Chapter 7

Discussion
Klaartje Kok
The PI3K signalling pathway is key in a multitude of cellular processes ranging from cell survival to cell death, highlighting its importance in cell biology. The class IA PI3K catalytic isoform p110δ is specifically expressed in leukocytes and plays important roles in allergy and immunity. The work presented in this thesis sought to further the understanding of (i) the regulation of the leukocyte-specific gene expression of p110δ; (ii) the actions of p110δ in innate immunity, using bone marrow-derived macrophages as a cell-based model and (iii) the function of p110δ PI3K in colitis and colitis-associated cancer using a murine colorectal cancer model. The results presented in this thesis are summarized and discussed below.

Expression of p110δ PI3K

Tissue distribution
The leukocyte-specific tissue distribution of p110δ PI3K is well documented [1-3]. A serious problem in the PI3K field however, is the lack of good quality antibodies, especially antibodies that are validated for use in immunohistochemistry. PI3K isoforms that based on Northern blot analysis were thought to be ubiquitously expressed (PI3K-C2α [4] and PI3K-C2β [5]) have been found to have very specific and selective tissue distribution based on studies using immunohistochemistry antibodies [6]. The use of p110α immunohistochemistry antibodies in ovarian carcinoma surprisingly showed the presence of p110α in the non-proliferating, rather than the proliferating cell compartment of the tumour [7]. Using reporter mice with a β-Gal/LacZ reporter gene inserted into the endogenous p110δ locus by homologous recombination, also p110δ was shown to have unexpected expression in tissue. Besides high expression in the hematopoietic compartment, p110δ was present at intermediate levels in the nervous systems [8]. Also malignant cells of non-leukocyte origin such as melanoma and breast cancer tissue can express fair amounts of p110δ [9] and most other tissues express p110δ at low levels. Future studies with high quality immunohistochemistry antibodies should be employed to further elucidate the exact cellular distribution of p110δ within tissue.

Altered expression
Levels of p110δ are very stable and not subject to acute cellular stimulation. The only circumstances thus far under which p110δ levels have been found to be upregulated are hypertension and cancer. p110δ expression is upregulated in arteries of hypertensive rats [10-12], with immunohistochemistry indicating that p110δ is located
in the smooth muscle layer of the arteries [10,11]. However, in these studies, vessel homogenates were used to monitor p110δ expression, which does not allow to exclude that increases in p110δ expression are related to increased leukocyte infiltration. Increased p110δ mRNA expression was found in glioblastoma [13] while in neuroblastoma both p110δ mRNA and protein levels were shown to be increased [14]. It remains unknown how increased expression of p110δ is established in cancer.

**Regulation of p110δ gene expression**

The data presented in chapter 3 of this thesis are the first to show how the expression of p110δ PI3K regulated at the transcriptional levels and that the p110δ gene expression is mediated by a leukocyte-specific PIK3CD promoter. p110δ arises from 4 distinct transcripts in mouse, and two in human, with distinct upstream untranslated exons which we named exon -1, -2a, -2b, -2c and -2d (-1, -2a and -2b in human). These untranslated exon are located up to 81 kb upstream of the translational start codon in exon 1. All cell types can express the distinct p110δ mRNA transcripts but leukocytes express a greater diversity of transcripts and significantly higher amounts of the individual transcripts. The p110δ transcript containing exon -2a is most abundantly expressed. We have identified a region that contains a highly conserved transcription factor (TF) binding cluster. This cluster in conserved between species and is located within exon -2a in mouse and immediately upstream of exon -2a in human. This region contains at least 4 binding sites for TFs that are associated with regulation of haematopoiesis and expression of leukocyte-specific genes, namely ETS, IRF, NFAT and LEF [15-31]. The TF cluster in exon -2a has enhanced promoter activity in leukocyte compared to non-leukocyte cell lines and thus contains the PIK3CD promoter region that accounts for the predominant leukocyte expression of p110δ. Future work is required to understand which TFs are critical in driving PIK3CD gene expression and to clarify if and how cancer cells that express enhanced levels of p110δ are able to use this promoter. Since 25% of promoters are located at the 3’ end of genes rather then at the 5’ end it is highly likely that p110δ expression will be subject to additional levels of control rather than by simple proximal promoter elements.

