

The Tyrosine Kinase Inhibitors Imatinib and Dasatinib Reduce Myeloid Suppressor Cells and Release Effector Lymphocyte Responses

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Abstract

Immune escape mechanisms promote tumor progression and are hurdles of cancer immunotherapy. Removing immunosuppressive cells before treatment can enhance efficacy. Tyrosine kinase inhibitors (TKI) may be of interest to combine with immunotherapy, as it has been shown that the inhibitor sunitinib reduces myeloid suppressor cells in patients with renal cell carcinoma and dasatinib promotes expansion of natural killer-like lymphocytes in chronic myeloid leukemia (CML). In this study, the capacity of dasatinib and imatinib to reduce myeloid suppressor cells and to induce immunomodulation *in vivo* was investigated *ex vivo*. Samples from CML patients treated with imatinib ($n = 18$) or dasatinib ($n = 14$) within a Nordic clinical trial (clinicalTrials.gov identifier: NCT00852566) were investigated for the presence of CD11b⁺CD14⁻CD33⁺ myeloid cells and inhibitory molecules

(arginase I, myeloperoxidase, IL10) as well as the presence of natural killer cells, T cells (naïve/memory), and stimulatory cytokines (IL12, IFN γ , MIG, IP10). Both imatinib and dasatinib decreased the presence of CD11b⁺CD14⁻CD33⁺ myeloid cells as well as the inhibitory molecules and the remaining myeloid suppressor cells had an increased CD40 expression. Monocytes also increased CD40 after therapy. Moreover, increased levels of CD40, IL12, natural killer cells, and experienced T cells were noted after TKI initiation. The presence of experienced T cells was correlated to a higher IFN γ and MIG plasma concentration. Taken together, the results demonstrate that both imatinib and dasatinib tilted the immunosuppressive CML tumor milieu towards promoting immune stimulation. Hence, imatinib and dasatinib may be of interest to combine with cancer immunotherapy. *Mol Cancer Ther*; 14(5); 1181–91. ©2015 AACR.

Introduction

Immunotherapy of cancer has been in the limelight during the past few years due to the success of, for example, immunomodulatory antibodies such as ipilimumab (anti-CTLA-4) for malignant melanoma (1). Intense research in tumor immunology has revealed the interplay between tumor cells, its stroma, and the

immune system. It is now recognized that tumor cells avoid destruction by natural killer (NK) cells and T cells by releasing immunosuppressive molecules as well as molecules that promote the differentiation of myeloid suppressor cells and regulatory T cells (Treg). Myeloid suppressor cells are a heterogeneous group of myeloid cells that are increased in most cancer patients (2). They utilize different mechanisms for suppressing immune responses, including upregulation of the enzyme Arginase 1 (Arg1) leading to inhibition of T cells and other immune cells (3, 4). Moreover, myeloid suppressor cells can affect the immune system by induction or recruitment of Tregs (5). Tregs modulate the immune system by inhibition of effector T cells as well as NK cells, dendritic cells, and B cells (6). Immune evasion hampers antitumor immune reactions and is therefore since 2011 recognized as one of the hallmarks of cancer (7). Thus, immune evasion strategies need to be considered during the development of immunotherapy.

Most immunotherapies in clinical trials are currently used together with a preconditioning strategy to reduce the level of suppressive immune cells. For example, cyclophosphamide given before treatment or metronomic during an extended period of time reduces the presence of Tregs (8). The tyrosine kinase inhibitor (TKI) sunitinib used for patients with renal cell carcinoma was primarily used because of its capacity to inhibit VEGF signaling but it was soon recognized that hampering myeloid suppressor cells was part of the mechanism of action (9). This inhibition has been linked to the suppression of STAT-3 signaling (10) a feature shared also with other TKIs such as imatinib and

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dasatinib (11, 12). TKIs have been used to treat patients with chronic myeloid leukemia (CML), as they target the constitutively active tyrosine kinase bcr/abl present only in the CML tumor cells. The TKIs imatinib and dasatinib are both very effective for CML treatment and are well tolerated but in some patients, inflammation of unknown origin arises, implicating an effect on the immune system (13). The high response rate is usually attributed to a direct effect of TKIs on the malignant cells. However, both *in vitro* (14–16) and *in vivo* (17–19) studies show that TKIs can modulate cells of the immune system, possibly affecting antitumor immunity of treated patients.

In this article, we sought to investigate the capacity of imatinib and dasatinib to reduce myeloid suppressor cells and their suppressive mediators *in vivo* to further understand their mechanism of action in CML as well as to validate their capacity to function as a preconditioning regimen prior, or during, immunotherapy of cancer.

Materials and Methods

Patient and control subject samples

Samples from 32 patients were obtained from the "Randomized Study Comparing the Effect of Dasatinib and Imatinib on Malignant Stem Cells in Chronic Myeloid Leukemia (NordCML006)" (clinicalTrials.gov identifier: NCT00852566). In this open labeled study, newly diagnosed chronic phase CML patients were randomized to a starting dose of 100 mg dasatinib once daily or 400 mg imatinib once daily. Eighteen patients treated with imatinib and 14 patients treated with dasatinib were analyzed for immune escape mechanisms and related markers. Plasma samples were taken at inclusion (pre, $n = 27$), at one ($n = 23$), three ($n = 13$), and six ($n = 23$) months after initiation of the treatment (PTI). PBMCs were also taken before treatment ($n = 30$) and after 1 ($n = 31$) and 6 ($n = 30$) months PTI. Samples were prepared and cryopreserved at the different study sites and cryopreserved samples were shipped to Uppsala, Sweden. The study was performed according to the declaration of Helsinki and all patients gave their written informed consent. The clinical study, including this spin-off study, was approved by the medical products agencies as well as the regional research ethics committees in the participating countries. PBMCs were obtained by Ficoll gradient separation (GE Healthcare). All samples had been cryopreserved before analysis.

Flow cytometry

Cryopreserved CML PBMCs from pre, 1, and 6 months PTI as well as cryopreserved PBMCs from control subjects were thawed in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% human serum albumin and 1% penicillin/streptomycin. IMDM and penicillin/streptomycin were from Life Technologies. Human serum albumin was from Octapharma AB. To remove clumps of dead cells after thawing and washing in supplemented IMDM medium, cells were run through a pre-separation filter (Miltenyi Biotec). Before staining for flow cytometry, unspecific binding was blocked by adding Fc-receptor blocking reagent (Miltenyi Biotec). To identify different populations of cells, extra- and intracellular staining were performed. Blocked cells were stained with specific antibodies and corresponding isotype controls. To identify CD11b⁺CD14⁻CD33⁺ myeloid cells, the cells were stained with α -CD11b-PE/Cy7 (clone: ICRF 44), α -CD14-Alexa Fluor 700 (clone: M5E2), and α -CD33-APC (clone: WM-53). These cells were also stained with

