Expression patterns of chemokine receptors on circulating T cells from myelodysplastic syndrome patients

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Introduction

Chemokines are a group of small (8–14 kDa) cytokines that exert key roles in the regulation of cell migration, proliferation and survival, and that are involved in many biological processes including antigen-presentation and angiogenesis.1,2 Chemokines are divided into four main groups based on their molecular structure and bind to heptahelical G-protein coupled receptors. Diverse stimuli of host or pathogen origin can induce the release of cytokines as well as the expression of chemokine receptors on a wide range of cells, including immunocompetent and hematopoietic cells.3,4 An aberrant chemokine release profile can be observed in many malignancies5–7 as well as in several autoimmune and inflammatory diseases.8

Myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal hematopoietic stem cell diseases. The International Prognostic Scoring System is used for prognostic evaluation of MDS patients, and—based on the percentage of bone marrow blasts, number of cytopenias and karyotype—distinguishes between the four risk groups: low, intermediate-1, intermediate-2 and high.9 Depending on age, the median survival of these patients ranges from 0.3 to 11.8 y. In addition, the WHO classification10 discriminates between different MDS subtypes based on (1) peripheral blood cytopenia, (2) bone marrow dysplasia in one or more of the major myeloid cell lines, (3) the number of blasts in peripheral blood and bone marrow and (4) the appearance of ring sideroblasts. Common features of all MDS subtypes are ineffective hematopoiesis, increased risk of developing acute myeloid leukemia and augmented prevalence of immune deregulation.

The only curative treatment for all types of MDS is allogeneic stem cell transplantation.11 However, abnormalities in the cellular and humoral immune system have been described in MDS patients independent of their subtype, and immunomodulating drugs (e.g., 5-azacitidine and lenalidomide) have therefore been investigated as potential therapies.12–14 Recent studies have also shown that chemokine expression levels are of prognostic value for MDS patients,15,16 yet little is known about the expression pattern of chemokine receptors in MDS. Based on these premises, we wanted to investigate whether T-cell chemokine expression levels differed in healthy individuals as compared with low- and high-risk MDS patients. T cells from MDS patients are usually studied starting from cryopreserved material. Investigating chemokine receptor expression is challenging in this context, since internalization may occur at low temperatures. In the present study, we investigated the chemokine receptor repertoire on fresh peripheral blood lymphocytes from 31 (22 low-risk and 9 high-risk) patients affected by MDS. Chemokine receptor expression was studied in defined T-cell subsets using eight-color flow cytometry. MDS patients exhibited quantitative differences in peripheral lymphocyte subpopulations. In addition, T cells obtained from MDS patients expressed a chemokine receptor pattern suggesting a dominance of mature and activated T cells. This is illustrated by increased levels of CCR3, CCR5, CX3CR1 and/or by a decreased abundance of CCR7 in defined T-cell subsets. The T-cell subset distribution appears to differ between the peripheral blood and the bone marrow of MDS patients, suggesting a preferential recruitment of specific T-cell subsets to the latter compartment. Alteration in chemokine receptor expression can develop over time even in patients that are considered clinically stable. Elevated expression levels of CXCR4 by CD8+ cells were associated with prolonged patient survival and reduced numbers of bone marrow blasts. We conclude that immunological abnormalities in MDS also involve chemokine receptors on different subsets of T cells, and that these changes may have a prognostic value.
work, we characterized the expression pattern of chemokine receptors on fresh T cells obtained from peripheral blood samples of an unselected group of MDS patients, focusing on various T-cell subpopulations.

**Results**

The frequency of total circulating T cells and most T-cell subsets do not differ between MDS patients and healthy individuals. The relative numbers of total T cells and various lymphocyte subsets were determined in the peripheral blood of MDS patients and healthy controls (Table 1). The abundance of total CD3+ T, natural killer (NK) and NKT cells was estimated as the percentage of the total lymphocyte population and did not differ between MDS patients and healthy subjects. The CD4+ or CD8+ T-cell subsets were determined as the percentage of CD3+ cells, and these subsets did not differ between patients and healthy individuals either (Table 1; Fig. 1).

Thereafter, multiple CD4+ and CD8+ T-cell subsets were estimated as the percentage of total CD4+ and CD8+ T cells, respectively. The percentage of CD4+ terminal effector cells was significantly increased in low-risk MDS patients as compared with healthy subjects (p = 0.03), whereas all the other CD4+ and CD8+ T-cell subsets did not differ between our MDS patients and healthy individuals. To conclude, we observed no major differences in the relative abundance of total T cells and various T-cell subsets when comparing our cohort of high- and low-risk MDS patients with healthy people, the only exception being a proportion of CD4+ terminal effector cells in low-risk MDS patients (Fig. 1).

