Lymphodepletion in the Apc$^{Min/+}$ mouse model of intestinal tumorigenesis

Short title: Lymphodepletion in the Apc$^{Min/+}$ mouse

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Abbreviations used in this paper: APC, adenomatous polyposis coli; DN, double negative; DP, double positive; SP, single positive; wt, wild-type.

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ABSTRACT

Germline mutations in the *Adenomatous polyposis coli* tumour suppressor gene cause a hereditary form of intestinal tumorigenesis in both mice and man. Here we show that in *Apc<sup>Min/+</sup>* mice which carry a heterozygous germline mutation at codon 850 of *Apc*, there is progressive loss of immature and mature thymocytes from approximately 80 days of age with complete regression of the thymus by 120 days. In addition, *Apc<sup>Min/+</sup>* mice show parallel depletion of splenic NK cells, immature B cells and B progenitor cells in bone marrow due to complete loss of IL-7 dependent B cell progenitors. Using bone marrow transplantation experiments into wild-type recipients, we have shown that the capacity of transplanted *Apc<sup>Min/+</sup>* bone marrow cells for T and B cell development appears normal. In contrast, although the *Apc<sup>Min/+</sup>* bone marrow microenvironment supported short-term reconstitution with wild-type bone marrow, transplanted *Apc<sup>Min/+</sup>* animals subsequently underwent lymphodepletion. CFU-F colony assays revealed a significant reduction in colony-forming mesenchymal progenitor cells in the bone marrow of *Apc<sup>Min/+</sup>* mice compared with wild-type animals prior to the onset of lymphodepletion. This suggests that an altered bone marrow microenvironment may account for the selective lymphocyte depletion observed in this model of familial adenomatous polyposis.
INTRODUCTION

The *Adenomatous polyposis coli* (*APC*) tumour suppressor gene is mutated in the hereditary form of colorectal cancer, familial adenomatous polyposis (FAP) and in the majority of sporadic colorectal cancers in humans.\(^1\)\(^-\)\(^3\) FAP individuals inherit one mutant *APC* allele and tumorigenesis proceeds following loss of the second *APC* allele. In sporadic colorectal cancers, tumorigenesis involves somatic inactivation of both *APC* alleles.

Much of our insight into the functions of *APC* has come from analysis of murine models. The mouse Apc protein exhibits 90% amino acid (aa) homology to human APC.\(^4\) Several different mouse models of intestinal polyposis exist, each harbouring different truncating mutations of the *Apc* gene. The *Apc\(^{Min/+}\)* mouse is one of the most commonly used models to investigate the molecular mechanisms of intestinal tumorigenesis. *Apc\(^{Min/+}\)* mice carry a heterozygous germline mutation at codon 850 of the *Apc* gene\(^4\) and loss of the normal *Apc* allele in intestinal epithelium precedes adenoma formation.\(^5\) These animals develop adenomas along the length of the small intestine and colon. *Apc\(^{Min/Min}\)* homozygotes arrest development at approximately 5 days *post coitum* and die *in utero*.\(^6\)

Apc is a large 310 kDa protein characterised by the presence of several different functional domains that mediate interactions with numerous protein partners.\(^7\) Apc is postulated to have many functions including roles in microtubule dynamics, cell cycle control, cell adhesion and chromosomal stability.\(^8\) However, the tumour suppressor function of Apc appears to lie in its regulation of cellular levels of the proto-oncogene β-catenin.\(^9\)\(^-\)\(^11\) β-catenin has dual roles, in both cadherin-mediated cell adhesion and in cell signaling as a transcriptional effector of the canonical Wnt signaling pathway. In this pathway, Apc forms part of a high molecular weight phosphorylation/destruction complex with axin, glycogen synthase kinase (GSK)-3β, protein phosphatase 2a and GBP/Frat1 that targets β-catenin for
degradation via the ubiquitin/proteasome system. In response to a Wnt signal, β-catenin escapes phosphorylation by the Apc complex and translocates to the nucleus, where it activates expression of target genes through the Tcf/Lef-1 transcription factors. Apc can also shuttle between nucleus and cytoplasm further regulating the subcellular localisation and turnover of β-catenin. In the intestinal epithelium loss of function of Apc promotes tumorigenesis via constitutive activation of β-catenin/Tcf-4 mediated transcription of downstream targets including the growth promoting genes c-Myc and cyclin D1.

