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ORIGINAL RESEARCH

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ABSTRACT

Changes in the immune system induced by tyrosine kinase inhibitors (TKI) have been shown to positively correlate with therapy responses in chronic myeloid leukemia (CML). However, only a few longitudinal studies exist and no randomized comparisons between two TKIs have been reported. Therefore, we prospectively analyzed the immune system of newly diagnosed CML patients treated with imatinib (n = 20) or bosutinib (n = 13), that participated in the randomized BFORE trial (NCT02130557). Comprehensive immunophenotyping, plasma protein profiling, and functional assays to determine activation levels of T and NK cells were performed at diagnosis, 3, and 12 months after therapy start. All results were correlated with clinical parameters such as Sokal risk and BCR-ABL load measured according to IS%.

At diagnosis, low Sokal risk CML patients had a higher frequency of cytotoxic cells (CD8 + T and NK cells), increased cytotoxic potential of NK cells and lower frequency of naïve and central memory CD4 + T cells. Further, soluble plasma protein profile divided patients into two distinct clusters with different disease burden at diagnosis. During treatment, BCR-ABL IS% correlated with immunological parameters such as plasma proteins, together with different memory subsets of CD4+ and CD8 + T cells. Interestingly, the proportion and cytotoxic potential of NK cells together with several soluble proteins increased during imatinib treatment. In contrast, no major immunological changes were observed during bosutinib treatment. In conclusion, imatinib and bosutinib were shown to have differential effects on the immune system in this randomized clinical trial. Increased number and function of NK cells were especially observed during imatinib therapy.

Introduction

There are currently five approved tyrosine kinase inhibitors (TKIs) for the treatment of chronic phase chronic myeloid leukemia (CP CML). Imatinib was first launched approximately 15 years ago, soon followed by the second-generation TKIs, dasatinib, nilotinib, and bosutinib, and last, the third-generation TKI, ponatinib.[1] All the TKIs, with the exception of ponatinib, are now approved as first-line treatments in CML.[2,3] The advances in the treatment of CML with different TKIs have led to a vast decrease in patients who are overall unresponsive or intolerant to therapy, as they may be treated with several different TKIs sequentially.[4] Today, CP CML patients are likely to have a normal life expectancy, but the majority face a life-long exposure to TKIs. Recent evidence shows, however, that up to half the patients who have achieved an optimal therapy response are able to discontinue their treatment without relapse, which may be partly due to the immunological changes observed during the treatment.[5,6]

TKIs are not entirely selective for BCR-ABL1 kinase inhibition because they also inhibit a variety of other kinases that may ultimately cause off-target effects. Notably, the second-generation inhibitors bosutinib and dasatinib both inhibit Src-kinases, which are important mediators of T-cell function.[7,8]

In contrast, imatinib and nilotinib have a much more narrow inhibition profile.[7] Earlier data from us and others have shown that dasatinib, in particular, has significant effects on the BCR-ABL-negative healthy immune cell compartment of CML patients both *in vivo* and *in vitro*, [5,9–15] whereas the immunomodulatory effects of imatinib have been previously shown especially in CML (as reviewed by Zitvogel et al.)[16] and gastrointestinal stromal tumors and recently also in neuroblastoma .[13,16–18] However, no systematic analysis of the immunological effects of the other second-generation TKI bosutinib inhibiting SRC family kinases at clinically relevant concentrations has yet been performed. Therefore, we aimed to characterize the immunological changes during frontline bosutinib and imatinib treatments, and analyze how these changes would correspond with the primary clinical parameters, such as the Sokal risk and BCR-ABL IS%, in order to understand the overall effects on the immune system in CP CML patients.

Results

CML patients with low Sokal risk score present a more active immune system at diagnosis

In order to study the effects of disease burden on the immune system, we first divided our CP CML cohort (n = 33) into three groups based on their Sokal risk score at diagnosis: low (n = 11), intermediate (n = 15), and high-risk (n = 6) (Tables 1 and 2). The immune system before the start of treatment was studied by extensive phenotyping, functional assays to measure the potential activity of both T and NK cells, and measuring 92 different plasma analytes by a high-throughput, multiplex immunoassay.

The highest median percentage of the main cytotoxic NK cell population (CD3negCD56+ CD16+) from the lymphocytes was observed in the low (12.1%) and intermediate Sokal risk groups (12.9%), compared to the high-risk patients (6.5%; p = .06 and 0.07) (Figure 1(a)). The low-risk patients had significantly less CD3 + T cells from lymphocytes (68.6% vs. 76.6%; p = .002)

Table 1. Characteristics of bosutinib-treated patients (n = 13).

No	Age at dg.	Sex (f/m)	Arm	Sokal risk group	BCR-ABL IS% at dg.	BCR-ABL IS% at 3m	BCR-ABL IS% at 12m
Bo1	46	f	Bosutinib	Inter.	107,8	3,1	0,029
Bo2	32	f	Bosutinib	High	16,9	0,008	neg
Bo3	30	m	Bosutinib	Low	82,0	1,51	0,0016
Bo4	42	f	Bosutinib	Inter.	99,6	21,36	switched to Im
Bo5	62	f	Bosutinib	High	77,9	20,04	switched to Im
Bo6	54	m	Bosutinib	Inter.	93,88	0,26	0,0086
Bo7	35	f	Bosutinib	Low	74,9	0,15	neg
Bo8	77	f	Bosutinib	Inter.	24,2	switched to Im	switched to Im
Bo9	28	f	Bosutinib	Low	98,1	7,98	0,019
Bo10	30	m	Bosutinib	Low	19,6	0,20	discontinued
Bo11	60	f	Bosutinib	Inter.	92,5	16,9	0,012
Bo12	52	m	Bosutinib	Inter.	76,7	1,22	0,049
Bo13	62	f	Bosutinib	Low	79,0	discontinued	discontinued
Median [range]	**46**	**Male, 31%**		**Low 38%**	**79**	**1,51**	**0,01042**
	[28–77]			**Inter. 46%**	**[16,9–107,8]**	**[0,008 − 21,36]**	**[neg −0,049]**
				High 15%			

Abbreviations: dg, diagnosis; f, female; Im, Imatinib; Inter., intermediate; IS, international scale; m, male.

Table 2. Characteristics of imatinib-treated patients (n = 20).

No	Age at dg.	Sex (f/m)	Arm	Sokal risk group	BCR-ABL IS% at dg.	BCR-ABL IS% at 3m	BCR-ABL IS% at 12m
Im1	30	f	Imatinib	Inter.	34,1	12,1	0,157
Im2	67	f	Imatinib	Inter.	79,7	5,0	0,019
Im3	59	f	Imatinib	High	27,1	2,4	0,497
Im4	47	m	Imatinib	Low	18,9	0,53	0,0068
Im5	55	f	Imatinib	Inter.	22,8	3,66	discontinued
Im6	62	m	Imatinib	Inter.	50,5	9,79	0,672
Im7	65	f	Imatinib	Inter.	NA*	NA*	NA*
Im8	55	f	Imatinib	Low	92,6	23,14	0,363
Im9	58	f	Imatinib	High	93,3	discontinued	discontinued
Im10	70	m	Imatinib	Inter.	65,2	5,13	0,013
Im11	43	m	Imatinib	Inter.	84,1	2,16	0,103
Im12	31	m	Imatinib	Inter.	20,8	2,91	0,038
Im13	81	m	Imatinib	NA**	NA**	NA**	NA**
Im14	59	f	Imatinib	High	62,3	26,71	2,589
Im15	71	m	Imatinib	Inter.	63,7	41,4	0,009
Im16	44	m	Imatinib	Low	117,6	10,51	0,506
Im17	51	f	Imatinib	Low	85,2	8,48	0,074
Im18	73	m	Imatinib	High	12,6	1,97	0,133
Im19	75	m	Imatinib	Low	3,1	0,80	0,0078
Im20	51	m	Imatinib	Low	104,1	28,65	32,8
Median [range]	**58**	**Male 55%**		**Low 32%**	**74,7**	**8,48**	**0,103**
	[30–81]			**Inter. 47%**	**[3,1–117,6]**	**[0.53–28.65]**	**[0,0068–32,8]**
				High 21%			

