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Daniel Zhang April 10, 2024

The Impact of *Ptpn11*(Shp2)E76K Mutation on Macrophage Phagocytosis

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Abstract

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Juvenile myelomonocytic leukemia (JMML) is an aggressive pediatric myeloproliferative disorder characterized by the overproduction of myelomonocytic cells, with approximately 34% of non-syndromic JMML cases arising from somatic mutations in *Ptpn11*, the gene encoding SHP-2. (Tartaglia et al. 2003; Loh et al. 2004) Macrophage-mediated phagocytosis is essential for the immune system's response to infections and the clearance of cellular debris. This study investigates the phagocytic activity of bone marrow-derived macrophages (BMDMs) from wild type and Shp2 E76K mice.

Macrophage-mediated phagocytosis is essential for the immune system's response to infections and the clearance of cellular debris. This study evaluated the phagocytic activities of BMDMs from wild type and Shp2 E76K mice against lineage-negative (Lin-) cells, HL60 cells, and E. coli particles utilizing a flow cytometry-based assay. BMDMs were cultivated in DMEM enriched with L-cell conditioned medium to promote differentiation. The phagocytic response was analyzed by exposing macrophages to varying ratios of Lin- and HL60 cells, as well as E. coli BioParticles.

Our findings revealed that WT macrophages exhibited a significantly greater phagocytic rate towards HL60 cells and E. coli, highlighting their pivotal role in immune defense and the facilitation of antigen presentation, essential for triggering adaptive anti-tumor immunity. In contrast, the *Ptpn11*E76K mutation in macrophages did not significantly affect their phagocytic capacity against Lin- progenitor cells. Notably, it was not the mutations in the macrophages, but rather the Lin- target cells harboring the *Ptpn11*E76K mutation, that led to increased phagocytosis, suggesting an enhanced phagocytic attraction or identification due to the mutation. We hope to deepen our understanding of leukemia pathogenesis through the thorough exploration of these findings.

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Introduction

The aggressive children myeloproliferative disorder known as juvenile myelomonocytic leukemia (JMML) is distinguished by an excess production of myelomonocytic cells. (Cai et al. 2020) The progenitor colonies in JMML patients feature monocytic cells at all stages of differentiation, ranging from blast forms to promonocytes, monocytes, and macrophages. This unique trait suggests that JMML is not caused by a total halt in differentiation, as seen in acute leukemias. Rather, it arises from a diversion of hematopoietic differentiation towards the monocytic lineage. (Chan et al. 2009) 34% of non-syndromic JMML is caused by somatic mutations in *Ptpn11*. (Tartaglia et al. 2003) Gain-of-function mutations in *Ptpn11* can disrupt mitosis and cytokinesis, leading to chromosomal instability and significantly heightened risk of DNA damage-induced cancers. Shp2 localizes to key cellular structures involved in mitosis, including the kinetochore, centrosome, spindle midzone, and midbody and mouse embryonic fibroblasts harboring *Ptpn11* gain-of-function mutations exhibit a weakened mitotic checkpoint. (Liu et al. 2016) The most prevalent and active Shp2 mutation is the E76K gain of function mutation. (Zhang et al. 2018) Ptpn11, also known as Shp-2, encodes for 2 N-terminal Src homology 2 domains (N-SH2) and a non receptor protein tyrosine phosphatase (PTP) that is involved in relaying signals of downstream of growth factor receptors to control a variety of responses, such as migration, differentiation, and proliferation. (Feng 1999) Typically, the PTP domain's catalytic site is blocked by N-SH2 and is only activated when ligand binding interrupts this inhibitory interaction leading to increased Ras/MAPK pathway activation. In JMML, *Ptpn11* mutations interfere with the inhibitory interaction between N-SH2 and PTP domains leading to increased access to the catalytic site and a gain of function. (Chang et al. 2014) SHP-2's function in transmitting signals from hematopoietic growth factor receptors to Ras and research linking hyperactive Ras to the development of JMML pinpoint *Ptpn11* as the gene that may be mutated in JMML cases without anomalies in RAS. (Loh et al. 2004)

Macrophages are indispensable to the body's immune defenses, orchestrating a range of responses from pathogen elimination to lymphocyte regulation. They showcase their protective prowess through the

phagocytosis of parasites and microbes, a fundamental process in immune defense. Beyond this, macrophages are central to activating and proliferating lymphocytes, including T- and B-cells, upon encountering antigens and foreign cells. This ability underscores their critical role in both the innate and adaptive immune responses. (Elhelu 1983) Originating from progenitors in the yolk sac and fetal liver during mouse embryonic development, macrophages diversify into self-sustaining, tissue-resident populations across various organs. After birth, monocytes from the bone marrow are brought in to supplement these resident groups and respond to additional needs arising from inflammation, infection, and changes in metabolism. Despite differences in their origins and locales, all macrophages engage in mRNA and protein synthesis, fulfilling specific and systemic functions vital for organ health and immune defense. (Gordon and Martinez-Pomares 2017)

The act of engulfing pathogens signifies the initiation of the body's primary immune mechanism by macrophages, paving the way for a sophisticated adaptive immune response. They employ a selective array of receptors, such as the mannose receptor, to distinguish between harmful invaders and the body's cells, targeting universal pathogen markers. Pathogens become targets for phagocytosis either through broad coating by complement proteins or specific tagging by antibodies, facilitated by complement and Fc receptors. (Aderem and Underhill 1999) Macrophages can also directly bind and ingest bacteria. By recognizing a broad spectrum of bacterial components, receptors facilitate the precise identification and elimination of pathogens. (Peiser et al. 2000) Macrophages also exhibit function in cancer by helping as important immune effectors for therapeutic antibodies. They are equipped with different types of Fcγ receptors, positioning them as powerful cells capable of eradicating tumors through antibody-dependent phagocytosis. It has shown that the ability of macrophages to engulf and digest cancer cells is a key mechanism behind the effectiveness of numerous antibodies that are sanctioned for cancer treatment. (Weiskopf and Weissman 2015)

Building on the fundamental understanding of macrophages' role in immune defense and their capacity for phagocytosis, we were interesting in the effects of the *Ptpn11*(Shp2)E76K mutation on macrophage phagocytic activity, particularly in the context of bacterial and cancer cell targets. To this

end, E. coli and HL60 cells, representing common bacterial pathogens and human leukemia cells respectively, were chosen as targets to assess the mutation's impact. The selection of these targets allowed for a comprehensive evaluation of the macrophages' ability to engulf and destroy both microbial invaders and malignant cells.