The Wnt signalling pathway is an evolutionarily conserved mechanism that governs cell fate decisions and patterning during embryogenesis and in adult tissues. Wnt ligands are secreted glycoproteins which act on members of the Frizzled and low density lipoprotein (LDL) receptor families to activate the canonical Wnt pathway. In the mouse at least 18 different Wnt genes have been identified, which show overlapping and/or cell-specific patterns of expression. Several Wnt genes are expressed in haemopoietic tissues and recently Wnt signaling has been shown to regulate haemopoiesis by effects on haemopoietic and stromal cells. Tcf-1 and Lef-1, the nuclear endpoints of Wnt signaling are expressed in developing T and B cells. Tcf-1−/− animals show thymic depletion and a block in T cell maturation. Lef-1−/− mice die shortly after birth and exhibit defects in pro-B cell proliferation and survival.

The emerging roles of Wnt signaling and Tcf-1/Lef-1 driven transcription in the immune system suggest that Apc, as a negative regulator of Wnt signaling, may also function in the differentiation of the lymphoid lineage. Apc protein is found in thymus, in lymphocytes and lymphoblastic cell lines although Apc mutations have not been linked to any haematological malignancies. In transgenic mice, over-expression of Axin, a component of the Apc phosphorylation complex, results in abnormal
thymus and a block in normal development of T lymphocytes again suggesting that components of the Wnt pathway, including Apc, may regulate lymphocyte development.

We have been investigating Apc function at the earliest stages of intestinal tumorigenesis using the \(Apc^{Min/+}\) mouse as a model. We noted that in addition to polyposis these animals exhibit thymic and lymph node atrophy which commences at approximately 80 days old. The similarity in phenotype between \(Tcf-1^{-/-}\) and \(Lef^{-/-}\) mice and the \(Apc^{Min/+}\) mouse with respect to lymphoid development, suggested that Apc may also play a role in haemopoiesis. However, a number of other factors are known to cause thymic involution. Several lines of evidence suggest that solid tumour growth itself is associated with immune down regulation in both humans and in animal models. Thymic atrophy in mammary tumour-bearing mice is associated with severe depletion of double positive (DP) T cells. Thymic involution has also been reported in mice with large colorectal carcinoma. In patients with sporadic colorectal cancer, a dramatic depletion of lymphocytes has been reported with a selective suppression of cytokines that is apparently related to tumour burden. Severe thymic atrophy can also occur secondary to various causes including stress malnutrition, graft-versus-host reactions, immunosuppressive or cytotoxic drugs and chronic viral infection especially with HIV.

To determine the nature of the lymphocyte defect in the \(Apc^{Min/+}\) mouse and relevant functional effects of Apc mutation, we characterised the lymphodepletion in these animals. Using bone marrow transplantation experiments, we have shown that there is no intrinsic defect in repopulating haemopoietic stem and progenitor cells of \(Apc^{Min/+}\) mice. However, there is a marked reduction in the incidence of mesenchymal progenitor cells (MPC) in the bone marrow of \(Apc^{Min/+}\) mice suggesting that the lymphodepletion observed in these animals may be related to an altered bone marrow microenvironment.
Materials and Methods

Animals

C57BL/6J-Apc\textsuperscript{Min/+} mice and wild-type (wt) C57BL/6J littermates were obtained at 6-8 weeks of age from The Jackson Laboratory (Bar Harbor, ME, USA). A breeding colony was then established in-house and offspring genotyped using allele-specific PCR of tail DNA, as described previously.\textsuperscript{39} All animals were housed in isolators and shown to be specific pathogen-free by bacteriological and serological testing. Experiments in the UK were undertaken under Home Office project and personal licence approval.

Histology

Specimens of thymi and lymph nodes were fixed in 4\% (w/v) paraformaldehyde prior to embedding in paraffin wax. Paraformaldehyde-fixed paraffin-embedded sections were mounted on glass slides and stained with haematoxylin and eosin (H and E) or Geimsa, by standard protocols.

Flow cytometry

Single cell suspensions were prepared from thymi, spleens and bone marrow, by ammonium chloride lysis. Monoclonal antibodies used were rat anti-mouse CD4-FITC, CD8-PE, B220-FITC, rat anti-mouse IgM-heavy chain-biotin (all from Serotec, Kidlington UK), SCA-1-FITC, GR-1-biotin, TER-119-PE, NK-1.1-PE (all from Becton Dickinson, Oxford, UK) and hamster anti-mouse CD3-FITC (Caltag, Burlingame, USA). Secondary antibodies were PE or Cy5-conjugated Neutravidin (a gift from Dr R.A. Jones, Leeds, UK) or Streptavidin-red 670 (Invitrogen, Paisley, UK).

Fluorescent staining was carried out for 15 minutes on ice followed by one wash with PBS supplemented with 2\% FCS and 0.1\% NaN\textsubscript{3} (Sigma. Poole, UK). In some experiments propidium
iodide (PI) (Sigma) at 4µg/ml was used for live-cell gating. Analyses were performed using Epics XL/MCL and dual-laser FACScan flow cytometers.