Abbreviations: dg, diagnosis; f, female; Inter, intermediate; IS, international scale; m, male, NA*, not available, atypical transcripts; NA** Not available, screening failure.
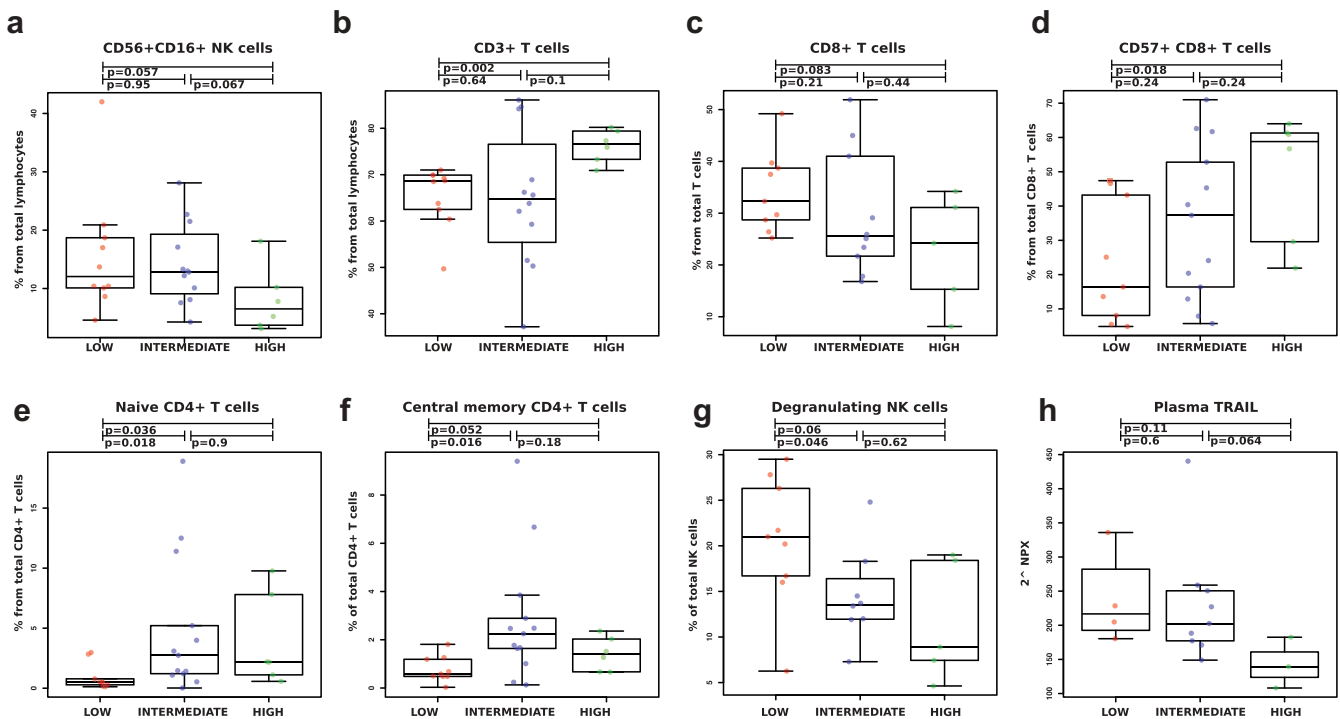
Figure 1. Low Sokal risk CML patients present a more active immune system. Effects of disease burden on the immune system were studied by first dividing the patients (n = 33) into three groups (low, intermediate, and high risk) based on their Sokal score at diagnosis and then by performing phenotyping and functional assays as well as measuring plasma proteins. Low and intermediate-risk patients had a higher percentage of NK cells (CD3negCD56+ CD16+) from lymphocytes than high risk patients (a). The low-risk patients had also lower frequency of CD3 + T cells from lymphocytes (b), higher percentage of CD8+ from total T cells (c), and a lower proportion of CD57 positive CD8 + T cells (d). Moreover, the low-risk patients had also the lowest frequency of naïve (e) and central memory CD4 + T cells (f). Also, the degranulation of NK cells was highest in the low-risk patients after in vitro stimulation (g). Plasma protein measurements revealed that the highest concentration of TRAIL in the plasma at diagnosis was also observed in the low-risk patients (h). TRAIL level is expressed as Normalized Protein eXpression (NPX) values, an arbitrary unit on log$_2$-scale. Non-parametric Wilcoxon signed-ranked test was performed to compute the differences among three groups and data are presented as boxplots, including the median values for each parameter.

(Figure 1(b)). The low-risk patients also had the highest percentage of CD8 + T cells from the CD3 + T cells (median 32.3%) compared to high-risk patients (24.2%; p = .08) (Figure 1(c)). Consequently, low-risk patients had less CD4 + T cells (median 59.8%) compared to the intermediate (70.2%; p = .31) and high-risk patients (72.1%; p = .04) (data not shown). Further, low-risk patients had significantly less CD57 expressing CD8 + T cells (median 16.4%) compared to the rest of the Sokal groups (intermediate 37.3%; p = .24 and high risk 58.8%; p = .02) (Figure 1(d)). When comparing the memory status of T cells by expression of CCR7 and CD45RA, [19] we observed that the low-risk patients had significantly less naïve CCR7+ CD45RA+ CD4 + T cells (median for low risk 0.5%, and intermediate 2.7%; p = .018 and high risk 2.2%; p = .036) and central memory CCR7+ CD45RAneg CD4 + T cells (median for low risk 0.6%, and intermediate 2.3%; p = .016 and high risk 1.4%; p = .052) (Figure 1(e,f)). No significant differences were observed in the absolute numbers for CD3+, CD4+, and CD8 + T cells, and CD3negCD56+ CD16+ NK cells (supplementary Figure 1).

We further analyzed the function of the NK cells from a proportion of the patients (low n = 9, intermediate n = 9 and high risk n = 5) by stimulating the cells with the target cell line K562 and measuring the degranulation from CD107a/b expressions. The low Sokal risk patients had more functional NK cells (median 21.0%) at diagnosis when compared to the intermediate-risk (13.6%, p = .05) and high-risk patients (8.9%, p = .06) (Figure 1(g)). Representative case of CD107 expression in

unstimulated and K562-stimulated NK cells is shown in supplemental Figure 2(a). Interestingly, the comparison of unstimulated NK cells at diagnosis revealed a higher baseline degranulation in the low-risk patients (supplemental Figure 2(b)).

The analysis of plasma proteins revealed a higher concentration of TRAIL, a ligand that induces apoptosis, in low and intermediate-risk Sokal patients in comparison to the high-risk patients (p = .11 and 0.06; Figure 1(h)).

Correlation of immunological parameters with BCR-ABL IS% at diagnosis and during treatment

