

Prognostic impact of immune cell recovery following first-line chemotherapy in acute myeloid leukemia

Ana Beatriz Pinto



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Ana Beatriz Sousa Pinto

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Orientador: Professor Doutor José Carlos Lemos Machado

Professor catedrático

Faculdade de Medicina da Universidade do Porto

Investigador Sénior e Coordenador do Grupo

Intercellular Communication and Cancer

Instituto de Patologia e Imunologia Molecular da

Universidade do Porto (IPATIMUP)

Instituto de Investigação e Inovação em Saúde (i3S) da

Universidade do Porto

Coorientador: Doutor Carlos Alberto Trindade Resende

Investigador Júnior do Grupo

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Universidade do Porto



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ABSTRACT

Acute myeloid leukemia (AML) is an aggressive myeloid malignancy, and the most frequently diagnosed leukemia in adults. Standard intensive chemotherapy still constitutes the backbone of first-line AML therapy, yet a considerable percentage of patients ultimately relapse. The immune system has long been shown to be capable of fully eliminating chemotherapy-resistant AML cells, as exemplified by the *graft-versus-leukemia* effect observed in patients treated via hematopoietic stem cell transplantation, one of the only therapies to offer a possibility of cure. The post-chemotherapy dynamics of immune replenishment, as well as its impact in residual AML elimination, have not yet been fully explored. In this pilot study, we quantified several immune subpopulations from peripheral blood samples of AML patients obtained after first-line chemotherapy, and compared these values to those obtained from a cohort of healthy individuals. In general, AML patients maintained a state of immune depletion throughout intensive chemotherapy and subsequent initial recovery, although some populations remained with healthy-comparable levels in all analyzed timepoints. Moreover, the prognostic impact of the frequencies of these immune subsets in samples collected after induction, after consolidation and approximately three months following intensive chemotherapy was also evaluated, and the quantification of the CD4⁺ CD45RA⁻ CD27⁻ (effector memory-like) T cell subset after induction chemotherapy was identified as a prognostic marker of superior survival. These findings suggest that some immune subsets quickly recover from intensive therapy-related depletion and may be involved in anti-leukemia responses, leading to delayed disease progression. These results provide further evidence of the potential advantage of coordinated administration of chemotherapy and immunotherapy, and a deeper understanding of the post-chemotherapy dynamics of immune replenishment may help identify preferential targets within specific treatment timepoints that could be amplified or emulated through novel or improved immunotherapeutic designs.

RESUMO

A leucemia mieloide aguda (LMA) é uma agressiva doença mieloide maligna, sendo a leucemia mais frequentemente diagnosticada em adultos. A quimioterapia intensiva convencional é ainda considerada a principal opção de terapia contra LMA; no entanto, uma grande percentagem dos doentes tratados eventualmente recidiva. O sistema imune é capaz de eliminar por completo células de LMA que resistiram à quimioterapia de primeira linha. Tal potencial é exemplificado pelo efeito enxerto-contra-leucemia associado à transplantação de células estaminais hematopoiéticas, atualmente uma das únicas terapias que oferece possibilidade de cura. A dinâmica de recuperação imunológica pós-quimioterapia, bem como o seu impacto na eliminação de células de LMA residuais, não foram ainda explorados por completo. Neste estudo preliminar, quantificámos algumas subpopulações imunes em amostras de sangue periférico de doentes com LMA extraídas após quimioterapia de primeira linha, e procedemos à comparação destes valores com os obtidos em amostras de dadores saudáveis. Em geral, os doentes permaneceram num estado de depleção imunológica ao longo das fases de quimioterapia e na recuperação inicial; contudo, algumas subpopulações apresentaram-se em todos os momentos em quantidades equiparáveis às dos indivíduos saudáveis. Adicionalmente, foi também avaliado o impacto prognóstico de contagens imunes em amostras recolhidas nos diferentes *timepoints* de quimioterapia intensiva, e foi possível identificar a frequência absoluta de células T CD4⁺ CD45RA⁻ CD27⁻ (*effector memory-like*) após quimioterapia de indução como um marcador preditivo de melhor sobrevivência. Este estudo parece indicar que algumas células imunes recuperam rapidamente após quimioterapia intensiva, e podem ainda estar envolvidas em repostas imunológicas contra a LMA, levando a um atraso na progressão da doença. Estes resultados comprovam a potencial vantagem de combinação de quimioterapia com imunoterapia, pelo que uma compreensão mais pormenorizada dos padrões de recuperação imune poderá ajudar a identificar componentes celulares específicos presentes numa particular fase do tratamento e cujas eventuais funções antileucémicas poderão ser amplificadas ou mimetizadas através de novos ou melhorados esquemas de imunoterapia.