**Colony assays**

For CFU-Mix and Pre-B cell colony assays, 5x10⁴ bone marrow cells were seeded in duplicate in complete media MethoCult™ M3434 and MethoCult™ M3630 respectively (StemCell Technologies, Vancouver, Canada) and colonies were scored on day 7. For CFU-F colony assays, 5x10⁵ bone marrow cells were seeded in duplicate in murine Mesencult™ basal medium with mesenchymal stem cell stimulatory supplements (StemCell Technologies, Vancouver, Canada) and colonies scored on day 14.

**Bone marrow transplantation**

Wild-type female C57BL/6J animals were lethally irradiated twice with 4.75 Gray from a Caesium-137 source (CS-Quelle) at an interval of 4 hours. 2x10⁶ bone marrow cells from male Apc<sup>Min/+</sup> donors were then transplanted intravenously into the tail vein of irradiated recipient mice.

Female Apc<sup>Min/+</sup> mice were sub-lethally irradiated twice with 3 Rads and were transplanted with 2x10⁶ bone marrow cells from male wild-type donors into the tail vein. All transplanted mice received neomycin (0.16 g per 100ml) in drinking water for 4 weeks following irradiation. Animals were killed at different times post–transplantation and the thymi, spleens and bone marrow were analysed by flow cytometry and Southern blotting.
Results

Thymic and lymph node atrophy in Apc\textsuperscript{Min/+} mice

Apc\textsuperscript{Min/+} mice develop adenomas along the length of the small intestine and colon. In our colony, Apc\textsuperscript{Min/+} mice develop a total of 53±4 (mean±SEM) adenomas by 130 days of age.\textsuperscript{39} In addition to intestinal tumorigenesis, we observed that Apc\textsuperscript{Min/+} mice had a small or absent thymus when compared with wt littermates. One hundred day old animals showed a variable reduction in the size of the thymus of between approximately 60 and 90% when compared with wt littermates (Fig. 1A). Both lobes of the thymus appeared equally affected. The rate of thymic atrophy was variable in Apc\textsuperscript{Min/+} siblings and commenced at approximately 80-90 days of age with complete regression of the thymus in most animals by 120 days. The size and morphology of the thymus in 27 day old animals appeared normal (data not shown). The thymus was affected in all Apc\textsuperscript{Min/+} animals observed whilst none of the wt animals observed had affected thymi.

Histological analysis of thymi from 100 day old Apc\textsuperscript{Min/+} mice revealed cortical thinning of thymocytes in all of the animals observed and complete depletion of cortical and medullary thymocyte populations in the most advanced stages of thymic atrophy (Fig. 1B). In thymic remnants, small lymphocytes having small condensed nuclei not typical of thymocytes were present along with stromal epithelial cells (Fig. 1B). The epithelial cells in Apc\textsuperscript{Min/+} thymi at early stages of atrophy appeared normal as judged by immunohistochemistry with an anti-β-catenin antibody, showing a well established epithelial network which appeared more prominent than in wt animals due to cortical thinning (data not shown). There was no evidence of lipid-laden macrophages in sections of Apc\textsuperscript{Min/+} thymi, a feature commonly associated with thymic involution induced by glucocorticoids or by stress in normal animals.\textsuperscript{40}
In addition to the thymic atrophy in $Apc^{Min/+}$ animals, we also observed lymph node atrophy. The Inguinal lymph nodes from different 100 day old $Apc^{Min/+}$ mice showed a variable reduction in size with associated depletion of the paracortical T cell population (Fig. 1C). The combined thymus and lymph node phenotype observed in $Apc^{Min/+}$ mice is therefore consistent with a loss of lymphocyte populations in these animals.

*Depletion of the double positive T cell population in $Apc^{Min/+}$ mice*

The thymic atrophy with initial loss of the immature cortical thymocyte population in $Apc^{Min/+}$ animals suggested that there may be a disruption in thymocyte development. To determine the nature and stage of any developmental defect, we performed two colour flow cytometry using anti CD4 and anti CD8 antibodies on thymocytes from mice of different ages. Prothymocytes enter the thymus as CD4/CD8− double negative (DN) cells and then pass through a stage with low CD8+ expression before up-regulating both receptors to become CD4+/CD8+ double positive (DP). Following further maturation, DP thymocytes downregulate one of the co-receptors to become CD4+ or CD8+ single positive (SP) cells. SP cells migrate out of the thymus to populate peripheral lymphoid organs.41

At 26 and 83 days of age, $Apc^{Min/+}$ mice had similar levels of SP and DP T cells as wt littermates (Fig. 2). However, by 100 days of age in $Apc^{Min/+}$ mice there was complete loss of the DP thymocyte population (Fig. 2). This was followed by loss of the more mature CD4− and CD8+ SP population between 100 and 120 days. This suggests that in the $Apc^{Min/+}$ mouse there is a defect in T cell development at the DP stage or earlier.