To further evaluate the effects of disease burden on the immune system, we next correlated the immunological parameters to BCR-ABL at diagnosis, then at 3 and 12 months after therapy. We performed a rank correlation between the BCR-ABL IS% score (all patients combined) and characterized the immune system with phenotyping, functional assays, and plasma cytokine analyses. At diagnosis, both patient groups had similar leukemic cell burden; the median BCR-ABL IS% was 79 in the bosutinib and 75 in the imatinib group (Tables 1 and 2; p = .29). After 3 and 12 months on treatment, bosutinib-treated patients had better treatment responses with significantly lower BCR-ABL levels (at 3 months 1.51 vs. 8.48, p = .046 and at 12 months 0.01042 vs. 0.103, p = .0069, Tables 1 and 2).
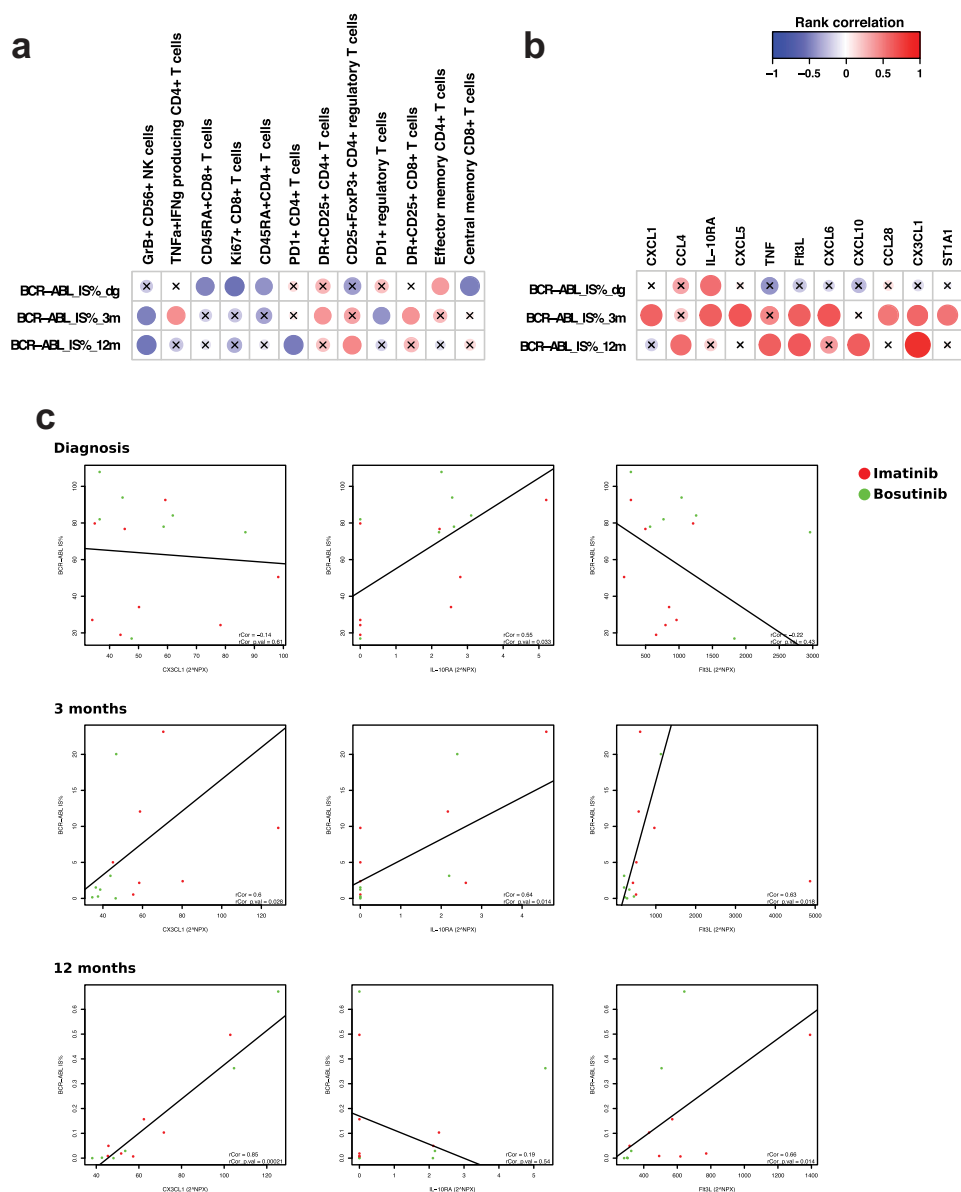
Figure 2. BCR-ABL burden correlates with immunological parameters during treatment time. Spearman rank correlation analysis revealed several significant hits when comparing BCR-ABL IS% at diagnosis, then 3 and 12 months after treatment start with multiple phenotyping parameters (a) as well at plasma proteins (b). The scale indicates correlation coefficient values: positive correlations are observed with red color and negative with blue color. Nonsignificant results are marked with an X. Individual correlation plots with regression were analyzed for CX3CL1, IL-10RA, and Flt3L at baseline, 3 months and 12 months (c). One outlier (abnormally high BCR-ABL IS%) was removed from the analysis. Bosutinib-treated patients are presented in the figures in green and imatinib-treated patients in red. Protein levels are expressed as Normalized Protein eXpression (NPX) values, an arbitrary unit on log₂-scale. Regulatory T cells were gated as CD45+ CD3+ CD4+ CD25high FoxP3+, memory subsets of T cells were defined by expression of CCR7 and CD45RA, and cytokine-producing T cells refer to TNF-alpha and interferon-gamma-producing CD4 + T cells after *in vitro*-stimulation.

At diagnosis, a significant negative correlation to the IS% score was observed in CD45RA+ CD8+ and CD45RA+ CD4 + T cells, Ki67+ CD8 + T cells and central memory CD8 + T cells (Figure 2(a)), suggesting that patients with higher tumor burden have more suppressed immune system at diagnosis.

Interestingly, both at 3 and 12 months GrB+ CD56+ NK cells showed significant negative correlation to disease burden suggesting that cytotoxic NK cells are associated with better treatment responses (lower BCR-ABL IS%). Moreover, positive correlation to the IS% score was observed cytokine-producing CD4 + T cells at 3 months (representative case of TNF-alpha and IFN-gamma production in unstimulated and

stimulated T cells are shown in supplemental Figure 3, patient Im4, Table 2). Other immune cell populations correlated only at 3- or 12-month time-points hindering definitive conclusions to be drawn (Figure 2(a)).

From the plasma analysis (normalized with the values from healthy controls), we found a positive correlation at diagnostic IS% with only IL-10RA (Figure 2(b)). However, at 3 months after the initiation of therapy, positive correlations were observed with the following cytokines: CXCL1, IL-10RA, CXCL5, Flt3L, CXCL6, CCL28, CX3CL1, and ST1A1. At the 12-month time-point, positive correlations between the IS% and CCL4, TNF-α, Flt3L, CXCL10, and CX3CL1 were detected (Figure 2(b)).

To further study the link between leukemic cells and cytokines, we next performed individual correlation plots with the BCR-ABL IS% for the most significant findings from the rank correlation analysis, namely CX3CL1, IL-10RA and Flt3L (Figure 2(c)).

The frequency and cytotoxic potential of NK cells increase during imatinib treatment

As NK cells are indicated to have a role in positive therapy response to imatinib and dasatinib, [5,6,20,21] we next studied the evolution of the cells during the two treatments by phenotyping the NK cells and performing a degranulation assay from the pre-treated and follow-up samples (3 and 12 months after initiation of treatment).

