Normal intrinsic Th1/Th2 balance in patients with chronic phase chronic myeloid leukemia not treated with interferon-α or imatinib

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Background and Objectives. CD4+ T helper cells are an integral part of effective immune responses against various malignancies; however in tumor-bearing patients they are frequently functionally unresponsive. T helper cells of patients with chronic myeloid leukemia (CML), analyzed as part of mononuclear cell fractions, show a loss of signaling molecules, a compromised Th1 cytokine production and a shift towards a non-productive Th2 state. The underlying mechanism is unknown and may involve intrinsic T cell defects as well as indirect effects mediated by leukemia or antigen-presenting cells. The purpose of the present study was to analyze the intrinsic cytokine-producing capacity of purified CML T helper cells in the absence of other cell types.

Design and Methods. *Untouched* CD4+ T cells with a purity of more than 90% were isolated from 10 patients with Ph+ chronic phase CML on maintenance treatment with hydroxyurea. The cells were isolated by density gradient centrifugation followed by immunomagnetic depletion of leukemia and accessory cells. The *ex vivo* cytokine-producing capacity of CML T helper cells in response to polyclonal stimulation with anti-CD3 and anti-CD28 was then compared to that of cells purified from matched healthy volunteers.

Results. T helper cells purified from CML patients produced comparable amounts of the Th1 cytokines interleukin (IL)-2 and interferon (IFN)-γ as cells purified from healthy volunteers. Likewise, no difference between CML and control T helper cells was found with respect to the Th2 cytokines, IL-4 and IL-13, as well as the immunomodulatory cytokine, IL-10.

Interpretation and Conclusions. In the absence of leukemia and accessory cells, the intrinsic cytokine-producing capacity of CML T helper cells is normal. A Th2 shift was not detected, and the predominant presence of an IL-10-producing, immunosuppressive T helper cell subset could be excluded.

Key words: chronic myeloid leukemia, T-helper cells, Th1, Th2, IL-10.

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Chronic myeloid leukemia (CML) is a myeloproliferative disease, in which malignant transformation of a pluripotent hematopoietic stem cell leads to clonal proliferation of myeloid cells at various stages of differentiation. Transformation is due to the constitutive tyrosine kinase activity of the fusion protein bcr/abl, expressed as a consequence of the t(9;22) chromosomal translocation (Philadelphia chromosome, Ph).[1] The natural course of CML can be divided into three stages: a relatively long and indolent chronic phase is followed by a short accelerated phase, in which a progressively increasing number of immature cells can be found, and which, if untreated, invariably leads to blast crisis and death. Treatment options include chemotherapy, interferon-α (IFN-α) and the tyrosine kinase inhibitor imatinib (Gleevec); however, to date the only proven curative treatment modality for CML remains allogeneic stem cell transplantation.[2,3]

There is considerable evidence that immune mechanisms, and in particular T cells, have a major impact on control of the disease in CML. For example, depletion of T cells in allogeneic stem cell grafts results in an increased probability of relapse.[4] Likewise, relapse after allogeneic stem cell transplantation can be cured by donor lymphocyte infusions (DLI).[5] Antigen-specific, leukemia-reactive T cells can be found in CML patients after successful treatment with IFN-α or allogeneic stem cell transplantation and likely contribute to the maintenance of their remission status.[6] The importance of immune surveillance mechanisms for the control of the malignant clone is further emphasized by the fact that bcr/abl transcripts can be detected at low levels in healthy individuals as well as in CML patients with long-term remissions,[7,8] and by the observation that expression of certain HLA types is associated with a reduced risk of developing CML.[9]

CD4+ T helper cells are being increasingly recognized as a central component of effective immune responses against malignant tumors,[10,11] but their role in CML is less clear. Their immunotherapeutic relevance is suggested by the fact that they are an integral part of donor lymphocyte infusions,[12] and that antigen-specific, MHC class II-restricted cytotoxic CD4+ T-cell clones can be generated *in vitro*.[13,14] However, as described for patients bearing solid tumors,[15] CD4+ T cells of CML patients appear to be dysfunctional. They do not respond to stimulation with bcr/abl-derived peptides[16,17] and, compared to T cells of healthy individuals, show decreased expression of the T-cell receptor (TCR)-ζ chain,[18,19] as well as a reduced capacity to

Table 1. Patients' characteristics.

