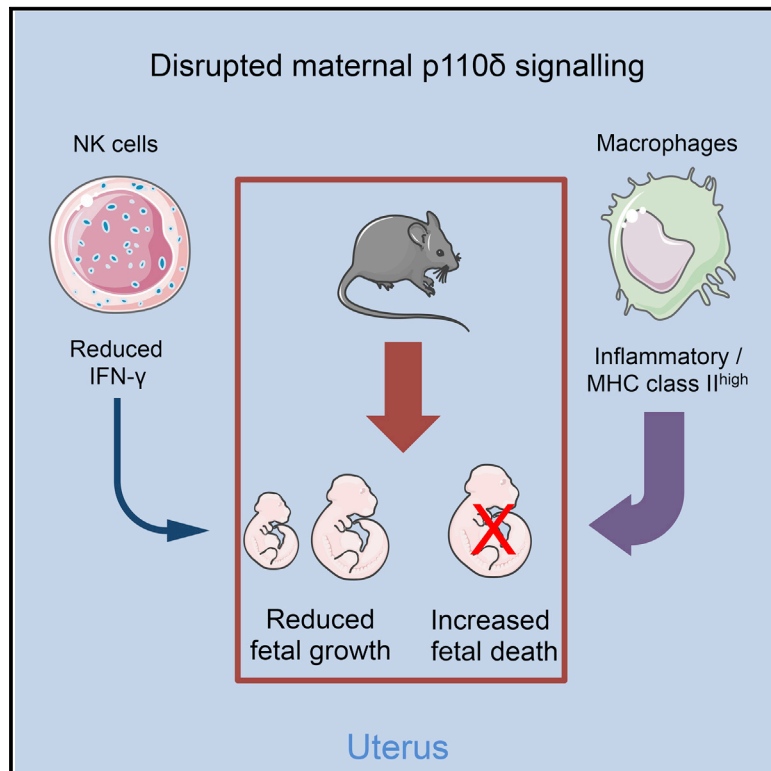


Disrupted PI3K p110 δ Signaling Dysregulates Maternal Immune Cells and Increases Fetal Mortality In Mice

Graphical Abstract



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In Brief

Kieckbusch et al. show that a key leukocyte signaling molecule, p110 δ , regulates uterine immune cells. Genetic p110 δ inactivation in mice reduced NK but increased macrophage function, thus impeding uterine arterial remodeling and enhancing local inflammation during pregnancy. Imbalanced adaptation of maternal immune cells curtailed fetal development and increased perinatal morbidity.

Highlights

- Genetic inactivation of p110 δ in pregnant mice perturbs maternal immune cells
- Uterine NK cells produce less cytokines, resulting in fetal growth restriction
- Inflammatory macrophages are overrepresented, resulting in increased fetal loss



Disrupted PI3K p110 δ Signaling Dysregulates Maternal Immune Cells and Increases Fetal Mortality In Mice

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SUMMARY

Maternal immune cells are an integral part of reproduction, but how they might cause pregnancy complications remains elusive. Macrophages and their dual function in inflammation and tissue repair are thought to play key yet undefined roles. Altered perinatal growth underpins adult morbidity, and natural killer (NK) cells may sustain fetal growth by establishing the placental blood supply. Using a mouse model of genetic inactivation of PI3K p110 δ , a key intracellular signaling molecule in leukocytes, we show that p110 δ regulates macrophage dynamics and NK-cell-mediated arterial remodeling. The uterus of dams with inactive p110 δ had decreased IFN- γ and MHC class II^{low} macrophages but enhanced IL-6. Poor vascular remodeling and a pro-inflammatory uterine milieu resulted in fetal death or growth retardation. Our results provide one mechanism that explains how imbalanced adaptations of maternal innate immune cells to gestation affect offspring well-being with consequence perinatally and possibly into adulthood.

INTRODUCTION

Class I phosphoinositide 3-kinases (PI3K) convert phosphatidylinositol-(4,5)-phosphate to phosphatidylinositol-(3,4,5)-phosphate (PIP₃). PIP₃ acts as a second messenger by recruiting proteins to the plasma membrane, where they activate signaling pathways that promote proliferation, survival, differentiation, and chemotaxis (Okkenhaug, 2013). PI3K can be subdivided into class I A and I B based on structural similarity. Class I A PI3K form heterodimers of p85 regulatory subunits and one of three isoforms of the catalytical p110 subunit (p110 α , p110 β , and p110 δ). While p110 α and p110 β are ubiquitously expressed, in mice, p110 δ expression is low or absent in most cell types but high in leukocytes (Vanhaesebroeck et al., 1997).

PI3K p110 δ is a key mediator of natural killer (NK) cell maturation and function, and absence of p110 δ signaling leads to reduced cytokine release, aberrant maturation, and incorrect trafficking to peripheral organs, including the uterus during pregnancy (Guo et al., 2008; Kim et al., 2007; Saudemont

et al., 2009; Tassi et al., 2007). In macrophages and other myeloid cells, the PI3K pathway has two opposing roles. While it promotes tissue infiltration and signal transduction for cytokine secretion in the early phases of inflammation, PI3K signaling also provides negative feedback inhibition and is thus involved in the resolution of inflammation and the prevention of “collateral damage” (Fukao and Koyasu, 2003; Günzl et al., 2010). As a result, inactivation of p110 δ leads to enhanced responses downstream of Toll-like receptors (Aksoy et al., 2012; Uno et al., 2010), increased production of pro-inflammatory cytokines such as interleukin-6 (IL-6) (Liu et al., 2009), upregulation of macrophage activation markers such as CD86 and major histocompatibility complex (MHC) class II in vitro, and chronic inflammation (Uno et al., 2010). Conversely, deletion of PTEN, which acts antagonistically to the PI3K pathway by degrading PIP₃, diminishes macrophage inflammatory responses (Sahin et al., 2014).

In mice, blastocyst implantation provokes a localized, progesterone-driven tissue reaction that causes stromal cells to proliferate extensively and form decidual cells (decidualization) (Dey et al., 2004). While the decidua grows, the vasculature feeding toward the developing fetoplacental unit changes from its conventional, arterial phenotype characterized by a thick smooth muscle wall to high-capacity, low-resistance vessels. Both processes are disrupted in mice lacking either NK cells (Ashkar et al., 2000; Barber and Pollard, 2003) or interferon- γ (IFN- γ) signaling (Ashkar and Croy, 1999), establishing a key role for IFN- γ produced by uterine NK (uNK) cells in reproduction. Uterine macrophages are a specialized myeloid subset whose spatial distribution and population dynamics are regulated by local colony stimulating factor (CSF)-1 signaling: macrophages and CSF-1 are highly abundant in the myometrium but relatively sparse in the decidua (Collins et al., 2009; Tagliani et al., 2011). CSF-1 signaling also blocks macrophage maturation at the transition from MHC class II^{low} to MHC class II^{high} cells (Tagliani et al., 2011). Little is known about the endogenous cues that activate or inhibit uterine macrophages, but excessive activation by exogenous stimuli is usually prevented (Erlebacher, 2013). Unrestrained macrophage activation can result in fetal mortality (Haddad et al., 1995; Robertson et al., 2006).