During imatinib treatment, the median proportion of CD56 + CD16+ NK cells drastically increased (dg 3.9% vs. 3 months 9.9% $p < .0001$; vs 12 months 13.8% $p = .0003$). In contrast, no significant changes were observed in the NK cells during bosutinib treatment (dg 4.6%, 3 months 8.5%, 12 months 5.8%) (Figure 3(a)). The cytotoxic potential of the NK cells, as measured by the expression of the degranulation marker CD107ab in NK cells after stimulation with target cell line K562, significantly increased after the start of imatinib treatment when compared to the samples from diagnosis (median at dg 16.9% vs. 3 months 29%, $p = .0017$; vs. 12 months 25.1%, $p = .03$) (Figure 3(b,c)). No significant changes were observed during bosutinib treatment (median at dg 22.8%, 3 months 16%, 12 months 34.9%). Representative case (Im3 in Table 2) of CD107 expression in unstimulated NK cells and K562-stimulated cells is shown in supplemental Figure 2(a).
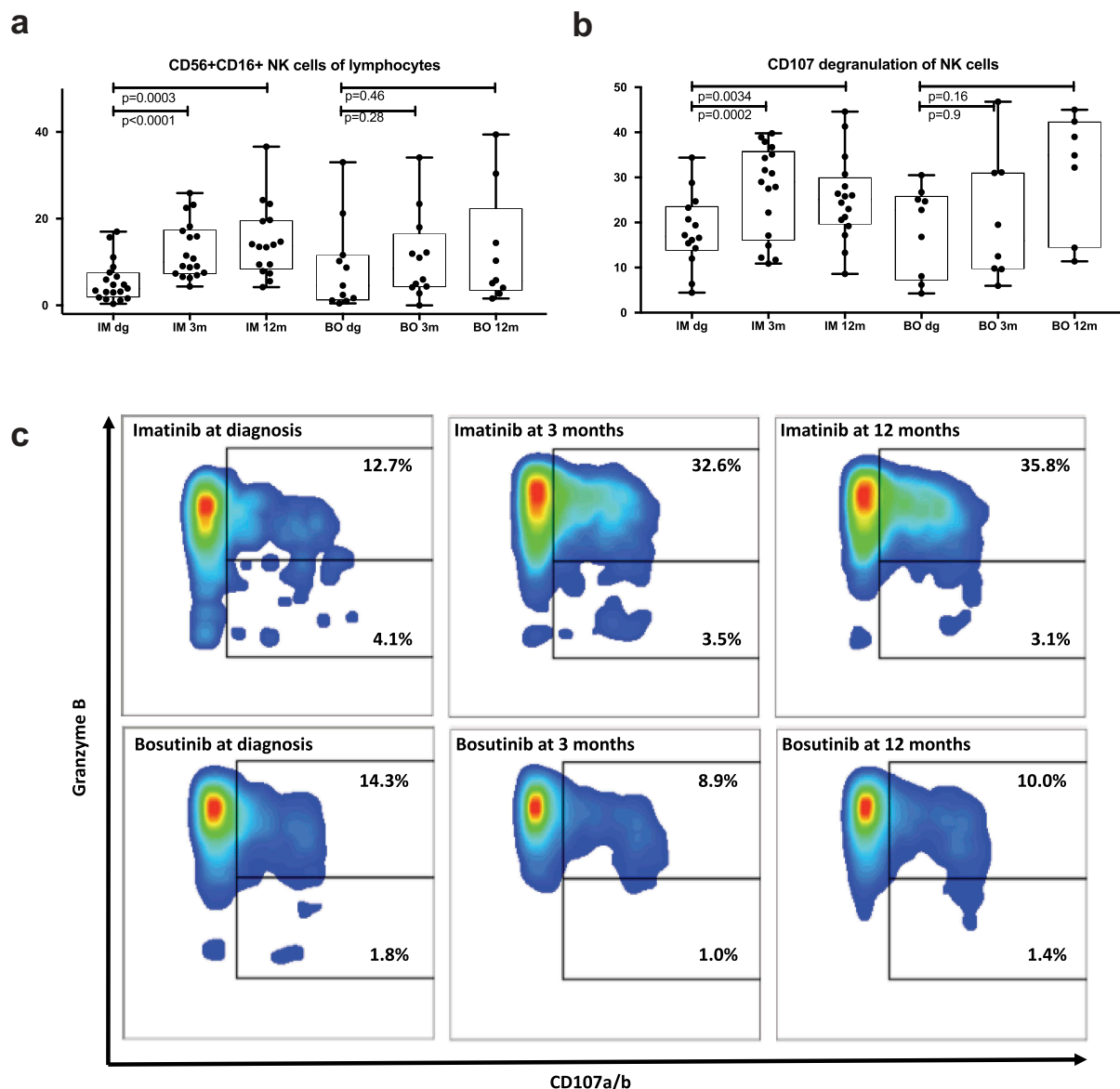
Figure 3. **Imatinib therapy increases the proportion and function of NK cells**. NK cells were characterized by flow analysis and functional assays. Imatinib-treatment significantly increases the proportion of NK cells from lymphocytes (a), as well as increase their responsiveness to K562-stimulation measured by degranulation (b-c). Non-parametric Wilcoxon signed-ranked test was performed to find the difference in functional assay at different timepoint and data are presented as boxplots.

Plasma cytokine profile changes during imatinib treatment

We next studied the changes in the soluble plasma proteins and receptors in both treatment arms. Plasma samples from 8 imatinib- and 8 bosutinib-treated patients (all time-points) and 10 healthy volunteers (6 females, 4 males, mean age 51.7 years) were analyzed using the Proseek® Multiplex Inflammation immunoassay. Protein levels were expressed as Normalized Protein eXpression (NPX) values, an arbitrary unit on $\log_2$-scale.

At diagnosis, the patients had significantly higher amounts of uPA ($p = .0001$), OSM ($p = .00004$), CD5 ($p = .0002$) and Flt3L ($p = .03$) compared to the healthy controls (Table 3). While comparing the patient groups and healthy controls, we observed that at 3 months, imatinib significantly increased the amount of soluble CXC3L1 ($p = .007$ compared to BO, $p = .3$ compared to healthy). CX3CL1 continued to increase in imatinib patients after 12 months of treatment ($p = .01$ compared to BO, $p = .07$ compared to healthy). At diagnosis, CX3CL1 levels were similar in the imatinib and bosutinib groups ($p = .80$). In contrast, MCP-1 was slightly higher at diagnosis in those patients who started on imatinib treatment (median for imatinib 586.7 and for bosutinib 370.4, $p = .10$), and although MCP-1 levels increased during both treatments, imatinib patients had still higher levels at 3 months ($p = .01$ compared to BO, $p = .01$ compared to healthy) and at 12 months ($p = .02$ compared to BO, $p = .004$ compared to healthy). Also, TWEAK increased during both TKIs, with a steeper rise during imatinib (at 3 months; $p = .02$ compared to BO, $p = .01$ compared to healthy; at 12 months $p = .16$ compared to BO, $p = .006$ compared to healthy) treatment. Similar results were observed with TRAIL (at 3 months; $p = .04$ compared to BO, $p = .3$ compared to healthy; at 12 months $p = .28$ compared to BO, $p = .08$ compared to healthy) (Figure 4(a,b), Table 3, supplemental Figure 4). No differences at diagnosis were observed for TWEAK and TRAIL when comparing the two patient cohorts ($p = .44$ and 0.50).

Unsupervised clustering reveals distinct patient subsets

Different immune cell subclasses were clustered with unsupervised hierarchical clustering to detect specific cell phenotypes that associate with each other or with clinical factors such as Sokal risk score, BCR-ABL IS% and study drug. All data were normalized to diagnosis median values, in order to visualize treatment-induced changes for each parameter.

At diagnosis, three distinct patient subgroups were formed. Interestingly, the majority of low Sokal risk patients clustered based on the low levels of terminally differentiated effector CD8 + T cells, CD57+ CD8 + T cells (same as seen in Figure 1(d)) and GrB+ CD8 + T cells (highlighted in Figure 5). The same sets of CD8 + T cells clustered together also at 3 and 12-month time-points. At 12 months, a cluster including mostly (6/7) imatinib-treated patients was formed based on the increased amount of the same CD8 + T cell

populations (highlighted in Figure 5). In addition, one patient who was randomized to bosutinib arm but switched to imatinib due to side-effects already before the 3-month time-point was in the same cluster with the imatinib patients (Bo8, marked with an asterisk in the annotation).

Same unsupervised clustering analysis, with 3 m and 12 m results normalized to diagnosis median values, was performed with the plasma proteins at each timepoint. Notably, cytokine profiles at diagnosis divided patients into two distinct subgroups (Figure 6). Interestingly, the median BCR-ABL IS% clearly differed between the two clusters at diagnosis (34.1 vs. 80.4). The cluster with higher BCR-ABL IS% levels had increased amount of several different cytokines. At 12 months, a distinct cluster of 10 patients was formed by 6 imatinib-treated patients and two bosutinib-treated patients who had switched to imatinib before the sampling (marked with asterisk in the annotation in Figure 6). In addition, a group of soluble plasma proteins clearly decreased after TKI start, namely CD5, CD6, CD244, OSM, IL-10RA, IL-4, and TGF-alpha (marked with blue arrows). In contrast, CCL28, SCF, TWEAK, CX3CL1, IL-12B, and CCL25 (marked with red arrows) markedly increased after TKI start (Figure 6).

