Author’s response to reviews

Title: Infliximab therapy together with tyrosine kinase inhibition targets leukemic stem cells in chronic myeloid leukemia

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Author’s response to reviews:

Dear Members of the editorial board,

We thank the reviewers and members of the editorial board for the review of our manuscript. We have made specific changes to the paper in order to carefully address the concerns that were pointed out by the reviewers and this resulted in the addition of 6 new figures and 1 revised figure. All text changes in the revised manuscript are marked in yellow and are included in this rebuttal letter. Deleted text passages appear only in this letter and are indicated by a curly bracket. We hope that the revised manuscript is now acceptable for publication in BMC Cancer.

Thank you and best regards,

Mirle Schemionek on behalf of all coauthors.
Reviewer reports:

Hsin-An Hou (Reviewer 1): It is well known that CML leukemic stem cells (LSCs) persist even in patients responding well to TKI therapy. LSCs with dysregulated capacities of self-renewal and survival are thought to be the root of leukemia and responsible for the relapse. Dr Herrmann et al demonstrated that ectopic expression of BCR-ABL could induce TNFα expression and in vitro exposure to TNFα antibody (infliximab, IFX) reduced clonogenic growth of CML. IFX therapy further boosted TKI-induced therapeutic effect in serial transplantation model. The manuscript is well written and would be of interest to the readers in this field.

Thank you.

(A) Since TNFα is a highly pleiotropic cytokine involved in wild spectrum of physiological processes that control inflammation and oncogenic/anti-tumor responses, do you have data suggesting TNFα could activate Treg and MDSC function, creating a tumor-supportive inflammatory niche in CML. Knockdown of TNFα could suppress tumor immune invasion in in vivo model.

In our study, we have focused on the effect of TNFα on the malignant cell itself and have so far not looked at immunomodulatory functions. However, indeed there are data available supporting the idea of a TNFα associated tumor-promoting niche in CML and we have therefore now added the following text to the discussion part of the manuscript (p 19, l 475):

“As a pleiotropic cytokine, TNFα is involved in pro- as well as anti-inflammatory processes and immunosuppressive mechanisms. In this regard, TNFα has been shown to impair conventional T cell survival [46] or promote immunosuppressive cells, such as myeloid-derived suppressor cells (MDSC) [47, 48] or regulatory T cells (Tregs) [49, 50]. Along this line, IFX therapy in sarcoidosis patients has been shown to reduce elevated frequency of Tregs [51]. Interestingly, CML patients show elevated levels of MDSCs [52, 53] and Tregs [54]. Moreover, CML derived MDSCs themselves have been suggested as a source of TNFα [55], tempting to speculate that TNFα inhibition could also impact on CML biology, not only by direct effects on the malignant stem cell itself but also by supporting a tumor promoting niche.”

(B) Did the authors also perform TNFα knockdown by shRNA or siRNA?

In order to follow an approach that would be more close to a potential clinical application we have here decided to target TNFα via drug treatment and have not performed shRNA or siRNA-mediated reduction, at this stage of the project. However, we do agree that this would be an interesting tool e.g. for identification of the important cell population being responsible for elevated TNFα levels in patients (malignant cells themselves, hematopoietic cell subsets or non-hematopoietic niche cells?) for upcoming projects.

(C) In a clinical observational study, IFX has been suspected to promote tumor development, as illustrated by occurrence of hematologic malignancies in the course of treatment with anti-TNFα (Arthritis Rheum 2002;46:3151-8). A diametrically opposed effect of IFX on the course of CML was previously reported (Ann Rheum Dis 2005;64:509-10 and Leuk
Do you have any comments while applying the combination therapy in clinical practice? As I am aware, there is no such ongoing clinical trial.

While studying the fundamental mechanisms of stem cell persistence in the first place we would envision that these findings might translate into a clinical application. In this respect, it is important to note that an anti-inflammatory therapeutic approaches in general bears a certain risk of off-target effects. The report mentioned by the reviewer describes 8 cases of lymphoma development in rheumatoid arthritis and Crohn’s disease patients, within 8 weeks of treatment in median (Arthritis Rheum 2002;46:3151-8). Although the relatively short latency between IFX application and lymphoma development is prominent, it is worth mentioning that the selected patient cohort has per se a higher risk of lymphoma development and this has to our knowledge not been reported for CML patients in the area of TKI therapy. If there is a perspective of TNFα inhibition in CML patients, one could attempt to first achieve a major molecular response using standard TKI therapy and then inhibit TNFα for a restricted period. In this scenario, TNFα inhibition could be applied for a relatively short period, e.g. in those patients that would be eligible for TKI stop, aiming to test if this approach may enhance treatment free remission rates in CML patients.

