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Pin1 as a Biomarker of ER+ Breast Cancers to Predict the Response to Tamoxifen and mTOR Inhibitors

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14. ABSTRACT

Previous work in our lab suggested a role for Pin1 in modulating LPS-stimulated IL-6 mRNA accumulation and protein secretion. We utilized primary Pin1 null MEFs to investigate the mechanism by which Pin1 modulates IL-6 production. We then aimed to determine a role for Pin1 (via regulation of IL-6 production) in modulating the sensitivity of breast cancer cells to chemotherapeutic agents. Our experiments, however, failed to produce reliable and convincing data to support a role for Pin1 in IL-6 production. Because of these results, we turned our attention to the observed *in vivo* inflammatory defects in our Pin1 null mice. We currently have evidence to support a role for Pin1 in modulating *in vivo* splenic Dendritic cell (DC) accumulation and *ex vivo* DC differentiation. Additionally, we find an elevation in the number of splenic Mac1+ granular cells in Pin1 null mice. Tumor-induced immunosuppression is a well-documented phenomenon, and is often associated with decreased numbers of circulating DC and increased numbers of circulating Mac1+ granular Myeloid-Derived Suppressor Cells (MDSC). For these reasons, we are interested in determining the ability of Pin1 null DC and Mac1+ granular cells to influence T cell function. We will also utilize a mouse model of mammary carcinoma to determine whether absence of Pin1 creates a more tumor-permissive immune environment for breast cancer growth.

15. SUBJECT TERMS
Pin1, LPS, IL-6, Inflammation, Dendritic Cell, Flt3 Ligand, GM-CSF, Stat3

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INTRODUCTION

The concept of tumor detection and elimination by a host's own immune system was introduced more than fifty years ago, but did not gain widespread recognition until genetically modified mice with immunodeficiencies were shown to be much more susceptible to tumor formation than immunocompetent mice. The resulting "tumor immunosurveillance" hypothesis is supported by data from immunosuppressed transplant patients, which exhibit an increased risk for developing cancer. Over the past two decades, it has become clear that tumor initiation and growth involve a complex series of interactions with the host immune system, and that tumor escape from immune destruction is mediated in part by the ability of the tumor to suppress T cell-mediated killing of tumor cells (*1*). This phenomenon has been documented in breast cancer patients, a majority of whom accumulate myeloid-derived suppressor cells (MDSC) that have been shown to inhibit T cell activation by a variety of mechanisms (*2-3*). Additionally, the number of circulating dendritic cells (DC), the main antigen-presenting cell type responsible for direct activation of naïve T cells, is frequently lower in breast cancer patients than in healthy individuals (*4-5*). Defects in immune system composition and function not only create a more tumor-permissive environment, but also impede the efficacy of cancer immunotherapies that attempt to induce a strong host response against the tumor (*6*). We believe that Pin1 plays a role in modulating the innate immune system, and hypothesize that loss of Pin1 will create a more tumor-permissive environment that supports breast cancer development. Our Pin1 null mice accumulate cells that resemble MDSC in their spleens. Additionally, we find that the loss of Pin1 impedes the *ex vivo* differentiation of DC from bone marrow, as well as the accumulation of mature DC (mDC) *in vivo* after immune challenge with bacterial lipopolysaccharide (LPS). We aim to determine whether these defects in the innate immune system of Pin1 null mice are able to alter T cell activation and to promote the engraftment of mammary tumors. Should Pin1 prove to be an important modulator of immune-mediated anti-tumor responses, combination therapies could be designed to manipulate Pin1 and/or its downstream targets in order to elicit a strong host immune response against breast tumors.

BODY

Updates for the Previous Statement of Work:

In the previous report, data was presented that supported a role for Pin1 in modulating IL-6 production in MEFs. Primary Pin1 null MEFs displayed a defect in both IL-6 mRNA induction as well as IL-6 protein secretion after being stimulated with LPS. Because IL-6 production has been implicated in both being predictive of breast cancer patient survival and conferring chemoresistance to breast cancer cell lines, we were interested in determining how Pin1 modulates its transcription (*7-9*).

Our previous data were generated from MEFs derived from one wild type (WT) embryo (denoted W6) and one litter-matched Pin1 null embryo (denoted K9). Experiments were repeated and the results confirmed several times using cells derived from these same two embryos. We then attempted to confirm our data in MEFs derived from different embryos. These experiments revealed that the production of IL-6 varies greatly between MEFs derived from multiple embryos, and we were unable to find a reproducible defect **(Figures 1A, 1B)**.

Because MEFs are derived from embryos, they may not accurately model physiological processes that occur in an adult animal. For this reason, we decided to utilize macrophages to assess the role of Pin1 in IL-6 production. One standard method for producing large of numbers of macrophages *ex vivo* is to isolate whole bone marrow and culture it in the presence of M-CSF for 5-7 days. We utilized this method to generate Bone Marrow-Derived Macrophages (BMDM) from WT and Pin1 null bone marrow and then measured LPS-stimulated IL-6 production. We found no defect in the ability of Pin1 null BMDM to secrete IL-6 protein or produce IL-6 mRNA **(Figures 2A, 2B)**. We also obtained primary macrophages from the peritoneal cavity of WT and Pin1 null mice. When these cells were stimulated *ex vivo* with LPS, we observed that Pin1 null cells had an early defect in IL-6 production after 3 hours (depicted in bar graphs with appropriate scaling) **(Figure 3A)**, but the extent of this defect was not maintained at later time points **(Figures 3A, 3B)**. We also measured IL-6 mRNA induction in peritoneal macrophages, and found a similar early defect in Pin1 null cells that was reversed at later time points **(Figure 3C)**.

Because of the unreliable data generated in MEFs and the lack of a robust phenotype in Pin1 null macrophages, we did not to carry out proposed experiments that would investigate the mechanism by which Pin1 modulates IL-6 production, or the influence of Pin1 expression on chemoresistance in breast cancer cell lines. Instead we chose to focus on the observed immune defects in our Pin1 null mice, which are further described in ***Revised Task 1***.

Data and Rationale Supporting the Revised Statement of Work:

REVISED TASK 1: Identify the immune cell type(s) responsible for attenuating inflammation in Pin1 null mice

Our lab has observed that Pin1 null mice challenged with an intraperitoneal (i.p.) injection LPS have an impaired immune response (unpublished data). Eighteen hours after i.p. administration of 15mg/kg LPS, serum was collected from WT and Pin1 null mice and analyzed for the presence of inflammation-related serum proteins. This screen revealed that Pin1 null animals had a reduction in nearly 2/3 of the 59 proteins measured (unpublished data). To understand how Pin1 might be modulating cytokine production *in vivo*, we sought to determine whether Pin1 null mice harbor impairments in immune cell localization to the peritoneal cavity or in the recruitment of cells to secondary lymphoid organs, such as the spleen. We first isolated cells from the peritoneal cavity of wild type and Pin1 null mice and stained for markers of granulocytes (GR-1) and macrophages (F4/80), two populations that are present in the peritoneum and express toll-like receptor 4, which binds and responds to LPS. We found both granulocytes and macrophages were present in the peritoneal cavity of Pin1 null mice, indicating that the defective response to LPS was likely not due to the absence of these cell types **(Figure 4)**. Next, we tested the function of peritoneal macrophages by stimulating them *ex vivo* with LPS and measuring the production of inflammatory cytokines. We found that Pin1 null peritoneal macrophages were fully capable of producing IL-6 mRNA and secreted protein **(Figures 3A-3C)** as well as TNFα mRNA **(Figure 3D)**.

