

RAS-p110 α signalling in macrophages is required for effective inflammatory response and resolution of inflammation

Reviewed Preprint

v3 • February 3, 2025

Revised by authors

Reviewed Preprint

v2 • November 14, 2024

Reviewed Preprint

v1 • April 12, 2024

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eLife Assessment

This **useful** study investigates the impact of disrupting the interaction of RAS with the PI3K subunit p110 α in macrophage function in vitro and inflammatory responses in vivo. **Solid** data overall supports a role for RAS-p110 α signalling in regulating macrophage activity and so inflammation, however for many of the readouts presented the magnitude of the phenotype is not particularly pronounced. Further analysis would be required to substantiate the claims that RAS-p110 α signalling plays a key role in macrophage function. Of note, the molecular mechanisms of how exactly p110 α regulates the functions in macrophages have not yet been established.

<https://doi.org/10.7554/eLife.94590.3.sa2>

Abstract

Macrophages are crucial in the body's inflammatory response, with tightly regulated functions for optimal immune system performance. Our study reveals that the RAS-p110 α signalling pathway, known for its involvement in various biological processes and tumorigenesis, regulates two vital aspects of the inflammatory response in macrophages: the initial monocyte movement and later-stage lysosomal function. Disrupting this pathway, either in a mouse model or through drug intervention, hampers the inflammatory response, leading to delayed resolution and the development of more severe acute inflammatory reactions in live models. This discovery uncovers a previously unknown role of the p110 α isoform in immune regulation within macrophages, offering insight into the complex mechanisms governing their function during inflammation and opening new avenues for modulating inflammatory responses.

Introduction

Phosphatidylinositol 3-kinases (PI3K) are a family of lipid kinases that phosphorylate phosphatidylinositides (PtdIns) at the 3'-hydroxyl group¹. Upon activation, PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP²) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP₃). PIP₃ serves as a second messenger that recruits proteins containing pleckstrin homology (PH) domains, such as Akt (also known as protein kinase B)^{2,3}. This activation of PI3K regulate various cellular functions, including cell proliferation, growth, survival, motility, inflammation, and metabolism, among others^{1,4}. In macrophages, the activation of PI3K-Akt signalling is crucial to restrict inflammation and to promote anti-inflammatory responses in Toll Like Receptors (TLR)-induced macrophages, contributing to macrophage polarization^{5,6}.

PI3Ks are heterodimeric lipid kinases composed of catalytic and adaptor/regulatory subunits that can be categorized into three classes based on their structures and substrate specificities^{3,7}. Class I catalytic isoforms, including p110 α , p110 β , p110 γ , and p110 δ , play essential roles in integrating signals from growth factors, cytokines, and other environmental cues. While p110 α and p110 β are ubiquitously expressed, p110 δ and p110 γ are largely restricted to the myeloid and lymphoid lineages⁷⁻⁹. While p110 α is primarily associated with cell growth regulation and survival in epithelial cells, it must be considered that this isoform is also expressed in immune cells, including macrophages. The role played by p110 α in macrophages is not well understood, although some studies have suggested that it might regulate the survival and the regulation of the phagocytic activity of macrophages¹⁰. In the context of cancer, impairment of RAS binding to p110 α in macrophages results in reduced recruitment of macrophages to the tumour site^{11,12}. Additionally, this disruption leads to a change in macrophage polarization, favouring a more proinflammatory M1 state¹². These findings suggest that p110 α plays a crucial role in regulating macrophage-dependent functions. However, despite these insights, the precise impact of p110 α on macrophage function and the underlying molecular mechanisms influencing the inflammatory response are not yet fully understood.

Inflammation is a complex and tightly regulated series of events triggered by various stimuli such as pathogens, harmful mechanical and chemical agents, and autoimmune reactions. The inflammatory response primarily occurs in vascularized connective tissues, involving a dynamic interplay of plasma components, circulating cells, blood vessels, and cellular and extracellular

factors^{13–17}. During inflammation, mediators released by recruited leukocytes orchestrate a response that aims to facilitate tissue repair and protect the body against harmful stimuli^{16–18}.

Macrophages play a vital role in the inflammatory response by performing functions such as antigen presentation, phagocytosis, and immunomodulation^{16, 17}. Their role begins with the active recruitment of monocytes from the bloodstream to the site of infections¹⁹, where they differentiate into macrophages and recognize microbes and cellular debris through specific mechanisms. Macrophages subsequently actively participate in phagocytosis, a vital process involving the internalization and elimination of pathogens. Microbial destruction predominantly takes place within lysosomes and phagolysosomes^{20–23}. At later stages of inflammation, macrophages contribute to the resolution of inflammation, thus preventing progression from acute to persistent inflammation that would cause additional tissue damage.

In this study we have used a combination of cell biology techniques and animal models to better understand the role of RAS-dependent activation of p110 α at the different stages of the inflammatory response. Our findings show that RAS-p110 α signalling plays a key role in the initial stages of inflammation, facilitating the extravasation of monocytes from the bloodstream by promoting the necessary cytoskeletal changes. Subsequently, RAS-p110 α has a crucial role in lysosomal acidification and activation of cathepsins, which are indispensable for efficient degradation of lysosomal cargo; when these functions are impaired, prolonged acute inflammatory responses and delayed resolution steps are observed. These results significantly enhance our understanding of the complex mechanisms governing the immune response to inflammation, emphasizing the pivotal role played by RAS-p110 α signalling in orchestrating proper monocyte extravasation and maintaining optimal lysosomal function.

Results

Disruption of RAS-p110 α causes prolonged and more acute responses to inflammatory stress *in vivo*

Previous data suggested that, in a tumoral setting, somatic disruption of RAS-p110 α prevents macrophage recruitment to tumours^{11, 12} and favours polarization of macrophages to a proinflammatory phenotype¹², suggesting a possible role for p110 α in macrophage function. To determine whether RAS-dependent activation of p110 α participates in innate or adaptive immune responses to inflammation in macrophages, we used an established mouse model designed for the tamoxifen-inducible disruption of RAS binding to p110 α ^{11, 24} (**Figure 1-Supplement 1A**). This mouse model introduces two-point mutations, T208D and K227A, in the RAS Binding Domain (RBD) of the endogenous *Pik3ca* allele (*Pik3ca*^{RBD}), enabling the selective disruption of the RAS-p110 α interaction²⁴. Wild-type (*Pik3ca*^{WT}) and *Pik3ca*^{RBD} mice were bred with mice containing a floxed *Pik3ca* allele²⁵, along with a strain containing a conditional Cre recombinase (Cre-ERT2) allele targeted to the ubiquitously expressed Rosa26 locus. The resulting *Pik3ca*^{WT/Flox} and *Pik3ca*^{RBD/Flox} mice displayed no discernible phenotype and exhibited behavior consistent with *Pik3ca*^{WT} mice^{11, 24}. Activation of Cre-recombinase by tamoxifen led to the excision of the floxed *Pik3ca* allele, resulting in mice expressing either one *Pik3ca*^{WT} allele (*Pik3ca*^{WT/-}) or one *Pik3ca*^{RBD} allele (*Pik3ca*^{RBD/-})¹¹. This inducible genetic manipulation strategy allows us to selectively disrupt the RAS-p110 α interaction in a controlled and temporally regulated manner, providing a valuable tool for dissecting the contributions of this pathway to immune responses in the context of inflammation.

Bone marrow derived macrophages (BMDMs) were generated from tibias and femurs of *Pik3ca*^{WT/Flox} and *Pik3ca*^{RBD/Flox} mice, ensuring efficient removal of the floxed allele (**Figure 1-Supplement 1B**). Subsequently, these BMDMs were induced towards a proinflammatory state

through stimulation with LPS and IFN- γ . To assess the impact of RAS-p110 α binding deficiency on inflammatory intracellular signalling, we first examined Akt activation. The results revealed a decrease in Akt activation levels under inflammatory conditions in BMDMs lacking RAS-p110 α binding, while no discernible change was observed in ERK activation, another well-known RAS effector (**Fig. 1A** and **Figure 1-Supplement 1C** and **D**). Interestingly, the decrease in Akt activation was accompanied by a decrease in the activation of NF- κ B (**Fig. 1B**).

Given the role of p65 in the expression of pro-inflammatory cytokines and chemokines²⁶, we next analyzed the expression of various inflammation mediators using a cytokine array in unstimulated and LPS- and IFN- γ -stimulated macrophages. Results showed that, under unstimulated conditions, RAS-PI3K disruption decreases the expression of IP-10, MIP-1 α (Ccl3), JE (Ccl2/MCP1), IL-16, and IL-12p70, with no upregulated cytokines observed (**Figure 1-Supplement 1E**). The downregulation of IP-10, MIP-1 α , and JE indicates an impaired ability to recruit monocytes, macrophages, and T cells to sites of inflammation. This suggests that macrophages lacking RAS-PI3K interaction may have a reduced capacity to mount a robust immune response, particularly in recruiting and activating essential immune cells needed to combat pathogens or initiate inflammation.

Regarding LP +IFN- γ stimulated macrophages, results showed a decrease in the expression of IL-1 β and IL-17, key drivers of pro-inflammatory responses, and an upregulation of IL-7, Ilra, JE, BLC, I-309, Eotaxin, and G-CSF (**Figure 1-Supplement 1F**). Elevated levels of these factors are associated with enhanced chemotactic signals and regulatory functions. Thus, the cytokine and chemokine expression profile observed in RAS-PI3K deficient macrophages suggests impaired pro-inflammatory responses and altered immune cell recruitment patterns, potentially influencing the resolution of inflammation and tissue repair processes.

Next we assessed whether the absence of RAS binding to p110 α affects the ability of BMDMs to acquire a pro-inflammatory state characterized by increased expression of markers such as CD80, CD86, and MHCII^{27, 28}. To assess this, *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs were stimulated with LPS+IFN- γ , followed by flow cytometry analysis. Expression levels of CD80 (**Figure 1-Supplement 1G**), CD86 (**Figure 1-Supplement 1H**), and MCHII (**Figure 1-Supplement 1I**) were examined. No significant differences were observed in the expression levels of any of these markers between the two genotypes under study.

Consequently, our next objective was to investigate whether in vivo disruption of RAS-p110 α might lead to altered inflammatory responses. To address this, we administered tamoxifen to 10-12-week-old *Pik3ca*^{WT/flox} and *Pik3ca*^{RBD/flox} mice with tamoxifen and, after a two-week interval, conducted a paw swelling assay. In this assay, zymosan (10 μ g/ μ l) or PBS was injected into the hind paws of *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} mice with paw thickness measured at regular intervals over a 5-day period. The results revealed a significant increase in paw inflammation in *Pik3ca*^{RBD/-} mice compared to *Pik3ca*^{WT/-} mice, evident from the earlier time points measured and persisting throughout the experiment (**Fig. 1D and 1E**). Paws from the *Pik3ca*^{WT/flox} and *Pik3ca*^{RBD/flox} mice, which had not received tamoxifen, exhibited comparable levels of inflammation (**Figure 1-Supplement 1J**). This reaffirms that the absence of p110 α triggers a significant alteration in the inflammatory response and confirms that *Pik3ca*^{WT/flox} and *Pik3ca*^{RBD/flox} mice do not show any phenotype, as previously described^{11, 24}. Additionally, analysis of the blood sedimentation rate, a reliable indicator of systemic inflammation levels²⁹, showed a lower basal sedimentation ratio in *Pik3ca*^{RBD/-} mice under PBS-treated conditions (**Figure 1-Supplement 1K**). Following zymosan injection, both genotypes exhibited an increase in sedimentation rate, but the rise was more pronounced in *Pik3ca*^{RBD/-} mice, indicating a heightened systemic inflammatory response. These findings underscore that disruption of RAS-p110 α interaction results in an exacerbated inflammatory state, reflected in both localized paw inflammation and systemic inflammatory mediator levels.

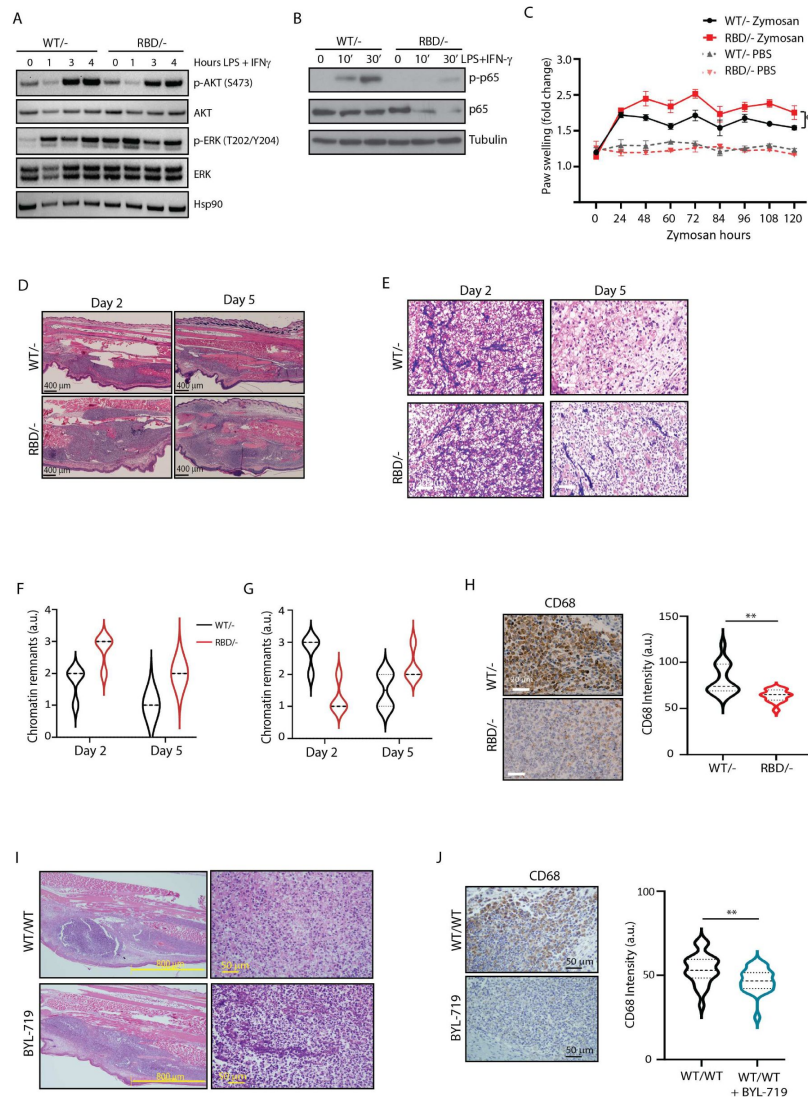


Figure 1.

***Pik3ca*^{RBD/-} mice have impaired responses to inflammatory insults.**

(A) Western blotting showing activation of Akt and ERK in *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs activated towards a pro-inflammatory state with LPS and IFN- γ at the indicated time points; (B) Western blotting showing activation of NF- κ B (p65) in *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs activated towards a pro-inflammatory state with LPS and IFN- γ at the indicated time points; (C) *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice were injected with zymosan or PBS in the back-hind paws and inflammation was measured and plotted over time, *Pik3ca*^{WT/-} PBS n = 6; *Pik3ca*^{RBD/-} PBS n = 5; *Pik3ca*^{WT/-} Zymosan n = 4; *Pik3ca*^{RBD/-} Zymosan n = 4; Error bars indicate mean $\pm$ SEM. Significance using 2-way ANOVA test: **p < 0.01 (D) Representative H&E images of the inflamed area of *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} paws injected with zymosan at the indicated times; (E) Representative images of cellularity present in the inflamed abscess of *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} paws injected with zymosan at the indicated times; (F) Graph showing quantification of loose chromatin present in the inflamed abscess; (G) Representative images of macrophages (CD68 positive cells) present in the inflamed abscess of *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} paws injected with zymosan and quantification of macrophages (CD68 positive cells) present; (H) Representative H&E images of the inflamed area and cellularity of *Pik3ca*^{WT/WT} paws and *Pik3ca*^{WT/WT} paws treated with BYL-719 injected with zymosan. (I) Representative images and quantification of macrophages (CD68 positive cells) present in the inflamed abscess of *Pik3ca*^{WT/WT} paws and *Pik3ca*^{WT/WT} paws treated with BYL-719 injected with zymosan and quantification of macrophages (CD68 positive cells). Error bars indicate mean $\pm$ SEM. Significance using Student's t test: **p < 0.01.

To delve deeper into the inflammatory response induced by zymosan, we collected paw samples at two- and five-days post-injection and conducted Haematoxylin & Eosin (H&E) studies. This approach enabled a comprehensive analysis, allowing us not only to scrutinize the early stages of inflammation but also to monitor the subsequent resolution phase of the inflammatory process. Paws obtained from mice injected with zymosan for 2 days displayed extensive areas of damaged connective tissue in both *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} paws (**Fig. 1D** [↗](#)). Notably, the inflamed region in *Pik3ca*^{RBD/-} mice was larger compared to control samples. After 5 days of zymosan injection, there was a significant reduction in the inflamed area, although it remained comparatively larger in the paws from *Pik3ca*^{RBD/-} mice (**Fig. 1D** [↗](#)). This observation suggests a prolonged and heightened inflammatory response in mice lacking RAS-p110α interaction, emphasizing the role of this interaction in the regulation of inflammatory processes.

