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Transient Potential Receptor Melastatin-2 (TRPM2) Does Not Influence Murine MLL-AF9 Driven AML Leukemogenesis or in Vitro Response to Chemotherapy

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Abstract

Transient potential receptor melastatin-2 (TRPM2) is a non-selective cationic, Ca²⁺ permeable transmembrane pore that is preferentially expressed in cells of the myeloid lineage, and modulates signaling pathways converging onto NF-κB. This is of potential interest for AML therapy, as NF-κB signaling is emerging as a key pathway mediating drug resistance and leukemia initiating cell survival in AML, and inhibition of NF-κB signaling has been shown to be synergistic with chemotherapy. TRPM2 is overexpressed in AML compared to normal bone marrow, with highest levels in the FAB M3-6 subtypes. To determine the effect of loss of Trpm2 in a defined genetic model, we established MLL-AF9 driven AML on a *Trpm2*^{-/-} genetic background. *Trpm2*^{-/-} MLL-AF9 leukemias displayed reduced NF-κB phosphorylation and nuclear translocation. *In vivo* primary and secondary recipients of *Trpm2*^{-/-} MLL-AF9 leukemias showed increased latency compared to recipients of wild type leukemia cells. However, the difference in latency was small, and lost in tertiary transplants. The effect of loss of *Trpm2* in a BCR-ABL/NUP98-HOXA9 fusion model was even smaller. Given reports that loss or inhibition of TRPM2 enhanced killing by DNA damaging agents in neuroblastoma, breast and prostate cancer cell lines, we exposed *Trpm2*^{-/-} and *Trpm2*^{wt} primary MLL-AF9 leukemias to doxorubicin, cytarabine and etoposide, but found no difference in IC50. The in vitro response to decitabine was also unaffected. In summary, TRPM2 does not seem to play a major role in myeloid leukemogenesis. In addition, loss of TRPM2 does not augment the cytotoxicity of standard AML chemotherapeutic agents.

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Conflict of interest statement

The authors report no conflict of interest.

Keywords

TRPM2; NF- κ B; MLL-AF9; leukemia

Introduction

Rearrangements of the Mixed Lineage Leukemia gene *MLL* are found in ALL, AML and treatment related AML (tAML). NF- κ B signaling recently emerged as playing an important role in *MLL*-rearranged leukemia (1, 2). The NF- κ B subunit RELA localizes to *MLL*-fusion target genes, and knockdown of RELA prolonged survival in murine models of *MLL*-rearranged leukemia (1). Similarly, overexpression of a dominant negative I κ B α in syngeneic murine AML models driven by MLL-AF9, MOZ-TIF or BCR-ABL/NUP98-HOXA9 resulted in prolonged survival (2). A plethora of inhibitors of NF- κ B signaling have been reported (3). These include compounds with broad biological activity such as anti-oxidants, anti-inflammatory agents or proteasome inhibitors, and specific inhibitors of IKK phosphorylation / degradation. However, NF- κ B is a broadly active pathway, and IKK inhibitors may have off target effects (3). Inhibition of NF- κ B specifically in leukemia cells may therefore be desirable.

Transient receptor potential melastatin 2 (TRPM2; TRPC7, LTRPC2) has been reported to modulate NF- κ B signaling. TRPM2 is a non-selective cationic, Ca²⁺-permeable pore and contains a unique C-terminal region containing ADP-ribose (ADPR) hydrolase activity (4–9). The highest expression levels of TRPM2 are observed in cells of neuronal origin and in the myeloid lineage (4, 8, 10, 11). *Trpm2*^{−/−} animals are viable and fertile, but exhibit reduced cytokine production (12). The *MLL*-rearranged AML cell line THP-1 (grown in differentiating conditions) requires TRPM2 for the LPS-induced production of several cytokines (11). In the U937 AML cell line, siRNA knockdown of TRPM2 impaired H₂O₂-mediated Ca²⁺ influx, diminishing nuclear translocation of NF- κ B and production of IL-8 (13).