**Action of p110δ PI3K**

Systemic immunity comprises several control mechanisms regulating distinct phases of inflammatory responses. PI3K regulate diverse innate responses in various cell type including dendritic cells and mast cells and influence adaptive immunity. In the work presented in chapter 5 of this thesis, we have studied the involvement of p110δ PI3K
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in the regulation of inflammatory signals mediated downstream of the TLR4 receptor. To study the actions of p110δ in innate immunity we used a bone-marrow derived macrophage (BMDM) cell model. BMDMs with and without p110δ catalytic activity (p110δ^{WT/WT} or WT and p110δ^{D910A/D910A} or D910A) were compared in their signal transduction events following stimulation with LPS.

We first tested a new peptide array technology for studying the activity of all kinases of whole cell lysates, the kinome and concluded that this metabolic array is a useful method to determine the enzymatic activities of a large group of kinases, thus offering a valuable method to determine the enzymatic activities of a large group of kinases (chapter 4). This peptide array was employed to compare phosphorylation events in BMDMs from WT vs. D910A mice. The results, presented in chapter 5, revealed a modulatory role for p110δ PI3K in LPS-dependent signalling. p110δ PI3K has an inhibitory role with respect to the Rac- and Rho- dependent pathways, p38 MAP kinase and JNK signalling, but simultaneously an stimulatory role in Syk, p42/p44 MAP kinase and protein kinase B-dependent pathways. This dichotomal role of p110δ in controlling macrophage LPS signalling was reflected in the fact that cytokine secretion was not altered between the two groups (data not shown). Ongoing work in the host laboratory is focusing on studying the role of p110δ PI3K in macrophages and other innate immune cell compartments.

Function of p110δ PI3K

In chapter 6, the function of p110δ was studied in a murine colitis-associated cancer model. D910A mice develop mild caecal and rectal colitis, but only in the presence of intestinal flora. Hence the colitis seen in D910A mice is a consequence of reduced tolerance towards the commensal flora. This was confirmed ex vivo, where exposure to lipopolysaccharide lead to increased inflammatory cytokine secretion and reduced anti-inflammatory cytokine secretion in D910A colons. We conclude that lack of active p110δ leads to defective immune suppression mechanisms. This notion was supported by observed diminished amounts of regulatory T cells (Tregs) in the periphery of D910A mice.

Due to the causal link between inflammation and cancer, D910A mice were examined for their susceptibility to colorectal cancer. Using the AOM/DSS colitis-associated cancer model we show that although D910A mice suffer from more severe colitis, they do not develop more cancer. Given that intestinal inflammation under these conditions exhibits strict correlation with increased tumourigenesis [32-34], this indicates that in fact the absence of active p110δ offers relative protection against cancer. Also this effect is possibly established through diminished function of D910A.
regulatory T cells. This hypothesis is supported by the following: new treatment strategies for melanoma focus on diminishing regulatory T cell activity to enhance anti-tumour immunity. CTLA-4 antibody are used to reduce numbers of peripheral regulatory T cells [35]. Importantly a side effect of this treatment is the induction of IBD. Thus suppression of inflammatory responses may aid malignant cells in suppression of anti-tumour immunity. These findings have important consequences for design of future therapy for IBD, as those that involve long-term increased regulatory T cell activity (e.g. chronic pre/probiotic therapy [36-38], L. lactis producing interleukin-10 [39], or mesenchymal stem cell therapy [40] may carry increased risk of colon cancer if a degree of inflammation remains present. Finally the notion that lack of active p110δ does not carry increased risk of colon cancer is highly relevant in light of the development of p110δ inhibitors for the treatment of allergy.

In conclusion, in this thesis we have (i) identified a key PIK3CD promoter in the leukocyte-specific expression of p110δ (ii) shown that p110δ PI3K has a dual role in innate immune signalling in macrophages and (iii) show that lack of active p110δ leads to increased inflammation but offers relative protection against colitis-associated cancer, thus providing increased insight into the expression, action and function of p110δ PI3K.
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