MYELOID NEOPLASIA

Autocrine TNF-α production supports CML stem and progenitor cell survival and enhances their proliferation

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Key Points

- Autocrine TNF-α production by CML stem/progenitor cells is not BCR-ABL kinase-dependent and provides survival signals.
- Targeting TNF-α production by stem/progenitor cells might be exploited therapeutically, especially in combination with tyrosine kinase inhibitors.

Chronic myeloid leukemia (CML) stem cells are not dependent on BCR-ABL kinase for their survival, suggesting that kinase-independent mechanisms must contribute to their persistence. We observed that CML stem/progenitor cells (SPCs) produce tumor necrosis factor-α (TNF-α) in a kinase-independent fashion and at higher levels relative to their normal counterparts. We therefore investigated the role of TNF-α and found that it supports survival of CML SPCs by promoting nuclear factor κB/p65 pathway activity and expression of the interleukin 3 and granulocyte/macrophage-colony stimulating factor common β-chain receptor. Furthermore, we demonstrate that in CML SPCs, inhibition of autocrine TNF-α signaling via a small-molecule TNF-α inhibitor induces apoptosis. Moreover TNF-α inhibition combined with nilotinib induces significantly more apoptosis relative to either treatment alone and a reduction in the absolute number of primitive quiescent CML stem cells. These results highlight a novel survival mechanism of CML SPCs and suggest a new putative therapeutic target for their eradication. (*Blood*. 2013; 122(19):3335-3339)

Introduction

Disease persistence in chronic phase (CP) chronic myeloid leukemia (CML) patients receiving tyrosine kinase inhibitor therapy is caused by a population of leukemic stem cells (LSCs)[1,2] which are not *BCR-ABL* oncogene addicted,[3,4] highlighting the need to identify novel therapeutic targets for their eradication. Autocrine production of interleukin 3 and granulocyte-colony stimulating factor by CML stem/progenitor cells (SPCs) resulting in STAT5 activation and growth factor (GF)-independent growth has been reported, suggesting that this mechanism is relevant to BCR-ABL-induced transformation.[5] Tumor necrosis factor-α (TNF-α) is a pleiotropic GF whose role in hemopoiesis is highly dependent on cell context, its concentration, and the presence of other GFs, with both inhibitory and stimulatory effects reported.[6-8] Although originally described as cytotoxic to cancer cells, given its ability to induce apoptosis,[9] TNF-α is often produced by malignant and immune cells present in the inflammatory reaction surrounding tumors.[10,11] Regardless of its source, TNF-α can contribute to tumorigenesis by creating a tumor-supportive inflammatory microenvironment and through direct effects on malignant cells.[12] A role has already been reported for autocrine TNF-α produced by $JAK2^{V617F+}$ cells in supporting the growth of myeloproliferative neoplasm patients' $CD34^+$ cells while inhibiting normal $CD34^+$ cell growth.[13] In CML, it has been shown that TNF-α concentration is higher in bone marrow (BM) supernatants derived from $BCR\text{-}ABL^+$ transgenic mice compared with wild-type mice. Moreover, LSCs from $BCR\text{-}ABL^+$ mice proliferate more compared with wild-type counterparts when cultured in the presence of TNF-α at the concentrations detected in the BM of leukemic mice.[14] More recently, BCR-ABL-mediated upregulation of inflammatory pathway receptors (including TNF-α) has been shown to promote CML LSC self-renewal through upregulation of p150 isoform of the RNA editing enzyme ADAR1.[15] Here we investigated TNF-α production and its putative role as a survival and proliferative signal in primary human CML SPCs.

Materials and methods

Nilotinib (NL) was supplied by Novartis. The small-molecule TNF-α inhibitor[16] and human recombinant TNF-α were purchased, respectively, from Merck Chemicals and New England BioLabs. The study was approved by the West of Scotland Research ethics committee, number 10/S0704/2. Plasma and primary cells were obtained after consent, according to the Declaration of Helsinki, from blood and leukapheresis samples of CML and lymphoma patients without BM involvement as normal controls. $CD34^+$ enrichment and in vitro culture in physiologic (for CML cells) or high (for normal cells) GF-supplemented serum-free medium and colony-forming cell (CFC) assays were performed as previously described.[3] Sorting into $CD34^+$ $CD38^-$ and $CD34^+$ $CD38^+$ cells and detection of *BCR-ABL* fusion in $CD34^+$ $CD38^-$ CML cells