*Depletion of pro- and pre-B cells in $Apc^{Min/+}$ mice*
The early defect in T cell development coupled with the lymph node atrophy observed in Apc<sup>Min/+</sup> mice raised the possibility that the defect manifested itself prior to T and B cell lineage separation and that B cell development may also be affected.

B cell development can be divided into distinct phases in mice that can be characterised by the expression of the B220 and IgM surface markers. Development proceeds through the pro- and pre- B cell stages (B220<sup>+</sup>, IgM<sup>-</sup>) to immature (B220<sup>+</sup>, IgM<sup>+</sup>) and recirculating mature B cells (B220<sup>++</sup>, IgM<sup>+</sup>). Analysis of lymphocytes in bone marrow of Apc<sup>Min/+</sup> and wt animals using two colour flow cytometry with anti B220 and anti IgM antibodies showed a progressive decrease in the proportions of B220<sup>+</sup> B cells in Apc<sup>Min/+</sup> animals compared with wt littermates (Fig. 3). Upto a 50% reduction in the number of B220<sup>+</sup> cells was evident in 100 and 120 day old animals. This depletion was due to a progressive decrease in the pro- and pre- B cells and immature B cells compared with wt littermates. By 100 days of age, Apc<sup>Min/+</sup> animals had a markedly reduced number of pro- and pre B cells suggesting failure to renew the early B cell population.

To establish that the block in B cell development in Apc<sup>Min/+</sup> mice occurred prior to the pre-B cell stage, we measured the number of IL-7 dependent B cell progenitors in the bone marrow of 100 day old Apc<sup>Min/+</sup> and wt mice. Apc<sup>Min/+</sup> mice had 9.6±3.9 (mean +/- standard deviation) pre B cell progenitors per 10<sup>5</sup> cells compared with 56.6±10.6 (n=3) in bone marrow of wt littermates. This suggested that the block in B cell maturation occurs at the IL-7 dependent stage of B cell development prior to the pre-B cell stage.
Myeloid and erythroid differentiation is normal in the bone marrow of Apc\textsuperscript{Min/+} mice

To determine if there was a parallel impairment in bone marrow myelo- and/or erythropoiesis in Apc\textsuperscript{Min/+} mice, we measured the total numbers of clonogenic progenitors (CFU-GEMM, CFU-GM and BFU-E) in a mixed colony assay. No significant difference in total myeloid and erythroid progenitor cell numbers from Apc\textsuperscript{Min/+} and wt littermates was observed (Fig 4). In order to examine whether myelopoiesis was affected we examined macrophage differentiation in Apc\textsuperscript{Min/+} and wt littermates by flow cytometric analysis after staining of bone marrow cells with the macrophage progenitor specific antibodies ER-MP12 and ER-MP20.\textsuperscript{43} No significant differences were observed (data not shown). Myeloid and erythroid maturation from clonogenic progenitors was also not impaired as proportions of both Gr-1\textsuperscript{+} (monomyeloid) and TER119\textsuperscript{+} (erythroid) cells in Apc\textsuperscript{Min/+} bone marrow remained at similar levels as wt littermates at all stages (data not shown).

\textit{Apc}\textsuperscript{Min/+} haemopoietic stem cells have lymphoid differentiation capacity in vivo

The parallel defects in T and B cell development observed in Apc\textsuperscript{Min/+} mice closely resembled those described in Tcf-1\textsuperscript{-/-} and Lef-1\textsuperscript{-/-} mutant mice respectively, albeit with a later time of onset. This striking similarity suggested that Apc might function directly in T and B cell development. The T and B cell developmental defects in Apc\textsuperscript{Min/+} mice could arise from an intrinsic defect in haemopoietic precursors cells or alternatively from changes in the bone marrow microenvironment.

To determine whether the Apc\textsuperscript{Min/+} genotype had a direct effect on repopulating stem and progenitor cells, we assessed the capacity of Apc\textsuperscript{Min/+} bone marrow cells from different aged mice to reconstitute the haemopoietic system of wt mice. Bone marrow from 63 day old male donor Apc\textsuperscript{Min/+} mice i.e. prior to the onset of lymphodepletion and from 100 day old male Apc\textsuperscript{Min/+} donors i.e. at an advanced stage of
lymphodepletion, was transplanted into lethally irradiated wt females. The T cell population in thymi and spleens and the B cell populations in bone marrow and spleens were characterised by flow cytometry (Fig. 5). Genomic Southern blot analysis on samples isolated at 11 months post transplantation using a Y-chromosome specific probe showed that the donor contribution was between 80 and 100 % (data not shown).

