ARTICLE

Immune composition and its association with hematologic recovery after chemotherapeutic injury in acute myeloid leukemia

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Chemotherapy-induced bone marrow (BM) injury is a significant cause of morbidity and mortality in acute myeloid leukemia (AML). Time to hematologic recovery after standard ("7 + 3") myeloablative chemotherapy can vary considerably among patients, but the factors that drive or predict BM recovery remain incompletely understood. Here, we assessed the composition of innate and adaptive immune subsets in the regenerating BM (day 17) after induction chemotherapy and related it to hematologic recovery in AML. T cells, and in particular the CD4 central memory (CD4CM) T-cell subset, were significantly enriched in the BM after chemotherapy, suggesting the relative chemoresistance of cells providing long-term memory for systemic pathogens. In contrast, B cells and other hematopoietic subsets were depleted. Higher frequencies of the CD4CM T-cell subset were associated with delayed hematopoietic recovery, whereas a high frequency of natural killer (NK) cells was related to faster recovery of neutrophil counts. The NK/CD4CM ratio in the BM after chemotherapy was significantly associated with the time to subsequent neutrophil recovery (Spearman's $\rho = -0.723$, $p < 0.001$, false discovery rate <0.01). The data provide novel insights into adaptive immune cell recovery after injury and identify the NK/CD4CM index as a putative predictor of hematopoietic recovery in AML.

HIGHLIGHTS

- Chemotherapy induces deep B-cell depletion with relative resistance of T-cell subsets.
- CD4CM- and NK-cell frequencies are associated with time to hematopoietic recovery.
- Immune composition may help predict duration of myelosuppression in AML.

Chemotherapy is the main type of treatment for patients with acute myeloid leukemia (AML). The current standard of care for patients, if they are eligible, is conventional "7 + 3" induction therapy consisting of cytarabine and anthracyclines [1,2]. This therapeutic modality is typically complicated by a prolonged phase of myelosuppression, resulting in considerable morbidity and mortality, particularly because of opportunistic infections. While the duration of bone marrow (BM) suppression, including neutropenia, may vary substantially among patients, little is known about the factors that support hematopoietic recovery in this setting. Also, we currently lack tools to predict the pace of BM recovery after chemotherapeutic injury.

To achieve hematologic recovery after chemotherapeutic injury, residual normal hematopoietic stem/progenitor cells (HSPCs) in the BM need to replenish mature blood cells. HSPC behavior is tightly regulated by its microenvironment, the so-called HSPC niche [3]. Many cell types contribute to the HSPC niche, including T cells as suggested by some studies [4–10]. The findings in these studies imply that lymphocyte subsets may play a role in hematopoietic recovery following AML chemotherapy, but how lymphocyte subsets are affected by intensive chemotherapy remains largely unknown.

Here, we interrogated the composition of cells constituting innate and adaptive immunity in the regenerating BM of AML patients after intensive chemotherapy and relate it to hematologic recovery.

METHODS

BM Aspirates

BM aspirates from AML patients (average: 58 years, range: 20–75 years) were collected at 17 days (range: 16–19 days) after start of remission induction chemotherapy (7 + 3 schedule of chemotherapy with cytarabine and anthracycline). Control marrow was obtained by aspiration from donors for allogeneic transplantation (average: 64.8 years, range: 47–78 years) after patients provided written informed consent. The use of human samples with informed consent was approved by the institutional review board of the Erasmus Medical Center (Rotterdam, Netherlands) in accordance with the Declaration of Helsinki.

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Cell isolation and flow cytometry

Mononuclear cells (MNCs) were isolated from BM aspirates as previously reported [11]. Briefly, cryopreserved BM aspirates were thawed in a water bath at 37°C. Subsequently, BM aspirates underwent centrifugation and were washed once with phosphate-buffered saline (PBS) + 0.5% fetal calf serum (FCS).