Hematopoietic stem cells (HSCs) are undifferentiated, self-renewing cells capable of giving rise to all blood cell types, while lineage-negative (Lin-) cells are a more differentiated subset that excludes cells committed to specific blood cell lineages, but they are still pluripotent. In this study, Lin- cells were chosen as the target for assessing macrophage phagocytic activity due to their close resemblance to HSCs, which are sparse in availability. Most HSCs are dormant or in a quiescent condition. (Cheng et al. 2000) During steady-state hematopoiesis, only a tiny percentage of HSCs enter the cell cycle to either self-renew or differentiate into new progeny. (Wright et al. 2001) For hematopoietic regeneration and long-term hematopoiesis to be maintained, there must be a delicate balance between quiescence and activation in this cell pool. When stem cells lose their quiescence or dormancy, abnormal activation and an increase in apoptosis follow, which over time can lead to stem cell exhaustion and impairments in repopulation capacity. (Nakamura-Ishizu et al. 2014)

In a zebrafish embryo model, macrophages make contact with stem cells as soon as they enter the bone marrow niche and probe their cell surfaces to detect stress. Macrophages engulf and kill stem cells with high levels of a stress-activated protein. Hematopoietic stem and progenitor cell proliferation was reduced by ERK/MAPK inhibition without reducing macrophage interactions. (Wattrus et al. 2022) Macrophages exert quality control over which specific stem cells produce everlasting blood production by controlling death and division. (Hagedorn et al. 2021) It was also found that activating mutations in *Ptpn11* within the mouse bone marrow environment significantly promote myeloproliferative neoplasm (MPN) development and progression by adversely affecting HSCs. Specifically, these mutations in mesenchymal stem/progenitor cells and osteoprogenitors attract monocytes that in turn hyperactivate HSCs via interleukin-1β and other cytokines, intensifying MPN. (Dong et al. 2016)

Building on this, the experimental design focused on dissecting the nuances of macrophage-stem cell interactions, particularly how macrophages with the *Ptpn11*E76K mutation compared to their wild type (WT) counterparts in engaging with target cells possessing the same genetic variances. The aim was to not only discern differences in phagocytic activity between mutated and non-mutated macrophages but also to understand how these macrophages affect and interact with Lin- target cells that are either WT or *Ptpn11*E76K mutated. This comparative approach was intended to shed light on the variances in phagocytic behavior that could be directly attributed to the genetic configurations of both the immune cells and their targets, providing insights into how genetic mutations influence the immune system's ability to respond to malignancies and infections.

Methods

Culturing of Bone Marrow-Derived Macrophages (BMDMs)

Preparation of L-Cell Conditioned Medium:

L929 cells were cultured in RPMI medium supplemented with 10% FBS. Upon reaching confluence, the supernatants were collected, filtered to remove any cellular debris, and aliquoted into 50 ml tubes. These aliquots were then stored at -80°C until needed for the macrophage culture medium.

Harvesting and Plating:

Bone marrow cells were harvested from the femurs and tibias of mice and cultured to differentiate into bone marrow-derived macrophages (BMDMs) using a protocol optimized for promoting macrophage growth. The medium used for culturing consisted of DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) to prevent bacterial contamination. Additionally, 20% L-cell conditioned medium was included to provide colony-stimulating factor-1 (CSF-1) derived from L929 cells, an essential factor for macrophage differentiation.

The entire bone marrow from a mouse was harvested and then placed into a single 100 mm culture dish. The cells underwent overnight culture to facilitate attachment. On the subsequent day, non-adherent cells were collected and transferred to a new 100 mm dish, a method that effectively isolated progenitor cells from the adherent phagocytes and fibroblasts that remained on the original culture dish. To ensure optimal growth conditions and nutrient availability, the culture medium was refreshed every three days. Once the cells reached confluency, they were split, with the adherent cells being detached using a cell lifter, and then gently resuspended by pipetting to achieve a homogeneous cell suspension. The cultured macrophages were then allowed to differentiate for a period ranging from 7 to 14 days, with phagocytosis assays primarily conducted on the eighth day.

Phagocytosis Assay and Flow Cytometry of BMDMs with Lineage-Negative Cells

On Day 0, the procedure commenced with the isolation of lineage-negative (Lin-) cells from both WT and Shp2 E76K mice, utilizing the mouse lineage depletion kit (MACS) for the purpose. Following this, mouse bone marrow (BM) was prepared by harvesting BM from the hind limbs of mice. This was achieved using a 3 ml syringe fitted with a 22G needle, which flushed 3 ml of PBS into a 15 ml culture tube. The resulting cell suspension underwent centrifugation for 8 minutes at 300g, after which the supernatant was carefully aspirated away. The cell pellet was then gently resuspended in 1 ml of RBC lysing buffer and incubated for 1 minute. Subsequently, 4 ml of PBS was added, and after another round of centrifugation at 1300 rpm for 5 minutes, the supernatant was completely aspirated, leaving behind the prepared cells.

In addition, preparations were made for the buffers and solutions required for the next steps. A staining buffer was prepared using PBS, which was then supplemented with 2% FBS and 2 mM EDTA, and kept on ice until needed. The Red Blood Cell (RBC) Lysis Solution was formulated by mixing 0.15 M ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA, readying all necessary components for the experimental procedures that were to follow.

Lineage Depletion Using Magnetic Beads

The cell pellet was initially resuspended in 30 µl of staining buffer, with careful attention paid to breaking up any clumps that had formed. To this suspension, 10 µl of a Biotin-Antibody Cocktail from the Miltenyi Biotec Lineage Cell Depletion Kit was added. This cocktail is composed of biotin-conjugated monoclonal antibodies targeting CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), and Ter-119, ensuring a broad spectrum of cell lineage identification. Following this addition, the cell-antibody mixture was incubated for 15-30 minutes at a temperature of 4°C to allow for sufficient antibody binding to the targeted cell surface markers.

After incubation, the cells were washed with 300µl of PBS and centrifuged at 300g for 5 minutes to remove any unbound antibodies. The pellet was then resuspended in an additional 35 µl of staining buffer, and 15 µl of Anti-Biotin MicroBeads were added. This mixture underwent a further incubation period of 20-40 minutes at 4°C, allowing the MicroBeads to bind to the biotinylated antibodies attached to the cells. Following another washing step with 300µl of PBS and centrifugation at 1300 rpm for 5 minutes, the cell pellet was resuspended in 500 µl of staining buffer.

This prepared cell suspension was then applied to an LS column positioned within a magnetic field, a step designed for the negative selection of cells. The lineage-negative cells were collected as the flow-through from the column, with a typical yield being around 1x10^6 cells per mouse. This process effectively isolated the desired lineage-negative cells for further experimental analysis.

Plating of Bone Marrow-Derived Macrophages (BMDMs):

Bone marrow-derived macrophages (BMDMs) were initially seeded at a density of 1x10^5 cells/ml using 100 µl of DMEM, which was supplemented with 20% L-cell conditioned medium (this medium is referred to as the starving medium) into a 96-well plate. This setup was done 16 hours prior to the start of the phagocytosis assay to ensure that the BMDMs had adequate time to adhere and condition within the starving medium.

On Day 1, the lineage-negative (Lin-) cells were centrifuged, the media removed, and the cells were then resuspended. Carboxyfluorescein succinimidyl ester (CFSE) was added to the Lin- cells dissolved in PBS, and they were incubated for 10 minutes in a CO2 incubator to label them fluorescently. Following this, the cells were centrifuged at 1300 rpm for 5 minutes and washed three times with PBS to remove excess CFSE. The Lin- cells were counted, diluted in medium without fetal bovine serum (FBS) to achieve the desired density, and 100 µl of this cell suspension was added to the wells containing the BMDMs. This was done to maintain a macrophage to Lin- cell ratio ranging from 1:1 to 1:3.

