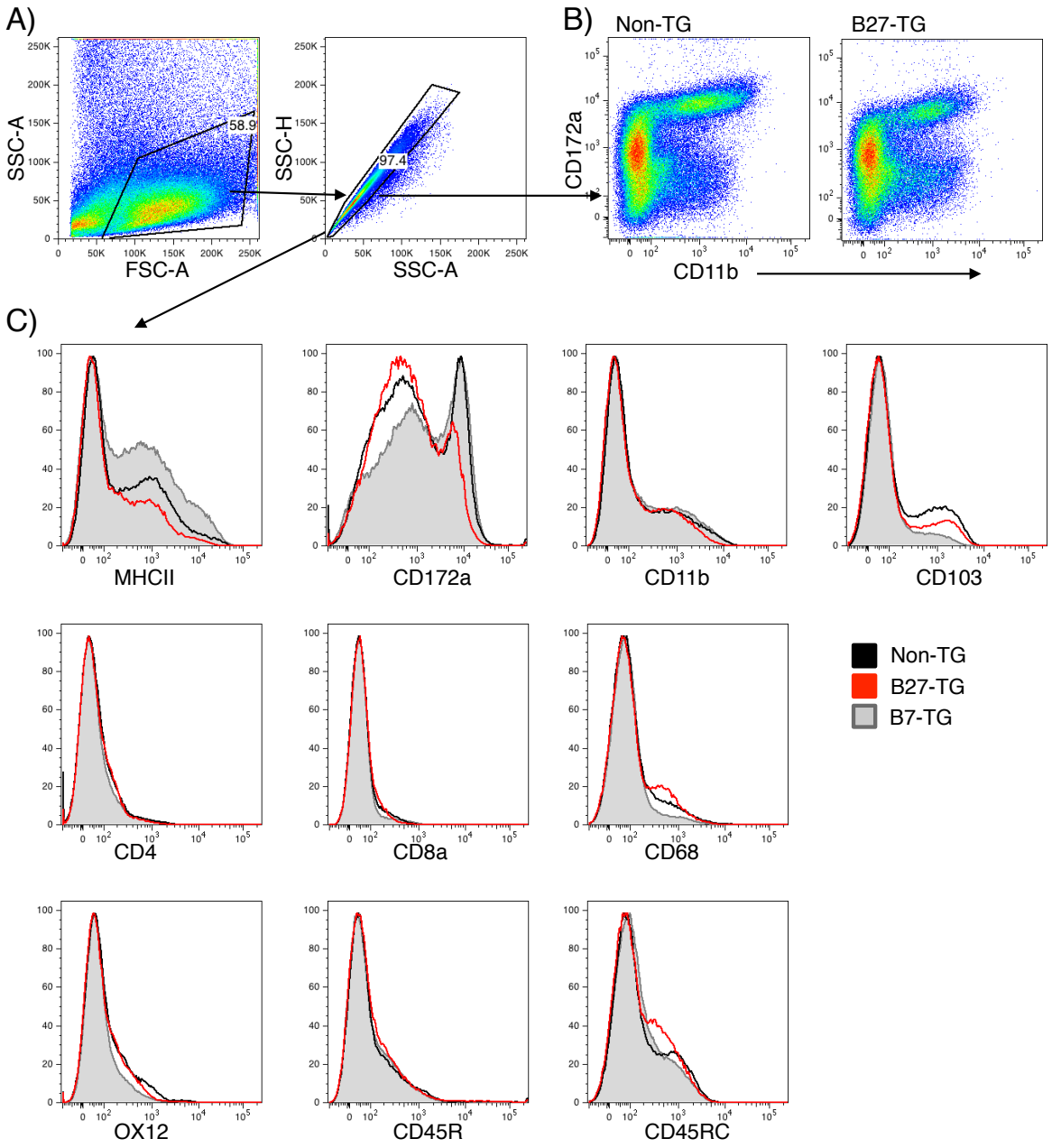


**Figure 6.18 Phenotype of BMDCs cultured with hFlt3L**

BM cells from B27-TG, B7-TG and non-TG animals were cultured with 100 ng/ml hFlt3L for 7 days, and on day 7 cells were harvested and stained for various markers as shown in figure. The expression level of these markers were compared between the animals. This experiment has only been performed once.

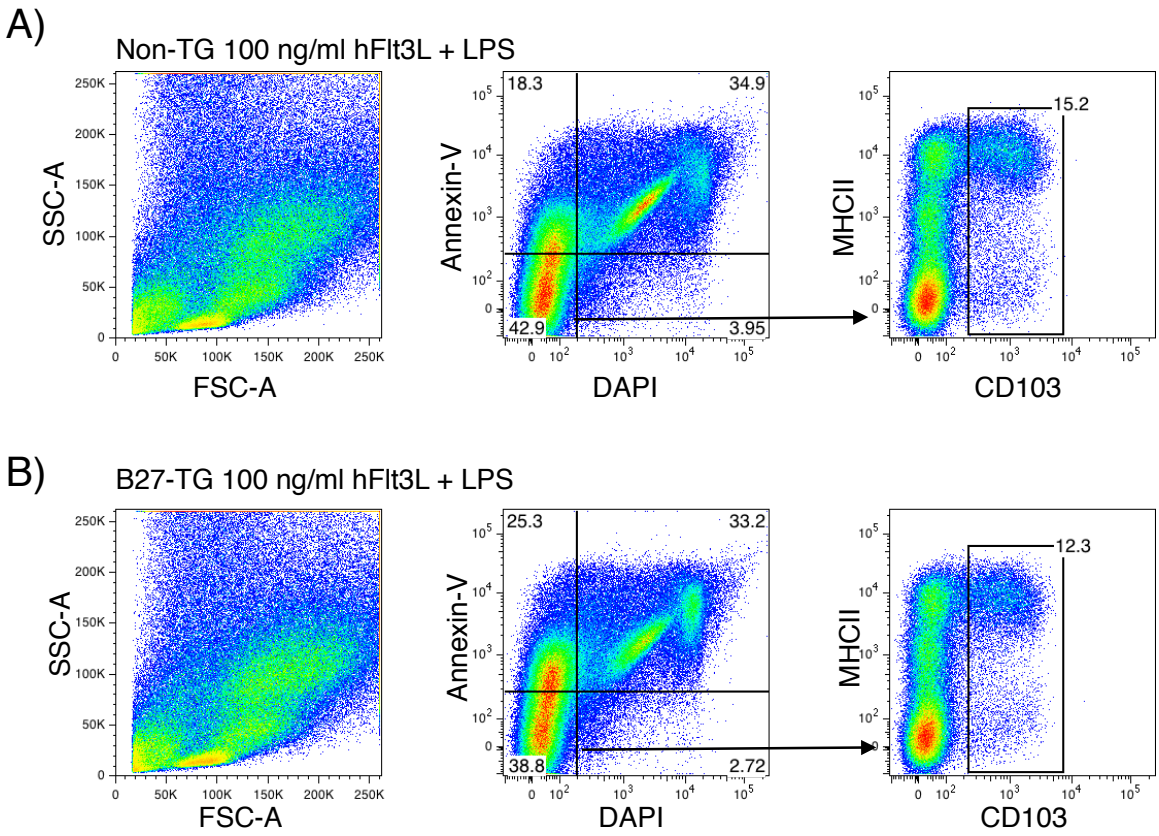


**Figure 6.19. Viability and phenotype of BMDCs cultured with 100 ng/ml hFlt3L and stimulated with LPS**

BM cells were collected form non-TG and B27-TG animals, were cultured for 6 days in the presence of 100 ng/ml hFlt3L, and were treated with LPS on day 6. On day 7, the cells were harvested and stained with DAPI and antibodies for Annexin-V, MHCII and CD103. The phenotype and viability of both non-TG (A) and B27-TG (B) BMDCs were analysed by FACS. Figure is a representative of 3 individual experiments.