The selective expression of p110 δ in leukocytes has made it an attractive pharmacological target to treat hematological malignancies and reduce unwanted immune responses in the context

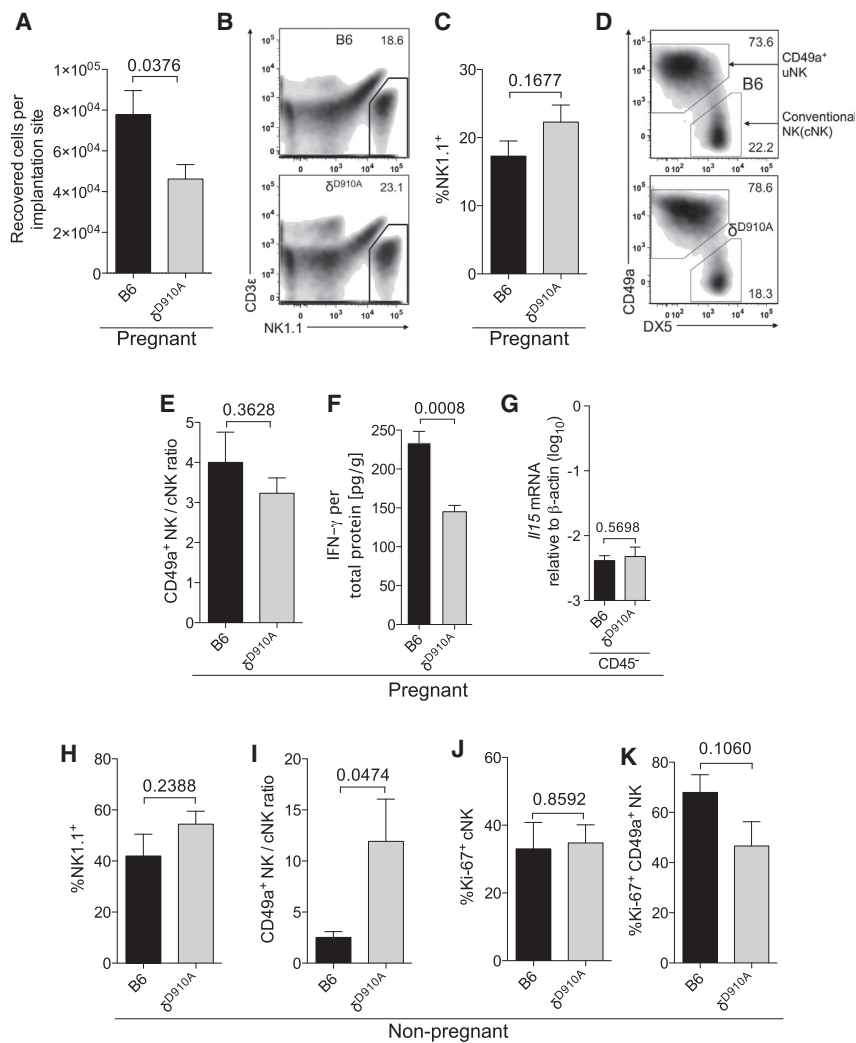


Figure 1. Maternal PI3K p110 δ Inactivation Affects IFN- γ Production In Utero

(A) Recovered live cells per implantation site after enzymatic digestion. (B) Representative density plot of CD3⁻ NK1.1⁺ cells as a fraction of leukocytes, gated on live CD45⁺ singlets. (C) Quantification of recovered live CD3⁻ NK1.1⁺ cells as % of leukocytes (gated as in B). (D) Representative density plot of CD49a and DX5 expression in utero, gated on live CD45⁺ CD3⁻ NK1.1⁺ cells. (E) Ratios of CD49a/DX5-positive cells. (F) Assessment of IFN- γ in uterine tissue; n = 5 mice per group. (G) Quantification of *Ilf15* expression in non-hematopoietic cells; n = 3 mice per group. (H–K) Assessment of uNK cells in non-pregnant mice: (H) frequency of NK1.1⁺ cells, (I) relative contribution of CD49a⁺ NK and cNK, and their ability to proliferate (J and K). n = 6 mice per group from two independent experiments. Indicated is the maternal genotype. All females were mated with B6 males. p values are from unpaired, two-tailed Student’s t tests. Data in (A), (C), and (E–K) are means \pm SEM; n = 6–7 mice from five independent experiments. See also Figures S1 and S2.

RESULTS

Maternal PI3K p110 δ Inactivation Affects IFN- γ Production In Utero

In mice, uNK cell-derived IFN- γ is key for the vascular changes the gravid uterus needs to undergo to ensure ample and constant blood supply to the fetoplacental unit (Ashkar and Croy, 2001; Ashkar et al., 2000; Kieckbusch et al., 2014). We and others have previously shown that PI3K

of autoimmunity and allergy (Rodon et al., 2013). Maternal immune adaptations during pregnancy are instrumental for the development of the growing fetus (Erlebacher, 2013; Moffett and Colucci, 2014). We used transgenic knockin mice carrying a kinase-dead point mutation in the gene coding for p110 δ (δ^{D910A}) as a genetic model for inactivation of the p110 δ pathway in order to investigate the effects of unbalanced maternal immune responses during gestation. We show that maternal p110 δ signaling is required for the normal production of IFN- γ in the gravid uterus and the associated NK-driven remodeling of the maternal vasculature feeding toward the fetoplacental unit. Fetuses carried by δ^{D910A} females were growth retarded and showed increased mortality in utero. Underpinning this fetal demise was a local inflammatory response characterized by increased abundance of MHC class II^{high} expressing macrophages and IL-6. After birth, the surviving offspring displayed rapid catch-up growth. These findings demonstrate that maternal p110 δ signaling is required for reproductive fitness and stress the importance of correct immune regulation during gestation.

p110 δ regulates NK cell development, function, and trafficking (Guo et al., 2008; Kim et al., 2007; Saudemont et al., 2009; Tassi et al., 2007). The role of this isoform in reproductive immunology, however, has not yet been well characterized.

NK cells from δ^{D910A} mice are known to have a reduced migratory potential to the pregnant uterus in a competition situation with wild-type NK cells (Saudemont et al., 2009). We therefore hypothesized that there may be NK cell abnormalities in δ^{D910A} mice that could underpin pregnancy complications and set out to investigate the effect of p110 δ inactivation in this context. δ^{D910A} females displayed reduced overall cellularity during pregnancy (Figure 1A), but the abundance of NK1.1⁺ CD3⁻ cells among CD45⁺ cells was comparable in δ^{D910A} and B6 controls (Figures 1B and 1C). Recently, NK cells found in peripheral tissues, including the uterus, have been distinguished into “tissue resident” and “conventional” NK (cNK) cells, which migrate to these organs (Sojka et al., 2014). Tissue resident NK cells display low or no expression of DX5 (CD49b) but are instead CD49a⁺. These resident cells would not be expected to be affected to the same degree by impaired NK trafficking in δ^{D910A} mice and

thus blur the picture. Accordingly, we delineated NK1.1⁺ CD3⁻ cells into CD49a⁺ cells (mainly comprising tissue-resident cells) and cNK subsets to resolve their relative frequencies in δ^{D910A} mice at midgestation (Figures 1D and 1E). The ratio of both cell population was similar in both strains, further demonstrating that the decreased migratory potential of NK cells from δ^{D910A} mice does not translate into overall changes in NK abundance in utero. uNK cells require IL-15 (Barber and Pollard, 2003) and produce >90% of IFN- γ in uterine tissues, and this cytokine is both necessary and sufficient to induce complete vascular remodeling (Ashkar and Croy, 1999, 2001; Ashkar et al., 2000). Reduced activation of uNK cell subsets leads to decreased uNK IFN- γ production (Kieckbusch et al., 2014), and the PI3K p110 δ /Akt pathway is known to be crucial for the normal release of IFN- γ by NK cells (Guo et al., 2008; Kim et al., 2007). In line with this reported evidence for peripheral NK cells, we found a marked reduction of IFN- γ in decidua and MLAp (myometrial lymphoid aggregate of pregnancy) by >35% (Figure 1F; 233.6 pg/g \pm 14.79 versus 145 pg/g \pm 8.24). We found no difference in *I115* expression by non-hematopoietic cells at the maternal fetal interface (Figure 1G). The uNK cell maturation profile differs from that of peripheral NK cells (Kieckbusch et al., 2014), and insufficient maturation might underpin reduced levels of tissue IFN- γ . However, we could not find absence of uNK cell maturation in δ^{D910A} females (Figure S1). PI3K p110 δ is known to be important for T and B cell development and function (Bilancio et al., 2006; Okkenhaug et al., 2002; Patton et al., 2006; Putz et al., 2012; Soond et al., 2010). No difference in the cellularity of any lymphocyte subset investigated was found (Figure S2). To investigate the possibility of an NK defect in these mice further, we extended our evaluation to non-pregnant mice. While we found no difference in the overall size of the NK compartment (Figure 1H), CD49a⁺ NK were overrepresented in non-pregnant δ^{D910A} mice (Figure 1I). Lack of p110 δ signaling did not affect the potential of either uNK cell subset to proliferate, as both displayed indistinguishable levels of Ki-67 positivity (Figures 1J and 1K). Together, these data demonstrate the importance of PI3K p110 δ in uNK cell IFN- γ production but show that its function is dispensable for maintaining normal uterine lymphocyte cellularity during pregnancy.

