Gaq signaling is required for the maintenance of MLL-AF9 induced AML


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Title: Gaq signaling is required for the maintenance of MLL-AF9 induced AML

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LETTER TO THE EDITOR

Gaq signaling is required for the maintenance of MLL-AF9 induced AML

Self-renewal and differentiation block are two key features of leukemic stem cells (LSCs) in MLL-rearranged AML, which can originate from either hematopoietic stem cells (HSCs) or granulocyte macrophage progenitors (GMPs). We have previously demonstrated that β-catenin is essential for the establishment of MLL LSCs, where MLL-AF9 activates β-catenin, one role of which is to confer self-renewal capacity to GMPs necessary for malignant transformation (1). The effect of β-catenin appears to be largely context dependent and driven by its upstream components that fine-tune the signal to modulate different functional programs downstream of β-catenin (2). The targeting of critical upstream components could therefore serve as a potential strategy to effectively block β-catenin signaling. However, our understanding of upstream signaling events leading to β-catenin activation in leukemia still remains poor.

G proteins and G protein-coupled receptors have been implicated in playing crucial roles in multiple cancers, where specific members of this family influence self-renewal and tumorigenesis largely through activation of β-catenin signaling (2). The G protein subunit Gaq has recently been identified as a direct target of MLL-AF4 (3), and Dot1l-mediated H3K79 methylation marks are enriched at the Gaq locus in MLL-AF9 LSCs (4), indicating a role for Gaq in MLL leukemia.

To determine if Gaq is functionally important in MLL AML, we first examined the effects of Gaq inhibition on active β-catenin expression and colony-forming capacity of pre-LSCs derived from MLL-AF9 transduced KLS (HSC-enriched Lin− CD127− Sca-1+ c-Kit+) and GMPs (Lin− CD127− Sca-1+ c-Kithigh CD16/32high CD34+) (1). These pre-LSCs are a recognized early stage of leukemia development that subsequently acquires additional events in vivo leading to a full-blowed leukemia (1). Stable knockdown of Gaq in pre-LSCs was achieved by lentiviral-mediated delivery of short hairpin RNA (shGaq). We found that all three individual shGaq reduced Gaq expression in KLS-derived pre-LSCs (KLSpref-LSC) and GMP-derived pre-LSCs (GMPpref-LSC) and as a result, Gaq knockdown led to a significant decrease in the levels of active β-catenin and colony formation (Figures 1a, 1b).

We next explored the effect of pharmacological inhibition of Gaq on pre-LSCs and leukemic cells. In the presence of 40 μM GP antagonist 2A (GP), a selective inhibitory peptide of Gaq protein (5), KLSpref-LSC exhibited a significant decrease in β-catenin expression associated with a 50% reduction in colony formation (Figure 1c; Figure S1). We then assessed the antagonistic effects of GP on GFP+ leukemic cells sorted from fully developed primary MLL AML originating from KLS or GMPs (KLS/GMPleukemic). The inhibitory effects observed for KLSpref-LSC and KLSleukemic cells (Figures 1c, 1d) were similar while GMPleukemic cells appeared to be more sensitive to GP treatment (Figure 1e).
We next tested the leukemogenic inhibitory potential of GP inhibition in vivo, by pre-treating leukemic cells ex vivo with GP (40uM) for 2 - 4 days, and subsequently transplanting viable cells into sub-lethally irradiated recipient mice. In vivo BrdU labeling analysis at 8 days post-injection showed that 2-day GP pre-treatment led to impaired cell proliferation in both KLSleukemic and GMPleukemic cells (Figures 1f, 1g). Consistent with the in vitro clonogenic assays (Figures 1d, 1e) we observed a slightly more pronounced reduction in proliferation for GMPleukemic cells. Further survival analysis showed that 4-day GP pre-treatment was able to promote a small but significant enhancement of survival in mice transplanted with GMPleukemic cells (Figure 1h), and genetic depletion of Gaq also significantly delayed the onset of leukemia induced by GMPleukemic cells (Figure 1i). Together, this provides the first evidence that Gaq signaling contributes to maintenance of fully-developed AML. Furthermore, we found that the inhibitory effect of GP on human MLL-AF9 AML cells might largely depend on the expression levels of endogenous β-catenin, as we observed that GP selectively suppressed the growth of human THP-1 AML cells (Figure 1j) that endogenously express β-catenin (6), but had no effects on MOLM-13 cells (Figure 1j) as well as other human AML cell lines (e.g. ML2 carrying the MLL-AF6 translocation, data not shown) in which β-catenin expression is undetectable (6). In contrast, ABT-199, a selective Bcl2 inhibitor, had a β-catenin-independent effect and inhibited the growth of these AML cell lines (Figure 1j).