Patient no.	Age (years)	Sex	Disease stage	Time from diagnosis (months)	Treatment	WBC count ($\times 10^9$/L)	Lymphocyte count ($\times 10^9$/L)
1	26	F	CP in PHR	5	Hydroxyurea	12.8	2.3
2	27	F	CP in PHR	3	Hydroxyurea	19.6	3.2
3	65	M	CP in PHR	20	Hydroxyurea	5.8	1.5
4	74	F	CP in PHR	45	Hydroxyurea	21.3	2.8
5	65	M	CP in PHR	6	Hydroxyurea	1.7	0.8
6	52	M	CP in PHR	2	Hydroxyurea	5.8	1.0
7	71	M	CP in CHR	60	Hydroxyurea	3.2	0.9
8	43	M	CP in CHR	58	Hydroxyurea, Mini-ICE*	5.0	1.7
9	51	M	CP in CHR	59	Hydroxyurea, IFN-α, Bu, Cy°	1.8	0.8
10	31	M	CP in PHR	2	Hydroxyurea	12.8	2.4

*CP: chronic phase; PHR/CHR, partial/complete hematologic remission; *Mini-ICE (idarubicin, cytarabine, etoposide) treatment was administered 2 years prior to the experiment; °IFN-α, busulfan and cytarabine treatment was terminated 7 months prior to the experiment.*

secrete the Th1 cytokines IFN-γ and interleukin (IL)-2.[20-22] In a recent study, T helper cells of CML patients, stimulated *ex vivo* with phorbol ester and calcium ionophore, were shown to produce less IFN-γ and IL-2, but more IL-10 compared to control cells, suggesting a systemic Th2 shift in these patients.[22] Notably, whereas all patients in this study have been treated with IFN-α, only those patients who responded to this treatment by achieving a complete cytogenetic remission, recovered their normal cytokine profile.

Because of the low number of T cells in chronic phase CML patients, the isolation of sufficiently pure $CD4^+$ T-cell populations is difficult. In the studies mentioned above, intrinsic T-cell defects could not be separated from indirect effects on T helper cell function because of the massive presence of contaminating leukemia cells in the mononuclear cell fractions used. The purpose of the present study was to approach the mechanism of T helper cell dysfunction in CML by analyzing their intrinsic cytokine-producing capacity in the absence of leukemia and antigen-presenting cells. We employed an immunomagnetic depletion procedure to isolate untouched $CD4^+$ T cells of CML patients to a purity of more than 90%, and compared their cytokine profile in response to polyclonal stimulation with anti-CD3 and anti-CD28 to that of cells isolated from matched healthy donors.

Design and Methods

Patients

After obtaining informed consent, peripheral blood was collected from CML patients, and at the same time from healthy volunteers, closely matched for age and sex. All CML patients were in Ph^+ chronic phase, received hydroxyurea as a maintenance treatment, and, according to published response criteria,[23] were in complete or partial hematologic remission (Table 1). With the exception of patient #9 (who had terminated treatment seven months before the experiment was performed), none of the patients had been pretreated with IFN-α for the following reasons: intended allogeneic stem cell transplantation (patients #1, 2, 5, 6, 10), age/performance status (patients #4, 7), and treatment by an external institution prior to referral to our department (patients #3, 8). None of the patients had been pretreated with imatinib.

Isolation of $CD4^+$ T cells

To avoid potential bias by day-to-day experimental variation, samples from patients and matched controls were drawn and processed in pairs and stimulated side-by-side under identical conditions. Peripheral blood was drawn into heparin-containing vacutainer tubes, diluted 1:1 with phosphate-buffered saline without calcium and magnesium (Gibco BRL, Karlsruhe, Germany) and separated over a 1.077 g/mL Ficoll-Hypaque density gradient (Lymphoprep™, Nycomed, Oslo, Norway) by centrifugation for 20 min at 800× g at room temperature. Peripheral blood mononuclear cells (PBMCs) were collected, washed, resuspended in RPMI 1640 containing 10% fetal calf serum (Gibco) and counted. Untouched $CD4^+$ T helper cells at a purity of at least 90% were negatively isolated by depletion of leukemia and accessory cells using the MACS $CD4^+$ T-cell isolation kit (Miltenyi,

Bergisch-Gladbach, Germany) according to the instructions of the manufacturer, with the exception that the antibody cocktail of the kit was supplemented with additional anti-CD33 and anti-CD15 microbeads (Miltenyi) to optimize depletion of the leukemia cells. The resulting cellular population was washed, counted and analyzed for purity by fluorescence activated cell sorting (FACS) using fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labeled monoclonal antibodies against human isotype and CD3, CD4, CD19, CD14, CD15, and CD33 antigens. In one patient (patient #10) a second round of depletion with anti-CD15 microbeads was performed, because FACS analysis after one round showed contamination of the $CD4^+$ T-cell population with residual $CD15^+$ myeloid cells.