Before adding the Lin- cells, the medium from the BMDMs was aspirated, and the wells were washed with PBS to prepare for co-culture. Lin- cells suspended in the starving medium were then added to the wells. The co-culture was incubated for 2 hours at 37°C in a CO2 incubator to allow interactions between the BMDMs and Lin- cells.

Post-incubation, the cells were collected into Eppendorf tubes using 100 µl PBS supplemented with 2% FBS and 2mM EDTA, with scraping employed to lift the cells. An additional wash with PBS, FBS, and EDTA was performed, with this wash also being added to the tubes to ensure the collection of all cells. Following centrifugation and the discarding of the supernatant, the cells were stained with macrophage markers CD11b and F4/80 to identify the macrophages in the mixture. Each sample was stained with 100 µl of dye solution and incubated at 4°C for 30-45 minutes.

DAPI (4',6-diamidino-2-phenylindole) diluted in PBS was added at a volume of 150 µl per tube to distinguish dead cells by their inability to exclude the dye. Finally, the stained cells were transferred to a 96-well plate, making them ready for analysis by flow cytometry, which would allow for the detailed examination of the macrophage and Lin- cell interactions and the efficiency of phagocytosis.

Phagocytosis Assay with HL60 Cells

The methodology for culturing BMDMs and subsequent phagocytosis assays described previously was done similarly with HL60 cells as target cells for phagocytosis. The following steps outline the procedures for the preparation of HL60 cells and their co-culture with BMDMs for the phagocytosis assay.

The preparation of HL60 target cells involved culturing the cells to the desired level of confluence in RPMI 1640 medium that was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Once the HL60 cells reached confluence, they were collected and then

prepared for staining with Carboxyfluorescein succinimidyl ester (CFSE), using the identical protocol that was applied for the lineage-negative cells to ensure consistency in the labeling process.

After being labeled with CFSE, the HL60 cells were introduced into the wells containing bone marrow-derived macrophages (BMDMs). This was done using a volume of 100 µl per well, thereby establishing multiple macrophages to HL60 cell ratios. The specific ratios employed were 1:1, 1:2, and 1:3, chosen to evaluate the extent of phagocytic engagement across different cellular proportions.

Phagocytosis Assay with pHrodo™ Green E. coli BioParticles™

For the phagocytosis assay utilizing pHrodo™ Green E. coli BioParticles™, the overall procedure mirrored the one described for CFSE-labeled cells, with modifications to accommodate the unique properties of the BioParticles™. CFSE labeling was not necessary as these particles emit fluorescence upon successful phagocytosis.

Two particle concentrations, 50 mg/ml and 25 mg/ml were added for co-culture with the BMDMs for 2 hours. To avoid artificial acidification of the particles, which could result in false-positive fluorescence, the incubation was carried out at 37 degrees Celsius without the addition of 5% CO2, deviating from the standard cell culture conditions. This adjustment ensured that the fluorescence signal observed was exclusively due to the phagocytosis of the BioParticles™ by the macrophages.

Flow Cytometry Analysis of Macrophage Phagocytosis

Flow cytometry is a tool for assessing various cellular characteristics. In this study a gating strategy was employed to quantify the phagocytic activity of macrophages using flow cytometry. The software allows for the creation of hierarchical gating strategies and provides tools for quantifying populations of interest.

Macrophages were cultured and co-incubated with CFSE-labeled target cells under specific experimental conditions designed to facilitate phagocytosis. Following incubation, cells were washed and stained with a panel of fluorescently labeled antibodies and viability dyes suitable for subsequent flow cytometric analysis.

The flow cytometric analysis followed a sequential gating strategy to accurately identify and analyze macrophages that have phagocytosed target cells:

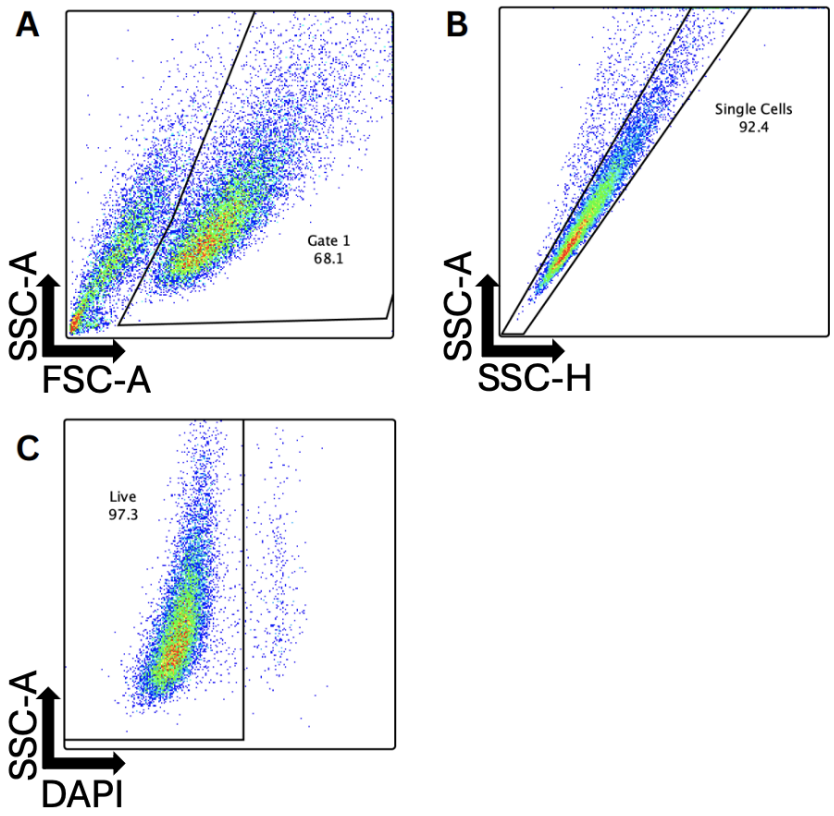

Figure 1. Flow Cytometry Gating Strategy for Cell Size, Singlet Discrimination, and Viability

As illustrated in Figure 1A, The first gating step involved Forward Scatter (FSC) and Side Scatter (SSC) parameters, which differentiate cells based on their size and internal complexity respectively. Larger cells, likely macrophages, can be distinguished from smaller cells and debris.

In Figure 1B, using Side Scatter Height (SSC-H) and Side Scatter Area (SSC-A), singlets were gated to ensure accurate cell counts and to avoid doublets or clusters of cells that could confound the analysis. A linear relationship between SSC-H and SSC-A indicates that a particle is a singlet, or a single cell, because the height and area of the scatter signal should scale proportionally for individual cells passing through the laser beam. This proportionality means that as cells increase in size or granularity, both the height and area of the scatter signal increase in a predictable, linear manner. Non-linear relationships suggest the presence of doublets or clusters. This step is crucial to ensure that events representing phagocytosis are true interactions between a macrophage and a target cell, rather than two cells merely attached to one another.

The cells were then gated based on viability using a DAPI stain detected in the Pacific Blue (PB-450) channel, shown in Figure 1C. DAPI penetrates and stains the DNA of dead cells, allowing for the exclusion of non-viable cells that may exhibit nonspecific fluorescence.