The cellular composition within the inflammatory abscess offers crucial insights into the severity and progression of the disease. Close examination with the pathologist revealed features indicative of an acute inflammatory response, including an inflammatory abscess with elevated numbers of polymorphonuclear cells, primarily neutrophils, and macrophages displaying altered cell shape and increased cell death, accompanied by fibrin and chromatin deposition (**Fig. 1E and F** [↗](#)). Notably, *Pik3ca*^{RBD/-} mice exhibited lower numbers of macrophages, a larger necrotic area with increased chromatin remnants, and reduced fibrin content compared to the paws from *Pik3ca*^{WT/-} mice (**Fig. 1E and F** [↗](#)). By day 5, paws from *Pik3ca*^{WT/-} mice showed a significant increase in the number of macrophages, a decrease in polymorphonuclear cells, and a nearly complete resolution of the necrotic area, indicating the initiation of the resolution phase. Moreover, there were abundant activated fibroblasts, suggesting active production of new connective tissue. In contrast, paws from *Pik3ca*^{RBD/-} mice exhibited a delayed healing process characterized by a larger central area of polymorphonuclear cells, abundant fibrin deposits, reduced numbers of infiltrating macrophages, and limited fibroblast activity (**Fig. 1E and F** [↗](#)). These findings collectively indicate an imbalance in the inflammatory response and a slower progression towards resolution in the absence of RAS-p110α interaction, emphasizing the pivotal role of this interaction in orchestrating an effective and timely resolution of inflammation.

To evaluate the presence of macrophages within the inflammatory lesion, we performed specific immunohistochemical (IHC) analysis using the macrophage-specific marker CD68 in paws from day 5, where more cellular preservation and initiation of the healing process had been observed. A notable decrease in the number of CD68-positive cells was observed in the inflamed abscess region of *Pik3ca*^{RBD/-} mice (**Fig. 1G** [↗](#)).

To further confirm the involvement of p110α signalling in the acute inflammatory response, *Pik3ca*^{WT/WT} mice were subjected to daily treatment with BYL719 (Alpelisib), a specific inhibitor of p110α isoform. After an initial 48-hour treatment period, the mice received injections of zymosan or PBS into their back-hind paws and were sacrificed 2 days later for analysis. Similar to what we had observed in *Pik3ca*^{RBD/-} mice, the inflamed area in BYL719-treated mice exhibited a larger extension than the inflamed area from non-treated mice (**Fig. 1H** [↗](#) and **Figure 1-Supplement 1L** [↗](#)). The central necrotic region was significantly larger in the BYL719-treated mice, and it contained large amount of apoptotic polymorphonuclear cells, lower number of macrophages, and increased deposits of chromatin (**Fig. 1H** [↗](#)) resembling the observations from *Pik3ca*^{RBD/-} mice. Immunostaining for CD68 revealed a reduction in the number of macrophages present in the inflammatory abscess upon inhibition of p110α signalling with BYL719 treatment (**Fig. 1I** [↗](#)).

In summary, our findings highlight that both genetic disruption of RAS-p110α interaction and pharmacological inhibition of p110α contribute to an expanded inflamed area and central necrotic region, concurrently reducing macrophage infiltration in a zymosan-induced inflammation model. These results collectively underscore the pivotal role of the p110α isoform of PI3K in orchestrating the resolution of inflammatory responses, emphasizing its significance in modulating the dynamics and outcomes of inflammatory processes.

Disruption of RAS binding to p110 α impairs the number of inflammatory monocytes in blood and spleen

Given the decrease in macrophages observed in the inflammatory abscess, we next set out to determine whether disruption of RAS binding to p110 α had an effect on the number of monocytes circulating in the blood of adult mice. *Pik3ca*^{WT/flox} and *Pik3ca*^{RBD/flox} mice were treated with tamoxifen at 12-14 weeks of age and 4 weeks later, blood was collected by cardiac puncture and immune populations were analysed by flow cytometry. To assess the effects of RAS-p110 α disruption on immune populations, we employed a flow cytometry gating strategy as described in the Methods section. Briefly, CD45⁺ cells were initially gated to define the overall immune cell population. Within this population, B cells were identified by CD19⁺ CD11b⁻ staining, and T cells were defined by CD3⁺ expression. Further differentiation of T cell subtypes (CD4⁺, CD8⁺, and double-negative T cells) was conducted based on CD4 and CD8 markers within the CD3⁺ cell population. Additionally, myeloid cells were gated as CD11b⁺ cells, with further identification of granulocytes and monocytes based on Ly6G and Ly6C expression. Specifically, granulocytes/neutrophils were characterized as Ly6G⁺ Ly6C⁺, classical monocytes as Ly6G⁻ Ly6C⁺, and alternative monocytes as Ly6G⁻ Ly6C⁻ (**Figure 2-Supplement 2A** [↗](#)). We found a decrease in the number of circulating classical (or inflammatory) monocytes (Ly6C^{Hi}/Ly6G⁻/CD11b⁺ cells) in *Pik3ca*^{RBD/-} mice (**Fig. 2A** [↗](#)) and no changes were observed in non-classical (or non-inflammatory) monocytes (Ly6C^{Lo}/Ly6G⁻/CD11b⁺ cells) (**Fig. 2B** [↗](#)). Together with the decrease in inflammatory monocytes, we observed an increase in the number of neutrophils (Ly6C⁻/Ly6G⁺/CD11b⁺ cells) (**Figure 2-Supplement 3A** [↗](#)). We did not detect differences in the numbers of T-cells (CD3⁺, CD8⁺ or CD4⁺) (**Figure 2-Supplement 3B** [↗](#)) or B-cells (CD19⁺) (**Figure 2-Supplement 3C** [↗](#)) in the blood after the disruption of RAS binding to p110 α . We also analysed these same cell populations in *Pik3ca*^{WT/flox} and *Pik3ca*^{RBD/flox} mice and no differences were found (data not shown).

Since splenic monocytes resemble their blood counterparts³⁰ [↗](#), we next aimed at determining whether splenic monocyte population would also be altered after disruption of RAS p110 α interaction. Flow cytometry from *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice was performed following the same gating strategy as described for blood samples (**Figure 2-Supplement 2B** [↗](#)). As observed in circulating blood, the number of classical monocytes (Ly6C^{Hi}/Ly6G⁻/CD11b⁺) in the spleen decreased after disruption of RAS-p110 α interaction (**Fig. 2C** [↗](#)) and no differences were found in non-classical activated monocytes (Ly6C^{Lo}/Ly6G⁻/CD11b⁺) (**Fig. 2D** [↗](#)). We also checked the levels of resident macrophages present in the spleen and results showed that spleens from the *Pik3ca*^{RBD/-} mice had a decrease in the number of differentiated macrophages (F4/80⁺/CD11b⁺/CD45⁺) (**Fig. 2E** [↗](#)). Analysis of macrophages in the white and red pulp of the spleen indicated that *Pik3ca*^{RBD/-} mice had a significant decrease in the macrophage population in the former region (**Fig. 2F and 2G** [↗](#)). No differences were found in the number of granulocytes (**Figure 2-Supplement 3D** [↗](#)), B-cells (**Figure 2-Supplement 3E** [↗](#)) or T-cells (**Figure 2-Supplement 3F** [↗](#)) present in the spleen between *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} mice. There were no differences in spleen size from *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} mice either (**Figure 2-Supplement 3G** [↗](#)).

Given the decrease in the number of inflammatory monocytes and macrophages observed in blood and spleens of *Pik3ca*^{RBD/-} mice, we wondered if disruption of RAS activation of p110 α could lead to a decrease in myeloid bone marrow precursors. Myeloid lineage descends from a common myeloid progenitor (CMP) in bone marrow and traverse into blood as mature cells³¹ [↗](#). CMP differentiates into the Granulocyte-Macrophage Progenitor (GMP) and the Megakaryocyte-Erythroid Progenitor (MEP). The CMP can generate all types of myeloid colonies, whereas the GMP or the MEP produces only granulocyte macrophage (GM) or megakaryocyte erythrocyte (MegE) lineage cells, respectively (**Figure 2-Supplement 3H** [↗](#)). No significant differences were noted in

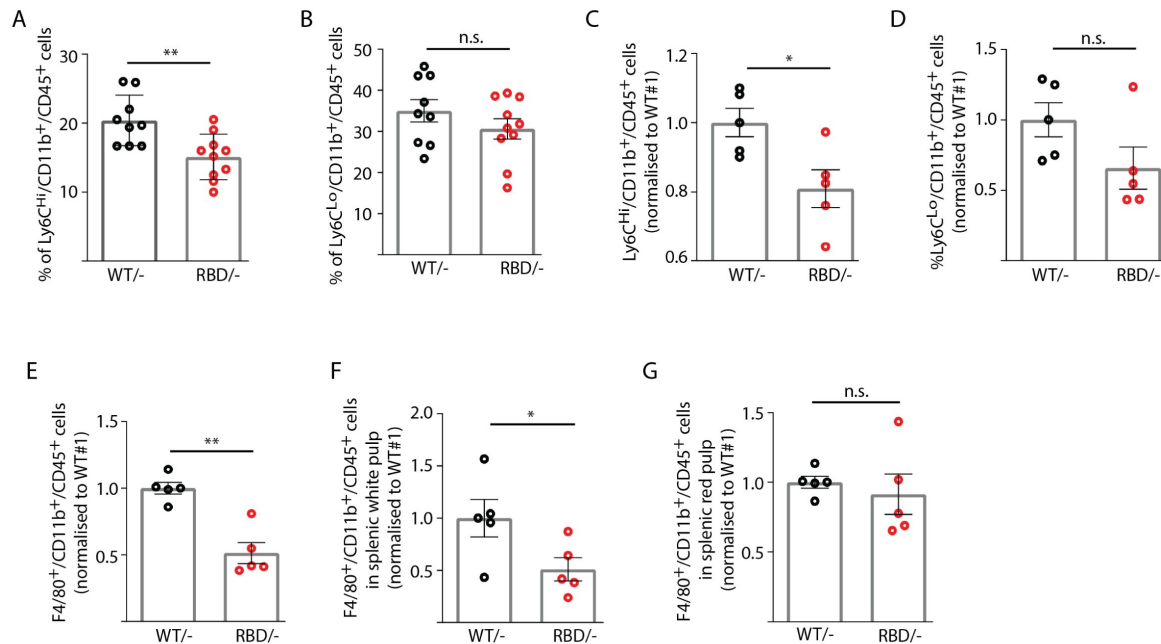


Figure 2.

Disruption of RAS-p110a interaction decreases the number of inflammatory monocytes in blood and spleen.

Twelve-week-old mice were treated with tamoxifen and after four weeks, flow cytometry analysis was performed to determine (A) inflammatory monocytes in circulating blood; (B) alternatively activated monocytes in circulating blood; (C) inflammatory monocytes in spleen; (D) classically activated monocytes in spleen; (E) macrophages in spleen; (F) macrophages in spleen's white pulp; (G) macrophages in spleen's red pulp. Data are presented as percentage of positive cells for the indicated markers. Black dots represent data from *Pik3ca*^{WT/-} mice and red dots represent data from *Pik3ca*^{RBD/-} mice. Each dot represents an individual mouse. Error bars indicate mean ± SEM. Significance using Student's t test: * p < 0.05; **p < 0.01.

the quantity of CMP progenitors within the bone marrow *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} mice (**Figure 2-Supplement 3I-L**). This observation suggests that the variations observed in blood and spleen parameters are not attributable to impairments in progenitor fate.

Finally, bone marrow precursors from *Pik3ca*^{RBD/flox} and *Pik3ca*^{WT/flox} mice were differentiated into macrophages *in vitro*, and the number of bone marrow-derived macrophages (BMDM) was determined by flow cytometry analysis. No differences were found in the number of BMDMs obtained from *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} mice (**Figure 2-Supplement 3M**), suggesting that the disruption of RAS-p110α signalling do not interfere with the ability of bone marrow precursors to differentiate to macrophages.

Disruption of RAS binding to p110α impairs monocyte transendothelial extravasation in response to inflammatory cues

The decrease in classical monocytes observed in the inflammatory abscess of paws from *Pik3ca*^{RBD/-} mice may indicate a decreased ability to mount an effective immune response. We carried out transwell assays since they are widely used to quantify transendothelial migration. Fibroblasts were seeded in the lower chamber to provide a continuous supply of Ccl2³², as this cytokine is well known for its ability to drive chemotaxis of myeloid cells under inflammatory conditions³³. *Pik3ca*^{RBD/-} or *Pik3ca*^{WT/-} BMDMs were seeded in the upper chamber of the transwell. Additionally, we stimulated *Pik3ca*^{RBD/-} or *Pik3ca*^{WT/-} BMDMs with lipopolysaccharide (LPS) and Interferon gamma (IFN-γ) to mimic/recapitulate the proinflammatory phenotype typically associated with bacterial infection that causes macrophage activation³⁴. Results showed that in both unstimulated and LPS+IFN-γ stimulated BMDMs, a lower number of *Pik3ca*^{RBD/-} BMDMs were able to go through the trans-well pore membranes compared to *Pik3ca*^{WT/-} BMDMs (**Fig. 3A**). As expected, when BMDMs were stimulated towards a proinflammatory phenotype, a decrease in their migratory ability is observed³⁵.

Extravasation entails the migration of monocytes through the endothelium^{36, 37}, so we conducted random migration assays of *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs growing in matrigel coated plates under unstimulated conditions or activated towards an inflammatory phenotype by addition of LPS and IFN-γ to the media. Analysis of the data revealed that, under pro-inflammatory conditions, *Pik3ca*^{RBD/-} BMDMs displayed a decrease in migration speed (**Fig. 3B**). Migration was also evaluated in *Pik3ca*^{WT/WT} BMDMs treated with BYL-719. Results confirmed a decrease in migration speed of macrophages upon treatment with BYL-719 (**Fig. 3B**) indicating that inhibition of p110α, either genetically or pharmacologically, reduces the ability of macrophages to migrate under inflammatory conditions.

To determine if disruption of RAS binding to p110α impairs monocyte ability to extravasate through the endothelium *in vivo* in response to an inflammatory stress, we analysed monocyte extravasation through the mesenteric vein in response to intraperitoneal Ccl2 injection. It is well established that loss of p110α function leads to significant impairment in endothelial and lymphatic system^{12, 38}, so in order to determine if monocytes from *Pik3ca*^{RBD/-} mice presented an alteration in extravasation, we generated chimeric mice in which only the bone marrow were defective in RAS binding to p110α (**Fig. 3C**). For this, bone marrow from *Pik3ca*^{RBD/-} or *Pik3ca*^{WT/-} mice was injected through the tail vein of irradiated LysM-GFP donor mice³⁹. Engraftment of *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} bone marrow could be followed by disappearance of the eGFP signal in donor mice (**Fig. 3D** and **Figure 3-Supplement 4A**).

Neutrophils (but no monocytes) were depleted using an anti-GR1 antibody (**Fig. 3E** and **Figure 3-Supplement 4B**) to avoid interference with monocyte extravasation. Intraperitoneal injection of Ccl2 was performed in the peritoneum of the chimera mice to induce extravasation of monocytes through the mesenteric vein and rolling, adhesion and extravasation was measured by intravital microscopy. Flow cytometry analysis demonstrated no differences in Ccr2 expression

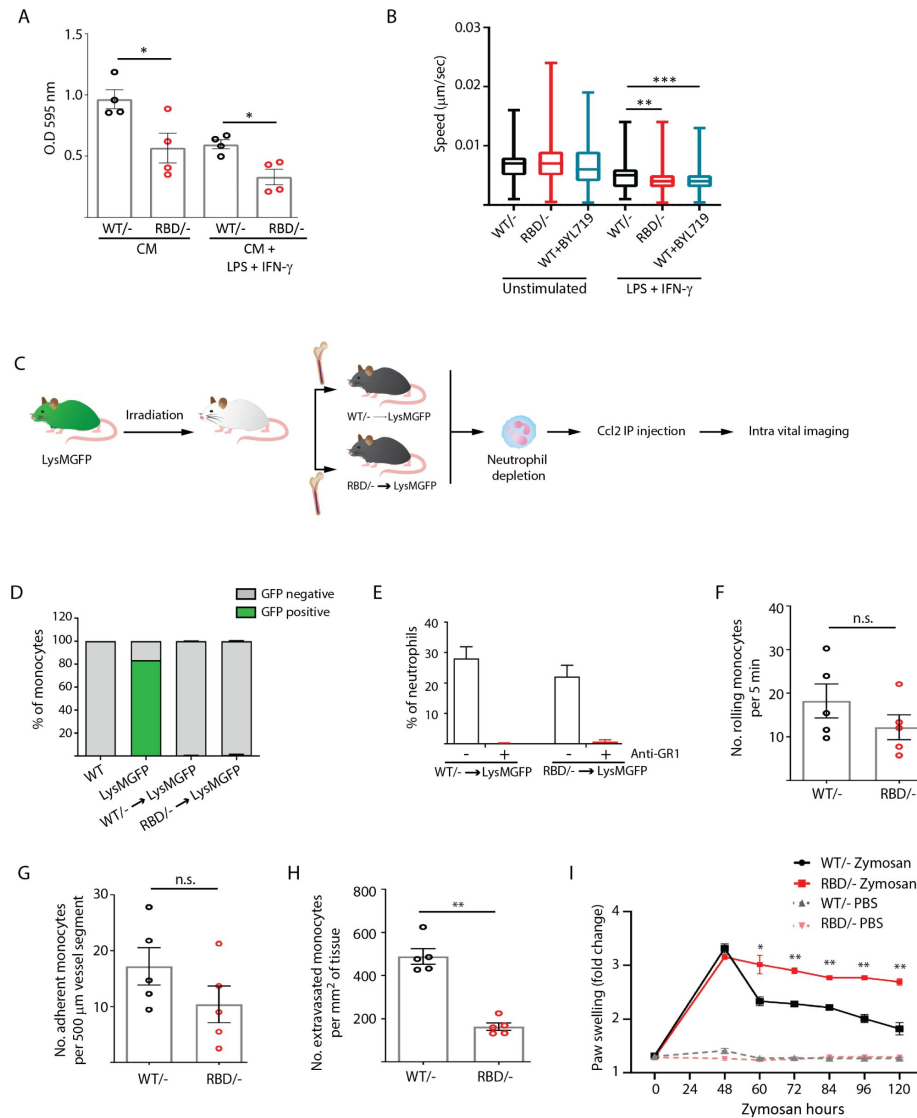


Figure 3.