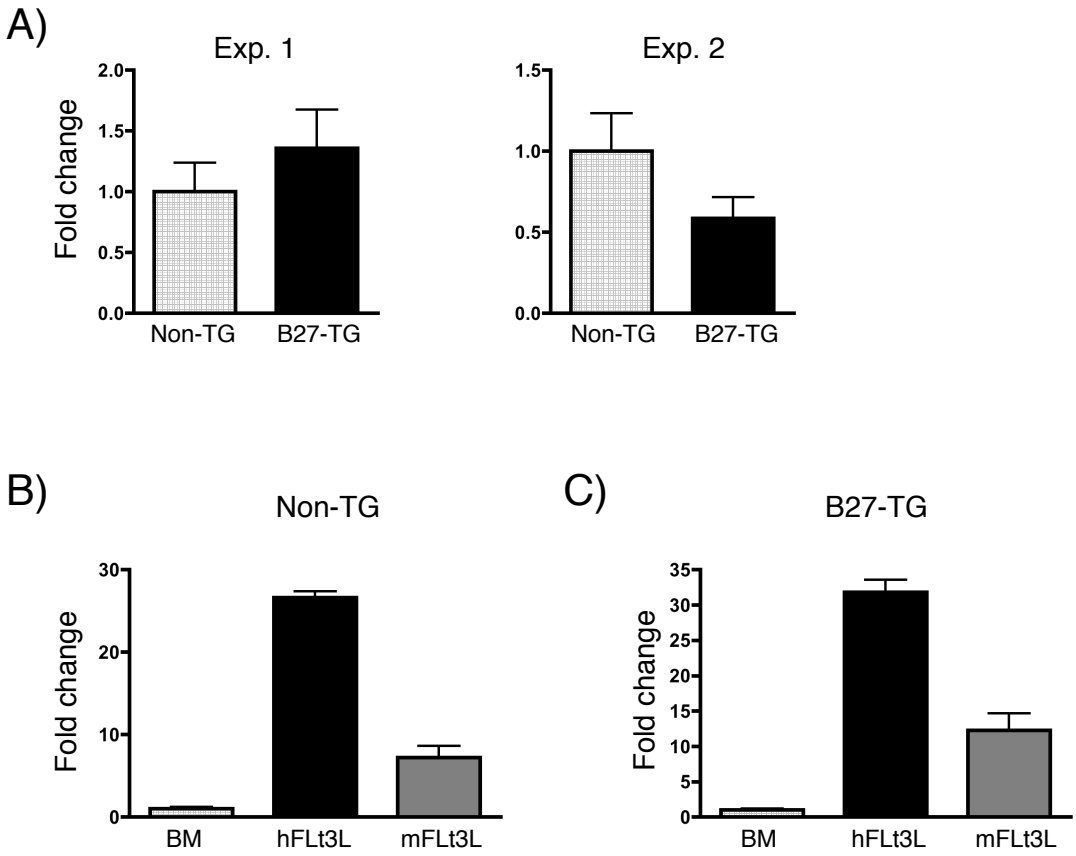


**Figure 6.20 No difference in the Flt3 receptor RNA expression between non-TG and B27-TG animals**

mRNA was isolated from either freshly isolated BM cells or from BMDCs grown for 7 days with either 10 % mFlt3L or 100 ng/ml hFlt3L, and reverse transcribed into cDNA. The level of Flt3 receptor RNA was analysed by Q-PCR for both non-TG and B27-TG cells. (A) Two individual experiments showing the expression of Flt3 receptor on freshly isolated BM cells from non-TG and B27-TG animals. Figure shows the fold change for B27-TG BM cells when non-TG BM sample has been set to 1. Expression of Flt3 receptor was also analysed on BMDCs grown with either mFlt3L or hFlt3L for non-TG (B) and B27-TG (C). In both cases Flt3L receptor RNA expression in the freshly isolated BM sample has been set to 1, and other values indicate the fold change shown as fold change.

The alternative method of using GM-CSF and IL-4 generates DCs of a more inflammatory type, not the steady state DCs that the lymph DCs represent (60). Curiously, when GM-CSF and Flt3L were used together, B27-TG BMDCs showed significantly improved viability compared to BMDCs grown with only mFlt3L. This could indicate there is a defect in the development of Flt3L dependent DC populations, but that the development of inflammatory DCs would not be affected in these animals.

Phenotypic analysis revealed no great differences between non-TG and B27-TG BMDCs. Even though Flt3L cultured BMDCs are said to more closely resemble the steady-state DC populations (288), they are not identical to DC populations in vivo. One notable difference to *in vivo* DCs is the expression of CD4. *In vivo*, a population of rat DCs express CD4, and in L-DCs CD4 is co-expressed with CD172a (273). Only a very few $CD4^+$ cells were seen in mFlt3L BMDC cultures. However, it should not be attempted to draw direct correlations between *in vitro* and *in vivo* DCs, as *in vivo* DCs receive a constant flow of information in the form of chemokines, cytokines and interactions with other cells, whereas *in vitro* the conditions are much more restricted.

BMDCs from both non-TG and B27-TG animals can be divided into three populations according to their expression of MHCII and CD103: $MHCII^-$ $CD103^-$ (DN), $MHCII^+$ $CD103^-$ ($CD103^-$) and $MHCII^+$ $CD103^+$ ($CD103^+$) populations. Further phenotypic characterisation of Flt3L derived BMDCs revealed that the three BMDC populations differed somewhat in their expression of L-DC subset markers CD11b and CD172a. Only the $CD103^+$ population was positive for both CD11b and CD172a, whereas the other two populations were more homogeneous. As all lymph DC subsets are positive to some degree for CD172a, it is likely that the $CD103^+$ population most closely resembles the *in vivo* L-DCs. No differences, however, were seen between non-TG and B27-TG BMDCs. The percentage and number of $CD103^+$ cells were compared between the non-TG and B27-TG animals and between the different culture conditions. It was hypothesised that Flt3L would induce more $CD103^+$ cells than the combination of Flt3L and GM-CSF, but this was not the case as B27-TG BMDC cultures with both Flt3L and GM-CSF contained more $CD103^+$ cells than Flt3L only cultures. However, BMDCs cultured with GM-CSF and IL-4 contain very few $CD103^+$ cells (Milling, unpublished data), suggesting that the $CD103^+$ DC population is indeed derived from Flt3L-dependent precursors. These results indicate, therefore, that in B27-TG animals Flt3L alone is not sufficient to support the survival and development of DCs, but addition of GM-CSF rescues the cells and augments their survival. Or alternatively, GM-CSF could be working on its own and generating

inflammatory-type DCs. In fact, examination of mice deficient in both Flt3L and GM-CSF revealed that these mice had fewer DCs than either single knockouts, and also the numbers of BM precursors were reduced (289). In the same publication it was observed that GM-CSF and Flt3L are both required for steady state regulation of dermal DC populations, especially the $CD11b^+$ subset, in the skin (289). Furthermore, it has also been demonstrated that $CD103^+$ $CD11b^+$ LP DCs are reduced both in GM-CSF and Flt3L deficient mice, suggesting they need both cytokines for their differentiation (290). As the dermal $CD11b^+$ cells can also be identified in the LNs, and $CD103^+$ DCs consist of DCs migrating from LP into MLN, these data indicate that some migrating DC populations require both Flt3L and GM-CSF for their development. The molecular mechanisms by which GM-CSF affects DC differentiation have been recently reviewed elsewhere (291). Curiously, as shown in chapters 4 and 5, it is the $CD103^+$ $CD172a^{lo}$ migratory DC subset that is reduced in B27-TG animals. Thus, it could be possible that the B27-TG DCs have a defect in Flt3 signalling, but they can be rescued by GM-CSF. It would be important to determine the levels of circulating Flt3L and GM-CSF in these animals to establish if there differences between non-TG and B27-TG animals. As it is difficult to reliably identify DC subsets in other rat tissues, it could be that there are missing or reduced DC subsets in other tissues as well. Thus, at the present it is not known how exactly Flt3L and GM-CSF affect *in vivo* DC populations in the rat.