Disease-associated alterations in the expression of chemokine receptors by circulating T cells affect different lymphocyte subsets in high- and low-risk MDS patients. The T-cell expression of various chemokine receptors was examined in high-risk (n = 9) and low-risk (n = 22) MDS patients, as well as in 18 healthy control individuals. The results for total CD3+ T cells and for CD4+ and CD8+ T-cell subsets are summarized in Table 2 and illustrated in detail in Figure 2. Both high- and low-risk MDS patients showed increased levels of circulating CD3+ T cells. However, when the analysis was focused on CD4+ and CD8+ T-cell subsets separately, only low-risk patients were found to exhibit increased levels of CD3+CD8+CCR3+ cells, whereas for high-risk patients only exhibited an increase in CD3+CD4+CCR3+ cells. Thus, an augmented expression of CCR3 was observed in different T-cell subsets in low-risk and high-risk MDS patients.

We also analyzed the expression of eight other chemokine receptors in T cells from MDS patients and healthy subjects. Among high-risk patients, CX3CR1 expression was altered similarly to that of CCR3; that is, the levels of CD3+CX3CR1+ cells were significantly increased, an effect stemming from the increase of CD3+CD4+CX3CR1+ cells in the presence of normal levels of CD3+CD8+CX3CR1+ cells. The CD8+ T-cell subset of high-risk patients also exhibited an increased expression of CCR5 and CXCR4 (Table 2; Fig. 2). On the other hand, low-risk patients showed increased levels CX3CR1+ cells in the CD4+ T-cell compartment whereas the CD8+ subset exhibited decreased levels of both CCR4 and CCR7 (Table 2; Fig. 2). Thus, the expression of several chemokine receptors is altered in T cells from MDS patients.
A subset of low-risk MDS patients exhibit high levels of CD8+ terminal effector cells. There was a wide variation in the frequency of various CD4+ and CD8+ T cell subsets among MDS patients (Table 1). Five low-risk patients exhibited CD8+ terminal effector cell levels exceeding the variation range of healthy individuals. These patients also showed a decreased CD4/CD8 ratio, as well as decreased portions of the CD8+ naïve and central memory subsets, and reduced CD3+CXCR4+ and CD8+CCR7+ cells as compared with other low-risk patients (p < 0.01, p = 0.02, p = 0.03, p = 0.02 and p = 0.01, respectively). There was no significant difference in treatment and clinical parameters between the groups. Thus, a minority of the low-risk patients show an expansion of the terminal effector subset, with chemokine receptor expression in line with normal maturation.

Chemokine receptor expression marginally changes over time in clinically stable patients. We were able to repeat the analyses described above after 12 months for 10 low-risk patients. Eight of these patients experienced stable disease during this period, as judged by clinical criteria and peripheral blood counts, whereas two patients manifested disease progression. Overall, their T-cell subset levels and T-cell chemokine receptor expression pattern did not vary to consistent extents over time. We also compared these parameters focusing on those eight patients that exhibited a clinically stable disease (median time since diagnosis 42 mo; range 24–120 mo) (Fig. 3). In this setting, we observed decreased levels of circulating CD4+CCR4+ cells after one year (p = 0.03) whereas there was a trend for increased CD4+CCR3+ and CD4+CCR5+ cells (p = 0.05). Thus, low-risk MDS patients with clinically stable disease appear to manifest only minor variations in T-cell subset levels and T-cell chemokine receptor expression.

T-cell subset distribution differs between the bone marrow and the peripheral blood in low-risk MDS patients. We obtained bone marrow samples for the analysis of the T-cell populations from five low-risk MDS patients. The frequency of CD3+ T cells...
The chemokine receptor profile of bone marrow T cells differed from that of their circulating counterparts and was characterized by (1) increased levels of CD3+CCR2+ (p = 0.02), CD4+CCR2+ (p = 0.04), CD8+CCR2+ (p < 0.01), CD4+CCR3+ (p = 0.04) cells; and (2) decreased levels of CD3+CCR6+ (p = 0.01), CD4+CCR6+ (p = 0.01), CD3+CXCR3+ (p = 0.04) and CD8+CXCR3+ (p = 0.02) cells.

To summarize: (1) there seems to be a selective recruitment of circulating T cells to the bone marrow compartment in low-risk MDS patients; (2) the bone marrow exhibits an increased frequency of CCR2+ T cells, bearing a chemokine receptor whose expression is increased in T cells from low-risk MDS patients (Table 2); (3) in MDS patients, some circulating T-cell subsets did not differ between the peripheral blood and the bone marrow, but the abundance of various T-cell subsets did so:

- Decreased levels of CD4+ and increased levels of CD8+ T cells were detected in the bone marrow as compared with the peripheral blood (p = 0.02 and p = 0.01). Thus, the CD4+:CD8+ T-cell ratio differs in these two hematopoietic compartments.
- Bone marrow CD4+ central memory cells were decreased and CD4+ terminal effector cells were increased (both p = 0.001), compared with peripheral blood.
- Bone marrow CD8+ naïve and central memory cells were decreased (p = 0.02 and p < 0.01, respectively), compared with peripheral blood.