We next looked at immune cell populations in the spleens of control and LPS-treated mice. We first noticed that Pin1 null mice exhibited elevated numbers of granular cells in their spleens (indicated by their high side scatter, or SSC), a condition that remained unchanged upon administration of LPS **(Figures 5A, 5B)**. When data from both HBSS-treated mice and LPS-treated mice are combined, the difference in granular cells becomes more highly significant

(Figure 5C). More than 80% of the granular (high SSC) cells express the myeloid marker Mac1 **(Figure 6B)**, which accounts for the increase in the total number of cells expressing Mac1 in Pin1 null mice **(Figure 6A)**. When the granular cell population is excluded from analysis, the increase in Mac1+ cells is eliminated **(Figure 6C)**. In conclusion, we have found that Pin1 null mice produce elevated numbers of Mac1+ granular cells in their spleens under basal and LPS-stimulated conditions.

We also quantified the number of mDC that accumulated in the spleens of WT and Pin1 null mice after LPS challenge. Compared to WT mice, Pin1 null mice accumulated only half as many mDC in their spleens in response to LPS. This trend is also present in control (HBSS) mice, but does not reach statistical significance **(Figures 7A, 7B**).

Remaining Experiments:

Future experiments in *Revised Task 1* will more thoroughly define the granular cells in the spleens of our Pin1 null mice by staining splenocytes with specific granulocyte markers, such as Ly6G and GR-1, and then analyzing by flow cytometry. We will also determine if the increase in granular cells is specific to the spleen, or if it extends to other compartments, such as the circulation and the bone marrow. Finally, we will measure the circulating concentrations of Flt3L and G-CSF after LPS challenge, as these two growth factors may influence DC differentiation and granulocyte differentiation, respectively.

REVISED TASK 2: Determine the mechanism by which Pin1 regulates immune cell function

After discovering defects in the granular cell population and the DC population in the spleens of Pin1 null mice, we sought to determine the cellular mechanism by which these defects arise. Altered cell numbers could be the result of changes in proliferation, cell death, migration, and/or differentiation. We know that Flt3L is required for the normal production of DC, as mice lacking either Flt3L or its receptor, Flt3, exhibit impaired production of peripheral DC (*10-11*). Additionally, acute ablation of CD11c+ DC in mice has been shown to give rise to a myeloid proliferative syndrome (*12*). It is therefore possible that the increase in Mac1+ granular cells in our Pin1 null mice may be a consequence of the defect we see in DC accumulation. Since an impaired response to Flt3L would be consistent with the alterations we see in both DC and granular cell populations in our Pin1 null mice, we first investigated the ability of Pin1 null cells to differentiate in response to Flt3L. We isolated whole bone marrow from WT and Pin1 null mice. A fraction of the bone marrow was stained for progenitor populations, and the remaining cells were cultured *ex vivo* in the presence of Flt3L. After 9 days of culture, the number of non-adherent cells was determined, and the percentage of dead cells was quantified by staining with propidium iodide. Cells were also stained for myeloid and DC markers and analyzed by flow cytometry.

We were unable to detect any statistically significant defects in progenitor cell populations in freshly isolated bone marrow cells from Pin1 null mice **(Figures 8A-8C)**. We did, however, find that Pin1 null bone marrow cells were defective in their ability to differentiate into Mac1+ DC in the presence of Flt3L **(Figure 9A)**. This was not due to changes in survival or cell death **(Figures 10A, 10B)**. It has been shown that bone marrow can also differentiate into Mac1- DC in the presence of Flt3L (*13*). When we assessed the ability of Pin1 null bone marrow to produce Mac1- DC, we found a similar defect **(Figure 9B)**.

Dendritic cells can also be generated *ex vivo* by culturing bone marrow in the presence of GM-CSF. It is thought that BMDC produced by Flt3L are similar to steady-state DC *in vivo*, while BMDC produced by GM-CSF are similar to DC that arise from monocytes under acute inflammatory conditions *in vivo* (*14*). Because Pin1 null mice exhibit defects in DC accumulation under LPS-stimulated inflammatory conditions, we wanted to assess the ability of Pin1 null bone marrow to differentiate in response to GM-CSF. We found that Pin1 null bone marrow exhibited an impaired ability to differentiate into Mac1+ DC in the presence of GM-CSF as well **(Figure 11)**.

To address whether there exists a general defect in the ability of Pin1 bone marrow to differentiate, we also cultured Pin1 null bone marrow cells in the presence of M-CSF, a potent inducer of macrophage differentiation. In contrast to the defects we found in the production of DC, Pin1 null bone marrow was fully capable of differentiating into Mac1+F4/80+ macrophages **(Figure 12)**. Because Pin1 null bone marrow exhibits reduced differentiation into DC, but no impairment in its differentiation into macrophage, we conclude that Pin1 specifically modulates DC differentiation.

Remaining Experiments:

It is important to determine if the defect in splenic mDC accumulation in Pin1 null mice is due to abnormalities other than differentiation, such as decreased proliferation of DC precursors or increased cell death in response to LPS. To address these possibilities *in vivo*, we will co-administer BrdU with LPS (or vehicle) to WT and Pin1 null mice. After 18 hours, splenocytes will then be harvested from spleens and stained with anti-BrdU to quantify the number of proliferating cells. Splenocytes will also be stained with propidium iodide and Annexin V to determine the amount of cell death. Cells will be analyzed by flow cytometry.

We have already identified a defect in the ability of Pin1 null bone marrow to give rise to DC *ex vivo*. We would now like to determine whether those DC that do arise from Pin1 null bone marrow are functionally normal. Function will be assessed by loading WT and Pin1 null BMDC with ovalbumin (ova) and then measuring their ability to activate T cells isolated from T cell receptor (TCR) transgenic mice, which have been engineered to produce T cells that express an ova-specific TCR. T cell activation will be determined by measuring T cell proliferation and the secretion of the T cell-derived cytokine IL-2.

Stat3 and β-catenin have both been identified as important regulators of DC differentiation (*15-16*). Because Pin1 has previously been reported to regulate these two proteins, we plan to determine if they are altered in our model of bone marrow differentiation (*17-18*). We will generate BMDC from whole bone marrow and then perform western blots (WB) to determine the level of total and phosphorylated Stat3 and β-catenin. Additionally, we will use rt-qPCR to measure the induction of Stat3 and β-catenin target genes, such as Socs3 and VEGF. Because it may be difficult to detect changes in a mixed cell population of which mature DC comprise only a small percentage, we will also deplete bone marrow of lineage positive cells and then stimulate the cells acutely with Flt3L or GM-CSF and measure the induction of Stat3 and β-catenin phosphorylation by WB.

REVISED TASK 3: Determine the ability of Pin1 to influence breast tumor formation through regulation of the inflammatory response

Remaining Experiments:

Studies have shown that patients with breast tumors, as well as other solid tumors, often exhibit decreased numbers of mature DCs and instead accumulate immature DCs in their peripheral blood and lymph nodes (*19-20*). It has also been documented that the DCs that do arise in breast cancer patients are functionally impaired (*5, 21-22*). Not only do DCs from cancer patients fail to upregulate the co-stimulatory molecules necessary to activate T cells, those that retain an immature or undifferentiated phenotype are actually capable of inducing T cell tolerance (*20*).

In addition to exhibiting defects in the DC compartment, breast cancer patients also accumulate myeloid-derived suppressor cells (MDSC) (*3*). These cells utilize multiple mechanisms to suppress T cell activation and anti-tumor responses, thereby becoming a major obstacle in the design of effective breast cancer immunotherapies (*23*). Tumor-bearing mice also produce MDSCs, which are identified by their expression of the granulocyte marker GR-1 and the myeloid marker Mac1 on their cell surface (*24-25*). Their ability to mediate tumor formation has been demonstrated by adoptively transferring MDSCs from tumor-bearing mice into tumor-free mice, where they suppressed host T cell responses and promoted tumor engraftment (*26*).