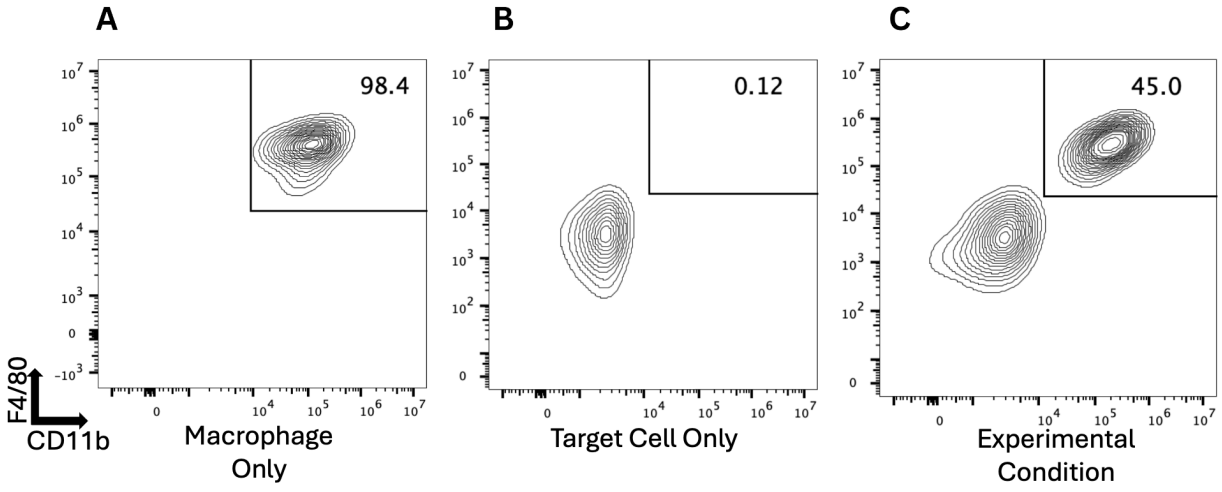

Figure 2. Gating of Phagocytic Macrophage Populations Using CD11b-PCy-7 and F4/80-APC Markers

The macrophage population was identified using two specific markers: CD11b conjugated with PerCP-Cy5.5 and F4/80 conjugated with Allophycocyanin (APC). These markers are used in distinguishing macrophages from other cell types in the samples. As shown in Figure 2A, The top left plot serves as the positive control, showing the expected expression of the markers on macrophages. In contrast, Figure 2B acts as the negative control, demonstrating less expression of these markers on target cells. Figure 1C plot depicts an experimental condition where both populations are present. By comparing the expression levels of CD11b and F4/80 in this plot to the controls, it's possible to accurately gate the macrophages within the experimental sample.

This gating strategy is then consistently applied across all experimental samples to ensure uniformity in the analysis of marker expression on macrophages. By utilizing the defined expression levels of CD11b and F4/80 as benchmarks from the controls, the macrophages are accurately gated within each experimental condition, maintaining a standard methodology for comparative assessment.

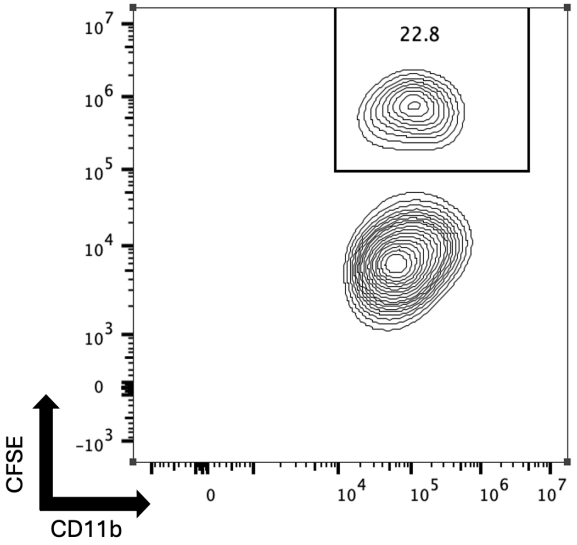

Figure 3. Gating of Phagocytic Macrophages

The final step focused on the functional assessment of phagocytosis. Macrophages that had engulfed CFSE-labeled target cells were identified by their fluorescence in the FITC channel. The percentage of macrophages that had phagocytosed Lin- cells was calculated by comparing the FITC

fluorescence of the sample to a control macrophage population that had not been exposed to CFSE-labeled cells, shown in Figure 3. The percentage of phagocytosis was determined by dividing the number of FITC-positive macrophages by the total number of macrophages and multiplying by 100.

To ensure the comparability of the flow cytometry data across all experimental samples, the gating strategies were standardized. Each gate, once determined, was uniformly applied to every sample within the experiment. This consistent application of gates allows for the direct comparison of results. It ensures that any observed differences in macrophage behavior, such as phagocytic activity, are due to the experimental conditions rather than inconsistencies in sample analysis.

Results

Phagocytosis Efficiency of Wild Type and *Ptpn11*E76K Mutated Macrophages against pHrodo™ Green E. coli BioParticles™

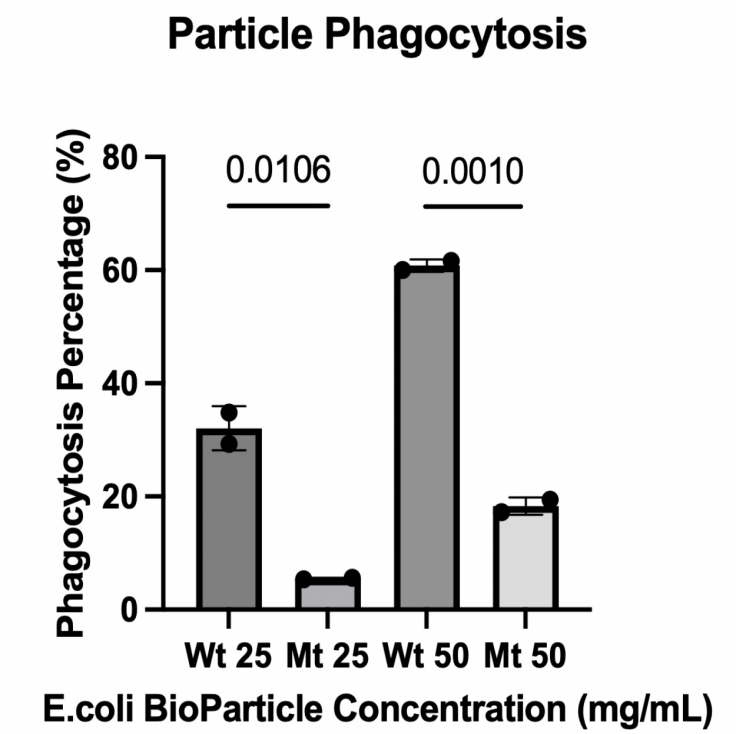

Figure 4. Phagocytic Uptake of pHrodo™ Green E. coli BioParticles™ by Wild Type and *Ptpn11*E76K Mutated Macrophages at Different Particle Concentrations

Macrophages are a critical component of the immune system, functioning as first responders to infection by phagocytosis of foreign particles, such as bacteria like *Escherichia coli* (E. coli). This process involves the engulfment of pathogens into phagosomes, which fuse with lysosomes to degrade the contents, a crucial step in innate immunity and subsequent antigen presentation. However, genetic mutations can potentially impair this capability of macrophages, leading to compromised immune responses.