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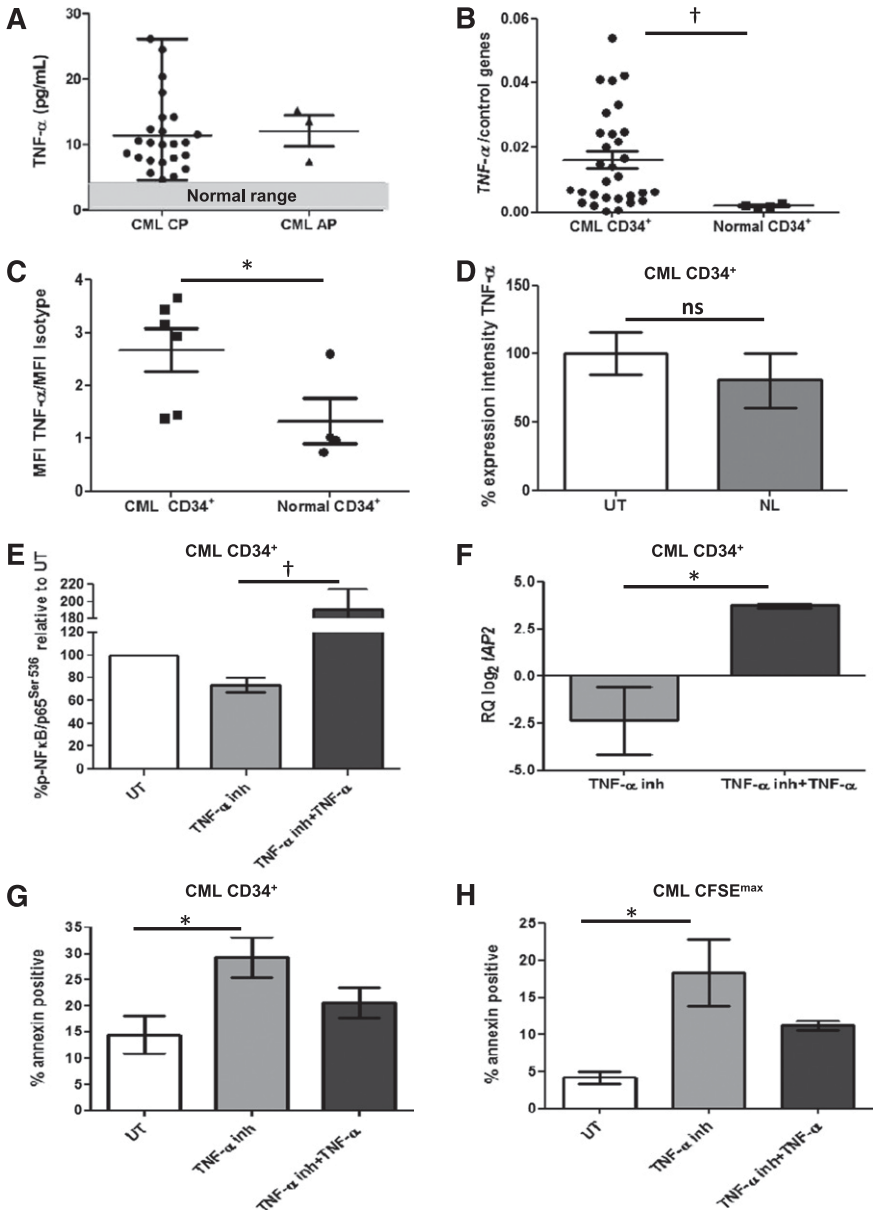