Maternal p110 δ Inactivation Impedes Normal Decidualization and Arterial Remodeling

In mice, decidualization is accompanied by the establishment of a highly organized lymphoid structure adjacent to the decidua (MLAp) and distal to the invading trophoblast. Both decidualization and MLAp growth are impaired in the absence of uNK cells (Ashkar and Croy, 2001; Barber and Pollard, 2003; Guimond et al., 1997). Visual examination of H&E-treated sections showed both smaller decidual and MLAp areas on midsagittal uterine sections, indicating impaired decidualization and myometrial abnormalities in δ^{D910A} females (Figure 2A). Quantification of the volumes of both compartments showed a reduction in size (Figures 2B and 2C), which correlated with the observed reduced overall cellularity (Figure 1A). Strikingly, implantation sites from δ^{D910A} females also displayed a marked reduction in vessel size (Figure 2D) and in the ratio between vessel and lumen areas, which is a measure of arterial remodeling (Figure 2E). Both are NK cell-driven

processes mediated by IFN- γ (Ashkar et al., 2000). This was confirmed by visualizing smooth muscle actin in the decidua (Figure 2F). While the thick muscle wall surrounding the spiral arteries had almost completely disappeared in controls, it was still prominent in δ^{D910A} females at gestational day 9.5 (gd9.5). To ascertain that this phenotype was leukocyte-specific, we evaluated expression of the *pik3cd* gene (which codes for p110 δ) in non-hematopoietic cells at the maternal-fetal interface. CD45⁻ cells showed very low levels of transcripts close to the detection threshold and comparable to those in the mouse fibroblast cell line NIH 3T3 (Figure 2G) that was used as a negative control due to its reported low *pik3cd* expression (Kok et al., 2009).

Taken together, these results demonstrate that maternal p110 δ signaling in leukocytes is important for normal decidual and myometrial development during pregnancy and that it is essential for arterial remodeling and a normal blood supply to the fetoplacental unit.

Maternal p110 δ Is Required for Normal Fetal Growth

Next, we investigated the downstream effects of the suboptimal intrauterine environment on the developing fetuses. Fetuses carried by δ^{D910A} females showed significantly lower weights, which was detectable as early as gd14.5. This reduction in fetal growth resulted in offspring that was >13% smaller than in control pregnancies just before term (1.227 g \pm 0.006 g versus 1.064 g \pm 0.015 g at gd18.5). Reduction of mean fetal weight at gd18.5 was due to differences in weight distribution (Figure 3B). Strikingly, there was a >15-fold increase in growth-retarded fetuses defined as within or below the fifth percentile of B6 controls (Figure 3C). Placental growth was very similar in conceptuses from δ^{D910A} females and B6 controls apart from a subtle increase in placental weight at gd16.5 (Figure 3D). Accordingly, absence of maternal p110 δ signaling lead to a reduction in the capacity of placentas of similar size to support the same fetal growth, suggesting reduced placental efficiency throughout gestation (Figure 3E). In these experiments, both δ^{D910A} females and control B6 females were mated with B6 males, resulting in heterozygous δ^{D910A} offspring (δ^{D910A} \times B6)F1 in one group and homozygous wild-type (WT) offspring in the other. To exclude a possible gene dose effect caused by only one functional copy in (δ^{D910A} \times B6)F1 fetuses rather than a maternal effect of p110 δ inactivation, we repeated these experiments using the reverse cross (B6 females mated with δ^{D910A} males) to produce isogenic fetuses in samples and controls (shown as “maternal \times paternal” genotype, (δ^{D910A} \times B6)F1 and (B6 \times δ^{D910A})F1). Figure 3F shows that isogenic heterozygous fetuses ($\delta^{D910A/WT}$) showed different fetal weights at gd18.5 that depended on whether or not maternal PI3K p110 δ signaling was absent, thus strongly suggesting a maternal rather than a fetal phenotype in these matings. Potential gene dose effects may also affect the placenta, which has the same genotype as its fetus. To this end, histological examination showed no overt differences in the fetomaternal exchange area (labyrinth zone, Figures 3G and 3H), but a slight increase in the size of the junctional zone that borders this area was observed (data not shown). To exclude differences in maternal nutrient allocation that might affect fetal growth in δ^{D910A} mothers, we also assessed the availability of soluble factors in maternal serum. We

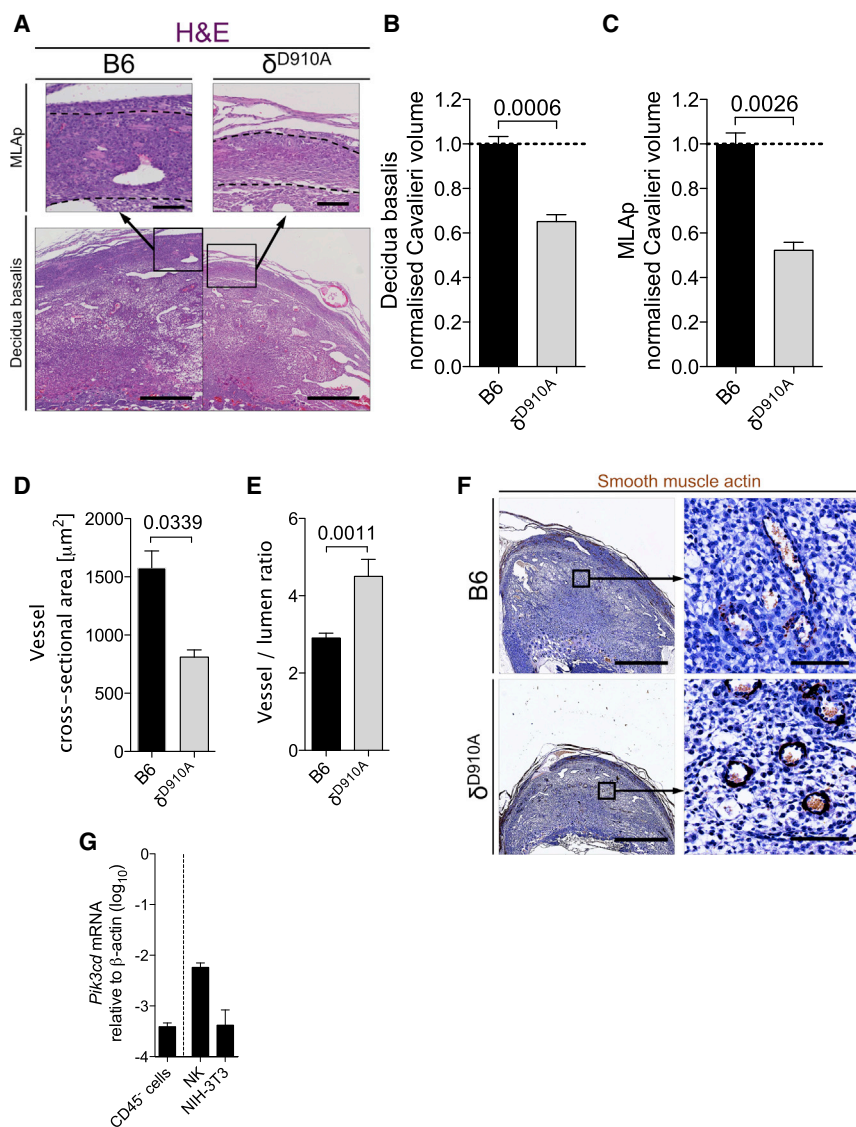


Figure 2. Maternal p110 δ Inactivation Impedes Normal Decidualization and Arterial Remodeling

(A) H&E staining of gd9.5 uterine tissue shows smaller MLAp (top) and decidual (bottom) areas in δ^{D910A} females. Scale bars represent 100 μ m (top) and 500 μ m (bottom). Dashed line demarcates the boundaries between MLAp and surrounding tissues.