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LIST OF ABBREVIATIONS

ABL1	ABL proto-oncogene 1
ADCC	Antibody-dependent cell-mediated cytotoxicity
ALAL	Acute leukemia of ambiguous lineage
AML	Acute myeloid leukemia
ANOVA	Analysis of variance
APC	Antigen-presenting cell or Allophycocyanin
APL	Acute promyelocytic leukemia
Arg II	Arginase II
ASXL1	Additional sex combs-like transcriptional regulator 1
BCOR	BCL-6 co-repressor
BCR	Breakpoint cluster region protein or B cell receptor
bZIP	Basic leucine zipper domain
CBFB	Core-binding factor subunit beta
CD	Cluster of differentiation
CEBPA	CCAAT/enhancer-binding protein alpha
CHIP	Clonal hematopoiesis of indeterminate potential
CI	Confidence interval
CLIP	Class II-associated invariant chain peptide
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CR	Complete remission
CREBBP	CREB-binding protein
CRT	Calreticulin
CT	Chemotherapy
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DAMP	Damage-associated molecular pattern
DDX41	DEAD-box helicase 41
DEK	DEK proto-oncogene
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule 1

DNMT3A	DNA methyltransferase 3 alpha
ELN	European LeukemiaNet
EVI1	Ecotropic virus integration site 1
EZH2	Enhancer of zeste homolog 2
FasL	Fas ligand
FBS	Fetal bovine serum
FISH	Fluorescence <i>in situ</i> hybridization
FITC	Fluorescein isothiocyanate
FLT3	Fms-like tyrosine kinase 3
FMO	Fluorescence minus one
FoxP3	Forkhead box protein P3
FSC	Forward scatter
Gal-9	Galectin-9
GATA2	GATA-binding protein 2
GBD	Global Burden of Disease
GMP	Granulocyte-monocyte progenitor
HC	Healthy controls
HLA	Human leukocyte antigen
HMA	Hypomethylating agent
HPV	Human papillomavirus
HR	Hazard ratio
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
ICD	Immunogenic cell death
IDH (1 and 2)	Isocitrate dehydrogenase (1 and 2)
IDO-1	Indoleamine 2,3-dioxygenase 1
IFN-γ	Interferon gamma
IL	Interleukin
ITD	Internal tandem duplication
KAT6A	Lysine acetyltransferase 6A
KIR	Killer immunoglobulin-like receptor
KMT2A	Lysine methyltransferase 2A

LDAC	Low-dose cytarabine
LMPP	Lympho-myeloid primed progenitor
LSC	Leukemic stem cell
MDS	Myelodysplastic syndrome
MECOM	MDS1 and EVI1 complex locus protein
MEP	Megakaryocyte-erythroid progenitor
MHC	Major histocompatibility complex
MLLT3	Myeloid/lymphoid or mixed-lineage leukemia; translocated to, 3
MPAL	Mixed-phenotype acute leukemia
MPN	Myeloproliferative neoplasm
MPP	Multipotent progenitor
MRD	Minimal residual disease
MRTFA	Myocardin-related transcription factor A
MYH11	Myosin heavy chain 11
N/A	Not applicable/not available
NaCl	Sodium chloride
NCR	Natural cytotoxicity receptor
NK	Natural killer
NKG2A	Natural killer group 2, member A
NKG2D	Natural killer group 2, member D
NKG2DL	Natural killer group 2, member D ligand
NKp44	Natural killer protein 44
NKp44L	Natural killer protein 44 ligand
NKR	Natural killer cell receptor
NPM1	Nucleophosmin 1
NUP98	Nucleoporin 98
NUP214	Nucleoporin 214
OS	Overall survival
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1