Eishi Ashihara (Reviewer 2): The authors demonstrated that Infliximab therapy with TKI suppresses LSC in CML. These findings are very interesting and informative for clinical scientists in Onco-Hematology. However, the following points are needed to be clarified with additional experiments:

1. Does BCR-ABL1 signal correlate with TNF-α signal in CML-LSCs/progenitors? If Yes, how does it correlate? If No, how is TNF-α signal activated?

We thank the reviewer for bringing up this point and we have now analysed 1) TNFα signaling in the absence and presence of Bcr-Abl inhibition and have studied 2) the expression of the cytokine itself in the malignant cell population. We have implemented the data as Figure 1f-i in the manuscript and added the following text to the results part (p 10, l 240):
malignant kinase activity. Next, we studied TNFα expression in LSK cells from SCLtTA/Bcr-Abl mice, that were first induced to express Bcr-Abl for 25 days and then reverted to not express Bcr-Abl for 48 days [4]. Expression of TNFα in these previously malignant LSK cells was reverted to normal level (Figure 1i).”

Moreover, we have added the following text to the discussion section (p 17, l 421):

“Although we clearly show elevated TNFα expression in Bcr-Abl positive cells, our data also suggest that this is dependent on the malignant kinase, at least in a murine myeloid progenitor cell line. Likewise, we observed reduction of TNFα expression in LSK cells upon long-term reversion of Bcr-Abl expression. However, this could also be due to re-expansion of Bcr-Abl negative LSK cells upon inhibition of the kinase as we have studied the expression of TNFα 48 days after Bcr-Abl reversion in this model. In primitive human LSCs, TKI persistent TNFα expression has been demonstrated [14, 33]. Yet, additional cell populations could contribute to elevated TNFα levels that are observed in CML mice and patients. This also ties in with the recent finding that CML-derived osteoblasts show elevated levels of TNFα expression, in the SCLtTA/Bcr-Abl model [34].”

2. On inhibition of TNF-α signal, was the proliferation of CML-LSCs/progenitors suppressed or was apoptosis induced in CML-LSCs/progenitors?

We have now studied the effect on apoptosis and proliferation using the murine myeloid progenitor cell line 32D Bcr-Abl. We have added the data as additional Figure 2f and 2g to the manuscript and adjusted the text in the results part as follows (p 12, l 296):

“To study the mechanism resulting in impaired CML cell biology upon TNFα inhibition we applied 32D Bcr-Abl cells that were subjected to TNFα antibody treatment, nilotinib therapy or the combination, upon adding or not adding physiological TNFα concentration. As expected, apoptosis was significantly induced upon nilotinib treatment but not further enhanced upon additional TNFα targeting (Figure 2f). TNFα targeting alone was not sufficient to induce apoptosis in these CML cells. Using the same model, we subsequently analyzed the effect on proliferation and here we could observe a significant reduction upon TNFα targeting alone. This was evident in CML cells independent of TNFα addition, likely because of TNFα expression per se in these cells (Figure 1d). Nilotinib treatment by itself largely abolished proliferation in this model and thus there was no additional effect observable (Figure 2g).”

Reviewer 2 (Reviewer 3): PEER REVIEWER ASSESSMENTS:

OBJECTIVE - Full research articles: is there a clear objective that addresses a testable research question(s) (brief or other article types: is there a clear objective)?
Yes - there is a clear objective
DESIGN - Is the current approach (including controls and analysis protocols) appropriate for the objective?

Yes - the approach is appropriate

EXECUTION - Are the experiments and analyses performed with technical rigor to allow confidence in the results?

Yes - experiments and analyses were performed appropriately

Statistics - Is the use of statistics in the manuscript appropriate?

Yes - appropriate statistical analyses have been used in the study

INTERPRETATION - Is the current interpretation/discussion of the results reasonable and not overstated?

Yes - the author's interpretation is reasonable

OVERALL MANUSCRIPT POTENTIAL - Is the current version of this work technically sound? If not, can revisions be made to make the work technically sound?

Probably - with minor revisions

PEER REVIEWER COMMENTS:

GENERAL COMMENTS: The manuscript addresses relevant clinical problem in leukemia treatment.

The experiments are well designed and most of the experiments are well executed. Overall, the manuscript is presented well. The experimental methods are explained adequately and used appropriate statistical analysis for evaluating the results. Most of the conclusions are appropriately drawn from the results.

Thank you.

REQUESTED REVISIONS:
The length of the abstract is relatively long. The authors may consider deleting words from the abstract to make the abstract more concise. Authors may particularly reduce words from the background section of the abstract.