α -CD40-FITC (clone: HB-14). Some patients and control subjects had very low levels of CD11b⁺CD14⁻CD33⁺ myeloid cells, thus, for these individuals CD40 expression on the CD11b⁺CD14⁻CD33⁺ myeloid cells could not be analyzed. NK cells were stained with α -CD3-FITC and α -CD56/CD16-PE NK cell cocktail (clones: UCHT-1 and 3G8/MEM-188) and were defined as CD3⁻CD56/CD16⁺ cells. To exclude dead cells from the analysis of CD11b⁺CD14⁻CD33⁺ myeloid cells and NK cells, cells were stained with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation (Life Technologies), according to the manufacturer's instructions. Cells that excluded the fluorescent dye were considered live cells. For staining of different T-cell phenotypes, α -CD3-FITC (clone: UCHT-1), α -CD8-APC (clone: SK-1, BD Biosciences), α -CD45RA-APC/Cy7 (clone: HI-100), and α -CCR7-PerCP/Cy5.5 (clone: G043H7) were used. CD8⁺ naïve T cells were identified as CD3⁺CD8⁺CD45RA⁺CCR7⁺, CD8⁺ memory T cells were identified as CD3⁺CD8⁺CD45RA⁻. To exclude dead cells from T-cell phenotype staining, 50 ng 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Life Technologies) was added to the tubes immediately before acquisition in the flow cytometer. Cells that did not include DAPI were considered live cells. Tregs were stained by the surface markers α -CD3-PerCP (clone: UCHT-1), α -CD4-FITC (clone: OKT-4), α -CD127-PE (clone: HIL-7R-M21, BD Biosciences) then the cells were fixed and permeabilized with the FOXP3 Fix/Perm buffer set according to the instructions from the manufacturer (BioLegend). After permeabilization and blocking with normal mouse serum (Cedarlane), intracellular FoxP3 was stained with an α -FoxP3-Alexa Fluor 647 antibody (clone: 206D). Tregs were defined as CD3⁺CD4⁺CD127⁻FoxP3⁺ cells. The isotype control antibodies IgG1-PE (clone: MOPC-21), IgG1-APC (clone: MOPC-21), IgG2b-brilliant violet 421 (clone: MPC-11), IgG1-FITC (clone: MOPC-21), and IgG1-Alexa Fluor 647 (clone: MOPC-21) were used to identify background staining. All specific antibodies and isotype controls were from BioLegend unless otherwise stated. Cells were analyzed on LSRII (BD Biosciences) and data was evaluated in Flow Jo software from Tree star. Before gating of specific cell subsets, dead cells and duplets were removed by gating (see Supplementary Figs. S1–S3).

Plasma analyses

Plasma samples were investigated for the presence of Arg1 using an ELISA from Hycult Biotech according to the manufacturer's instructions. Arg1 can be released from dying erythrocytes. Hence, to exclude the contribution of Arg1 from dying erythrocytes, hemolytic plasma samples were excluded from the analysis. IFN γ and IL10 were measured by proinflammatory 9-plex Ultra-Sensitive kit from Meso Scale Discovery Inc. MIG and IP10 were analyzed by a service by Myriad RBM Inc using a custom human MAP multiplex panel, whereas soluble CD40L, myeloid peroxidase (MPO), and IL12 were analyzed by a service by Olink Bioscience AB, using the ProSeek Multiplex Oncology⁹⁶ \times ⁹⁶. ELISA, Meso Scale, and Myriad analyses gave concentrations of analytes per mL plasma without correlation to plasma proteins. Olink Bioscience AB's assay detects relative values to compare differences among groups.

Proliferation assay

The suppressive effect of CML myeloid cells on healthy donor T-cell proliferation was determined in a 3H-thymidine assay. Briefly, leukocytes were collected from three newly

Table 1. Patient characteristics

Pat. no.	Age	Gender	Sokal group	EUTOS group	WBC ($\times 10^6$)	Treatment	BCR-ABL >10% ^a	MMR month 12	Dose intensity (%)	Adverse events, grade	Conc. med.
1	43	M	LR	LR	28	Ima	No	No	100	Leukopenia, 2	No
2	55	F	LR	LR	75	Ima	No	No	96	Leukopenia, 3	G-CSF
3	60	F	IR	LR	32	Dasa	No	Yes	100	Anemia, 2	No
4	45	M	LR	LR	32	Dasa	No	Yes	100	Leukopenia, 2 Anemia, 1	No
5	51	F	IR	HR	40	Dasa	No	Yes	100	No	No
6	62	M	LR	LR	26	Ima	No	Yes	99	No	No
7	40	F	LR	LR	11	Dasa	No	Yes	100	Thrombopenia, 2	No
8	66	M	LR	LR	30	Ima	No	No	100	Leukopenia, 2 thrombopenia, 2	No
9	39	M	LR	LR	18	Ima	No	Yes	100	No	No
10	71	F	IR	HR	14	Dasa	No	Yes	100	Leukopenia, 2 thrombopenia, 2	No
11	61	F	LR	LR	20	Dasa	No	Yes	100	Anemia, 1	NSAID
12	52	F	IR	LR	109	Ima	No	No	100	Leukopenia, 3	G-CSF
13	60	M	HR	LR	288	Ima	Yes	No	100	Leukopenia, 2 thrombopenia, 4	G-CSF
14	63	M	LR	LR	22	Ima	No	No	100	No	No
15	44	F	IR	LR	164	Dasa	No	Yes	100	Leukopenia, 2	No
16	45	F	LR	LR	39	Dasa	No	Yes	100	No	No
17	73	F	IR	LR	45	Ima	No	Yes	100	No	No
18	47	F	LR	LR	46	Dasa	No	Yes	100	Neutropenia, 3	G-CSF
19	57	F	LR	LR	38	Ima	No	No	100	No	No
20	51	F	LR	LR	74	Ima	No	Yes	100	No	NSAID
21	71	M	IR	LR	37	Ima	No	Yes	100	Leukopenia, 2	No
22	58	M	LR	LR	43	Dasa	No	Missing ^b	64	Pleural effusion, 2	G-CSF, steroid
23	37	M	LR	LR	165	Ima	Yes	No	100	Neutropenia, 2	No
24	61	M	HR	HR	336	Dasa	No	Yes	100	No	No
25	63	F	HR	LR	20	Dasa	No	Yes	100	No	No
26	67	F	HR	HR	70	Ima	No	Yes	92	Leukopenia, 2	No
27	43	M	LR	LR	154	Ima	No	No	100	No	No
28	28	F	IR	LR	270	Dasa	No	No	95	No	No
29	45	F	LR	LR	25	Dasa	No	Yes	96	Leukopenia, 3	No
30	55	M	LR	LR	65	Ima	No	Yes	100	No	No
31	59	F	IR	LR	97	Ima	Yes	No	100	No	No
32	43	M	LR	LR	110	Ima	Yes	No	100	No	No

NOTE: Sokal group: LR, low risk (score <0.8); IR, intermediate risk (score 0.8–1.2); HR, high risk (score >1.2). EUTOS group: LR, low risk (score ≤ 87); HR, high risk (score >87).