Bone marrow grafts of 63 and 100 day old donors sustained long term T and B lymphoid reconstitution in thymus, spleen and bone marrow of transplanted animals when analysed at 337 days post transplantation. This showed that Apc\textsuperscript{Min/+} bone marrow could differentiate and maintain T and B cells in a wt environment. Furthermore, as the 100 day old Apc\textsuperscript{Min/+} bone marrow graft was capable of lymphoid-lineage reconstitution, this suggested that lymphodepletion in Apc\textsuperscript{Min/+} mice may be due to an intrinsic stromal cell defect or alternatively to failure of the complete bone marrow microenvironment to support cell survival and/or differentiation.

WT bone marrow can repopulate Apc\textsuperscript{Min/+} mice in vivo but is still subject to lymphodepletion

To further determine if the lymphodepletion observed in Apc\textsuperscript{Min/+} animals was due to failure of bone marrow or thymic stroma to support B and T cell differentiation, wt bone marrow cells were injected into sub-lethally irradiated Apc\textsuperscript{Min/+} animals prior to the onset of lymphodepletion. This would determine firstly if the animals could be reconstituted and secondly, if the reconstituted immune system would undergo lymphodepletion. Sub-lethal rather than lethal irradiation was used to ensure survival of the relatively fragile Apc\textsuperscript{Min/+} animals.
Bone marrow from 49 day old male wt mice was used to transplant sub-lethally irradiated 51 day old female \(Apc^{\text{Min/+}}\) mice. Flow cytometric analysis of thymi, spleens and BM of transplanted animals at 93 days of age (42 days post-transplantation) showed that \(Apc^{\text{Min/+}}\) animals were reconstituted with all T and B cell populations to the same extent as wt animals (data not shown). Genomic Southern blotting of samples isolated at 6 weeks post transplantation using a Y-chromosome specific probe showed that the donor contribution was between 80 and 100 % (data not shown). This suggests that the bone marrow and thymic stroma of \(Apc^{\text{Min/+}}\) mice was capable of supporting short-term T and B cell reconstitution.

Flow cytometric analysis of 143 day old transplanted animals (92 days post transplantation) revealed a decrease in the number of DP T cells in thymus with a relative increase in the population of CD4\(^+\) and CD8\(^+\) SP cells (Fig. 6). CD4\(^+\) and CD8\(^+\) T cells were also reduced in the spleens of recipient \(Apc^{\text{Min/+}}\) animals. Similarly, the number of mature B cells in spleen was also reduced. In recipient \(Apc^{\text{Min/+}}\) bone marrow, the number of pro- and pre- B cells was reduced by approximately 86% compared with wt recipients. This pattern of depletion mirrored that observed in untreated \(Apc^{\text{Min/+}}\) animals. These results show that the reconstituted T and B cell populations are subject to lymphodepletion by 142 days of age. The depletion of T and B cell populations was not as advanced as that observed in the untreated 120 day old \(Apc^{\text{Min/+}}\) mice (Fig. 2 and 3). However, as all of the animals tested (n=3) showed lymphodepletion albeit at various stages, this suggests that the onset of depletion in these animals was delayed. \(Apc^{\text{Min/+}}\) control animals which were sub-lethally irradiated without transplantation also had a reduced level of lymphodepletion at the same age (data not shown).

_Depletion of NK cells in \(Apc^{\text{Min/+}}\) mice_
The fact that $Apc_{Min/+}$ mice transplanted with wt bone marrow were fully reconstituted and then underwent lymphodepletion suggested that the depletion might be due to change in the bone marrow microenvironment. One of the major factors regulating the bone marrow microenvironment is the cytokine milieu. IL-7 is crucial for murine T and B lymphopoiesis and the primary source of IL-7 in the bone marrow are stromal cells of non-haemopoietic origin. In contrast, IL-7 is not essential for the development of NK cells and IL-7$^{-/-}$ mice have an increased frequency of NK cells in spleen. We therefore examined NK cell development in $Apc_{Min/+}$ mice to determine if defective IL-7 signalling may play a role in the observed lymphodepletion in these animals.

Splenocytes were stained with anti NK1.1 and anti CD3 antibodies and defined as NK1.1$^{+}$ CD3$^{-}$ (Fig 7). At 36 and 63 days of age, $Apc_{Min/+}$ mice had similar levels of NK cells and CD3$^{+}$ T cells as wt littermates. However, there was a progressive loss of NK cells in $Apc_{Min/+}$ mice of approximately 58% and 72% by 110 and 120 days of age respectively. This was accompanied by depletion of the splenic CD3$^{+}$ T cell population. This shows that there is a parallel and progressive depletion of T, B and NK cells in $Apc_{Min/+}$ mice which suggests that defective IL-7 signaling cannot alone account for the observed phenotype.