Disruption of RAS-p110a interaction impairs transendothelial extravasation to sites of inflammation.

(A) Graph indicating the quantification of *Pik3ca*^{RBD/-} and *Pik3ca*^{RBD/-} BMDMs passing through 8 μm membrane pore transwells for 24 hours. Membrane attached macrophages on the lower part of the transwell were stained with crystal violet and quantified. Each dot represents an independent experiment. Error bars indicate mean ± SEM; (B) Random migration of *Pik3ca*^{WT/-}, *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/WT} BMDMs treated with BYL719 (500 ng/ml) was analysed by time-lapse video microscopy and cell tracing in the presence or absence of LPS (100 ng/ml) and IFN-γ (20 ng/ml); (C) Schematic representation of myeloid chimera generation strategy; (D) Graph showing level of bone marrow reconstitution with *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} myeloid lineage; (E) Graph quantifying the number of neutrophils in the blood of chimera mice treated with anti-GR1 (25 μg/mouse/day); (F-H) Intra vital imaging quantification of the number of: (F) rolling monocytes per 5 minutes; (G) adherent monocytes per 500 μm of vessel segment; (H) extravasated monocytes per mm² of tissue. Each dot represents an individual mouse. (I) Chimera *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice were injected with zymosan or PBS in the back-hind paws and inflammation was measured and plotted over time, *Pik3ca*^{WT/-} PBS n = 5; *Pik3ca*^{RBD/-} PBS n = 5; *Pik3ca*^{WT/-} Zymosan n = 5; *Pik3ca*^{RBD/-} Zymosan n = 5.

Statistical significance was obtained using Mann-Whitney test (A-H) or 2-way ANOVA test (I): n.s. non significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

between *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} monocytes (**Figure 3-Supplement 4C**). We observed that blocking RAS-p110α interaction did not induce a decrease in the number of rolling monocytes (**Fig. 3F**) or in the number of monocytes that adhere to the endothelium (**Fig. 3G**). However, when extravasation was measured, data showed that disruption of RAS binding to p110α caused a significant decrease in the number of monocytes that were capable of extravasating through the endothelium of the mesenteric vein (**Fig. 3H**).

Additionally, in order to determine whether the differences observed in the inflammatory response of *Pik3ca*^{RBD/-} mice were due to lack of monocyte extravasation, we repeated the zymosan paw swelling assay in the chimera mice. As previously, we observed that disruption of RAS-p110α only in the immune system led to higher inflammatory response and a delay in the initiation of the resolution phase (**Fig. 3I**).

Disruption of RAS-p110α activation in macrophages induces changes in cytoskeleton reorganization

During transendothelial migration, leukocytes undergo cytoskeletal rearrangements that allow them to squeeze through the tight spaces between endothelial cells and enter the underlying tissue^{40, 41}. Therefore, we next sought to determine whether the differences observed in *Pik3ca*^{RBD/-} BMDMs extravasation and migration were attributable to altered cytoskeletal dynamics. To do so, we first aimed at examining cell shape and spread in *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs in pro-inflammatory conditions after LPS+IFN-γ treatment. Treatment with LPS+IFN-γ induced an increase in cell spread in both *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs when compared to their respective unstimulated counterparts (**Fig. 4A and 4B**). However, cell spread in LPS+IFN-γ activated *Pik3ca*^{RBD/-} BMDMs was significantly decreased compared to that observed *Pik3ca*^{WT/-} BMDMs (**Fig. 4A and 4B**). Additionally, *Pik3ca*^{RBD/-} BMDMs were more elongated and did not acquire the typical rounded shape known to be induced in macrophages after treatment with LPS+IFN-γ⁴² (**Fig. 4A and 4C**). We also analysed cell height and results showed that *Pik3ca*^{RBD/-} BMDMs are higher both in unstimulated conditions and after LPS+IFN-γ stimulation (**Fig. 4D**).

Actin is a bona fide regulator of cell shape⁴³, so we next aimed at exploring the actin cytoskeleton in *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs. Actin fractionation assays were carried out in unstimulated or LPS+IFN-γ activated *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs. Our results revealed that disruption of RAS-p110α binding caused a decrease in the F-actin pool in unstimulated BMDMs (**Fig. 4E**), with no changes observed in the G-actin pool when compared to matched controls (**Fig. 4F**). Analysis of actin dynamics after activation with LPS+IFN-γ showed that, in proinflammatory conditions, the F-actin pool is increased, as expected⁴⁴ (**Fig. 4E**). However, actin polymerization was significantly active in *Pik3ca*^{RBD/-} BMDMs, leading to a striking increase in F-actin when compared to that observed in controls (**Fig. 4E**). In parallel, a decrease in the G-actin pool in *Pik3ca*^{RBD/-} BMDMs was observed, suggesting an increase in the stabilization of F-actin after disruption of RAS binding to p110α (**Fig. 4F**).

We next sought to investigate whether the enhanced F-actin stabilization corresponded to increased cellular rigidity. Consequently, we measured the elastic modulus of *Pik3ca*^{RBD/-} BMDMs, revealing a significant increase in cell stiffness following RAS-p110α disruption under both basal and proinflammatory conditions (**Fig. 4G**). To validate that the heightened cell stiffness was attributed to p110α, we treated *Pik3ca*^{WT/WT} BMDMs with BYL-719 and observed an amplified elastic modulus in these cells when stimulated with LPS+IFN-γ (**Fig. 4G**), confirming that the inhibition of p110α indeed results in augmented cellular rigidity.

These observations led us to hypothesize that phagocytosis, which heavily relies on actin rearrangement, might also be affected in *Pik3ca*^{RBD/-} BMDMs. To test this hypothesis, we assessed the ability of *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs to phagocytose fluorescent microspheres,

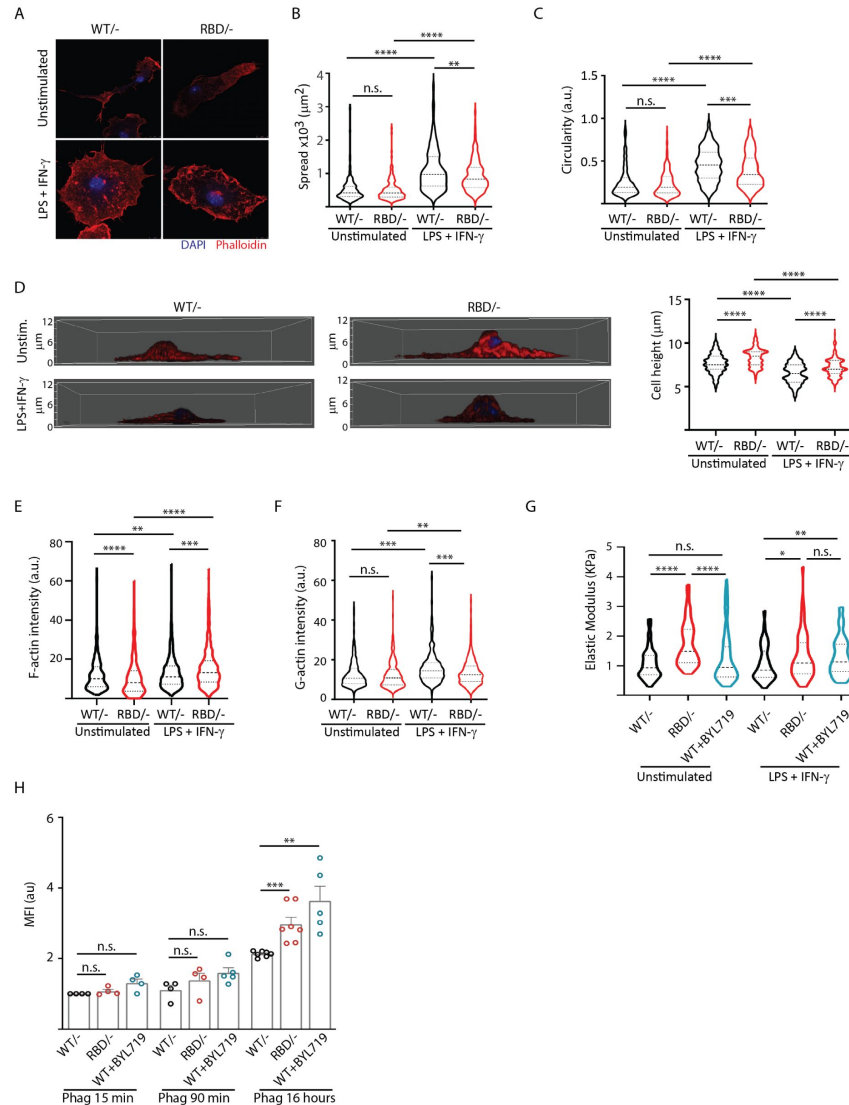


Figure 4.

Disruption of RAS-p110a signalling impairs BMDM ability to remodel their cytoskeleton in response to inflammatory cues.

(A) Representative images of *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs unstimulated or activated with LPS+ IFN-γ; (B) violin plot quantifying spread area. IF images of *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs co-stained with phalloidin and DAPI were used to analyse spread area. Results are represented as a violin plot. Three independent biological replicates were analysed (n ≥ 250 total cells); (C) Quantification of cell circularity of *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs unstimulated or activated with LPS+ IFN-γ. Quantification was performed using the same images from panel (A); (D) Representative 3D projections of *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs unstimulated or activated with LPS+IFN-γ and violin plot showing quantification of the corresponding cell height. 3D projections and cell height were analysed using same images used in panel (A). (E) Violin plot representing F-actin pool in *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs unstimulated or activated with LPS+ IFN-γ. Three independent biological replicates were analysed (n ≥ 250 total cells); (F) Violin plot representing G-actin pool (measured by DNase staining) in *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs unstimulated or activated with LPS+ IFN-γ. Three independent biological replicates were analysed (n ≥ 250 total cells); (G) Graph showing stiffness (elastic modulus) of *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs; (H) Graph showing phagocytosis of apoptotic cells (efferocytosis) over time in *Pik3ca*^{WT/-}, *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/WT} BMDMs treated with BYL719 BMDMs.

Statistical significance was obtained using Mann-Whitney test: n.s. non-significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Borrelia burgdorferi, and apoptotic cells. The results revealed varying outcomes depending on the target. When phagocytosing 1 μm non-opsonized green-fluorescent beads, *Pik3ca*^{RBD/-} BMDMs were less efficient at engulfing the microspheres compared to their WT counterparts (**Figure 4-Supplement 5A** [↗](#)). Similar results were obtained when *Pik3ca*^{WT/WT} BMDMs were treated with BYL-719 (**Figure 4-Supplement 5A** [↗](#)). In contrast, no significant differences were observed between *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs in their ability to phagocytose *Borrelia burgdorferi* (**Figure 4-Supplement 5B** [↗](#)).

Lastly, we evaluated the phagocytosis of apoptotic cells by *Pik3ca*^{RBD/-} BMDMs over extended periods. There were no differences in the initial uptake of apoptotic cells between *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs. However, at later time points, *Pik3ca*^{RBD/-} BMDMs showed an accumulation of phagocytosed particles. Similar results were obtained when *Pik3ca*^{WT/WT} BMDMs were treated with BYL-719. These data suggest a delay in processing and degradation, indicating a potential role for RAS-p110 α in the later stages of phagocytosis (**Fig. 4H** [↗](#)).

In summary, our findings suggest that loss of RAS-p110 α interaction leads to increased actin polymerization, resulting in stiffer and less deformable cells, which impairs phagocytic efficiency for certain targets. Specifically, RAS-p110 α is important for the effective phagocytosis of non-biological particles and may also play a role in the proper processing and degradation of phagocytosed apoptotic cells. The differential effects on various phagocytic targets highlight the complexity of RAS-p110 α 's role in macrophage biology and underscore the importance of cytoskeletal flexibility in efficient phagocytosis.

Disruption of RAS-p110 α activation impacts the secretome of macrophages

Given our previous observations, we hypothesized that the cytoskeletal alterations observed after disruption of RAS-p110 α interaction, may significantly impact the secretory functions of macrophages. The accumulation of apoptotic material in *Pik3ca*^{RBD/-} BMDMs suggests a disruption in normal phagocytic processing, which could influence the release of cytokines and other inflammatory mediators. Thus, we analysed the secretome of *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs both under steady state condition and during phagocytosis. We utilized apoptotic LKR10 cells, a murine lung cancer cell line, as the substrate in our phagocytosis assay. LKR10 cells were exposed to cisplatin for 16 h and apoptosis was confirmed by Western Blot (**Figure 5-Supplement 6A** [↗](#)). Our goal was to create a more physiologically relevant experimental setting that more closely mimics the complex nature of the inflammatory response. For the secretome analysis, macrophages were incubated with or without apoptotic cells for 16 h. Culture supernatants were collected, clarified, and subjected to label-free quantitative proteomics analysis.

A total of 127 peptides corresponding to 105 proteins (Supplementary File 1) showed differential expression between *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDM secretomes at steady state conditions. Additionally, 359 peptides corresponding to 210 proteins (Supplementary File 2) were present at significantly different levels in the secretomes of *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDM during phagocytosis of apoptotic cells. Next, we compared these peptides with the list of secreted proteins available at The Human Protein Atlas, and removed those that do not correspond to secreted proteins. After this step, 18 proteins were found to be differentially secreted by *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDM in steady state conditions (**Fig. 5A** [↗](#)), and 38 by *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDM during phagocytosis (**Fig. 5B** [↗](#) and **Figure 5-Supplement 6B and 6C** [↗](#)). Surprisingly, most proteins were secreted at lower levels in *Pik3ca*^{RBD/-} BMDMs, independently of the conditions under study. 12 proteins were differentially secreted in both experimental conditions under study (**Fig. 5C** [↗](#)).

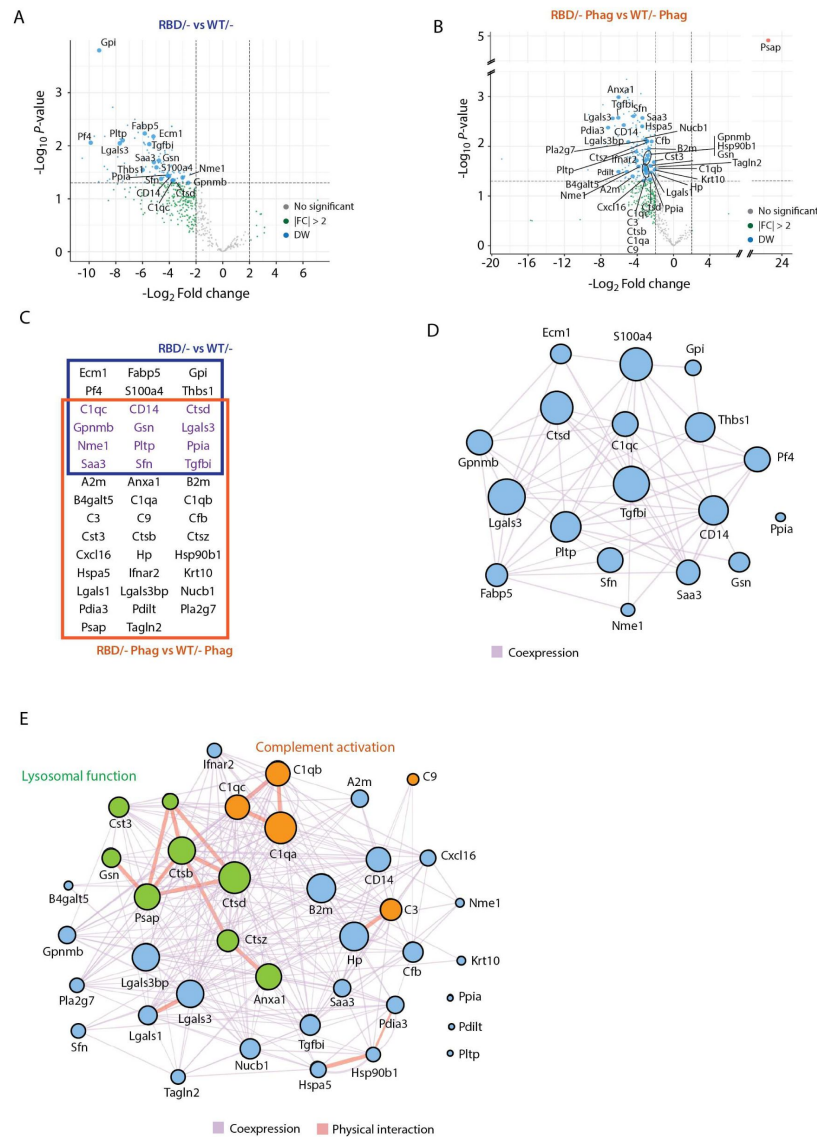


Figure 5.

