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Letter to the Editor

The dynamic of *TNF* and *IL6* gene expression in chronic myeloid leukemia patients reveals early responders to imatinib*

1. Introduction

Imatinib, the first *BCR-ABL1* tyrosine kinase inhibitor (TKI), revolutionized the management of chronic myeloid leukemia (CML) and remains as the preferred first-line therapy for newly diagnosed patients [1]. Besides its direct onco-kinase inhibition, cumulative data suggest that TKI treatment exert concomitant immunomodulatory effects [2–5].

The main anti-tumor effector cells of the immune system, including natural killer (NK) cells and T cells, play a direct role controlling hematological malignancies in the host, including CML [6]. In the innate immune response against malignant cells, NK cells exert a potent cytotoxicity and release TNF-α and IFN-γ cytokines ($CD56^{bright}$ NK subset), enabling dendritic cells (DC) to prime the adaptive immunity [7]. The fundamental ability of CD8+ cytotoxic T lymphocytes (CTL) to kill leukemia cells is accompanied by a Th1 type response of CD4 + T cells along with an optimum production of the associated cytokines such as TNF-α and IFN-γ [7]. The role of other Th subsets, including Th17, in cancer is discussed with conflicting reports related to pro- or anti-tumor effects [8,9]. The IL-6 is produced by different cell types and seems to promote an anti-tumor Th17 response in the context of TGF-β1 [9].

A broadly compromised immune system has been described in CML patients at time of diagnosis supporting for a suppression of anti-CML immune responses. These abnormalities include an expansion of the suppressive immune cell populations and dysfunctional effector NK- and T-immune responses [3,10,11]. On TKI treatments, CML patients seem to re-activate their immune system restoring the effector-mediated immune surveillance. TKI leads the restoration of NK function, Th1/ CTL responses, and increased the number and antigen-presenting cell function of DC [4,11–13]. Also, the immune-stimulatory effects of TKI include decreasing the suppressive immune cells functions [3,4,10,11].

Most of above mentioned findings have been studied by different approaches: directly in CML-stem cells, in the marrow plasma and/or the peripheral counterpart with heterogeneous criteria for timing or the assessed response (hematological, cytogenetic or molecular) to TKI. TNF-α, IFN-γ and IL-6 are fundamental mediators involved in the immune and inflammatory response. There are, however, few studies that evaluate possible immune-pathological events related to their expression at diagnosis and during treatment in CML regarding molecular response at each time point according to current guidelines [1], and longitudinally studies are scarce or missing [7]. Herein we describe the dynamic of *TNF*, *IFNG* and *IL6* gene expression, on the same sample used to monitor the molecular response to imatinib.

2. Materials and methods

In this study we analysed 161 peripheral blood samples of CML patients – from the registry of the *Laboratorio de Genética Hematológica, Instituto de Medicina Experimental/ Academia Nacional de Medicina* – and of 26 healthy donors. Fifty-eight individual samples, at diagnosis or under imatinib 400 mg at 3, 6, 12 months or ≥ 4 years, and multiple samples of 34 serially followed patients were classified according to the molecular response at each time point [1] (Supplementary Table S1). All individuals gave informed consent for participation in the study, which was approved by our institutional ethics committee.

An aliquot of the stored sample used to monitor *BCR-ABL1* level as previously reported [14], was used for this study. cDNA was synthesized from 1 μg of total RNA using random hexamer primers (Biodynamics) and M-MLV (Moloney murine leukemia virus) reverse transcriptase (Promega), following the recommended protocol of the enzyme. *TNF*, *IFNG* and *IL6* gene expression was quantified using a Rotor-Gene Q cycler (Qiagen) with the SYBR Green PCR Select Master Mix (Applied Biosystems). Relative quantification to *GAPDH* was calculated applying the comparative Ct method. Primer sequences are detailed in Supplementary Table S2.

Mann-Whitney U/ Kruskal-Wallis tests were applied to evaluate independent groups and Wilcoxon test to paired samples. Statistical analyses were performed using the InfoStat software v17 (Universidad de Córdoba, Argentina). All tests were two-sided, and p-values < 0.05 were considered statistically significant. The heatmap plot was obtained using R package version 3.4.1.