PD-L1	Programmed cell death ligand 1
PE	Phycoerythrin
PE-Cy7	Phycoerythrin-cyanine7
PerCP-Cy5.5	Peridinin-chlorophyll-protein-cyanine5.5
PML	Promyelocytic leukemia
PR	Partial remission
Prog	Progression
PRR	Pattern recognition receptor
RARA	Retinoid acid receptor alpha
RBM15	RNA-binding motif protein 15
RCF	Relative centrifugal force
RFS	Relapse-free survival
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT-qPCR	Real-time quantitative polymerase chain reaction
RUNX1	RUNX family transcription factor 1
RUNX1T1	RUNX1 partner transcriptional co-repressor 1
SF3B1	Splicing factor 3b subunit 1
SRSF2	Serine/arginine-rich splicing factor 2
SSC	Side scatter
STAG2	Stromal antigen 2
Tc	T cytotoxic
TCR	T cell receptor
TEMRA T cell	Terminally differentiated effector memory T cell expressing CD45RA
TET2	Tet methylcytosine dioxygenase 2
Tfh	T follicular helper
TGF-β	Transforming growth factor beta
T_H	T helper
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domain

TIM-3	T cell immunoglobulin and mucin domain-containing protein 3
TKD	Tyrosine kinase domain
TNF-α	Tumor necrosis factor alpha
TP53	Tumor protein p53
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cell
U2AF1	U2 small nuclear RNA auxiliary factor 1
UHCSJ	University Hospital Center of São João
V	Variable
WHO	World Health Organization
WT1	Wilms tumor protein 1
ZRSR2	Zinc finger CCCH-type, RNA-binding motif and serine/arginine-rich protein 2
$\alpha\beta$	Alpha-beta
$\gamma\delta$	Gamma-delta

Background

BACKGROUND

Acute myeloid leukemia

Definition and epidemiology

Acute myeloid leukemia (AML) is a type of hematological cancer defined by an uncontrolled expansion of abnormal immature cells of myeloid lineage (AML blasts) that accumulate in the bone marrow – where it leads to a severe disruption of normal hematopoietic function –, as well as in peripheral blood (1, 2). It is also the most prevalent acute leukemia among the adult population, accounting for 3 to 4 times more cases than acute lymphoblastic leukemia (2, 3). The Global Burden of Disease (GBD) study estimated that, in 2019, more than 197 000 individuals worldwide were living with AML, and almost 125 000 were newly diagnosed with this disease (4). In Portugal, these numbers were estimated to be around 510 and 470, respectively (4).

Median age at the time of AML diagnosis is 68 years (5), making AML a condition that predominantly affects older adults; moreover, this impact is not restricted to AML incidence, as its prognosis is also poorer – while 35 to 40% of AML patients 60 years or younger are cured from this disease, this number corresponds to only 5 to 15% for those older than 60 (3, 6). Because of its heterogeneity regarding molecular features and clinical behavior, AML is characterized by a diversity of subtypes associated with varying outcomes, and recent AML research efforts have been directed towards the development of more specific therapies targeting individual alterations (7); however, it appears that their benefit has only yet reached the younger patients (2). Nevertheless, and independently of age, AML is still currently associated with high mortality, which corresponded to 94 000 AML-related deaths globally and 400 in Portugal in 2019 (4).

Risk factors

For more than 70% of AML patients, there is not a clear factor that is identified as a probable cause for disease onset (2, 8). For the remaining 30%, however, a known preceding condition is suspected to, directly or indirectly, have contributed to AML etiology. These cases correspond to secondary AML, with 20% having progressed from a prior hematological malignancy, such as a myelodysplastic syndrome (MDS) or a myeloproliferative neoplasm (MPN), and almost 10% being the result of treatment for an

antecedent malignancy (8). The latter, also classified as therapy-related AML, typically emerges 1 to 7 years after exposure to ionizing radiation or cytotoxic chemotherapy agents, namely topoisomerase II inhibitors and alkylating agents (8).

AML mostly emerges as a *de novo* leukemia, resulting from the aging-associated accumulation of molecular alterations that drive the malignant transformation of hematopoietic progenitors; thus, older individuals are inherently conditioned to a higher risk of AML development (9). Nevertheless, other risk factors have been found to potentially impact AML leukemogenesis, including obesity (10), smoking (11) and benzene exposure (12). On the other hand, some hereditary and congenital conditions, such as bone marrow failure syndromes, familial syndromes associated with mutations in *CEBPA*, *GATA2* and *RUNX1* (respectively, CCAAT/enhancer-binding protein alpha, GATA-binding protein 2, and RUNX family transcription factor 1), and Down syndrome, although uncommon, also offer predisposition for AML (13).