Interfering with TRPM2 mediated Ca²⁺ signaling may thus allow to modulate NF- κ B activity specifically in myelomonocytic cells, including AML. The fact that *Trpm2*^{−/−} mice are viable and fertile suggests that a substantial therapeutic window for TRPM2 inhibition. Furthermore, knockdown or inhibition of TRPM2 in neuroblastoma, breast and prostate cancer cell lines resulted in decreased proliferation and was synergistic with chemotherapy (14–17). Induction of NF- κ B by doxorubicin has been reported as a resistance mechanism to chemotherapy in a myelomonocytic AML cell line (18). This raises the possibility that modulating TRPM2 signaling could increase the effect of standard chemotherapy in AML cells while sparing normal hematopoietic stem and early progenitor compartments.

In order to assess the role of TRPM2 in AML leukemogenesis, growth, survival and drug resistance, we utilized the MLL-AF9 and BCR-ABL/NUP98-HOXA9 induced leukemia models established on a defined genetic loss of function background for *Trpm2*.

Methods

For detailed experimental procedures please refer to the supplemental materials.

Trpm2 knockout mice

Animals were maintained at the Animal Research Facility at the University of Colorado Anschutz Medical Campus. Animal experiments were approved by the Internal Animal Care and Use Committee. Trpm2 deficiency in *Trpm2* knockout mice (Knowles et al, 2011) was functionally validated by patch clamp electrophysiology experiments. *Trpm2*^{-/-} animals were maintained on a C57BL/6 background.

Generation of murine leukemias

Lin⁻Sca-1⁺c-Kit⁺ (LSK) cells were transduced with MSCV-MLL-AF9-IRES-GFP, or MSCV-BCR-ABL-GFP and MSCV-NUP98-HOXA9-YFP, sorted, and transplanted into sublethally irradiated recipients (2×10⁵ cells/mouse). For secondary and tertiary transplants, GFP⁺ cells from MLL-AF9 leukemic mice were isolated and transplanted after brief *in vitro* culture.

Cytotoxicity Assays

Doxorubicin (Pfizer), etoposide, decitabine and cytarabine (Sigma-Aldrich) were added at the indicated concentrations. After 72 hours, necrotic cells were labeled with propidium iodide and total cell number was measured on Guava (Millipore).

Cell Growth & Colony Assays

Cell growth and viability were followed by serial cell counts. For colony assays, leukemia cells were plated in methylcellulose M3234 (Stem Cell Technologies) containing IL3, IL6 and SCF (PeproTech).

Western Blotting

Antibodies for NF-κB detection were phospho-p65 3033p and p65 82420 (Cell signaling).

Data analysis and statistical methods

All data are expressed as means ± s.e.m. Statistical analysis of *in vivo* survival was performed using Kaplan-Meier estimates.

Results and Discussion

TRPM2 expression in hematopoietic development and AML

We analyzed publicly available data sets for TRPM2 expression during normal hematopoiesis to confirm the expression pattern reported in the literature. TRPM2 expression is low in stem and early progenitor compartments. It is first upregulated in granulocyte macrophage progenitors (GMP), and rises with increasing myeloid differentiation (Figure 1A). Furthermore, TRPM2 expression is higher in AML compared to normal bone marrow and lymphoid malignancies (Figure 1B). Within AML, higher levels

are observed in more differentiated FAB subtypes (FAB M3-6, Figure 1C). This suggests that TRPM2 is expressed in AML cells and its inhibition could be a means to modulate NF- κ B.