Our Pin1 null mice harbor two defects that may impact the formation of mammary tumors: decreased DC and increased Mac1+ granular cells. Although these immune alterations correlate with tumor incidence and are not normally detected in healthy individuals, it is possible that individuals already harboring these immune defects would be at a higher risk for developing breast tumors. Additionally, if loss of Pin1 is responsible for giving rise to these defects, we believe that manipulation of Pin1 or its downstream targets could be useful in designing therapies to combat tumor-mediated immune suppression.

We have already observed that our Pin1 null mice are defective in recruiting mature DC to their spleens during LPS-induced inflammation, and that Pin1 null bone marrow does not differentiate normally *ex vivo*. Because the DC that arise in cancer patients are impaired in their ability to activate T cells, we would like to test the ability of freshly isolated Pin1 null DC to induce T cell activation. We will isolate CD11c+ splenic DC directly from the spleens of WT and Pin1 null mice, load them with ovalubumin (ova), and then co-culture them with ova-specific T cells from transgenic mice. T cell activation will be measured by T cell proliferation and the production of the T-cell-derived cytokine IL-2.

MDSC isolated from humans and mice have been shown to inhibit T cell activation *ex vivo*. We will measure the ability of Pin1 null, Mac1+ granular cells to suppress or inhibit T cell activation by isolating Mac1+ granular cells from the spleens of WT and Pin1 null mice and adding them into culture with ova-specific T cells that have been activated either by non-specific CD3 ligation or by prior culture with WT ova-loaded DC. T cell activation will again be assayed by measuring T cell proliferation and IL-2 production.

To understand how the immune alterations of our Pin1 null mice affect breast cancer development, we will induce mammary tumors in our mice using the EO771 tumor cell line. EO771 cells are derived from a spontaneously occurring estrogen receptor positive mammary adenocarcinoma that developed in a C57BL/6 mouse. When implanted into the mammary fat pads of syngeneic C57BL/6 mice, these cells will give rise to highly invasive mammary tumors that eventually metastasize to the lung (*27*). In order to follow the development of mammary

tumors *in vivo*, we will first transduce the EO771 cells with a luciferase reporter before injecting them into the mammary fat pad of WT or Pin1 null mice. This will allow us to track tumor growth via bioluminescent imaging. Additionally, tail vein bleeds will be carried out every two weeks following tumor cell inoculation, and blood cells will be stained and analyzed by flow cytometry to determine the numbers of circulating CD11b+GR1+ MDSC and CD11c+ DC. Upon sacrifice, tumors will be removed, embedded in paraffin, and stained with hematoxylin and eosin (H&E) to determine tumor type and the extent of immune cell infiltration. Additionally, spleens will be removed and both DC and MDSC will be purified and assayed for their ability to induce or suppress T cell activation, as described above.

KEY RESEARCH ACCOMPLISHMENTS

- Pin1 modulates the accumulation of splenic mDC in response to LPS challenge *in vivo*.

- Pin1 regulates the production of Mac1+ granular cells in the spleens of mice.

- Pin1 plays a specific role in the differentiation of bone marrow into DC, but not macrophages.

REPORTABLE OUTCOMES

The Role of Pin1 in the Immune Response. Barberi TJ, Racioppi L, and Means AR. Oral presentation at the 18th Annual Graduate Student Symposium in Biological Sciences, Duke University, Durham, NC (November 13, 2009).

The Role of Pin1 in the Immune Response. Barberi TJ, Racioppi L, and Means AR. Poster presentation at the Department of Pharmacology and Cancer Biology Annual Retreat, Duke University, Durham, NC (September 25, 2009).

CONCLUSION

We have identified defects in the immune composition of Pin1 null mice. The loss of Pin1 leads to an abnormal accumulation of Mac1+ granular cells in the spleens of mice under both steady-state and inflammatory conditions. Additionally, we find that Pin1 null mice fail to accumulate mDC in their spleens after LPS challenge. We believe that these defects may be due, in part, to an inability to undergo normal DC differentiation when Pin1 is absent. This is supported by experiments indicating that Pin1 null bone marrow is defective in producing DC when cultured in the presence of either Flt3L or GM-CSF, despite being fully capable of producing macrophages when cultured with M-CSF.

Tumor-induced immune suppression is a well-documented phenomenon that not only supports tumor survival, but also impedes the efficacy of breast cancer immunotherapies (*24, 28*). Immune suppression is achieved by both the inhibition of DC differentiation as well as the accumulation of MDSC, two changes that ultimately function to reduce T cell-mediated anti-tumor responses (*23, 29*). Because our Pin1 null mice harbor immune system defects resembling

those found in tumor-bearing patients, we believe that Pin1 may play a role in modulating the immune response to mammary tumor formation. If determined to be the case, manipulation of Pin1 and/or its targets may prove useful for reversing tumor-mediated immunosuppression in breast cancer patients.

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APPENDICES

None.

SUPPORTING DATA

Figure 1. IL-6 Secretion is Variable in MEFs Derived From Different Embryos

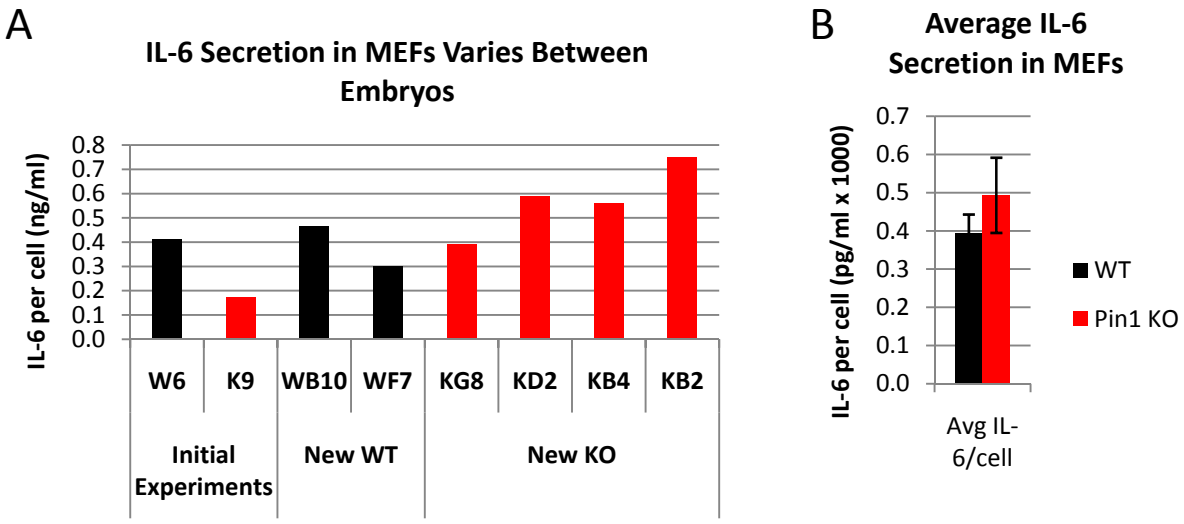

Figure 1. A) and B) MEFs were seeded at 400,000 cells/well into each well of a 6 well plate and rested for 24 hours. Cells were then stimulated with 0.1ug/ml LPS, and media was collected after 6 hours. IL-6 protein secretion was quantified by IL-6 ELISA and normalized by the number of cells present at the end of the experiment. **B)** Individual IL-6 levels from **A)** were averaged and graphed. Error bars indicate the standard error.

