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Chapter 5

The absence of functional PI3Kγ prevents leukocyte recruitment and ameliorates DSS-induced colitis in mice

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Abstract

Phosphatidylinositol-3-kinase gamma (PI3Kγ) is the major PI3K that is activated in response to chemoattractants. It is responsible for the migration of leukocytes from the bloodstream to sites of injury or infection. Constant migration of new leukocytes to the intestinal mucosa may be an important factor in maintenance of inflammation and tissue damage in inflammatory bowel disease (IBD). Reducing this influx, for example by inhibition of PI3Kγ, might therefore be a potential goal for therapy. Here we investigated the role of PI3Kγ in the migration of leukocytes to sites of intestinal inflammation. We induced colitis in mice with a point mutation that inactivates PI3Kγ enzymatic activity (‘kinase-dead’) by oral administration of dextran sodium sulphate (DSS). Mice were treated with 1.5% DSS for one week and effects on cytokine production, leukocyte recruitment and disease severity were examined. Both clinical and histological parameters showed that the severity of colitis was significantly reduced in PI3Kγ-kinase-dead mice compared to controls. Although mutant mice had a less severe colitis than controls they produced significantly more pro-inflammatory Th1 cytokines such as Il-12, Tnfα and Ifnγ and more Il-10. PI3Kγ mutant mice showed increased numbers of resident macrophages and T cells in the colonic lamina propria in an unstressed condition but failed to recruit new leukocytes to the mucosa upon treatment with DSS despite the increased cytokine levels. These results suggest that PI3Kγ plays a critical role in lamina propria leukocyte trafficking and that loss of PI3Kγ-activity ameliorates DSS induced colitis in mice.
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Introduction

The major goal in the treatment of patients with inflammatory bowel disease is to achieve and maintain remission of disease activity. One of the strategies that seems particularly attractive for remission maintenance but may also be effective for remission induction is to interfere with mucosal leukocyte recruitment. Several compounds that target leukocyte recruitment are currently under clinical investigation.1 One of the critical factors in the migration of leukocytes to a site of inflammation or injury is the cell’s response to chemotactic factors. Chemokines are small proteins responsible for chemotaxis in inflammation. Chemokines act on 7-span transmembrane receptors that are coupled to small G-proteins in the cytoplasm. Binding of a ligand to its G-protein coupled receptor (GPCR) switches the G-protein from an inactive (GDP bound) to an active (GTP bound) state and activates its downstream signaling pathways such as the RAS-RAF-MAPK and Phosphatidylinositol-3-kinase (PI3K)-AKT pathways.2 Signaling through PI3Ks is critical for directed migration of leukocytes in response to stimulation of G-protein coupled chemokine receptors. PI3Ks consist of a complex of regulatory and catalytic subunits.3 PI3Kγ is one of the four isoforms of the catalytic subunits and is exclusively activated through G-protein coupled receptors4, 5 in response to chemotactic stimuli.6-10 PI3Kγ is expressed throughout the cell lineages of the hematopoietic system. PI3Kγ mutant mice are viable and reproduce normally but show phenotypes when their immune system is challenged.7, 9, 10 PI3Kγ plays a crucial role in the movement of leukocytes from the bloodstream to sites of injury or inflammation in response to chemokines.9-16 Besides its role in the recruitment of leukocytes, PI3Kγ is also involved in the regulation of T cell proliferation, activation and differentiation7, 10, 17, 18 and plays an important role in GPCR-induced respiratory burst in neutrophils7, 19 and in mast cell degranulation.20

In mouse models of several chronic inflammatory diseases, the absence of, or blocking of, PI3Kγ has previously been shown to ameliorate disease activity.21-25 PI3Kγ therefore seems a promising target for the treatment of various inflammatory and auto-immune diseases. Here we used mice with a point mutation that inactivates PI3Kγ enzymatic activity (‘PI3Kγ-kinase-dead mice’) to investigate the role of PI3Kγ on leukocyte migration in dextran sodium sulphate (DSS) induced colitis in mice.