LPS treatment of BMDCs revealed that the $CD103^+$ cells from both non-TG and B27-TG animals upregulated their expression of MHCII, thus further supporting the fact that these cells could be the *in vitro* equivalent of migrating L-DCs. The number of $CD103^+$ cells was not different between LPS treated and non-treated cultures, indicating that $CD103^+$ DCs do not proliferate in response to LPS, but just upregulate their MHCII expression. As could be expected because of the reduced overall viability of B27-TG BMDCs, after LPS stimulation B27-TG BMDCs had significantly decreased proportion of live cells compared to non-TG cells, but there was no difference in the absolute number of live cells/ well between non-TG and B27-TG BMDCs. However, when Flt3L+GM-CSF cultured BMDCs were stimulated with LPS, B27-TG DCs again demonstrated differential responsiveness to GM-CSF when compared to non-TG. Thus, B27-TG cells seem to respond differently to GM-CSF than non-TG cells, again indicating there could be a developmental problem with Flt3L-dependent B27-TG BMDCs, but not with the GM-CSF derived DCs. There were no great differences in the phenotype of B27-TG and non-TG $CD013^+$ BMDCs when expression of MHCII, CD11b, CD172a and CD25 were analysed before and after LPS stimulation. B27-TG $CD103^+$ DCs did seem to have constantly lower levels of all markers

investigated, but they did respond to LPS stimulation as non-TG cells. Expression levels of CD80 and CD86 were not measured, however, so it is not clear if there could be a difference in the expression of these markers between non-TG and B27-TG animals after LPS stimulation.

It has been reported that the unusual biochemical properties of the HLA-B27 molecule can lead to accumulation of misfolded HLA-B27 protein in the ER, as discussed in detail in the Introduction. An increase in incorrectly assembled proteins can lead to ER stress, and to activation of unfolded protein response (UPR) where the cells try to re-establish normal homeostasis (247). However, if ER stress is severe or chronic, these responses can lead to apoptosis or increased inflammatory signalling (282). Thus, it could be that B27-TG BMDCs were under ER stress and dying by apoptosis. Caspases are central molecules for the initiation of apoptosis, and a compound called Q-VD can inhibit three main caspase pathways: 9/3, 8/10 and 12 (284). We tested whether B27-TG BMDCs could be rescued by treatment with Q-VD. Indeed, when cells were treated with various concentrations of Q-VD, we observed that the proportion of viable B27-TG cells increased in a concentration dependent manner. The best results were achieved when 10 μM Q-VD was used, with a significant improvement in the viability of B27-TG BMDCs compared to non-treated cells, and in addition the FSC and SSC characteristics of the cells became similar to non-TG cells. Even when using this very high concentration of Q-VD, no increased cell death was observed in the non-TG BMDCs, indicating that Q-VD is not toxic.

Another compound called salubrinal was also tested. Salubrinal inhibits dephosphorylation of a molecule called elF2α, which is involved in the activation the UPR pathway, and thus salubrinal can inhibit ER-induced apoptosis (285). No improvement in the viability of B27-TG BMDCs was noticed when salubrinal was added to the cultures, despite a wide a range of concentrations used. However, it has been reported that salubrinal is not effective at inhibiting apoptosis in all cell types or cell lines, and thus it could be that it does not work effectively on the BMDCs (292). Since Q-VD improved the viability of cells and salubrinal did not, it could be argued that in the B27-TG BMDCs apoptosis is not caused by ER stress. However, ER stress has been suggested to activate especially the mitochondrial intrinsic caspase pathway as well, connecting these two mechanisms (293). It is also possible that I failed to discover the right concentration of salubrinal to use, and that is why no improvement in the viability of B27-TG BMDCs was seen. As Q-VD was so effective at inhibiting cell death *in vitro* and it did not show signs of toxicity even at very high concentrations, it might be possible to use it for treatment of B27-TG animals *in*

*vivo,* and to observe if this would have an effect on disease symptoms, or even if it would rescue the missing CD172a$^{lo}$ L-DC subset.

As was mentioned before, ER stress does not always lead to apoptosis, but it can also affect the functions of APCs either directly or indirectly. For example, co-culture of cells treated with the ER stress-inducing agent thapsigargin with mouse BMDCs leads to increased production of inflammatory cytokines IL-6, IL-23 and IL-12p70 by the DCs, although the latter two are only induced if LPS is also added to the cultures (294). Furthermore, thapsigargin-induced ER stress in human monocyte derived DCs induced increased production of IL-23 when LPS was added to cultures (295), and in addition increased IL-23 production by LPS treated macrophages from AS patients has been observed (253). A similar phenomenon has also been observed in B27-TG rats, where increased LPS dependent IL-23 production by macrophages (252), as well as increased Th17 cells have been noted (144, 252). Taking into account that all these results suggest a function-modifying role for ER stress, rather than apoptosis induction, the functions of surviving B27-TG and non-TG BMDCs were investigated, using the MLR as readout. Unlike L-DCs from B27-TG animals, B27-TG BMDCs grown with mFlt3L were very poor at driving CD4 T cell proliferation. Even when Q-VD was added to the cultures for the duration of the 5-day MLR co-culture to increase viability, no improvement was noticed in the capability of B27-TG BMDCs to drive cell proliferation. A more refined MLR method was also used, where DCs were co-cultured with flow sorted naïve T cells. In this instance, the B27-TG BMDCs were grown in the presence of Q-VD for 7 days, and Q-VD was also added to the DC-T cell co-cultures for the duration of MLR. However, B27-TG DCs again did not succeed in driving proliferation on naïve T cells.

Taking into account the information above suggesting that DCs and macrophages only show increased production of cytokines when also stimulated with LPS, it would be interesting to find out if the situation would change if these BMDCs were activated with LPS before the MLR. Migrating L-DCs from B27-TG were as good as non-TG L-DCs at inducing T cell proliferation, as shown in the previous chapter, which might be explained by the different microenvironments the L-DCs and BMDCs develop in. Also, taking into account the observed improvement in viability of B27-TG BMDCs when GM-CSF was used together with mFlt3L, it would be interesting to compare DCs grown with mFlt3L only to cells grown with both mFlt3L and GM-CSF. However, these results do suggest a specific HLA-B27 dependent defect of mFlt3L cultured BMDCs in inducing T cell

proliferation, as non-TG BMDCs were perfectly capable at driving T cell activation and proliferation.

In the above experiments total flow sorted live DCs were used. However, it was noted earlier that when LPS was added to the BMDC cultures, only the $CD103^+$ cells upregulated their expression of MHCII. Thus, when the three BMDCs subsets were sorted and used for MLRs individually, it was noted that, as expected, the $CD103^+$ cells were far more capable at inducing proliferation of naïve T cells than the two other populations. But as the three BMDC populations also differed in their abilities to induce T cell proliferation, it is clear that these are indeed separate populations. In the previous chapter it was shown that B27-TG L-DCs were less capable at driving proliferation of sorted naïve T cells, and thus these results indicate that both *in vivo* and *in vitro* $CD103^+$ DCs from B27-TG animals behave in a similar fashion.