T-cell stimulation

$CD4^+$ T cells were stimulated in 24-well plates (BD Falcon, Biosciences Discovery Labware, Bedford, MA, USA) at a concentration of 1×10^6 cells/mL with plate-bound monoclonal antibodies against human CD3 (200 ng/mL) and human CD28 (1 μg/mL) antigens (BD Biosciences Pharmingen, San Diego, CA, USA).

ELISA assays

Supernatants were collected 48 hours after stimulation, centrifuged, and tested for cytokine content. Levels of IL-10 and IL-2 were determined using commercial ELISA kits (Quantikine, R&D Systems, Wiesbaden, Germany) according to the instructions of the manufacturer. Levels of IFN-γ, IL-4 and IL-13 were determined using monoclonal antibody pairs (coating antibody and biotinylated detecting antibody) specific for human IFN-γ, IL-4 and IL-13 (BD Biosciences Pharmingen, San Diego, CA, USA) and standard protocols. Briefly, 96-well plates (BD Falcon, Biosciences Discovery Labware) were coated overnight at 4°C with the coating antibody (1 μg/mL) diluted in phosphate-buffered saline (PBS), washed twice with PBS containing 0.01% Triton X-100 (Sigma-Aldrich, Deisenhofen, Germany) and blocked for 2 hours at room temperature (RT) with PBS containing 2% bovine serum albumin (Sigma) and 0.002% sodium azide (Sigma). After washing the plates twice, serial dilutions of supernatants and cytokine standards were added and incubated overnight at 4°C. The plates were then washed four times, after which the biotinylated detecting antibody (1 μg/mL) was added and incubated for 45 minutes at RT. With intermediate washing of the plates (six times each), horseradish peroxidase-conjugated streptavidin (Zytomed, Berlin, Germany) and tetramethylbenzidine substrate (Dako, Hamburg, Germany) were sequentially added. The resulting photometric reaction was finally stopped by adding 0.18 M sulfuric acid, and the absorbance read at 450 nm. The sensitivity of the assays was 7, 15, 4, 30 and 240 pg/mL for IL-2, IL-4, IL-10, IL-13 and IFN-γ, respectively.

Statistical analysis

Results are expressed as mean ± standard deviation. Statistical differences between patients and healthy volunteers were determined for each cytokine by the two-tailed Mann-Whitney test.

Results

To analyze the intrinsic cytokine-producing capacity and Th1/Th2 balance of CML T cells independently of potential confounding effects of IFN-α treatment, for our experiments we selected a homogeneous group of patients with Ph^+ chronic phase CML on maintenance treatment with hydroxyurea who, except for one patient (for details see the *Design and Methods* section), had not been pretreated with IFN-α (Table 1). As expected, the mononuclear cell fractions of these patients after Ficoll-Hypaque density gradient centrifugation still contained a considerable number of contaminating leukemia cells (Figure 1, left panels). These cells, via the production of IL-10[24] or other regulatory mediators, have been postulated to deviate T helper cell cytokine expression from a productive Th1 to a non-productive Th2 phenotype.[22] Prior to the stimulation of the T cells, we therefore depleted leukemia as well as accessory cells using a cocktail of immunomagnetic beads, resulting in a population of $CD4^+$ T helper cells with a purity of at least 90% (Figure 1, right panels). To avoid potential bias from day-to-day experimental variation, cells of a given patient and the patient's matched control were always isolated and processed in pairs, allowing direct side-by-side comparison.