Abbreviations: Ima, imatinib; Dasa, dasatinib.

^aBCR-ABL >10% at month 3; MMR, major molecular response; BCR-ABL is $\leq 0.1\%$; grading of adverse events is according to CTCAE.

^bMMR at 6 months.

diagnosed CML patients at three occasions. Because of poor viability of CML cells after freeze/thaw cycles, the cells were treated with a dead cell removal kit that reduce the number of dead cells before further analyses (Miltenyi Biotec). The myeloid fraction was selected by CD3⁺ sorting using MACS beads and collecting the flow through CD3⁻ fraction (Miltenyi Biotec). The myeloid CD3⁻ fraction was irradiated with 40 Gy and used as allogeneic stimulation in a T-cell proliferation assay. Allogeneic healthy donor CD3⁺ cells were sorted from PBMCs by MACS beads and were then mixed with the CML myeloid CD3⁻ fraction in a 1:1 ratio and cultured in triplicates in a round-bottom 96-well plate. ³H-thymidine (1 uCi; PerkinElmer) was added to the wells and the cells were cultured for 3 days before they were harvested to filters and counts per minute (cpm) were measured by a Wallac b-counter (PerkinElmer Life Science). As a positive proliferation control in each of the three experiments, the myeloid CD3⁻ fraction from healthy donors was used to stimulate the same allogeneic CD3⁺ T cells.

Statistical analysis

The Student *t* test with Welsh correction for possibly unequal variances was used to determine statistical differences between

unpaired groups while Wilcoxon matched-pairs signed rank test was used to determine differences between pre and PTI samples. One-way ANOVA was used to calculate differences when more than two samples were compared and those groups were then post tested using Wilcoxon for matched pairs. For correlation analysis, the nonparametric Spearman rank correlation was assessed. All statistical analyses were made with Prism Software (GraphPad Software Inc.).

Results

Patient characteristics

A summary of patients' baseline characteristics is shown in Table I. The median age of the patients was 55 years with the dasatinib patients being slightly younger. In the dasatinib-treated group, more patients were in the Sokal and Eutos intermediate and high risk. Only 5 patients (patient number 13, 15, 24, 28, and 31) had spleen enlargement at diagnosis. The dose intensity was generally high (as seen in Table 1) and pauses in treatment were due to hematologic toxicity in all cases but patient no 22 who developed pleural effusion on dasatinib treatment. After 12 months of TKI treatment, 59% of the patients had a major

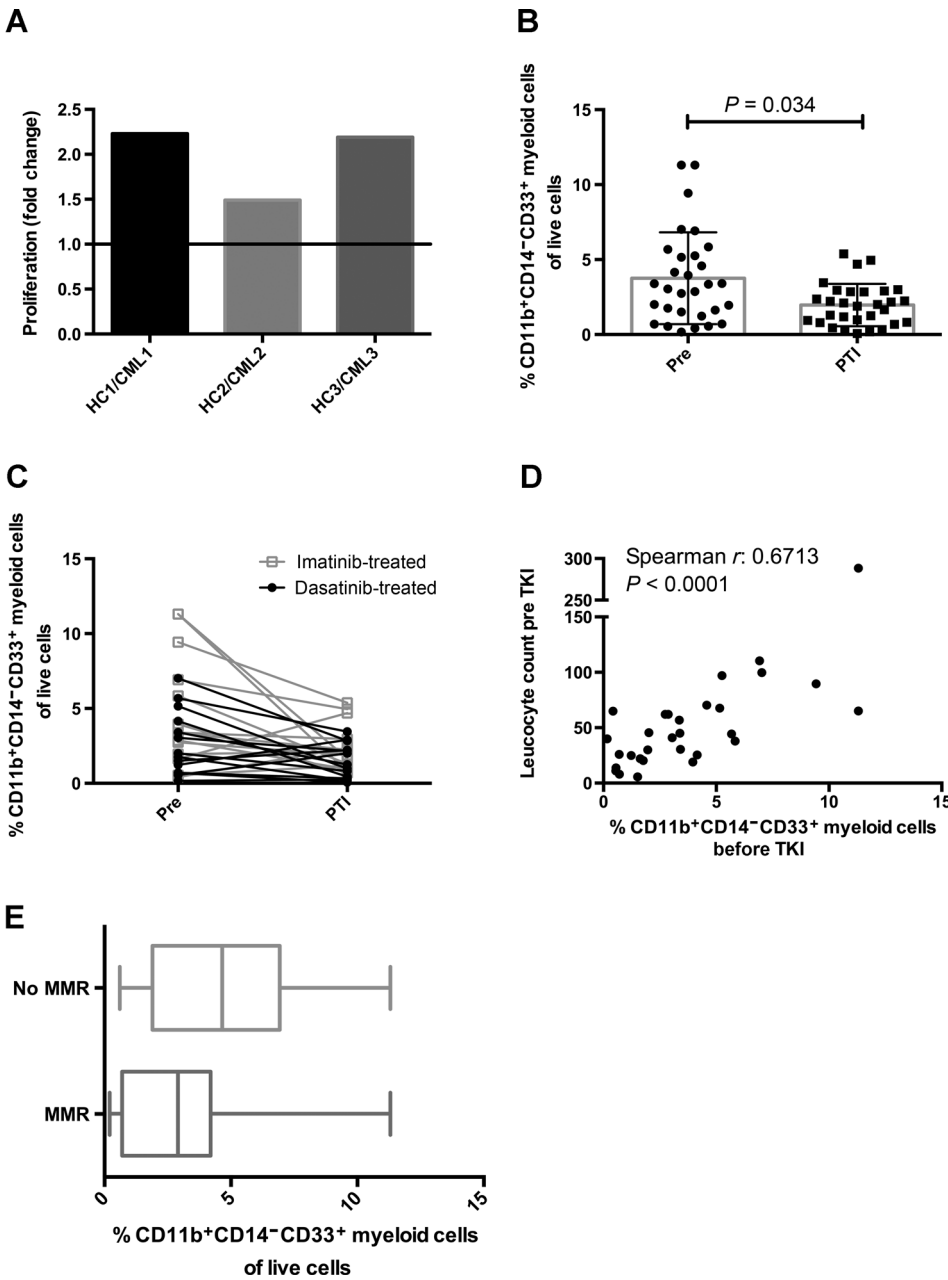


Figure 1. CD11b⁺CD14⁻CD33⁺ myeloid cells after TKI treatment. Myeloid cells (CD3⁻ PBMCs) from 3 CML patients and 3 healthy controls (HC) were irradiated and cocultured with allogeneic healthy donor CD3⁺ T cells in three separate experiments. Proliferation was measured using thymidine incorporation. A, the fold change of T cells stimulated with allogeneic healthy donor myeloid cells compared with allogeneic CML-derived myeloid cells is shown. PBMCs from CML patients pre- and 6 months after TKI therapy initiation (PTI) were analyzed by flow cytometry. B, the difference in myeloid suppressor cells pre and PTI is shown while in C the levels in patients treated with imatinib (square) and dasatinib (dot), respectively, are shown. Statistical significance was calculated using the Wilcoxon test for nonparametric, paired samples. The CD11b⁺CD14⁻CD33⁺ myeloid cells were correlated to the leucocyte count before TKI therapy (D), or compared between in the MMR and no MMR groups at 12 months (E). Statistical significance was calculated using the Spearman correlation test for nonparametric testing. P values < 0.05 were considered significant. Error bars, median with interquartile range.

molecular response (MMR). In-depth analysis of the clinical results have been published previously (20).