In light of this, the impact of the *Ptpn11*E76K mutation on the phagocytic function of macrophages has not been examined. To investigate this, we conducted an experiment to compare the phagocytic efficiency of wild type macrophages with those harboring the *Ptpn11*E76K mutation using pHrodo™ Green E. coli BioParticles™ as a target. These fluorescently tagged biological particles serve as a direct quantification method for phagocytosis, as they fluoresce upon the acidification that occurs within phagosomes, allowing for a precise measure of phagocytic uptake.

We quantitatively assessed the phagocytosis efficiency of WT and *Ptpn11*E76K macrophages with pHrodo™ Green E. coli BioParticles™ as the target. The experiment was carried out using two concentrations of BioParticles™: 25 mg/mL and 50 mg/mL.

As shown in Figure 4, a higher concentration of 50 mg/mL, WT macrophages demonstrated a mean phagocytosis percentage of 60.8%, indicating robust phagocytic activity. On the other hand, *Ptpn11*E76K mutated macrophages presented a substantially reduced mean phagocytosis percentage of 18.3% at this concentration, suggesting a significant impairment in phagocytic function attributed to the Ptpn11E76K mutation. This difference is statistically significant (p-value = 0.0010).

When the concentration of BioParticles™ was lowered to 25 mg/mL, there was a general decrease in phagocytic activity for both macrophage types. WT macrophages showed a mean phagocytosis percentage of 32.05%, whereas the *Ptpn11*E76K mutated macrophages had a mean of 5.495% (p-value = 0.0106), reinforcing the observation of diminished phagocytic capacity in the presence of the *Ptpn11*E76K mutation (Figure 4).

Phagocytosis Efficiency of Wild Type and *Ptpn11*E76K Mutated Macrophages against Human Promyelocytic Leukemia Cells (HL60)

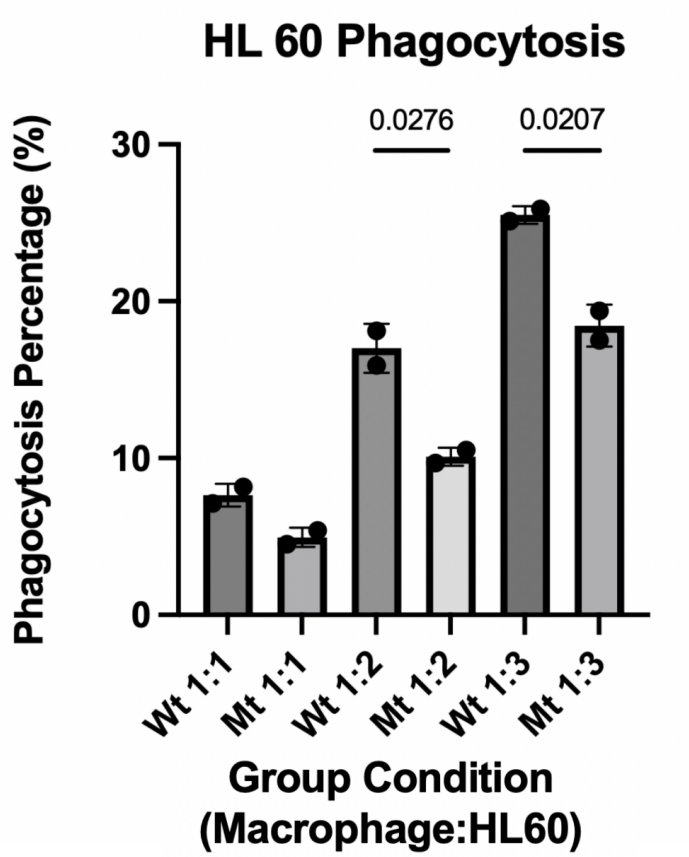

Figure 5. Phagocytotic Comparison between Wild Type and *Ptpn11*E76K Mutated Macrophages Engulfing HL60 Cells

Macrophages play a critical role not only in recognizing and eliminating pathogens but also in engulfing tumor cells. After phagocytosis, macrophages can present tumor-specific antigens, thereby initiating the induction of adaptive antitumor immunity (Zhou *et al.* 2021). There is a growing interest in targeting macrophage phagocytosis as a strategy for cancer immunotherapy. Given their potential to both directly attack tumor cells and catalyze an immune response, macrophages offer a promising avenue for enhancing the efficacy of treatments aimed at combating cancer.

Thus, we investigated the phagocytic abilities of WT and *Ptpn11*E76K mutated macrophages when challenged with HL60 cells, a human promyelocytic leukemia cell line. HL60 cells serve as an ideal model for this study due to their cancerous properties and the opportunity they provide to examine how

genetic variations in macrophages, such as the *Ptpn11*E76K mutation, may influence the efficiency of tumor cell clearance. The HL60 cells were added to macrophage cultures at ratios of 1:1, 1:2, and 1:3 (Macrophage:HL60) to compare the phagocytic percentages across these two macrophage genotypes at different cell ratios.

At the 1:1 ratio, WT macrophages demonstrated a mean phagocytosis percentage of 7.635% with a standard deviation of 0.728, whereas *Ptpn11*E76K mutated macrophages exhibited a mean percentage of 4.95% (Figure 5). This initial comparison indicates a reduced phagocytic ability in the *Ptpn11*E76K mutated macrophages relative to the WT macrophages.

When the ratio was adjusted to 1:2, the mean phagocytosis percentage for WT macrophages increased to 17%, and *Ptpn11*E76K mutated macrophages showed a mean percentage of 10.095% with a standard deviation 0.573 (Figure 5).

Further increasing the HL60 cell ratio to 1:3, the mean phagocytosis percentage for WT macrophages rose to 25.5%, while *Ptpn11*E76K mutated macrophages displayed a mean of 18.45% (Figure 5).

The statistical significance of the differences between WT and *Ptpn11*E76K mutated (Mt) macrophages was assessed at the varying HL60 cell ratios. The analysis revealed that at the 1:1 macrophage to HL60 cell ratio, the difference in phagocytic activity between WT and Mt macrophages was not statistically significant due to the percent phagocytosis in this condition being too low to establish a difference. However, when the ratio was increased to 1:2, a statistically significant difference was observed, with a p-value of 0.0276. This significance was further sustained at a higher ratio of 1:3, where the p-value was reported as 0.0207. Both of these p-values fall below the standard significance threshold of 0.05, indicating that the differences in phagocytic activity between WT and Mt macrophages at these ratios are unlikely to be due to random chance.

The trend across different ratios indicates that WT macrophages consistently maintain a higher phagocytotic percentage compared to *Ptpn11*E76K mutated macrophages, suggesting an inherent difference in phagocytic ability that is influenced by the *Ptpn11*E76K mutation. Despite the variation in HL60 cell ratios, WT macrophages show superior phagocytosis in all conditions tested. This differential in phagocytic performance highlights the potential functional impairment in macrophages due to the Ptpn11E76K mutation.