Leukemic hematopoiesis

Throughout time and as individuals age, hematopoietic stem cells (HSCs), multipotent progenitors (MPPs) or common myeloid progenitors (CMPs) may acquire genetic alterations that result in a form of competitive advantage in comparison with other cells (14-16). Upon self-renewal and further differentiation, those alterations become sustained and define a clone of altered cells that dominate the overall hematopoietic populations (14). The occurrence of this process, known as clonal hematopoiesis (Figure 1), can be detected in older, healthy individuals, thereby being referred to as clonal hematopoiesis of indeterminate potential (CHIP), given that these mutations do not generate aberrantly-behaving hematopoietic cells (14). The most common of these alterations include mutations in genes associated with epigenetic mediators, such as *DNMT3A* (DNA methyltransferase 3 alpha), *TET2* (Tet methylcytosine dioxygenase 2) and *ASXL1* (additional sex combs-like transcriptional regulator 1) (14, 15).

Even though these mutations, by themselves, do not define any pathological condition, they are often detected in MDS and AML cases, indicating that subsequent molecular modifications may trigger an evolution into the realm of hematological malignancy (15). Therefore, CHIP can potentially be a preleukemic state that later evolves into AML (16).

Preleukemic stem cells can, therefore, develop molecular alterations that further induce the generation of leukemic stem cells (LSCs). Furthermore, quiescent LSCs give rise to AML progenitors that retain self-renewal capability while also originating immature

(but slightly more differentiated), highly proliferative AML blasts (16, 17). These cells, which also lack functional senescence and apoptosis mechanisms, constitute the bulk of AML (16, 17). The driver, pan-AML molecular aberrations that trigger this leukemic hematopoiesis include mutations in the *NPM1* (nucleophosmin 1), *FLT3* (Fms-like tyrosine kinase 3), *CEBPA*, *RUNX1*, and *WT1* (Wilms tumor protein 1) genes, as well as chromosomal abnormalities, such as the *CBFB* (core-binding factor subunit beta)-*MYH11* (myosin heavy chain 11) or *RUNX1-RUNX1T1* (RUNX1 partner transcriptional co-repressor 1) fusion genes (18). Of note, and as previously mentioned, MDS itself can also progress into (secondary) AML (16), thus possibly representing a preleukemic state as well; however, the genetic alterations that trigger this conversion are not necessarily the same, with some *de novo* AML mutations being rare in secondary AML, and vice-versa (16, 18).

Upon treatment, particularly with intensive chemotherapy, some LSCs may prevail within minimal residual disease (MRD) and later restart the proliferation process, leading to a full-scale relapse. On the other hand, more differentiated AML cells can also develop a novel stem cell phenotype upon therapy-induced stress, and, similarly, give rise to relapse (6, 16, 19).

