

**Autocrine TNF- $\alpha$  production supports CML stem and progenitor cell survival and enhances their proliferation**

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Running title: Autocrine TNF- $\alpha$  as a survival cue in CML

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**Key points**

- Autocrine TNF- $\alpha$  production by CML stem/progenitor cells (SPCs) is not BCR-ABL kinase-dependent and provides survival signals
- Targeting TNF- $\alpha$  production by CML SPCs might be exploited therapeutically especially in combination with tyrosine kinase inhibitors (TKIs)

**Abstract**

Chronic myeloid leukaemia (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 tumour necrosis factor-alpha (TNF- $\alpha$ ) in a kinase-independent fashion and at higher levels relative to their normal counterparts. We therefore investigated the role of TNF- $\alpha$  and found that it supports survival of CML SPCs by promoting NF $\kappa$ B/p65 pathway activity and expression of the interleukin-3 and granulocyte/macrophage-colony stimulating factor common  $\beta$ -chain receptor. Furthermore, we demonstrate that in CML SPCs inhibition of autocrine TNF- $\alpha$  signalling via a small molecule TNF- $\alpha$  inhibitor induces apoptosis. Moreover TNF- $\alpha$  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.

## Introduction

Disease persistence in chronic phase (CP) chronic myeloid leukaemia (CML) patients on tyrosine kinase inhibitor (TKI) therapy is caused by a population of leukaemic stem cells (LSCs)<sup>1,2</sup> which are not BCR-ABL oncogene addicted<sup>3,4</sup>, thus 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<sup>5</sup>. Tumour necrosis factor-alpha (TNF- $\alpha$ ) is a pleiotropic GF whose role in haemopoiesis is highly dependent on cell context, its concentration and the presence of other GFs, with both inhibitory and stimulatory effects reported<sup>6-8</sup>. Although originally described as cytotoxic to cancer cells given its ability to induce apoptosis<sup>9</sup>, TNF- $\alpha$  is often produced by malignant and immune cells present in the inflammatory reaction surrounding tumours<sup>10,11</sup>. Regardless of its source, TNF- $\alpha$  can contribute to tumourigenesis by creating a tumour-supportive inflammatory microenvironment and through direct effects on malignant cells<sup>12</sup>. A role has already been reported for autocrine TNF- $\alpha$  produced by JAK2<sup>V617F+</sup> cells in supporting the growth of myeloproliferative neoplasm patients' CD34<sup>+</sup> cells, while inhibiting normal CD34<sup>+</sup> cell growth<sup>13</sup>. In CML it has been shown that TNF- $\alpha$  concentration is higher in bone marrow (BM) supernatants derived from BCR-ABL<sup>+</sup> transgenic compared to wild-type mice. Moreover LSCs from BCR-ABL<sup>+</sup> mice proliferate more compared to wild-type counterparts when cultured in the presence of TNF- $\alpha$  at the concentrations detected in the BM of leukaemic mice<sup>14</sup>. More recently, BCR-ABL-mediated upregulation of inflammatory pathway receptors (including TNF- $\alpha$ ) has been shown to promote CML LSC self-renewal through upregulation of p150 isoform of the RNA editing enzyme ADAR1<sup>15</sup>. Here we investigated TNF- $\alpha$  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- $\alpha$  inhibitor<sup>16</sup> and human recombinant TNF- $\alpha$  were purchased respectively from Merck Chemicals and New England BioLabs. Plasma and primary cells were obtained following consent, according to the Declaration of Helsinki, from blood and leukapheresis samples of CML and lymphoma

patients without BM involvement as normal controls. CD34<sup>+</sup> enrichment, *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<sup>3</sup>. Sorting into CD34<sup>+</sup> CD38<sup>-</sup> and CD34<sup>+</sup> CD38<sup>+</sup> cells and detection of BCR-ABL fusion in CD34<sup>+</sup> CD38<sup>-</sup> CML cells by fluorescence in situ hybridisation were performed as previously reported<sup>17</sup>. ELISA was carried out using the Invitrogen Human UltraSensitive TNF- $\alpha$  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<sup>3,17</sup>. 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 (list of antibodies and gene expression assays used in supplemental materials). Statistical analysis was done by Student *t*-test for matched samples, Mann-Whitney test for unpaired samples and one-way ANOVA with post-hoc testing for multiple comparisons.