Phagocytosis Efficiency of Wild Type and *Ptpn11*E76K Mutated Macrophages against Different Experimental Conditions for Lineage Negative (Lin-) Cells

	WT Lin-	Ptpn11E76K Lin-
WT Macrophages	Condition A	Condition B
Ptpn11E76K Macrophages	Condition C	Condition D

Table 1. Experimental Setup for the Assessment of Phagocytic Activity of Wild Type and Ptpn11E76K Mutated Macrophages against Wild Type and *Ptpn11*E76K Mutated Lineage-Negative Target Cells

Hematopoietic stem cells are undifferentiated, self-renewing cells capable of giving rise to all blood cell types, while lineage negative cells are a more differentiated subset that excludes cells committed to specific blood cell lineages, but they are still pluripotent. In this study, Lin- cells were chosen as the target for assessing macrophage phagocytic activity due to their close resemblance to HSCs, which are sparse in availability. Given the limited quantity of cells that can be extracted from a single mouse's femur and tibia, it is not feasible to obtain a sufficient number of HSCs to conduct this experiment. Lin- cells, therefore, serve as the most viable proxy, allowing for a practical approach to study the interactions between macrophages and cells within the bone marrow niche. This choice is

underpinned by the crucial role of macrophages in the bone marrow, where, according to recent findings, they make early contact with stem cells, search their surfaces for stress signals and can selectively engulf ones exhibiting high levels of stress-activated proteins. This interaction suggests macrophages play a significant role in hematopoietic stem and progenitor cell regulation, impacting their proliferation and quality control within the niche by modulating their death and division. (Wattrus et al. 2022 and Hagedorn et al. 2021)

The experimental design was to examine the nuances of macrophage-stem cell interactions. The aim was to compare not only the differences between WT and *Ptpn11*E76K mutated macrophages but also to assess how these macrophages interact with WT and *Ptpn11*E76K mutated Lin- target cells. This approach was intended to illuminate the potential variances in phagocytic behavior attributable to the genetic makeup of both the macrophages and the target cells.

Following this rationale, four distinct conditions were established to investigate the impact of the *Ptpn11*E76K mutation on phagocytic activity between macrophages and lineage-negative target cells. As shown in Table 1, Condition A served as the control group, where wild type macrophages were exposed to wild type lineage-negative target cells, establishing a baseline for phagocytic activity without the influence of any mutations. Condition B involved exposing wild type macrophages to lineage-negative target cells that possessed the *Ptpn11*E76K mutation. In Condition C, macrophages carrying the *Ptpn11*E76K mutation were exposed to wild type lineage-negative target cells. Finally, Condition D examined the scenario where both the macrophages and the lineage-negative target cells carried the *Ptpn11*E76K mutation.

CONDITION (MACROPHAGE:LIN-)	PHAGOCYTOSIS (%)	STANDARIZED VALUE
Condition A (Wt:Wt)	11.0	1
Condition B (Wt:Mt)	26.8	2.44
Condition C (Mt:Wt)	8.46	0.77
Condition D (Mt:Mt)	42.3	2.94

Table 2. Example Calculated Phagocytic Activity Percentage Across Experimental Conditions in Macrophages

In order to account for variabilities inherent to distinct experimental runs, such as the fact that cells for each experiment were derived from different individual mice, the value of phagocytosis efficiency was normalized to condition A. This normalization involved dividing the phagocytosis rates of all conditions by the value obtained in Condition A. For example, in Condition A (Table 2), which is our reference standard, the phagocytosis rate was recorded as 11.0. Condition B, exhibited a phagocytosis rate of 26.8, which when divided by the Condition A value yields a normalized value of approximately 2.44 (26.8/11.0 = 2.44) (Table 2). This reflects a more than two-fold increase in phagocytic activity in Condition B compared to Condition A.

Continuing with this approach, Condition C shows a phagocytosis rate of 8.46, resulting in a normalized value of about 0.77 when divided by Condition A. Similarly, Condition D indicates a phagocytosis rate of 32.3, which normalizes to approximately 2.94 relative to Condition A (Table 2).

These calculated values provide a standardized metric for comparing phagocytic activity across all conditions, thereby mitigating the impact of experimental variations and ensuring that comparisons are based on the intrinsic differences in phagocytosis rates rather than external variables.

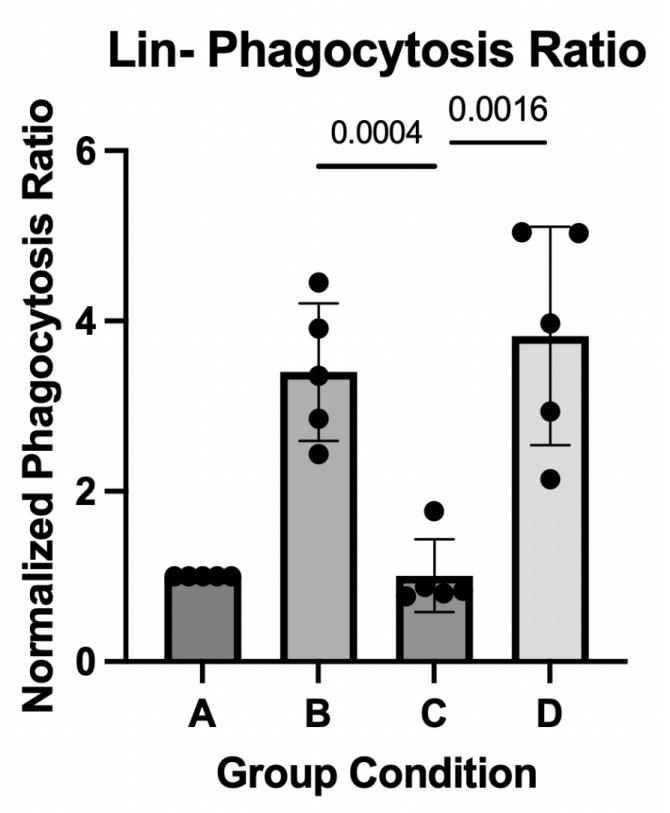

Figure 6. Comparison of Normalized Phagocytosis Ratios Across Different Experimental Conditions for Lineage-Negative Cells

Following the standardization of the phagocytosis rates against Condition A, the data from five separate experiments were averaged to obtain a measure of phagocytic activity across different experiments. This approach served to integrate results from multiple experimental runs, each using cells derived from different individual mice. By averaging the normalized values, the analysis mitigated the impact of any single-run anomalies and allowed us to make direct comparisons of phagocytic activity across different experimental runs.

Condition A, involving WT macrophages with WT lineage-negative (Lin-) target cells, the phagocytosis ratio is always going to be 1, establishing the baseline for all experiments.