Cytokine production by $CD4^+$ T helper cells from CML patients (*CML T helper cells*) and matched healthy volunteers (*control T helper cells*) in response to polyclonal stimulation with anti-CD3 and anti-CD28 antibodies is graphically depicted in Figures 2–4. Cytokine production by both CML and control T helper cells was characterized by considerable interindividual variability, resulting in a large range of values for all cytokines tested (Figures 2-4). In a previous report, when T cells were stimulated within mononuclear cell fractions, Th1 cytokine production by chronic phase CML T helper cells was lower that that by control cells, whereas production of the Th2 cytokine IL-10 was enhanced.[22] In contrast, purified T helper cells isolated from CML patients and healthy volunteers did not differ in their capacity to produce Th1 cytokines, as they secreted similar amounts of IL-2 (CML, mean 60±86 ng/mL, range 2 to 246 ng/mL,

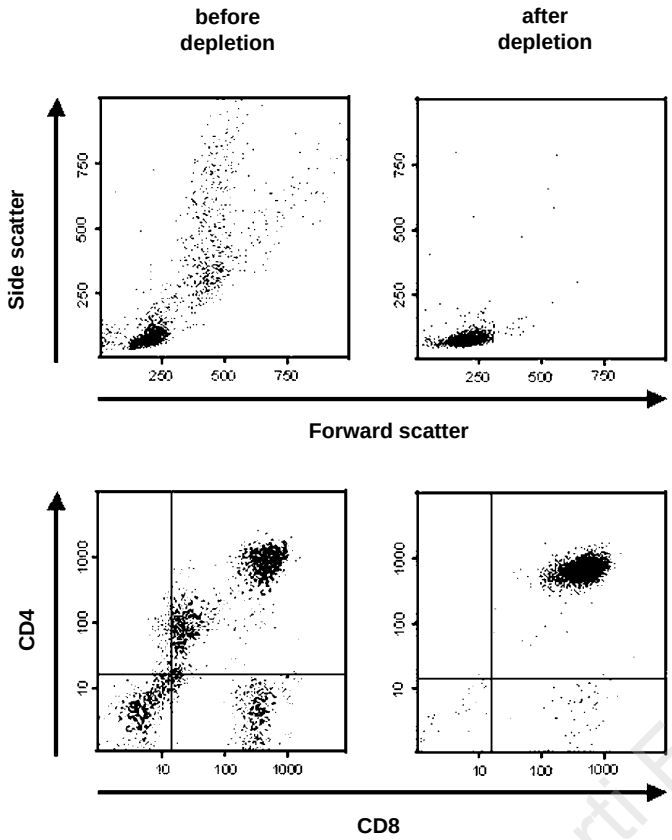

Figure 1. Purification of CD4⁺ T helper cells by immunomagnetic depletion of leukemia and accessory cells from mononuclear cell fractions of Ph⁺ chronic phase CML patients. The figure shows FACS scans (top, forward and sideward scatter; bottom, CD3 and CD4 fluorescence) of a representative patient (patient 2). Left, mononuclear cell fraction after Ficoll-Hypaque density gradient centrifugation, before depletion with immunomagnetic beads; right, the same cells after depletion. See the *Design and Methods* section for details.