Normal blood counts are achieved faster in bosutinib-treated patients

Complete blood counts (percentages and absolute counts) were compared between the two groups of patients at diagnosis, and then at 3 and 12 months. No differences were observed in between the groups at diagnosis, but at 3 months after therapy start, bosutinib-treated patients had significantly higher absolute numbers of leukocytes ($p = .009$), monocytes ($p = .001$), neutrophils ($p = .013$) and platelets ($p = .041$) (Figure 7(a,b)), whereas in some imatinib-treated patients the blood counts were below the lower normal limit. Further at 12 months, the leukocytes ($p = .014$), monocytes ($p = .032$) and neutrophils ($p = .022$) were still significantly higher in the bosutinib group (Figure 7(a,c)). No significant differences were observed in the percentage or absolute counts of lymphocytes between the two groups at either time-point (Figure 7(a)).

No changes were observed in the blood cell counts after imatinib or bosutinib intake

The second-generation TKI, dasatinib, is known to induce a rapid mobilization of lymphocytes to the peripheral blood after drug intake.[22] In order to study whether similar changes would occur after imatinib or bosutinib intake, differential blood counts were taken before and after 2 h of drug intake at 3- and 12-month time-points. In contrast to what was observed in dasatinib-treated CML patients, no significant changes were observed in the hemoglobin and the absolute blood cell counts (leukocytes, erythrocytes, platelets, neutrophils, eosinophils, basophils or lymphocytes; supplemental Figure 5). A significant increase was observed only in the absolute monocyte counts ($p = .027$) at 3 months, and absolute neutrophil counts ($p = .0029$) at 12 months after imatinib admission (supplemental Figure 5).

Table 3. Protein levels were expressed as Normalized Protein eXpression (NPX) values, an arbitrary unit on $\log_2$-scale, and shown here as median values for each studied group.

Baseline

	Healthy	CML	Healthy vs CML	Function
uPA	760.4	1446.6	**0.0001**	a serine protease
MCP-1	463.9	487.5	0.70	a chemokine secreted by monocytes, macrophages, and dendritic cells
TRAIL	183.2	195.4	0.86	a cytokine that upon binding to its different receptors can activate apoptosis
CST5	113.1	99.9	0.82	cysteine proteinase inhibitor
OSM	4.5	27.6	**0.00004**	both a pro- and an anti-inflammatory cytokine
FGF-5	2.4	2.6	0.70	growth factor involved in regulation of cellular proliferation and differentiation
CD5	13.0	23.1	**0.0002**	a membrane-associated glycoprotein
Flt3L	440.5	824.9	**0.03**	cytokine, involved in mobilization and differentiation of hematopoietic stem cells
CCL28	1.8	1.7	0.70	a chemokine, chemotactic activity on T and B cells
DNER	150.4	127.3	0.11	activator of the NOTCH1 pathway
CX3CL1	61.4	48.9	0.53	a chemokine, chemotactic function for T cells and monocytes
TWEAK	229.5	254.9	0.35	a cytokine, induces multiple signaling pathways that lead to apoptosis

3 months

	Healthy	Imatinib	Bosutinib	BO vs IM	BO vs healthy	IM vs healthy	Function
uPA	760.4	943.8	675.9	**0.03**	0.20	**0.05**	a serine protease
MCP-1	463.9	656.6	442.2	**0.01**	1.00	**0.01**	a chemokine secreted by monocytes, macrophages, and dendritic cells
TRAIL	183.2	206.8	172.0	**0.04**	0.20	0.32	a cytokine that upon binding to its different receptors can activate apoptosis
CST5	113.1	86.6	110.2	0.08	0.97	0.15	cysteine proteinase inhibitor
OSM	4.5	5.3	9.3	**0.02**	**0.004**	0.27	both a pro- and an anti-inflammatory cytokine
FGF-5	2.4	3.0	2.2	0.07	0.20	0.24	growth factor involved in regulation of cellular proliferation and differentiation
CD5	13.0	16.8	13.8	**0.05**	0.24	**0.01**	a membrane-associated glycoprotein
Flt3L	440.5	541.1	307.5	**0.05**	0.32	**0.04**	cytokine, involved in mobilization and differentiation of hematopoietic stem cells
CCL28	1.8	2.0	1.7	0.13	0.36	0.32	a chemokine, chemotactic activity on T and B cells
DNER	150.4	138.0	116.9	**0.03**	**0.002**	0.41	activator of the NOTCH1 pathway
CX3CL1	61.4	64.6	41.2	**0.007**	**0.03**	0.36	a chemokine, chemotactic function for T cells and monocytes
TWEAK	229.5	331.1	259.1	**0.02**	0.97	**0.02**	a cytokine, induces multiple signaling pathways that lead to apoptosis

12 months

|	Healthy	Imatinib	Bosutinib	BO vs IM	BO vs healthy	IM vs healthy	Function
uPA	760.4	976.0	737.8	0.06	0.70	**0.03**	a serine protease
MCP-1	463.9	733.3	525.9	**0.02**	0.20	**0.004**	a chemokine secreted by monocytes, macrophages, and dendritic cells
TRAIL	183.2	237.1	215.1	0.28	0.51	0.08	a cytokine that upon binding to its different receptors can activate apoptosis
CST5	113.1	87.3	113.0	**0.05**	0.83	0.17	cysteine proteinase inhibitor
OSM	4.5	7.9	14.3	0.23	**0.006**	**0.04**	both a pro- and an anti-inflammatory cytokine
FGF-5	2.4	3.3	2.8	**0.05**	0.46	**0.01**	growth factor involved in regulation of cellular proliferation and differentiation
CD5	13.0	17.9	15.5	0.06	0.12	**0.001**	a membrane-associated glycoprotein
Flt3L	440.5	612.3	320.5	0.06	0.51	**0.006**	cytokine, involved in mobilization and differentiation of hematopoietic stem cells
CCL28	1.8	2.4	2.2	0.23	0.24	**0.009**	a chemokine, chemotactic activity on T and B cells
DNER	150.4	150.0	138.7	0.19	0.27	0.83	activator of the NOTCH1 pathway
CX3CL1	61.4	87.3	46.9	**0.01**	0.32	0.07	a chemokine, chemotactic function for T cells and monocytes
TWEAK	229.5	374.6	300.4	0.16	0.24	**0.006**	a cytokine, induces multiple signaling pathways that lead to apoptosis
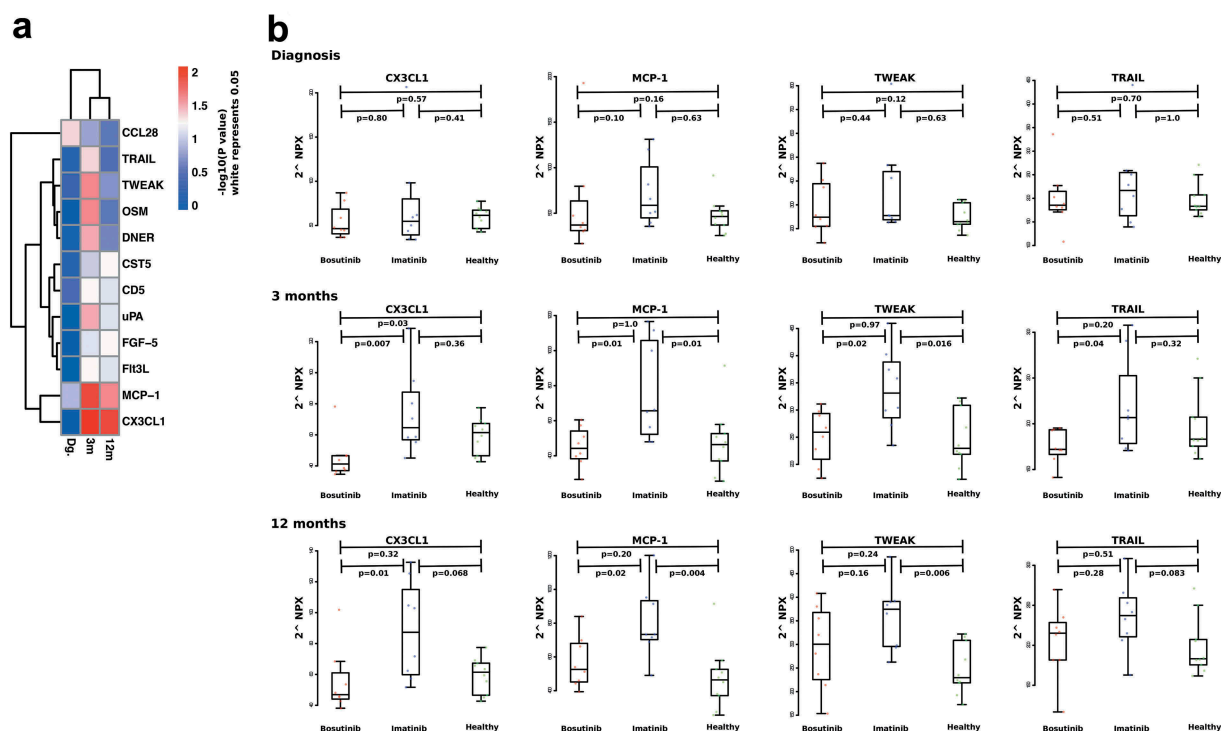
Figure 4. Differences of plasma protein at diagnosis and during TKI therapy. A *p*-value heatmap presenting significant differences between imatinib- and bosutinib-treated patients' plasma proteins (a). Imatinib-treated patients have a significant increase in MCP-1, CX3CL1. TWEAK and TRAIL at both 3-month and 12-month time-points (b). Non-parametric Wilcoxon signed-ranked test was performed to compare differences between treatments and data are presented as boxplots. Protein levels are expressed as Normalized Protein eXpression (NPX) values, an arbitrary unit on $\log_2$-scale.