(B and C) Volume estimation of decidual and MLAp compartments using the Cavalieri method.

(D and E) Quantification of vessel size (D) and vessel/lumen ratio as readout for vascular remodeling (E). Data are representative of four litters with $n = 13$ –14 implantation sites.

(F) Smooth muscle actin staining shows incomplete vascular remodeling in the absence of maternal p110 δ signaling. Scale bar, 100 μ m. Indicated is the maternal genotype and all females were mated with B6 males. p values for the effect of maternal genotype from a mixed model approach, taking the clustering of observations by litter into account.

(G) Relative expression of the gene coding for p110 δ (*Plk3cd*) in non hematopoietic CD45⁺ cells and NK cells (positive control) as well as the NIH 3T3 fibroblast cell line (negative control). $n = 3$ mice per group. All data are presented as means \pm SEM. MLAp, mesometrial lymphoid aggregate of pregnancy.

found a subtle decrease in non-fasting glucose levels at the end of gestation that was pregnancy specific, as we found non-pregnant δ^{D910A} to have slightly elevated glucose levels (Figure 3I). No significant differences were observed in the levels of triglycerides or cholesterol (data not shown). Taken together, these data show that maternal p110 δ is crucial for normal fetal growth in utero.

Fetal Death and Accelerated Postpartum Growth in the Absence of Maternal p110 δ Signaling

To further investigate the effect of maternal p110 δ inactivation on reproductive success, we assessed the litter sizes from δ^{D910A} females (Figure 4A). The number of viable conceptuses remained similar up until midgestation, demonstrating that maternal p110 δ signaling is dispensable for implantation. This changed in the second half of gestation, where >35% of fetuses in δ^{D910A} females died (litter sizes were 7.76 ± 0.2 in wild-type females

versus 5 ± 0.38 in δ^{D910A} females), further emphasizing the detrimental effect of maternal p110 δ inactivation for fetal well-being. Litter sizes in the reverse cross were normal, demonstrating that absence of maternal p110 δ signaling, rather than a fetal effect of the heterozygous δ^{D910A} mutation, was responsible for this (Figure 4B). Importantly, *Rag2*^{-/-}*Il2rg*^{-/-} (*Rag2*^{-/-} γ c^{-/-}) mice that lack all lymphocytes including NK cells show a reduction in fetal growth (Croy et al., 2011; Kieckbusch et al., 2014) but no decrease in the number of viable implantation sites (Figure 4B). This suggested that while the defect in NK cells described here may be sufficient to explain the reduction in fetal weight and arterial remodeling, it was likely that other, non-lymphoid cells were also affected by maternal p110 δ inactivation and contributed to the fetal demise.

Low birth weight predisposes to adult diseases such as metabolic disease, cardiovascular disease, and cancer (Barker, 2004). Excessive growth postpartum (“catch-up” growth) following fetal growth restriction is associated with additional risk factors, including decreased cognitive functions and short lifespan (Fisher et al., 2006; Geiger et al., 2012; Ozanne and Hales, 2004). To investigate if maternal p110 δ inactivation might contribute to this increased risk, we followed the growth trajectory of ($\delta^{D910A} \times$ B6)F1 and (B6 \times δ^{D910A})F1 offspring. Pup weights from both crosses were indistinguishable starting from postpartum day 7 (pp7) (Figure 4C; data not shown),

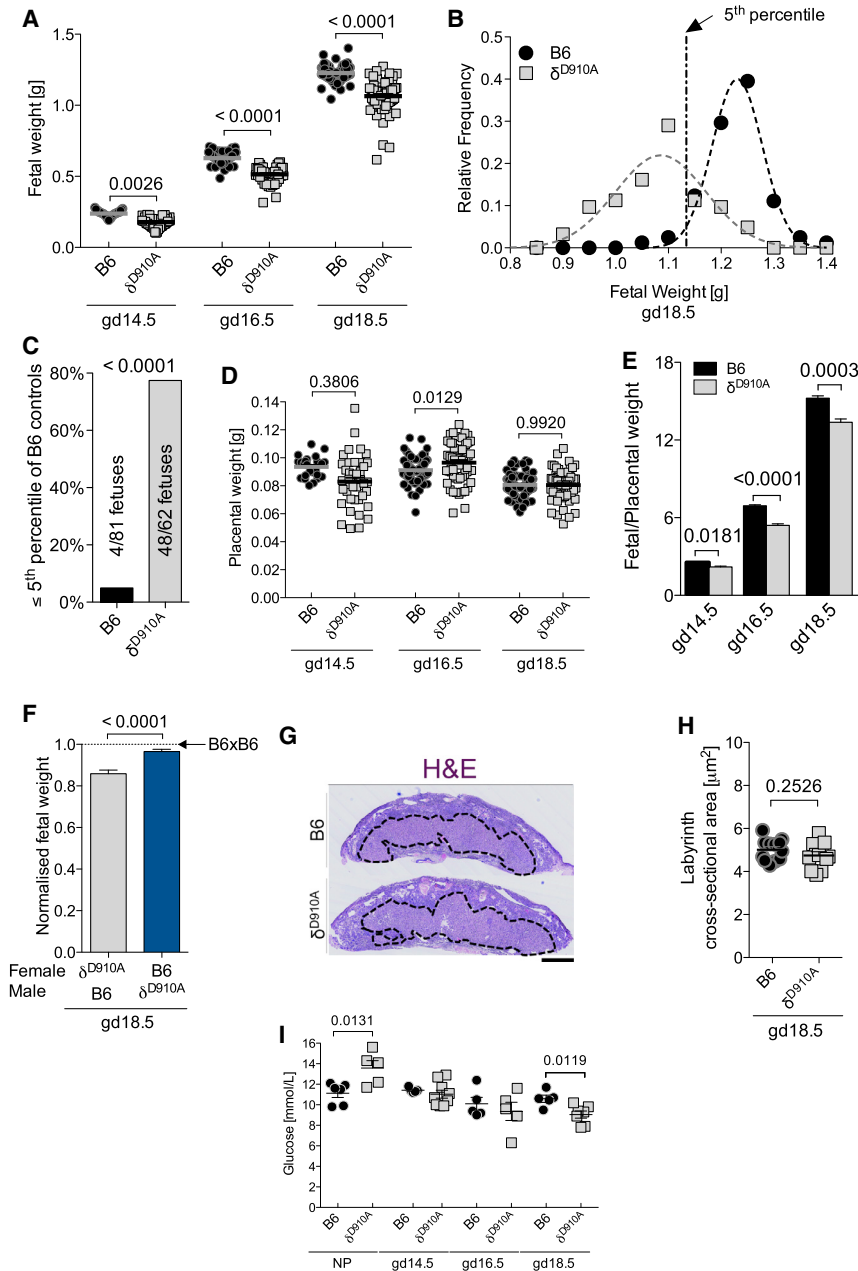


Figure 3. Maternal p110 δ Is Required for Normal Fetal Growth

(A) Fetal weights of conceptuses from either B6 of δ^{D910A} females mated with B6 males (means \pm SEM). Data are from 9–14 litters per maternal genotype per time point.

(B and C) Analysis of the distribution of fetuses from B6 of δ^{D910A} females. Data are from 13 or 14 litters, dashed line shows fitted Gaussian distribution. p value from Fisher's exact test comparing the fraction of growth restricted fetuses (weights within or below the fifth percentile of controls).

(D) Placental weights of conceptuses from either B6 of δ^{D910A} females mated with B6 males (means \pm SEM). Data are from 9–14 litters per maternal genotype per time point.

(E) Ratio of fetal/placental weight from (B) and (D) showing placental efficiency.

(F) Comparison of fetal weight between isogenic fetuses from the depicted crosses normalized to B6 controls (mean \pm SEM). Data from 4–14 litters. (A, D, E, and F) p values for the effect of maternal genotype from a mixed model approach taking the clustering of observations by litter into account.