Mesenchymal progenitor cells are reduced in the bone marrow of $Apc_{Min/+}$ mice

Many of the factors, both secreted and cell associated, that are required for the self-renewal and differentiation of HSC, are produced by the bone marrow stroma. The stroma is a complex tissue composed of a heterogeneous population of vascular and connective tissue cell types. Bone marrow stroma also contains a rare population of MPC (sometimes referred to as mesenchymal stem cells) that
are capable of self-renewal and can differentiate along multiple mesenchymal cell lineages.\textsuperscript{49} MPC play a vital role in HSC survival, proliferation and differentiation. MPC from whole bone marrow grow as clonal populations derived from a single precursor termed the colony forming unit-fibroblast (CFU-F).\textsuperscript{50,51} To determine if the observed lymphodepletion was due to cellular changes in the $Apc^{Min/+}$ bone marrow stroma, we determined the numbers of CFU-Fs in bone marrow of wt and $Apc^{Min/+}$ mice.

The number of CFU-Fs derived from bone marrow of $Apc^{Min/+}$ mice was significantly lower than that of wt animals at 36, 63 and 87 days of age i.e. at stages prior to and at the onset of lymphodepletion (Fig. 8). There was on average a 5-fold decrease in the number of colony forming cells in $Apc^{Min/+}$ bone marrow compared with wt animals. There was no difference in the number of colony forming cells in $Apc^{Min/+}$ mice of different ages. In wt animals the number of CFU-Fs increased from 36 to 63 days of age and then remained constant (Fig. 8).
Discussion

The *Apc* tumour suppressor gene codes for a large multi-domained protein which is ubiquitously expressed.\(^7\) It is mutated in several different tumours of epithelial origin but its function in haemopoietic tissues has not been previously investigated. We show here that *Apc\(^{Min/+}\)* mice in addition to intestinal tumorigenesis show progressive loss of immature and mature thymocytes from 83 days of age with complete atrophy of the thymus by 120 days. This is accompanied by a parallel and progressive depletion of B cell progenitors and immature B cells in bone marrow, atrophy of lymph nodes and depletion of the NK cell population.

As *Apc* plays a pivotal role in transduction of the Wnt signalling pathway, we considered that the *Apc\(^{Min/+}\)* lymphoid phenotype may be related to defective Wnt signaling. Wnt signalling has been shown to be important for T and B cell development and for self-renewal of HSC.\(^26\) In *Tcf-1* knockout mice, DP thymocytes fail to develop from immature SP thymocytes\(^27\) although in these animals unlike in *Apc\(^{Min/+}\)* mice, T cell maturation deteriorates progressively from birth and halts completely around 6 months of age.\(^27,28\) Mice lacking both *Tcf-1* and *Lef-1* have a complete block in T lymphopoiesis.\(^52\) *Lef-1\(^{-/-}\)* animals die two weeks after birth and *Lef1\(^{-/-}\)* pro-B cells show reduced cell proliferation and increased apoptosis both *in vitro* and *in vivo*.\(^24\) In contrast, Wnt signaling in stromal cells has been shown to inhibit B cell lymphopoiesis. Yamane et al.\(^25\) showed in vitro that B cell cultures supplemented with Wnt 3a completely inhibited B cell development and that this effect was mediated through stromal cells.

The *Apc\(^{Min/+}\)* T and B cell defects described here at first sight resembled those described for *Lef-1\(^{-/-}\)* and double knockout *Lef-1\(^{-/-}\)/*Tcf-1\(^{-/-}\)* cells, showing a similar stage specific block in development. However, in *Apc\(^{Min/+}\)* mice there was no obvious defect in T and B cell progenitors prior to 83 days of age. Using...
bone marrow transplantation experiments, we showed that there was no apparent intrinsic defect in ApcMin/+ bone marrow stem cells and progenitors as they are capable of long term T and B cell reconstitution in wt mice. This suggests that normal Wnt/Lef-1/Tcf signaling pathways are active in these populations. In contrast, Tcf-1 deficient bone marrow was unable to reconstitute lethally irradiated recipients.28 Furthermore, 100 day ApcMin/+ bone marrow grafts taken from animals at an age when lymphodepletion was advanced could also support long term lymphoid reconstitution. This shows that haemopoietic stem cell populations as well as T and B progenitor cells capable of short term reconstitution, were present in the ApcMin/+ bone marrow at this time and in a wt environment could proliferate and differentiate normally. This suggests that the ApcMin/+ allele does not disrupt Lef-1/Tcf action in haemopoietic progenitor and stem cell populations and that lymphodepletion may therefore be related to changes in the bone marrow microenvironment.