Secretome analysis of *Pik3ca^{RBD/-}* BMDMs suggested a defect in complement activation and lysosomal function.

(**A** and **B**) Volcano plots of secretome analysis from *Pik3ca^{WT/-}* and *Pik3ca^{RBD/-}* BMDMs in nonstimulated conditions (**A**) or phagocytosing apoptotic cells (**B**). The x-axis shows the log2 FC of each identified protein and the y-axis the corresponding -log10 P value. Statistically significant peptides with FC ≥ 2 and p-value < 0.05 are in blue; peptides that do not pass this threshold are in grey; peptides with FC ≥ 2 but p-value ≥ 0.05 are in green; (**C**) Venn diagram showing the overlap of *Pik3ca^{RBD/-}* vs *Pik3ca^{WT/-}* BMDMs differentially expressed proteins at rest and during phagocytosis of apoptotic cells. Proteins displayed in the blue square represents peptides differentially expressed in resting BMDMs, the brown square represents peptides differentially expressed during phagocytosis, and the peptides differentially expressed in both conditions are displayed in purple; (**D** and **E**) Network analysis of significantly expressed proteins identified in the secretome analysis of *Pik3ca^{RBD/-}* vs *Pik3ca^{WT/-}* BMDMs in steady state conditions (**D**) or during phagocytosis of apoptotic cells (**E**). The nodes represent individual proteins, and the edges represent known interactions between proteins, either coexpression (purple) or physical interaction (thick pink). The size of each node reflects the significance of differential expression. Proteins in green have been implicated in lysosomal function, while proteins in orange are members of the complement cascade.

Functional analysis of differentially secreted proteins in unstimulated BMDMs showed no significant pathways related to these group of proteins (**Fig. 5D**). However, differentially secreted proteins in phagocytosing conditions were involved in two main biological processes: complement activation (C1qa, C1qb and C1qc connected through physical interaction and C3, C9 and Cfb through coexpression) and lysosome function, mainly cathepsins (Ctsd, Ctsb, Ctsz, Cst3, Psap, Anxa1 and Gsn) (**Fig. 5E**). Together, the complement cascade and lysosome function work in concert to provide an effective defence against pathogens and promote overall maintenance of cellular homeostasis^{22, 45–47} and data from the secretome analysis of *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs suggests that RAS activation of p110α may play a crucial role in the regulation of both response pathways. Moreover, these findings align with previous observations showing an accumulation of phagocytosed material in *Pik3ca*^{RBD/-} BMDMs, indicating a potential disruption in the processing and degradation of engulfed targets.

Disruption of RAS-p110α signalling leads to altered lysosomal function

We next aimed to functionally validate the proteomics data suggesting that RAS-p110α activation regulates lysosomal function in macrophages. First, we performed immunofluorescence analysis of lysosomal-associated membrane protein 1 (LAMP1) to assess lysosomal biogenesis and function in *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs. Analysis of LAMP1 in *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs showed a decrease in LAMP1 expression in steady state conditions (**Fig. 6A**) suggesting a decrease in the number of lysosomes after disruption of RAS-p110α interaction. However, *Pik3ca*^{RBD/-} showed increased levels of Lamp1 expression when subjected to phagocytosis of apoptotic cells, suggesting an increase in the number of phagolysosomes present in these cells.

We hypothesised that the increase in Lamp1 expression in BMDMs lacking RAS-p110α interaction during phagocytosis could be attributed to aberrant lysosomal function and phagolysosome retention. Thus, we next evaluated lysosomal activity by using the lysosomotropic dye lysotracker red coupled with epifluorescence analysis, since it is specifically taken up by acidic organelles. As such, its accumulation is proportional to the number of acidic vesicles. Data analysis showed that disruption of RAS-PI3K in BMDMs leads to a significant decrease in lysotracker uptake, both in unstimulated conditions and also after activation with LPS+IFN-γ (**Fig. 6B**). Similar results were obtained with control BMDMs treated with BYL719, the p110α specific inhibitor. Data analysis showed a decrease in the uptake of lysotracker after p110α inhibition (**Fig. 6B**), further suggesting that loss of p110α function in BMDMs results in altered lysosomal pH. To confirm that lysosomal pH of *Pik3ca*^{RBD/-} BMDMs was less acidic, we next stained *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs with Green lysosensor, a pH-sensitive dye that exhibit a pH-dependent increase in fluorescent intensity upon lysosomal acidification. As shown in **Fig. 6C**, *Pik3ca*^{RBD/-} BMDMs presented attenuated fluorescent intensity both in unstimulated and activated when compared with that in the control group, indicating that their lysosomal content is less acidic than in *Pik3ca*^{WT/-} BMDMs.

Considering that *Pik3ca*^{RBD/-} lysosomes were less acidic, we next investigated the expression level and activation of some of the cathepsins identified in the secretome analysis (**Fig. 5**) by western blotting. Cathepsins play a critical role in lysosomal protein degradation. They are initially synthesized as inactive precursors and are activated through proteolysis in the lysosome at a low pH⁴⁸. We found a reduction in the expression and activation of cathepsin D and cathepsin B in *Pik3ca*^{RBD/-} BMDMs upon stimulation with LPS+IFN-γ (**Fig. 6D**). This observation suggests that the impaired lysosomal pH observed in *Pik3ca*^{RBD/-} BMDMs could potentially account for the observed decrease in cathepsin activity.

Acidification of lysosomes and cathepsin activation are critical steps for activation of the resolute stage of the inflammatory response, so we next evaluated the ability of *Pik3ca*^{RBD/-} BMDM's lysosomal compartment to degrade internalised particles. We set up another phagocytosis

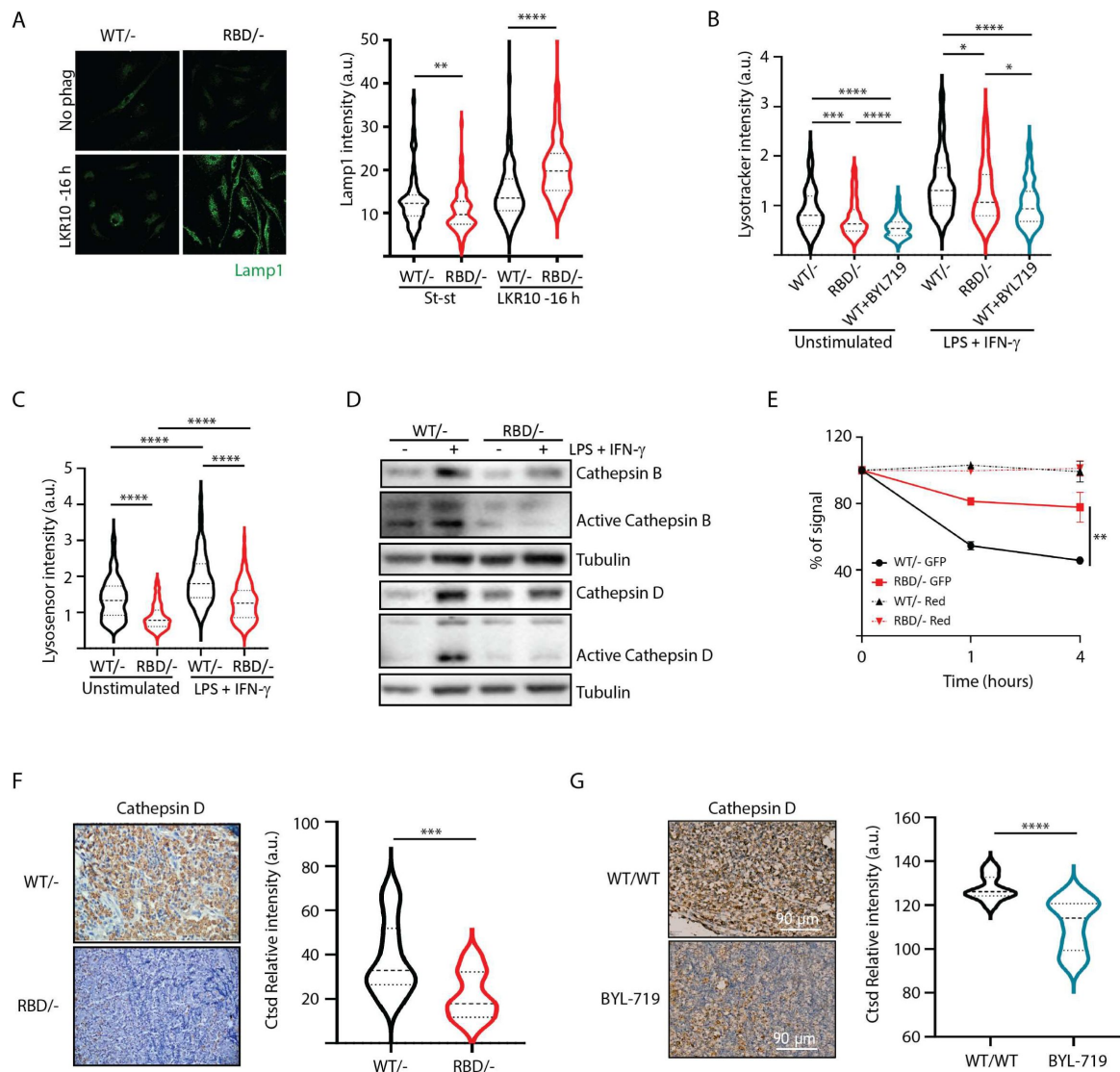


Figure 6.

Disruption of RAS-p110a activation in BMDMs leads to abnormal lysosomal function

(A) Representative IF images and quantification analysis of lamp intensity in *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs in steady state conditions and during phagocytosis of apoptotic LKR10 cells. Three independent biological replicates were analysed (n ≥ 250 total cells). Error bars indicate mean ± SEM; **(B)** Quantification analysis of lysosomal function in *Pik3ca*^{WT/-}, *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/WT} BMDMs treated with BYL719 BMDMs unstimulated or activated with LPS + IFN-γ using LysoTracker staining. Three independent biological replicates were analysed (n ≥ 250 total cells); **(C)** Violin plot displaying lysosome pH acidity in unstimulated and LPS+IFN-γ stimulated *Pik3ca*^{WT/-} (black), *Pik3ca*^{RBD/-} (red) and *Pik3ca*^{WT/WT} BMDMs treated with BYL719 (Blue) BMDMs, as determined by LysoSensor staining. Three independent biological replicates were analysed (n ≥ 250 total cells); **(D)** *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} BMDMs were activated with LPS and IFN-γ and expression and activation of Cathepsin B and D were blotted; **(E)** Quantification of phagocytosed apoptotic cells in *Pik3ca*^{WT/-}, *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/WT} BMDMs treated with BYL719 BMDMs. Apoptotic cells were labelled with a red tracker and allowed to be phagocytosed by control, *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/WT} BMDMs treated with BYL719 for 16 hours, measuring the amount of cell tracker that was internalized at different time points. Each dot of the graph indicates a different independent experiment. Error bars indicate mean ± SEM; **(F)** Representative image and quantification of Cathepsin D expression in the inflammatory abscess of *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} paws injected with zymosan; **(G)** Representative image and quantification of Cathepsin D expression in the inflammatory abscess of paws from control and *Pik3ca*^{WT/WT} mice treated with BYL719 injected with zymosan.

Statistical significance was obtained using Mann-Whitney test: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

assay in which *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs would phagocytose apoptotic cells that were transduced with GFP (which is pH sensitive⁴⁹) and also labelled with Celltracker Red CMTPX (pH insensitive). *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} BMDMs were allowed to engulf these apoptotic cells for 16 h and after this time, apoptotic cells were eliminated by washes and BMDMs were analysed by flow cytometry at different time points to measure GFP signal. Our results showed that GFP signal is lost significantly faster in control BMDMs than in *Pik3ca*^{RBD/-} BMDMs (**Fig. 6E**). As expected, we did not observe any decay in the red tracker signal. Collectively, these data evidence that *Pik3ca*^{RBD/-} BMDMs exhibit a significant impairment in removing engulfed particles, that can be attributed to the absence of lysosome acidification.

We have shown a delayed clearance of apoptotic cells in *Pik3ca*^{RBD/-} mice after zymosan injection (**Fig. 1**). This, together with previous data took us to analyse the levels of Cathepsin D in the inflamed abscess from the paws of *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} mice. Results showed a significant decrease in the levels of Cathepsin D in the inflamed area of *Pik3ca*^{RBD/-} mice (**Fig. 6F**) and BYL719-treated mice (**Fig. 6G**).

Lysosomal digestion plays a pivotal role in activation of resolutive programs by mediating PPAR activation^{50, 51}. Analysis of PPAR δ and PPAR γ expression in phagocytosing macrophages showed a significant decrease in the expression of PPAR δ , but no PPAR γ in *Pik3ca*^{RBD/-} BMDMs (**Figure 6- Supplement 7A**), suggesting that resolutive programs might not be activated effectively.

In summary, our findings underscore the crucial role of RAS-p110 α signalling axis in maintaining the balance of the inflammatory response and promoting timely resolution, providing further evidence for the significant involvement of RAS-p110 α signalling in the response to inflammatory stimuli.

Discussion

In this study, we provide compelling evidence that disruption of RAS-p110 α signalling or chemical inhibition of p110 α impairs response to inflammatory stresses due to defects in both monocyte extravasation during the early stages of the inflammatory response and decreased lysosomal function during the later stages (**Fig. 7**). Although RAS-p110 α signaling disruption affects all the hematopoietic cells, several lines of evidence indicate that macrophages play a central role in the observed phenotype. In our chimera mice experiments, neutrophil depletion did not alter the reduced transendothelial extravasation, suggesting that macrophages are the primary cell type involved. Furthermore, in the paw edema model, macrophages were the population exhibiting the most significant functional defects. Together, these findings, along with the specific deficiencies observed in myeloid populations, support a predominant role of macrophages in mediating the impaired inflammatory response. Nevertheless, we acknowledge that other myeloid cells, such as dendritic cells or additional immune populations, may also contribute to the global phenotype and further analysis should be performed to address this.

Monocyte extravasation during the inflammatory response is a critical step that allows immune cells to reach the site of infection or injury¹⁹. Impairment on this process causes slower or inadequate immune response, as macrophages are crucial for detecting and engulfing pathogens or cellular debris at the site of inflammation, as well as delayed resolution of inflammation, resulting in prolonged inflammation and subsequent tissue damage^{52, 53}.

Our data shows that RAS binding to p110 α is involved in macrophage extravasation by modulating actin dynamics. During monocyte extravasation, actin filaments form actin-rich protrusions that are essential for monocyte migration across the endothelium^{41, 54}. Our data show that, during

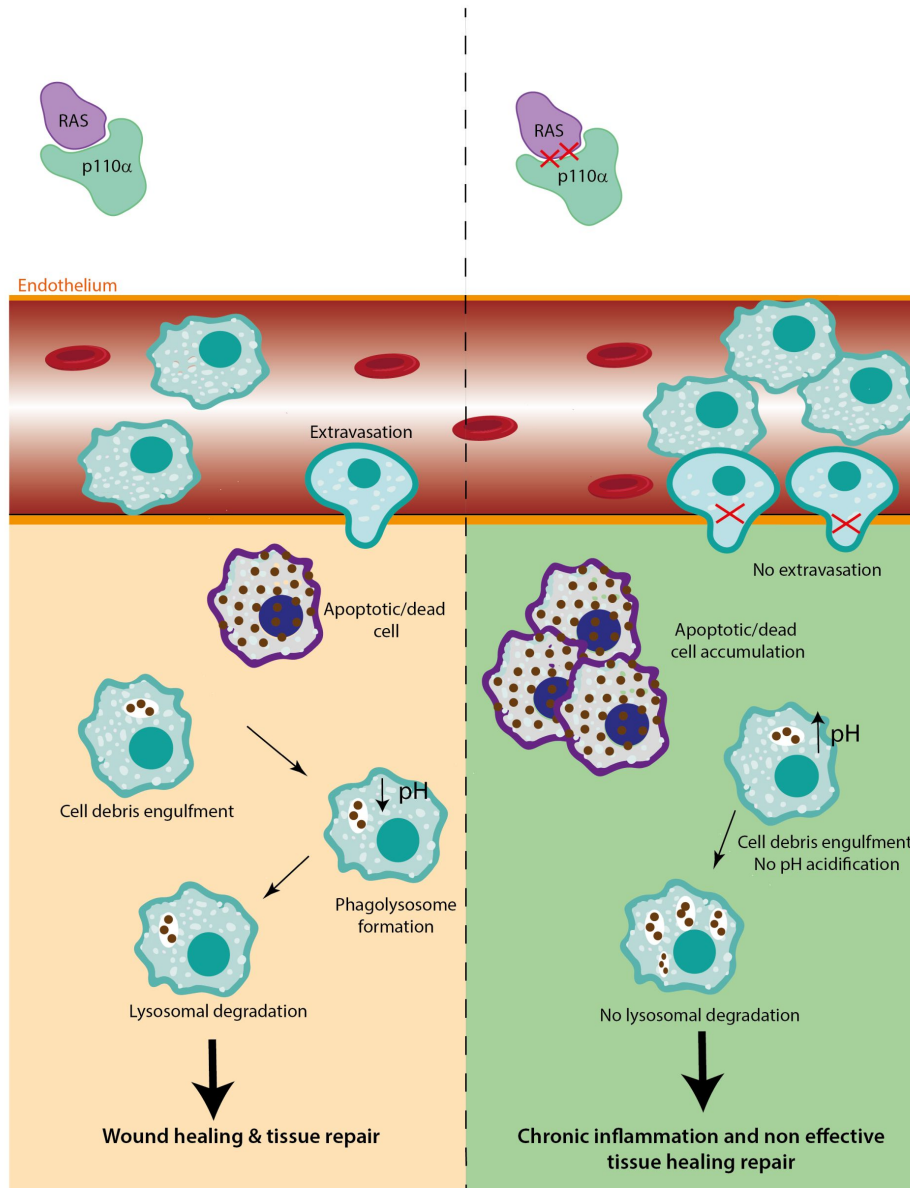


Figure 7.