The concentrations of cytokines produced from the MLRs with $CD103^+$ DCs were also measured to assess whether B27-TG and non-TG BMDCs induce differentiation of T cell with different phenotypes. It was noted that B27-TG BMDCs induced significantly more IL-17F and lower levels of IFN-γ compared to non-TG cells. The concentrations of cytokines varied between individual experiments, but always showed the same trend; B27-TG BMDCs induce more IL-17, even though they stimulate much less $CD4^+$ T cell proliferation. In contrast, non-TG BMDCs induce more IFN-γ. Of course, some of these MLR cytokines could be DC derived, such as IL-6, as it is not possible to differentiate which cytokines come from what cells exactly. Thus, to confirm the findings indicated above, and to confirm the cytokines are truly T cell derived, T cells from the MLR co-cultures could be stained intracellularly with antibodies for IFN-γ and IL-17. However, we have not been successful with either rat IL-17 or IFN-γ intracellular staining in our laboratory, despite attempts over a number of years, with a number of protocols using published antibodies.

The cytokine results differ from those seen in the previous chapter when *ex vivo* L-DCs were used, but that result was from one experiment and should be repeated to evaluate whether BMDCs and L-DCs truly differ in their functions. These functional experiments do reveal that not all BMDCs are equally capable of inducing proliferation of naïve $CD4^+$ T cells, and thus in the future greater care should be taken when using BMDCs for experiments. Also, B27-TG BMDCs are significantly less capable at inducing T cell proliferation than non-TG BMDCs, and non-TG and B27-TG BMDCs stimulate

production of different cytokines. These results add to the existing knowledge about how defects in B27-TG DCs may induce inflammatory disease in B27-TG animals.

As many investigators use recombinant human Flt3L instead of mouse Flt3L to culture rat BMDCs, it was also decided to see if it the species of origin of Flt3L would affect the viability B27-TG BMDCs. hFlt3L and mFlt3L have differences at both nucleic acid and protein level. The *mFlt3L* gene is about 4 kb long, whereas the *hFlt3L* gene is 5.9 kb, and the main translational product for both *mFlt3L* and *hFlt3L* is a transmembrane protein, which is cleaved to generate the soluble, bio-active form of Flt3L (296). The human and mouse Flt3L proteins are of similar length, with 235 and 231 aa respectively, and have 72 % homology (296). However, while the extracellular domains of human and mouse Flt3L are similar, the cytoplasmic domains only share 52 % sequence homology. Furthermore, mice have an additional membrane-associated isoform of Flt3L that is biologically active, and resistant to proteolytic cleavage (296). Flt3LR is also structurally different between the two species (296). In spite of these differences, hFlt3L has been successfully used in mice both *in vivo* and *in vitro* to expand DC populations (60, 69).

When BM cells were cultured with 100 ng/ml hFlt3L, it was immediately noticed that the viability of B27-TG BMDCs was better than when mFlt3L was used. B27-TG BMDCs still had a lower proportion and number of live cells, but the difference to non-TG animals was much less marked than when mFlt3L was used. In addition, when a much higher concentration (10 μg/ml) of hFlt3L was used, the difference between B27-TG and non-TG BMDCs was smaller still. This led us to hypothesize that the 10 % mFlt3L used had a lower than 100 ng/ml concentration of Flt3L, and thus maybe B27-TG BMDCs require a higher concentration of Flt3L to develop properly. Unfortunately I did not analyse the concentration of Flt3L in the cell culture supernatant that was used for the experiments, nor did I use a lower concentration of hFlt3L, which could have also addressed this issue. However, data from a colleague (Laura Ford, University of Glasgow, personal communication) indicated that the 10 % mFlt3L used for the experiments here has a concentration of about 160 ng/ml. If this is true, it indicates that B27-TG BMDCs respond differently to mFlt3L and hFlt3L, and importantly B27-TG cells have a defect in responding to the mFlt3L. There is some data to support a different response of murine DCs to human and murine Flt3L (297). In this study, mice were given *in vivo* the same concentration of either mFlt3L or hFlt3L, and splenic DC populations were compared. It was observed that hFlt3L caused a much bigger increase in DC numbers compared to mFlt3L. The authors could not provide an explanation for this observation. However, no

differences in the yield or viability of non-TG BMDCs grown with either mFlt3L or hFlt3L were noticed, suggesting that at least non-TG rat cells are perfectly capable of responding to both mFlt3L and hFlt3L. Furthermore, there are no differences between cells grown with mouse or human Flt3L in the response to LPS treatment, and in addition the phenotype of mFlt3L and hFlt3L grown cells is very similar, suggesting again that rat cells are capable of using both human and mouse Flt3L. As it was mentioned at the beginning of this section, mFlt3L and hFlt3L are indeed different, and thus it could be that there is a difference in Flt3R between B27-TG and non-TG animals, causing B27-TG to be less responsive to mFlt3L. Unfortunately, there was not enough time to carry out functional studies to compare the ability of hFlt3L and mFlt3L derived BMDCs to induce T cell proliferation and cytokine production.

Since experiments with mFlt3L did suggest there might be difference in the way non-TG and B27-TG BMDCs respond to Flt3L, it was decided to investigate by Q-PCR if the receptor for Flt3L was expressed at the same level on both non-TG and B27-TG animals. There was no difference in the level of Flt3 receptor on freshly isolated non-TG or B27-TG BM cells. However, it still possible that there could be differences between DC precursors from non-TG and B27-TG animals, but unfortunately it is not possible to identify and sort these pre-cursors from rats to carry out more detailed studies. When BM cells were cultured with either mFlt3L or hFlt3L, and the expression of Flt3 receptor was then determined on these mature BMDCs, it was first noticed that Flt3 receptor was more highly expressed on cells from both Flt3L cultures when compared to fresh BM samples. But, in addition, it was also observed that both non-TG and B27-TG cells grown with hFlt3L showed about 20-fold higher level of Flt3 receptor expression than cells grown with mFlt3L. Again, this could indicate that the 10 % mFlt3L supernatant used contains less than 100 ng/ml of Flt3L. Or that mFlt3L is not as effective as the human equivalent. However these experiments should be carried out several more times to obtain more biological replicates, and thus confirm if this pattern is consistent. It would also be useful to determine the levels of Flt3L and maybe also GM-CSF in the serum of the animals to see if there are any differences between non-TG and B27-TG animals.

In conclusion, these experiments have revealed a possible developmental problem in the mFlt3L-derived B27-TG BMDCs, which can be alleviated by GM-CSF. This developmental problem was revealed when a significantly higher number of BMDCs from B27-TG animals were observed to be either dead or undergoing apoptosis in mFlt3L cultures. This phenomenon could be reversed by addition of caspase inhibitor Q-VD into

the cultures, providing evidence that B27-TG BMDCs were dying by apoptosis. Furthermore, it was shown that mFlt3L-derived B27-TG BMDCs also had a functional defect, as B27-TG $MHCII^{+}CD103^{+}$ DCs were significantly worse at inducing proliferation of naïve T cells in a MLR compared to non-TG cells. Despite this defect, more IL-17 and less IFN-γ were found in the supernatants collected from the MLR co-cultures with B27-TG DCs than with non-TG DCs. These data agree with a recent publication demonstrating that B27-TG splenic DCs induce more Th17 cells than non-TG splenic cells *in vitro* (144). In addition, it was also recently shown that increased numbers of Th17 cells are present in the colonic lamina propria of B27-TG animals compared to non-TG animals (252). Thus, as in many other inflammatory diseases, Th17 cells have also been implicated in the pathogenesis of B27-TG animal model of SpA. As an increased concentration of IL-17 was observed in the B27-TG DC co-cultures, it could be that a defect in these DCs could drive differentiation of increased numbers of Th17 cells, and thus lead to inflammatory disease. However, discovering the exact role of these cells in the B27-TG animal model is not straightforward due to the lack of available antibody reagents, and the absence of knockout or additional transgenic animal models commonly used in murine research.