### Table 2. Expression of chemokine receptors by CD3+, CD4+ and CD8+ lymphocytes from healthy controls and MDS patients

|                | Control                | Low-risk MDS          | High-risk MDS          |
|----------------|------------------------|-----------------------|------------------------|
|                | Median | Range      | Median | Range      | P | Median | Range      | P |
| **CD3+**       |        |            |        |            |    |        |            |    |
| CCR2           | 0.1    | (0.0–1)    | 0.2    | (0.0–7)    | 0.02 | 0.1    | (0–0.2)   | Ns |
| CCR3           | 0.7    | (0.0–1)    | 1.4    | (0.4–4)    | < 0.01 | 1.5    | (0.7–13)  | < 0.01 |
| CCR4           | 20     | (7–53)     | 15     | (3–38)     | Ns | 15     | (10–25)   | Ns |
| CCR5           | 24     | (13–52)    | 28     | (10–51)    | Ns | 32     | (16–46)   | Ns |
| CCR6           | 27     | (8–47)     | 19     | (6–55)     | Ns | 19     | (3–44)    | Ns |
| CCR7           | 42     | (8–70)     | 31     | (8–71)     | Ns | 31     | (9–49)    | Ns |
| CXCR3          | 39     | (10–53)    | 40     | (6–60)     | Ns | 39     | (9–72)    | Ns |
| CX3CR1         | 32     | (8–62)     | 37     | (11–63)    | Ns | 43     | (23–58)   | Ns |
| **CD4+**       |        |            |        |            |    |        |            |    |
| CCR2           | 0.1    | (0.0–0.4)  | 0.1    | (0.0–1)    | Ns | 0.1    | (0–0.2)   | Ns |
| CCR3           | 0.6    | (0.0–1)    | 0.8    | (0.2–5)    | Ns | 4      | (0.4–18)  | < 0.01 |
| CCR4           | 23     | (3–58)     | 17     | (5–40)     | Ns | 19     | (5–27)    | Ns |
| CCR5           | 20     | (11–42)    | 24     | (7–47)     | Ns | 27     | (8–48)    | Ns |
| CCR6           | 29     | (12–52)    | 24     | (10–56)    | Ns | 27     | (18–46)   | Ns |
| CCR7           | 47     | (10–68)    | 41     | (8–74)     | Ns | 42     | (13–59)   | Ns |
| CXCR3          | 36     | (18–50)    | 40     | (8–60)     | Ns | 32     | (9–85)    | Ns |
| CX3CR1         | 36     | (9–65)     | 41     | (12–64)    | Ns | 44     | (21–64)   | Ns |
| **CD8+**       |        |            |        |            |    |        |            |    |
| CCR2           | 0.1    | (0–0.3)    | 0.2    | (0–2)      | Ns | 0.1    | (0–0.2)   | Ns |
| CCR3           | 0.7    | (0–2)      | 1.4    | (0.4–9)    | < 0.01 | 1.6    | (0.5–9)   | Ns |
| CCR4           | 13     | (2–35)     | 8      | (0.5–50)   | 0.02 | 17     | (0–32)    | Ns |
| CCR5           | 43     | (16–73)    | 48     | (24–77)    | Ns | 58     | (27–74)   | 0.03 |
| CCR6           | 4      | (1–37)     | 7      | (0.4–58)   | Ns | 5      | (0.8–15)  | Ns |
| CCR7           | 26     | (2–70)     | 11     | (2–52)     | 0.03 | 13     | (3–31)    | Ns |
| CXCR3          | 47     | (23–84)    | 57     | (3–89)     | Ns | 55     | (14–76)   | Ns |
| CX3CR1         | 28     | (7–63)     | 33     | (9–76)     | Ns | 39     | (35–53)   | 0.04 |
| **CD3+**       |        |            |        |            |    |        |            |    |
| CCR2           | 0.1    | (0.0–1)    | 0.2    | (0.0–7)    | 0.02 | 0.1    | (0–0.2)   | Ns |
| CCR3           | 0.7    | (0.0–1)    | 1.4    | (0.4–4)    | < 0.01 | 1.5    | (0.7–13)  | < 0.01 |
| CCR4           | 20     | (7–53)     | 15     | (3–38)     | Ns | 15     | (10–25)   | Ns |
| CCR5           | 24     | (13–52)    | 28     | (10–51)    | Ns | 32     | (16–46)   | Ns |
| CCR6           | 27     | (8–47)     | 19     | (6–55)     | Ns | 19     | (3–44)    | Ns |
| CCR7           | 42     | (8–70)     | 31     | (8–71)     | Ns | 31     | (9–49)    | Ns |
| CXCR3          | 39     | (10–53)    | 40     | (6–60)     | Ns | 39     | (9–72)    | Ns |
| CX3CR1         | 32     | (8–62)     | 37     | (11–63)    | Ns | 43     | (23–58)   | Ns |

The studies included 18 healthy controls, 22 low-risk and 9 high-risk MDS patients. The percentage of various lymphocyte populations were estimated as follows: (1) total CD3+ T lymphocytes as the percentage of total lymphocytes; (2) CD4+ and CD8+ T cells as the percentage of the total CD3+ T cells. All p values refer to a statistical comparison between high- or low-risk MDS patients and healthy controls.
bear decreased levels of CCR6 (Table 3), and the bone marrow also contains reduced levels of CCR6+ T cells; and (4) the decreased frequency of CXCR3+ T cells in the bone marrow of MDS seems not associated with a significantly altered expression of CXCR3 on circulating cells (Table 2), suggesting that bone marrow T-cell recruitment is not only dependent on the chemokine receptor repertoire but also on other factors, e.g., local chemokine gradients.