Discussion

In principle, the immunological off-target effects of TKIs may improve or impair the overall therapy outcome in CML patients.[23] The immune system, notably NK cells, has recently been linked with successful treatment discontinuation.[5,6,20,21] Therefore, several groups have lately attempted to understand the immunological effects of TKIs in CML patients and their impact on therapy responses. Especially, the SRC family of kinases and other signaling pathways involved in cellular activity of the immune system may play an important role for secondary, immune-mediated effects of TKIs. Still, no longitudinal studies have been performed to characterize the immunomodulatory effects of first-line imatinib and bosutinib in CP CML patients and how these changes correlate with the remaining disease burden.

Two large phase III clinical trials, BELA (NCT00574873) and BFORE (NCT02130557), have recently shown that bosutinib is superior to imatinib in the first-line setting regarding MMR rate at 12 months. Also, the complete cytogenetic response rate at 12 months was significantly higher in the bosutinib arms.[24–26] These results are likely to be explained by bosutinib's higher activity against BCR-ABL and various imatinib-resistant mutations.[27] Bosutinib also has a wider TK inhibitory profile than imatinib and thus more resembles dasatinib, another second-generation TKI, in the sense that for example, both inhibit the SRC kinase family.[7,8] Interestingly, previous reports from our group and others have shown significant immunomodulatory effects of dasatinib.[5,9–15,22] With this in mind, it is surprising that we

did not detect immunomodulatory effects in the bosutinib-treated CML patients. In contrast, we observed that imatinib treatment induced multiple immunological changes. As seen in our study cohort as well as in the clinical trials, imatinib treatment resulted in slower therapy responses and recovery of the bone marrow, while bosutinib-treated patients mostly achieved quicker therapy responses and faster regain of normal bone marrow functions. We are not able to exclude the possibility that because bosutinib causes faster therapy responses, there is no need for the immune system to activate and clear remaining leukemic cells. In contrast, slower therapy responses induced by imatinib could leave time for the immune system to act after the initial immune suppression observed at diagnosis is reverted.[28] However, although dasatinib induces a more rapid molecular response than imatinib, significant immunological changes are observed during the treatment.[10] Therefore, it is also possible that imatinib has direct immunological effects in CML similarly as shown in gastrointestinal stromal tumors and neuroblastoma patients.[13,17,29]

The initial fraction of leukemic stem cells at diagnosis is known to have an impact on the overall therapy outcome,[30,31] but also on the immune system, causing significant malfunction and suppression of the lymphocytes in CML patients.[28,32–34] High Sokal risk CML patients have also been reported to have higher numbers of myeloid-derived suppressor and PD1-expressing CD8 + T cells.[35] In accordance, we show here, that patients with low and intermediate Sokal risk scores have more active immune systems compared to those in the high-risk category. This was particularly true for NK cells, which were
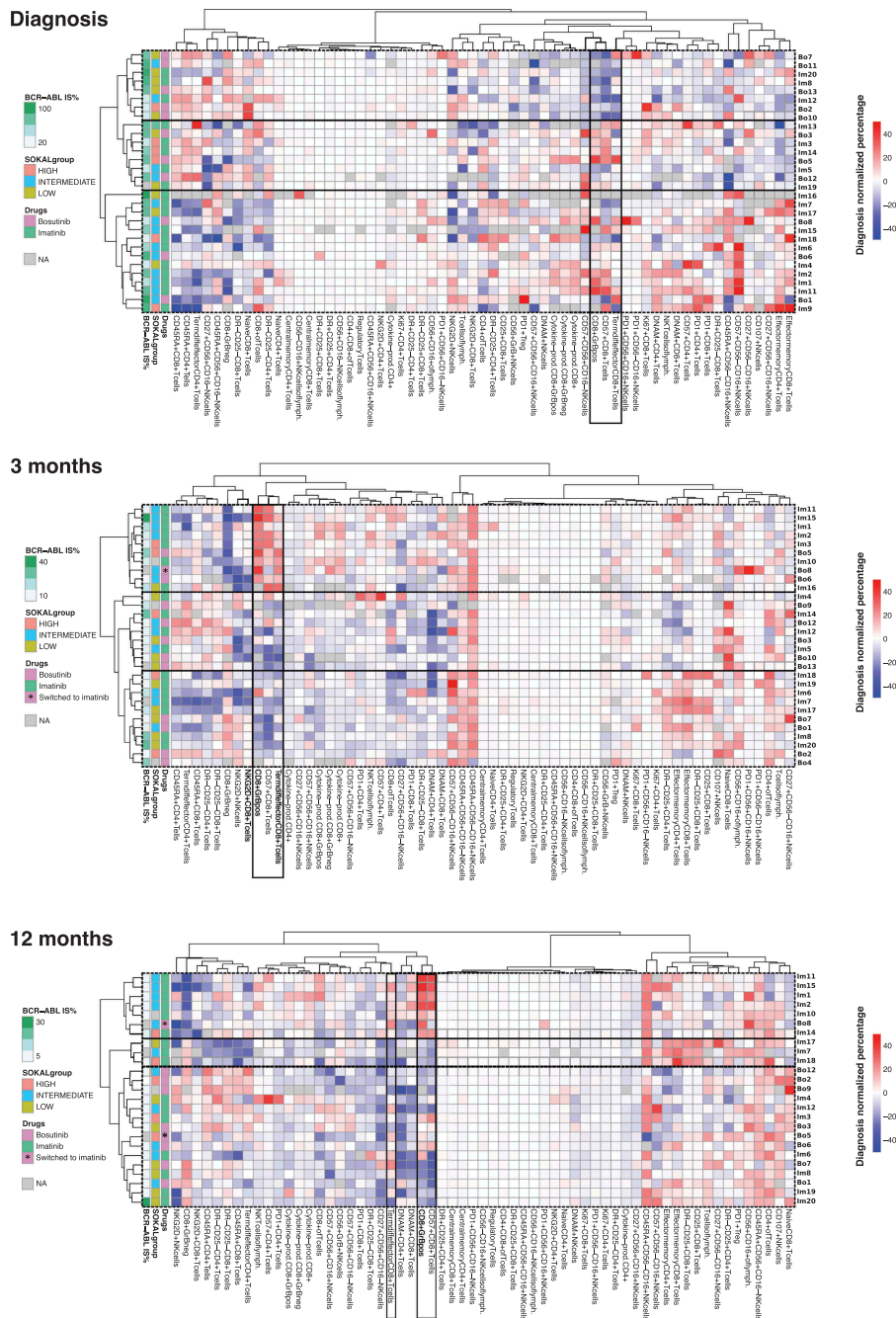
Figure 5. Unsupervised hierarchical clustering of immune cell subsets. Flow cytometry data for each timepoint were normalized to diagnosis median values and heatmap was drawn based on Euclidean distance and unsupervised hierarchical clustering methods for each timepoint independently. Annotations were added for Sokal risk at diagnosis, BCR-ABL IS% at each timepoint and study drug. Low Sokal risk patients at diagnosis clustered based on terminally differentiated effector CD8 + T cells, CD57+ CD8 + T cells and GrB+ CD8 + T cells, which are highlighted in each heatmap. At 12-month time-point, the majority of imatinib-treated patients cluster based on their T and NK cells phenotypes. Patients who started on bosutinib and switched to imatinib treatment during the 12-month follow up are marked with an asterisk.