In an attempt to dissect the mechanism by which Gaq inhibition causes impairment in MLL leukemogenesis, we performed gene-expression analysis using RNA from shGaq transduced KLSleukemic cells and found that several mitochondrial complex 1 genes (i.e. mt-Nd2, mt-Nd4l, mt-Nd5) were down-regulated in Gaq knockdown cells, while the stress response and DNA damage repair gene Gadd45a was up-regulated (Figure 2a). The link between Gaq mRNA expression and the expression of mitochondrionally-encoded Nd2 and Nd4l genes was confirmed in a panel of primary human AML samples (n = 84; Table S1). Furthermore, activation of Gadd45a has been shown to occur as a result of activation of the FoxO family of transcription factors in response to oxidative stress (7). Taken together, these data suggest a possible link between Gaq and mitochondrial function; therefore, we next sought to test whether Gaq inhibition affects the response of leukemic cells to mitochondrial stress. We first treated leukemic cells with the mitochondrial complex 1 inhibitor rotenone, which has been reported to increase ROS production triggering apoptosis (8). Our results showed that ROS levels were slightly elevated in rotenone-treated KLSleukemic cells and were dramatically increased in rotenone-treated GMPleukemic cells (Figure 2b). Gaq knockdown also dramatically increased ROS levels in GMPleukemic cells (Figure 2c), suggesting a crucial role for Gaq activation in protecting GMPleukemic cells against oxidative stress. Gaq activity is important in GMPleukemic cells for maintaining basal oxidative phosphorylation (OXPHOS) levels and mitochondrial ATP production as Gaq inhibition, by either GP treatment or Gaq knockdown, significantly reduced the oxygen consumption rate (OCR, indicative of OXPHOS) and ATP levels in these cells, with a slightly reduced effect in KLSleukemic cells (Figures 2d, 2e, 2f, 2g). Such a functional role for Gaq is in keeping with the positive correlation between expression of Gaq and levels of mitochondrial complex 1 gene expression, supporting the hypothesis that Gaq expression and function may be closely
coupled to mitochondrial function in AML cells. It is worth noting that in MLL leukemic cells Gaq inhibition had similar effects to rotenone treatment on induction of ROS and suppression of OCR and colony formation (Figures 2b, 2d, 2h).

In this study, we report a previously unknown role for Gaq in maintenance of MLL leukemia. Previous studies implicate Gaq as a MLL fusion target whose expression correlates with H3K79 methylation in MLL LSCs (3, 4). The effect of Gaq inhibition on repression of MLL leukemic cell growth largely depends on endogenous β-catenin activity. This is consistent with our previous findings that aberrant activation of β-catenin is essential for self-renewal of MLL LSCs, whereas inhibition of β-catenin impairs LSC formation and MLL leukemia development (1, 9).

Here we describe a potential mechanism linking mitochondrial function to leukemogenesis via Gaq signaling activation (Figure S2). LSCs in AML rely primarily on OXPHOS to generate ATP for energy (10), and our results show that Gaq inhibition can impair the energy-generating capacity of MLL leukemic cells, increase ROS levels and lead to activation of a stress response associated with activation of Gadd45a. Our data revealing reduced β-catenin levels following Gaq inhibition are consistent with the effects of Gaq on mitochondrial activity being mediated via β-catenin activity, as recent studies have shown the crucial role for β-catenin in mitochondrial energy metabolism (11, 12), and β-catenin is also critical in HSC for suppression of ROS levels (12). MLL AML has a particular dependence on β-catenin activity (1) and the findings presented here raise the possibility that targeting of β-catenin and mitochondrial metabolism via Gaq may be a potential approach to reduction of leukemogenesis in MLL AML. Of note, while human AML represents a heterogeneous disease, the correlation of Gaq expression with expression of mitochondrially-encoded Nd2 and Nd4l genes provides support that Gaq-regulation of mitochondrial function may also be important in other AML subtypes. Whether activity of Gaq and/or OXPHOS in human AML cells, or levels of mitochondrial complex 1 genes, are predictive of response to chemotherapy is still under investigation. Gaq-mediated signaling is likely to cooperate with other pathways to drive leukemogenesis. In particular, FLT3-ITD mutation accelerates the onset of MLL-AF9-induced AML and correlates with poor outcome in AML patients (13). We have previously demonstrated that down-regulation of Gadd45a via FLT3-ITD contributes to the myeloid differentiation block in AML (14), and strikingly the regulator of G protein signaling 2 (Rgs2), a potent negative regulator of Gaq function (15), has also been shown to be a critical target gene down-regulated in FLT3-ITD-induced AML (16).

It is essential to investigate the mechanisms involved in G protein-associated regulation of β-catenin signaling in AML LSCs, as a number of studies have now shown that G-protein coupled signaling can be targeted therapeutically (15) and such an approach may provide clinical benefit for MLL AML patients. Future studies will identify predominant transducers in Gaq signaling and further define the role of β-
catenin down-regulation, FoxO transcription factors and Gadd45a in the stress response associated with Gaq inhibition.

References


Figure Legends

Figure 1: Gaq is required for maintenance of MLL leukemia.

(a) Western blot analysis of Gaq knockdown efficiency and beta-catenin expression (total beta-catenin versus phosphorylated/inactive beta-catenin) in scrambled control (Scr) versus 3 independent Gaq shRNAs (shGaq#1, shGaq#2, shGaq#3) transduced KLSpre-LSC and their respective colony formation. A reduced level of total beta-catenin (beta-catenin), concomitant with no change in phosphorylated/inactive beta-catenin (p-beta-catenin), indicates the activation of beta-catenin. The percentage of colonies at the third round of replating is shown.