The level of CD11b⁺CD14⁻CD33⁺ myeloid cells decreased after TKI treatment

We have previously shown that CD11b⁺CD14⁻CD33⁺ myeloid suppressor cells are increased in Sokal high-risk CML patients (21). To demonstrate the suppressive capacity of myeloid cells in CML patients, we depleted the CD3 lymphocytes from three newly diagnosed patients and three healthy controls and tested the ability of the remaining myeloid cell population to inhibit allogeneic T-cell proliferation. Allogeneic T cells expanded vigorously upon coculture with healthy donor myeloid cells, whereas

the allogeneic T cells showed less proliferative capacity when cultured with myeloid cells from CML patients (Fig. 1A). As TKIs may reduce the presence of such cells, the level of CD11b⁺CD14⁻CD33⁺ myeloid cells in CML patients pre- and postinduction of imatinib or dasatinib therapy was investigated. The median level of these cells in PBMCs before treatment was 3.2% (range 0.2–11.3%, Fig. 1B, gating strategy in Supplementary Fig. S1). After 6 months of treatment, the levels of CD11b⁺CD14⁻CD33⁺ myeloid cells were significantly decreased ($P = 0.0034$). In Fig. 1C, the responses to either imatinib or dasatinib are displayed for each patient. There was no difference in this cell population between imatinib- and dasatinib-treated patients. In solid tumors, the number of circulating myeloid suppressor cells correlates

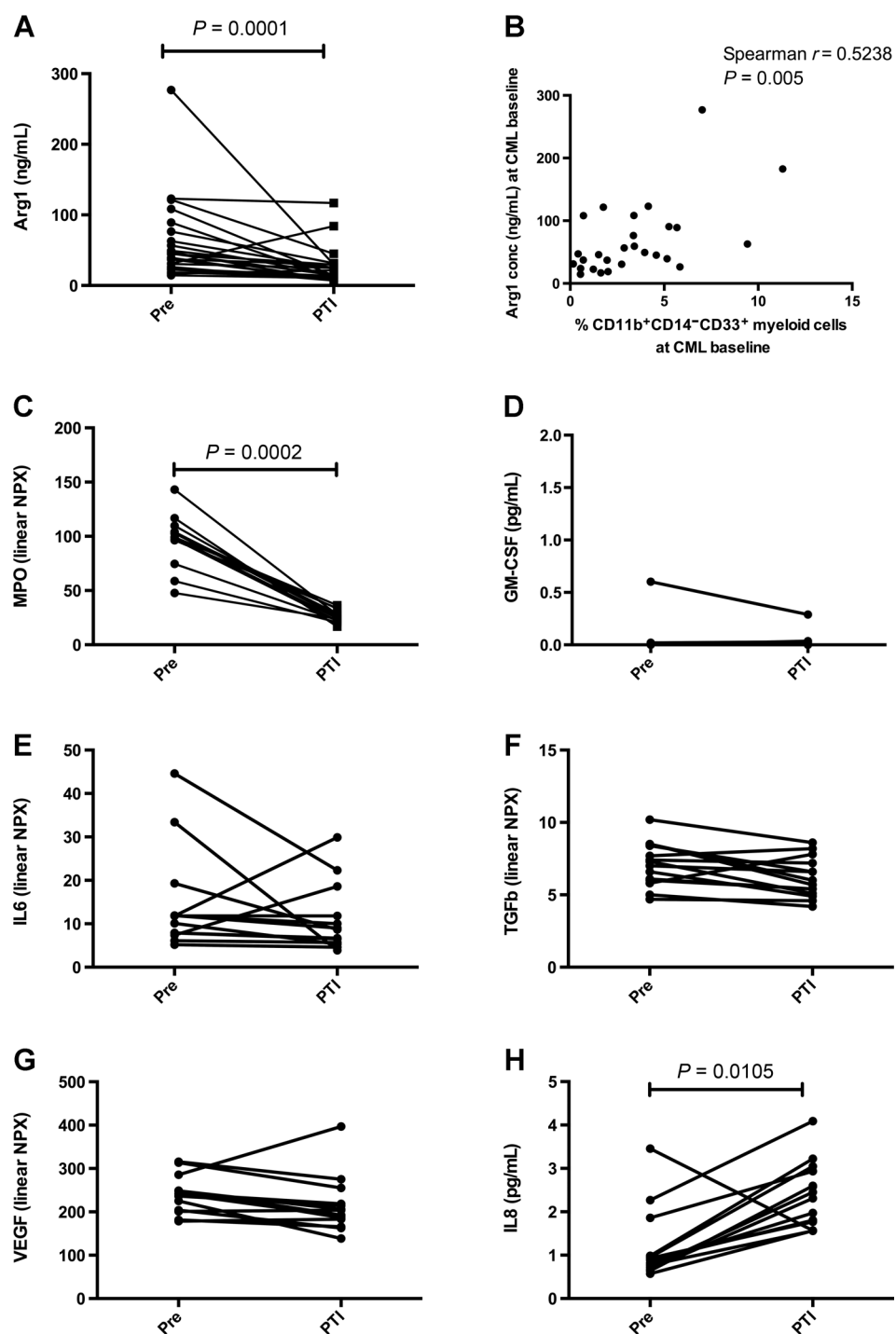
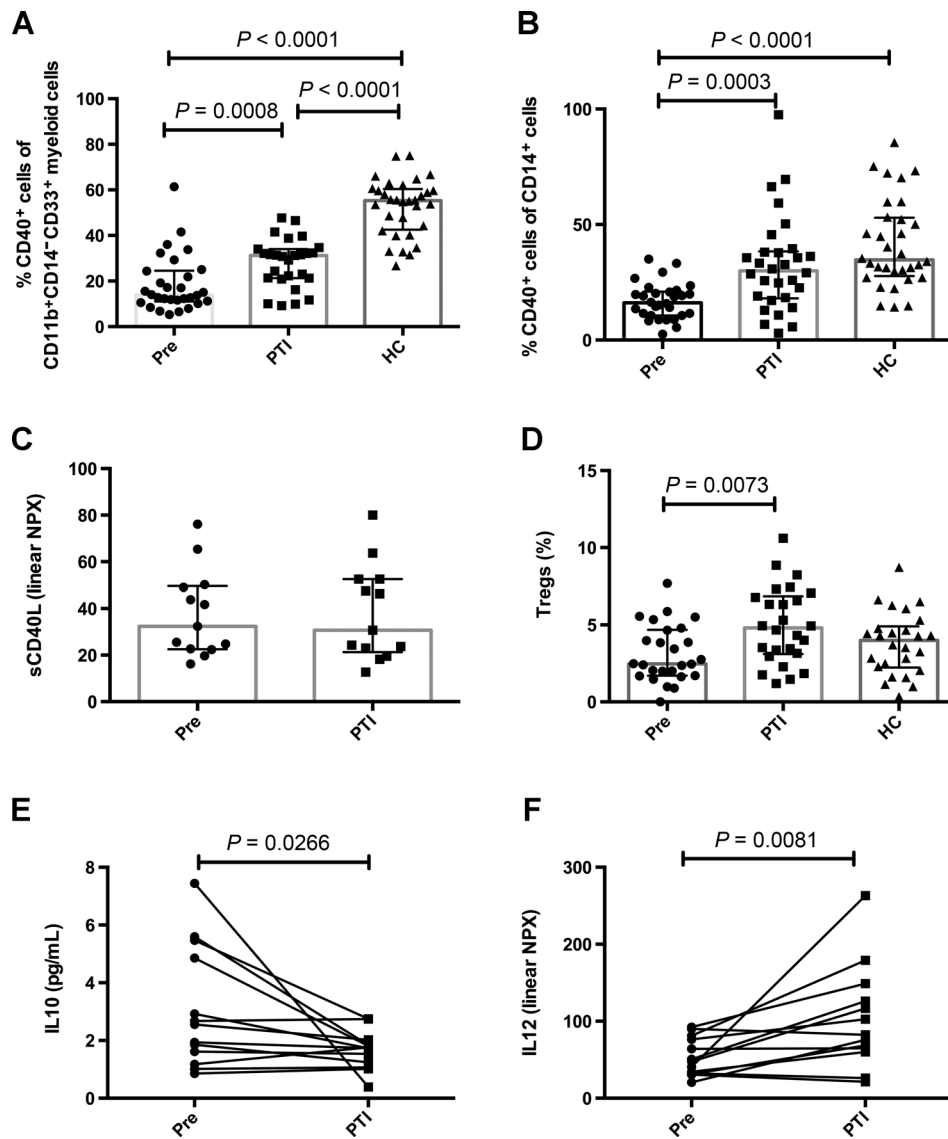