Also, it is important to understand how these data could relate to the human disease. In humans *in vitro*, only monocyte derived DCs can be examined, and thus it is not possible to directly translate the findings made here about rat BMDCs into humans. Nor is it feasible, at least not in our laboratory, to test BMDCs from humans. In addition, due to the difficulty in obtaining tissue samples, in humans circulating blood DCs are most commonly studied. In rodents however, these DCs are present in much lover frequency than in humans and are rarely studied, and in rats it is not possible to identify them, further limiting direct comparison of results from rat and human studies. However, much progress has been made recently in the identification of various human blood DC populations, their functions and possible similarities with DCs from other species. In our laboratory, in addition to detailed phenotypical analysis of human blood DCs, DC populations from healthy controls and AS patients are being compared. We are hoping these exciting studies will add to the current knowledge of human DC biology, as well as hopefully reveal the role HLA-B27 has in DC function and disease pathology in patients with SpA.

# 7. Final discussion

In the preceding chapters several aspects of the SpA inflammatory disease both in humans and in B27-TG animals have been discussed in detail. In addition, I have discussed the relevance of my results to published data. Thus, in this final chapter I would like to present two models to address how HLA-B27 could contribute to development of inflammatory disease both in the animal model and in HLA-B27$^+$ SpA patients. These models are consistent with the data presented in previous chapters, but are not necessarily directly related and aim to take a wider view of the events leading to overt disease. The first model focuses on the cellular events that could lead to inflammatory disease in rats, but some aspects could also be relevant to human disease. As several events at the molecular level have been proposed to contribute to development of inflammatory disease, especially in the animal model, the second model aims to combine several aspects of these events in to a framework to aid in the design of future experiments.

## 7.1 Model 1: Loss of a tolerogenic DC subset drives or exacerbates disease in B27-TG animals

As shown in chapter 4, migrating intestinal DCs in rats can be divided into three subsets, which all migrate from the intestine into the MLN even in steady state. All subsets are MHCII$^+$ CD103$^+$, but differ in their expression of CD11b and CD172a. These subsets are: CD11b$^{lo}$ CD172a$^{hi}$ (CD172a$^{hi}$), CD11b$^+$ CD172a$^{int}$ (CD172a$^{int}$) and CD11b$^{int}$ CD172a$^{lo}$ (CD172a$^{lo}$). B27-TG rats lack the CD172a$^{lo}$ subset of these DCs in the intestinal draining lymph, as shown in chapter 4, and the proportion of this same subset was also decreased in the MLNs of B27-TG animals (Chapter 5). The lymph defect was detected on rats over 10 weeks old, when these animals already have established disease, raising the question whether the DC defect is caused by the inflammatory disease in these animals. The reduced proportion of CD172a$^{lo}$ DCs in the MLNs was also originally observed on animals over 10 weeks old, however, I have subsequently discovered that the subset was already reduced in 6-week old animals. Animals of this age do not display inflammatory symptoms, suggesting that the DC defect precedes the onset of clinical inflammation. Thus, the lack of this subset could contribute to disease development in these animals. As has been discussed in detail throughout this thesis, the CD172a$^{lo}$ subset is thought to be involved in induction and maintenance of peripheral tolerance, as it has been noted to carry self-antigen from the intestine to the MLN. In the absence of inflammation, DCs migrating

from the intestine are in a semi-mature state, expressing high MHCII, but only low or intermediate levels of co-stimulatory molecules. In the MLN, these DCs could present these "non-pathogenic" Ags to T cells without engaging co-stimulatory molecules, leading to induction of anergy or Treg cells in the responding Ag-specific naïve T cells. This phenomenon of peripheral tolerance is illustrated in Figure 7.1 (points 1-3), which depicts the situation in non-inflamed intestine of a non-TG animal. Both mice and human $CD103^+$ MLN DCs have been shown to be uniquely capable of inducing generation of $FoxP3^+$ Treg cells, and rat DCs have also been shown to be able to do this. In addition, $CD103^+$ DCs in the MLN are able to imprint the responding T cells with gut homing molecules CCR9 and $\alpha4\beta7$. This aspect of $CD103^+$ DC function has not yet been investigated on rat DCs, but on-going studies in our laboratory are addressing this issue. After interacting with T cells, DCs remain in the MLN and probably die there. T cells and other lymphocytes, on the other hand, leave the MLN via efferent lymphatics, and return to circulation (Figure 7.1; point 4.). As these cells have been imprinted with gut homing molecules, they will eventually home back to the intestinal sites (Figure 7.1; 5.). These events create a homeostatic balance in the healthy intestine, where DCs maintain tolerance to harmless pathogens, but are still able to mount a rapid response to pathogenic antigens.

Figure 7.2 depicts the possible events taking place in B27-TG intestine. B27-TG animals lack the $CD172a^{lo}$ subset of DCs in lymph, which has been implicated in tolerance induction and maintenance (Figure 7.2; 1). At present, it is not known if this subset is present in intestinal tissue, but fails to migrate out to the MLN, or if there is a problem in the development of this specific DC subset from DC precursors. In either case, the result is that this subset is significantly reduced both in lymph, and in the MLN of B27-TG animals. The two remaining DC subsets have been previously shown to have a more activated phenotype compared to $CD172a^{lo}$ subset, and also respond differently to TLR stimulation, indicating that these DCs are more immunogenic. Thus, it could be that these two subsets carrying antigen from self, food or commensal bacteria in to the MLN could present these harmless antigens in an immunogenic fashion, leading to development of active immune response rather than tolerance (Figure 7.2; 2+3). Th17 cells specifically have been recently linked to development of inflammatory disease both in B27-TG rats and in humans, and thus it could be that these DCs induce Th17 differentiation over Th1 or Th2 differentiation. Supporting this are the results from chapter 6, where B27-TG $CD103^+$ BM derived DCs stimulated less proliferation, but more IL-17F production from naïve $CD4^+$ T cells than non-TG DCs. In addition, splenic DCs from B27-TG animals have been shown to induce

more IL-17 production (144). We have not yet investigated the function of $CD103^+$ DCs from the B27-TG MLN.

In addition to skewing towards Th17 induction, several other B27-TG DC defects have been reported, and these seem to specifically centre around DC – T cell interactions. Thus, it could be that B27-TG $CD103^+$ DCs have additional defect in imprinting homing molecules on T cells. As was discussed above, DCs in MLNs would normally induce gut homing molecules on the cells they activate. However, defective DCs could induce other homing molecules, such as CCR4 for skin homing. This would lead to cells activated in response to intestinal antigens leave the MLN, and home to other tissues where they could potentially be involved in pathogenesis (Figure 7.2; 4). This would explain the various symptoms observed both in SpA patients and B27-TG rats. Some evidence for this defective function of B27-TG DCs has emerged from studies in our lab (Drs Aude Aumeunier and Dawn Firmin, unpublished observations). However, some effector cells would also home back to the intestine, and contribute to development of the intestinal pathology (Figure 7.2; 5). Supporting this, increased colonic $Il\text{-}17^+$ APCs have been observed in B27-TG rats compared to non-TG animals (252). This could lead to a positive feedback loop, where Th17 cells induce more intestinal inflammation, leading to increased activation and migration of DCs to MLNs, and further generation of inflammatory cells. Data in chapter 4, however, demonstrates that there is in fact decreased number of DCs in the B27-TG lymph at 0-24h timepoint. This could be a result of chronic inflammation, where increased DCs did migrate at the acute phase, are not subsequently replenished properly from the precursors. As SpA patients often present with intestinal inflammation or lesions, it could be that some microbial events in the intestine, together with a susceptible phenotype, lead to development of inflammatory disease. This has been shown to be the case for development of reactive arthritis, but no direct evidence exists for other SpA disease. Understandably, investigation of human DC functions is more complicated as intestinal tissue samples are not readily available. Thus, research is mostly limited to peripheral blood DCs, whose relationship with their tissue counterparts is not entirely clear. However, increased circulating $IL\text{-}17^+$ $CD4^+$ T cells have also been reported in AS patients, thus indicating that these cells might have a role in the human disease as well.