(G) H&E stainings of placentas at gd18.5 show no overt differences in the maternal/fetal exchange region (labyrinth, dashed line). Scale bar, 1 mm.

(H) Quantification of cross-sectional area of labyrinth zone in gd18.5 placentas. Data representative of 12 placentas from four litters per cross (means \pm SEM).

(I) Non-fasting blood glucose levels prior to and during pregnancy.

Data are means \pm SEM (H and I); p values are from unpaired, two-tailed Student's t tests.

Maternal p110 δ Signaling Prevents Uterine Inflammation

As the NK phenotype could explain differences in fetal growth, but not in litter size, we extended our focus to myeloid cells. A gating strategy adapted from Tagliani et al. (2011) was used to identify macrophages, neutrophils, Ly6C^{high} monocytes (a precursor to uterine macrophages), and dendritic cells (DCs) (which are recruited to the uterus by uterine macro-

demonstrating accelerated growth in the sensitive postnatal period.

In order to assess if the observed phenotype was restricted to primiparous females, we obtained breeding records from two animal facilities (Figures 4D, 4E, and S3). We found that maternal p110 δ signaling was essential for normal litter sizes regardless of parity. This finding was reproduced also in OT-II *Rag2*^{-/-} δ^{D910A} mice which lack B cells and are incapable of generating fetal-specific T cells (Figure 4F), suggesting that dysregulation in these cells does not contribute to increased fetal loss. Together, these data show that maternal p110 δ inactivation affects fetal well-being.

phages; Figure 5A). We found that all myeloid subsets except neutrophils were significantly underrepresented during pregnancy (Figure 5B). Using immunohistochemistry, we confirmed that the abundance of F4/80⁺ was indeed lower in both decidua and MLAP of δ^{D910A} females around midgestation (Figure 5C).

Uterine macrophages can be grouped into two subsets based on their expression of MHC class II (Tagliani et al., 2011): a subset that expresses low levels of MHC class II (MHC-II^{low}), which has been reported to have higher mRNA levels of genes associated with alternatively activated (M2) macrophages, and a second subset that expresses high levels of MHC class II (MHC-II^{high}) and has a transcription profile of genes more

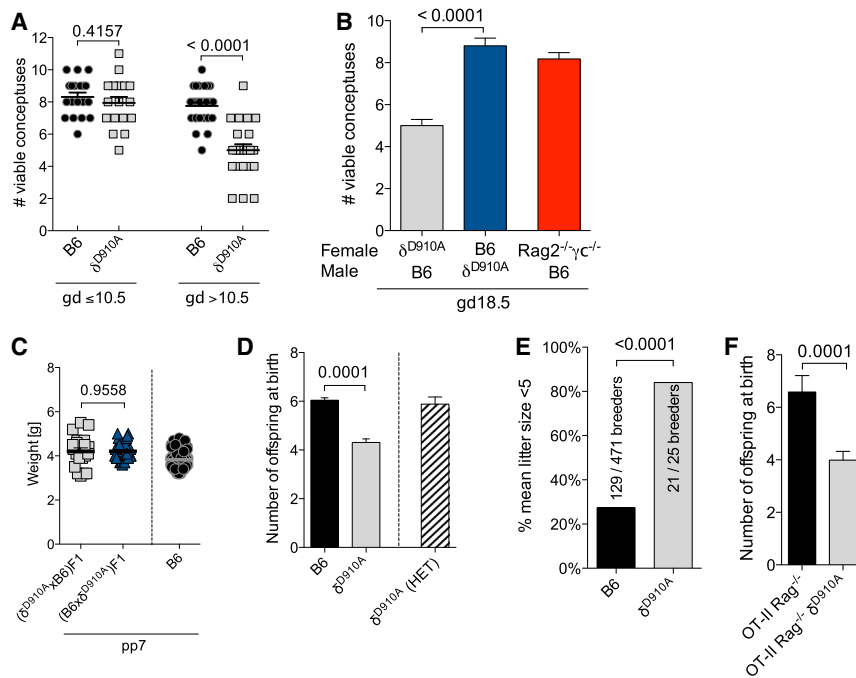


Figure 4. Fetal Death and Accelerated Postpartum Growth in the Absence of Maternal p110 δ Signaling

(A) Assessment of the number of viable implantation sites in litters from females of the depicted genotype mated with B6 males before and after midgestation; n = 17–29 litters.

(B) Assessment of the number of viable implantation sites of the indicated crosses; n = 5–14 litters per group.

(C) Comparison of isogenic offspring from matings with maternal or paternal p110 δ inactivation. Data are from six to eight litters per parental cross.

(D) Summary of breeding records showing the mean litter size of breeding females at birth (indicated is the maternal genotype). Data are means \pm SEM; p values are from unpaired, two-tailed Student's t tests.

(E) Quantification of the fraction of multiparous breeders with a mean litter size <5. p values are from Fisher's exact test.

(F) Assessment of offspring survival in $\Delta D910A$ mice lacking B cells and T cells that can recognize fetal antigens; n = 31–60 litters per group. Data are means \pm SEM; p values are from unpaired, two-tailed Student's t tests.

Data in (D) and (E) are representative of 25 and 471 breeders, respectively, corresponding to 102–1,055 litters.

See also Figure S3.

associated with the M1 type of macrophages. Given the striking reduction in macrophage abundance, we investigated their phenotype further. In $\Delta D910A$ females, the MHC-II^{high} subset was relatively overrepresented among macrophages (Figures 5D and 5E), suggesting an imbalance toward a more inflammatory uterine milieu in these mice during pregnancy. In contrast, no subset imbalance was seen for DCs (CD11b^{high} versus CD11b^{low}) or monocytes (MHC-II⁺ versus MHC-II⁻; Figures 5F and 5G). Apart from IFN- γ , uNK cells can also produce other immunomodulatory factors, including CSF-1, a key cytokine for uterine macrophage population dynamics, so that unbalanced uNK activation could potentially be responsible for the macrophage phenotype. To explore this possibility, we assessed the expression of *csf1* and *ccl5* (whose gene product is RANTES that could regulate uNK cell migration). For *csf1*, we found the highest relative amount of mRNA in non-hematopoietic cells, but at similar levels in both strains across cell types (Figure 5H). Expression of *Ccl5* was also similar between strains, but uNK cells had higher expression than CD45⁻ cells (Figure 5I). To further investigate a possible regulatory role of uNK or other lymphoid cells on macrophages, we also assessed the phenotype of myeloid cells in $Rag2^{-/-}\gamma c^{-/-}$ mice which showed neither disruption of macrophage numbers nor reduction in DC or monocytes despite the total absence of NK cells (Figures 5J and 5K). We did, however, find increased numbers of uterine neutrophils, thus excluding the possibility that an increase of these cells by itself can cause fetal loss. Together, these results demonstrate changes in uterine myeloid cells including a more inflammatory macrophage phenotype in the absence of p110 δ signaling. The results also suggest that these changes are not due to NK cell dysregulation.

Local, but Not Systemic, Inflammation Underpins Fetal Growth Restriction and Increased Fetal Mortality

The PI3K pathway is known to be important for the resolution of inflammation mediated by myeloid cells (Günzl et al., 2010), and the p110 δ subunit of PI3K has been shown to induce a negative feedback mechanism that dampens macrophage activation downstream of Toll-like receptors (Aksoy et al., 2012). The overrepresentation of MHC-II^{high} macrophages raised the question as to whether an increased inflammatory response could be responsible for the increase in fetal loss. To test whether changes in the relative abundance of macrophage subsets translate into a more potent inflammatory response, we assessed macrophage effector functions (IL-6 production and CD107a surface exposure as a proxy for degranulation). Macrophages in $\Delta D910A$ mice showed a significantly higher responsiveness even after overnight culture (Figures 6A and 6B). IL-6 has been reported to be involved in fetal loss (Zenclusen et al., 2003), and following its increased production by uterine macrophages in the absence of p110 δ signaling, we wanted to directly assess whether this correlated with overall higher levels of this cytokine in decidua and myometrium. We found a significant increase in IL-6 levels in situ (Figure 6C), but no change in IL-10 (Figure 6D), which is associated with an anti-inflammatory response. Tissue levels of tumor necrosis factor (TNF) and IL-1 β were below the detection threshold in both strains (data not shown). Together, these results show a dysregulation of macrophage dynamics and function that was associated with upregulation of IL-6, suggesting a more inflammatory intrauterine environment.