Figure 2. Arg1 and MPO after TKI treatment. Expression of cytokines connected to myeloid suppressor cells. Plasma samples at pre and 6 months PTI were analyzed for Arg1 using ELISA (A) where after the Arg1 level pretreatment was correlated to CD11b⁺CD14⁻CD33⁺ myeloid cells at base line (B). Plasma samples were further analyzed for MPO (C), GM-CSF (D), IL6 (E), TGFβ (F), VEGF (G), and IL8 (H) before and at 3 months PTI using ProSeek (C-G) and MesoScale (H). NPX, normalized protein expression. Statistical significances were calculated using Wilcoxon test for paired samples. *P* values <0.05 were considered significant. Error bars, median with interquartile range.

with metastatic tumor burden and clinical cancer stage (22). Likewise, in CML patients, the leukocyte count (reflecting the tumor burden of a patient) before treatment correlated with the CD11b⁺CD14⁻CD33⁺ myeloid cells (Spearman *r*: 0.6713, *P* < 0.0001; Fig. 1D). However, there was no correlation between MMR values and MDSCs, and no significant difference could be calculated when comparing the MDSC levels in the MMR group versus no MMR at 12 months (Fig. 1E). Arg1 and MPO are suppressor molecules connected to myeloid cells. After 6 months

of TKI therapy, the median Arg1 concentration in patient plasma was significantly decreased (Fig. 2A; *P* = 0.0001). The concentration of Arg1 before treatment was correlated to the number of CD11b⁺CD14⁻CD33⁺ myeloid cells (Fig. 2B; Spearman *r*: 0.5238, *P* = 0.005). On a fraction of the patients (*n* = 13, dasatinib *n* = 5; imatinib *n* = 8), the MPO concentration before and 3 months PTI was measured. MPO was significantly decreased after treatment initiation (Fig. 2C; *P* = 0.0002). Furthermore, growth factors connected to the development of MDSCs such as

**Figure 3.**

Tregs and CD40 expression on cells in peripheral blood of CML patients after TKI treatment. A, CD40 expression was determined on myeloid suppressor cells ($CD11b^+CD14^-CD33^+$) and on monocytes ($CD14^+$); B) using flow cytometry in CML patients and healthy controls pre and at 6 months PTI. C, the plasma concentration of soluble CD40L was evaluated using ProSeek. NPX refers to normalized protein expression. D, Tregs ($CD3^+CD4^+CD127^-FoxP3^+$) were quantified using flow cytometry pre and PTI in CML patients and healthy controls. The plasma concentration of IL10 (E) and IL12 (F) was determined using MesoScale 9-plex kit and Proseek, respectively. Statistical significances were calculated using Wilcoxon test for paired samples and Student *t* test with Welch correction for unpaired calculations (e.g., comparison to healthy controls). *P* values < 0.05 were considered significant. Error bars, median with interquartile range.

GM-CSF, IL6, TGF β , VEGF were not significantly different (Fig. 2D–G). GM-CSF was not detected in most patients. However, IL8 was significantly enhanced by TKI therapy (Fig. 2H).

CD40 expression on $CD11b^+CD14^-CD33^+$ myeloid cells

To investigate the status of the myeloid cells in CML patients after TKI therapy, the immunomodulatory receptor CD40 was evaluated. Stimulation of myeloid suppressor cells with soluble monomeric CD40L has recently shown to increase their suppressive capacity (23). Interestingly, before therapy, the CD40 expression was significantly lower on both myeloid suppressor cells ($CD11b^+CD14^-CD33^+$; $P < 0.0001$) and monocytes ($CD14^+$; $P < 0.0001$) compared with the CD40 levels in healthy individuals. However, the total number of myeloid suppressor cells is low in healthy individuals and this may reflect the high level of CD40. After initiating TKI therapy, CD40 increased on the myeloid suppressor cells (pre vs. post: $P = 0.0008$) and on the monocytes (pre vs. post: $P = 0.0003$; Fig. 3A and B). However, the level of sCD40L in plasma ($n = 13$) was not affected by TKIs (Fig. 3C).