## **Results and discussion**

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

TNF- $\alpha$ 's pleiotropic effects are secondary to its ability to activate both proapoptotic and prosurvival signals<sup>18</sup>. Amongst the latter, the NF $\kappa$ B/p65 transcription factor is particularly relevant. Upon NF $\kappa$ B/p65 expression, TNF- $\alpha$  is unable to induce apoptosis because it simultaneously activates NF $\kappa$ B/p65 which promotes, amongst others, the expression of the inhibitor of apoptosis protein (IAP) family. IAPs block the proapoptotic caspase-8 activation

also induced by TNF- $\alpha$  so that in their presence the net output of TNF- $\alpha$  signalling is to promote survival and proliferation of its target cells<sup>19,20</sup>. IAP2 in particular is directly activated by NF $\kappa$ B/p65 and in turn activates it through a positive feedback loop<sup>20</sup>. CML cells express a constitutively active NF $\kappa$ B/p65<sup>21</sup> and treatment of CML CD34<sup>+</sup> cells with TNF- $\alpha$  inhibitor - which promotes subunit disassembly of the TNF- $\alpha$  trimer<sup>16</sup>- reduced phosphorylation levels of NF $\kappa$ B/p65 on the activating serine 536<sup>22</sup> (although to a moderate extent suggesting residual NF $\kappa$ B/p65 phosphorylation was present possibly due to BCR-ABL kinase activity) and of its upstream inhibitor I $\kappa$ B $\alpha$  - which is degraded when phosphorylated<sup>18</sup> - on serine 32/36. Moreover consistent correlative changes in IAP2 gene expression were observed. These effects were rescued by adding TNF- $\alpha$  to the culture (Figure 1E, F; supplemental Figure 2). TNF- $\alpha$  also exerts stimulatory effects on normal haemopoiesis indirectly by inducing interleukin-3 and granulocyte/macrophage-colony stimulating factor common  $\beta$ -chain receptor (CSF2RB) expression in normal CD34<sup>+</sup> cells<sup>23</sup>. We observed that CSF2RB gene and protein expression were higher in CML relative to normal SPCs and downregulated by TNF- $\alpha$  inhibitor, with these effects again rescued by TNF- $\alpha$  (supplemental Figure 3). Together these results suggest that autocrine TNF- $\alpha$  could act as a survival and proliferative signal in CML CD34<sup>+</sup> by inducing NF $\kappa$ B/p65 activity and CSF2RB expression. Consistent with this hypothesis, TNF- $\alpha$  inhibitor reduced proliferation and increased apoptosis levels in CML CD34<sup>+</sup> cells, including within the TKI resistant quiescent (CFSE<sup>max</sup>) population<sup>17,24</sup>, with TNF- $\alpha$  again rescuing this phenotype (Figure 1G, H). Similar effects were not seen in normal CD34<sup>+</sup> cells which express lower/negligible levels of autocrine TNF- $\alpha$  suggesting that the results observed in CML CD34<sup>+</sup> cells were secondary to autocrine TNF- $\alpha$  inhibition (supplemental Figure 4).

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

These observations support the hypothesis that, similarly to the effects reported in JAK2<sup>V617+</sup> myeloproliferative neoplasms<sup>14</sup>, autocrine TNF- $\alpha$  promotes survival and proliferation in CML CD34<sup>+</sup> cells and that interference with TNF- $\alpha$  production/signalling could be exploited therapeutically for their eradication. Moreover as TNF- $\alpha$  acts as a prosurvival signal only in the presence of an active NF $\kappa$ B/p65, its autocrine production could also be directed towards apoptosis induction by inhibiting NF $\kappa$ B/p65 signals through IAP inhibitors (such as SMAC mimetics), as already shown in other cancer models<sup>25</sup>. A detailed characterisation of the effects of autocrine GFs produced by CML SPCs can help identifying novel therapeutic targets for their eradication.

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### **Authorship Contributions**

P.G. designed and performed research, analysed 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; H.G.J. and T.L.H. designed research, interpreted data and wrote the manuscript.