The role of RAS-p110α in macrophage-mediated inflammatory responses.

In the presence of functional RAS-p110α, monocytes efficiently migrate through the endothelium in response to inflammatory signals. Following extravasation, monocyte-derived and resident macrophages clear apoptotic cells via phagocytosis and lysosomal degradation, facilitating resolution of inflammation. In contrast, loss of RAS-p110α leads to cytoskeletal changes that hinder monocyte transendothelial migration. Additionally, resident macrophages exhibit impaired degradation of phagocytosed particles, resulting in unresolved inflammation and more severe acute inflammatory responses.

extravasation, RAS-p110 α signalling regulates actin dynamics so monocytes are able to squeeze through endothelial cells⁴¹,⁴³.

The disruption of RAS-p110 α in macrophages emerges as a pivotal determinant in cellular mechanics, elucidating a cascade of events resulting in increased F-actin levels. The increase in cytoskeletal components, particularly F-actin, causes a notable increase in cell stiffness and a concurrent decrease in cell deformability. The orchestration of these changes underscores the intricate balance in cytoskeletal dynamics. Notably, the regulatory role of Rho-GTPases comes into focus as potential mediators of this phenomenon. Rho-GTPases are well-known mediators of actin cytoskeletal rearrangements⁵⁵, and their dysregulation is known to impact cell mechanics⁵⁶,⁵⁷. The observed increase in cell stiffness and deformability, may thus be governed by the modulation of Rho-GTPase activity. This intricate interplay provides a compelling avenue for further investigation into the molecular mechanisms through which RAS-p110 α disruption influences Rho-GTPases, ultimately shaping the biomechanical properties of macrophages and may open avenues for therapeutic interventions targeting cytoskeletal dynamics in macrophages.

Monocyte extravasation shares many features with monocyte egression from the bone marrow. During monocyte egress, monocytes also undergo changes in cytoskeleton dynamics to detach from the sinusoidal endothelial cells and to migrate through the endothelial fenestrae and basement membrane into the bone marrow sinusoids⁵⁸. Thus, the decrease in the number of classical monocytes in the *Pik3ca*^{RBD/-} mice in blood may be due, at least in part, to a defect in the extravasation process. This result may also explain, at least partially, the lack of macrophage recruitment to lung tumours found in previous studies using the RBD mouse model¹¹,¹².

Our findings also revealed a crucial role of RAS-p110 α activation in the acute phase of the inflammatory response by regulating effective lysosomal degradation of phagocytosed and engulfed material. Phagocytosis constitutes a vital step in the inflammatory response, whereby phagosomes bind to lysosomes to form phagolysosomes in which pathogens are eradicated to facilitate an appropriate host response. This response encompasses antigen presentation to engage T-cell responses, secretion of inflammatory mediators that guide the adaptive immune response, and initiation of tissue repair mechanisms²²,⁴⁷. Our data provide evidence that the activation of RAS-p110 α signalling pathway is involved in the critical process of lysosomal acidification, which is essential for the efficient degradation of internalized particles and the activation of proteolytic enzymes, ultimately resulting in the formation of fully functional lysosomes. Consequently, lack of lysosome acidification impairs the expression and activation of important proteases such as cathepsin B and cathepsin D. The proper functioning of lysosomes is essential for mounting a robust response to inflammatory stress. Lysosomes play a central role in the breakdown of pathogens and dead cells by providing the necessary degradative enzymes and maintaining an acidic environment that facilitates the degradation of engulfed particles²⁰,²¹. When lysosomal function is compromised in macrophages, the degradation of phagocytosed material becomes impaired, leading to the accumulation of toxic debris. This accumulation subsequently triggers inflammation and causes damage to the surrounding tissues, leading to chronic inflammation and appearance of lysosomal storage diseases (LSD)⁵⁹,⁶⁰.

Our data showed that the disruption of Ras-p110 α in macrophages presents a multifaceted impact in inflammatory response, notably manifesting as a reduction in NF- κ B activation and cathepsin expression coupled with diminished lysosomal function. NF- κ B, a pivotal regulator of inflammatory responses⁶¹,⁶², is intricately linked to lysosomal dynamics, with lysosomes playing a crucial role in modulating NF- κ B signalling through the degradation of relevant molecules⁶³,⁶⁴. Additionally, the observed decrease in NF- κ B activation aligns with the decline in lysosomal function, which has previously been suggested in previous reports⁶⁵,⁶⁶. This bidirectional influence, where perturbations in lysosomal function coincide with alterations in NF- κ B activity, underscores the intricate and contextdependent nature of this regulatory network, suggesting a plausible mechanistic interdependence. Our data suggest that disruption in

Ras-p110 α signalling cascade could trigger downstream effects impacting both NF- κ B activity and lysosomal integrity. This intricate nexus between Ras-p110 α signalling, NF- κ B activation, cathepsin expression, and lysosomal function underscores the complexity of cellular regulatory networks. Further analysis of the molecular pathways connecting these observations holds the potential to reveal novel insights into the coordinated regulation of inflammatory and lysosomal processes in macrophages. Thus, further investigations are necessary to decipher the causal relationships and unravel the broader implications of Ras-p110 α disruption in shaping these intricate cellular responses during the inflammatory response.

The possible functional link between the increased actin polymerization and lysosomal dysfunction observed in *Pik3ca*^{RBD/-} mice remains an unanswered question. Lysosome acidification and actin polymerization are tightly interconnected processes that have pivotal roles in numerous cellular functions^{67–69}. Studies have demonstrated that lysosome acidification can influence actin polymerization dynamics through the activation of specific actin-binding proteins and the modulation of actin-regulatory proteins^{67, 70}. Conversely, disruption in lysosome acidification, such as impaired proton pump activity or lysosomal storage disorders, have been associated with changes in actin polymerization and the organization of the cytoskeleton. Notably, it has been reported that depolymerization of F-actin plays a crucial role in assembling the macromolecular components of the acidification machinery in nascent endosomes⁷¹. Therefore, the intricate relationship between lysosome acidification and actin polymerization suggests a potential reciprocal influence, where perturbations in one process could impact the other. Further investigation is required to determine the precise regulatory mechanisms by which p110 α influences both lysosome acidification and actin polymerization, whether it occurs in a linear manner or through separate pathways.

This study provides valuable insights into the mechanisms that govern the immune response to inflammation, particularly emphasizing the essential role of RAS-p110 α signaling. Our results underscore the pivotal role of RAS-p110 α signaling in both the initiation and resolution of inflammation. The impaired ability of RAS-PI3K-deficient monocytes and macrophages to effectively migrate, initiate a robust inflammatory response, and resolve inflammation highlights the critical importance of this pathway in maintaining immune homeostasis. The persistent inflammation observed in these models may contribute to the development and perpetuation of chronic inflammatory conditions. Given the centrality of p110 α in both initiating and resolving inflammation, its dysfunction could be a key factor in the chronic, dysregulated inflammation characteristic of diseases such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, or systemic lupus erythematosus.

The potential of p110 α as a therapeutic target in these conditions is especially intriguing. The recent identification of a p110 α small molecule activator⁷² offers a promising tool to explore this avenue. By transiently enhancing p110 α function, it may be possible to promote the resolution of inflammation and thereby alleviate the symptoms and progression of these inflammatory diseases. Further research into this therapeutic strategy could open new pathways for treating chronic inflammatory disorders, providing much-needed relief for patients affected by these debilitating conditions.

Methods

Animal studies

For removal of *Pik3ca*-floxed allele, *Pik3ca*^{RBD/lox} and *Pik3ca*^{WT/flox} mice⁶⁴ were given 3.2 mg tamoxifen (Sigma) dissolved in 80 μ l of corn oil by oral gavage once per day during 3 consecutive days. Efficiency of tamoxifen treatment was routinely performed by genotyping for the presence of the floxed allele.

For **paw oedema studies** *Pik3ca*^{RBD/-} and *Pik3ca*^{WT/-} mice⁶⁴ were divided into groups of four two weeks before oedema induction. Before inducing the paw oedema, the mice were anesthetized with 4% isofluorane. To induce the oedema, mice received ipsilateral i.pl. injection (30 µl) of either zymosan (10 µg/µl, Sigma Aldrich) or PBS into the back-hind paw. Injection of 0.1mg/kg Buprenorphine (NOAH, Vetergesic) was given for pain prevention. Paw thickness was measured using a calliper every hour during the first 6 hours after injection and then at 8, 10 hours and twice per day afterwards. Buprenorphine was injected twice per day during the length of the experiment.

Animals were randomly assigned to experimental groups to ensure unbiased allocation and minimize potential confounding factors. Animals were randomly assigned to groups with similar numbers, ensuring all were born on the same date to control for age-related factors. No formal sample size calculation was conducted. Blinding was implemented during data collection and analysis to reduce bias, with researchers unaware of the group allocations when assessing outcomes. Inclusion and exclusion criteria were pre-established to ensure consistency; animals showing pre-existing health conditions were excluded from the study to maintain experimental integrity; only females were included in this study. Mice were kept, managed, and sacrificed in the NUCLEUS animal facility of the University of Salamanca according to current European (2007/526/CE) and Spanish (RD 1201/2005 and RD53/2013) legislation. All experiments were approved by the Bioethics Committee of the Cancer Research Center. All animal procedures were conducted in accordance with the guidelines and humane endpoints established in our animal experimental license to minimize pain, suffering, and distress. Interventions such as Buprenorphine were employed as needed, and procedures were designed to be minimally invasive. Animals were monitored daily for expected and unexpected signs of pain or distress, with specific criteria—such as weight loss, lethargy, abnormal grooming—set as humane endpoints to ensure early intervention if required. No unexpected adverse events occurred during the study.

Isolation, culture and treatments of BMDM

Bone marrow cells from tibias and femurs of 12–14-week-old *Pik3ca*^{RBD/Lox} mice and *Pik3ca*^{WT/Lox} littermates were cultured with DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/mL streptomycin, 2 mM L-Glutamine and 20 ng/mL M-CSF for 7 days. 4-hydroxytamoxifen (Sigma Aldrich) (100 nM) was added to culture media on day 3 to eliminate *Pik3ca*-Lox allele. The differentiated BMDM were then detached using cell dissociation buffer (C5914-100, Merck) and cultured in DMEM supplemented with 10% FBS, 100 µg/mL streptomycin, 2 mM L-Glutamine, 20 ng/mL M-CSF for unstimulated BMDMs. For macrophage polarization towards an inflammatory phenotype 20 ng/ml IFNγ (Peprotech) and 100 ng/ml LPS (Sigma Aldrich) was added to culture media.

Generation of chimeric animals

Chimeric mice exhibiting WT or RBD deficient leukocytes were generated by lethal irradiation with 5.5 Gy twice, 4 h apart of LysM GFP recipient animals (mice exhibiting endogenously GFP fluorescent monocytes and neutrophils) followed by an injection of bone marrow cells (1.5×10^6 cells/recipient i.v.) from C57BL/6 WT or RBD donor mice. Chimerism was then assessed 4 weeks later by flow cytometry from blood samples (reconstitution of 99.8 ± 0.2 % and 99.8 ± 0.1 % for WT and RBD deficient donor cells, respectively; n =5 mice per group).

Neutrophil depletion

Neutrophil depletion of chimeric mice was induced by intraperitoneal injection of anti-GR1 25µg/mouse/day for 3 days). Numbers of blood circulating monocytes and neutrophils were quantified by flow cytometry pre- and post-depletion. Neutrophils were found to be reduced by

99.5%, whilst this anti GR1-depleting protocol had no effect on blood monocyte proportion (n=5 mice/group).

Brightfield intravital confocal microscopy

Mesenteric inflammation was induced following intraperitoneal injection of mouse recombinant CCL2 (500ng/mouse in 500uL of PBS). Six hours later, anesthetized chimeric mice (150 mg/kg ketamine, 7.5 mg/kg xylazine, i.p.) were placed in supine position on a heating pad (37 °C) for maintenance of body temperature. The mesenteric vascular bed was exteriorized, placed on a purpose-built stage of an upright brightfield microscope (Zeiss Axioskop). Mesenteries were superfused with warmed (37 °C) Tyrode's solution (Sigma). After a 5-min equilibration period, analysis of leukocyte-endothelium interactions was made in at least 9 (and up to 16) randomly selected segments (100 µm in length) of post-capillary venules (20–40 µm in diameter) for each mouse. Leukocyte rolling was quantified by counting the number of rolling cells passing a fixed transversal line in the middle of the vessel segment for 5 min. Leukocyte adhesion (stationary position of the cell for 30 s or longer) was quantified along a 100 µm vessel length and data were normalized as the number of cells per 500µm vessel segments. Leukocyte extravasation response was quantified within 50 µm on either side of the 100 µm vessel segment in the perivenular tissue; and data were normalized as the number of extravasated leukocytes per mm² of extravascular tissue. At the end of the analysis period, mice were humanely killed by cervical dislocation.

BMDM elastic modulus

The elastic modulus of BMDMs was quantitatively assessed utilizing Atomic Force Microscopy (AFM). Specifically, cells were cultured on cover glass slides, which were subsequently positioned in a specialized BIO-AFM setup integrated with an inverted optical microscope (Nikon TE2000). The BIO-AFM was equipped with a V-shaped, four-sided pyramidal silicon nitride tip (Bruker AFM Probes) to facilitate accurate force measurements. To avoid localized variations and ensure data representativeness, no more than three cells were selected from a single field of view for mechanical characterization, and these cells were never contiguous. Subsequently, the stiffness of each individual cell was characterized through measurements obtained at three perinuclear points. For each point, force-displacement (F vs. z) curves were captured (10 µm amplitude at a speed of 5 µm/s). The determination of the elastic modulus was performed based on the analysis of force-displacement curves. A total of five curves were recorded for each perinuclear point at an indentation depth of 300 nm. Data were analysed by fitting the curves to the Hertz model as previously described^{73, 74}. Finally, the elastic modulus for each cellular population under distinct experimental conditions was calculated based on a minimum of fifteen measurements per independent cell, with each condition comprising at least ten independent cells (n ≥ 10).

Cytokine arrays

Bone marrow-derived macrophages (BMDMs) were cultured in 6-well plates at a density of 1×10^6 cells per well. Cytokine production was analyzed in unstimulated and M1 macrophages using commercial mouse cytokine array (R&D Systems, #ARY006). Cells were lysed at 4°C for 30 minutes using a lysis buffer containing 1% Igepal CA-630, 20mM Tris-HCl (pH 8.0), 137mM NaCl, 10% glycerol, 2mM EDTA, 10µg/ml Aprotinin, 10µg/ml Leupeptin, and 10µg/ml Pepstatin, following the manufacturer's protocol. Lysates were then centrifuged at 16,100 RCF for 15 minutes at 4°C. The supernatant was collected, and protein concentration was determined using a Qbit 2.0 Fluorometer. A total of 1400µg of protein was diluted to a final volume of 1ml with Array Buffer 6, followed by the addition of 0.5ml Array Buffer 4 and 15µl of the reconstituted Mouse Cytokine Array Panel A Detection Antibody Cocktail. The samples were mixed and incubated for one hour on a shaker at room temperature. Nitrocellulose membranes from the mouse cytokine array were blocked with Array Buffer 6 for one hour at room temperature on a shaker. The blocking buffer was then removed, and the sample mixture was added to the arrays, which were incubated overnight at 4°C on a shaker. Membranes were washed with Wash Buffer provided by the

manufacturer. Membranes were then incubated with 1× Streptavidin-HRP in Array Buffer 6 for 30 minutes at room temperature. After further washing, the Chemi Reagent Mix was applied, and signals were detected using an Amersham Imager 600 (GE Healthcare, #29-0981-07 AC).