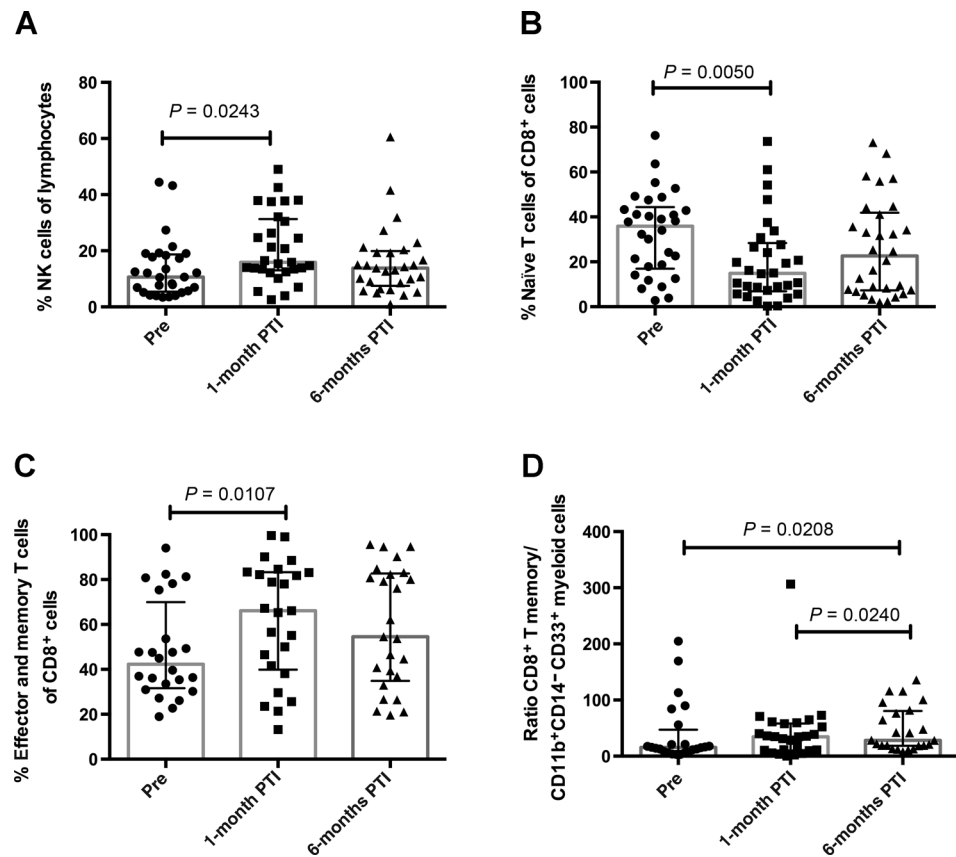
Tregs ($CD3^+CD4^+FoxP3^+CD127^-$) were increased 6 months after initiation of TKI therapy ($P = 0.0073$) but the percentage of Tregs in treated patients was not significantly different from healthy controls (Fig. 3D). Instead, the Treg-associated cytokine IL10 was decreased in patient plasma 3 months posttreatment ($n = 13$, $P = 0.0266$; Fig. 3E), whereas the concentration of the Th1 effector cell stimulator IL12 was increased ($n = 13$, $P = 0.0081$; Fig. 3F). IL12 is released from activated myeloid cells such as mature dendritic cells (DC) that promote T- and NK cell activation indicating that the $CD3^+CD4^+FoxP3^+CD127^-$ cells may not reflect true Tregs but merely recently activated T cells that may also transiently express FoxP3. Collectively, these findings point to immune activation rather than increased immunosuppression and the level of lymphocytes and lymphocyte-associated molecules were thus investigated.

Activation of Th1 effector cells and cytokines by TKI therapy

It has previously been shown that NK cells in CML patients are activated after dasatinib therapy initiation (19). In our cohort of

Figure 4.

The number of NK cells and experienced T cells in CML patients. The level of NK cells ($CD3^- CD56^+ / CD16^+$; A) and T cells (B: naïve $CD3^+ CD8^+ CD45RA^+ CCR7^+$; C: experienced $CD3^+ CD8^+ CD45RA^-$) was analyzed in CML patients before, at 1 month, and at 6 months PTI using flow cytometry. The ratio of T memory cells and myeloid suppressor cells ($CD11b^+ CD14^- CD33^+$) was calculated and displayed in D. Statistical significances were first calculated using one-way ANOVA for comparison of all three paired groups. This test showed that there was a difference between the groups NK cells and naïve T cells. To calculate the exact difference between the pre and a specific time point Wilcoxon test for paired samples was used. For the analysis of experienced T cells, some time points were not analyzed by flow cytometry and, hence, the one-way ANOVA could not be performed. In the figure, the significant difference is only calculated using paired sample Wilcoxon test. P values <0.05 were considered significant. Error bars, median with interquartile range.



patients, the NK cells expanded and demonstrated a significant increase after 1 month but not at 6 months PTI (Fig. 4A; $P = 0.0243$). This effect was seen in both imatinib- and dasatinib-treated patients. Five of 6 patients with the highest NK cell levels were in MMR at 12 months but the cohort is unfortunately too small for statistical correlations to MMR. Naïve $CD8^+$ T cells ($CD3^+ CD8^+ CD45RA^+ CCR7^+$) were decreased after 1 month of TKI treatment (Fig. 4B; $P = 0.0050$) and most patients also stayed at this lower level after 6 months of treatment. In contrast, experienced (effector and memory, $CD3^+ CD8^+ CD45RA^-$) $CD8^+$ T cells were instead increased after 1 month of TKI treatment initiation similarly to the NK cells (Fig. 4C; $P = 0.0107$). Interestingly, the ratio of experienced T cells to myeloid suppressor cells was increased after treatment initiation indicating a shift in the T cell:suppressor cell balance in favor of active T-cell responses (Fig. 4D).

Because both NK cells and T cells release $IFN\gamma$ upon activation and killing of target cells, the levels of $IFN\gamma$, the monokine induced by $IFN\gamma$ (MIG) and $IFN\gamma$ -induced protein 10 (IP10) were investigated in a cohort of patients ($n = 13$) 3 months after initiation of TKI therapy from plasma. $IFN\gamma$ was below detection limit for most patients before treatment but was detected in several of the patients after TKI therapy initiation and the increase of $IFN\gamma$ was significant (Fig. 5A; $P = 0.0273$). Likewise, MIG was increased PTI (Fig. 5B; $P = 0.0015$), whereas IP10 was stable (Fig. 5C). To investigate whether high MIG and $IFN\gamma$ levels PTI are good markers for ongoing lymphocyte activity, we correlated the MIG and $IFN\gamma$ levels to the presence of experienced T cells and NK

cells in our samples. Interestingly, high levels of MIG and $IFN\gamma$ PTI correlated to high levels of experienced T cells in patients before treatment (Fig. 5D and E; $P = 0.0034$ and $P = 0.0485$, respectively) and they tended to be correlated to the level of experienced T cells 1 month PTI (Fig. 5F and G). However, NK cells did not correlate to the presence of experienced T cells or to the molecules $IFN\gamma$ and MIG (Fig. 5H-J).