When examining Condition B, where WT macrophages were paired with *Ptpn11*E76K Lin- target cells, we found that the normalized phagocytosis ratio fluctuated between 2.436 and 4.454. The average ratio was 3.401, signifying a generally increased phagocytic response compared to the control condition. This heightened activity suggests a distinct interaction dynamic when WT macrophages engage with mutated Lin- target cells .(Figure 6)

In Condition C, the focus was on macrophages with the *Ptpn11*E76K mutation exposed to WT Lin- target cells. Here, the phagocytosis ratio spanned from 0.769 to 1.767, averaging at 1.009. The proximity of the mean ratio to the baseline of Condition A implies that the *Ptpn11*E76K mutation in macrophages does not significantly alter their ability to phagocytose WT Lin- cells. (Figure 6)

Lastly, Condition D analyzed the interaction where both macrophages and Lin- target cells harbored the *Ptpn11*E76K mutation. We observed a broad range of phagocytic ratios from 2.936 to 5.031, with a mean of 3.824. The data from this condition underscore a marked elevation in phagocytic activity and suggest greater variability in how the mutated macrophages handle similarly mutated Lin- target cells. (Figure 6)

Upon analysis, conditions involving mutated Lin- target cells (B and D) revealed an increased phagocytic ratio relative to the control, suggesting that the *Ptpn11*E76K mutation in the target cells augments phagocytic activity by macrophages. However, Condition C, characterized by the *Ptpn11*E76K mutation in macrophages, demonstrated a mean phagocytic ratio similar to that of the control, implying that this specific mutation within the macrophages does not significantly alter the phagocytosis of WT Lin- target cells.

From a statistical perspective, a significant elevation in the phagocytic ratio was observed in both Conditions B and D compared to Condition C, with p-values of 0.0004 and 0.0016, respectively. These results strongly suggest that the *Ptpn11*E76K mutation in Lin- target cells notably enhances their phagocytosis by WT macrophages. In contrast, the same mutation in the macrophages does not appear to impact their phagocytic capability towards WT target cells, as the average ratio in Condition C was in close accordance with the control.

Discussion

Our results indicated that *Ptpn11*E76K macrophages display a significantly lower phagocytic rate when exposed to both HL60 cells and E. coli particles compared to WT macrophages. This observation aligns with the established role of macrophages as fundamental to the immune system, they can identify and phagocytose foreign particles, including bacteria like Escherichia coli. Phagocytosis is an essential mechanism for not only the clearance of pathogens but also for initiating antigen presentation. Phagocytosis of tumor cells allows macrophages to process and present tumor-specific antigens, which is a vital trigger for the induction of adaptive antitumor immunity.

Interestingly, when assessing the phagocytic interaction between macrophages and Lin- progenitor cells, the distinction between WT and *Ptpn11*E76K mutated macrophages was not as pronounced as observed with the particle and HL60 experiments. This finding is intriguing, as it suggests that the presence of the *Ptpn11*E76K mutation does not substantially impair the macrophages' ability to phagocytose progenitor cells compared to the more marked effect seen with other targets. This could imply that the signals or markers on progenitor cells that macrophages respond to are affected by the *Ptpn11*E76K mutation.

However, this is in line with the behavior observed where macrophages initiate contact with stem cells upon entry into the bone marrow niche, specifically probing for stress indicators on the cell surfaces. (Wattrus et al. 2022) The preferential phagocytosis of mutated Lin- cells over wild type cells in the experiments suggests that the mutation may serve as a signal akin to the stress-activated proteins, guiding macrophages in the selection and removal of potentially compromised cells from the pool of progenitor cells. (Wattrus et al. 2022)

Moreover, it is proposed that macrophages are instrumental in quality control within the hematopoietic system, not only by removal of certain cells but also by potentially stimulating division in others. (Hagedorn *et al.* 2023) The reduced proliferation of hematopoietic stem and progenitor cells following ERK/MAPK pathway inhibition, without diminishing macrophage interactions, provides a

valuable insight into the supportive role macrophages may play. This study suggests that even when the function of macrophages is altered by a mutation, they retain some capacity to discern and respond to mutated cells, potentially impacting the balance between cell death and division. (Hagedorn *et al.* 2023)

The observed preference for phagocytosis of mutated Lin- cells could also be indicative of a more pronounced recognition signal, potentially linked to altered cell surface markers or intracellular changes that are detected by macrophages. This specificity suggests an adaptive response to maintain cellular integrity, with macrophages responding to indicators of cellular abnormality.

Collectively, these studies, along with the current work, underscore the intricate role of macrophages as gatekeepers in stem cell biology. Further research should aim to decode the molecular signals and pathways through which macrophages discern cellular stress or mutation in stem cells. Understanding these processes could be pivotal in harnessing macrophages for therapeutic strategies aimed at regulating stem cell populations, with implications for treating a variety of hematologic conditions and enhancing regenerative medicine.

References

Aderem, Alan, and David M. Underhill. "Mechanisms of Phagocytosis in Macrophages." *Annual Review of Immunology* 17, no. Volume 17, 1999 (April 1, 1999): 593–623. https://doi.org/10.1146/annurev.immunol.17.1.593.

Cai, Zhigang, Chi Zhang, Jonathan J. Kotzin, Adam Williams, Jorge Henao-Mejia, and Reuben Kapur. "Role of lncRNA Morrbid in PTPN11(Shp2)E76K-Driven Juvenile Myelomonocytic Leukemia." *Blood Advances* 4, no. 14 (July 22, 2020): 3246–51. https://doi.org/10.1182/bloodadvances.2020002123.

Chan, Rebecca J., Todd Cooper, Christian P. Kratz, Brian Weiss, and Mignon L. Loh. "Juvenile Myelomonocytic Leukemia: A Report from the 2nd International JMML Symposium." *Leukemia Research* 33, no. 3 (March 1, 2009): 355–62. https://doi.org/10.1016/j.leukres.2008.08.022.

Chang, Tiffany Y., Christopher C. Dvorak, and Mignon L. Loh. "Bedside to Bench in Juvenile Myelomonocytic Leukemia: Insights into Leukemogenesis from a Rare Pediatric Leukemia." *Blood* 124, no. 16 (October 16, 2014): 2487–97. https://doi.org/10.1182/blood-2014-03-300319.

Cheng, T., N. Rodrigues, H. Shen, Y. Yang, D. Dombkowski, M. Sykes, and D. T. Scadden. "Hematopoietic Stem Cell Quiescence Maintained by P21cip1/Waf1." *Science (New York, N.Y.)* 287, no. 5459 (March 10, 2000): 1804–8. https://doi.org/10.1126/science.287.5459.1804.

Dong, Lei, Wen-Mei Yu, Hong Zheng, Mignon L. Loh, Silvia T. Bunting, Melinda Pauly, Gang Huang, et al. "Leukaemogenic Effects of Ptpn11 Activating Mutations in the Stem Cell Microenvironment." *Nature* 539, no. 7628 (November 10, 2016): 304–8. https://doi.org/10.1038/nature20131.

Elhelu, Mohamed A. "The Role of Macrophages in Immunology." *Journal of the National Medical Association* 75, no. 3 (March 1983): 314–17.

Feng, G. S. “Shp-2 Tyrosine Phosphatase: Signaling One Cell or Many.” *Experimental Cell Research* 253, no. 1 (November 25, 1999): 47–54. https://doi.org/10.1006/excr.1999.4668.

Gordon, Siamon, and Luisa Martinez-Pomares. “Physiological Roles of Macrophages.” *Pflügers Archiv - European Journal of Physiology* 469, no. 3 (April 1, 2017): 365–74. https://doi.org/10.1007/s00424-017-1945-7.

Gupta, Aditya Kumar, Jagdish Prasad Meena, Anita Chopra, Pranay Tanwar, and Rachna Seth. “Juvenile Myelomonocytic Leukemia-A Comprehensive Review and Recent Advances in Management.” *American Journal of Blood Research* 11, no. 1 (February 15, 2021): 1–21.