Immunofluorescence

BMDM were fixed using 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked for 1 h with 3% BSA in PBS before incubation with the primary antibodies, used at a 1:100 dilution: Lamp1 (#553792, BdPharmigen), Deoxyribonuclease I-Alexa Fluor™ 488 Conjugate (#D12371, Invitrogen, 1:2000). To stain actin cytoskeleton, Alexa Fluor™ 647 Phalloidin (Invitrogen, 1:10 000) was directly added to the primary antibody mixture. Alexa Fluor 488- or Alexa Fluor 555-conjugated secondary antibodies (Invitrogen) were used to detect the indicated proteins at a 1:1000 dilution. Cells were counterstained with DAPI on the mounting solution (ProLong Gold Antifade Reagent with DAPI, Invitrogen). Images were taken using a Zeiss LSM510 confocal microscope or Leica DM6 B THUNDER Imager 3D Tissue.

Transwell migration assay

Transwell migration assays were carried out using the 6.5 mm Transwell® with 8.0 µm Pore Polyester Membrane Insert (Corning). 9×10^4 MEFs from wild type mice were used as a chemo-attractant to encourage macrophage migration. 8×10^5 BMDM were seeded in the transwell. Transwells were performed following the manufacturer instructions.

Random migration assay

For random migration assays, BMDMs were seeded in 24-well plates coated with matrigel (0.5mg/ml) and labelled using CellTracker™ Red CMTPX Dye 1 µM (ThermoFisher) for 30 minutes. 24 hours later LPS + IFN-γ was added when necessary. Triplicates of each condition and genotype were prepared. Time-lapse imaging was carried out for 24 h. One image was taken every 10 min within the same well using a Nikon microscope driven by Metamorph (Molecular Devices, Chicago, IL, USA). A total of 80100 cells per condition were tracked using the Fiji plugin Trackmate. Pre-processing was done using Mexican hat filter 3.0 radius to increase particle detection. Images were segmented using the fluorescence channel with the Laplacian of the Gaussian detector with a 30 µm estimated particle diameter, a 10.0 threshold and median filter option selected. Segmented objects were linked from frame to frame with a Linear Assignment Problem (LAP) tracker with 45 µm frame-to-frame linking distance and 2 frame gap closure. Criteria for track acceptance were track duration at least the 90% of the video. Tracks were visually inspected for completeness and accuracy of the tracking.

Secretome mass spectrometry

Samples for secretome analysis were prepared as previously described¹³⁰. In brief, 100µg of proteins were digested into peptides using trypsin and peptides were desalted using Oasis HLB extraction cartridges (Waters UK Ltd) and eluted with 50% acetonitrile (ACN) in 0.1% Trifluoroacetic acid (TFA).

Dried peptides were dissolved in 0.1% TFA and analysed by nano ACQUITY liquid chromatography (Waters Corp., Milford, MA, USA) coupled on-line to a tandem LTQ Orbitrap XL, mass spectrometer (Thermo Fisher Scientific)¹³¹. Gradient elution was from 5% to 25% buffer B in 180 min at a flow rate 300nL/min with buffer A being used to balance the mobile phase (buffer A was 0.1% formic acid in water and B was 0.1% formic acid in ACN). The mass spectrometer was controlled by Xcalibur software and operated in the positive mode. The spray voltage was 1.95 kV and the capillary temperature was set to 200 °C. The LTQ Orbitrap XL was operated in data dependent mode with one survey MS scan followed by 5 MS/MS scans. Label-free quantitative proteomics analysis was performed using three independent biological samples per group. Additionally, each

sample was analysed in technical duplicates. To ensure robust quantitative analysis, we utilized LTQ Orbitrap XL tandem mass spectrometry (MS/MS) to generate six distinct mass spectral profiles from each group.

MS raw files were converted into Mascot Generic Format using Mascot Distiller (version 2.3.0) and searched against the SwissProt database (release December 2015) restricted to human entries using the Mascot search daemon (version 2.3.1). Allowed mass windows were 10 ppm and 600 mmu for parent and fragment mass to charge values, respectively. Variable modifications included in searches were oxidation of methionine, pyro-glu (N-term) and phosphorylation of serine, threonine and tyrosine.

Spectral counting quantification method relies on the number of times peptides are identified by tandem mass spectrometry (with expectancy value <0.05) from a given protein. Spectral counts were obtained from Mascot result (DAT) files using a python script written in house in the Mascot Parser Toolkit environment (version 2.4.x).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE⁷⁵ partner repository with the dataset identifier PXD057794 and 10.6019/PXD057794.

Proteomic data analysis

The proteomic data obtained consisted of 6,844 peptides and 30 samples: Pik3ca^{WT/-} and Pik3ca^{RBD/-} BMDMs in steady state conditions (labelled as cell samples: WT/- and RBD/-), phagocytosing apoptotic cells (labelled as cell samples: WT/-Phag and RBD/-Phag), and the apoptotic LKR10 cells alone (labelled as LKR). For each of these samples, proteomic experiments were performed with 6 replicates (3 biological replicates $\times$ 2 technical replicates), yielding a data set of 30 samples.

The first analytical step was to remove all peptides for which there was no information contained in the proteomic raw data matrix and the peptides for which 85% or more of the signal values were missing. All these peptides were specific of mouse proteins and in many cases were unique. The corresponding proteins were annotated and labelled together with each measured peptide. Next, low-quality samples were also removed, testing the overall signal per sample to identify if there were clear outliers with very low signal or with a very different signal distribution. Comparison of the overall signal distributions of the 30 samples (comparing boxplots) and identified 3 samples that were very different were obtained and discarded (WT/-Phag_s3r2 (sample 3, replicate 2), RBD/-_s2r1 and LKRc_s1r1). These 3 samples showed a median signal in their distributions that deviated $>20\%$ from the median signal of the distributions of all other samples.

Differential expression analysis for each peptide of each protein were next performed. The algorithm used to carry out this analysis was *limmaVoom* within EdgeR R package^{76, 77}. Prior to this analysis, a Bartlett test was performed to see the homogeneity of variances, verifying that for this data we cannot consider equality of variances and this factor was included in the differential expression algorithm. With this algorithm, normalization factors to use *a-posteriori* were calculated and, transformation and calculation of the variance weights was performed. The model to fit before using *Voom* as specified since it uses the variances of the model's residuals (observed - fitted). Finally, an estimation of the contrast for each feature tested (i.e. each peptide) was carried out using the *Empirical Bayes* approach in *limma* as previously described⁷⁸. Peptides were ordered by the p.value of the *limma* test considering significant peptides changed only with a p.value below 0.05 and with a $\log_2(\text{Fold-Change}) > |2|$. All these analyses were performed using the statistical computing language R and packages or libraries obtained from R-cran (cran.r-project.org) or Bioconductor (www.bioconductor.org).

Cytoscape software (v3.9)⁷⁹ including GeneMania app⁸⁰ was then used to generate and visualize protein-protein networks of the significantly altered proteins selected in secretome analysis of unstimulated and phagocytosing BMDMs. This tool provides information on protein-protein associations based in co-expression studies and also based in physical interaction studies.

Western blot analysis

Immunoblot was performed per a general western-blot protocol (Abcam). Total protein was extracted using Cell Lysis Buffer (Cell Signaling Technology) supplemented with cOmplete mini protease inhibitor cocktail (Roche), 50 mM sodium fluoride and 1 mM of PMSF. Protein was quantified using Bradford Method (Bio-Rad). 20 µg of protein was separated by SDS-PAGE and transferred to 0.2 µm pore-size PVDF membranes (Sigma-Aldrich). Blots were probed using the following antibodies, at a concentration 1:1000 unless otherwise stated: cathepsin B (12216-1-AP, Proteintech), cathepsin D (21327-1-AP, Proteintech), α-tubulin (ab15246, Abcam; concentration 1:5000). Horseradish peroxidase-conjugated secondary antibodies (Amersham) were used (1:5000) and detected using an enhanced chemiluminescent substrate (Amersham). Signal was detected using an iBright 1500 System (Invitrogen).

Flow cytometry analysis

Single-cell suspensions from cultured cell, spleen or blood monocytes were generated from mice, washed twice in staining buffer and incubated with 1:100 Fc-block (BD Biosciences, #553142) diluted in FACS buffer. Cells were subjected to surface antibody staining with labelled antibodies diluted in staining buffer for 30 min at 4 °C: CD3-PE-Cy7 (#100328, Biolegend), CD4-BV605 (#100548, Biolegend), CD8-APC (#100712, Biolegend), CD19-PerCP-Cy5.5 (#115534, Biolegend), CD45-BV785 (#103149, Biolegend), Ly6C-PerCP-Cy5.5 (#128012, Biolegend), Ly6C-E450 (#48-5932-82, eBioscience), Ly6G- AF700 (#56-5931-82, eBioscience), CD11b-BV650 (#101239, Biolegend), F4/80-PE-Cy7 (#123114, Biolegend), F4/80-PE (#123110, Biolegend), CCR2 (CD192)-PE-Vio @ 770 (#130-108-724, Miltenyi Biotec). After incubation, cells were washed in staining buffer and analysed immediately. For all staining, isotype controls were used.

The gating strategy for analysing distinct cellular populations began by isolating cells positive for the CD45 marker. Subsequently, B cells, identified by their positivity for CD19 and negativity for CD11b, were selected. To determine the frequency of T cells, the CD45⁺ cell population, devoid of both CD19 and CD11b, underwent examination for CD3 expression. CD3⁺ cells were further categorized based on CD4 and CD8 expression, distinguishing CD4⁺, CD8⁺, and double-negative (DN) T cells. Following the application of these gating criteria across all samples, the percentages of CD45-positive cells expressing CD19 and T cells (CD3⁺) within the CD45⁺ population were calculated. Additionally, myeloid populations were analysed by quantifying the proportion of CD11b⁺ cells. To distinguish between circulating monocytes and granulocytes, we assessed their expression of Ly6G (granulocytes) and Ly6C proteins (monocytes). Classical monocytes were characterized by negativity for Ly6G and positivity for Ly6C, while alternative monocytes lacked both markers, and neutrophils/granulocytes displayed weak positivity for Ly6C and positivity for Ly6G.

Samples were acquired on a BD LSR FORTESA FACS or FACS Aria III machine that uses FACS DIVA software (BD Biosciences). Compensation was performed using 1 drop of Ultracomp ebeads (eBioscience) in 300 µl of FACS buffer. 1 µl of each antibody used in the pool was mixed with 100 µl of compensation beads solution and acquired. A total of 50000 cells per mouse were analysed. Analysis was performed with FlowJo software (FlowJo V10.4). Once the different pools were compensated samples were acquired.

Phagocytosis assay

For phagocytosis assay, BMDMs in suspension in DMEM without FBS were labelled with 1:200 red cell tracker (Molecular Probes) for 30 minutes at 37°C. Cells were then centrifuged for 5 minutes at 300g at 4°C, supernatant was removed and labelled BMDMs were washed twice with 5ml of PBS and plated overnight in complete DMEM containing 20 ng/ml M-CSF. On the following day, 100 ng/ml LPS (Sigma Aldrich) was added to BMDMs overnight.

LKR10 cells (murine lung cancer cell line) were stained with red cell tracker (Molecular Probes) as previously described for macrophages. Stained cells were plated in complete DMEM medium and, after 12 hours, 50 µM Cisplatin (MCE MedChemExpress) was added to the media and left overnight. BMDMs were then incubated with apoptotic cancer cells at a 1:2 ratio and cultured at 37 °C for different time points in DMEM supplemented with 10% FBS.

Flow cytometry data were acquired using a BD LSR FORTRESSA FACS instrument with FACS DIVA software (BD Biosciences) and analysed using FlowJo V10.4 software. A minimum of 2×10^5 events were acquired and analysed. Data analysis and interpretation was done using FlowJo software (FlowJo V10.4).

Image analysis

Confocal images were post-processed and analysed using Fiji distribution of ImageJ version 1.53q. Cell shape descriptors such as “aspect ratio” (AR), “circularity” (C) and “cell area” were measured using Fiji. Specifically, aspect ratio is calculated as $(\text{major axis} \times \text{minor axis} - 1)$ therefore representing solely the degree of elongation, whereas circularity is calculated as $[4\pi \times (\text{area} \times \text{perimeter} - 2)]$, thus representing the degree of similarity to a circumference with a value ranging from 0 to 1 (perfect circle).

Histology

Tissue was fixed using 4% formaldehyde for 48h, dehydrated and paraffin-embedded. Sections (3 µm) were cut and stained using hematoxylin-eosin. For immunodetection, citrate pH 6 buffer was used for antigen retrieval. Staining was used using the following primary antibodies: CD68 (ab125212, Abcam, 1:200), cathepsin B (12216-1-AP, Proteintech, 1:100), cathepsin D (21327-1-AP, Proteintech, 1:400). Dako EnVision+ System HRP labelled Polymer secondary antibodies (Dako) were used, and DAB+ Substrate Chromogen System (Dako) was used for color development.

For the quantification of loose chromatin remnants in paw edema images, the pathologist assigned a score ranging from 1 to 3 based on the chromatin content within the inflammatory abscess. A score of 1 indicates low chromatin content, occupying less than 30% of the abscess area, while a score of 3 represents high chromatin content, covering over 60% of the abscess area.

Statistical Analysis

All experiments were conducted with at least three independent biological replicates, each containing technical triplicates. For statistical analysis, we used the Mann-Whitney test to compare control and RBD groups as a non-parametric approach suitable for two independent groups. Additionally, two-way ANOVA was used to evaluate interaction effects between factors in our experimental design, enabling comparisons across multiple groups and conditions

Supplementary Files

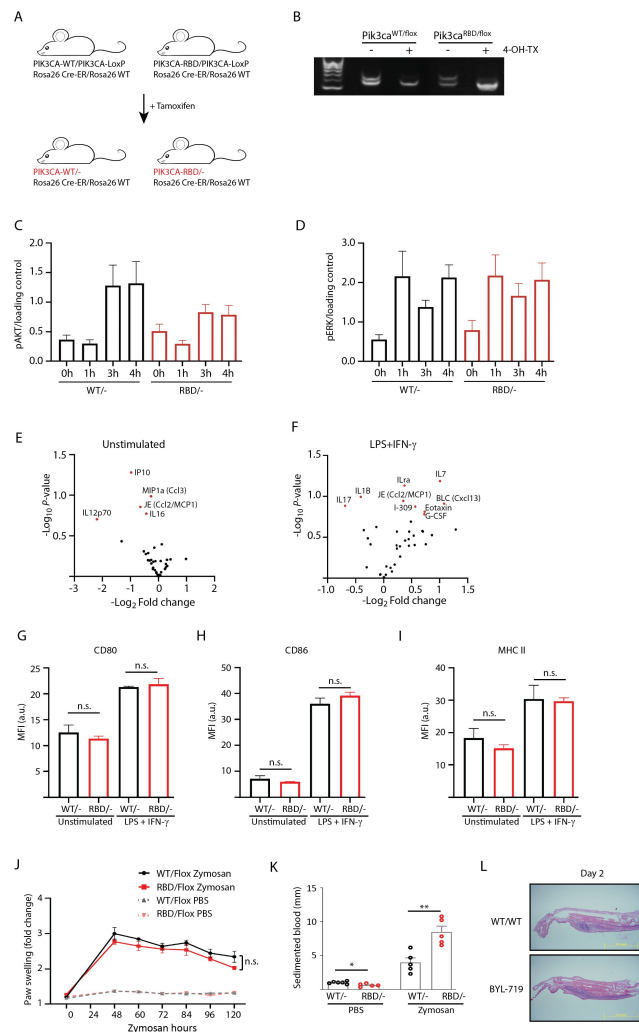


Figure 1-Supplement 1.

***Pik3ca*^{RBD/-} mice have more extended inflamed tissue after zymosan injection**

(A) Schematic representation of the mouse model used in which the interaction of p110α with RAS was disrupted by the introduction of two-point mutations, T208D and K227A, into the endogenous *Pik3ca* gene (*Pik3ca*RBD). Wild-type (*Pik3ca*WT) and *Pik3ca*RBD mice were bred with mice containing a floxed *Pik3ca* allele and a mouse carrying a conditional Cre recombinase (Cre-ERT2) allele targeted to the ubiquitously expressed *Rosa26* locus; (B) Representative PCR showing efficiency of floxed allele removal after treatment with 4-hydroxytamoxifen for 48 hours; (C) Graph quantifying p-AKT signal from 3 independent WB and normalized against its loading control; (D) Graph quantifying p-ERK signal from 3 independent WB and normalized against its loading control; (E) Volcano plots corresponding to identified cytokines differentially expressed in unstimulated BMDMs; (F) Volcano plots corresponding to identified cytokines differentially expressed in LPS + IFN-γ BMDMs; (G) (H) (I) *Pik3ca*RBD/- and *Pik3ca*RBD/- BMDMs were stimulated with LPS+IFN-γ and expression of (G) CD80; (H) CD86; and (I) MHCII were analysed by flow cytometry (*Pik3ca*RBD/- n = 5; *Pik3ca*WT/- n = 3). Error bars indicate mean ± SEM. Significance using Mann-Whitney test: n.s. no-significant.; (J) *Pik3ca*WT/Flox and *Pik3ca*RBD/Flox mice were injected with zymosan or PBS in the back-hind paws and inflammation was measured and plotted over time, *Pik3ca*WT/Flox n = 5; *Pik3ca*RBD/Flox PBS n = 5; *Pik3ca*WT/Flox Zymosan n = 5; *Pik3ca*RBD/Flox Zymosan n = 5; Error bars indicate mean ± SEM. Significance using 2way ANOVA test: n.s. non-significant (K) Blood from the same mice was obtained by cardiac puncture prior culling and blood sedimentation was measured. The clear layer formed at the top was used for data quantification. Black dots represent data from *Pik3ca*WT/- mice and red dots represent data from *Pik3ca*RBD/- mice. Each dot represents an individual mouse. Error bars indicate mean ± SEM. Significance using Student's t test: * p < 0.05; **p < 0.01. *Pik3ca*WT/- PBS n = 6; *Pik3ca*RBD/- PBS n = 5; *Pik3ca*WT/- Zymosan n = 4; *Pik3ca*RBD/- Zymosan n = 4; (L) Representative images showing zymosan-induced inflammatory area in *Pik3ca*WT/WT mice and *Pik3ca*WT/WT mice treated with BYL-719.