Next, BM aspirates were divided in two and were stained with one of two antibody cocktails: Cocktail 1 was used to determine the frequencies of monocytes, B cells, T cells, and natural killer (NK) cells, and cocktail 2 was used to determine the frequencies of naïve and memory T-cell subsets. For cocktail 1, we used CD14 (Catalog No. 560180, BD Pharmingen, San Diego, CA) and a multitest mixture (Catalog No: 342417, BD Pharmingen) containing CD45, CD3, CD16/CD56, and CD19, while for cocktail 2, we used CD45 (Catalog No. 11-9459-42, eBioscience, San Diego, CA), CD45RA (Catalog No: 46-0468-42, eBioscience), CD3 (Catalog No. 12-0039-42,21, eBioscience), CD4 (Catalog No. 317443, BioLegend, San Diego, CA), CD8 (Catalog No. 561423, BD Pharmingen), and CD62L (Catalog No. 17-0629-42, eBioscience). The BM aspirates were then mixed 1:10 with 1× IOTest3 Lysing Solution (Catalog No, A07799, Beckman Coulter, Indianapolis, IN) to lyse red blood cells.

Flow-cytometric analyses were performed using a BD FACSCanto II (BD Biosciences, San Jose, CA, USA) and data were analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

Statistics

To compare the mean differences between different (patient) groups, the Mann−Whitney

U test was applied. To measure the degree of correlation between immune subsets and hematologic recovery, Spearman's rank correlation coefficient was used. Concomitantly, corresponding p values were also calculated. In addition, p values were adjusted for multiple comparisons when needed using the Benjamini−Hochberg procedure and are expressed as false discovery rates (FDRs).

Software

Statistical analyses were performed using IBM SPSS 25 (IBM, Armonk, NY). Graphs were generated using Prism 5 (GraphPad Software, San Diego, CA). Final figures were completed in Adobe Illustrator CC.

RESULTS

Selective depletion of B cells and relative persistence of T-cell subsets in AML after intensive chemotherapy

Bone marrow aspiration during recovery after chemotherapy (17−21 days after start of treatment) is standard practice to assess the patient's response status by enumerating the frequency of remaining leukemic blasts.

To study the lymphocyte composition in regenerative marrow, BM aspirates of 28 AML patients were obtained 17 days after start of the first cycle of remission induction therapy (Supplementary Table E1, online only, available at www.exphem.org). All patients exhibited an "empty marrow," with <5% myeloblasts, and a complete remission/complete remission with incomplete hematologic recovery (CR/CRi) was documented for all patients following subsequent BM examinations after the treatment cycle. These "regenerative" BM samples without morphologic evidence of residual disease were compared with steady-state BM from healthy donors ($n = 7$) using flow cytometry. This approach was useful in the determination of immune subset frequencies, although it did not allow determination of absolute counts for each population studied.

First the overall contribution of all lymphocytes to the hematopoietic compartment was determined. Hematopoietic cells (marked by CD45 expression) were identified and subsequently classified into granulocytes (lacking CD14 expression and marked by high side scatter [SSC]), monocytes (marked by CD14 expression and low SSC) [12], and lymphocytes (lacking CD14 expression and marked by low SSC) with flow cytometry (Figure 1A). The frequency of lymphocytes was markedly increased compared with healthy BM (91.5% ± 1.8% vs. 22.2% ± 3.8%, FDR < 0.001 by Mann−Whitney U test and adjusted for multiple comparisons using the Benjamini−Hochberg procedure) (Figure 1B), suggesting that lymphoid cells are relatively chemoresistant. Conversely, the frequency of granulocytes in the regenerative marrow was much lower in comparison to marrow under steady-state conditions (4.2% ± 1.5% vs. 72.5% ± 5.3%, FDR <0.001) (Figure 1C), consistent with the view that chemotherapy depletes neutrophils [13]. Monocyte frequency remained unaltered (4.3% ± 1.0% vs. 5.2% ± 1.6%, FDR >0.05) (Supplementary Figure E1A, online only, available at www.exphem.org).

To evaluate the lymphocyte subset composition in regenerative BM, we dissected the lymphoid compartment by analyzing expression of CD16/CD56 (NK- and NKT-cell markers) [14], CD3 (T-cell and NKT-cell marker) [15], and CD19 (B-cell marker). Flow cytometric assessment revealed that within the lymphoid compartment, T cells were enriched in regenerating marrow compared with healthy marrow (78.2% ± 2.5% vs. 62.8% ± 4.3%, FDR <0.05), seemingly at the cost of B-cell frequency (3.4% ± 1.0% vs. 20.6% ± 3.6%, FDR <0.001) (Figure 1D−F). NKT-cell frequency within the lymphoid compartment was also increased (6.0% ± 1.0% vs. 2.6% ± 0.5%, FDR >0.05), though this finding was not significant when corrected for multiple comparisons (Figure 1D,H). NK-Cell frequency varied widely in the regenerative marrow between AML patients, although overall, it was not significantly different from healthy control BM (8.1% ± 1.4% vs. 7.9% ± 0.6%, FDR >0.05) (Figure 1D,G). These findings indicate that within the lymphoid fraction, specific subsets of lymphocytes may be more susceptible (B cells) or resistant (T cells) to chemotherapy compared with other subsets, in line with previous reports [13,16,17].