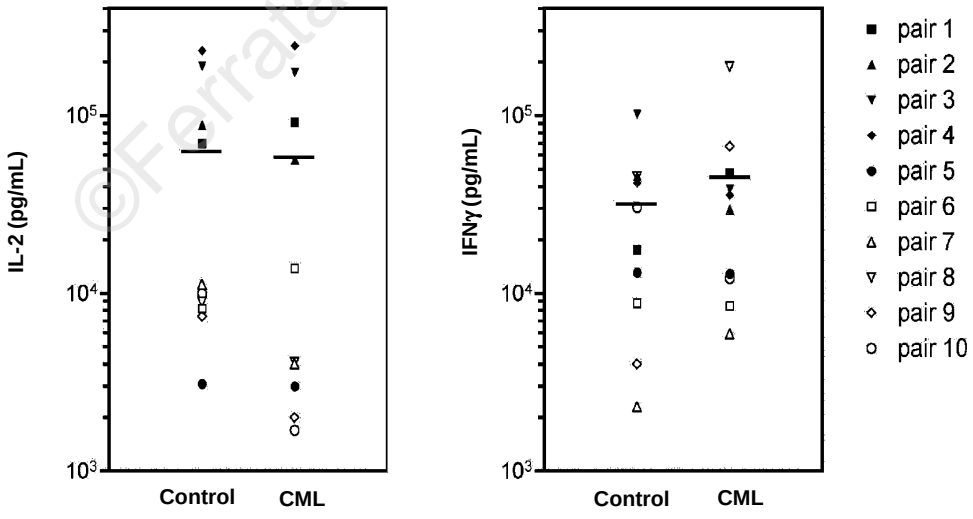

Figure 2. Th1 cytokine production by purified T helper cells of CML patients and healthy controls. CD4⁺ T cells were negatively purified by density gradient centrifugation and immunomagnetic depletion of leukemia and accessory cells, and stimulated as described in *Design and Methods*. Supernatants were tested for IL-2 (left panel) and IFN-γ (right panel) content by ELISA. Mean values for each population and cytokine are indicated with a horizontal line. CML patients versus matched controls, p = 0.48 (IL-2) and 0.74 (IFN-γ). Note the logarithmic scale of the y-axis.

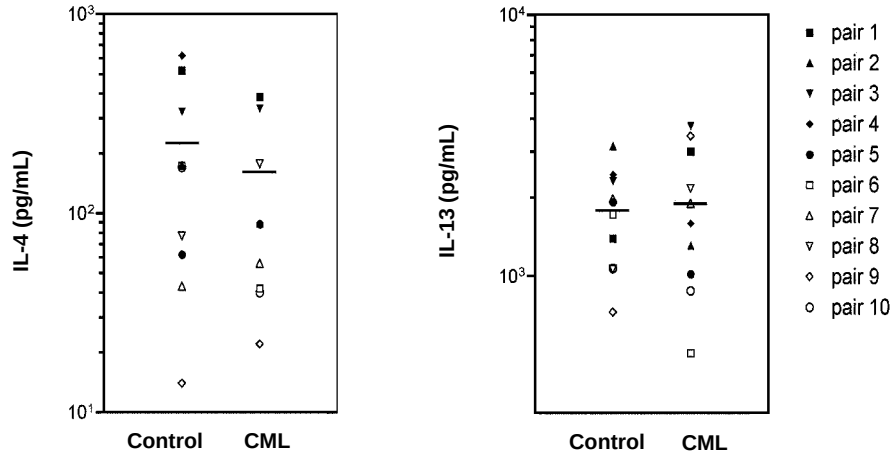

Figure 3. Th2 cytokine production by purified T helper cells of CML patients and healthy controls. CD4+ T cells were negatively purified by density gradient centrifugation and immunomagnetic depletion of leukemia and accessory cells, and stimulated as described in Design and Methods. Supernatants were tested for IL-4 (left panel) and IL-13 (right panel) content by ELISA. Mean values for each population and cytokine are indicated with a horizontal line. CML patients versus matched controls, p = 0.68 (IL-4) and 1.00 (IL-13). Note the logarithmic scale of the y-axis.

versus control, 63±84 ng/mL, range 3 to 232 ng/mL; p = 0.48) and IFN-γ (CML, mean 45±54 ng/mL, range 6 to 189 ng/ml, versus control, 31±30 ng/mL, range 2 to 102 ng/ml; p = 0.74) upon stimulation (Figure 2).

To assess Th2 cytokine production in CML and control T helper cells, we first tested the cytokines IL-4 and IL-13, which are hallmarks of Th2 cells.[25] In contrast to the results obtained in mononuclear cell fractions,[22] Th2 cytokine secretion by purified CML T helper cells was not enhanced compared to secretion by control cells (Figure 3). IL-4 production of CML T helper cells ranged from 22 to 386 pg/mL (mean 162±149 pg/mL) and was therefore not notably different from the amounts secreted by control cells (mean 218±207 pg/mL; range 14 to 619 pg/mL; p = 0.68). Likewise, levels of IL-13 found in supernatants of CML T helper cells (mean 1955±1115 pg/mL, range 508 to 3748 pg/mL) were comparable to those detected in supernatants of control cells (mean 1778±740 pg/mL, range 729 to 3149 pg/mL; p = 1.00). IL-10 is a cytokine produced by Th2 cells,[25,26] but it is also characteristic of a distinct, recently described subpopulation of T helper cells with immunosuppressive properties.[27] The elevated IL-10 levels previously reported for *ex vivo*-stimulated CML T helper cells[22] may therefore indicate a true Th2 shift; alternatively, the elevated levels may indicate the predominant presence of this immunoregulatory subpopulation of T helper cells in CML patients. However, as shown in Figure 4, IL-10 production by purified CML T helper cells