Figure 2. Pin1 null Bone Marrow-Derived Macrophages Exhibit a Normal Response to LPS

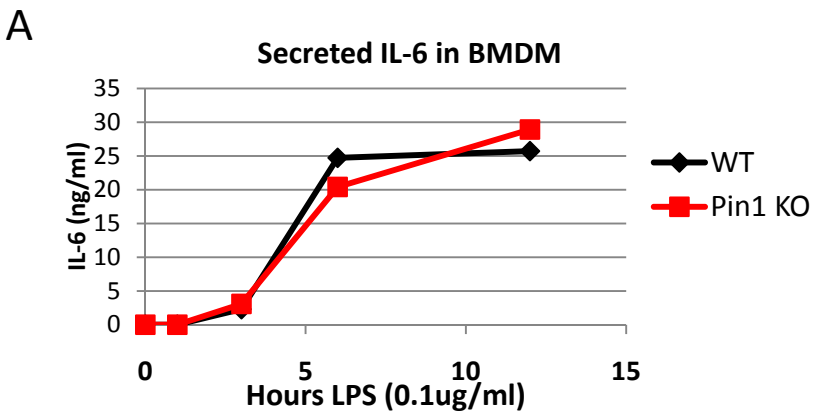

Figure 2. Pin1 null Bone Marrow-Derived Macrophage Exhibit a Normal Response to LPS

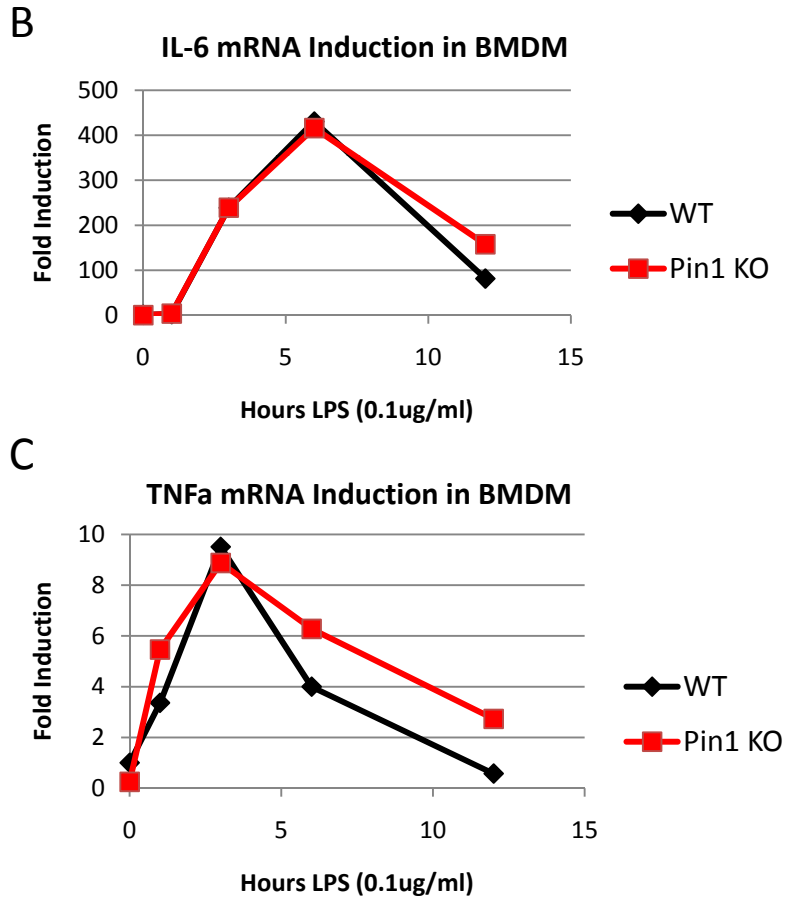

Figure 2. Bone marrow was isolated from femurs and tibia and plated into 6 well plates at $2X10^6$ cells/well in the presence of L929-conditioned media containing M-CSF for 7 days. Non-adherent cells were removed and adherent cells (macrophages) were stimulated with 0.1ug/ml LPS for 0 to 12 hours. **A) and B)** Media was collected and secreted IL-6 was measured by ELISA. **C) and D)** RNA was isolated from adherent cells, reverse transcribed into cDNA, and rt-qPCR was used to quantify IL-6 and TNFa mRNA expression. Values were normalized by Cyclophilin A expression.