The CD4CM T-lymphocyte fraction is increased in regenerative BM

T cells can be further subdivided into CD4- and CD8-expressing T cells which in turn can be grouped into naïve (N), central memory (CM), effector memory (EM), and CD45RA re-expressing (TEMRA) cells based on CD62L and CD45RA expression [18]. Dissection of the T-cell compartment revealed that the majority of the T-cell subset frequencies within the lymphoid compartment did not significantly differ between regenerative marrow and healthy BM (Figure 1I; Supplementary Figure E1B−H, online only, available at www.exphem.org). Only the frequency of the CD4CM T-cell subset was significantly increased in regenerating marrow compared with steady-state BM (25.8% ± 2.7% vs. 14.2% ± 1.5%, FDR <0.05) (Figure 1I,J).

Taken together, these results suggest that T cells (and, in particular, the CD4CM subset), NK cells and NKT cells are relatively resistant to

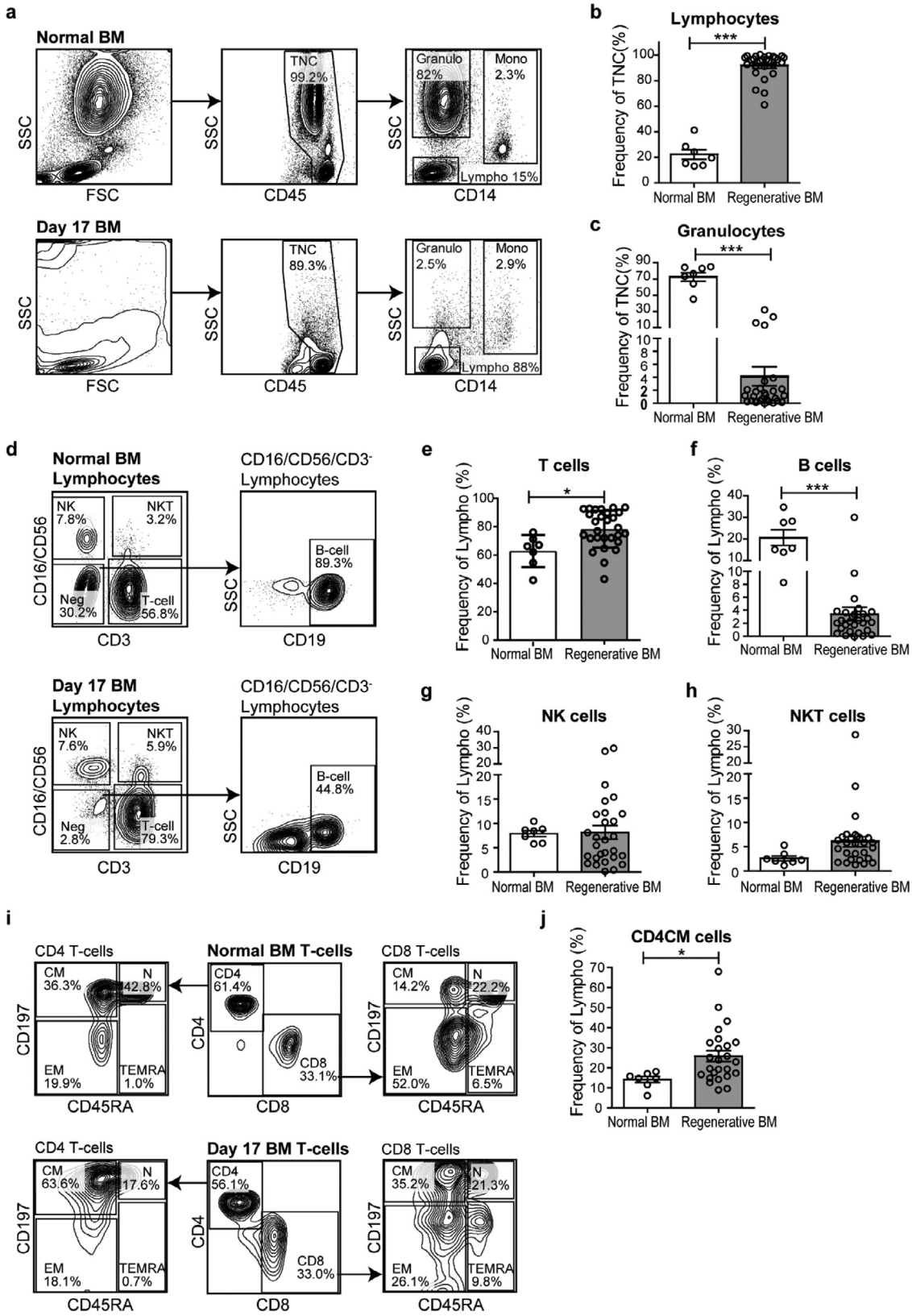

Figure 1 Composition of immune subsets in the BM on recovery after intensive chemotherapy in AML. (A) Representative flow cytometric plots depicting normal steady-state BM from healthy individuals (upper panels) and BM on regeneration, 17 days after

chemotherapy in comparison to B cells, with the latter being severely reduced within the lymphoid fraction after induction chemotherapy. This is in line with previous studies that found a dramatic depletion of B cells in the peripheral blood of cancer patients after chemotherapy [13,16,17].

The frequency of CD4CM T and NK cells is associated with hematopoietic recovery

Next, we related the frequency of immune subsets to the time needed for hematologic recovery. Hematologic recovery was defined as the time from start of chemotherapy to achievement of an absolute neutrophil count (ANC) $\geq 500/mm^3$. Eight patients were excluded from analysis because they were discharged from the hospital before reaching this ANC threshold (Supplementary Table E1, online only), precluding accurate determination of time to recovery. The average time to neutrophil recovery in the studied subset of patients was 24 days (range: 18–30 days). Higher frequencies of T cells within the lymphoid fraction were significantly associated with a delayed neutrophil recovery (Spearman's $\rho = 0.633$, $p < 0.01$, FDR <0.05) (Figure 2A). In particular, the frequency of the CD4CM T-cell fraction seemed to be relevant, as only this T-cell subset was significantly associated with neutrophil recovery (Spearman's $\rho = 0.613$, $p < 0.01$, FDR <0.05) (Figure 2B).