$\Delta D910A$ mice are known to develop mild inflammatory bowel syndrome in adulthood (Okkenhaug et al., 2002). Accordingly,

we tested if the inflammatory phenotype observed in utero was only seen in the local environment or due to possible systemic inflammation and unrelated to pregnancy. To this end, we found similar serum levels of IL-6, TNF, and IL-10 in the second half of gestation, clearly showing that the inflammatory uterine milieu is restricted locally and not associated with acute systemic inflammation during pregnancy (Figure 6). We did find increased IL-6 levels in one out of six females at gd14.5 and one out of seven females at gd18.5. However, these did not correlate with increases in TNF, and in order to be causally linked to the phenotype described here, we would expect all females to show increases in IL-6 at the end of gestation (gd18.5). We also assessed serum IL-1 β with very low overall abundance and a similar number of samples below the detection threshold of 0.3 pg/ml in both strains (data not shown). Progesterone withdrawal is a potent inducer of fetal loss (Yang et al., 2000). Lastly, to investigate whether reduced progesterone levels might contribute to the phenotype in δ^{D910A} females, we evaluated the levels of this hormone in the serum late in gestation. We found no evidence for a reduction in progesterone due to p110 δ inactivation but instead found a small but significant increase (Figure 6H). Together, these data show a heightened inflammatory environment in utero in the absence of systemic inflammation or progesterone dysregulation.

DISCUSSION

Little is known about the mechanisms that orchestrate the dynamic changes that uterine innate immune cells undergo during gestation. Our results identify a key role for maternal PI3K p110 δ signaling in the immune regulation during pregnancy. This isoform is absent in most tissues but highly expressed in leukocytes (Vanhaesebroeck et al., 1997). Due to its function in many immune cells and the importance of leukocytes for successful pregnancy, the mechanisms by which p110 δ signaling impacts reproductive success are multifactorial. Maternal p110 δ inactivation affected the outcome of pregnancy through two pathways: one mediated by poor uNK function leading to reduced arterial remodeling and fetal growth restriction and one characterized by a heightened inflammatory uterine milieu underpinning increased fetal mortality (Figure S4). PI3K p110 δ has been implicated in smaller litter sizes in a previous study using knockout mice on a 129 background (Li et al., 2013). In that study, the authors suggested that this was due to differences in ovarian hormone responsiveness of these *pik3cd*^{-/-} mice. The study of class IA PI3K is complicated by the fact that the absence of one subunit affects the expression levels of other subunits (Okkenhaug and Vanhaesebroeck, 2001), which is not the case for the kinase-dead mutation in δ^{D910A} mice (Okkenhaug et al., 2002). Our results demonstrate that maternal p110 δ does not alter the amount of implanted blastocysts that gestate to midpregnancy but rather fetal growth and survival after this time point.

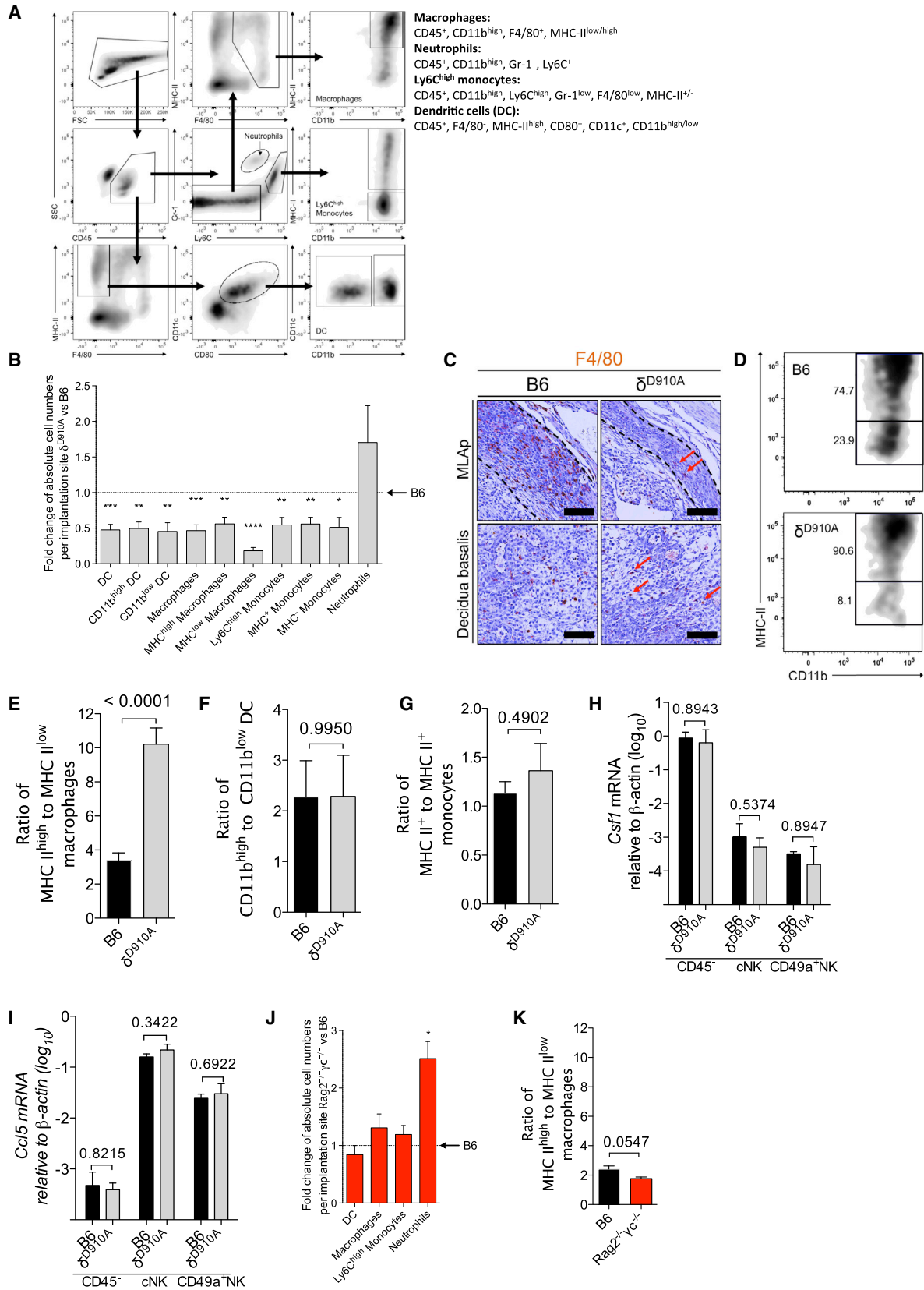
In non-pregnant mice, we found an overrepresentation of CD49a⁺ uNK cells. Based on our previous work that showed defective migration of δ^{D910A} NK cells to the uterus upon transfer to pregnant mice (Saudemont et al., 2009), we hypothesized to

find decreased numbers of cNK cells in pregnant δ^{D910A} mice, but both their fraction and total number in implantation sites at midgestation were normal in δ^{D910A} mice. Moreover, transcripts of genes encoding the key cytokine IL-15 and the chemokine CCL5 were normal in δ^{D910A} mice. Our results presented here suggest that compensatory mechanisms other than overt differences in proliferation or migration are in place that lead to similar uNK cell abundance in both strains.

While we were unable to detect a clear difference in the uterine lymphocyte profile of δ^{D910A} mice during pregnancy, we did notice an overall higher variance in some leukocyte subsets compared to B6 controls (e.g., T cells in Figure S2 and neutrophils in Figure 5B). The reason for this is unclear, but it might be due to the fact that the results are generated by pooling implantation sites from individual mice, in which the observed phenotype may be at different stages. Another factor is that we used pooled cell suspensions from decidua and myometrium (MLAp) to evaluate overall leukocyte dynamics in utero. This might obscure smaller differences that occur in one compartment, but not in the other, especially given their known differences in leukocyte composition (Doisne et al., 2015; Tagliani et al., 2011).