Material and Methods

Gene Targeting of PI3Kγ in mice

PI3Kγ-kinase-dead mice were previously generated. In these mice the wild-type PI3Kγ locus was replaced by a chimeric minigene containing a mutated form of the human cDNA, in which the Lys833 was converted into Arg, followed by a neomycin resistance
gene (Neo') cassette sandwiched between loxP sequences. Heterozygous mice were crossed with Balancer Cre mice to delete the Neo' cassette.\textsuperscript{26}

**Colitis model and assessment of inflammation**

Colitis was induced in C57Bl/6 PI3K\textsuperscript{-} kinase-dead and wild-type mice, all similar in age and weight and at least 8 weeks old, by adding 1.5% DSS (TdB Consultancy AB, #DB001) to their drinking water for 7 days and allowing them to drink ad libitum. Each morning the bodyweight of the mice was determined. On day 8, one day after the end of DSS treatment, the mice were sacrificed. Weight loss was calculated as the percentage difference between the original bodyweight and the actual bodyweight on any particular day. Blood loss and stool consistency were scored according to a standard scoring system adapted from Cooper et al.\textsuperscript{27} Scores were defined as follows: Bodyweight loss: 0 = <1%, 1 = 1% to 5%, 2 = 5% to 10%, 3 = 10% to 15%, 4 = >15%. Blood loss: 0 = negative, 2 = positive, 4 = gross bleeding. Stool consistency: 0 = normal, 2 = loose stools, 4 = diarrhea. The average of the scores for bodyweight loss, blood loss and stool consistency was defined as the Disease Activity Index (DAI). Colons were cut in half over the length, saving one half for histology and one half for protein lysate.

**Histology and Immunohistochemistry**

Histology of standard H&E slides was scored in a blinded fashion by a gastrointestinal pathologist (FJtK), who examined the degree of inflammation by scoring edema, crypt loss, influx of inflammatory cells, fibrosis and ulceration. Each parameter was scored with a number from 0 (normal) to 3 (extensive). For immunohistochemical staining slides were deparaffinized, dehydrated and immersed in 1.5% H\textsubscript{2}O\textsubscript{2} in phosphate-buffered saline (PBS) for 30 minutes. Different methods of antigen retrieval were used for the different antibodies. For the F4/80 staining slides were cooked at 100°C for 10 minutes in 0.1 M sodium citrate (pH 6), for the CD3 staining they were cooked for 10 minutes in Tris/EDTA buffer (10 mM Tris, 1 mM EDTA (pH 9)). For the Ly6G staining antigen retrieval was performed by incubation of the slide in 0.025% pepsin in 0.1M HCl at 37°C for 15 minutes. Subsequently slide were blocked with Teng-T (10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% [vol/vol] Tween-20, pH 8.0) for 30 minutes, followed by incubation overnight at 4°C with the primary antibody in PBS with 0.1% Triton X-100 and 1% bovine serum albumin. The CD3 antibody (1:100) was from DAKO (A0452), the F4/80 antibody (1:200) was from BMA Biomedicals, (#T-2006) and the B220/CD45R antibody (1:500) was from hybridoma cells, clone RA3-6B2. For neutrophil-staining we used a Ly6G antibody (1:1000) from BD Pharmingen (553127). For detection of F4/80 and B220 an avidin–biotin detection system was used. Secondary antibodies were from Dako (1:200) (E0468), diluted in PBS with 10% human serum and incubated for one hour at room temperature (RT). Slides were then incubated for one hour with horseradish peroxidase-conjugated avidin–biotin complex (DAKO K0355). For detection of CD3 and Ly6G PowerVision Plus Poly-HRP detection system from Immunologic (DPVB+110HRP) was used for detection of the primary antibody. Peroxidase activity was detected with
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Sigma Fast 3,3-diaminobenzidine Tablets (Sigma, D4293). Sections were counterstained with hematoxylin, dehydrated and mounted with Pertex (Histolab 00801). Macrophages and T cells were counted blindly in 10 intercrypt spaces per mouse.

Measurement of cytokine production
Frozen colonic tissue was homogenized on ice in Greenberger Lysis Buffer (150 mM NaCl, 15 mM Tris, 1 mM MgCl₂·H₂O, 1 mM CaCl₂, 1% Triton) with protease inhibitor cocktail from Roche (11697498001), pH 7.4), diluted 1:1 with PBS. Homogenates were incubated on ice for 1 hour and centrifuged at 14000 rates per minute for 10 minutes. Protein concentrations of Il-12, Ifnγ, Tnfα, Il-10, Mcp-1 and Il-6 were measured in supernatants by cytometric beads array multiplex assay (BD Biosciences, San Jose, CA, USA).

Ethical Considerations
The study was approved by the Institutional Animal Care and Use Committee of the University of Torino.