3. Results and discussion

Alterations in the cytokine levels and dysfunctional immune cells are likely limiting the anti-leukemia immune response in CML patients at diagnosis [3,10,11]. In accordance with a basal immune suppression, the expression levels of the effector cytokines *TNF*, *IFNG* and *IL6* were significantly diminished at diagnosis when compared with healthy controls ($p = 0.0336$, $p < 0.0001$ and $p = 0.0007$, respectively) (Fig. 1A). Our findings are in agreement with a previous report that demonstrated a strongly suppression of CD3+ cells producing TNF-α and INF-γ in whole blood samples of newly diagnosed CML patients [15]. Also, several studies have shown that NK-cell proportions among lymphocytes were decreased [11], and that T cells were functionally impaired displaying a decreased TCRζ-chain expression, a limited cytotoxic activity and a diminished production of Th1-cytokines (*i.e.* INF-γ and TNF-α) [7].

Besides the direct anti-tumor effect of *BCR-ABL1* TKI, data from

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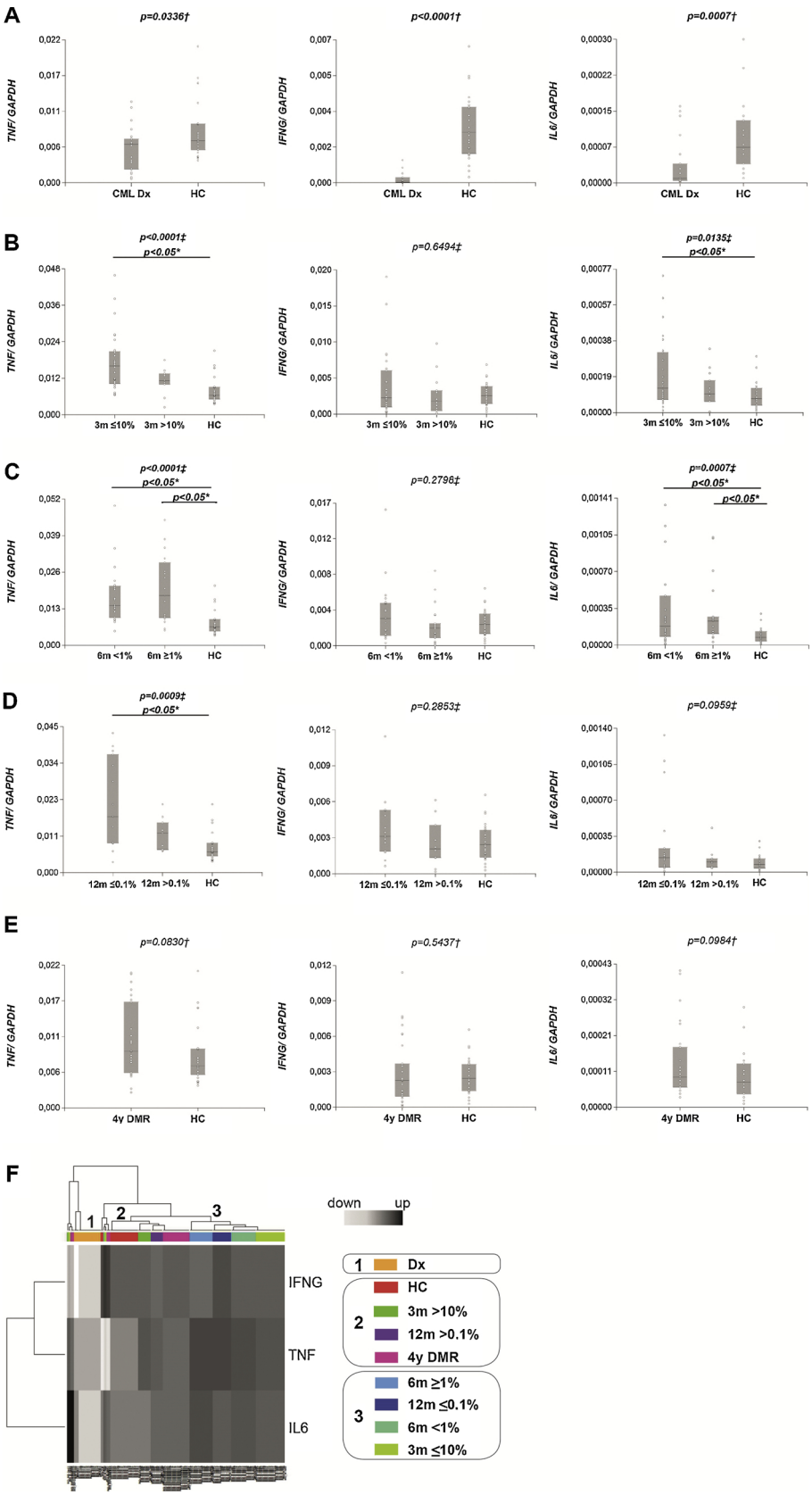