The chemokine receptor expression pattern of CD8+ T cells correlates with MDS patient prognosis. We have correlated prognostic parameters and T-cell chemokine receptor expression patterns in our patients. Such prognostic parameters were the IPSS score and the variables that used to estimate this score (bone marrow blast counts and peripheral blood cytopenia). The following observations were made:

• CXCR4. When all MDS patients were included in the analysis, there were significant correlations between the bone marrow blast counts and CXCR4 expression levels in circulating CD8+ central memory (p < 0.01) and effector memory (p < 0.01) T cells. A correlation between blast counts in the bone marrow and CXCR4 expression by CD8+ effector memory cells was also detected when low-risk patients were analyzed separately. Furthermore, we observed significant correlations of patient survival with the levels of circulating CD8+CXCR4+ T cells (p < 0.05), as well as with CXCR4 expression by CD4+ central memory T cells (p < 0.05). These observations suggest an association between CXCR4 expression and MDS prognosis.

• CCR3. When all MDS patients were included in the analysis, the levels of circulating CD4+CCR3+ cells were significantly correlated with the IPSS score. There was no correlation between CCR3 expression and neutrophil counts when the whole patient population was investigated, but among low-risk patients we observed a significant correlation between neutrophil counts and the levels of circulating CD3+CCR3+ T cells (p < 0.01).

• CCR7. When including all patients in the analysis, the level of circulating CD8+CCR7+ T cells inversely correlated with neutrophil counts in the peripheral blood (p = 0.02).

Finally, we investigated whether the expression pattern of chemokine receptors exhibited any correlation with the level of circulating lymphocytes in MDS patients. First, we observed significant correlations between the levels of peripheral blood lymphocytes and the levels of CD8+CCR5+ T cells, when all MDS patients were included in the analysis (Table 2).
observed between the lymphocyte levels in the peripheral blood and (1) the levels of CD8⁺CCR7⁺ (p < 0.01) T cells as well as the CCR3 expression levels of CD4⁺ T cells (p = 0.02) when all MDS patients were included in the analysis; and (2) the abundance of CD8⁺CCR7⁺ cells when low-risk patients were studied separately (p = 0.01).

Discussion

This is the first study to describe altered chemokine receptor expression patterns in T cells from MDS patients. These patients may have autoimmune manifestations, and immune mechanisms seem to be involved in the pathogenesis of the disease, at least for a subgroup of patients. Several T-cell abnormalities have been detected, including alterations in the CD4⁺:CD8⁺ T-cell ratio, skewed T-cell subsets, T H17/ regulatory T cells imbalance and clonal expansion of autoreactive cytotoxic T cells.¹³,¹⁴

Even though MDS patients are heterogeneous, they share fundamental biological and clinical characteristics.¹⁰ We investigated unselected patients that were consecutively admitted to our department. This group represents patients requiring specialist consultation from a defined geographical area. Our aim was to investigate whether common immunological characteristics can be detected in such a group of unselected patients.

Our study was based on the analysis of cells prepared immediately after sampling and not on cryopreserved cells. Cryopreserved materials usually include a minor cell population of necrotic/apoptotic cells even when optimal techniques for