Results

Absence of PI3Kγ enzymatic activity protects against the development of colitis
We found that mice lacking functional PI3Kγ lost significantly less weight than the wild-type mice. At the end of the experiment PI3Kγ-kinase-dead mice had gained 2.43% of their initial bodyweight whereas the wild-type mice had lost 2.19% of their initial bodyweight (Figure 1A). The PI3Kγ-kinase-dead mice showed less intestinal bleeding and had a more solid stool consistency (Figure 1B). The disease activity index, which summarizes these parameters, showed that the PI3Kγ-kinase-dead mice developed a significantly less severe colitis than the wild-type mice (Figure 1C). H&E staining of the

Figure 1. Absence of functional PI3Kγ reduces the severity of DSS colitis in mice
(A) Mice lacking functional PI3Kγ were protected against weight loss induced by treatment with DSS compared to the wild-type mice. (P=0.0035). (B) The PI3Kγ-kinase-dead mice showed less intestinal blood loss (P=0.0035) and had less diarrhea (P=0.029). (C) The disease activity index was lower in the PI3Kγ-kinase-dead mice (P=0.0075) (n=10 per group). Results are representative of two independent experiments with the same results.
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colonic tissue showed an increasing severity of colitis from the proximal to the distal colon. No difference was seen in the amount of surface area involved in inflammation between the two groups. A significant reduction in the degree of edema, crypt loss and mononuclear cell infiltration was seen in the PI3K-kinase-dead mice; granulocytes were almost absent in both groups and very little fibrosis and ulceration were seen in either group (Figure 2).

Figure 2. Histological parameters for inflammation
(A) Scoring histological parameters for inflammation showed that edema, crypt loss and mononuclear cell infiltration were significantly reduced in the PI3K-kinase-dead mice ($P=0.0066$, $P=0.046$ and $P=0.0071$ respectively); the amount of granulocytes, fibrosis and ulceration were reduced, but not significantly ($P=0.72$, $P=0.66$ and $P=0.27$ respectively). (n=10 per group). (B) H&E staining of the colons of both wild-type and mutant mice shows that inflammation was less prominent in the PI3K-kinase-dead tan in wild type mice. The mutant mice have less edema (left panels), reduced crypt loss and a decreased amount of inflammatory cells (right panels).

Increased cytokine production in PI3K-kinase-dead mice
PI3K-kinase-dead mice developed a significantly less severe colitis than wild type mice. We determined the production of several cytokines with a role in (DSS) colitis to examine if cytokine production paralleled the activity of disease. Concentrations of inflammatory cytokines were examined in colonic homogenates of the mice using a cytokine bead array. Surprisingly, although they had significantly less severe colitis on histology, the levels of Il-12, Tnfα and Ifnγ were significantly higher in PI3K-kinase-dead mice compared to wild-type mice (Figure 3A-C). Levels of the anti-inflammatory cytokine Il-10 were also increased in PI3K-kinase-dead mice (Figure 3D). Mcp-1 and Il-6 were not significantly different between groups (Figure 3 E,F).

Aberrant leukocyte trafficking in PI3K-kinase-dead mice
To examine if PI3K modulates leukocyte recruitment in the DSS model we studied numbers of T cells (CD3), B cells (B220), macrophages (F4/80) and neutrophils (Ly6G) in wild type and PI3K-kinase-dead mice with and without DSS treatment. We found that
Figure 3. Increased production of both Th1 cytokines and Il-10 in colon homogenates of PI3Kγ-kinase-dead mice

Six different cytokines were measured by a CBA on colon homogenates. (A-F). A significant increase was observed in levels of Il-12, TNFα and IFNγ ($P=0.0015$, $P=0.0006$ and $P=0.043$ respectively). Expression of the anti-inflammatory cytokine Il-10 (D) was significantly increased ($P<0.0001$). (F,G) Mcp-1 and Il-6 were reduced, but this was not significant ($P=0.18$ and $P=0.18$ respectively). (n=10 per group).
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in untreated PI3Kγ-kinase-dead mice the basal number of macrophages and T cells were significantly increased (Figure 4). Treatment of wild type mice with DSS resulted in a strong influx of both macrophages (96% increase, Figure 5A) and T cells (145% increase, Figure 5B). In contrast, there was no influx of macrophages (1% decrease, Figure 5A) or T cells (12% decrease, Figure 5B) in response to treatment with DSS in the PI3Kγ-kinase-dead mice.

B-cells were mainly observed in the follicles (Figure 5C left). Throughout the rest of the lamina propria B-cells were very scarce in both wild-type mice and PI3Kγ-kinase-dead mice (Figure 5 right). Neutrophils (Figure 5D) were almost absent in both wild-type and PI3Kγ-kinase-dead mice before and after treatment with DSS (using tissue of inflamed lung as a positive control). In conclusion, PI3Kγ-kinase-dead mice had increased numbers of macrophages and T cells in their colonic lamina propria at baseline but completely fail to recruit new macrophages and T cells in response to treatment with DSS.