Conversely, an inverse relation was observed between the frequency of NK cells in the regenerative BM and hematologic recovery. Patients with higher frequencies of NK cells achieved an ANC of 500 cells/mm^3 blood (Figure 2C) faster than patients with lower levels of NK cells (Spearman's $\rho = -0.478$, $p = 0.03$), although not significantly so after correcting for multiple comparisons (FDR >0.05).

Subsequently, we asked if we would find a stronger association between immune subsets and hematologic recovery by using the NK/CD4CM index, consisting of the two immune subsets' frequencies that exhibited the strongest association with neutrophil recovery. Indeed, patients with higher NK/CD4CM index scores (proportionally higher NK cell levels relative to CD4CM T-cell levels) reached an ANC of 500 cells/mm^3 blood (Figure 2D) significantly faster than patients with lower NK/CD4CM scores (Spearman's $\rho = -0.723$, $p < 0.001$, FDR <0.01).

Similarly, the association of the NK/CD4CM index with platelet recovery (defined as a count $\geq 50{,}000/mm^3$) after starting chemotherapy was examined. Of the 28 patients, 6 were excluded from analysis because they were discharged from the hospital before reaching this platelet threshold (Supplementary Table E1, online only), precluding accurate determination of time to recovery. Two other patients were excluded because of the lack of information on their T-cell memory subset frequencies. Congruent with the notion that a NK/CD4CM index is associated with hematologic recovery, patients with a higher NK/CD4CM index score reached a platelet count of $50{,}000/mm^3$ (Figure 2E) much faster than patients with a lower NK/CD4CM index score (Spearman's $\rho = -0.595$, $p < 0.01$, FDR <0.05).

DISCUSSION

Persistent myelosuppression and immunosuppression following chemotherapy predispose cancer patients to opportunistic infections and constitute a major cause of morbidity and mortality. The nature of the chemotherapy-induced immunosuppression is incompletely understood, and the dynamics of immune subsets in the BM after chemotherapeutic injury have remained largely elusive. Here, we provide data on immune cell composition in the BM following exposure to myeloablative chemotherapy. The data reveal relative preservation of T-cell subsets in comparison to other immune cells with a significant association with hematopoietic recovery.