(b) Western blot analysis of Gaq and beta-catenin expression in Scr, shGaq#1, shGaq#2 or shGaq#3 transduced GMPpre-LSC and their respective colony formation.

(c) Colony formation of KLSpre-LSC treated with control (water, H2O), 20 μM GP or 40 μM GP for 5 days, and western blot analysis of beta-catenin expression in KLSpre-LSC treated with 40 μM GP.

(d, e) Colony formation of KLSleukemic cells (d) or GMPleukemic cells (e) treated with 20 μM GP or 40 μM GP for 5 days. *, P < 0.05; **, P < 0.01; ***, P < 0.0005; ****, P < 0.0001.

(f, g, h) Bar graphs representing quantitative analysis of the data obtained from in vivo BrdU incorporation cell proliferation assays, indicative of 5 independent experiments for KLSleukemic cells (f) and 6 independent experiments for GMPleukemic cells (g). GFP" leukemic cells, which were sorted from fully developed primary MLL AML originating from KLS or GMPs, were pre-treated ex vivo with 40 μM GP for 2 - 4 days and subsequently transplanted into 2nd recipient mice for in vivo BrdU cell proliferation assays at 8 days post-injection (f, g), and Kaplan–Meier analysis of mouse survival (h).

(i) Kaplan-Meier survival curves of mice transplanted with GMPleukemic cells expressing Scr versus shGaq#2.
(j) Growth inhibition in human AML cell lines after treatment with 40 µM GP or 1 µM ABT-199 for 72 hr, determined by AlamarBlue cell proliferation assay.

***, $P < 0.01$; ****, $P < 0.0005$; *****, $P < 0.0001$; NS, not significant ($P > 0.05$).

**Figure 2:** Gaq inhibition target leukemic cells by impairing mitochondrial OXPHOS.

(a) Heat map showing differential expression of genes in Scr versus shGaq transduced KLS\textsuperscript{leukemic} cells (Blue = down-regulated, Red = up-regulated) with $p$-value $\leq 0.01$ and fold change cutoff of $\geq 1.9$.

(b, c) Mitochondrial ROS, as evaluated by the mitochondrial-specific redox probe MitoSox in KLS\textsuperscript{leukemic} or GMP\textsuperscript{leukemic} cells treated with control (DMSO), 25 nM rotenone or 50 nM rotenone for 24 h (b), or transduced with Scr versus shGaq (c).

(d, e) KLS\textsuperscript{leukemic} or GMP\textsuperscript{leukemic} cells treated with 40 µM GP or 1 µM rotenone for 48 h (d), or transduced with Scr versus shGaq (e). OCR was measured over a 2 h period and plotted as normalized % values of baseline (control) values for each sample.

(f, g) ATP levels in KLS\textsuperscript{leukemic} or GMP\textsuperscript{leukemic} cells treated with 40 µM GP for 48 h (f), or transduced with Scr versus shGaq (g).

(h) Colony formation of KLS\textsuperscript{leukemic} or GMP\textsuperscript{leukemic} cells treated with 25 nM rotenone or 50 nM rotenone for 5 days.
Figure 1.

**KLS\text{pre-LSC}**

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**GMP\text{pre-LSC}**

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**KLS\text{pre-LSC} - GP treatment**

- **H_2O 40µMGP**
- **β-cat**
- **Actin**

**KLS\text{leukemic} - GP treatment**

- **H_2O 10µM 20µM 40µM**
- **% colonies**

**GMP\text{leukemic} - GP treatment**

- **H_2O 10µM 20µM 40µM**
- **% colonies**

**Primary MLL AML**

- **Ex vivo GP pre-treatment (2 - 4 days)**
- **2nd transplant**
- **In vivo BrdU assays (8 days post-injection)**
- **Survival**

**KLS\text{leukemic} - 2nd transplant**

- **% BrdU+ cells**

**GMP\text{leukemic} - 2nd transplant**

- **% BrdU+ cells**

**Human AML cells - GP treatment (72hr)**

- **% Cell growth**

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Figure 2.

(a) Heatmap showing expression levels of various proteins, including Gadd45a, mt-Nd4, mt-Nd5, and mt-Nd2.

(b) Graph showing ROS levels under Rotenone treatment for KLSleukemic and GMPleukemic cells. DMSO 25nM and 50nM comparisons are depicted.

(c) Graph showing ROS levels under shGaq treatment for KLSleukemic and GMPleukemic cells.

(d) Graph comparing normalized OXPHOS levels under GP treatment for KLSleukemic and GMPleukemic cells.

(e) Graph comparing normalized OXPHOS levels under shGaq treatment for KLSleukemic and GMPleukemic cells.

(f) Graph showing ATP levels under GP treatment for KLSleukemic and GMPleukemic cells.

(g) Graph showing ATP levels under shGaq treatment for KLSleukemic and GMPleukemic cells.

(h) Graph showing colonies formed under Rotenone treatment for KLSleukemic and GMPleukemic cells.