more frequent and also had a superior cytotoxic potential in the low risk compared to the high-risk patients. Interestingly, recent papers published by us and others have described an important role for NK cells in achieving a deep molecular response and the successful discontinuation of imatinib treatment.[5,6,21,36] Ilander et al. have shown that the proportion of NK cells was associated with molecular relapse-free survival as patients with a higher NK-cell percentage at the time of drug discontinuation had a better probability of staying in remission. In addition, Hughes et al. have recently reported that the quantitative and

functional defects of NK cells were observed at diagnosis, and later, those patients who were in MMR and MR4.5 had acquired more mature, cytolytic CD57+ CD62L-NK cell.[36] Interestingly, our present results show that both the percentage and the cytotoxic potential of the NK cells increased especially in the imatinib-treated patients, while bosutinib did not significantly affect the NK-cell frequency or their function.

Cytokines are small proteins that are important for cell-cell signaling and therefore, they represent useful readouts to study changes in the immune system. In our CML cohort,
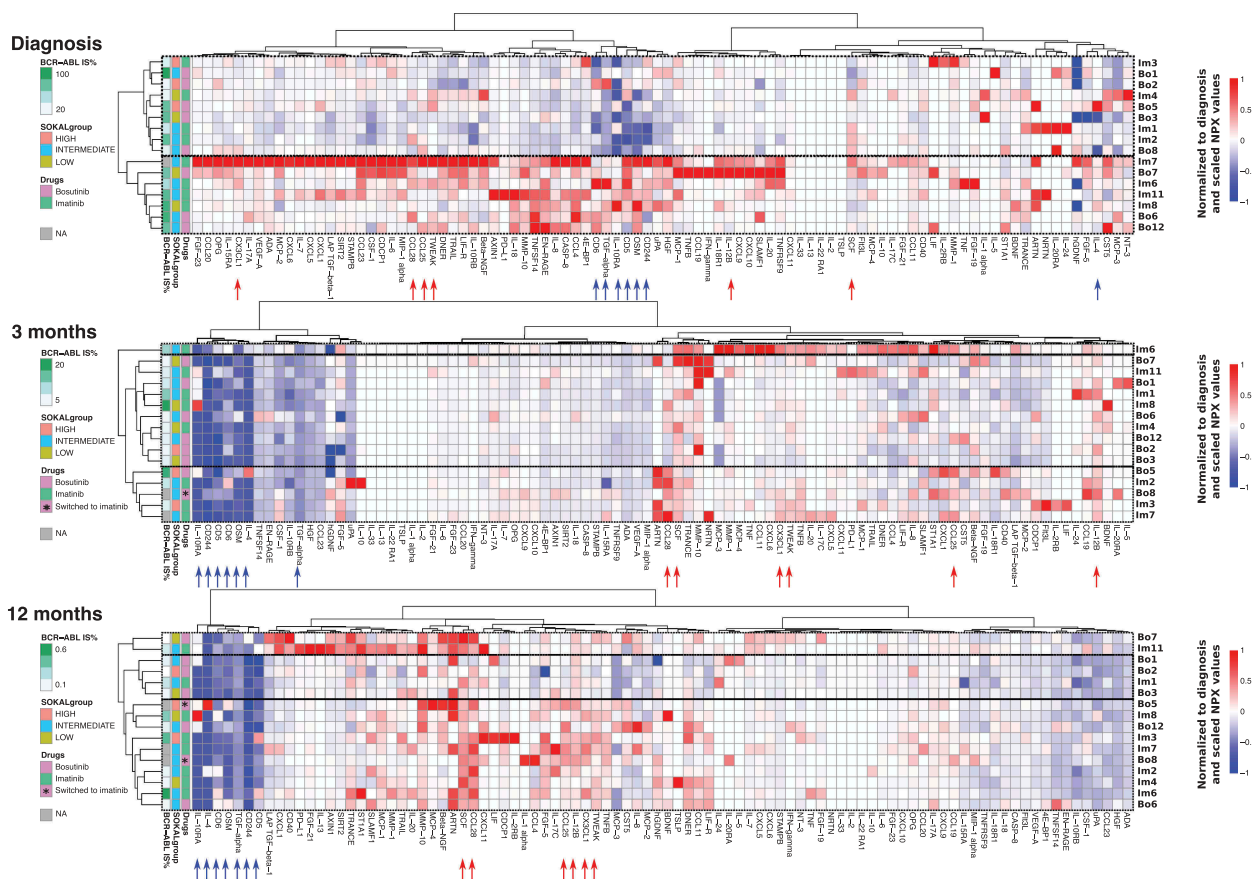
Figure 6. CML patients with high BCR-ABL IS% at diagnosis have distinct soluble plasma protein and receptor profile. Soluble plasma proteins were calculated as NPX values at diagnosis and after therapy start. All data were normalized to baseline median values, in order to visualize treatment-induced changes in each parameter. Heatmap analysis was performed including Euclidean distance and unsupervised hierarchical clustering methods for each timepoint independently, and annotations were added for Sokal risk at diagnosis, BCR-ABL IS% at each timepoint and study drug. At diagnosis, two clusters with different BCR-ABL IS% (median values 34.1 and 80.4) are observed with distinctive plasma protein profiles. After the therapy start, a group of soluble proteins and receptors clearly decreased after TKI start, namely CD5, CD6, CD244, OSM, IL-10RA, IL-4, and TGF-alpha (marked with blue arrows). On the contrary, CCL28, SCF, TWEAK, CX3CL1, IL-12B, and CCL25 (marked with red arrows) prominently increased after TKI start. Patients who started on bosutinib and switched to imatinib treatment during the 12-month follow up are marked with an asterisk.

we found that imatinib treatment led to elevated levels of several soluble proteins in the plasma, whereas bosutinib had minor effects. The most drastic differences between the two patient groups were observed at 3 months. TRAIL, TWEAK, CX3CL1, and MCP1 were significantly increased during imatinib treatment. These plasma proteins are either key players in cytotoxic pathways or function as chemoattractants for T cells among other cell types. Inflammatory cytokines belonging to tumor necrosis factor (TNF) family, such as TRAIL and TWEAK, are able to induce apoptosis in cancer cells while exhibiting little or no toxicity in normal cells.[37] Moreover, members of the TNF superfamily provide a communication complex that is essential in synchronizing multiple cell types into an effective host defense system against malignant cells. These pathways differentiate both innate and adaptive immune cells, and modulate stromal cells into microenvironments that are conducive to host defenses.[38]

Chemokines on the other hand induce chemotaxis in responsive cells. We observed that CX3CL1 levels in particular increased during imatinib treatment. CX3CL1 and its cognate receptor CX3CR1 are involved in the recruitment of

leukocytes to the inflamed vasculature.[39] CXC3L1 also induces active migration of NK cells and by blocking the CX3CL1/CXCR3 pathway it has been shown that the NK cells no longer were able to clear the cancer cells in a mouse tumor model.[40] We conclude that these elevated cytokine levels reflect an active immune system in the imatinib-treated patients, affecting mainly the NK cells, and this is also observed as an increased NK-cell percentage in these patients. Moreover, our analysis revealed that leukemic cell burden (as reflected by BCR-ABL IS%) during the treatment time correlated positively with several plasma proteins such as CCL4, CXCL1, CXCL5, CXCL6, CXCL10, CCL28, CX3CL1, IL-10RA, Flt3L, ST1A1, and TNF.