**Conflict of Interest Disclosures**

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.

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## Figure legends

### **Figure 1. Autocrine TNF- $\alpha$ production in CML SPCs is BCR-ABL kinase-independent, induces NF $\kappa$ B/p65 activity and promotes their survival**

(A) TNF- $\alpha$  blood plasma levels were measured by ELISA in CP (n=24) and accelerated phase (AP) (n=3) CML patients. Levels are expressed as pg/mL. Range of TNF- $\alpha$  blood plasma levels in normal controls (n=8) is shown in shaded area. (B) TNF- $\alpha$  mRNA expression levels were measured by qRT-PCR and normalised 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<sup>+</sup> cells. (C) TNF- $\alpha$  protein expression was measured by intracellular flow-cytometry in CML (n=6) and normal (n=4) CD34<sup>+</sup> cells and expressed as a ratio of the mean fluorescence intensity (MFI) of TNF- $\alpha$  antibody stained cells over the MFI of cells stained with a matched isotype control. (D) CML CD34<sup>+</sup> cells (n=4) were either left UT or treated with NL (5 $\mu$ M) for 48 hours and TNF- $\alpha$  protein expression was measured by intracellular flow-cytometry as explained in panel C. TNF- $\alpha$  expression levels in the NL treated cells were expressed as a percentage of UT. (E) CML CD34<sup>+</sup> cells (n=3) were either left UT or treated with TNF- $\alpha$  inhibitor (TNF- $\alpha$  inh) (3 $\mu$ M) or TNF- $\alpha$  inh (3 $\mu$ M) + TNF- $\alpha$  (1ng/mL). Levels of p-NF $\kappa$ B/p65<sup>Ser536</sup> were measured by intracellular flow-cytometry at 24 hours as described in panel C and expressed as percentage of UT. (F) IAP2 gene expression levels were measured at 24 hours by qRT-PCR following treatment as in E. Differences in gene expression levels following treatment were calculated using the  $2^{-\Delta\Delta C_t}$  method after normalisation within each sample of candidate gene expression levels against GAPDH and TBP expression levels. Relative quantification (RQ) of TNF- $\alpha$  mRNA expression following NL treatment was then plotted as  $\log_2$  of the  $2^{-\Delta\Delta C_t}$  values (with the UT cells having a value of 0 in the graph being the calibrator). (G) CML CD34<sup>+</sup> cells (n=5) were either left UT or treated with TNF- $\alpha$  inh (3 $\mu$ M) or TNF- $\alpha$  inh (3 $\mu$ M) + TNF- $\alpha$  (1ng/mL) for 72 hours. Percentage of apoptotic cells was measured by annexin staining. (H) CML CD34<sup>+</sup> cells (n=3) were CFSE stained and then cultured as in panel G for 72 hours. Percentage of apoptotic cells within the undivided (CFSE<sup>max</sup>) 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  $\pm$  standard error of the mean (SEM). Significance values: \*,  $P < 0.05$ ; †,  $P < 0.01$ ; ‡,  $P < 0.001$ ; ns, not significant.

**Figure 2. Effects of autocrine TNF- $\alpha$  inhibition in combination with NL on CML SPCs survival and proliferation**

(A) CML CD34<sup>+</sup> cells (n=3) were either left UT or treated with TNF- $\alpha$  inh (3 $\mu$ M), NL (5 $\mu$ M) 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) versus granulocyte/macrophage-colony forming unit (CFU-GM) - was recorded after 12 days culture. (B) CML CD34<sup>+</sup> cells (n=5) were cultured as in panel A for 72 hours and percentage of apoptotic cells was measured by annexin staining. (C) CML CD34<sup>+</sup> cells (n=4) were CFSE stained and then cultured as in panel A for 72 hours. Percentage of apoptotic cells within the undivided (CFSE<sup>max</sup>) population was measured as explained in Figure 1H. (D) Sorted CML, BCR-ABL<sup>+</sup> (by fluorescence in situ hybridisation) CD34<sup>+</sup> CD38<sup>-</sup> 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<sup>max</sup> population in each treatment arm. (F) CML CD34<sup>+</sup> cells (n=4) were treated for 72 hours as in panel A and the percentage of starting CD34<sup>+</sup> cells recovered within each division in each treatment arm was calculated by recording the number of viable cells seeded initially in each culture and their number following different treatment conditions and using levels of CFSE fluorescence to measure the percentage of cells within each division as explained elsewhere<sup>17</sup>. All data from independent experiments are presented as mean  $\pm$  SEM. Significance values; \*,  $P < 0.05$ ; †,  $P < 0.01$ ; ‡,  $P < 0.001$ .