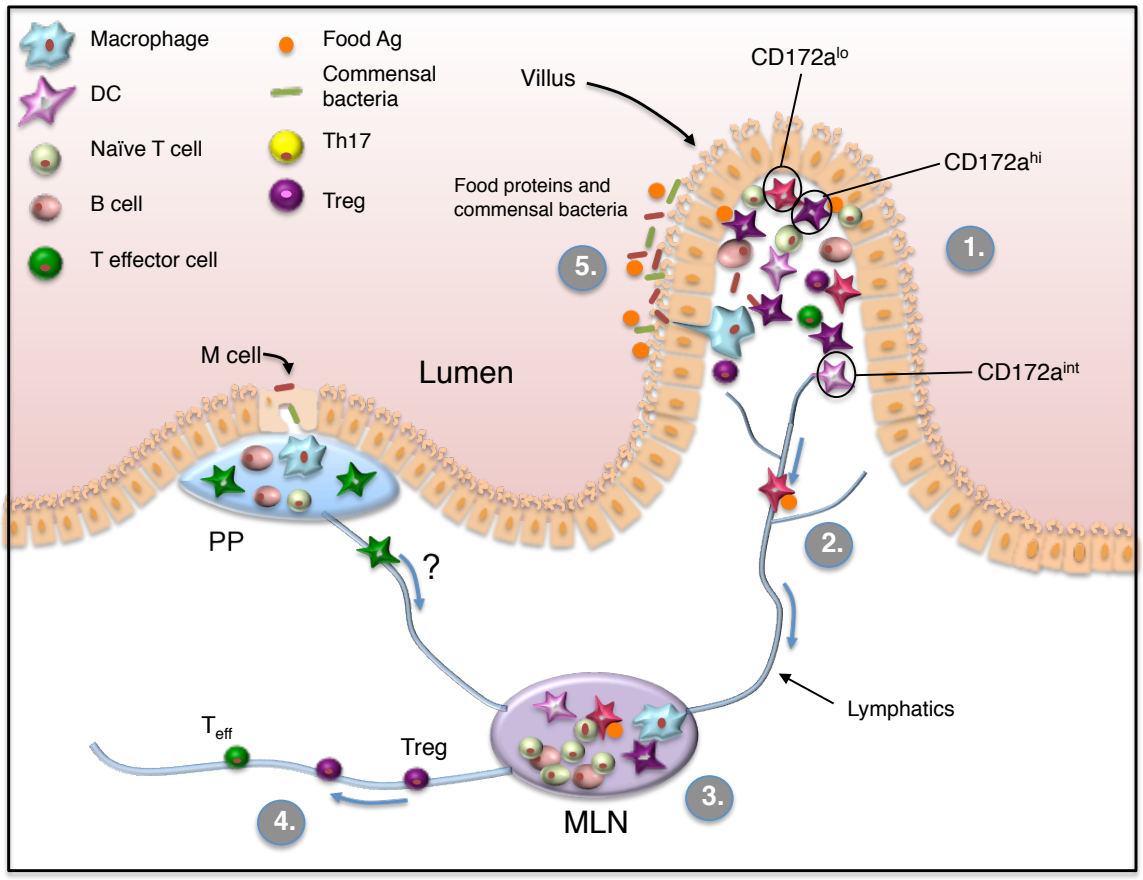


**Figure 7.1 Hypothetical non-TG intestine**

**(1)** In a non-TG healthy intestine, CD172a$^{lo}$, CD172$^{int}$ and CD172a$^{hi}$ rat DC subsets are all present. There is a balance between all the cell types, and no overt inflammation. **(2)** DCs migrate continuously from the intestine into the MLN via lymph, where they present Ag derived from self, food or commensal bacteria to naïve T cells. Specifically, the CD172a$^{lo}$ subset has been shown to carry self-Ag to MLNs. **(3)**. As these DCs are not activated and express low levels of co-stimulatory molecules, they induce T cells to either enter a state of anergy, or to differentiate into Tregs. Both these events contribute to peripheral tolerance to harmless antigens. Some effector T cells might also be generated. DCs in the MLN also induce expression of gut homing molecules, such as CCR9 and α4β7, on responding T cells. **(4)** T cells leave the MLN via efferent lymph, and return to the circulation. **(5)** Eventually, cells activated in the MLN return to intestinal sites.