Haemopoiesis is regulated by the complex interplay between the bone marrow microenvironment and stem and progenitor cells. Bone marrow stroma contains a heterogeneous population of cells which provides the required cytokines, adhesion molecules and matrix molecules for stem cell populations to self-renew and differentiate appropriately.47 Transplantation experiments using wt bone marrow into ApcMin/+ animals were performed to determine if the ApcMin/+ haemato-lymphoid microenvironment was capable of supporting reconstitution. As short term reconstitution of T and B cells was achieved, this suggested that the microenvironment could initially support seeding of transplanted bone marrow cells and sustain lymphoid development. However, by 143 days of age ApcMin/+ animals grafted with wt bone marrow of a similar age, showed the same pattern of lymphodepletion with loss of T and B cell progenitors in thymus, spleen and bone marrow. This suggests that the bone marrow microenvironment in ApcMin/+ animals cannot sustain lymphopoiesis.
BM stromal cells originating from MPC play a major role in supporting haemopoiesis in the bone marrow. Surprisingly, we observed that the number of colony forming MPC was markedly reduced in the bone marrow of $Apc^{Min/+}$ mice compared with wt animals. This reduction was already evident in young animals (36 days) at a time when the lymphocyte compartment appeared normal. There was no change in the number of CFU-Fs with age in $Apc^{Min/+}$ mice. This raises the possibility that the $Apc^{Min/+}$ genotype exerts an intrinsic effect on the development of mesenchymal progenitors in bone marrow. This could arise from a dominant negative effect of the truncated protein expressed from the $Apc^{Min}$ allele, from haploinsufficiency for full-length Apc protein, or by loss of Apc function via somatic mutation of the wt Apc allele. All three mechanisms have been suggested to de-regulate Wnt signalling. However, our own preliminary data (S. Holwell, personal communication) suggests that there is no loss of the wt $Apc$ allele in cultured MPC.

The reduction in MPC in $Apc^{Min/+}$ mice raises the possibility that Apc and/or Wnt signalling regulate proliferation and/or differentiation of mesenchymal progenitors. Bone marrow stromal cells express frizzled receptors and respond to WNT proteins\textsuperscript{25} and a number of Wnt proteins are found in bone marrow.\textsuperscript{24,53} In this context, it is interesting to note that TCF-4$^{-/-}$ animals have reduced numbers of epithelial stem cells in the intestine\textsuperscript{54} showing Wnt involvement in the regulation of stem cell compartments. Furthermore, in the haemopoietic system, stimulation of HSC and progenitors with soluble Wnt proteins or downstream activators of the Wnt signaling pathway leads to their expansion. Reduction of the MPC population in $Apc^{Min/+}$ mice suggest that Wnt signaling may play a similar role in the expansion/differentiation of MPC and that failure to expand the MPC population may underlie the lymphodepletion in these animals.
It is not yet clear why lymphodepletion in $Apc^{Min/+}$ mice starts at around 80 days of age whilst prior to then, T, B and NK cell development appears normal. The timing of onset may reflect a cumulative effect of a reduced MPC population over time, or alternatively that secondary factors may be involved. Physiological states including stress and nutritional deficiency may trigger lymphodepletion by exerting an additional effect on a fragile bone marrow microenvironment. At 80 – 90 days of age at the onset of lymphodepletion, $Apc^{Min/+}$ mice have macroscopically visible adenomas in the intestine which equates to a diameter greater than 1mm in size. From this stage onwards, these animals exhibit progressive physiological stress associated with tumour burden. It is possible that this factor may contribute to lymphodepletion. Bone marrow stromal cells have been shown to protect haemopoietic precursors from corticosteroid-induced apoptosis\textsuperscript{55} suggesting a possible relationship between tumour burden and an altered bone marrow compartment in $Apc^{Min/+}$ mice.

It is not known if the MPC population is altered or if lymphodepletion as observed in $Apc^{Min}$ mice, occurs in individuals with FAP who carry germline mutations in the $APC$ gene. Lymphopenia in patients with sporadic colorectal cancer has, however, been described and changes in cytokine levels were documented.\textsuperscript{35,36} The $Apc^{Min/+}$ mouse provides a unique model to investigate regulation of proliferation and differentiation of bone marrow MPC and to investigate the relationship between the bone marrow stroma, lymphodepletion and immune suppression relating to tumorigenesis \textit{in vivo}. 
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Figure 1; Thymic and lymph node atrophy in Apc\textsuperscript{Min/+} mice

A. Thymi from (a) wt and (b and c) Apc\textsuperscript{Min/+} animals at 100 days of age showing the reduction in size and variable rate of atrophy in Apc\textsuperscript{Min/+} mice.