| Table 3. Expression of the chemokine receptors CCR6 and CXCR4 on subsets of CD4⁺ and CD8⁺ T lymphocytes from healthy controls and MDS patients |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Control         | Low-risk MDS    | High-risk MDS   |
|                  | Median | Range  | Median | Range  | p     | Median | Range  | p     |
| **Expression of CCR6 on T-lymphocyte subsets** |     |        |       |        |       |       |        |       |
| CD4⁺ naive       | 4      | 0.9–8  | 3      | 0.5–12 | Ns    | 3      | 1.9–11 | Ns    |
| CD4⁺ central memory | 37    | 14–52  | 38     | 15–58  | Ns    | 40     | 31–57  | Ns    |
| CD4⁺ effector memory | 54    | 12–75  | 45     | 14–79  | Ns    | 49     | 21–66  | Ns    |
| CD4⁺ terminal effector | 5     | 1.0–29 | 2      | 0–24   | < 0.01 | 3      | 0.3–12 | Ns    |
| CD8⁺ naive       | 0.4    | 0.0–38 | 0.2    | 0.0–4  | Ns    | 0.5    | 0.0–2  | Ns    |
| CD8⁺ central memory | 3     | 1.0–27 | 5      | 0.0–25 | Ns    | 5      | 1.6–11 | Ns    |
| CD8⁺ effector memory | 14    | 1.0–42 | 15     | 0.4–87 | Ns    | 14     | 1.0–41 | Ns    |
| CD8⁺ terminal effector | 1.4   | 0.2–25 | 0.8    | 0.0–18 | Ns    | 0.5    | 0.1–10 | Ns    |
| **Expression of CXCR4 on T-lymphocyte subsets** |     |        |       |        |       |       |        |       |
| CD4⁺ Naive       | 73     | 55–89  | 79     | 28–95  | Ns    | 78     | 40–93  | Ns    |
| CD4⁺ central memory | 30    | 15–50  | 36     | 9–70   | Ns    | 37     | 29–64  | 0.03  |
| CD4⁺ effector memory | 13    | 6–49   | 16     | 3–51   | Ns    | 24     | 11–33  | Ns    |
| CD4⁺ terminal effector | 18    | 5–51   | 12     | 1.7–51 | Ns    | 19     | 5–58   | Ns    |
| CD8⁺ naive       | 65     | 27–92  | 64     | 15–94  | Ns    | 74     | 50–95  | Ns    |
| CD8⁺ central memory | 34    | 11–44  | 38     | 10–83  | Ns    | 53     | 25–62  | < 0.01 |
| CD8⁺ effector memory | 19    | 5–46   | 23     | 2–72   | Ns    | 34     | 13–76  | 0.02  |
| CD8⁺ terminal effector | 22    | 3–62   | 26     | 5–76   | Ns    | 39     | 5–50   | Ns    |

The studies included 18 healthy controls, 22 low-risk and 9 high-risk MDS patients. The percentage of various lymphocyte subpopulations were estimated as the percentage of total CD4⁺ and CD8⁺ T cells, respectively. All p-values refer to a statistical comparison between high- or low-risk MDS patients and healthy controls.
preservation and thawing are applied. In addition, chemokine receptors may be internalized during cryopreservation. Our methodological approach was chosen to ensure (1) maximal cell viability and (2) a receptor expression pattern maximally reflecting the in vivo situation.

We investigated the chemokine receptor expression pattern in defined T-cell subsets. CD3+ T lymphocytes are divided into the major helper CD4+CD8− and cytotoxic CD8+CD4− cell subsets, which can be further subdivided into naïve (CD62L−CD45RA−), central memory (CD62L−CD45RA+), effector memory (CD62L−CD45RA−) and terminal effector memory (CD62L−CD45RA−) T cells. All these subsets can be detected in MDS patients. Other studies have used CCR7 and not CD62L to define T-cell subsets, but since the main focus of our study were chemokine receptors, we decided not to use this marker to define cell subpopulations. Our findings in MDS patients were compared with those obtained in a group of matched healthy individuals (Table 2). Zou et al. have previously described a reduction in CD4+ and CD8+ naïve T cells and an increase in the abundance of effector memory and terminal effector subsets in MDS patients. We observed similar trends, in particular for CD8+ T cells; although they did not reach statistical significance. This discrepancy may stem from the fact that the average age of our patients was comparatively higher, as Zou et al. described that these differences were most clearly seen among young patients.

CCR7 is generally lost when T cells are activated to become terminal effector/effector memory cells. CCR5 and CCR3/CCR4 expression are associated with T cells, and CX3CR1 is specifically expressed on mature cells with high cytotoxic capacity, regardless of lineage. Chemokine receptor expression profiles can thus be used to define functionally distinct T-cell subsets. Our data indicating an increased T-cell expression of CCR3/CCR5/CX3CR1 and decreased expression of CCR7 (see Table 2) suggest that T cells from MDS patients have a more mature chemokine receptor profile. This is also consistent with previous studies describing increased levels of terminal effector cells in MDS patients.

Furthermore, high CCL5 serum levels have been reported in low-risk individuals affected by low-risk MDS and have been associated with prolonged patient survival. CCL5 is a ligand both for CCR3 and CCR5, which were increased in CD8+ T cells from low- and high-risk MDS patients, respectively. This altered receptor expression may hence contribute to the prognostic impact of circulating CCL5 levels. Finally, the ligands for CCR4 are CCL17 and CCL22, which can be released by normal dendritic cells as well as by myeloid leukemic blasts and leukemia-derived dendritic cells. Decreased CCR4 expression in low-risk MDS patients may hence inhibit the migration of T cells in the direction of CCL17/CCL22-producing cells.

There seems to be a similar variation in the expression of several chemokine receptors in CD4+ and CD8+ T cells (Fig. 2). This was further confirmed by correlation analyses. These observations are consistent with the hypothesis that similar mechanisms regulate chemokine receptor expression in these two T-cell subsets. Still, the differences between patients and control individuals allowed statistical significance to be reached only for certain receptors and/or patient sub-groups.