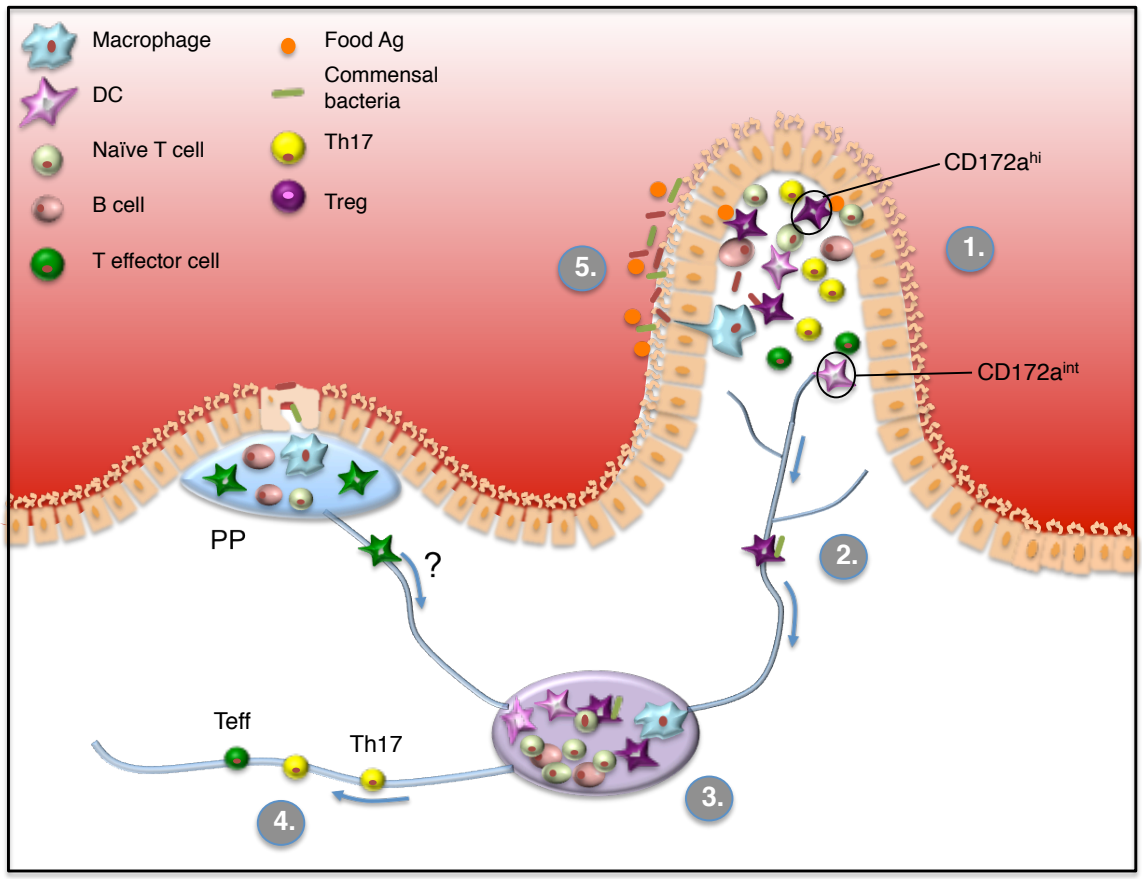


**Figure 7.2 Hypothetical B27-TG intestine**

**1)** B27-TG animals lack the subset of tolerogenic CD172a$^{lo}$ DCs. In the absence of these cells, the remaining CD172a$^{+}$ DC subsets carry Ag from the intestine into the MLN **(2)** and present Ag from self, food or commensal bacteria to naïve T cells. **(3)** The remaining DCs could be more activated, and can induce immune responses rather than tolerance, as Ag are presented to T cells together with co-stimulatory molecules. Specifically, Th17 cell differentiation can be induced. DCs can induce gut homing molecules on these cells, however defective B27-TG DCs could also imprint expression of homing molecules to tissues other than intestine, such as CCR4 for skin. **(4)** Thus, effector T cells primed with intestinal Ag can leave the MLN, and home back to various tissues, such as skin or synovium, explaining the emergence of peripheral inflammation in the rat. **(5)** Effector T cells homing back to the intestine can exacerbate the intestinal inflammation, and lead to even more inflammation.

## 7.2 Model 2: Molecular events leading to SpA disease pathogenesis

The cellular events presented above do not describe how the B27-TG DCs acquire their defective phenotype. In this section, I suggest molecular events that could lead B27-TG DCs and other cells to behaving differently from non-TG DCs. This model is unashamedly DC-centric. Thus, Figure 7.3 aims to demonstrate some of the molecular events that could contribute to either development or maintenance of inflammatory disease in rats and humans. The model is based on published results. However, some of the events have been published to take place outside SpA diseases, or in other systems, and have been brought together to illustrate events that could take place.

Firstly, HLA-B27 is a class I MHC molecule that behaves normally even in the transgenic animal system, and can thus present peptides on its surface to T cells. MHCI molecules normally interact with $CD8^+$ T cells, but as these cells appear to be redundant for disease in the animal model, alternative explanations have been proposed. For example, it has been shown that $CD4^+$ T cells from AS patients can bind to HLA-B27 molecules (257). In addition, it has been shown that HLA-B27 heavy chains can be expressed as β2m-free dimers on the cell surface (denoted $B27_2$) (249). These dimers are thought to be derived from heterotrimeric surface HLA-B27 molecules that are recycled through the endocytic pathway (298) (Figure 7.3; 1). It has been noted that KIR3DL1 and KIR3DL2 receptors expressed by NK cells and some T cells can recognise these unusual structures on the cell surface. KIR3DL1 can also bind to intact HLA-B27 molecules, in which case the interaction is dependent on the bound peptide. In contrast, interaction of both of these receptors with $B27_2$ is independent of the bound peptide sequence (264) (Figure 7.3; 2). Numbers of both $CD4^+$ T cells and NK cells expressing KIR3DL2 are increased in SpA patients compared to healthy controls (265, 299). KIR3DL2 is an inhibitory receptor shown to decrease IFN-γ production by NK cells upon binding their ligands (264, 299). In addition, $KIR3DL2^+$ cells from SpA patients are more activated, and *in vitro* culture with $B27_2$-expressing cells was shown to enhance their survival (265). As activated NK cells are potent killers, it could be that the increased resistance to apoptosis leads to increased numbers of NK cells in the tissues, which could contribute to pathogenesis in peripheral tissues. In addition, activated NK cells may induce DC maturation at sites of inflammation by a phenomenon called DC editing (300), and thus NK-DC interaction could lead to activation and maturation of DCs. The decreased IFN-γ secretion could be of importance as well in the context of Th17 versus Th1 polarisation, as IFN-γ is needed for Th1

polarisation, but not to Th17 cell differentiation. This would lead back to the proposed role of Th17 cells in SpA disease pathogenesis, which will also be discussed more below.

It has been shown that HLA-B27 molecule is prone to misfolding in the ER due to its slow maturation rate (248). This misfolding can create unusual heavy-chain homodimers in the ER, leading to accumulation of misfolded proteins in the ER, which has been demonstrated to cause ER stress in rats cells (250). ER stress triggers a specific cellular machinery, collectively called the UPR, which attempts to restore homeostasis in the cell (247). The UPR re-establish homeostasis, for example by decreasing protein translation in order to reduce the protein load in the ER. If these remedies are not successful, the cells can eventually die by apoptosis. However, in some cases ER stress can lead to production of pro-inflammatory cytokines via the NF-κB pathway. The molecular events leading to this outcome are not known at present. TLR activation can be linked to these events as well, as it has been noted that macrophages from B27-TG animals treated with the TLR4 ligand LPS firstly show more signs of UPR, and secondly produce increased levels of IL-23 (252). In addition, increased levels of IFN-β have been seen from TLR activated B27-TG macrophages (301). Furthermore, monocyte derived macrophages from AS patients showed markedly increased production of IL-23 upon LPS stimulation (253). However, the UPR has not been detected in human HLA-B27$^+$ cells. These events are depicted in Figure 7.3 at points 3 and 4. Thus far, no study has been conducted using DCs from either AS patients or B27-TG animals to investigate the effect ER stress has on these cells. In addition, we did not look for IL-23 in our experiments presented here, so it is not clear if similar events could be taking place in DCs. In addition, rat L-DCs have been shown to be hyporesponsive to TLR4 stimulation (189). These data do not however exclude the possibility that events like those described above could take place in DCs, perhaps driven by several TLR agonists. Intestinal DCs have constant exposure to several TLR ligands, and thus it could be that TLR ligation leads to increased DC activation, and thus to increased protein translation. In B27-TG cells, this would lead to accumulation of aberrant dimeric molecules in the ER, and would eventually lead to ER stress response.

Activated DCs can produce the cytokine IL-6, which is important in Th17 T cell differentiation (see introduction). In addition to IL-6, Th17 differentiation requires TGF-β, which is also required for differentiation of FoxP3$^+$ Tregs. However, in the presence of IL-6, Treg differentiation is inhibited, leading to induction of Th17 cells instead (Figure 7.3; 5a, b). As mentioned above, HLA-B27$^+$ cells can secrete more IL-23 in response to TLR stimulation, and increased expression of IL-23 has been detected in macrophages/DCs in

the colon of B27-TG animals (252), and also in the terminal ileum of SpA patients (302). IL-23 is an important cytokine involved in maturation and activation of Th17 cells (Figure 7.3; 6). Thus, aberrant behaviour of DCs and production of IL-23 can lead to increased numbers of Th17 cells. In fact, splenic DCs from B27-TG animals have been shown to induce more Th17 differentiation than non-TG DCs (144), and in addition, I noticed an increase in IL-17F cytokine concentration from MLRs with B27-TG BMDCs (chapter 6). Furthermore, increased numbers of IL-$17^+$ $CD4^+$ cells have been found in the colons of B27-TG animals (252). In humans, increased numbers of circulating IL-$17^+$ $CD4^+$ IL-$23R^+$ T cells have been found in the peripheral blood of SpA patients compared to healthy controls (267). In addition, these cells also express the KIR3DL2 receptor, and were shown proliferate when incubated *in vitro* in the presence of $B27_2$ expressing cells. Curiously, many of these cells were also shown to secrete IFN-γ in addition to IL-17, and the production of both cytokines was increased in response to IL-23. Recently, reports have emerged to suggest that Th17 cells differentiated in the presence of IL-6, IL-23 and IL-1β may acquire an "alternative" phenotype, and produce IFN-γ and IL-17 (303). In fact, it is these "double producers" that are often found at inflammatory sites, and are proposed to be more inflammatory than the "classical" Th17 cells (303). IL-23 can also have Th17-independent roles, as both DCs and macrophages can express IL-23R, leading to autocrine activation of the cells by IL-23, and leading to further inflammatory cytokine production (304).