Figure 3. Pin1 null Peritoneal Macrophage Exhibit a Normal Response to LPS

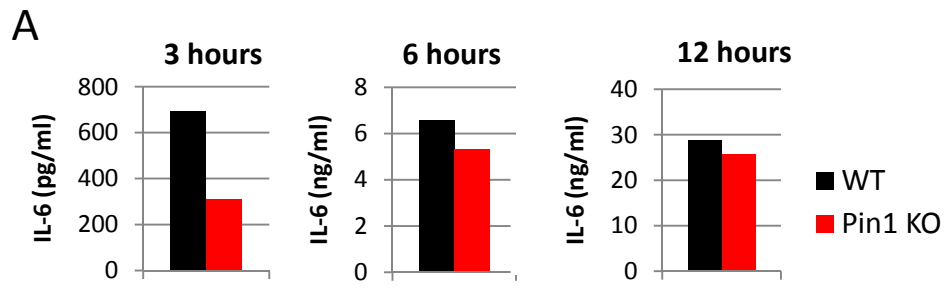

Figure 3. Pin1 null Peritoneal Macrophage Exhibit a Normal Response to LPS

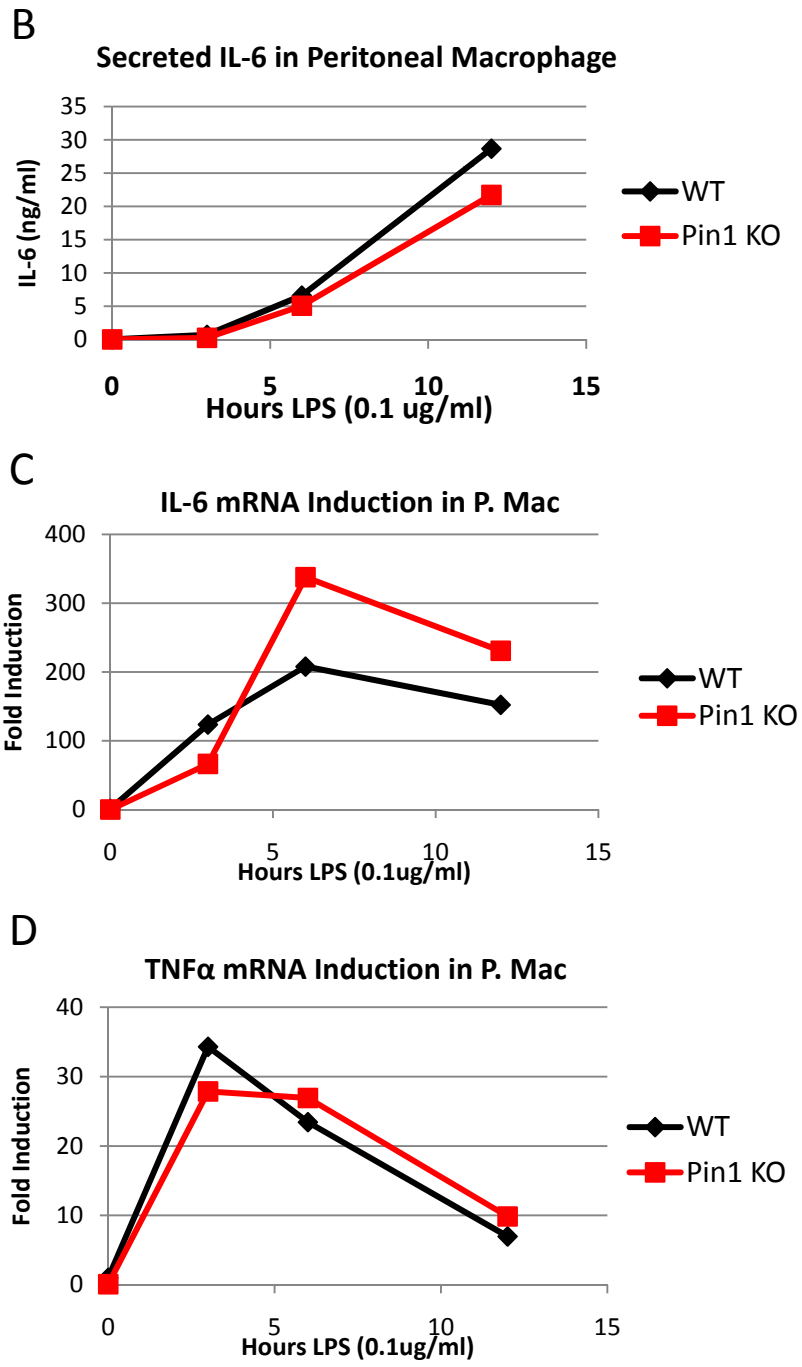

Figure 3. Peritoneal cells were isolated by peritoneal flush and put into culture in 6 well dishes. After resting 24 hours, non-adherent cells were removed and the remaining adherent cells were stimulated with 0.1ug/ml LPS for 0 to 12 hours. **A) and B)** Media was collected and secreted IL-6 was measured by ELISA. **C) and D)** RNA was isolated from adherent cells, reverse transcribed into cDNA, and rt-qPCR was used to quantify IL-6 and TNFa mRNA expression. Values were normalized by Cyclophilin A expression.

Figure 4. Peritoneal cavity of Pin1 null mice contains granulocytes and macrophages.

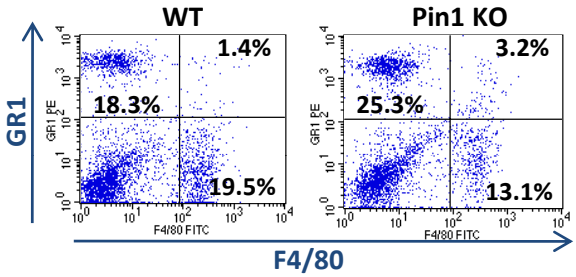

Figure 4. Peritoneal cells were isolated by peritoneal flush and stained for the granulocyte marker GR1 and the macrophage marker F4/80. Granulocytes are identified as GR1+F4/80- cells, and macrophages are identified as GR1-F4/80+ cells. Percentages represent the percentage of total Mac1+ cells.

Figure 5. Pin1 null mice accumulate granular cells in their spleens.

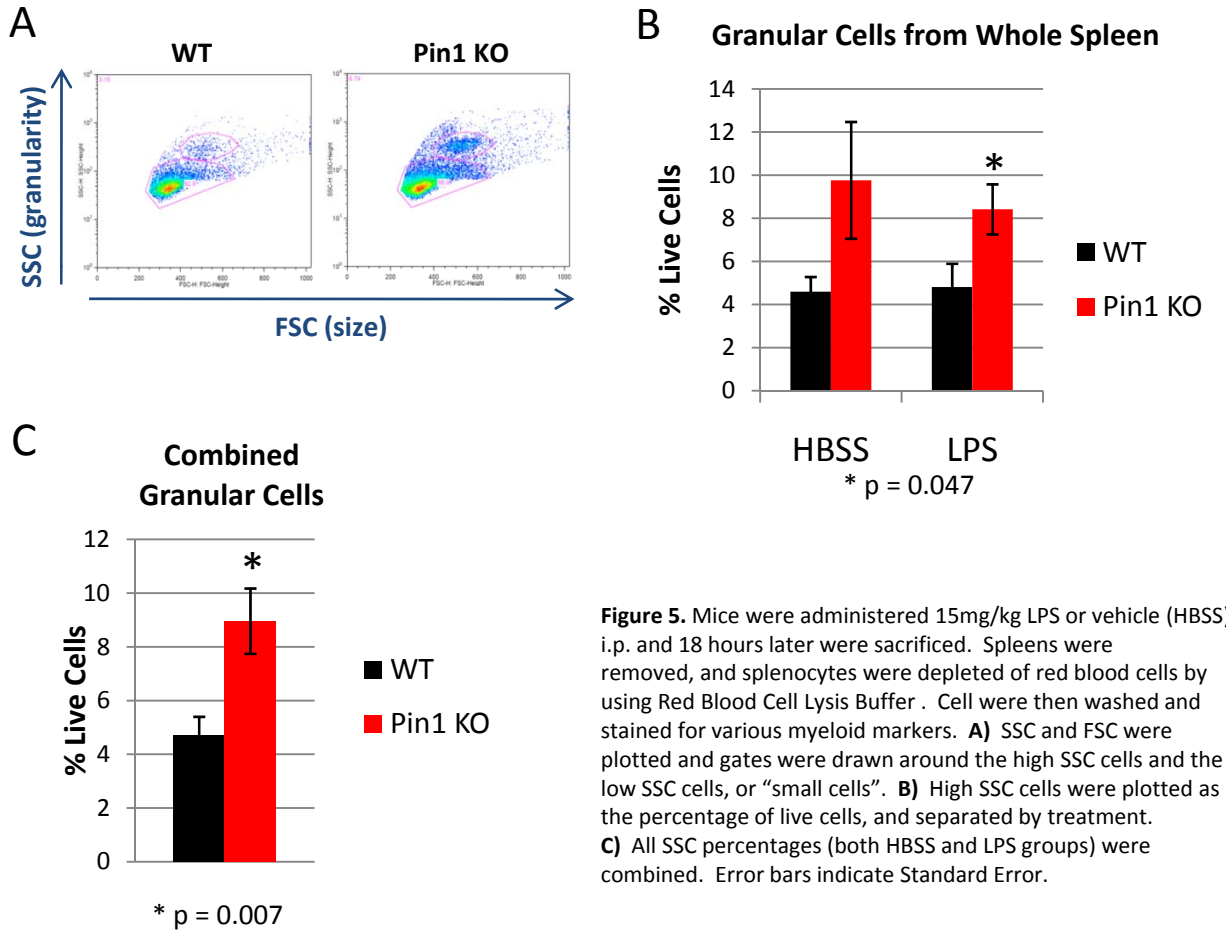

Figure 5. Mice were administered 15mg/kg LPS or vehicle (HBSS) i.p. and 18 hours later were sacrificed. Spleens were removed, and splenocytes were depleted of red blood cells by using Red Blood Cell Lysis Buffer . Cell were then washed and stained for various myeloid markers. **A)** SSC and FSC were plotted and gates were drawn around the high SSC cells and the low SSC cells, or "small cells". **B)** High SSC cells were plotted as the percentage of live cells, and separated by treatment. **C)** All SSC percentages (both HBSS and LPS groups) were combined. Error bars indicate Standard Error.