B. Thymic histology of (a) wt thymus and (b-d) Apc\textsuperscript{Min/+} thymi at 100 days of age. Three different thymi from the same age Apc\textsuperscript{Min/+} animals are shown demonstrating the initial depletion of cortical thymocytes and the variable rate and stages of atrophy. The most advanced stage of atrophy is shown in (d).

Sections are stained with H and E; C cortex, M medulla.
C. Lymph node histology of (a) wt and (b-d) $Apc^{Min/+}$ lymph nodes at 100 days of age. Inguinal lymph nodes from three different $Apc^{Min/+}$ animals at various stages of atrophy are shown demonstrating the progressive depletion of the paracortex.

Sections are stained with Geimsa. PC, paracortex, PF, primary follicle, GC germinal centre.
Figure 2; Depletion of the DP T cell population in thymi of *Apc^{Min/+}* mice.

Flow cytometry results for anti CD4^+ CD8^+ staining of thymocytes from the different stated ages for wt and *Apc^{Min/+}* mice are shown. Expression of surface markers on cells was detected using an Epics XL/MCL flow cytometer and analysed using the WinMDIv2.7 programme. FCS/SSC gating was used
to exclude debris and doublets and dead cells were gated out on the basis of PI positivity measured on the FL-3 channel. The percentages of cells in each gate are shown in each panel. Each panel is representative of data from at least three different animals.
Figure 3: B cell depletion in the bone marrow of Apc$^{Min/+}$ mice

To identify B cell maturation stages, cells were double stained with monoclonal antibodies B220 and anti IgM. For analysis, a lymphoid FCS/SSC gate was set up to exclude other cell lineages and the B cell maturation stages were identified as pro- and pre- B cells with a B220$^+$/IgM$^-$ phenotype, immature
B cells with a B220+/IgM+ phenotype and mature B cells with a B220+++/IgM+ phenotype. The proportions of cells present in each subpopulation are given as a percentage of the total B220 positive population. Results are representative of at least 3 different animals per group.
Figure 4; Myelo and erythropoiesis are similar in bone marrow of $Apc^{Min/+}$ and wt mice

The numbers of clonogenic erythroid and myeloid progenitor cells in a mixed colony assay are shown for mice of different ages. Numbers are shown as mean ± SEM.
Figure 5; Flow cytometric analysis of lymphocyte populations in wt mice transplanted with $Apc^{Min/+}$ bone marrow.

Analysis of animals transplanted with bone marrow from 63 (63d) or 100 (100d) day old male donors at 337 days post transplantation. Cells from thymus and spleen were stained with anti CD4 and anti CD8 antibodies (upper panel) and from spleen and thymus with anti B220 and anti IgM antibodies (lower panel). Only cells in the lymphocyte gate were analysed. The percentages of cells in each gate are shown in each panel. Results are representative of at least 3 different animals per group.
Figure 6; Flow cytometric analysis of lymphocyte populations in $Apc^{Min/+}$ mice transplanted with wt bone marrow

Analysis of animals transplanted with bone marrow from 49 day old male donors at 142 days post transplantation. Cells from thymus and spleen were stained with anti CD4 and anti CD8 antibodies (upper panel) and from spleen and thymus with anti B220 and anti IgM antibodies. Only cells in the lymphocyte gate were analysed. The percentages of cells in each gate are shown in each panel. Results are representative of 3 different animals per group.
Figure 7; NK cell depletion in the spleen of Apc<sup>Min/+</sup> mice

Representative dot plots of lymphocyte gated splenic cells stained with antibodies against mouse CD3 and NK1.1. NK cells are defined as the NK1.1<sup>+</sup> CD3<sup>-</sup> population contained within the top left gate.
The bottom right gate contains CD3$^{+}$ T cells. Numbers indicate the percentage of lymphocytes that fall within the gates shown. Plots shown are representative of at least 3 different animals.
Figure 8; Colony-forming mesenchymal progenitor cells are reduced in bone marrow of Apc^{Min/+} mice.

The numbers of clonogenic mesenchymal progenitor cells in a CFU-F assay are shown for mice of different ages. Numbers are shown as mean ± SEM. (* p ≤ 0.1: ** p ≤ 0.005: Student's t-test). n=3 at 36 days and 63 days, n=2 at 87 days.
Lymphodepletion in the Apc\textsuperscript{Min/+} mouse model of intestinal tumorigenesis

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