We have now shortened the background section of the abstract and deleted the text from the manuscript indicated in red (p 2, l 29):

“Abstract

Background: {The oncoprotein}Expression of Bcr-Abl {has an essential role in the development of} in hematopoietic stem cells is sufficient to cause chronic myeloid leukemia (CML) and {inhibition of the constitutively active kinase by} tyrosine kinase inhibitors (TKI) induce cytogenetic as well as molecular remission in the majority of CML patients. However, the disease driving stem cell population is not fully targeted by TKI therapy, and leukemic stem cells (LSC) capable of re-inducing the disease can persist. {A variety of inflammatory factors is altered within the malignant stem cell niche, with a beneficial effect for LSC.} Single-cell RNA-sequencing technology recently identified an enriched inflammatory gene signature with TNFα and TGFβ being activated in TKI persisting quiescent LSC. Here, we studied the effects of human TNFα antibody infliximab (IFX), which has been shown to induce anti-inflammatory effects in mice, combined with TKI treatment on LSC function.”

ADDITIONAL REQUESTS/SUGGESTIONS:

In the additional file 2, authors may consider using colored lines for clarity instead of different line patterns. This will help readers to easily understand the result.

We have now adjusted Additional Figure 2 as suggested by the reviewer.

Paolo Gallipoli (Reviewer 4): In this manuscript Herrmann and colleagues study the role of microenvironmental cues in survival of CML LSC using mostly a very elegant and well characterised mouse model. They specifically focus, based on previous literature in the field and their gene expression data, on the role of TNF-alpha and demonstrate a subtle but consistent in vitro and in vivo effect of targeting TNF-alpha signalling in supporting LSC survival and/or function above all in combination with standard BCR-ABL TKI treatment.

The manuscript is well written, the data are clearly presented and overall the conclusions appropriate.

Thank you.

My main comments are as follow:
1) Is TNF-alpha production in BCR-ABL expressing cells kinase dependent? It would be good if the authors could test this as it might help explaining the effects of combined Treatment.

This is also partially addressed by the response to Reviewer 2, point 1.

We have now analysed expression of TNFα in 32D Bcr-Abl cells upon imatinib treatment as well as LSK cells that we isolated from mice in that we first induced expression of Bcr-Abl and then reverted oncogenic expression (RNA from previously performed in vivo experiments published in Hamilton et al., Blood 2012, PMID 22184410). We have added the data as Figure 2h and Figure 2i to the manuscript and added the following text to the results section (p 11, l 252):

“We proceeded to test if TNFα secretion by the malignant clone is Bcr-Abl dependent. Inhibition of Bcr-Abl using imatinib significantly reduced TNFα expression in 32D Bcr-Abl cells (Figure 1h) suggesting that in this model TNFα levels depend on the malignant kinase activity. Next, we studied TNFα expression in LSK cells from SCLtTA/Bcr-Abl mice, that were first induced to express Bcr-Abl for 25 days and then reverted to not express Bcr-Abl for 48 days [4]. Expression of TNFα in these previously malignant LSK cells was reverted to normal level (Figure 1i).”

Moreover, we have added the following text to the discussion part of the manuscript (p 17, l 421):

“Although we clearly show elevated TNFα expression in Bcr-Abl positive cells, our data also suggest that this is dependent on the malignant kinase, at least in a murine myeloid progenitor cell line. Likewise, we observed reduction of TNFα expression in LSK cells upon long-term reversion of Bcr-Abl expression. However, this could also be due to re-expansion of Bcr-Abl negative LSK cells upon inhibition of the kinase as we have studied the expression of TNFα 48 days after Bcr-Abl reversion in this model. In primitive human LSCs, TKI persistent TNFα expression has been demonstrated [14, 33]. Yet, additional cell populations could contribute to elevated TNFα levels that are observed in CML mice and patients. This also ties in with the recent finding that CML-derived osteoblasts show elevated levels of TNFα expression, in the SCLtTA/Bcr-Abl model [34].”

2) The authors state that the effects of IFX in vitro are possibly not due to direct binding to TNF-alpha and one wonder if they could possibly be off target. This might explain why the effects of the murine TNF-alpha antibody MP6 are less obvious and only seen in the presence of TNF-alpha. The alternative explanation however is that MP6 is not as good therapeutic and ideally the authors should try the TNF-alpha rescue also in the IFX treated cells. This would allow one to see if indeed TNF-alpha plays a role in the effects seen with IFX regardless whether this is via direct binding or indirect effects.