Discussion

TKIs such as sunitinib decrease the level of myeloid suppressor cells in cancer and this may lead to enhanced antitumor immune responses. Therefore, TKIs may be of great interest to combine with active immunotherapy to increase efficacy. Indeed, sunitinib increased the efficacy of peptide-pulsed dendritic cells in a murine B16.OVA model (24). Other TKIs such as imatinib or dasatinib may share the same mechanisms to reduce suppressive cells and may thus be interesting candidates for preconditioning regimens. Both imatinib and dasatinib have been used extensively in CML and are well tolerated for extended periods of time. Moreover, they have been connected to immunologic actions. For example, expansion of a population of large granular lymphocytes of T- or NK cell origin in patients treated with dasatinib has been described (19). Expanded T cells were of both α/β and γ/δ type (25). This lymphocyte expansion was associated with good treatment responses, but also with side effects like pleuritis and colitis (19, 25). The cause of lymphocyte expansion remains unknown. Both a rapid decrease of tumor cells, and thereby a decrease in

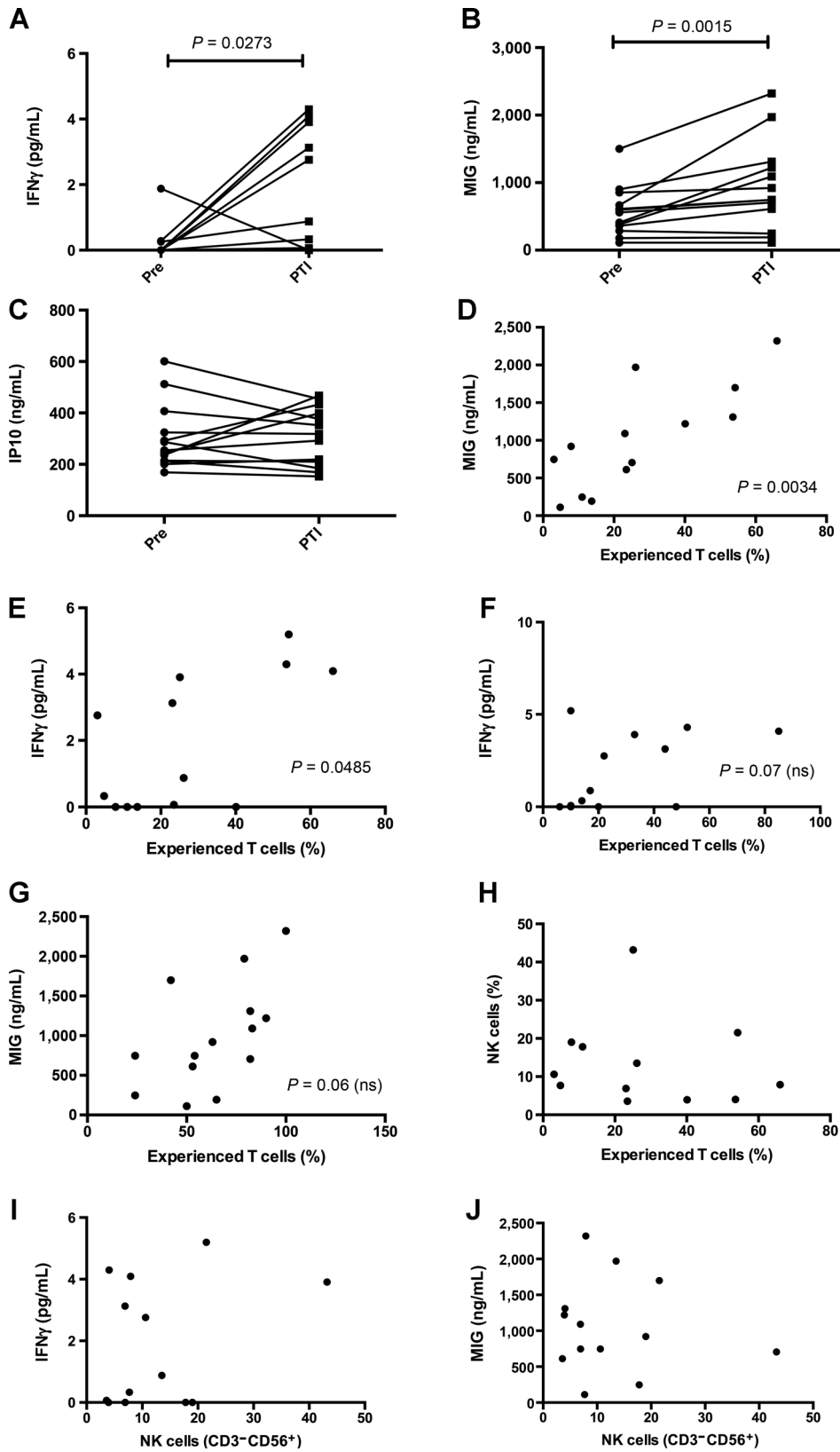


Figure 5.

NK- and T-cell-associated cytokines in CML patients. Plasma from CML patients ($n = 13$) was analyzed before and at 3 months PTI to determine the level of IFN γ (A), MIG (B), and IP10 (C). IFN γ was evaluated using an ultrasensitive 9-plex kit from MesoScale while MIG and IP10 were evaluated using a custom Myriad RBM kit. Statistical significances were calculated using Wilcoxon test for paired samples. MIG concentration PTI was correlated to experienced T cells pre and PTI (D and G) and IFN γ concentration PTI was correlated to experienced T cells pre and PTI (E and F). NK cell levels PTI were then correlated to experienced T-cell levels PTI (H), to IFN γ (I), and to MIG (J). Significant differences were calculated using Spearman correlation test for nonparametric samples. P values < 0.05 were considered significant.

potentially immunosuppressive cells, as the tumor cell per se may be immunosuppressive, as well as off-target effects on other tyrosine kinases present in immune cells may serve as explanations for the lymphocyte expansion. In a recent work, it was further demonstrated that dasatinib induced granzyme B in the expanded cells, demonstrating their ongoing activity (26). We have demonstrated previously that sunitinib upregulated CXCL10 and CXCL11 in tumor vessels which was accompanied by up to 18-fold increased infiltration of T cells in a melanoma model (27). Likewise, dasatinib was shown to enhance the effect of immunotherapy in melanoma partly by upregulating CXCL9, 10, and 11, as well as reducing the presence of both Tregs and MDSCs (28). The exact mechanisms have been difficult to dissect as PBMCs including T cells lose viability or reduce their proliferation upon *in vitro* culture with TKIs such as dasatinib (29, 30). It is likely that the continuous dose of TKIs in culture media has a negative effect on the immune cells that may not be seen in patients, as the dose is not constant. Furthermore, TKIs may affect other cell types *in vivo* that give secondary immune activating effects that may override possible negative effects of TKIs. For example, inhibiting the MDSCs may release expansion of lymphocytes and the overall TKI effect may be stimulatory. There may also be other not yet defined factors *in vivo* that protect the lymphocytes from the toxic effects of TKIs noted *in vitro*. In the present work, we therefore investigated the *in vivo* effect of imatinib and dasatinib on the immune system in patients with CML.