A small minority of our low-risk MDS patients received cytokine treatment (Table 4), which seemed to have only a minor influence on T-cell populations. Moreover, a subset of our patients was examined initially as well as after a one-year delay. The majority of these individuals had clinically stable disease, and most of them had also had stable disease for a relatively long time before the first sampling. Our results demonstrate that immunological alterations may develop over time even though patients are clinically stable.

We characterized T-cell subsets in the bone marrow, and for these experiments we used cryopreserved material. Cryopreservation and thawing may cause the internalization of membrane molecules and can also provoke a minor decrease in cell viability. However, the differences between our observations in the peripheral blood and bone marrow cannot be explained by a general effect induced by cryopreservation/thawing on bone marrow cells, because (1) when comparing relative values we observed an altered CD4+:CD8+ T-cell ratio, (2) we did not detect any increase in the double negative T-cell subset (i.e., effector memory cells) and (3) there was a selective increase in the expression of specific chemokine receptors. Although we emphasize that these results should be interpreted with care, in our opinion the differences that we observed are most likely due to true differences between circulating and bone marrow T-cell populations. Altered T-cell characteristics caused by several distinct, subset-specific cryopreservation/thawing-induced alterations seem less likely.

Our high-risk MDS patients showed increased CXCR4 expression in mature CD8+ T-cell subsets as compared with control individuals. Increased CXCR4 expression was also associated with prolonged survival in high- and low-risk patients, even though CXCR4 expression was not significantly increased among low-risk patients in general (Table 2). CXCR4 is normally expressed at high levels on naïve T-cells. This was also true in our high-risk MDS patients, but, in contrast to healthy individuals, these high-risk patients showed relatively high CXCR4 expression levels also in more-mature T-cell subsets (Table 3). CXCR4 binds the homeostatic ligand CXCL12, which is released by normal bone marrow stromal cells. Hence, the high expression levels of CXCR4 on mature cells from high-risk patients may suggest an abnormal maturation of effector cells, resulting in an increased homing potential to the bone marrow. This is further supported by the detection of increased CXCL12 levels in the bone marrow and plasma of MDS patients. Our study thereby suggests that the CXCL12/CXCR4 system has a dual function in human MDS patients: not only is CXCR4 expressed by CD34+ bone marrow cells in MDS patients and is important for survival and migration of these cells, as previously shown, but also CXCR4/CXCL12 is important for the immunoregulation and the recruitment of immunocompetent cells to the bone marrow.

To conclude, the chemokine receptor expression pattern on circulating T cells from MDS patients suggests a dominance of mature T cells as defined by the expression of CD45RA and CD62L. Our results as well as previous studies have demonstrated that there is a wide variation in the levels of circulating T-cell subsets in both MDS patients and healthy individuals,
Table 4. Patients included in the study, with patient and disease characteristics, treatment received and follow-up time in months

| #  | Gender | Age | Diagnosis          | WHO class | Cytopenia(s) | Cytogenetics | Treatment | Follow-up time |
|----|--------|-----|--------------------|-----------|-------------|-------------|-----------|----------------|
|    |        |     |                    |           |             |             |           |                |
| 1  | M      | 63  | 2002               | RCMD      | A, T        | NA          | BS        | 18.1*          |
| 2  | M      | 70  | 2009               | RCMD      | A, N, T     | NA          | BS        | 4.5*           |
| 3  | F      | 89  | 2005               | RARS      | A           | NA          | BS        | 33.0           |
| 4  | F      | 84  | 2000               | RARS      | A           | NA          | BS (E)    | 32.2           |
| 5  | M      | 88  | 2009               | RCMD      | A, N, T     | NA          | BS (E+G)  | 7.6*           |
| 6  | M      | 83  | 2009               | RCMD      | A           | 46 XY      | E+G       | 12.8*          |
| 7  | M      | 85  | 2007               | RCMD      | A, T        | 45XY, del(11)(q23) (2011) | BS | 34.1 |
| 8  | F      | 83  | 2008               | RCMD      | A, N        | NA          | G (E+G)   | 19.2*          |
| 9  | M      | 68  | 2009               | RCMD      | A           | 47XY+9[15]/47, idem, der(16)[1;16]/q(12;q11)/[18] | BS | 4.1*          |
| 10 | M      | 71  | 2009               | RCMD      | A           | 46 XY      | BS        | 30.7           |
| 11 | M      | 71  | 2008               | RCMD      | A, T        | 46 XY      | BS (E)    | 29.3           |
| 12 | F      | 85  | 2000               | RARS      | A           | NA          | E+G       | 29.0           |
| 13 | M      | 81  | 2008               | RARS      | A           | NA          | BS (E)    | 18.5*          |
| 14 | M      | 58  | 2009               | RCMD      | A           | 46 XY      | E (E+G+A) | 33.4           |
| 15 | M      | 67  | 2010               | RCMD      | N           | 46 XY, t(3;6) (q26q25)[27] (2011) | BS | 21.0 |
| 16 | M      | 85  | 2011               | RCMD      | A           | NA          | BS        | 5.0*           |
| 17 | M      | 73  | 2000               | RARS      | A           | NA          | BS        | 19.9           |
| 18 | M      | 93  | 2011               | RCMD-RS   | A           | NA          | E         | 1.3            |
| 19 | F      | 73  | 2012               | RAEB-1    | N, T        | 46XX       | BS        | 1.3            |
| 20 | F      | 85  | 2012               | RCMD      | A           | NA          | BS        | 0.8            |
| 21 | M      | 73  | 2011               | RCMD      | NT          | 46XY       | BS        | 0.6            |
| 22 | M      | 84  | 2012               | RCMD      | A           | 46XY       | BS        | 0.6            |
| 23 | M      | 80  | 2011               | RCMD      | A, N, T     | 46 XY      | BS        | Only BM        |
| 24 | M      | 65  | 2007               | RARS      | A           | 46 XY      | E+G       | Only BM        |
| 25 | M      | 55  | 2009               | RAEB-2    | A, N        | 46 XY      | BS (ASCT) | 3.0*           |
| 26 | F      | 57  | 2010               | RAEB-2    | A, N, T     | 46XX,de[5]/q(15q33)/9, idem,add(17) (p13), -22+mar(11) | BS | 0.5*          |
| 27 | F      | 68  | 2009               | RAEB-2    | A, N, T     | 46XX,del(12)(p11) | BS (A) | 18.8*          |
| 28 | M      | 66  | 2010               | RAEB-2    | A, N, T     | 46 XY      | BS (A)    | 30.7           |
| 29 | M      | 61  | 2010               | RAEB-2    | A, T        | 44-45 XY der[3](t1;13)(p32;27)-5del(6)(p23), -7,add(14)(p11), +add(14) (p11), del(18)(p20) | A | 4.2*          |
| 30 | M      | 69  | 2008               | RAEB-1    | A, N, T     | 46XY,del(12)(p12)[3]/46,XY[17] | A (G) | 21.1 |
| 31 | M      | 66  | 2011               | RAEB-2    | A, N, T     | 46 XY      | BS (A, ASCT) | 19.6 |
| 32 | M      | 71  | 2012               | RAEB-2    | A, N, T     | 47 XY +8 [10] | A | 1.3 |
| 33 | M      | 78  | 2011               | RAEB-2    | A, N        | 47 XY +11 [2] | BS | 0.8 |