We agree with the reviewer and the TNFα rescue in IFX treated primary murine cells would be a relevant approach to study the specific effect of IFX on TNFα. However, these experiments were performed using primary murine lineage negative cells that we isolated from the SCLtTA/Bcr-
Abl mouse model. Upon breeding, tetracycline has to be added to the drinking water in order to suppress Bcr-Abl expression, in this model. This is currently regarded as an animal experiment and requires approval from the authorities which would be months beyond the timeline that was given for this revision. However, we have implemented these experiments in a current proposal and could perform them upon approval, which could be expected within the next six months, in case the reviewer regards these experiments as essential.

3) In vivo experiments in figure 3 show only a mild phenotype with the Nilotinib + IFX combination which is surprising compared to the in vitro data in figure 2. Is this because TNF-alpha plays a bigger role in vivo than in vitro and IFX is not targeting it? Could the authors speculate as to why this is?

Figure 2a and 2b are looking at the same cell type in vitro (lineage negative cells) as Figure 3f does in vivo. As we do have a wt control group in the in vivo setting we would state that nilotinib is already quite effective in terms of reducing the malignant lineage negative cell population in vivo and reduces the level of expanded cells back to wt control level. Therefore, it could be difficult to unravel an additional effect of IFX in this cell population in vivo. This could be a result of longer duration of treatment in vivo (18 days) vs in vitro (3 days) and thus the effect of combined treatment would be primarily observable on the malignant cell population that is not fully targeted by nilotinib treatment alone (Figure 3g).

4) Why in the in vivo experiments the authors do not have a IFX only control arm ? They had it in vitro and showed efficacy on its own so ideally it should be shown for the in vivo experiments too?

We agree with the reviewer and it would be beneficial to study the effect of IFX therapy only in the CML mouse model. However, we have focused on combined TKI and IFX therapy. For clinical application, IFX therapy alone would most likely not be applicable to CML patients at diagnosis, due to the enormous success of TKI therapy. Yet, one could envision of targeting TNFα alone during TKI stop.

5) Why LSK % engraftment from the donor is so low in WT transplanted mice (figure 3g) given that the recipient were lethally irradiated and the total CD45.1 (donor) % is around 75% (figure3b)?

In Figure 3b we looked at the donor-derived cells within the PB 14 days after transplantation and in Figure 3g we studied the proportion of donor-derived cells in the BM-LSK cells 32 days after transplantation. The data indeed suggest that the wt donor-derived cells in particular within the BM-LSK compartment were significantly decreasing over time. Although we have irradiated the recipient mice using 10 Gy we here applied a single dose of irradiation (rather than using 2x5 Gy with a chronical interval of at least 4 hours). This could be responsible for reducing the efficiency in targeting low cycling cells, such as LSKs (as compared to more mature cells), which could in turn result in residual recipient hematopoietic immature cells that could compete
with the transplanted cells and over time re-expand. However, as we have irradiated all recipients using the same protocol (and actually they were irradiated altogether) we would still take the view that the comparison between Bcr-Abl positive and wt transplanted recipients is valid.

6) In the secondary transplant experiments of figure 4 the authors do not see any effects on survival with combination therapy. They speculate this is due to the high number of cells transplanted. Have they tried to lower the number of cells transplanted or a limiting dilution analysis to bring out this phenotype? Ideally this should be done as it would strengthen or disprove their conclusions. This is even more important as most of the effects the authors see in the secondary transplantation in the blood composition of the combined treatment animals is due to a reduction of the B220 population (figure4e) which one has to assume is not the malignant CML population in this model of chronic phase disease. The effects on the actual blasts (figure4c) with the combined treatment are only marginal compared to the nilotinib only arm. If the authors cannot perform these experiments, they should explain their findings taking into account all the above and discuss their current data more cautiously in terms of the effects on the leukemia initiating cell population. Also could the authors speculate as to why the B220 appears to be preferentially targeted by the combined treatment?

In our study, we showed impaired serial CFU potential of primary murine CML cells upon combined nilotinib and IFX therapy as compared to nilotinib therapy alone as well as a reduction of malignant LSK cells upon combined treatment in our primary recipients. However, we do agree with the reviewer that our serial BMT model does not comprehensively show significant reduction of the cell population being capable of re-inducing the disease in vivo, in the current design. Moreover, we have clarified that the B220 cells are not expected to reflect the predominant malignant clone. We have therefore changed the manuscript accordingly. Text that has been deleted from the manuscript is indicated in red (p 16, l 382):