Trpm2^{-/-} leukemias have lower levels of phospho NF- κ B and nuclear NF- κ B

To determine the effect of loss of *Trpm2* in a defined genetic model, we established AML on a *Trpm2*^{-/-} genetic background by retroviral introduction of MLL-AF9 into lin⁻ Sca1⁺ cKit⁺ (LSK) cells (Figure 2A). Consistent with the lack of Trpm2 expression in earlier hematopoietic compartments, the flow profiles for hematopoietic stem cells and progenitors were comparable between wild type and *Trpm2*^{-/-} mice (Figure S1A+B). MLL-AF9 transduced *Trpm2*^{wt} or *Trpm2*^{-/-} LSK cells were injected into sublethally irradiated recipients to establish leukemias. In MLL-rearranged differentiated human cell lines, knockdown of TRPM2 resulted in lower levels of active, phosphorylated and nuclear NF- κ B. NF- κ B has been reported to be induced as an escape mechanism to chemotherapy, including doxorubicin. We therefore assessed NF- κ B subcellular localization and phosphorylation in *Trpm2*^{wt} and *Trpm2*^{-/-} leukemias at baseline and after exposure to doxorubicin. Nuclear translocation (Figure 2B) and p65 phosphorylation (Figure 2C) of NF- κ B were lower in *Trpm2*^{-/-} MLL-AF9 leukemia cells, particularly after stimulation with doxorubicin, confirming that Trpm2 modulates NF- κ B in MLL-AF9 leukemia cells.

Primary recipients of Trpm2^{-/-} leukemias have a lower leukemic burden and extended survival

Recipients of *Trpm2*^{-/-} MLL-AF9 transduced LSK cells displayed a significantly lower leukemic burden and prolonged survival compared to recipients of wild type cells (Figure 2D). *Trpm2*^{-/-} leukemias (verified by genotyping, Figure S2A) were phenotypically similar to *Trpm2*^{wt} and displayed a myeloid phenotype (GR1⁺/Mac1⁺, data not shown). In addition to the longer latency, recipients of *Trpm2*^{-/-} MLL-AF9 transduced cells had a not statistically significant trend towards smaller spleen sizes, lower leukemic burden (WBC), and better preserved bone marrow function (measured as degree of anemia) at the time of death (Figures S2B-D). However, the difference between *Trpm2*^{wt} and *Trpm2*^{-/-} leukemias in primary recipients was small.

Loss of Trpm2 causes minimal effects in secondary and tertiary transplants

The increase in latency observed in *TRPM2*^{-/-} MLL-AF9 leukemias and the fact that Kuo et al (1) reported a profound effect of loss of RELA on leukemia initiating cells (LICs) raised the question whether our observation reflected a lower functional LIC frequency, and *TRPM2*^{-/-} MLLAF9 leukemias would eventually exhaust. We therefore performed secondary and tertiary transplants including cohorts transplanted with reduced cell numbers. Secondary recipients again showed a statistically significant decrease in leukemic burden on day 20, and a modest increase in latency (Figure 2E). However, this effect was lost in tertiary recipients (Figure 2F). We analyzed NF- κ B phosphorylation in leukemias isolated from tertiary recipients and found phospho-p65 levels restored to levels at or higher than that of wild type leukemias.

Loss of *Trpm2* causes minimal effects in a second murine leukemia model

In addition to *MLL*-rearranged leukemias, a critical role for NF- κ B signaling has been described in other models as well, including the very aggressive BCR-ABL/NUP98-HOXA9 model. We therefore sought to investigate whether knockout of *Trpm2* would influence leukemia latency in a second model. We transduced LSK cells from wild type and *Trpm2*^{-/-} mice with retroviral BCR-ABL-GFP and NUP98-HOXA9-YFP. Similar to our observations in the *MLL*-AF9 model, absence of *Trpm2* was associated with lower NF- κ B phosphorylation (Figure 2H). However, NF- κ B phosphorylation was not absent, and survival of recipients injected with *Trpm2*^{-/-} BCR-ABL/NUP98-HOXA9 transduced cells was not different from recipients of wild type cells (Figure 2I). Loss of *Trpm2* thus had no or only minimal effects on leukemogenesis in two independent syngeneic leukemia models.