Myeloablative chemotherapy resulted in an increase in the frequency of memory $CD4^+$ T cells, indicative of relative preservation of this subtype of T cells. The BM is an important reservoir for human memory T cells where they are maintained as resting cells in survival niches defined by IL-7-expressing stroma cells [19]. They provide long-term polyfunctional memory for systemic pathogens [20,21]. Our data indicate that these important immune cells are relatively resistant to chemotherapeutic injury (perhaps by virtue of their quiescent state in protecting niches), with an increase in their frequency within the lymphocyte compartment on chemotherapeutic challenge. Although function was not interrogated, data would be in line with the notion that long-term memory against systemic pathogens representing earlier infections may remain relatively intact after myeloablative therapy.

In contrast, B cells were significantly depleted in the marrow after chemotherapy, in line with earlier studies assessing circulating B-cell numbers after chemotherapy and relating this finding to impaired humoral immunity in patients [22,23]. Chemotherapy may directly target B cells by inducing apoptosis in these cells [24] or attenuating their protective vascular niches [25], as

commencing chemotherapy in AML patients (lower panels). Hematopoietic cells were selected by CD45 expression (middle panels). Granulocytes were identified by high SSC characteristics, monocytes by CD14 expression, and lymphocytes by lack of SSC properties and CD14 expression (right panels). (B,C) Frequencies of lymphocytes (b) and granulocytes (c) within the total living nucleated cells of normal BM ($n = 7$) and regenerative BM ($n = 28$). (D) Representative flow cytometric plots depicting lymphocyte composition in normal BM (upper panels) and BM on regeneration (lower panels). Lymphocytes were characterized by CD3 and CD16/CD56 expression, $CD16/CD56^-CD3^+$ cells were classified as T cells, whereas $CD16/CD56^+CD3^-$ and $CD16/CD56^+CD3^+$ cells were identified as NK and NKT cells, respectively. B cells were classified as $CD16/CD56^-CD3^-$ cells that expressed CD19. (E–H) Frequencies of T cells (E), B cells (F), NK cells (G), and NKT cells (H) within the lymphocyte compartment of normal BM ($n = 7$) and regenerative BM ($n = 28$). (I) Representative flow cytometric plots depicting T-cell composition in normal BM (upper panels) and BM on regeneration (lower panels). T cells were subdivided into CD4 and CD8 T cells (middle panels). Both CD4 and CD8 T cells were further classified as central memory (CM), effector memory (EM), CD45RA re-expressing cells (TEMRA), and naïve (N) subsets based on CD62L and CD45RA expression. (J) Frequency of CD4CM cells within the lymphocyte compartment of normal BM ($n = 7$) and regenerative BM ($n = 28$). *FDR < 0.05, ***FDR < 0.001, by Mann–Whitney *U* test and adjusted for multiple comparisons using the Benjamini–Hochberg procedure. *AML*=acute myeloid leukemia; *BM*=bone marrow; *FDR*=false discovery rate; *FSC*=forward scatter; *NK*=natural killer; *SSC*=side scatter.