## Figures

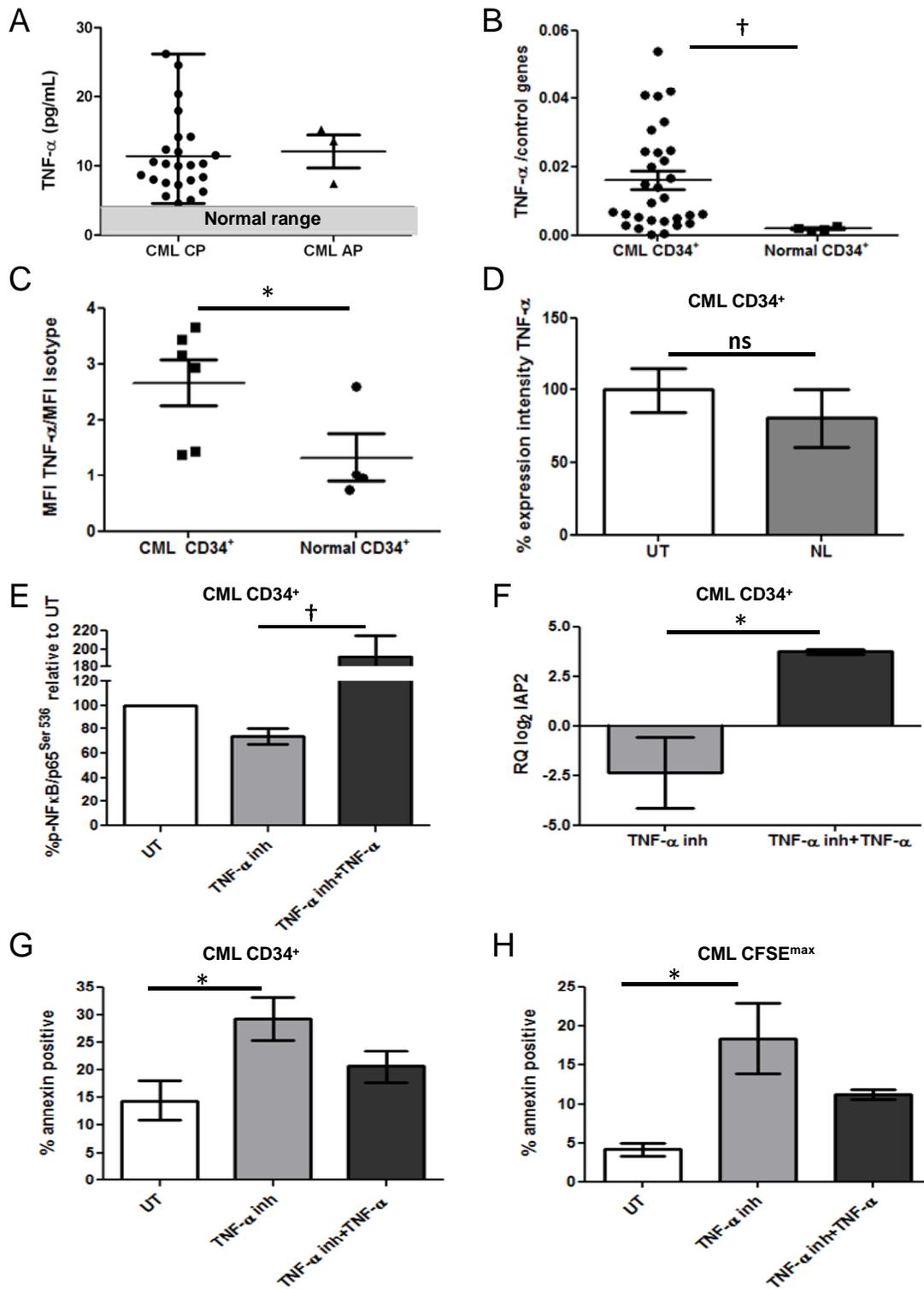