Genetic inactivation of *Trpm2* does not potentiate chemotherapy

Given reports that knockdown or inhibition of TRPM2 enhanced killing by cytotoxic agents in neuroblastoma, breast and prostate cancer cell lines (8, 19, 20), we next exposed *Trpm2*^{wt} and *Trpm2*^{-/-} *MLL*-AF9 leukemias to doxorubicin, cytarabine, and etoposide *in vitro*. *In vitro* growth rates in liquid culture and clonogenic potential in methylcellulose was similar between *Trpm2*^{wt} and *Trpm2*^{-/-} leukemias (Figure 3A+B, S3A+B). We also found no difference in efficacy of doxorubicin, cytarabine, or etoposide between *Trpm2*^{wt} and *Trpm2*^{-/-} leukemias (Figure 3C+D). We also tested the DNA demethylating agent decitabine, which was equally effective on *Trpm2*^{wt} and *Trpm2*^{-/-} leukemias. Loss of *Trpm2* therefore does not potentiate the toxicity of standard AML chemotherapeutic agents in this model.

Conclusion

The recent description of a critical role for NF- κ B in AML (2, 21, 22), particularly *MLL*-rearranged AML (1), the expression of TRPM2 in clinical M3-6 AML and *MLL*-rearranged leukemia cell lines, and the synergy between TRPM2 knockdown and chemotherapy in other cancer models suggested that TRPM2 might be an attractive therapeutic target in AML, and specifically *MLL*-rearranged leukemia. Furthermore, the fairly mild phenotype of *Trpm2* knockout mice suggested a large therapeutic window for TRPM2 inhibition. However, our data demonstrate no substantial role for TRPM2 mediated modulation of NF- κ B in *MLL*-rearranged leukemia.

The difference in results between our study and the studies by Kuo et al (1) and Kagoya et al (2) may be due to more profound inhibition of NF- κ B signaling following *RELA* knockdown and *IKK* inhibition/dominant negative than the modulation through *Trpm2* knockout. In fact, Yamamoto et al reports residual NF- κ B nuclear translocation independent of TRPM2 (13). Thus, residual *Trpm2* independent NF- κ B activity may explain the modest effect in primary transplants. In addition, it is likely that alternate Ca²⁺-entry pathways (for example store-operated Ca²⁺-entry) are compensating for the deficiency in *Trpm2*. This is supported by the restoration of NF- κ B phosphorylation in tertiary leukemias to levels at or above what we observed in leukemia cells with functional TRPM2. The fact that tertiary leukemias display restored NF- κ B phosphorylation also underscores the critical nature of this pathway for self-renewal as suggested by Kuo et al (1), and Kagoya et al (2).

Currently, over 800 compounds have been proposed to modulate NF- κ B signaling, directly or indirectly, in different biologic contexts (3, 23). In addition, NF- κ B is just one of several promising pathways currently being explored for targeted therapies. The number of pathways and molecules proposed to play a role in cancer by far exceeds what is feasible to test in clinical trials. Rigorous preclinical testing that eliminates less promising approaches therefore is critical to advance the development highly effective novel therapies. Despite a compelling rationale, our stringent genetic model suggests that TRPM2 only plays a minor role in AML, its absence is readily compensated for, and deletion of TRPM2 does not sensitize leukemia cells to chemotherapy. Our results will thus be helpful in guiding future efforts to modulate NF- κ B signaling in AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Transient potential receptor melastatin-2 (TRPM2) is expressed in myeloid cells..
- TRPM2 has been reported to modulate NF- κ B.
- NF- κ B is required for MLL-AF9 and BCR-ABL/NUP98-HOXA9 AML leukemia initiating cells.
- Nevertheless, loss of *Trpm2* only minimally affects leukemogenesis in these models.
- Loss of *Trpm2* also does not potentiate standard AML chemotherapy.