Fig. 1. Gene expression levels of the effector cytokines *TNF*, *IFNG* and *IL6* in CML patients at diagnosis and on imatinib therapy compared with healthy controls. **(A)** CML patients at basal. Imatinib-treated CML patients according to achieve or failure to achieve the optimal response at different time points: **(B)** 3 months, **(C)** 6 months, and **(D)** 12 months. **(E)** CML patients in sustained deep molecular response for, at least, 4 years. **(F)** Heatmap plot for the gene expression profile and the dendrogram distinguishing three main clusters. †Mann-Whitney U test, ‡Kruskal-Wallis test and *Kruskal-Wallis multiple comparisons. Box-plots specify the interquartile range and lines indicate the medians. Dx: at diagnosis; HC: healthy controls; DMR: deep molecular response.

multiple *in vitro* and *ex vivo* studies indicate that these TKI may also influence the anti-tumor immunity [3,4,10–13]. In this study, once imatinib therapy was initiated, those patients who achieved an early molecular response (EMR, *BCR-ABL1* ≤10% at 3 months) significantly increased their *TNF* and *IL6* gene expression when compared with healthy controls (Fig. 1B). At 6 months, the enhancement of *TNF* and *IL6* was observed in either responders (*BCR-ABL1* < 1%) or non-responders (≥1%) (Fig. 1C). Whereas, at 12 months the increase was only

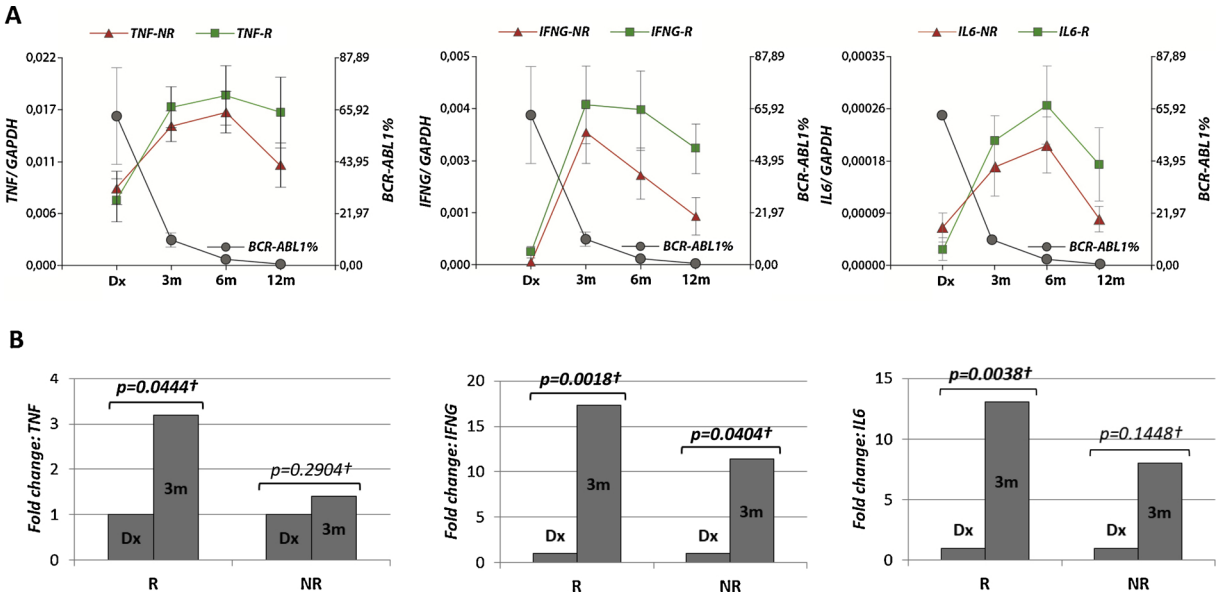

Fig. 2. Dynamic of the *TNF*, *IFNG* and *IL6* gene expression in a CML patient cohort divided into two groups based on achieved optimal molecular response to imatinib therapy. (A) Serial analysis describing the dynamic of cytokine gene expression against *BCR-ABL1* level over the first year on treatment. Graphs of multivariate profile indicate the mean ± standard error for each time point. **(B)** Fold change between patients at diagnosis and after 3 months on treatment (R: n = 8 and NR: n = 4). †Wilcoxon test for paired samples. Dx: at diagnosis; R: responder; NR: non-responder.