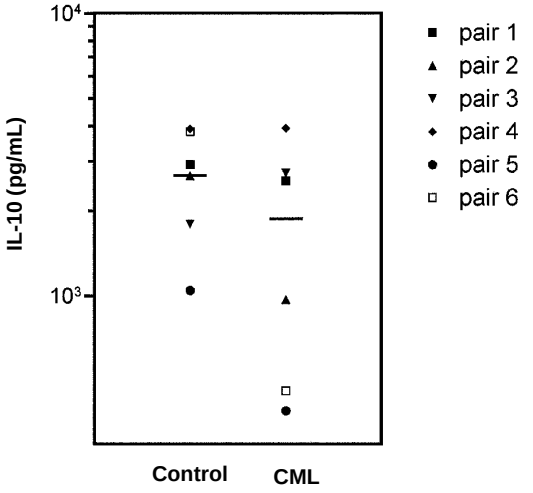

Figure 4. IL-10 production by purified T helper cells of CML patients and healthy controls. CD4+ T cells were negatively purified by density gradient centrifugation and immunomagnetic depletion of leukemia and accessory cells, and stimulated as described in Design and Methods. Supernatants were tested for IL-10 content by ELISA. Mean values for each population are indicated with a horizontal line. CML patients versus matched controls, p = 0.31. Note the logarithmic scale of the y-axis and that because of limitation of the sample material only six out of ten patients could be evaluated for this cytokine.

was not enhanced compared to that by control cells (CML, mean 1838±1440 pg/mL, range 393 to 3912 pg/mL, versus control, mean 2692±1124 pg/mL, range 1049 to 3908 pg/mL; $p = 0.31$), thereby excluding the latter possibility.

Collectively, the results indicate that the intrinsic cytokine-producing capacity of CML T helper cells is essentially normal and not characterized by a Th2 shift or the predominance of T-cell subsets with immunosuppressive properties.

Discussion

The central role of $CD4^+$ T helper cells in the immunological control of malignant cells has recently been demonstrated in a number of solid tumor models.[10,11] T helper cells are important for the priming of cytotoxic T cells, because they secrete cytokines such as IL-2 and activate antigen-presenting cells directly through interaction with CD40-CD40L receptors. Additionally, they have their own effector functions, partly attributed to the production of the Th1 cytokine IFN-γ which enhances MHC class I and II expression, has antiproliferative properties, and activates tumoricidal macrophages.[28] However, T helper cells in tumor-bearing patients are frequently functionally unresponsive.[15] This phenomenon is initially restricted to local, tumor-antigen-specific T cells, but at more advanced stages of tumor growth extends to a more global, non-specific systemic T-cell dysfunction. T-cell anergy in these patients is characterized by a loss of signaling molecules (e.g. the TCR-ζ chain and downstream tyrosine kinases), a reduced capacity to produce Th1 cytokines, and a shift towards a non-productive Th2 state, and imposes a significant barrier to tumor vaccination or other immunotherapeutic strategies.

A comparable dysfunction has been described for T helper cells of patients with CML stimulated *ex vivo* as part of mononuclear cell fractions.[16-22] The underlying mechanism is unclear and may involve intrinsic T-cell defects as well as indirect effects mediated by leukemia or antigen-presenting cells present in these fractions (compare Figure 1). The purpose of the present study was to assess the intrinsic function of CML T helper cells directly by using highly purified $CD4^+$ populations. We observed considerable variability in cytokine production in both the patients and the control volunteers, which may be partly due to the extensive *ex vivo* manipulation of the cells and/or a fluctuations of reagent activity during the study.

However, these potentially confounding factors were minimized by the design of the study, in which the cells from a patient and a sex- and age-matched control were always processed side-by-side and under strictly identical conditions. Consequently, the variability of cytokine production between matched patients and controls was much lower than the day-to-day variability between the experiments (Figures 2-4).