Figure 1. TRPM2 expression in hematopoiesis and AML – analysis of publicly available gene expression databases

A. TRPM2 expression in the hematopoietic system is confined to myeloid cells starting at the stage of the common myeloid progenitor (GMP). Data from gene expression commons (<https://gexc.stanford.edu>) (24).

B. TRPM2 expression in normal bone marrow, AML and ALL demonstrates highest levels in AML. Oncomine, Andersson Leukemia Dataset (25).

C. TRPM2 expression in AML FAB subgroups demonstrates high expression levels in more differentiated AML subtypes (FAB M3-6), including monoblastic and monocytic subtypes (FAB M4/5). Oncomine, Valk Leukemia Dataset (20).

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Figure 2. *Trpm2*^{-/-} MLL-AF9 leukemias are less aggressive than *Trpm2*^{wt} leukemias in primary and secondary, but not tertiary transplants

A. experimental design: LSK cells were isolated from *Trpm2*^{wt} or *Trpm2*^{-/-} mice and transduced with MSCV-MLLAF9-IRES-GFP. 48 hrs after transduction, cells were sorted for GFP and injected into sublethally irradiated recipients. Fully transformed leukemias were isolated from leukemic mice and subjected to downstream analysis.

B. Nuclear NF-κB and

- C.** phospho p65 were analyzed in *Trpm2^{wt}* and *Trpm2^{-/-}* leukemias at baseline and after exposure to Doxorubicin.
- D.** MLL-AF9 leukemic burden on day 20 as measured by flowcytometry as % GFP+ cell in the peripheral blood and Kaplan-Meier survival curve of primary recipients. n=9 *Trpm2^{wt}* and n=10 *Trpm2^{-/-}* recipients, 2 independent experiments.
- E.** MLL-AF9 leukemic burden on day 20 as measured by flowcytometry as % GFP+ cell in the peripheral blood and Kaplan-Meier survival curve of secondary recipients. n=12 mice/group from 4 different primary recipients, 2 independent experiments.
- F.** MLL-AF9 leukemic burden on day 20 as measured by flowcytometry as % GFP+ cell in the peripheral blood and Kaplan-Meier survival curve of tertiary recipients. n=6-7 mice/group from 3 different secondary recipients, 2 independent experiments.
- G.** phospho p65 was analyzed in *Trpm2^{wt}* and *Trpm2^{-/-}* tertiary MLL-AF9 leukemias and demonstrates restoration of NF-κB phosphorylation.
- H.** phospho p65 was analyzed in *Trpm2^{wt}* and *Trpm2^{-/-}* BCR-ABL / NUP98-HOXA9 transduced LSK cells.
- I.** Kaplan-Meier survival curve of primary recipients of *Trpm2^{wt}* or *Trpm2^{-/-}* BCR-ABL / NUP98-HOXA9 transduced LSK cells. n=8 recipients per group.

A Cell growth in liquid culture **B Cell growth in methylcellulose** **C Cytarabine** **D Doxorubicin** **E Etoposide** **F Decitabine**

Figure 3. In vitro growth and response of *Trpm2^{wt}* and *Trpm2^{-/-}* MLL-AF9 leukemias to doxorubicin, cytarabine, etoposide and decitabine

A. Cell growth in liquid culture of *Trpm2^{wt}* and *Trpm2^{-/-}* leukemias. $n=3$, p =not significant.

B. Serial replating in methylcellulose of *Trpm2^{wt}* and *Trpm2^{-/-}* leukemias. $n=3$, p =not significant.

C-F. *Trpm2^{wt}* and *Trpm2^{-/-}* MLL-AF9 leukemias were exposed to (C) cytarabine (D) doxorubicin (E) etoposide and (F) decitabine at the indicated concentrations. After 72 hrs, cell numbers and cell viability were assessed using automated cell counting and propidium iodide.

n=3-4 leukemias, 3-4 independent experiments performed in duplicate. p=not significant.

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