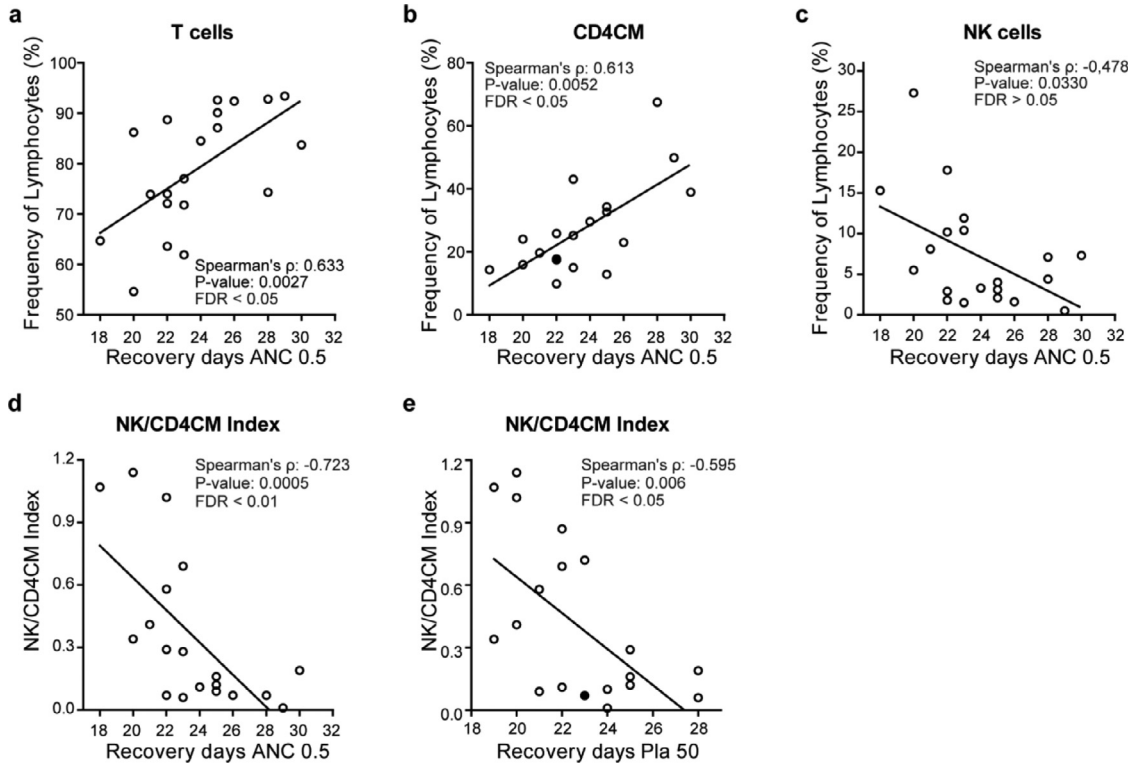

Figure 2 Frequency of CD4CM T and natural killer cells is associated with neutrophil recovery after chemotherapy in acute myeloid leukemia. (A) Representative flow cytometric plots depicting normal steady-state BM from healthy individuals (upper panels) and BM on regeneration, 17 days after commencing chemotherapy in AML patients (lower panels). Hematopoietic cells were selected by CD45 expression (middle panels). Granulocytes were identified by high side-scatter characteristics, monocytes by CD14 expression, and lymphocytes by lack of side-scatter properties and CD14 expression (right panels). (B,C) Frequencies of lymphocytes (B) and granulocytes (C) within the total living nucleated cells of normal BM (n=7) and regenerative BM (n = 28). (D) Representative flow cytometric plots depicting lymphocyte composition in normal BM (upper panels) and BM upon regeneration (lower panels). Lymphocytes were characterized by CD3 and CD16/CD56 expression, CD16/CD56-CD3+ cells were classified as T cells, whereas CD16/CD56+CD3-, and CD16/CD56+CD3+ cells were identified as NK and NKT cells, respectively. B cells were classified as CD16/CD56-CD3- cells that expressed CD19. (E–H) Frequencies of T cells (E), B cells (F), NK cells (G), and NKT cells (H) within the lymphocyte compartment of normal BM (n = 7) and regenerative BM (n = 28). (I) Representative flow cytometric plots depicting T-cell composition in normal BM (upper panels) and BM on regeneration (lower panels). T cells were subdivided in CD4 and CD8 T cells (middle panels). Both CD4 and CD8 T cells were further classified as central memory (CM), effector memory (EM), CD45RA re-expressing cells (TEMRA), and naïve (N) subsets based on CD62L and CD45RA expression. (J) Frequency of CD4CM cells within the lymphocyte compartment of normal BM (n = 7) and regenerative BM (n = 28). *FDR < 0.05, ***FDR < 0.001, by Mann–Whitney U test and adjusted for multiple comparisons using the Benjamini–Hochberg procedure. *AML*=acute myeloid leukemia; *ANC*=absolute neutrophil count; *BM*=bone marrow; *FDR*=false discovery rate; *FSC*=forward scatter; *PLA*=platelets; *NK*=natural killer; *SSC*=side scatter.

chemotherapeutic injury is known to disrupt endothelial niches, at least in murine studies [26–28].