Figure 1. Autocrine TNF-α production in CML SPCs is BCR-ABL kinase-independent, induces NFκB/p65 activity, and promotes their survival. (A) TNF-α blood plasma levels were measured by enzyme-linked immunosorbent assay in CP (n = 24) and accelerated phase (AP) (n = 3) CML patients. Levels are expressed as picograms per milliliter. The range of TNF-α blood plasma levels in normal controls (n = 8) is shown in the shaded area. (B) *TNF-α* mRNA expression levels were measured by qRT-PCR and normalized to the control genes *ATP5B*, *B2M*, *ENOX2*, *GUSB*, *TBP*, and *TYW1* mRNA expression levels in newly diagnosed CP CML (n = 30) and normal (n = 4) $CD34^+$ cells. (C) TNF-α protein expression was measured by intracellular flow cytometry in CML (n = 6) and normal (n = 4) $CD34^+$ cells and expressed as a ratio of the mean fluorescence intensity (MFI) of TNF-α antibody-stained cells over the MFI of cells stained with a matched isotype control. (D) CML $CD34^+$ cells (n = 4) were either left untreated (UT) or treated with NL (5 μM) for 48 hours, and TNF-α protein expression was measured by intracellular flow cytometry, as explained in panel C. TNF-α expression levels in the NL-treated cells were expressed as a percentage of UT cells. (E) CML $CD34^+$ cells (n = 3) were either left UT or treated with TNF-α inhibitor (TNF-α inh) (3 μM) or TNF-α inh (3 μM) + TNF-α (1 ng/mL). Levels of *p*-NFκB/$p65^{Ser536}$ were measured by intracellular flow cytometry at 24 hours, as described in panel C, and expressed as a percentage of UT. (F) *IAP2* gene expression levels were measured at 24 hours by qRT-PCR after treatment, as in panel E. Differences in gene expression levels after treatment were calculated using the $2^{-\Delta\Delta Ct}$ method, after normalization within each sample of candidate gene expression levels against *GAPDH* and *TBP* expression levels. Relative quantification (RQ) of *IAP2* mRNA expression after TNF-α inh treatment was then plotted as $\log_2$ of the $2^{-\Delta\Delta Ct}$ values (with the UT cells having a value of 0 in the graph being the calibrator). (G) CML $CD34^+$ cells (n = 5) were either left UT or treated with TNF-α inh (3 μM) or TNF-α inh (3 μM) + TNF-α (1 ng/mL) for 72 hours. Percentage of apoptotic cells was measured by annexin staining. (H) CML $CD34^+$ cells (n = 3) were CFSE stained and then cultured as in panel G for 72 hours. Percentage of apoptotic cells within the undivided ($CFSE^{max}$) population was measured by gating on the population double-positive for maximal CFSE expression and annexin staining. All data from independent experiments are presented as mean ± standard error of the mean. *$P < .05$; †$P < .01$; ‡$P < .001$; ns, not significant.

by fluorescence in situ hybridization were performed as previously reported.[17] Enzyme-linked immunosorbent assay was carried out using the Invitrogen Human UltraSensitive TNF-α kit (KHC3014), according to the manufacturer's protocol. Western blotting and flow cytometry for surface/intracellular protein, annexin, and carboxyfluorescein succinimidyl ester (CFSE) staining with percentage recovery calculations were performed as previously described.[3,17] Quantitative real-time–polymerase chain reaction (qRT-PCR) was undertaken using the Fluidigm BioMark HD System and TaqMan (Applied Biosystems) gene expression assays, as per manufacturer's instructions (a list of antibodies and gene expression assays used is provided in the supplemental Methods). Statistical analysis was done by Student *t*-test for matched samples, Mann–Whitney test for unpaired samples, and 1-way analysis of variance with post hoc testing for multiple comparisons.

Results and discussion

Having demonstrated that TNF-α plasma levels are consistently higher in samples from CML patients than in normal patients' samples, regardless of disease stage, we investigated *TNF-α* mRNA expression in a large cohort of CML SPCs and found that it was significantly elevated relative to normal SPCs. Although higher in CML mononuclear compared with $CD34^+$ cells (as expected, given TNF-α is normally produced by lymphocytes and macrophages), *TNF-α* mRNA levels were similar between the CML $CD34^+$ $CD38^-$ and $CD34^+$ $CD38^+$ cell fractions (Figure 1A-B; supplemental Figure 1A-B). We confirmed this finding at the protein level in a small group of samples and showed that autocrine TNF-α production by CML SPCs was not significantly reduced by treatment with NL at either the mRNA or protein level, suggesting it is not under the control of BCR-ABL kinase (Figure 1C-D; supplemental Figure 1C-E).

TNF-α's pleiotropic effects are secondary to its ability to activate both proapoptotic and prosurvival signals.[18] Among the latter, the nuclear factor κB (NFκB)/p65 transcription factor is particularly relevant. On NFκB/p65 expression, TNF-α is unable to induce apoptosis because it simultaneously activates NFκB/p65, which