Figure 6. Granular cells in the spleen of Pin1 null mice account for the increase in total numbers of splenic B220-Mac1+ cells

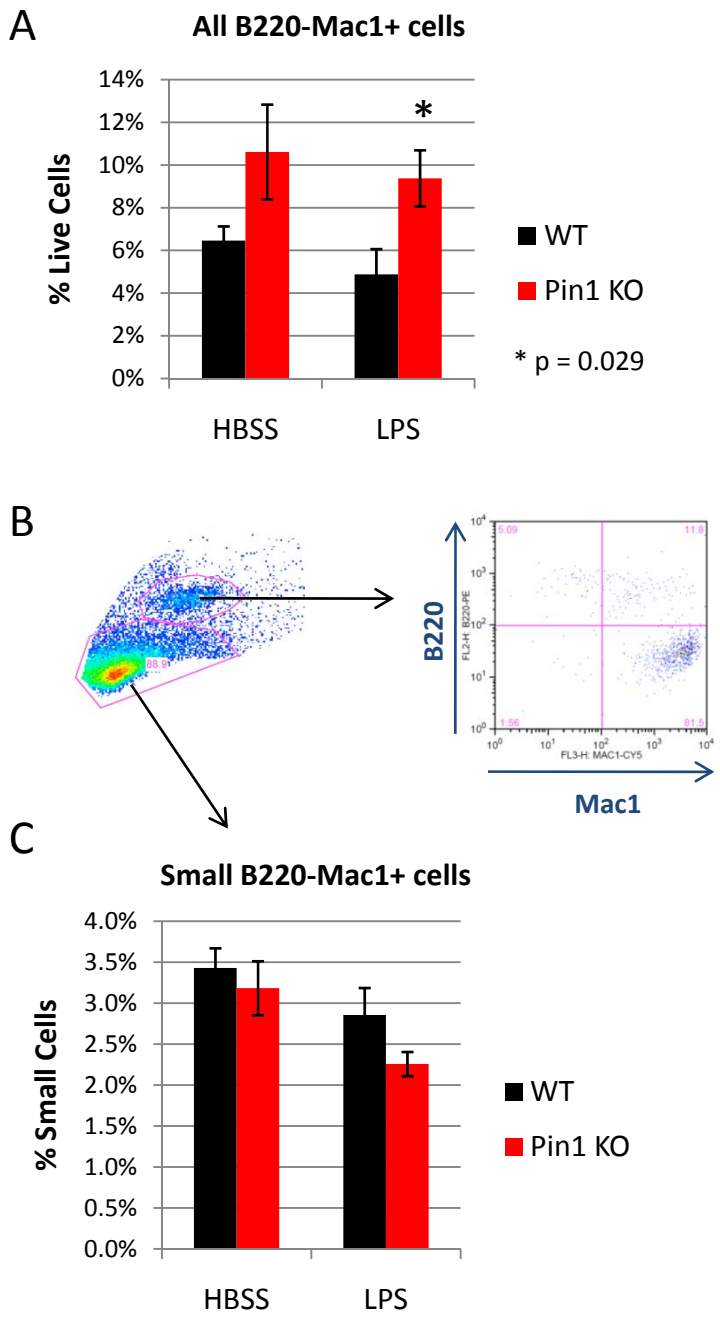

Figure 6. Mice were administered 15mg/kg LPS or vehicle (HBSS) i.p. and 18 hours later were sacrificed. Spleens were removed, and splenocytes were depleted of red blood cells by using Red Blood Cell Lysis Buffer . Cells were then washed and stained for various myeloid markers. **A)** B220-Mac1+ cells were averaged and plotted as the percentage of total live cells. **B)** High SSC cells were analyzed for the expression of B220 and Mac1 and found to be predominantly B220-Mac1+ cells. **C)** When high SSC cells are eliminated from analysis, the increase in B220-Mac1+ observed in total Pin1 null splenocytes in **A)** is eliminated. Error bars indicate Standard Error.

Figure 7. Loss of Pin1 impairs splenic mDC accumulation in response to LPS

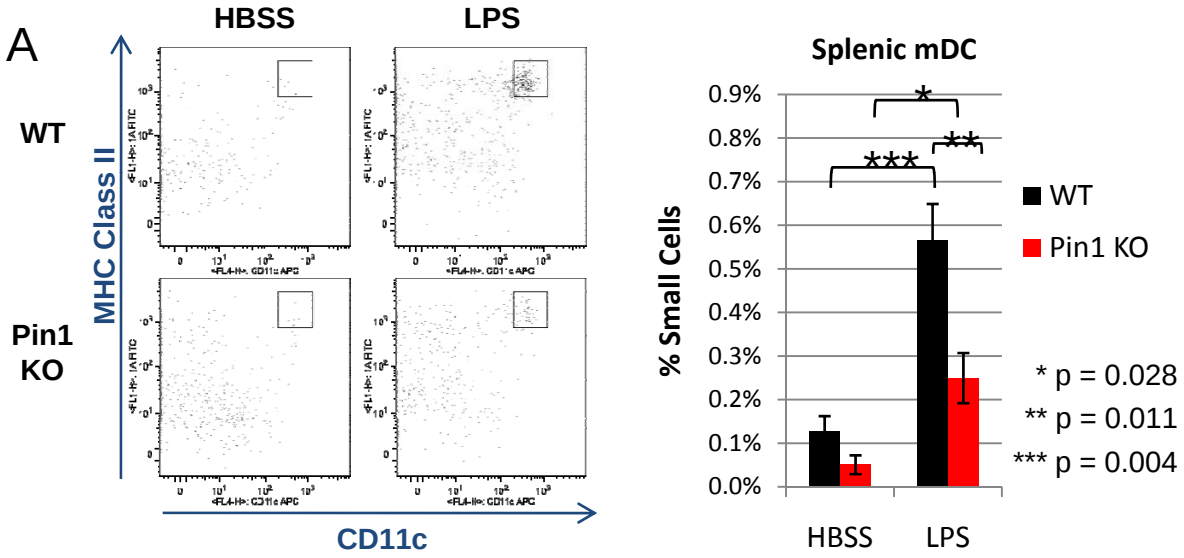

Figure 7. Mice were administered 15mg/kg LPS or vehicle (HBSS) i.p. and 18 hours later were sacrificed. Spleens were removed, and splenocytes were depleted of red blood cells by using Red Blood Cell Lysis Buffer . Cells were then washed and stained. Splenic mDC are plotted as a percentage of the small SSC cells (indicated in Figure 6) to avoid skewing based on increased numbers of granular cells. Error bars indicate Standard Error.

Figure 8. Pin1 null bone marrow has normal numbers of progenitors.

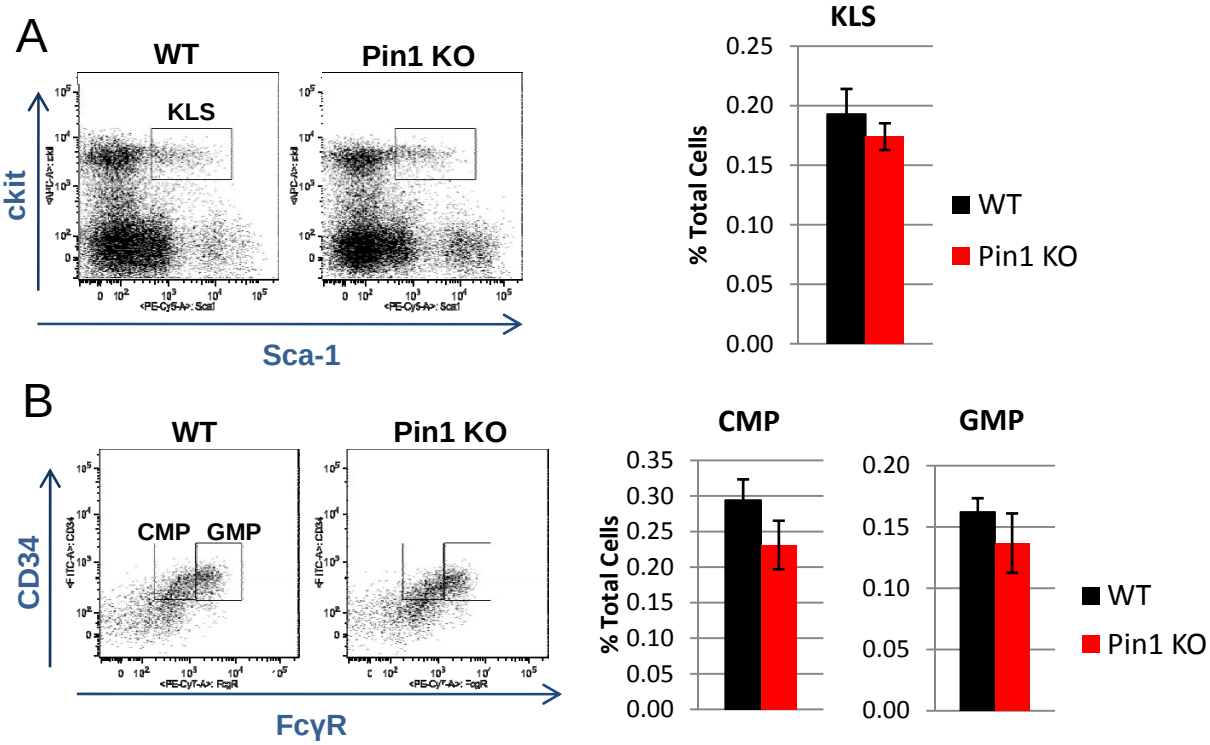

Figure 8. Pin1 null bone marrow has normal numbers of progenitors.