Interestingly, immune cell composition shortly after chemotherapeutic exposure was correlated with subsequent hematopoietic recovery in AML patients. In particular, the ratio between NK and CD4CM cells was strongly correlated to the duration of neutropenia in this cohort. The association between immune reconstitution and hematopoietic recovery may be a reflection of repopulation kinetics in the marrow in which the most resistant (CD4CM) cells increase after chemotherapeutic challenge with a decline in frequency on subsequent recovery of other immune subsets. In this context it is noteworthy that indeed NK cells are relatively short-lived cells (exhibiting a half-life of 7–10 days) [29], requiring constant replenishment by active cycling progenitors, likely intrinsically more vulnerable to chemotherapeutic injury. The underlying lineage specification of the bipotent NK/T-committed progenitor [30] implies that a shift in cell fate decision biased toward NK-cell generation may come at the cost of T-cell development [31,32], potentially further inversely coupling NK- and T-cell proportions within the lymphoid pool.

Alternatively (albeit not mutually exclusively), certain immune subsets may be active participants in the regeneration of HSPCs and their (myeloid) progeny. T cells have been reported to suppress granulopoiesis directly or indirectly by the release of hematopoietic-inhibitory factors [33,34], while NK cells may produce myelopoiesis-stimulating

factors such as interferon (IFN) γ [35], tumor necrosis factor (TNF) α [36,37], and granulocyte–macrophage colony-stimulating factor (GM-CSF) [38].

Of further interest, distinct T-cell subsets may have a role in the maintenance of hematopoietic stem cell (HSC) quiescence. In particular, Tregs have been implicated in HSC quiescence and their protection against oxidative stress [39]. The markers we used for discerning CD4 T-cell subsets did not allow the identification of Treg subsets, but it is conceivable that they play a role in HSC protection in the setting of chemotherapeutic exposure. In addition, it would be of interest to explore potential crosstalk between the CD4CM T-cell subset and HSPCs in this setting.

In addition to providing biologic insights and the instruction of future investigations toward immune cell contributions to hematopoietic recovery, our findings may have clinical relevance. Myelosuppression and persistent neutropenia are important determinants of treatment-related morbidity and mortality in AML. To date, no post-treatment prognostic factors have been identified predicting the duration of neutropenia in AML. Identification of an NK/CD4TM immune index predicting hematopoietic recovery allows future interrogation of its value to identify patients at increased risk for opportunistic infections. NK-Cell frequencies varied substantially between patients (Figure 1G) after chemotherapeutic exposure, and have been implicated in the direct killing of fungi by recognizing fungal pathogens and eliminating them by releasing lytic granules or by augmenting the antifungal host response [40]. It will be of interest to explore whether the extent of NK-cell depletion, in addition to neutropenia, predisposes patients to fungal infections. Insights may facilitate further tailoring of antifungal prophylaxis in AML treatment or the use of hematopoietic growth factors (G-CSF) in selected patients with low NK-cell constitution and predicted delayed recovery.

Taken together, the data provide novel insights into immune reconstitution after chemotherapeutic injury and implicate the balance between CD4CM and NK cells as a potential predictive factor for hematopoietic recovery in AML. Findings are anticipated to prompt future investigations into roles of specific immune subsets in HSPC recovery and instruct potential future tailoring of supportive treatment.

Conflict of interest disclosure

The authors declare that no conflicts of interest exist.