PI3K p110 δ has a critical role for B and T cell function, including regulatory T (Treg) cells (Ali et al., 2014; Liu et al., 2009; Okkenhaug et al., 2002; Patton et al., 2006), and it is thus possible that Treg cells are less effective at controlling effector responses in the uterus of δ^{D910A} mice. While fetal loss in δ^{D910A} females mated with syngeneic males was independent of adaptive B or T cells capable of responding to fetal antigens, we would hypothesize that p110 δ inactivation in allogeneic matings may be even more detrimental to fetal survival, as Treg function is crucial in this setting (Rowe et al., 2012; Samstein et al., 2012).

CSF-1 is a key player for orchestrating distribution and population dynamics of macrophages as well as DCs in utero, and mice lacking CSF-1 show mildly reduced litter sizes (Pollard et al., 1991; Tagliani et al., 2011). High MHC class II expression in macrophages correlates with a transcription profile of classically activated (M1) cells that are associated with inflammatory responses, whereas low class II expression is found on cells that express genes observed in alternatively activated macrophages (M2 group) that are involved in tissue remodeling and angiogenesis (Murray et al., 2014). The conversion of these M2-like cells to M1-like cells is normally blocked by CSF-1 signaling. Because p110 δ is the dominant PI3K isoform downstream of CSF-1 receptor in primary macrophages (Papakonstanti et al., 2008), it is conceivable that dampened CSF-1 signaling may contribute to the altered macrophage dynamics and the relative abundance of cells highly expressing MHC class II in δ^{D910A} mice. It has been speculated that MHC-II^{high} and MHC-II^{low} subsets resemble CD209⁻ and CD209⁺ macrophage subsets found in first trimester human endometrium based on similarities in gene expression (Erlebacher, 2013). In macrophages and other myeloid cells, p110 δ signaling is known to be crucial for the resolution of inflammation (Aksoy et al., 2012; Fukao and Koyasu, 2003; Günzl et al., 2010; Liu et al., 2009; Uno et al., 2010). This might explain the relative overrepresentation of macrophages with a more inflammatory phenotype; these



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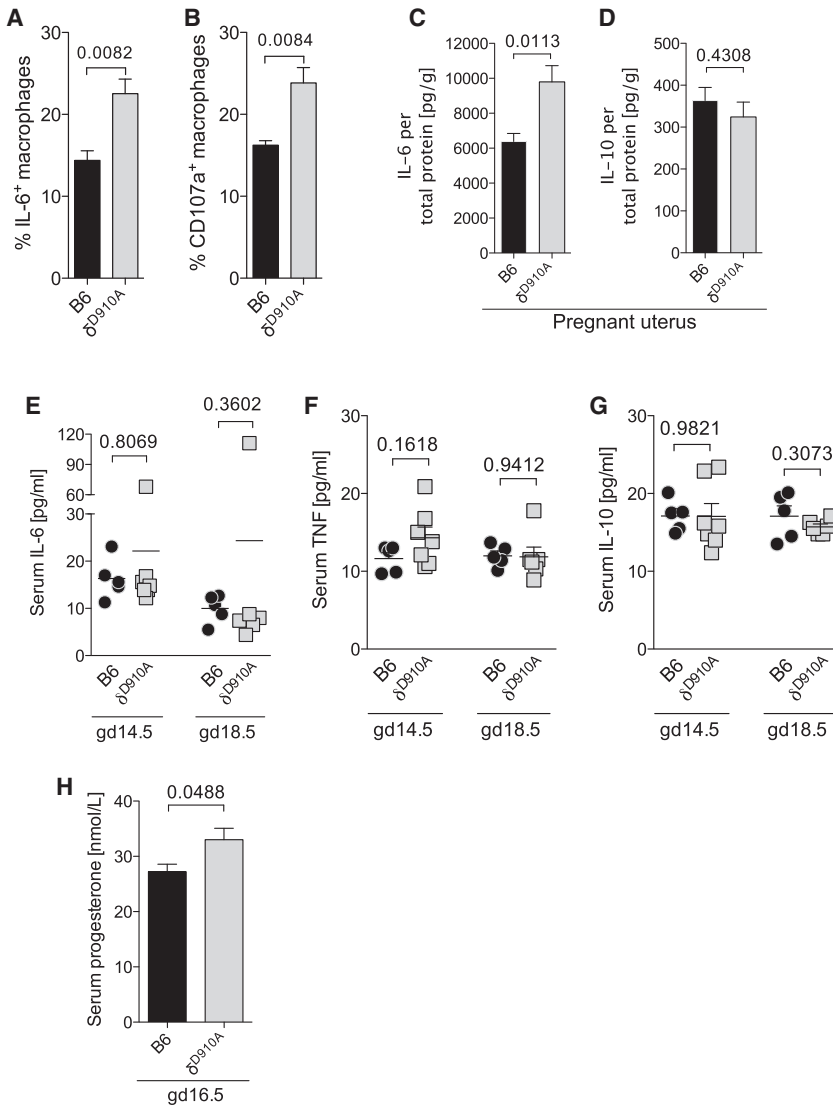


Figure 6. Local, but Not Systemic, Inflammation Underpins Fetal Growth Restriction and Demise

(A–D) Flow cytometry staining for intracellular IL-6 (A) and surface CD107a (B) on macrophages (adherent CD45⁺ CD11b⁺ F4/80⁺ cells) at gd10.5 after overnight culture. Data are from three independent experiments; n = 4 mice per group. (C) Quantification of tissue IL-6 and (D) IL-10; n = 5 mice per group. (E–H) Assessment of serum IL-6 (E), TNF (F), IL-10 (G), and progesterone (H); n = 5–7 per group. Data are means \pm SEM; p values are from an unpaired, two-tailed Student's t test.

cells need to receive their physiological cues in utero to function correctly. In the absence of p110 δ signaling, this activation becomes dysregulated, and instead of switching to their physiolog-

ical role in tissue remodeling, macrophages sustain persistent inflammation. Excessive immune activation has long been known to compromise pregnancy success and activation of the innate immune system by pathogen-derived danger signals can jeopardize fetal survival (Parant and Chedid, 1964; Xu et al., 2000). Although the underlying mechanisms are still not fully understood, it seems clear that the tissue damage mediated by infiltrating neutrophils and activated macrophages is not conducive to a uterine microenvironment capable of promoting adequate fetal growth. Pregnancy is not accompanied by general immunosuppression (Kourtis et al., 2014), however, and it is becoming increasingly clear that successful pregnancies involve a fine balance between activating those parts of the immune system involved in trophic functions (uNK-mediated arterial remodeling, macrophage involvement in tissue remodeling) and those components that are involved in host defense (Colucci and Kieckbusch, 2015). To this end, great care must be taken when it comes to clinical interventions with the aim of interfering with immune responses during gestation, as the spatio-temporal role of each leukocyte subset is far from understood.

Figure 5. Maternal p110 δ Signaling Prevents Uterine Inflammation

(A) Gating strategy for identifying macrophages, dendritic cells (DC), Ly6C^{high} monocytes, and neutrophils at gd10.5. (B) Fold change of absolute cell numbers per implantation site of the indicated populations in δ^{D910A} females compared to B6 controls. Data are means \pm SEM; p values compare the cell number in δ^{D910A} with the mean of controls. (C) Histological examination of abundance of F4/80⁺ cells in MLAp (top) and decida basal (bottom) of sections from gd9.5 pregnant females. Scale bars, 100 μ m. (D) Representative density plots showing MHC-II expression of uterine macrophages gated as in (A). (E) Relative abundance of MHC class II^{high} versus MHC class II^{low} macrophage populations. (F) Relative abundance of CD11b^{high} and CD11b^{low} DC subsets. (G) Relative abundance of MHC class II⁺ versus MHC class II⁻ Ly6C^{high} monocyte populations. Data are representative of five experiments using n = 6–7 mice per group. (H and I) Relative mRNA quantification for *Csf1* (H) and *Ccl5* (I); n = 3 mice per group. (J) Fold change of absolute cell numbers per implantation site of the indicated populations in Rag2^{-/-} γ c^{-/-} females compared to B6 controls. p values compare the cell number in Rag2^{-/-} γ c^{-/-} with the mean of controls. (K) Relative abundance of MHC class II^{high} versus MHC class II^{low} macrophage populations. Data are means \pm SEM; n = 4–6 mice per group. p values are from an unpaired, two-tailed Student's t test.