Discussion

PI3Kγ plays a crucial role in leukocyte motility and migration. Loss of the capacity to recruit inflammatory cells has previously shown to ameliorate disease in several mouse-models of immune-mediated diseases. Here we studied the role of PI3Kγ in DSS induced colitis in mice and found that mice that lack functional PI3Kγ have significantly reduced scores for both clinical and histological parameters. The reduced severity of colitis contrasted with the increased levels of the proinflammatory cytokines Tnfα, Ifnγ and Il-12 in the PI3Kγ-kinase-dead mice. Instead, the reduced severity of colitis seemed to correlate with a defect in leukocyte recruitment in PI3Kγ-kinase-dead mice in response to the treatment with DSS. We found that PI3Kγ-kinase-dead mice had higher baseline numbers of macrophages and T cells in the colonic lamina propria than wild-type mice. Treatment of wild type mice with DSS resulted in a prominent increase
Figure 5. Absence of functional PI3Kγ prevents recruitment of new leukocytes in response to treatment with DSS

(A,B) Immunohistochemical stainings for F4/80, a macrophage marker, and CD3, a T cell marker, in unchallenged mice and mice treated with DSS. (A) The strong influx of macrophages that is observed in wild type mice is completely abrogated in PI3Kγ-kinase-dead mice (P<0.0001, n=10 per group). (B) Recruitment of T cells was similarly completely absent in the PI3Kγ-kinase-dead mice (P<0.0001, n=10 per group). Immunological staining for B220 showed staining of B-cells in the follicles (C left panel), but very little cells in the rest of the colonic lamina propria (C right panel). (D) Immunological staining for Ly6G showed absence of neutrophils, using a pulmonary infiltrate as positive control (insert in D). Original magnifications: 100x (A,B), 40x (C left,D) and 200x (C right).
in the numbers of both macrophages and T cells, whereas recruitment of these cells was completely absent in the PI3Kγ mutant mice.

We induced a mild DSS colitis in PI3Kγ-kinase-dead and wild type mice. Loss of body weight, blood loss and diarrhea, three major clinical outcome parameters, were significantly reduced in PI3Kγ-kinase-dead mice. Histologically this correlated with a significantly reduced severity of crypt loss, edema and mononuclear cell infiltration. In several animal models of inflammation absence of functional PI3Kγ significantly reduced neutrophil influx, however the amount of neutrophils after 7 days of DSS treatment was very low in the colons of our mice. Neutrophil influx usually starts during the acute phase of inflammation and peaks 24 to 72 hours after onset of the inflammatory reaction. This could explain the absence of neutrophils after one week of treatment with DSS.

Although the DSS induced colitis was less severe in PI3Kγ mutant than in wild-type mice, we found that both the pro-inflammatory cytokines Tnfα, Ifnγ and Il-12 and the anti-inflammatory cytokine Il-10 were significantly increased in the mutant mice. This suggests a role for PI3Kγ in the negative regulation of these cytokines. This may fit well with the important role of PI3Kγ in G-protein coupled receptor signaling. It was shown in previous experiments by others that ligands for G-protein coupled receptors such as the chemokines MCP-1 to 4, fMLP and C5a and for example tetrahydrocannabinol and morphine suppress Th1 cytokine production. Also treatment with pertussis toxin, a Ga inhibitor, strongly increases production of both Th1 cytokine and Il-10 production in splenocytes and dendritic cells. A role for PI3K signaling as a downstream mediator of the negative regulation of these cytokines by G-protein coupled receptors was suggested by the finding that the PI3K-Akt signaling pathway negatively regulates Il-12 production in innate immune cells. Our results may therefore be explained by an important role of PI3Kγ in the negative regulation of both Th1 cytokines and Il-10 by Ga proteins downstream of G-protein-coupled receptors.