FIGURE 1

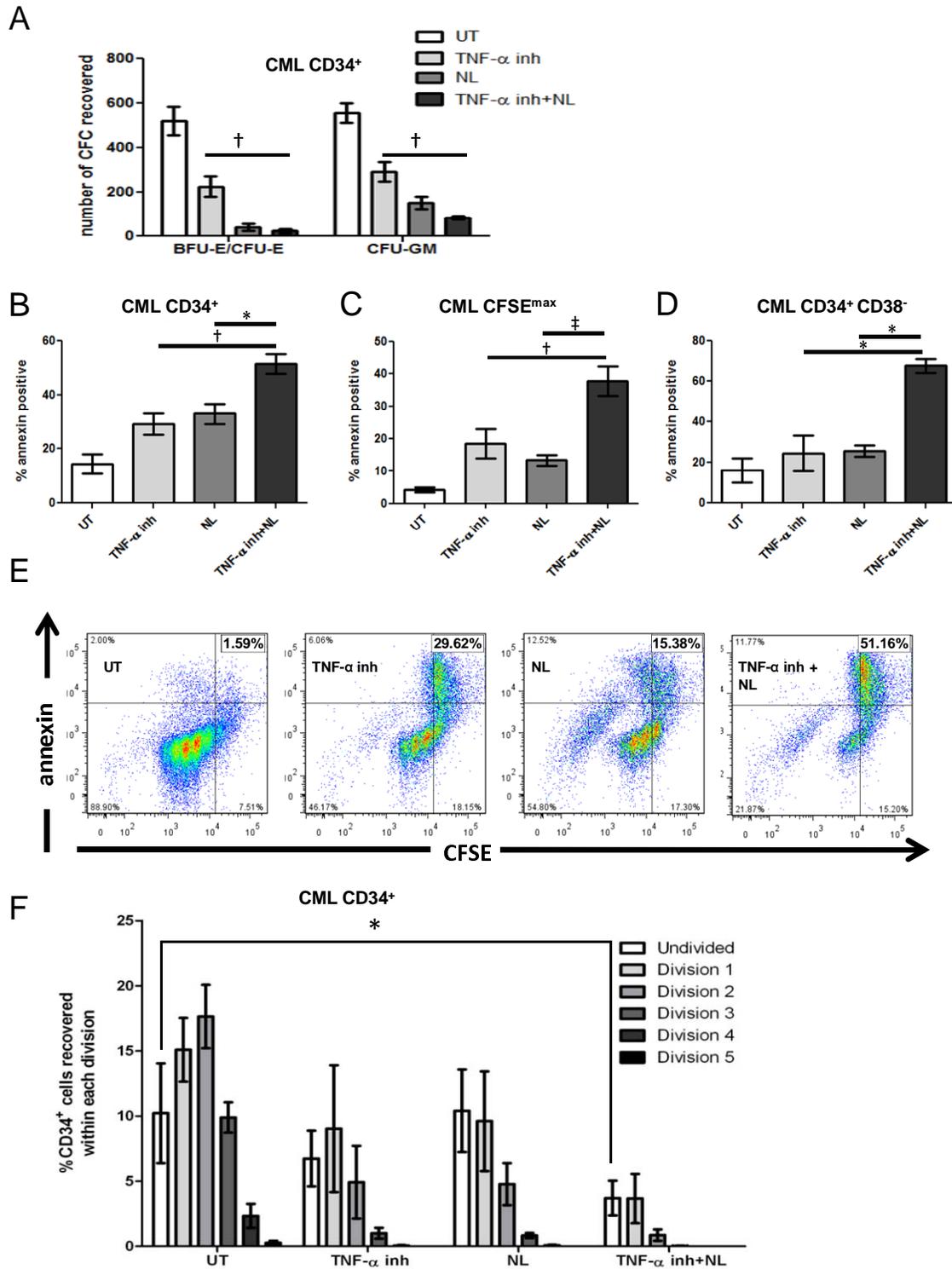


FIGURE 2