Hagedorn, Elliott J., Julie R. Perlin, Rebecca J. Freeman, Samuel J. Wattrus, Tianxiao Han, Clara Mao, Ji Wook Kim, et al. “Transcription Factor Induction of Vascular Blood Stem Cell Niches in Vivo.” *Developmental Cell* 58, no. 12 (June 19, 2023): 1037-1051.e4. https://doi.org/10.1016/j.devcel.2023.04.007.

Liu, Xia, Hong Zheng, Xiaobo Li, Siying Wang, Howard J. Meyerson, Wentian Yang, Benjamin G. Neel, and Cheng-Kui Qu. “Gain-of-Function Mutations of Ptpn11 (Shp2) Cause Aberrant Mitosis and Increase Susceptibility to DNA Damage-Induced Malignancies.” *Proceedings of the National Academy of Sciences* 113, no. 4 (January 26, 2016): 984–89. https://doi.org/10.1073/pnas.1508535113.

Locatelli, Franco, and Charlotte M. Niemeyer. “How I Treat Juvenile Myelomonocytic Leukemia.” *Blood* 125, no. 7 (February 12, 2015): 1083–90. https://doi.org/10.1182/blood-2014-08-550483.

Locatelli, Franco, Peter Nöllke, Marco Zecca, Elisabeth Korthof, Edoardo Lanino, Christina Peters, Andrea Pession, et al. “Hematopoietic Stem Cell Transplantation (HSCT) in Children with Juvenile Myelomonocytic Leukemia (JMML): Results of the EWOG-MDS/EBMT Trial.” *Blood* 105, no. 1 (January 1, 2005): 410–19. https://doi.org/10.1182/blood-2004-05-1944.

Loh, Mignon L. “Recent Advances in the Pathogenesis and Treatment of Juvenile Myelomonocytic Leukaemia.” *British Journal of Haematology* 152, no. 6 (2011): 677–87. https://doi.org/10.1111/j.1365-2141.2010.08525.x.

Loh, Mignon L., Shashaank Vattikuti, Suzanne Schubbert, Melissa G. Reynolds, Elaine Carlson, Kenneth H. Lieuw, Jennifer W. Cheng, et al. "Mutations in PTPN11 Implicate the SHP-2 Phosphatase in Leukemogenesis." *Blood* 103, no. 6 (March 15, 2004): 2325–31. https://doi.org/10.1182/blood-2003-09-3287.

Moritake, Hiroshi, Toshio Ikeda, Atsushi Manabe, Sachiyo Kamimura, and Hiroyuki Nunoi. "Cytomegalovirus Infection Mimicking Juvenile Myelomonocytic Leukemia Showing Hypersensitivity to Granulocyte–Macrophage Colony Stimulating Factor." *Pediatric Blood & Cancer* 53, no. 7 (December 15, 2009): 1324–26. https://doi.org/10.1002/pbc.22253.

Nakamura-Ishizu, Ayako, Hitoshi Takizawa, and Toshio Suda. "The Analysis, Roles and Regulation of Quiescence in Hematopoietic Stem Cells." *Development (Cambridge, England)* 141, no. 24 (December 2014): 4656–66. https://doi.org/10.1242/dev.106575.

Peiser, Leanne, Peter J. Gough, Tatsuhiko Kodama, and Siamon Gordon. "Macrophage Class A Scavenger Receptor-Mediated Phagocytosis of Escherichia Coli: Role of Cell Heterogeneity, Microbial Strain, and Culture Conditions In Vitro." *Infection and Immunity* 68, no. 4 (April 2000): 1953–63. https://doi.org/10.1128/iai.68.4.1953-1963.2000.

Shinjyo, Tetsuharu, Ryoko Kuribara, Takeshi Inukai, Hajime Hosoi, Taisei Kinoshita, Atsushi Miyajima, Peter J. Houghton, A. Thomas Look, Keiya Ozawa, and Toshiya Inaba. "Downregulation of Bim, a Proapoptotic Relative of Bcl-2, Is a Pivotal Step in Cytokine-Initiated Survival Signaling in Murine Hematopoietic Progenitors." *Molecular and Cellular Biology* 21, no. 3 (February 2001): 854–64. https://doi.org/10.1128/MCB.21.3.854-864.2001.

Tartaglia, Marco, Charlotte M. Niemeyer, Alessandra Fragale, Xiaoling Song, Jochen Buechner, Andreas Jung, Karel Hählen, Henrik Hasle, Jonathan D. Licht, and Bruce D. Gelb. "Somatic Mutations in PTPN11 in Juvenile Myelomonocytic Leukemia, Myelodysplastic Syndromes and Acute Myeloid Leukemia." *Nature Genetics* 34, no. 2 (June 2003): 148–50. https://doi.org/10.1038/ng1156.

US Department of Health and Human Services; National Cancer Institute. "Cancer Incidence and Survival among Children and Adolescents: United States SEER Program 1975-1995," 1999. https://doi.org/10.1037/e407432005-001.

Wattrus, Samuel J., Mackenzie L. Smith, Cecilia Pessoa Rodrigues, Elliott J. Hagedorn, Ji Wook Kim, Bogdan Budnik, and Leonard I. Zon. "Quality Assurance of Hematopoietic Stem Cells by Macrophages Determines Stem Cell Clonality." *Science (New York, N.Y.)* 377, no. 6613 (September 23, 2022): 1413–19. https://doi.org/10.1126/science.abo4837.

Weiskopf, Kipp, and Irving L Weissman. "Macrophages Are Critical Effectors of Antibody Therapies for Cancer." *mAbs* 7, no. 2 (March 4, 2015): 303–10. https://doi.org/10.1080/19420862.2015.1011450.

Wright, Douglas E., Amy J. Wagers, Anjali Pathak Gulati, Frances L. Johnson, and Irving L. Weissman. "Physiological Migration of Hematopoietic Stem and Progenitor Cells." *Science* 294, no. 5548 (November 30, 2001): 1933–36. https://doi.org/10.1126/science.1064081.

Yang, Zhenyun, Yiping Li, Fuqin Yin, and Rebecca J. Chan. "Activating *PTPN11* Mutants Promote Hematopoietic Progenitor Cell-Cycle Progression and Survival." *Experimental Hematology* 36, no. 10 (October 1, 2008): 1285–96. https://doi.org/10.1016/j.exphem.2008.04.016.

Zhang, Qian, Yao Li, Rongrong Zhao, Xinyi Wang, Chuling Fan, Youzhi Xu, Yakun Liu, Jiabin Li, and Siying Wang. "The Gain-of-Function Mutation E76K in SHP2 Promotes CAC Tumorigenesis and Induces EMT via the Wnt/β-Catenin Signaling Pathway." *Molecular Carcinogenesis* 57, no. 5 (2018): 619–28. https://doi.org/10.1002/mc.22785.

Zhou, Xuefei, Xiangrui Liu, and Leaf Huang. "Macrophage-Mediated Tumor Cell Phagocytosis: Opportunity for Nanomedicine Intervention." *Advanced Functional Materials* 31, no. 5 (January 27, 2021): 2006220. https://doi.org/10.1002/adfm.202006220.