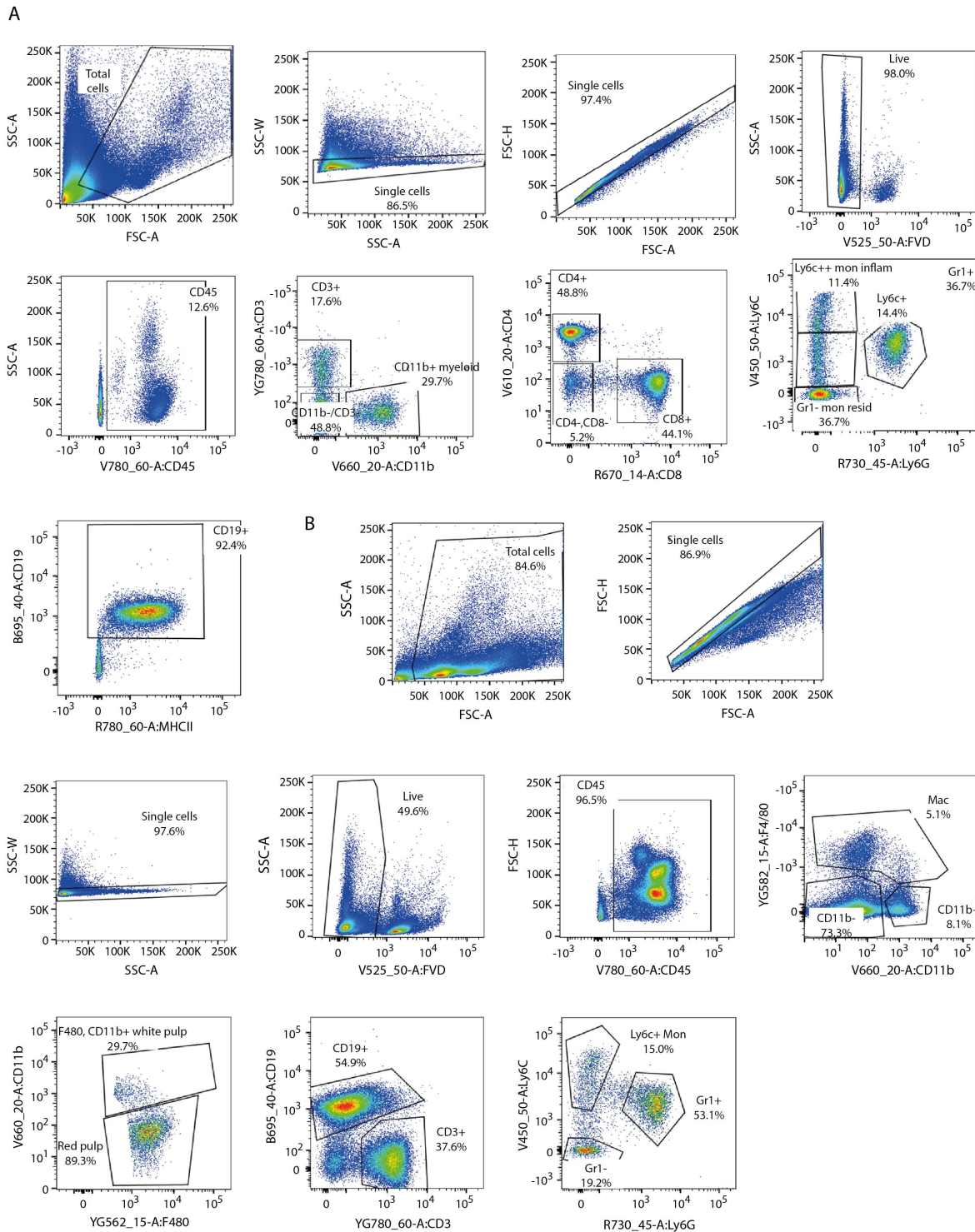


Figure 2-Supplement 2.

Gating Strategy for Immune Population Analysis in Blood and Spleen.

(A) Graphs showing gating strategy used to analyze immune populations in peripheral blood from *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice. **(B)** Graphs showing gating strategy used to analyze immune populations in spleens from *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice.

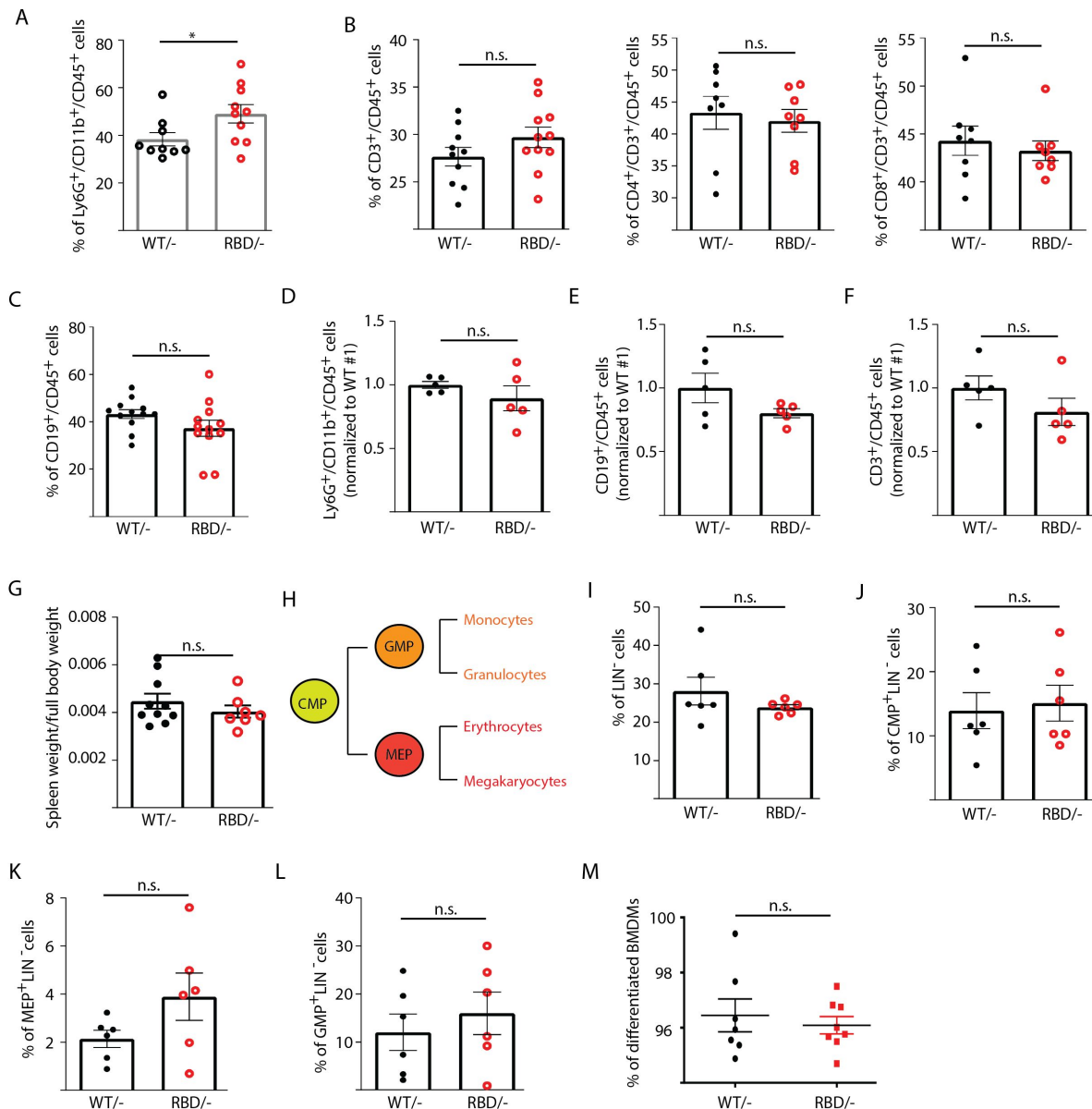


Figure 2-Supplement 3.

Disruption of RAS-p110 α interaction does not affect the number of B-cells or T-cells in blood or spleen

Twelve-week-old mice were treated with tamoxifen and after four weeks, flow cytometry analysis was performed to determine abundance of different immune populations. (A) Granulocytes in circulating blood; (B) CD3, CD4⁺ and CD8⁺ T-cells in circulating blood; (C) B-cells in circulating blood; (D) Granulocytes in spleen; (E) B-cells in spleen; (F) T-cells in spleen; (G) Spleen weight compared to full body; (H) Schematic representation of myeloid precursors differentiation (CMP-common myeloid progenitor; GMP-Granulocyte-monocyte progenitor; MEP-Megakaryocyte-erythroid progenitor); (I) Graph showing percentage of bone marrow cells progenitor cells (have not acquire any lineage marker, Lin⁻); (J) Graph showing percentage of CMP cells within progenitor cells; (K) Graph showing percentage of MEP cells within progenitor cells; (L) Graph showing percentage of GMP cells within progenitor cells; (M) Graph depicting % of differentiated macrophages obtained in culture from bone marrow precursors in the presence of m-CSF. Black dots represent data from *Pik3ca*WT/- mice and red dots represent data from *Pik3ca*RBD/- mice. Each dot represents an individual mouse. Error bars indicate mean $\pm$ SEM. Significance using Student's t test: n.s. non-significant.

Figure 3-Supplement 4.

***Pik3ca*^{RBD/-} monocytes do not extravasate through the endothelium.**

(A) Graph showing levels of neutrophil reconstitution in chimera's bone marrow. Blood from chimera mice was collected and GFP in granulocytes was analysed by flow cytometry to determine engraftment percentage. (B) Graph quantifying the number of monocytes in the blood of chimera mice treated with anti-GR1 (25µg/mouse/day) for 3 days. (C) Graph showing levels of expression of CCR2 in *Pik3ca*WT/- and *Pik3ca*RBD/- BMDMs detected by flow cytometry. Error bars indicate mean ± SEM. Significance using Mann-Whitney test: n.s. no-significant

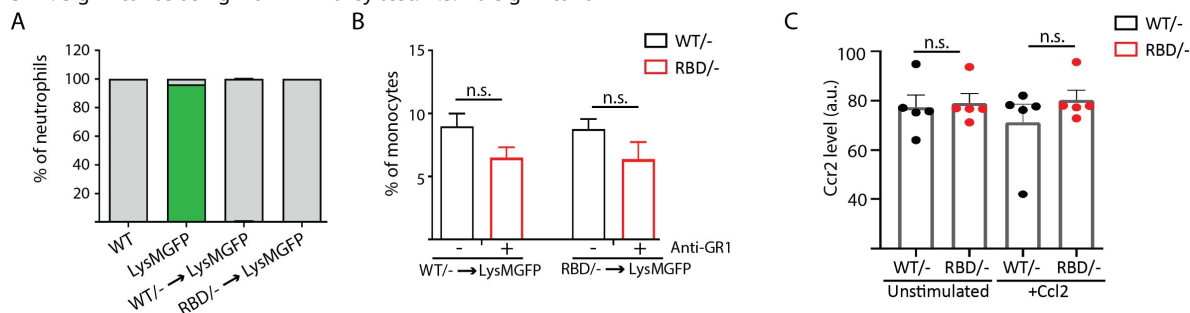


Figure 4-Supplement 5.

Disruption of RAS-p110α interaction has a differential role in phagocytosis.

(A) Graph representing the ability of *Pik3ca*RBD/- and *Pik3ca*RBD/- BMDMs to phagocyte sepharose beads. Data from 3 independent experiments are presented; (B) Graph representing the ability of *Pik3ca*RBD/- and *Pik3ca*RBD/- BMDMs to phagocyte *Borrelia burgdorferi*. Data from 6 independent mice are presented. Error bars indicate mean ± SEM. Significance using Mann-Whitney test: * p < 0.05; **p < 0.01; n.s. no-significant

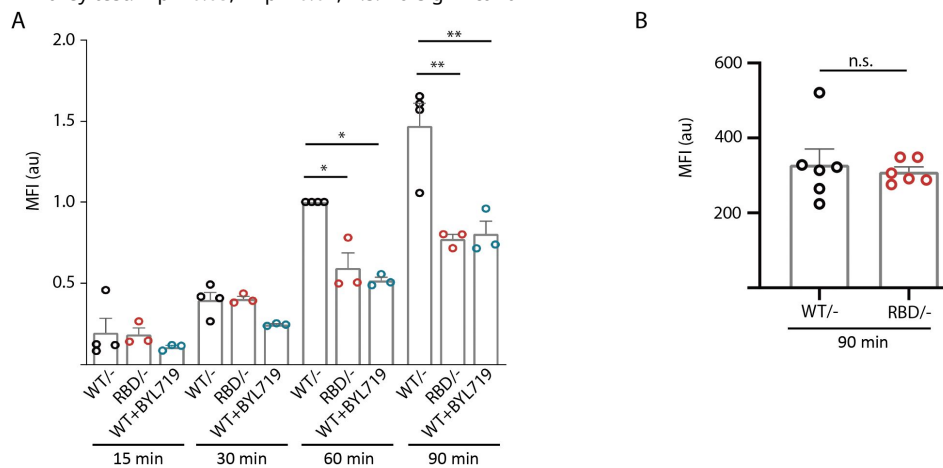


Figure 5-Supplement 6.

Disruption of RAS-p110α interaction alters the secretome of BMDMs.

(A) WB showing induction of apoptosis in LKR10 cell line treated with cisplatin for 16 hours; (B) Dendrogram showing hierarchical clustering of peptides found in the secretome analysis of *Pik3ca*^{RBD/-} and *Pik3ca*^{RBD/-} BMDMs in steady state conditions; (C) Dendrogram showing hierarchical clustering of peptides found in the secretome analysis of *Pik3ca*^{RBD/-} and *Pik3ca*^{RBD/-} BMDMs phagocytosing apoptotic cells.

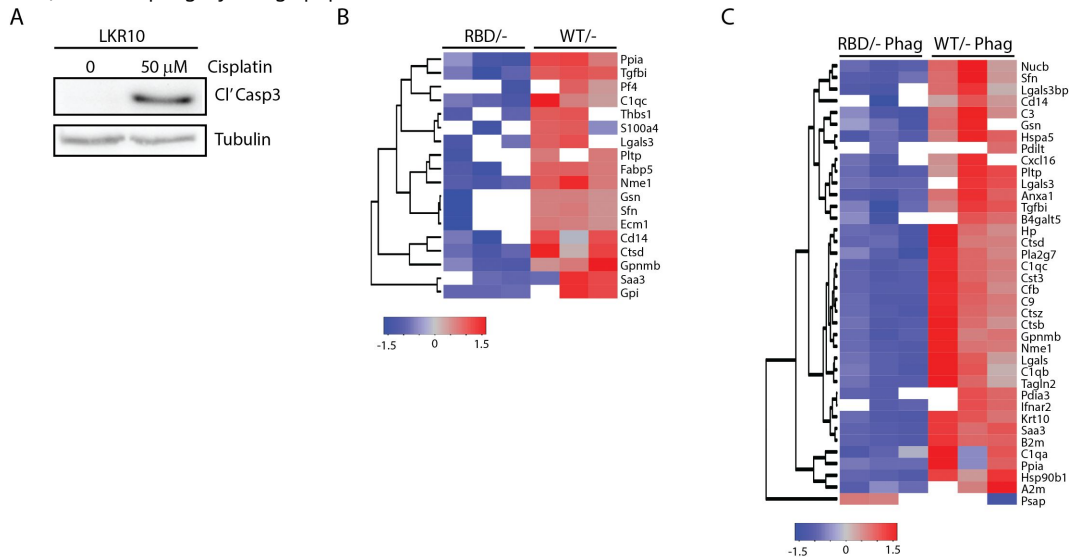
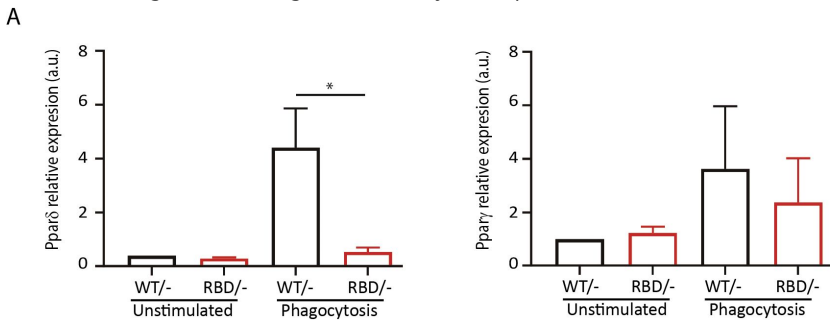


Figure 6-Supplement 7.