“Further analyses of the PB revealed that donor-derived blasts (CD45.1+; c-kit+) were increased in recipients of Bcr-Abl positive BM and there was a trend of less CD45.1+; c-kit+ cells being present in nilotinib re-transplanted mice that was further continued and reduced by 1.88-fold upon combinatory treatment (Figure 4c). Donor neutrophils (Gr1+; CD45.1+) were not increased in the nilotinib re-transplanted animals compared to re-transplanted nilotinib + IFX mice (Figure 4d). Instead, the donor-derived {malignant} re-expanding cells were positive for the B-cell marker B220 (Figure 4e) and there was a significant reduction in these cells due to the combinatory treatment as compared to nilotinib monotherapy (11.22-fold). Taken together these data {confirm} suggest that reduction of FACS positive malignant stem cells seen upon combined treatment in the primary recipients translates into a reduced tendency of these cells to re-expand upon serial transplantation. {early malignant cell expansion and shows that combined therapy is more potent in reducing disease initiating cells.} However, additional experiments would be required to fully address the effect on LSC function.”

Moreover, we have added the following text to the discussion (p 18, l 444):
“Upon serial transplantation, we observed a non-significant 1.88-fold reduction in donor-derived c-kit+ cells and a significant 11.22-fold reduction in donor-derived B220+ cells due to combined IFX and nilotinib treatment as compared to nilotinib treatment alone. While the reduction in blasts can be assigned to reduced CML disease the mechanism inducing B-cell reduction is unclear at present but it has been discussed that IFX can alter B-cell biology in treated patients [37, 38] suggesting that this could rather reflect an effect of the antibody treatment itself.”

Finally we have toned down our conclusion as follows (p 20, l 497):

“TNFα signaling is induced in CML stem cells and anti-inflammatory therapy elevates TKI induced clonogenic growth reduction. Compatible with this, anti-inflammatory therapy in CML mice enhances TKI induced decline of {LSC} LSK-cells confirming that successful targeting of {persisting} CML stem and progenitor cells can be enhanced via addressing their malignant microenvironment simultaneously.”

7) In the discussion, the authors provide some possible explanations on the mechanism behind the efficacy of IFX in combination with nilotinib and particularly its putative role in reducing IFN-gamma production in the spleen of the CML mice. Can the authors expand on that a bit more? Is this due to the effects of IFX on T-cell population/subsets? Given the effects seen in the secondary transplant on the B220 population, it might be nice to show also any effects on the T-cell population which is the likely source of IFN-gamma and other cytokines? Also they discuss the known effects of TNF-alpha on NFKB signalling and this prompts the obvious question if IFX has any effect on NFKB signalling itself. Have the authors looked at this as this might also explain the effects on cytokine production they already show with combination treatment?

We have now expanded the discussion and added the following text to manuscript (p 19, l 466):

“The mechanism inducing reduced IFNγ expression is unclear at present. However, it has been shown that IFX impairs the frequency of IFNγ-secreting cells. Natural killer cells in rheumatoid arthritis patients were reduced upon IFX therapy [44] and in ulcerative colitis patients derived cells, IFX treatment decreased the proliferation of CD4+ and CD8+ T-cells as well as their secretion level of IFNγ and TNFα, among other cytokines [45]. We have not studied the IFX effect on NK or T-cell populations in the SCLtTA/Bcr-Abl model, yet these data tempt to speculate that IFX-mediated activity on NK or T-cell subsets could also be involved in the pathophysiological effects observed in our study.”

Although we have not studied the role of IFX-mediated NF-KB signaling within the malignant cells we have now performed these experiments using the murine TNFα antibody MP6-XT22 (also implemented in our response to reviewer 2, point 1). Therefore, we have studied the effect of TNFα inhibition on the phosphorylation of IKBa, that is a negative regulator of NF-KB signaling. Phosphorylation of IKBa marks the protein for degradation via ubiquitination. Here we could show that MP6-XT22 treatment indeed decreases pIKBa (Figure 1f), suggesting that NF-KB signaling is subsequently inhibited.
Minor comments:

1) Page 7, line 158. Nilotinib concentration is stated as being 100mM which is far too high. I suspect this was 100nM as per figure legend and should be corrected. Similarly, page 15 line 361, und should be changed to and.

We thank the reviewer for bringing this to our attention and have corrected the nilotinib concentration in the manuscript. Indeed, we used 100nM throughout the complete study.

2) The authors state that the 2 mice treated with the combination therapy who died during the delivery of therapy did not show splenomegaly. Could they however explain the likely cause of death. I suspect this might have been infection or radiation effect but if it was due to the therapy is worth mentioning.

These mice did not show typical signs of radiation-associated death. As these mice died immediately after oral gavage, we would suspect rather a technical problem than an effect of the combined treatment.