Isoform-specific small molecule inhibitors for p110 δ are now in clinical trials. δ^{D910A} mice model long-term p110 δ inhibition, and our results suggest that such long-term inhibition may best be avoided during pregnancy.

The traditional model for inflammation-mediated fetal demise is injection with Toll-like receptor agonists such as poly(I:C) or lipopolysaccharide. This also triggers systemic immune system activation (Cotechini et al., 2014; Thaxton et al., 2013; Zhao et al., 2015). In our study, we observed local and spontaneous inflammation in utero with physiological levels of inflammatory cytokines in the periphery throughout gestation. This suggests that δ^{D910A} mice are indeed a good model to study the effects of uterine inflammation during pregnancy in greater detail, as it allows for complete dissociation of systemic and local inflammation. In conclusion, our results emphasize the complexity of maternal immune responses during pregnancy. This strengthens the notion that clinical interventions with the aim of dampening maternal immune responses do not have a scientific basis (Moffett and Colucci, 2014). Rather, multiple leukocyte subsets need to exert their functions in a balanced way.

EXPERIMENTAL PROCEDURES

Mice

All animal experiments were approved by the University of Cambridge Ethical Review Panel and carried out in accordance with Home Office Project License PPL 70-8222. C57BL/6 (B6) mice were purchased from Charles River UK. Mice with a kinase-dead mutation in the gene coding for PI3K p110 δ (δ^{D910A}) have been described before (Okkenhaug et al., 2002) and were backcrossed on a B6 background for >12 generations. OT-II *Rag2*^{-/-} δ^{D910A} (Ali et al., 2014) and alymphoid *Rag2*^{-/-} *Il2rg*^{-/-} (*Rag2*^{-/-} γ c^{-/-}) (Colucci et al., 1999) mice on B6 background have been described before. Mice were 8–12 weeks of age and showed no signs of colitis. For pregnancy experiments, female mice were randomly introduced to males and the timing of conception was determined by detection of a copulation plug representing gd0.5.

Flow Cytometry

Cell suspensions of uterine tissues were prepared using either mechanical dissociation followed by centrifugation through a Lympholyte M (Cedarlane) gradient according to the manufacturers recommendations or by enzymatic digestion using Liberase TM (Roche) as described previously (Collins et al., 2009). Cells were typed using fluorochrome-conjugated antibodies with specificity for CD45 (30-F11), NK1.1 (PK138), CD11b (M1/70), CD27 (LG.3A10), CD49a (HM α 1), CD49b (DX5), KLRG1 (MAFA), Ly6C (HK1.4), Gr-1 (RB6-8C5), I-A/I-E (M5/114.15.2), F4/80 (BM8), CD11c (N418), CD3 ϵ (145-2C11), CD4 (GK1.5), CD8a (53-6.7), CD19 (1D3), CD122 (5H4), NKp46 (29A1.4), IL-6 (MP5-20F3), CD107a (1D4B), and Ki-67 (B56) purchased from BD Pharmingen, BioLegend, or eBioscience. For intracellular and intranuclear antigens, Fix & Perm and Foxp3 staining buffer set (both eBioscience) were used, respectively. For estimation of total cell number and leukocyte composition, enzymatic digestion was preferred as it yielded higher amounts of non-lymphocytes and did not exclude cells based on size or density. For phenotyping of NK cells, mechanical dissociation followed by Lympholyte M was used in order to prevent degradation of surface proteins.

qRT-PCR

From gd10.5 and 9.5 pregnant females, cells of either non-hematopoietic (CD45⁻), conventional uNK (CD45⁺, CD3⁻, CD19⁻ NK1.1⁺, NKp46⁺, CD49a⁻) or tissue-resident uNK (CD45⁺, CD3⁻, CD19⁻ NK1.1⁺, NKp46⁺, CD49a⁺) phenotype were sorted using a FACSAria III (BD). Cell lysates were prepared with the Cells-to-CT 1-Step TaqMan Kit (Ambion, Life Technologies) and mRNA expression was quantified by qRT-PCR using an ABI PRISM 7900HT (Applied Biosystems) using commercially available TaqMan probes

(Mm00432686_m1, Mm01302427_m1, Mm00434210_m1, Mm00435674_m1 and Mm02619580_g1) for *Csf1*, *Ccl5*, *Il15*, *pik3cd*, and β -actin, respectively. Gene expression of each cell type from each mouse was assessed in triplicate. Transcript levels were normalized to β -actin using Δ C_T values.

In Vitro Culture

Single-cell suspensions from gd10.5 pregnant females were prepared by Liberase TM digestion and incubated in complete RPMI cell culture media for 2 hr. Non-adherent cells were removed and cells cultured overnight in fresh complete RPMI with brefeldin A (eBioscience) and monensin present for the final 4 hr.

Histology

Rat-anti mouse F4/80 was used at 1/100 (C1:A3-1, Ab Serotec) after proteinase K antigen retrieval and visualized using the ImmPress kit (Vector). Smooth muscle actin (DAKO 1A4) staining was performed after heat-induced antigen retrieval in citrate buffer using a 1/100 dilution according to the instructions of the mouse-on-mouse immunodetection kit (Vector). Histological examination of H&E stains for overt placental abnormalities was performed at gd18.5 by determining the area of the labyrinth zone on midsagittal sections through the placenta. All histology was carried out on formalin-fixed paraffin-embedded tissue.

Stereology

Tissue volume estimation using the Cavalieri method and assessment of spiral artery remodeling was performed using H&E-stained sections as described previously (Kieckbusch et al., 2015).

Cytokine Quantification in Tissue Lysates

Myometrial and decidual tissue from gd10.5 pregnant females was snap frozen on dry ice and the whole litter pooled. 150 mg tissue was added to 1 ml lysis buffer (150 mM NaCl, 20 mM Tris, 1 mM EGTA, 1% Triton X-100, 1/100 Phosphatase Inhibitor Cocktail 3 [Sigma], 1/100 Phosphatase Inhibitor II [Sigma], 2 \times Ultra Protease Inhibitor solution [Roche], and 2 mM PMSF) and processed with a tissue homogenizer on ice. The homogenate was incubated on ice with agitation for 30 min and spun at 20,000 \times g for 10 min. The supernatant was used for further analysis on the MesoScale Ultra Sensitive platform. Total protein concentration was determined using a Bradford assay (Pierce).

Blood and Serum Measurements

Non-fasting blood samples were taken immediately after cervical dislocation from the aorta. Blood glucose was measured immediately using a digital glucometer. Samples were centrifuged at 12,000 \times g for 5 min and supernatant stored at -80°C before being used for MesoScale Ultra Sensitive assays.

Statistics

Normally distributed, independent, and unmatched data were analyzed with two-tailed, unpaired Student's *t* tests. Data clustered by litter (fetal/placental weights, arterial remodeling) that violate the assumption of independence of data points were analyzed using a mixed-model approach taking the fixed effect of parental cross and the random effect of litter variability into account (Barber and Pollard, 2003; Kieckbusch et al., 2014). Categorical data were analyzed using Fisher's exact test. *p* < 0.05 was taken as statistically significant for all tests. Analyses were performed using GraphPad Prism, GraphPad Quick Calcs, and IBM SPSS.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.11.050>.

AUTHOR CONTRIBUTIONS

J.K. performed and designed experiments and wrote the manuscript, E.B. performed and designed experiments, D.A.H. performed experiments, and F.C.

conceived the study, designed experiments, and wrote the manuscript. All authors critically read the manuscript.

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