1 Age at first sample; 2 Year diagnosed with MDS; 3 RARS, refractory anemia with ringed sideroblasts; RCMD, refractory cytopenia with multilineage dysplasia; RAEB-1, refractory anemia with excess blasts-1; RAEB-2, refractory anemia with excess blasts-2; A, anemia; N, neutropenia; T, thrombocytopenia; NA, not acquired; 4 Treatment after sample in parentheses; BS, best supportive care including transfusions if needed; E, erythropoietin; G, G-CSF; A = azacitidine; 5 Follow-up in months since first sample, BM, bone marrow. *Patients 1, 6, 8 and 13 dead from unrelated disease, 2, 9 and 26 dead from other cancer, 5 and 16 dead from infection, 25 dead from GVHD, 27 and 29 dead from AML.

exhibiting a considerable overlap.27,30 The maturation of T cells in MDS patients does not seem to differ from that seen in normal activated lymphocytes, with the exception of increased CXCR4 levels, as observed in mature CD8+ T cell subsets of high-risk MDS patients.26,34 Targeting chemokine receptors with monoclonal antibodies or specific inhibitors may affect T-cell trafficking and thereby the interactions between immunocompetent and hematopoietic cells in the bone marrow of MDS patients.
Materials and Methods

Patients. The studies were approved by the regional ethical committee, REK Vest, REK number 3.2008.409. Patients constitute an unselected group of consecutive individuals diagnosed at our department, i.e., they represent the large majority of patients diagnosed with MDS from a defined geographic area. Patients with IPSS group low and intermediate-1 were considered as low-risk patients (n = 22; median age 82, range 58–93) and patients in intermediate-2 and high were defined as high-risk patients (n = 9; median age 66, range 56–75). For two additional low-risk patients, we were able to analyze bone marrow samples, but not peripheral blood samples. Data on these patients are not presented in the text, but included in Table 4. Only a minority of patients received erythropoietin and/or G-CSF at the time of sampling, but nearly half of the low-risk patients required such treatment later on.

The WHO MDS subtypes were represented as follows: refractory anemia with ringed sideroblasts (RARS; n = 5, 16%), refractory cytopenia with multilineage dysplasia (RCMD; n = 15, 48%), RCMD with ring sideroblasts (RMCD-RS; n = 1, 3%), refractory anemia with excess (5–9%) of blasts (RAEB-1; n = 2, 6%), refractory anemia with excess (10–19%) of blasts (RAEB-2; n = 8, 26%). In our study we defined high-risk MDS as WHO class RAEB-1 with disease progression within 6 months and WHO class RAEB-2. All other patients are referred to as low-risk MDS. Median follow-up time from first sampling was 78.7 weeks. For patients 23, 24, we had only bone marrow samples, and do not present follow up data other than that they were both alive at the end of follow-up for the rest of the cohort. Control samples were obtained from healthy volunteers (n = 18, median age 58 y with range 40–83 y).