Disruption of RAS-p110α interaction impacts expression of inflammation resolution mediators.

Pik3ca^{RBD/-} and *Pik3ca*^{RBD/-} BMDMs were set up to phagocyte apoptotic cells and expression of (A) *Pparδ* and (B) *Pparγ* was measured by qPCR.

Error bars indicate mean ± SEM. Significance using Mann-Whitney test: * $p < 0.05$



Acknowledgements

This work was supported by grants from the Spanish Ministry of Science and Innovation (RTI2018-099161-A-I00), Programa JAE-Intro ICU from CSIC (JAEICU-21-IBMCC-6), JCyL (CSI185-20), Marie Curie Initial Training Network on Tumour Infiltrating Myeloid Cell Compartment (PF7 MCA-ITN317445) and CRUK-Barts Cancer Centre Development Fund. This research was co-financed by FEDER funds. The CIC is supported by the Programa de Apoyo a Planes Estratégicos de Investigación de Estructuras de Investigación de Excelencia of Castilla y León autonomous government (CLC-2017-01) and AECC Excellence program Stop Ras Cancers (EPAEC222641CICS). The authors wish to thank the Pathology Unit, the Mouse Model Experimentation Unit, and the Advanced Cellular Analysis Unit at CIC for their assistance in carrying out this work.

Additional information

Materials availability Statement

All materials used in this study are available upon request. Researchers may contact Dr. E. Castellano (ecastellano@usal.es) for access. Most materials have no restrictions; however, access to the mouse model may require a Material Transfer Agreement (MTA) to protect intellectual property or for regulatory compliance. Dr. Castellano can provide details on terms and facilitate the transfer process.

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Reviewer #2 (Public review):

Summary:

Cell intrinsic signaling pathways controlling the function of macrophages in inflammatory processes, including in response to infection, injury or in the resolution of inflammation are incompletely understood. In this study, Rosell et al. investigate the contribution of RAS-p110 α signaling to macrophage activity. p110 α is a ubiquitously expressed catalytic subunit of PI3K with previously described roles in multiple biological processes including in epithelial cell

growth and survival, and carcinogenesis. While previous studies have already suggested a role for RAS-p110 α signaling in macrophage function, the cell intrinsic impact of disrupting the interaction between RAS and p110 α in this central myeloid cell subset is not known.

Strengths:

Exploiting a sound previously described genetically engineered mouse model that allows tamoxifen-inducible disruption of the RAS-p110 α pathway and using different readouts of macrophage activity in vitro and in vivo, the authors provide data consistent with their conclusion that alteration in RAS-p110 α signaling impairs various but selective aspects of macrophage function in a cell-intrinsic manner.

Weaknesses:

My main concern is that for various readouts, the difference between wild-type and mutant macrophages in vitro or between wild-type and Pik3caRBD mice in vivo is modest, even if statistically significant. To further substantiate the extent of macrophage function alteration upon disruption of RAS-p110 α signaling and its impact on the initiation and resolution of inflammatory responses, the manuscript would benefit from a more extensive assessment of macrophage activity and inflammatory responses in vivo.

In the in vivo model, all cells have disrupted RAS-p100 α signaling, not only macrophages. Given that other myeloid cells besides macrophages contribute to the orchestration of inflammatory responses, it remains unclear whether the phenotype described in vivo results from impaired RAS-p100 α signaling within macrophages or from defects in other haematopoietic cells such as neutrophils, dendritic cells, etc.

Inclusion of information on the absolute number of macrophages, and total immune cells (e.g. for the spleen analysis) would help determine if the reduced frequency of macrophages represents an actual difference in their total number or rather reflects a relative decrease due to an increase in the number of other/s immune cell/s.

Comments on revisions:

I thank the authors for addressing my comments.

- I believe that additional in vivo experiments, or the inclusion of controls for the specificity of the inhibitor, which the authors argue are beyond the scope of the current study, are essential to address the weaknesses and limitations stated in my current evaluation.
- While the neutrophil depletion suggests neutrophils are not required for the phenotype, there are multiple other myeloid cells, in addition to macrophages, that could be contributing or accounting for the in vivo phenotype observed in the mutant strain (not macrophage specific).
- Inclusion of absolute cell numbers (in addition to the %) is essential. I do not understand why the authors are not including these data. Have they not counted the cells?
- Lastly, inclusion of representative staining and gating strategies for all immune profiling measurements carried out by flow cytometry is important. This point has not been addressed, not even in writing.

<https://doi.org/10.7554/eLife.94590.3.sa1>

Author response:

The following is the authors' response to the current reviews.

| *Comments on revisions:*

I thank the authors for addressing my comments.

- I believe that additional in vivo experiments, or the inclusion of controls for the specificity of the inhibitor, which the authors argue are beyond the scope of the current study, are essential to address the weaknesses and limitations stated in my current evaluation.

We respectfully acknowledge the reviewer's concern but would like to reiterate that demonstrating the specificity of the inhibitor is beyond the scope of this study. Alpelisib (BYL-719) is a clinically approved drug widely recognized as a specific inhibitor of p110 α , primarily used in the treatment of breast cancer. Its selectivity for the p110 α isoform has been extensively validated in the literature.

In our study, we used Alpelisib to assess whether pharmacological inhibition of p110 α would produce effects similar to those observed in our genetic model, which is particularly relevant for the potential translational implications of our findings. Given the well-documented specificity of this inhibitor, we believe that additional controls to confirm its selectivity are unnecessary within the context of this study. Instead, our focus has been to investigate the functional role of p110 α activity in macrophage-driven inflammation using the models described.

We appreciate the reviewer's insight and hope this clarification addresses their concern.

- While the neutrophil depletion suggests neutrophils are not required for the phenotype, there are multiple other myeloid cells, in addition to macrophages, that could be contributing or accounting for the in vivo phenotype observed in the mutant strain (not macrophage specific).

We appreciate the reviewer's observation regarding the potential involvement of other myeloid cells. However, it is important to highlight that the inflammatory process follows a well-characterized sequential pattern. Our data clearly demonstrate that in the paw inflammation model:

- Neutrophils are effectively recruited, as evidenced by the inflammatory abscess filled with polymorphonuclear cells.
- However, macrophages fail to be recruited in the RBD model.

Given that this critical step is disrupted, it is reasonable to expect that any subsequent steps in the inflammatory cascade would also be affected. A precise dissection of the role of other myeloid populations would require additional lineage-specific models to selectively target each subset, which, as we have previously stated, would be the focus of an independent study.

While we cannot entirely exclude the contribution of other myeloid cells, our data strongly support the conclusion that macrophages are, at the very least, a key component of the observed phenotype. We explicitly address this point in the Discussion section, where we acknowledge the potential involvement of other myeloid populations.

- Inclusion of absolute cell numbers (in addition to the %) is essential. I do not understand why the authors are not including these data. Have they not counted the cells?

We appreciate the reviewer's concern regarding the inclusion of absolute cell numbers. However, as stated in the Materials and Methods section, we analyzed 50,000 cells per

sample, and the percentages reported in the manuscript are directly derived from this standardized count.

Our decision to present the data as percentages follows standard practices in flow cytometry-based analyses, as it allows for a clearer and more biologically relevant comparison of relative changes between conditions. This approach ensures consistency across samples and facilitates the interpretation of population dynamics during inflammation.

We would also like to clarify that all data are based on actual counts, and rigorous controls were implemented throughout the study to ensure accuracy and reproducibility. We hope this explanation addresses the reviewer's concern and provides further clarity on our approach.

- Lastly, inclusion of representative staining and gating strategies for all immune profiling measurements carried out by flow cytometry is important. This point has not been addressed, not even in writing.

We appreciate the reviewer's concern regarding the inclusion of absolute cell numbers. However, as stated in the Materials and Methods section, we analyzed 50,000 cells per sample, and the percentages reported in the manuscript are directly derived from this standardized count.

Our decision to present the data as percentages follows standard practices in flow cytometry-based analyses, as it allows for a clearer and more biologically relevant comparison of relative changes between conditions. This approach ensures consistency across samples and facilitates the interpretation of population dynamics during inflammation.

We would also like to clarify that all data are based on actual counts, and rigorous controls were implemented throughout the study to ensure accuracy and reproducibility. We hope this explanation addresses the reviewer's concern and provides further clarity on our approach.

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

This study by Alejandro Rosell et al. reveals the immunoregulatory role of the RAS-p110 α pathway in macrophages, specifically in regulating monocyte extravasation and lysosomal digestion during inflammation. Disrupting this pathway, through genetic tools or pharmacological intervention in mice, impairs the inflammatory response, leading to delayed resolution and more severe acute inflammation. The authors suggest that activating p110 α with small molecules could be a potential therapeutic strategy for treating chronic inflammation. These findings provide important insights into the mechanisms by which p110 α regulates macrophage function and the overall inflammatory response.

The updates made by the authors in the revised version have addressed the main points raised in the initial review, further improving the strength of their findings.

Reviewer #2 (Public review):

Summary:

Cell intrinsic signaling pathways controlling the function of macrophages in inflammatory processes, including in response to infection, injury or in the resolution of

inflammation are incompletely understood. In this study, Rosell et al. investigate the contribution of RAS-p110α signaling to macrophage activity. p110α is a ubiquitously expressed catalytic subunit of PI3K with previously described roles in multiple biological processes including in epithelial cell growth and survival, and carcinogenesis. While previous studies have already suggested a role for RAS-p110α signaling in macrophage function, the cell intrinsic impact of disrupting the interaction between RAS and p110α in this central myeloid cell subset is not known.

Strengths:

Exploiting a sound previously described genetically engineered mouse model that allows tamoxifen-inducible disruption of the RAS-p110α pathway and using different readouts of macrophage activity in vitro and in vivo, the authors provide data consistent with their conclusion that alteration in RAS-p110α signaling impairs various but selective aspects of macrophage function in a cell-intrinsic manner.

Weaknesses:

My main concern is that for various readouts, the difference between wild-type and mutant macrophages in vitro or between wild-type and Pik3ca^{RBD} mice in vivo is modest, even if statistically significant. To further substantiate the extent of macrophage function alteration upon disruption of RAS-p110α signaling and its impact on the initiation and resolution of inflammatory responses, the manuscript would benefit from a more extensive assessment of macrophage activity and inflammatory responses in vivo.

Thank you for raising this point. We understand the reviewer's concern regarding the modest yet statistically significant differences observed between wild-type and mutant macrophages in vitro, as well as between wild-type and Pik3ca^{RBD} mice in vivo. Our current study aimed to provide a foundational exploration of the role of RAS-p110α signaling in macrophage function and inflammatory response, focusing on a set of core readouts that demonstrate the physiological relevance of this pathway. While a more extensive in vivo assessment could offer additional insights into macrophage activity and the nuanced effects of RAS-p110α disruption, it would require an array of new experiments that are beyond the current scope.

However, we believe that the current data provide significant insights into the pathway's role, highlighting important alterations in macrophage function and inflammatory processes due to RAS-p110α disruption. These findings lay the groundwork for future studies that can build upon our results with a more comprehensive analysis of macrophage activity in various inflammatory contexts.

In the in vivo model, all cells have disrupted RAS-p100α signaling, not only macrophages. Given that other myeloid cells besides macrophages contribute to the orchestration of inflammatory responses, it remains unclear whether the phenotype described in vivo results from impaired RAS-p100α signaling within macrophages or from defects in other haematopoietic cells such as neutrophils, dendritic cells, etc.

Thank you for raising this point. To address this, we have added a paragraph in the Discussion section acknowledging that RAS-p110α signaling disruption affects all hematopoietic cells (lines 461-470 in the discussion). However, we also provide several lines of evidence that support macrophages as the primary cell type involved in the observed phenotype. Specifically, we note that neutrophil depletion in chimera mice did not alter transendothelial extravasation, and that macrophages were the primary cell type showing significant functional defects in the paw edema model. These findings, combined with specific deficiencies in myeloid populations, suggest a predominant role of macrophages in

the impaired inflammatory response, though we acknowledge the potential contributions of other myeloid cells.

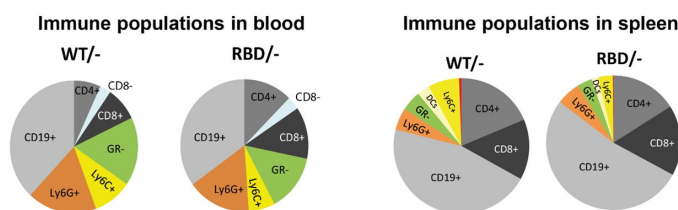
Inclusion of information on the absolute number of macrophages, and total immune cells (e.g. for the spleen analysis) would help determine if the reduced frequency of macrophages represents an actual difference in their total number or rather reflects a relative decrease due to an increase in the number of other/s immune cell/s.

Thank you for this suggestion. We understand the value of presenting actual measurements; however, we opted to display normalized data to provide a clearer comparison between WT and RBD mice, as this approach highlights the relative differences in immune populations between the two groups. Normalizing data helps to focus on the specific impact of the RAS-p110α disruption by minimizing inter-sample variability that can obscure these differences.

To further address the reviewer's concern regarding the interpretation of macrophage frequencies, we have included a pie chart that represents the relative proportions of the various immune cell populations studied within our dataset. Author response image 1 provides a visual overview of the immune cell distribution, enabling a clearer understanding of whether the observed decrease in macrophage frequency represents an actual reduction in total macrophage numbers or a shift in their relative abundance due to changes in other immune populations.

We hope this approach satisfactorily addresses reviewer's concerns by providing both a normalized dataset for clearer interpretation of genotype-specific effects and an overall immune profile that contextualizes macrophage frequency within the broader immune cell landscape.

Author response image 1.



Recommendations for the authors:

Reviewer #2 (Recommendations for the authors):

As proof of concept data that activation of RAS-p110α signaling constitutes indeed a putative approach for treating chronic inflammation is not included in the manuscript, I suggest removing this implication from the abstract.

Thank you for this suggestion. We have now removed this implication from the abstract to maintain clarity and to better reflect the scope of the data presented in the manuscript.

Inclusion of a control in which RBD/- cells are also treated with BYL719, across experiments in which the inhibitor is used, would be important to determine, among other things, the specificity of the inhibitor.

We appreciate the reviewer's suggestion to include RBD/- cells treated with BYL719 as an additional control. However, we would like to clarify that this approach would raise a different biological question, as treating RBD mice with BYL719 would not only address the

specificity of the inhibitor but also examine the combined effects of genetic and pharmacologic disruptions on PI3K pathway signaling. Investigating this dual disruption falls outside the scope of our current study, which is focused specifically on the effects of RAS-p110 α disruption.

It is also important to note that our RBD mouse model selectively disrupts RAS-mediated activation of p110 α , while PI3K activation can still occur through other pathways, such as receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs). Thus, inhibiting p110 α with BYL719 would produce broader effects beyond the inhibition of RAS-PI3K signaling, impacting PI3K activation regardless of its upstream source.

In addition, incorporating this control would require us to repeat nearly all experiments in the manuscript, as it would necessitate generating and analyzing new samples for each experimental condition. Given the scope and resources involved, we believe this approach is unfeasible at this stage of the revision process.

We hope this explanation is satisfactory and that the current data in the manuscript provide a rigorous assessment of the RAS-p110 α signaling pathway within the defined experimental scope.

| *Figure 3I is missing the statistical analysis (this is mentioned in the legend though).*

Thank you for pointing this out. We apologize for the oversight. The statistical analysis for Figure 3I has now been added.

| *Gating strategies and representative staining should be included more generally across the manuscript.*

Thank you for this suggestion. To address this, we have added a new supplementary figure (Figure 2-Supplement Figure 2) that illustrates the gating strategy along with a representative dataset. Additionally, a brief summary of the gating strategy has been included in the main text to further clarify the methodology.

| *It is recommended that authors show actual measurements rather than only data normalized to the control (or arbitrary units).*

Thank you for this suggestion. We understand the value of presenting actual measurements; however, we opted to display normalized data to provide a clearer comparison between WT and RBD mice, as this approach highlights the relative differences in immune populations between the two groups. Normalizing data helps to focus on the specific impact of the RAS-p110 α disruption by minimizing inter-sample variability that can obscure these differences.

To further address the reviewer's concern regarding the interpretation of macrophage frequencies, we have included a pie chart that represents the relative proportions of the various immune cell populations studied within our dataset. Author response image 1 provides a visual overview of the immune cell distribution, enabling a clearer understanding of whether the observed decrease in macrophage frequency represents an actual reduction in total macrophage numbers or a shift in their relative abundance due to changes in other immune populations.

We hope this approach satisfactorily addresses reviewer's concerns by providing both a normalized dataset for clearer interpretation of genotype-specific effects and an overall immune profile that contextualizes macrophage frequency within the broader immune cell landscape.

<https://doi.org/10.7554/eLife.94590.3.sa0>