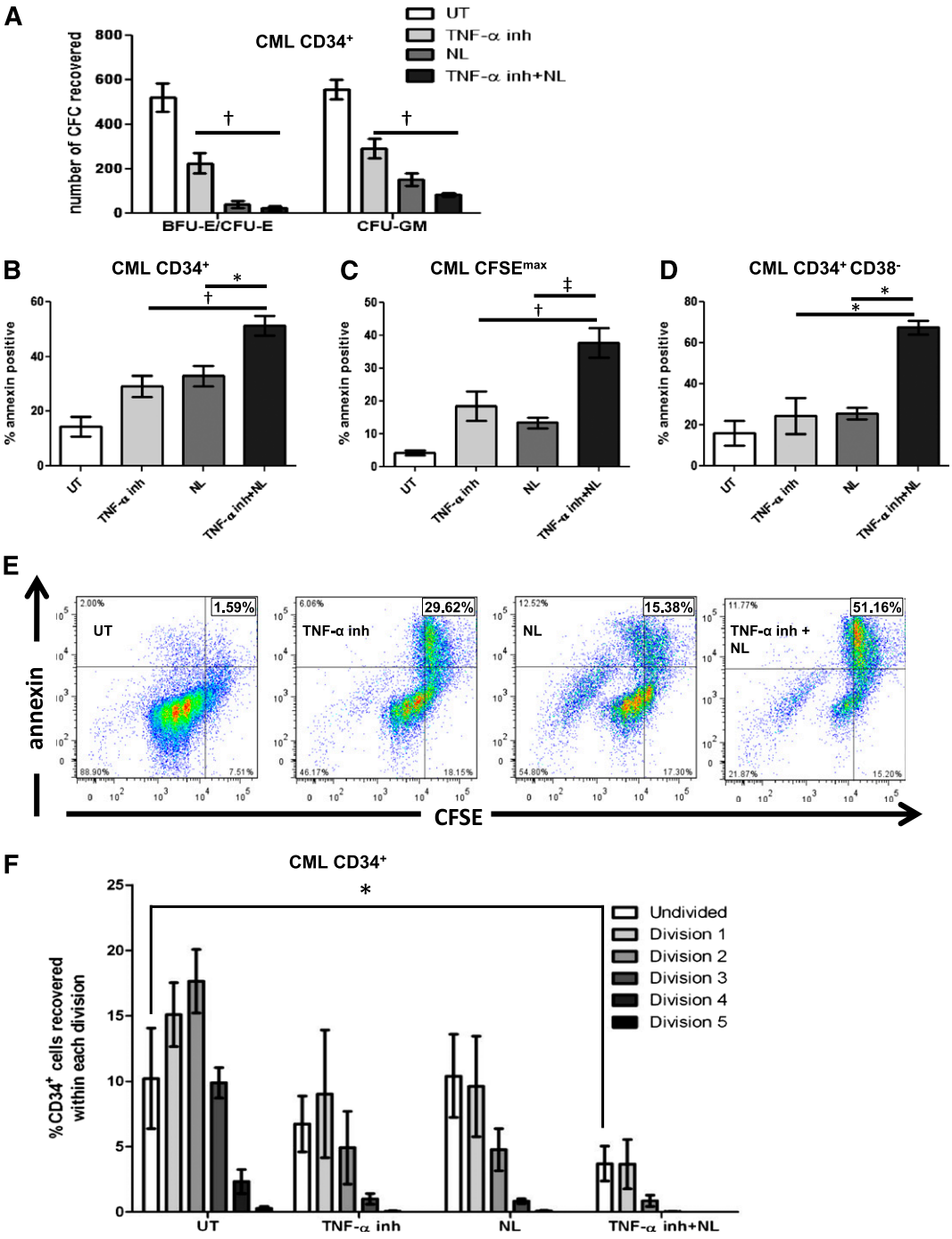

Figure 2. Effects of autocrine TNF-α inhibition in combination with NL on CML SPCs survival and proliferation. (A) CML CD34$^+$ cells (n = 3) were either left UT or treated with TNF-α inh (3 μM), NL (5 μM), or their combination for 72 hours before drug washout and plating in methylcellulose progenitor assays. CFC frequency based on their morphology (erythroid-burst-forming unit [BFU-E] and erythroid-colony-forming unit [CFU-E] vs granulocyte/macrophage-colony forming unit [CFU-GM]) was recorded after 12 days of culture. (B) CML CD34$^+$ cells (n = 5) were cultured as in panel A for 72 hours, and the percentage of apoptotic cells was measured by annexin staining. (C) CML CD34$^+$ cells (n = 4) were CFSE stained and then cultured as in panel A for 72 hours. Percentage of apoptotic cells within the undivided (CFSE^{max}) population was measured as explained in Figure 1H. (D) Sorted CML, *BCR-ABL*$^+$ (by fluorescence in situ hybridization), CD34$^+$ CD38$^-$ cells (n = 2) were cultured as in panel A for 72 hours. Percentage of apoptotic cells was measured by annexin staining. (E) Representative flow cytometry plot of CFSE and annexin double-staining showing levels of apoptosis within the CFSE^{max} population in each treatment group. (F) CML CD34$^+$ cells (n = 4) were treated for 72 hours, as in panel A, and the percentage of starting CD34$^+$ cells recovered within each division in each treatment group was calculated by recording the number of viable cells seeded initially in each culture and their number after different treatment conditions, using levels of CFSE fluorescence to measure the percentage of cells within each division, as explained elsewhere.[17] All data from independent experiments are presented as mean ± standard error of the mean. *$P < .05$; †$P < .01$; ‡$P < .001$.