In conclusion, the disease burden, including both Sokal risk at diagnosis and BCR-ABL IS% during treatment, correlates with multiple immunological factors, providing further insight into the role of the immune system in CML. In addition, we observed that imatinib treatment generated a more active immune system, which correlated with the BCR-ABL IS%. In contrast, no clear immunomodulatory effects were detected during bosutinib treatment, despite the wide kinase inhibition profile of the drug. We conclude that the immune system in CML patients
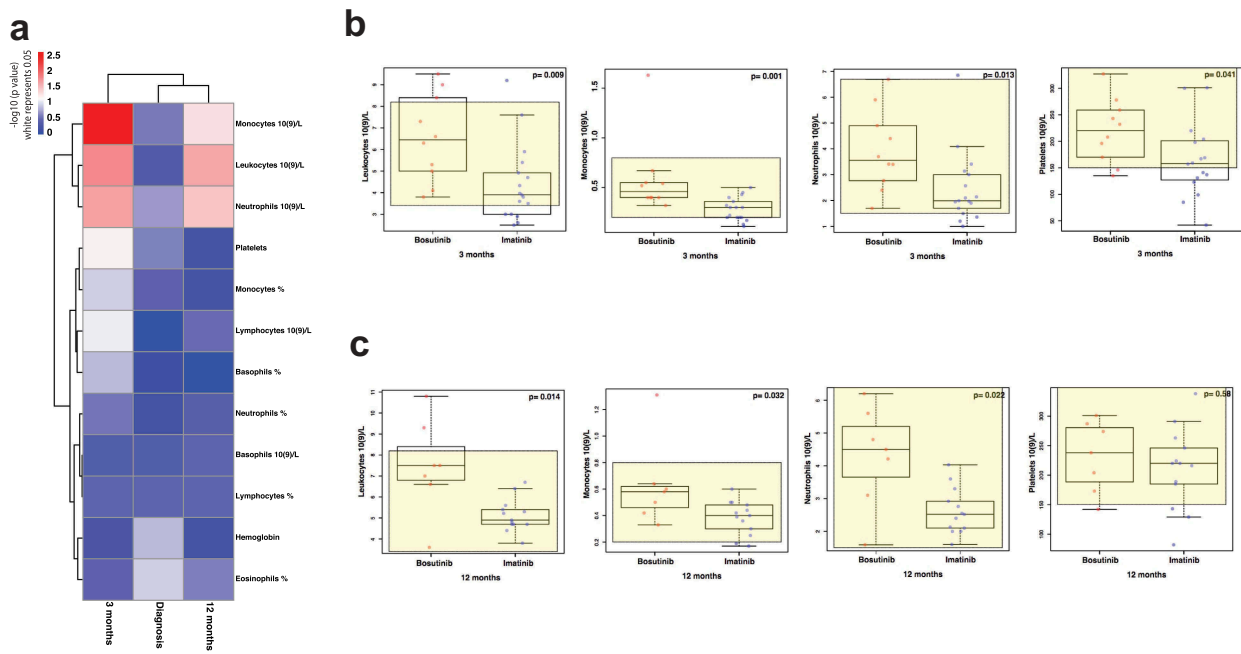
Figure 7. **Normal blood cell values are faster re-established during bosutinib-treatment**. A *p*-value heatmap presenting significant differences between imatinib- and bosutinib-treated patients' complete blood counts (a). At diagnosis, no differences were observed between the two groups of patients. Three months after therapy start, bosutinib-patients have higher absolute numbers of neutrophils, monocytes, platelets, and leukocytes (b). The same differences were observed also after 12 months of treatment (c). Non-parametric Wilcoxon signed-ranked test was performed to compare differences between treatments and data are presented as boxplots, showing the median value of each parameter.

with a low disease burden is more active and has a higher functional capacity than in patients with a higher disease load.

Patients and methods

Study patients and samples

Newly diagnosed CP CML patients were enrolled in the BFORE clinical trial (NCT02130557). Peripheral blood samples were obtained from imatinib- (n = 20) and bosutinib-treated (n = 13) patients at diagnosis (dg), as well as 3 and 12 months after the start of TKI therapy. Moreover, differential blood counts were taken before and 2-h post drug intake at 3 and 12 months to examine the direct effects of the drug intake on lymphocyte counts.[22] Peripheral blood mononuclear cells (PB MNCs) from all patients and healthy controls were separated by Ficoll-Paque gradient centrifugation (GE Healthcare, Buckingham, UK) and were freshly used or stored as live vials at −150°C.

All patients and healthy controls gave a written informed consent and the study was approved by the ethical committees of each local university hospital. The study was conducted in accordance with the Declaration of Helsinki.

Phenotyping

PB MNC were isolated with Ficoll from fresh blood and the cells were immediately stained for 15 min with surface markers (CD3, CD4, CD8, CD14 (Invitrogen), CD16, CD19 (Invitrogen), CD25, CD27, CD38, CD45, CD45RA, CD56, CD57, CCR7 (RnD), HLA-DR, NKG2D, and PD1). Intracellular stains using

FoxP3 (eBioscience) and Ki-67 antibodies were performed after permeabilizing the cells with the Transcription Factor Staining Buffer Set (eBioscience). A total of 50 000 lymphocytes were acquired with the FACS Verse and analyzed with FlowJo (Version 10.0.8r1, TreeStar) software. Dead cells were removed from analysis from SSC vs FSC and CD45 scatter. All antibodies were purchased from BD Biosciences (BD Biosciences, San Diego, CA, USA) unless mentioned otherwise.

NK-cell degranulation assay

Thawed PB MNCs were stimulated with the target cell line K562 at a 10:1 ratio. PB MNCs without target cells were used as negative controls. Cells were incubated for 6 h in the presence of antibodies containing degranulation markers, CD107a and CD107b. All cells were stained with CD45, CD3, CD16, and CD56. 50,000 CD45$^+$ lymphocytes were acquired with FACS Verse and analyzed with FlowJo.

Activation of T-cells

Thawed PB MNCs were stimulated with anti-CD3 (2.5 µg/ml), co-stimulatory anti-CD28 (1 µg/ml), anti-CD49d (0.5 µg/ml) (BD Biosciences), and incubated for 6 h at +37°C. PB MNCs without stimulation were used as controls. GolgiStop (BD Biosciences) was added to each well. After the incubation, cells were stained with the following antibodies: CD45, CD3, CD4, CD8. Subsequently, cells were permeabilized and fixed according to the Cytofix Cytoperm (BD Biosciences) kit

protocol. Intracellular stains were carried out with Granzyme B, and the cytokines IFN-γ and TNF-α, whereby 50 000 CD45$^+$ lymphocytes were analyzed with FACS Verse.

Soluble plasma protein and receptor analysis by multiplex immunoassay

ACD plasma samples from 8 imatinib-treated, 8 bosutinib-treated patients (all time-points), and 10 healthy volunteers (6 females, 4 males, mean age 51.7 years) were analyzed using the Proseek® Multiplex Inflammation immunoassay. Proseek® is a high-throughput, multiplex immunoassay that enables the analysis of 92 inflammation-related protein biomarkers simultaneously (Proseek Multiplex CVD, Olink Bioscience, Uppsala, Sweden). Protein levels were expressed as Normalized Protein eXpression (NPX) values, an arbitrary unit on log$_2$-scale.

Statistical analysis

For heatmaps, in the case of plasma protein data, the values measured in the patients were normalized using the plasma protein levels in healthy controls and scaled between −1 and +1. The Euclidean distance and complete clustering methods were used for the heatmap of both flow cytometry results and plasma protein data heatmap. The differences between the groups were analyzed using the non-parametric Wilcoxon signed-ranked test. The correlation between the data sets was computed using the Spearman rank correlation method.

Disclosure statement

PK has received honoraria from Novartis, BMS, Ariad, and Pfizer. UOS has received honoraria from Ariad. JR has received honoraria and research funding from Novartis and Bristol-Myers Squibb and honoraria from Ariad. HHjH has received honoraria from Pfizer, Novartis, Bristol-Myers Squibb, and Incyte. SM has received honoraria and research funding from Novartis, Bristol-Myers Squibb, Ariad, and Pfizer.

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