observed for *TNF* gene expression in responders (*BCR-ABL1* ≤0.1%) (Fig. 1D). Levels of these cytokines were restored to normal levels in patients who were in a sustained deep molecular response (MR 4.0 or deeper) for, at least, 4 years (Fig. 1E). The heatmap plot depicted a dendrogram that classified three main clusters according to the cytokine expression profile: the first cluster individualizing patients at basal with a diminished expression; the opposite main branch, included most patients with an optimal response at the specific time point with a higher expression, and, the central cluster composed by patients that failed to achieve a suitable response (Fig. 1F).

We have also studied a subpopulation of CML patients who were serially followed to get a longitudinal approach of the cytokine expression dynamic. This serial analysis described *TNF*, *IFNG* and *IL6* gene levels against *BCR-ABL1* transcript levels in responders and non-responders. We could observe that the expression of the three cytokines was diminished in CML patients at diagnosis, when *BCR-ABL1* transcript levels were higher, whereas increased cytokine levels were noted after imatinib initiation (Fig. 2A). Interestingly, we detected a statistically significant 3-folds increase of *TNF* ($p = 0.0444$) and 13-folds of *IL6* ($p = 0.0038$) between diagnosis and 3 months in those patients who achieve an EMR. The increase of *IFNG* was significant either in responders (17-folds, $p = 0.0018$) or non-responders (11-folds, $p = 0.0404$) (Fig. 2B). No significant differences were observed between 3 and 6 months, while other comparisons were not performed due to the limited number patients with complete register (see Supplementary Table S1). In addition, the slopes between diagnosis and 3 months were significantly non-zero (positive slopes) in concordance with a significantly increase of the gene expression only in patients with EMR (*TNF*: $p = 0.0175$, *IFNG*: $p = 0.0180$, *IL6*: $p = 0.0377$). Our results contribute to the characterization of a systemic anti-CML immune response differentiating responder patients to imatinib, however, they should be confirmed at protein level.

Although several data suggest an immune restoration in CML patients on TKI, there are few articles comparing the level of immune reactivation depending on response to TKI. A recovery of abnormalities in the frequencies of Th1, Th17 and Th22 cells were noticed in patients on imatinib therapy; however, without specifying neither time nor the response achieved [8]. Our results are in accordance with Söderlund et al. [16] that demonstrated a possible shift towards Th1-immunity at 3 months on TKI therapy, although they did not classify to the patients according to EMR [16]. Hematological remission was associated with a highly significant increase of CD3+ cells producing pro-inflammatory Th1 cytokines (*i.e.* INF-γ and TNF-α) [15] and this T cell response is sustained over time while in remission [12]. Also, patients with a complete cytogenetic response tend to a greater NK-cell reactivity [17].

Nievergall et al. [18] pointed TGF-α and IL-6 as novel biomarkers with high diagnostic plasma levels strongly predictive of subsequent failure to achieve EMR. However, they observed similar IL-6 plasma levels at basal or after 6 months under TKI to healthy donors without data at 3 months [18], where we could observe the statistical increase in responders. IL-6 plays various roles on modulating the activities of tumor and immune cells, but is unclear how it controls the host immune response in cancer patients. IL-6, in the context of TGF-β, drives primarily the differentiation of naive CD4 + T cells into Th17. The anti-tumor effect of Th17 cells seems to rely on IL-6 production by different cells, which is amplified by a synergic interaction with TNF-α [9]. Our data may support this context reflecting a down regulation of cytokines at basal with an increase in EMR patients enhancing the anti-tumor effect of TKI.

4. Conclusion

Our results are in agreement with a significant immune suppression in CML patients at diagnosis and an initial stimulatory effect on the immune system after imatinib initiation, especially in early responder patients. In addition, following the dynamic of *TNF*, *IFNG* and *IL6* gene expression on the same sample to monitor the molecular response to TKI may play a role as an immune biomarker allowing the differentiation of optimal from suboptimal responders. The individualization of accessible immune-biomarkers, which account for the systemic anti-CML immunity, might be of interest for future clinical trials combining TKIs with an active immunotherapy in order to increase tumor eradication.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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