A remarkable finding in the PI3Kγ mutant mice was the accumulation of leukocytes in the lamina propria in an unstressed condition. One interpretation of this finding may be that this accumulation is related to the established role of PI3Kγ in leukocyte migration. PI3Kγ may not only play a role in leukocyte recruitment in response to injury and inflammation but also regulate emigration of leukocytes from the lamina propria under physiological conditions. Although many details of the migration of leukocytes from the vasculature to a tissue are well characterized, we understand much less about the way leukocytes emigrate from a tissue into the lymphatic channels or back into the vasculature. Signaling by sphingosine-1-phosphate (S1P) through the S1P receptors is an important step in lymphocyte emigration from the lymphnodes and Fingolimod a drug that targets this pathway is an immuno-suppressant that has recently been successfully used in patients with multiplesclerosis. Since the S1P receptors are G coupled
receptors the accumulation of lymphocytes observed in the lamina propria of the PI3K mutant mice may at least in part be related to loss of functioning of S1P signaling. Interestingly, it has previously been shown that T cell emigration from the thymus is highly dependent on G\textsubscript{ai} signaling\textsuperscript{42, 43} suggesting that T cell recirculation requires G-protein coupled receptor signaling. The leukocytes of PI3K\textsubscript{γ}-mutant animals therefore seem to have at least two aspects of their phenotype in common with inhibition of G\textsubscript{ai} signaling by pertussis toxin, the altered cytokine profile and a defect in T cell tissue emigration. This may suggest an important role for PI3K\textsubscript{γ} downstream of G\textsubscript{ai} proteins. Alternative explanations for the accumulation of T cells in the lamina propria could be changes in the rate of proliferation and/or apoptosis of the lamina propria T cells. Even though basal levels of macrophages and T cells were higher in the PI3K\textsubscript{γ}-kinase-dead mice, there was a complete lack of macrophage and T cell influx after 7 days of treatment with DSS in these mice. The difference in influx of macrophages and T cells between the PI3K\textsubscript{γ} mutant mice and the wild-type mice is very likely the explanation for the reduced disease severity in the mutant mice.

At the moment drug development in the field of IBD is largely focused on inhibition of cytokines. With the exception of strategies directed against TNFα for which the mechanism of action remains under investigation this strategy has been mostly unsuccessful. It is important to realize that cytokines are messenger molecules that do not of course damage tissues and that it is the recruitment of inflammatory cells and their effector mechanisms that ultimately damage tissues. Such mechanisms include the production of oxygen radicals, activation of proteolytic enzymes and activation of complement cascades. An alternative therapeutic strategy targets the mechanisms effector cells require to be recruited to areas of inflammation. Examples are drugs that inhibit adhesive molecules required for the migration of leukocytes into inflamed tissue such as Natalizumab, an antibody against α4-integrin, or MLN02, an antibody against α4β7-integrin. Such compounds have shown promising results for the treatment of Crohn’s disease and Ulcerative colitis.\textsuperscript{1, 44-48} Even though three reports of progressive multifocal leukoencephalopathy in patients using Natalizumab\textsuperscript{49} have tempered initial enthusiasm for the use of this drug in IBD, blocking inflammatory cell migration and adhesion still seems a very promising target in the treatment of IBD. By impairing the movement of leukocytes towards the inflamed intestine, PI3K\textsubscript{γ} could possibly be an additional target in this group of drugs. However, to test this idea, further experiments on the role of PI3K\textsubscript{γ} in intestinal inflammation would first have to be done in other models of colitis, such as the TNBS model or a transfer model, that resemble the human situation of IBD more closely than the DSS model.

A study by González-Garzía et al.\textsuperscript{50} in which PI3K\textsubscript{γ} knock-out mice were treated with DSS is currently in press and shows similarities with the results described in this manuscript. The major technical difference between our experiments is that González-Garzía et al. use a mouse model in which the PI3K\textsubscript{γ} protein is absent whereas we use
a model in which the kinase activity is abolished by a point mutation and the (kinase-dead) PI3Kγ protein is normally expressed. The advantage of using a kinase-dead mouse is that it is perhaps more representative of the effects that are to be expected with a small molecule that inhibits PI3Kγ kinase activity. This is an important difference as PI3Kγ also has kinase independent roles as a scaffold protein and major difference have been observed in outcomes of PI3Kγ knock-out versus kinase-dead mice. Similar to our findings González-Garzía et al. found that the absence of functional PI3Kγ protects from DSS induced colitis and that in the PI3Kγ knock-out mice fail to recruit T cells and macrophages to the colon after treatment with DSS. This suggests that it is the kinase function of the protein that is responsible for the outcome in inflammation and recruitment of leucocytes. One of the major differences with the findings of González-Garzía et al is that they observed a decrease in Tnfα cytokine production in the PI3Kγ knock-out mice upon treatment with DSS, whereas we found a substantial increase in our mice. This suggests that kinase dependent versus independent roles of PI3Kγ regulate Tnfα expression in an opposite manner.

In summary, mice lacking functional PI3Kγ have higher baseline levels of macrophages and T cells in the lamina propria of their colons. They produce more inflammatory cytokines upon DSS-induced colitis than wild-type mice, but as PI3Kγ is needed for the motility of immune cells they seem not able to recruit these cells towards the site of inflammation. In PI3Kγ-kinase-dead mice this reduced influx of effector cells ameliorates disease severity in DSS induced colitis in mice.
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Reference List