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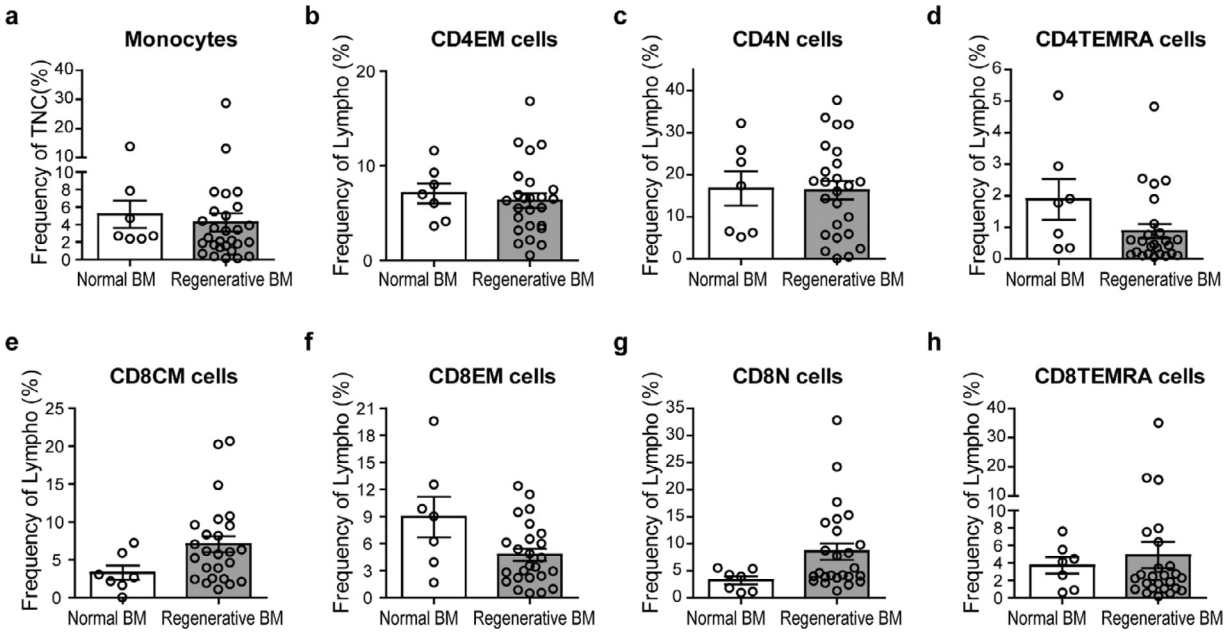
SUPPLEMENTAL TABLE 1

Table S1 Related to Figure 1 and 2. AML patient characteristics.

Sample ID	WHO diagnosis	Response	Age	Gender	ANC0.5 (days)	Pla50 (days)
1	AML with mutated NPM1	CR	65	Male	20	20
2	AML with myelodysplasia-related changes	CR	73	Male	30	**NA**
3	AML with mutated NPM1	CR	57	Male	**NA**	22
4	AML NOS	CR	53	Female	22	20
5	AML NOS	CR	70	Male	23	28
6	AML with mutated NPM1	CR	71	Male	**NA**	24
7	AML with mutated NPM1	CR	69	Male	24	22
8	AML NOS	CR	58	Female	25	21
9	MDS RAEB-2	CRi	72	Female	28	**NA**
10	Therapy related AML	CR	68	Male	20	19
11	AML with myelodysplasia-related changes	CR	68	Male	25	25
12	AML with mutated CEBPA	CR	33	Female	**NA**	24
13	AML with myelodysplasia-related changes	CR	70	Male	**NA**	23
14	Relapse AML	CR	59	Male	29	**NA**
15	AML with t(8;21)	CR	67	Male	**NA**	23
16	AML with t(8;21)	CR	37	Female	25	25
17	AML with mutated CEBPA	CR	27	Male	23	22
18	AML with mutated NPM1	CR	58	Female	26	23
19	AML NOS	CR	28	Female	22	23
20	AML with mutated NPM1	CR	35	Male	**NA**	28
21	AML with mutated NPM1	CR	67	Female	21	20
22	Relapse AML	CRi	67	Female	28	**NA**
23	AML with myelodysplasia-related changes	CR	69	Male	23	**NA**
24	AML with myelodysplasia-related changes	CR	69	Female	18	19
25	AML NOS	CRi	26	Female	**NA**	32
26	Therapy related AML	CR	65	Female	**NA**	**NA**
27	Therapy related AML	CR	65	Male	22	25
28	AML with mutated NPM1	CR	58	Female	22	21

BM was obtained from patients included in the HOVON103 and HOVON132 clinical trials. All patients dysplayed a CR/CRi (complete remission with incomplete hematologic recovery) at day 17 in subsequent marrow aspirations. Patient ID, WHO classification based on the 2017 ELN recommendations, therapy response, age, and gender are listed. The time needed to achieve 500 neutrophils per mm^3 blood after start of chemotherapy is also depicted. NA = not available.

SUPPLEMENTAL FIGURE 1



Supplemental Figure 1 Related to Figure 1. Frequencies of immune subsets in normal vs AML regenerative BM.

a. The frequency of monocytes within the total living nucleated cells in normal BM (n=7) and regenerative BM (n=28).

b-h. Frequencies of CD4EM T cells (b), CD4N T cells (c), CD4TEMRA T cells (d), CD8CM T cells (e), CD8EM T cells (f), CD8N T cells (g), and CD8TEMRA T cells (h) within the lymphocyte compartment in normal BM (n=7) and regenerative BM (n=28).