We have previously demonstrated that CML patients have an increased level of myeloid suppressor cells defined as CD11b⁺CD14⁻CD33⁺ cells. This phenotype was seen on both CD34⁺ and CD34⁻ cells in the peripheral blood indicating that not only healthy myeloid cells (CD34⁻) but also a proportion of the leukemic myeloid cells (CD34⁺bcr/abl⁺) may have a suppressor cell phenotype (21). In the current study, we demonstrated that both imatinib and dasatinib treatment efficiently decreased the suppressive peripheral CD11b⁺CD14⁻CD33⁺ myeloid cells. Simultaneously, suppressive molecules connected to myeloid cells such as Arg1 and MPO were significantly decreased. Before TKI therapy, the patients had reduced level of CD40 on their immature myeloid cells but, interestingly, the remaining CD11b⁺CD14⁻CD33⁺ myeloid cells upregulated CD40 on their surface after treatment initiation indicating that they may differentiate or even mature. CD40/CD40L signaling has shown to improve the capacity of myeloid suppressor cells to induce Tregs (31). There is a study demonstrating that stimulation of myeloid suppressor cells with soluble monomeric CD40L resulted in an increased capacity to suppress T-cell proliferation (23) as opposed to the induction of Th1 immunity noted when dendritic cells are stimulated via CD40L (32). In our patient cohort, there was no difference in sCD40L levels. However, the level of Tregs was significantly increased while its associated inhibitory molecule, IL10, was decreased in most patients even if the overall presence of IL10 in plasma was very low and the biologic role of these levels are not determined. We cannot exclude that the increase in FoxP3⁺ cells reflected effector T cells that transiently express FoxP3 even if we gated for the CD127⁻ population. In a study by Balachandran and colleagues, imatinib was shown to decrease Tregs in an animal model (33), which further supported that the increase of FoxP3⁺ cells in our cohort post TKI may not reflect true Tregs. The level of Tregs in CML patients before TKI therapy was not increased compared with healthy donors and, hence, the effect

on Tregs after initiating TKI may give different results in another type of cancer. For example, in patients with gastrointestinal stromal tumors (GIST), Treg levels were decreased after initiation of imatinib therapy (34). However, the patients had received imatinib during a median of 11.7 months, which is longer than our patients. In another study on GIST, imatinib was shown to increase M2 macrophages and thereby increasing IL10 (35), in our patients IL10 is decreased. Nevertheless, GIST patients have aC-KIT exon 11 mutation that may explain discrepancies to the effect of imatinib in CML. Alternatively, the effect of TKIs may be depending on the ongoing immune profile at the start of treatment. More studies to confirm the role of TKIs in different patient groups are clearly warranted. Upregulation of CD40 was also noted on other myeloid cells such as monocytes in our study. CD40 is an important receptor on myeloid cells and upon ligation they commonly mature and increase their expression of costimulatory molecules and inflammatory cytokines including IL12 which is important for activation of Th1 type of immune responses as mentioned above (32). In the CML patients, IL12 was indeed increased after TKI therapy initiation. Hence, the upregulation of CD40 on myeloid cell populations in treated patients may be of immunologic importance to release antitumor immune responses. This connection, however, needs to be experimentally confirmed.

We next investigated the presence of NK cells and T cells after TKI therapy. We confirmed that NK cells were expanded in some of the patients during TKI treatment and that there was no significant difference between imatinib or dasatinib therapy in our cohorts. However, the increase was significant after one month of TKI treatment and thereafter the number of NK cells was reduced to normal levels in most patients. Similarly, effector and memory T cells were significantly increased at 1 month after TKI therapy initiation in some patients but at the 6 months sampling, the level was normalized. Hence, there may be an initial stimulatory effect on the immune system, as the numbers of experienced T cells and NK cells increases, likely because of the reduction of suppressive factors. Nevertheless, this effect is lost with time, which may depend on the lack of incitements of immune activation, as the tumor burden is decreased to undetectable levels in most patients. Most patients that had initial high levels of experienced T cells remained high during TKI therapy. The ratio of T memory cells to naïve T cells was previously shown to be higher in patients that remained in MMR after treatment discontinuation compared with both control subjects and to patients that relapsed after therapy discontinuation (36). Correspondingly, in our study, the level of naïve T cells was reduced in favor of effector and memory T cells. Moreover, IFN γ was detected in patient plasma in some of the patients during TKI therapy indicating active NK or T-cell responses. IFN γ stimulates the production of MIG (37) and this monokine was also increased after initiation of TKI therapy. However, IP10 that is also induced by IFN γ was only increased in some patients in our cohort. As the patients with high T-cell numbers did not simultaneously have high NK cell numbers, it is possible that different patients respond to TKI therapy either with T cells or NK cells dependent on factors yet unknown.

In conclusion, the results in our study demonstrate that both imatinib and dasatinib can reduce immune escape mechanisms by decreasing the number of myeloid suppressor cells and inhibitory cytokines Arg1, MPO, and IL10. Moreover, upregulation of CD40 and IL12 production indicate ongoing adaptive immunity.

NK cells or T cells expand during the first month of treatment and IFN γ and MIG could be detected in plasma during TKI therapy. Even if the most important mechanism of imatinib and dasatinib therapy in CML is the direct effect on the tumor cells by inhibiting the bcr/abl kinase, the long-term effect may also be dependent on activated antitumor immune responses. Moreover, because of the potent reduction of immune escape, at the same time activating lymphocytes, and the relatively mild adverse events due to imatinib or dasatinib, these TKIs may be interesting options for preconditioning cancer patients before immunotherapy. For example, combining TKI with T-cell therapy may allow for a better *in vivo* persistence of the infused T cells. Furthermore, active immunotherapy such as peptide vaccinations may be boosted by TKIs due to the reduction of suppressive factors.

Disclosure of Potential Conflicts of Interest

H. Hjorth-Hansen reports receiving commercial research grants from Bristol-Myers Squibb, Merck, and Novartis, and is a consultant/advisory board member of Bristol-Myers Squibb and Novartis. J. Richter reports receiving commercial research grants from Bristol-Myers Squibb, Merck, and Novartis and received speakers bureau honoraria from Novartis and Bristol-Myers Squibb. S. Mustjoki reports receiving commercial research grants from Pfizer, Novartis, Bristol-Myers Squibb, and Merck; received speakers bureau honoraria from Bristol-Myers Squibb and Novartis; and is a consultant/advisory board member for Bristol-Myers Squibb and Novartis. A. Loskog reports receiving other commercial research support from Olink Biosciences AB and is a consultant/advisory board member for NEXTTOBE AB. U. Olsson-Stromberg reports receiving a commercial research grants from Bristol-Myers Squibb, Novartis, and Merck. No potential conflicts of interest were disclosed by the other authors.

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