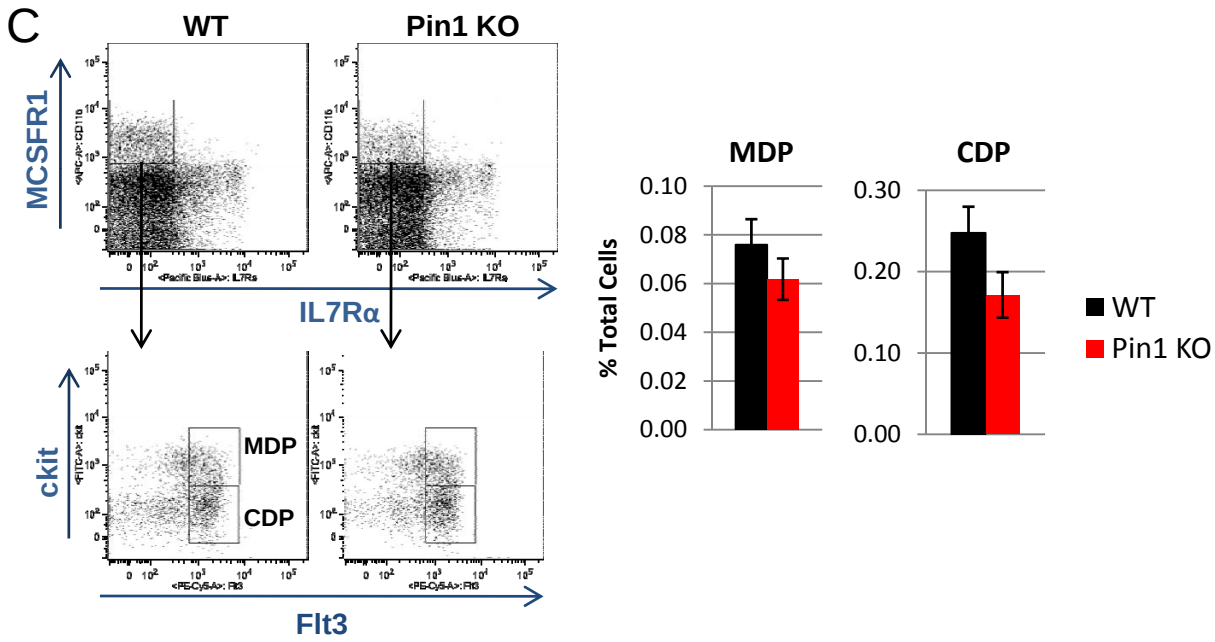

Figure 8. A-C) Mice were sacrificed and femurs and tibia were removed . Marrow was flushed out of the bones and red blood cells were lysed with Red Blood Cell Lysis Buffer. Cells were then washed, stained, and analyzed. Error bars indicate Standard Error.

Figure 9. Pin1 null bone marrow is defective in its ability to give rise to BMDC when cultured with Flt3L

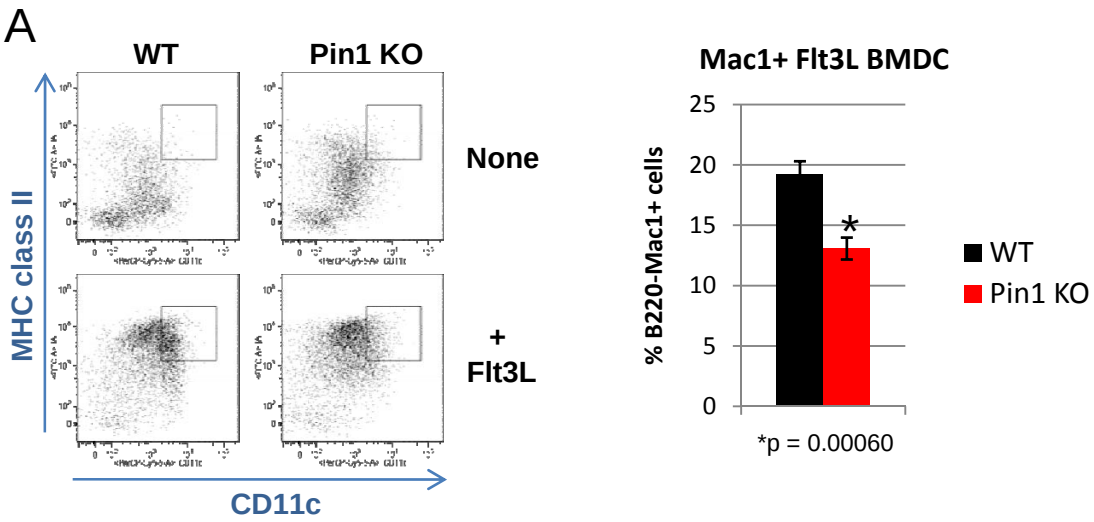

Figure 9. Pin1 null bone marrow is defective in its ability to give rise to BMDC when cultured with Flt3L

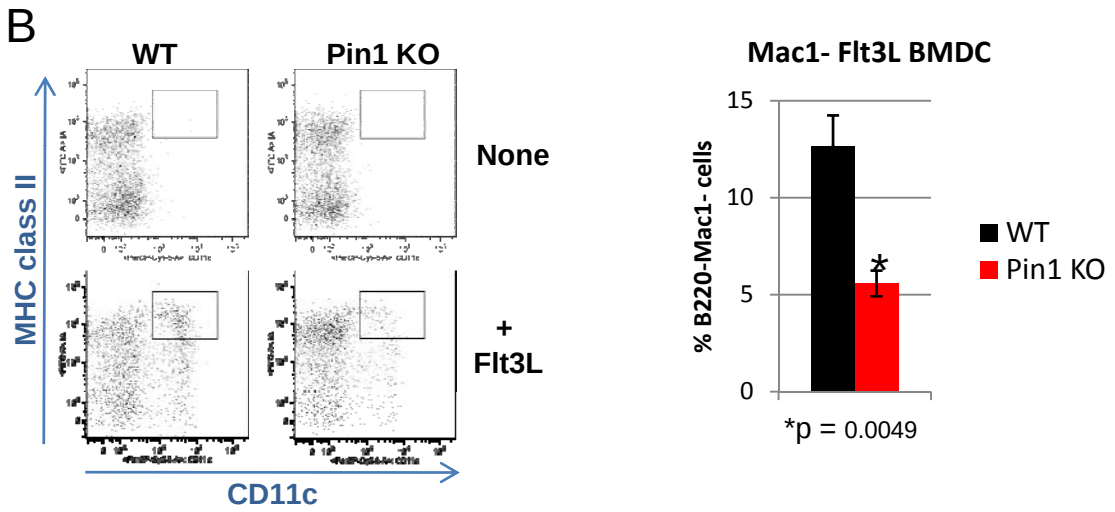

Figure 9. Mice were sacrificed and femurs and tibia were removed . Marrow was flushed out of the bones and red blood cells were lysed with Red Blood Cell Lysis Buffer. Cells were then washed and plated into 6 well dishes at 2 million cells/ml in the presence of 50ng/ml Flt3L for 9 days. Every 3 days, 2/5 of the media was removed and replaced with fresh media containing Flt3L. On day 9, non-adherent cells were removed, washed, and stained. Error bars indicate Standard Error.