In contrast to above-mentioned reports, our results demonstrate clearly that the cytokine-producing capacity of T helper cells purified from Ph^+ chronic phase CML patients is not different from that of healthy donors, as measured by the secretion of well-established Th1 and Th2 cytokines.[25] Our study thus rules out the presence of intrinsic T-cell defects, but was not designed to address the role of leukemia or accessory cells on CML T helper cell function. Furthermore, due to the distinct experimental settings used, our results cannot be readily applied on the findings of the above-mentioned studies. Therefore, at present, potential indirect mechanisms remain speculative and warrant further investigation. Several aspects must be considered. CML is a systemic disease in which all peripheral blood T cells are in intimate contact with the tumor cells.

The presence of a non-specific anergizing soluble factor secreted by the leukemia cells would, therefore, not only explain the reported dysfunction of CML lymphocytes, but also the fact that anergy is not restricted to antigen-specific T helper cells but is also apparent after polyclonal stimulation of T cells[22] as well as NK cells.[29] Indeed, CML cells produce IL-10,[24] a cytokine which is known to suppress cytokine expression by T cells.[30] A second (not mutually exclusive) possibility is that transformed antigen-presenting cells (APCs) of CML patients may adversely affect T helper cell function, for example by inducing an unproductive Th2 bias.[22] A rationale for the assumption that APCs are dysfunctional in CML is provided by the observation that B cells of CML patients lack ICSBP (interferon consensus sequence binding factor),[31] a transcription factor which is critical for IL-12 production and therefore Th1 differentiation.[32] Other mechanisms than those discussed are conceivable, and comparisons of T-cell function side-by-side in the presence and absence of leukemia cells, APCs or other cell types will be required to dissect the relative contribution of these cells to the induction of T helper cell anergy in CML patients.

Our finding of normal intrinsic T helper cell function in CML patients may have therapeutic implications. Current treatment modalities for CML include chemotherapy, IFN-α, the recently introduced tyrosine kinase inhibitor imatinib (Gleevec) and allogeneic bone marrow transplantation.[2,3] Among these, chemotherapy is only palliative, IFN-α is effective in only a small portion (about 10-20%) of patients, and Gleevec is promising but still unpredictable in its long-term effects, since the development of resistance has been described.[3] Hence, to

date the only proven curative treatment modality remains allogeneic stem cell transplantation, which is, however, associated with considerable treatment-related morbidity and mortality, and, because of advanced age or lack of a suitable donor, is only feasible in about 30% of patients.[3] For the majority of patients, therefore, the development of immune therapeutic strategies is desirable. Efforts to generate autologous, leukemia-reactive cytotoxic $CD4^+$ and $CD8^+$ T cells, either by *in vivo* vaccination or *ex vivo* priming, are promising but depend on the reversal of the above-mentioned state of T-cell anergy. The current study supports the feasibility of this concept, because it demonstrates that autologous T helper cells of CML patients are principally functional. Consequently, future efforts should focus on the identification of cell types and mechanisms responsible for the induction and/or maintenance of T-cell anergy in CML patients. Specific interference with these mechanisms may eventually facilitate immune therapeutic approaches for CML patients by restoring the cytokine-producing capacity of their autologous T cells.

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Pre-publication Report & Outcomes of Peer Review

Contributions

All authors made substantial contributions to the conception and design of this study, the interpretation of the data and the drafting and/or critical revision of the manuscript. AK and IH were additionally responsible for the physical realization of the experiments and data acquisition. The authors would like to thank Drs. M. Bornhäuser, U. Schuler and C. Thiede for helpful discussions and critical reading of the manuscript.

Disclosures

Conflict of interest: none.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Dr. Francesco Dazzi, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Dazzi and the Editors. Manuscript received March 24, 2003; accepted May 28, 2003.

In the following paragraphs, Dr. Dazzi summarizes the peer-review process and its outcomes.

What is already known on this topic

Anti-leukemia immune responses are of crucial importance in the control of chronic myeloid leukemia (CML), probably irrespective of the type of treatment used. There is evidence that CML patients exhibit abnormal T-cell function but it is not clear whether this is the cause or the effect of the hemopoietic malignancy.

What this study adds

This paper shows that CD4 T cells, purified from 10 CML patients, display a normal Th1 and Th2 cytokine pattern when leukemic and accessory cells are removed from the cell suspension. This suggests that the documented immunologic abnormalities might be the consequences of CML.

Caveats

The only limitation is that the analysis has been confined to the use of a polyclonal stimulus rather than to selective Th1 or Th2 stimulation.