Cytofluorometric analyses of chemokine receptors on T cells. Blood samples from MDS patients were acquired after written informed consent. Blood collected in EDTA was subject to red cell lysis using Pharmlyse (BD PharMingen) following the manufacturer’s instructions. To optimize chemokine receptor expression, samples were kept at room temperature until staining. We also analyzed bone marrow (BM) samples from 13 patients. BM was obtained from the posterior iliac crest and BM mononuclear cells (MNCs) were isolated by Ficoll-Hypaque (Nycodemed) gradient centrifugation and cryopreserved. For analysis, the cells were thawed and resuspended in StemSpan medium (StemCell Technologies, Inc.), and kept at room temperature.

Approximately 0.5 × 10^6 cells were added to cytofluorometry tubes containing the antibodies corresponding to five different panels. Antibodies against the following targets were used (conjugated fluorochrome is indicated): CD3 (Pacific Orange, UCHT1, CD0330 and APC-Cy7, SK7, 557832), CD4 (FITC, SK3, 345768), CD8 (Pacific Blue, RPA-T8, 558207), CD45RA (PE-Cy7, HI100, 560675), CD56 (PE, B159, 555516), CD62L (FITC, DREG-56, 555543), CCR3 (PE, 5E8, 558165), CCR4 (PE, 1G1, 551120), CCR5 (PerCP-Cy5.5, 1G1, 560726), CCR5 (PE-Cy7, 2D7/CCR5, 557752), CCR6 (PerCP-Cy5.5, CCR6, 560467), CCR7 (FITC, 3D12, 560548), CXCR3 (AlexaFluor®488, 1C6/CXCR3, 58047), CXCR4 (APC, 12G5, 555976), CX3CR1 (FITC, 2A9-1, 341606). All antibodies were mouse anti-human, except for anti-CCR7 and anti-CX3CR1 antibodies, which were rat anti-human. Anti-CD3 antibodies were purchased from Invitrogen, (Life Technologies Ltd), CX3CR1 from BioLegend and all other from BD Biosciences. Staining volume was 100 μL, and samples were incubated for 15 min on a shaker, in the dark, at room temperature. Cells were then washed twice with PBS, resuspended in 0.5% bovine serum albumin (BSA), and kept on ice until cytofluorometric analysis. Single stained beads (BD Biosciences) were used for compensation. The samples were analyzed on the same day using a FACSCanto II cytometer (BD Biosciences). An average of approximately 20,000 CD3+ cells were analyzed per antibody panel per low-risk patient, and approximately 47,000 CD3+ cells per high-risk patient. Compensation and data analysis were performed by means of the FlowJo 7.6 software (TreeStar Inc.). After setting the compensation, the lymphocyte gate was set by light scatter properties. Cells were then gated on CD3 positivity and CD8 positive and negative gates were set strictly. In one panel, gates were set by displaying CD4 against CD8, hence CCR2-CCR4 represent values in the CD4⁺CD8⁻ and CD8⁺CD4⁺ populations rather than in CD8⁺ or CD8⁻ cells. In some figures, the CD8⁺ population may be

![Figure 4. Gating strategy. The lymphocyte populations were identified by light scatter properties (side and forward scatter, upper left). T cells were then identified using CD3 expression (middle left), and CCR2-CCR4 expression was analyzed on cells gated on CD4 and CD8 expression (lower left), while CCR5–7, CXCR2–4 and CX3CR1 expression was evaluated upon CD8 gating (middle). T-cell subsets were gated using CD45RA and CD62L on CD8-stained cells (middle right), and these cells were also examined for CCR6 and CXCR4 expression. The gates for detectable chemokine receptor expression were set manually for each individual, and the layover of healthy subject (outline) over MDS patient (gray) is only illustrative. Examples are shown for CCR7 and CCR3 (top and bottom right, respectively).](image-url)
referred to as CD4+, for the sake of simplicity. Only the CCR2-CCR4 values were gated using a CD4 antibody. CD3+CD8+ and CD3+CD8− T-cell subset analysis was based on the expression of CD45RA and CD62L. Only CCR6 and CXCR4 expression were analyzed in these subsets. For chemokine receptors, the best cut point was found by looking at several subpopulations for shifts and to identify subsets with similar autofluorescence as negative controls. Each gate was adjusted for the chosen subpopulation separately, based on observed shifts. The gating strategy is shown in Figure 4.

**Statistical analyses.** Mann-Whitney non-parametric tests were used when comparing subset proportions and chemokine receptor expression between patients and healthy individuals. Wilcoxon Signed Ranks tests were used when comparing paired samples. Spearman correlation was applied to detect correlations between chemokine expression and clinical parameters. p values < 0.05 were considered statistically significant. Statistical analyses were done using IBM SPSS 19.0 (IBM).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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