Th17 cells produce several cytokines that have been indicated to have a role in pathogenesis. Firstly, Th17 can produce the growth factor and pro-inflammatory cytokine GM-CSF. In a recent publication it was shown that in the mouse model of EAE, IL-23 drives GM-CSF production by Th17 cells, and GM-CSF in turn drives IL-23 secretion by DCs (153) (Figure 7.3; 7). This would create a positive feedback loop, and lead to further increased numbers of Th17 cells. In addition, GM-CSF can drive the maturation and activation of both DCs and macrophages, thus leading to increased production of other inflammatory cytokines (275). In addition, GM-CSF can act on monocytes, which can differentiate into inflammatory DCs, further perpetuating the inflammatory response. It is also not known at present if there are differences in the concentration of serum GM-CSF between HLA-$B27^+$ SpA patients and healthy controls. Other Th17 derived cytokines can have widespread effects on a variety of cells types, leading to activation and IL-6 and TNF production from these cells, which would further drive the inflammatory response in a variety of target organs (Figure 7.3; 8).

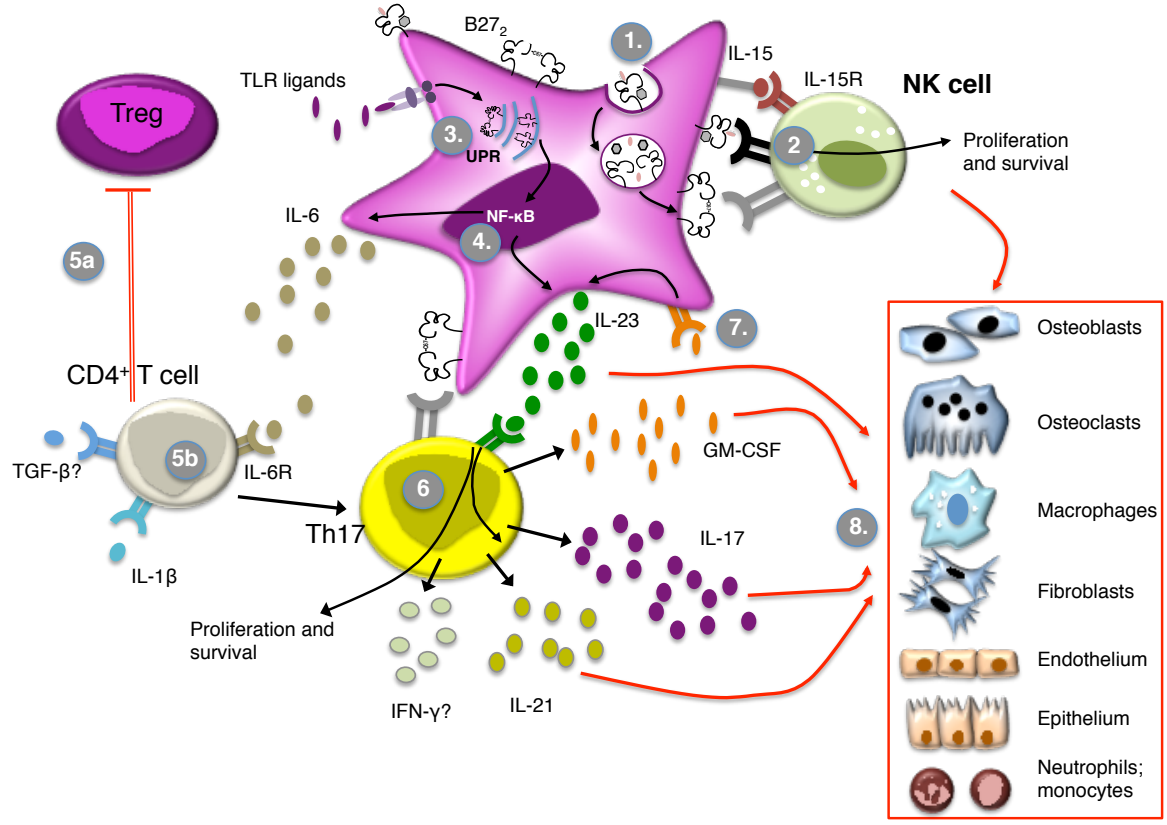


**Figure 7.3 Molecular events leading to SpA pathogenesis**

**(1)** HLA-B27 can form β2m free heavy chain homodimers ($B27_2$) on cell surface, and these are thought to be generated in the endosomes from recycled intact HLA-B27 surface molecules. **(2)** NK cells can express KIR3DL1 and KIR3DL2 receptors, which can bind both intact HLA-B27 molecules as well as $B27_2$ dimers. These interactions, with other activating factors such as IL-15, can lead to increased survival and proliferation of NK cells. **(3)** HLA-B27 molecule has a propensity to misfold in the ER, leading to accumulation of HLA-B27 heavy chain dimers in the ER. TLR stimulation increases the misfolding rate, and can lead to ER stress and UPR. **(4)** ER stress can also result in production of pro-inflammatory cytokines such as IL-6 and IL-23. **(5a)** IL-6 produced by DC can inhibit differentiation of naïve T cells into Tregs, even in the presence of TGF-β, and instead they can differentiate into Th17 cells **(5b)**. Presence of IL-1β can lead to generation of "alternative" Th17 cells, identified by their co-production of IFN$^-$γ and IL-17. **(6)** In addition, Th17 cells can express KIR3DL2 receptor, which can bind $B27_2$ dimers. IL-23 produced by the DC can induce proliferation and survival of Th17 cells. **(7)** Th17 cells can produce GM-CSF, which induces increased IL-23 production by the DC, thus creating a positive feedback loop. In addition, Th17 cells can produce other inflammatory cytokines, such as IL-21 and IL-17. **(8)** Th17 cytokines, and IL-23 alone, induce inflammatory changes in target cells and thus contribute to disease pathogenesis.

## 7.3 Conclusion

Experiments presented in this thesis have added to the existing pool of knowledge about role of DCs in the B27-TG rat model of SpA. Specifically they have provided intriguing new evidence for the possible role of intestinal dendritic cells in the HLA-B27-associated disease pathogenesis. Hopefully in the future we will be able to further investigate the role these DCs have in the animal model, and perhaps also confirm whether similar events could take place in the human SpA. The immunological events leading to the development of human SpA are far from clear, and very little is known about the role DCs have in this process. Taking into account the evidence that has emerged from the animal model, it would not be surprising to find out DCs have a central role in the human SpA pathogenesis as well. SpA treatment at present is not always very successful, and it is heavily relaying on the therapeutics used for treatment of RA or IBD, which are related, but different diseases with different immunological fingerprints. Thus it would be important to investigate if DCs could be targeted to offer more specialised next generation therapeutics for treatment of SpA.

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