promotes, among others, the expression of the inhibitor of apoptosis protein (IAP) family. IAPs block the proapoptotic caspase 8 activation, which is also induced by TNF-α, so that in their presence, the net output of TNF-α signaling is to promote survival and proliferation of its target cells.[19,20] IAP2 in particular is directly activated by NFκB/p65 and, in turn, activates it through a positive feedback loop.[20] CML cells express a constitutively active NFκB/p65,[21] and treatment of CML CD34$^+$ cells with TNF-α inhibitor (which promotes subunit

disassembly of the TNF-α trimer[16]) reduced phosphorylation levels of NFκB/p65 on the activating serine 536[22] (although to a moderate extent suggesting residual NFκB/p65 phosphorylation was present, possibly as a result of BCR-ABL kinase activity) and of its upstream inhibitor IκBα, which is degraded when phosphorylated,[18] on serine 32/36. Moreover, consistent correlative changes in *IAP2* gene expression were observed. These effects were rescued by adding TNF-α to the culture (Figure 1E-F; supplemental Figure 2). TNF-α also exerts stimulatory effects on normal hemopoiesis indirectly by inducing interleukin 3 and granulocyte/macrophage colony-stimulating factor common β-chain receptor (CSF2RB) expression in normal $CD34^+$ cells.[23] We observed that CSF2RB gene and protein expression were higher in CML relative to normal SPCs and were downregulated by TNF-α inhibitor, with these effects again rescued by TNF-α (supplemental Figure 3). Together, these results suggest that autocrine TNF-α could act as a survival and proliferative signal in CML $CD34^+$ by inducing NFκB/p65 activity and CSF2RB expression. Consistent with this hypothesis, TNF-α inhibitor reduced proliferation and increased apoptosis levels in CML $CD34^+$ cells, including within the tyrosine kinase inhibitor resistant quiescent ($CFSE^{max}$) population,[17,24] with TNF-α again rescuing this phenotype (Figure 1G-H). Similar effects were not seen in normal $CD34^+$ cells, which express lower/negligible levels of autocrine TNF-α, suggesting that the results observed in CML $CD34^+$ cells were secondary to autocrine TNF-α inhibition (supplemental Figure 4).

Because TNF-α production by CML SPCs was not BCR-ABL kinase-dependent and TNF-α inhibitor showed no off-target inhibition of BCR-ABL kinase activity (supplemental Figure 5), we investigated the effects of NL and TNF-α inhibitor in combination on CML SPCs. This combination reduced CML $CD34^+$ cells' CFC output and induced significantly higher levels of apoptosis relative to either treatment alone, including within $CFSE^{max}$ and $CD34^+$ $CD38^-$ cells (Figure 2A-E). Analysis of percentage recovery of starting cells, which relates the contribution of input cells to the surviving output cell number after drug treatment, confirmed that the NL and TNF-α inhibitor combination resulted in a significant depletion of the $CFSE^{max}$ cells relative to untreated cells (Figure 2F).

These observations support the hypothesis that, similar to the effects reported in $JAK2^{V617+}$ myeloproliferative neoplasms,[13] autocrine TNF-α promotes survival and proliferation in CML $CD34^+$ cells, and that interference with TNF-α production/signaling could be exploited therapeutically for their eradication. Moreover, as TNF-α acts as a prosurvival signal only in the presence of an active NFκB/p65, its autocrine production could also be directed toward apoptosis induction by inhibiting NFκB/p65 signals through IAP inhibitors (such as second mitochondria-derived activator of caspases [SMAC] protein mimetics), as already shown in other cancer models.[25] A detailed characterization of the effects of autocrine GFs produced by CML SPCs can help identifying novel therapeutic targets for their eradication.

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Authorship

Contribution: P.G. designed and performed research, analyzed and interpreted data, and wrote the manuscript; F.P., H.M., K.L., and E.K.A. performed research and reviewed the manuscript; R.B. and M.C. interpreted data and reviewed the manuscript; and H.G.J. and T.L.H. designed research, interpreted data, and wrote the manuscript.

Conflict-of-interest disclosure: P.G. has previously received travel grants from Bristol-Myers Squibb; R.B. has previously served in advisory boards and received honoraria from Novartis, Bristol-Myers Squibb, and Teva; and T.L.H. has previously received research funding from Novartis and Bristol-Myers Squibb. The remaining authors declare no competing financial interests.

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