Figure 10. Defects in the production of BMDC in Pin1 null bone marrow is not a consequence of changes in surviving cell numbers or the incidence of cell death.

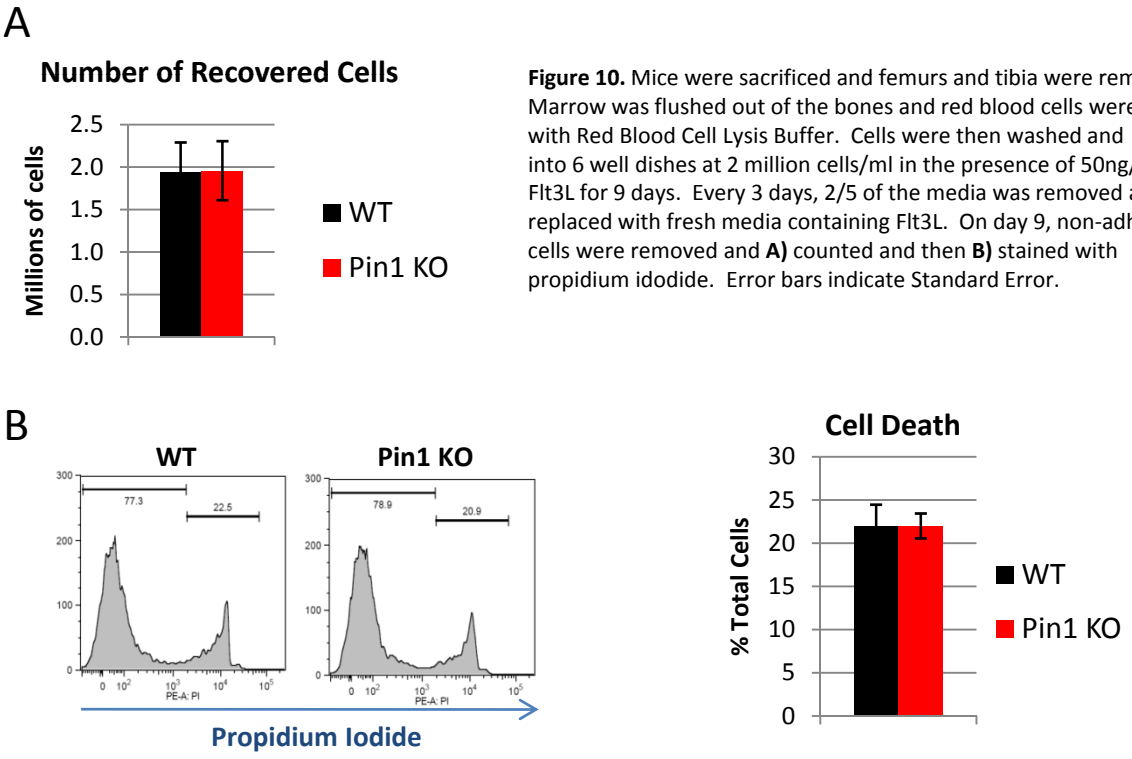

Figure 10. Mice were sacrificed and femurs and tibia were removed . Marrow was flushed out of the bones and red blood cells were lysed with Red Blood Cell Lysis Buffer. Cells were then washed and plated into 6 well dishes at 2 million cells/ml in the presence of 50ng/ml Flt3L for 9 days. Every 3 days, 2/5 of the media was removed and replaced with fresh media containing Flt3L. On day 9, non-adherent cells were removed and **A)** counted and then **B)** stained with propidium idodide. Error bars indicate Standard Error.

Figure 11. Pin1 null bone marrow is defective in producing BMDC when cultured in the presence of GM-CSF

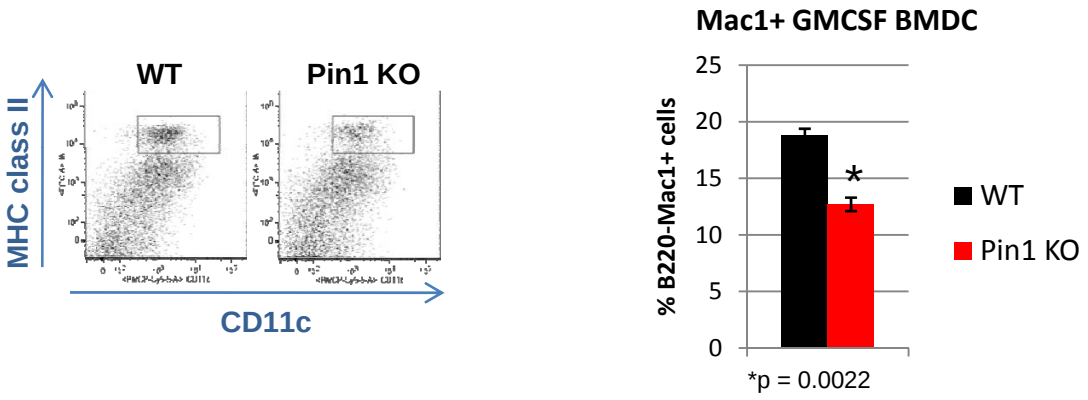

Figure 11. Mice were sacrificed and femurs and tibia were removed . Marrow was flushed out of the bones and red blood cells were lysed with Red Blood Cell Lysis Buffer. Cells were then washed and plated into 6 well dishes at 2 million cells/ml in the presence of 5ng/ml GM-CSF for 5 days. On day 3, 2/5 of the media was removed and replaced with fresh media containing GM-CSF. On day 5, non-adherent cells were removed, washed, and stained. Error bars indicate Standard Error.

Figure 12. Pin1 null bone marrow is capable of producing BMDM in response to M-CSF

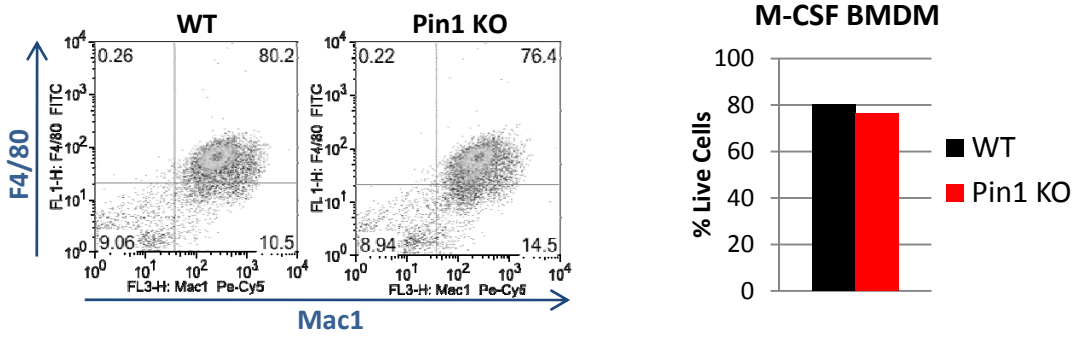

Figure 12. Mice were sacrificed and femurs and tibia were removed . Marrow was flushed out of the bones and red blood cells were lysed with Red Blood Cell Lysis Buffer. Cells from 3 WT mice and 3 Pin1 KO mice were pooled together and plated into 6 well dishes at 2 million cells/ml in the presence of 15ng/ml M-CSF for 7 days. On day 7, adherent cells were collected and stained. Because cells were pooled from multiple mice, no statistical analysis was performed.