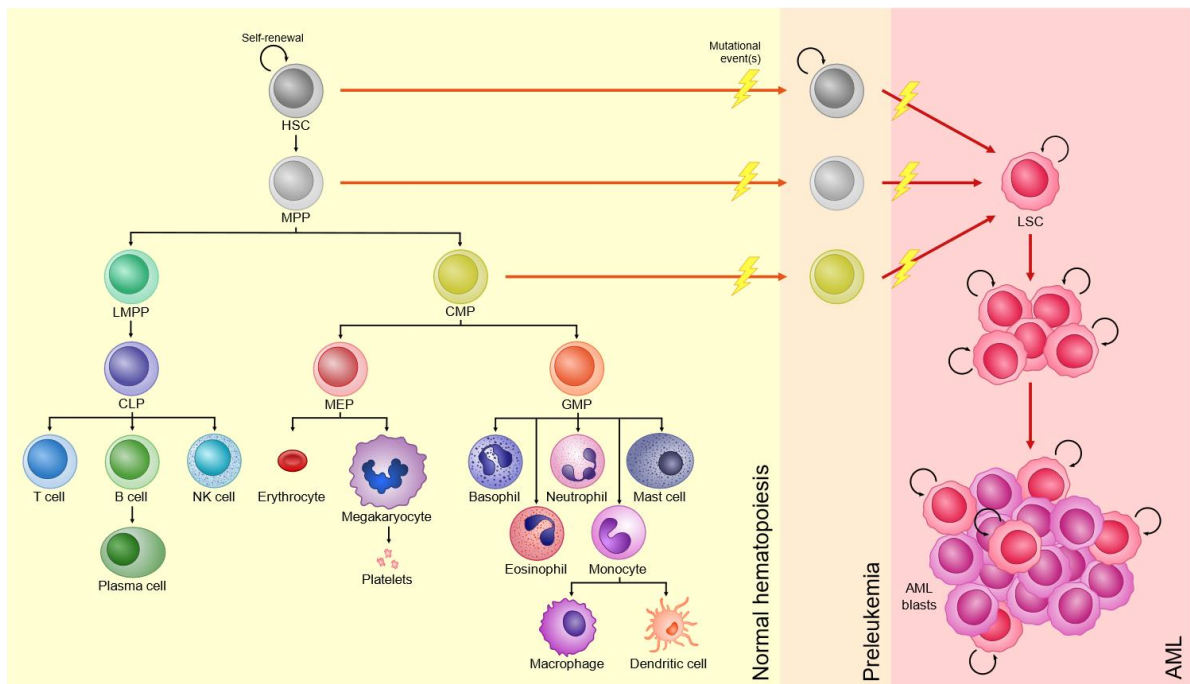


Figure 1. Normal and leukemic hematopoiesis (AML). HSC, hematopoietic stem cell; MPP, multipotent progenitor; LMPP, lympho-myeloid primed progenitor; CLP, common lymphoid progenitor; NK, natural killer; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-monocyte progenitor; LSC, leukemic stem cell.

Clinical presentation and diagnosis

Undiagnosed AML patients typically present with generalized symptoms, such as tiredness, decreased appetite and difficulty breathing (2, 20). Additionally, due to direct suppression by AML cells, normal hematopoiesis is impaired (9), often resulting in other common symptoms, such as anemia, bleeding, bruising and infections (20). Occasionally, patients exhibit renal failure due to the occurrence of tumor lysis syndrome (20).

A complete peripheral blood cell analysis typically reveals extensive cytopenia, with low neutrophil, erythrocyte and platelet counts, and, while blood counts of at least 20% myeloid blasts immediately suggest an AML diagnosis, further bone marrow biopsy evaluation is still required for confirmation and subtype classification (2). Briefly, and in general, AML diagnosis confirmation typically requires a quantification of at least 20% blasts in the bone marrow or blood, and immunophenotyping is performed to distinguish between myeloid or lymphoid lineages (2, 21). Furthermore, molecular cytogenetic assays, namely conventional karyotyping and fluorescence *in situ* hybridization (FISH), are conducted to identify specific chromosomal abnormalities contemplated in the different AML subtypes (21). Additionally, screening for mutations in specific genes, particularly *NPM1*, *FLT3*-ITD (*FLT3*-internal tandem duplication) and *FLT3*-TKD (*FLT3*-tyrosine kinase domain), *CEBPA*, *TP53* (tumor protein p53), *ASXL1*, *BCOR* (BCL-6 co-repressor), *DDX41* (DEAD-box helicase 41), *EZH2* (enhancer of zeste homolog 2), *IDH1* and *IDH2* (isocitrate dehydrogenases 1 and 2), *RUNX1*, *SF3B1* (splicing factor 3b subunit 1), *SRSF2* (serine/arginine-rich splicing factor 2), *STAG2* (stromal antigen 2), *U2AF1* (U2 small nuclear RNA auxiliary factor 1), and *ZRSR2* (zinc finger CCCH-type, RNA-binding motif and serine/arginine-rich protein 2), is also performed for subtype classification or prognostic purposes (21). Importantly, identification of AML-defining, recurrent genetic abnormalities lowers the requiring blast percentage to at least 10%, with the exception of the *BCR::ABL1* fusion gene, which still implies 20% blood or marrow blasts (counts of 10 to 19% correspond to a diagnosis of chronic myeloid leukemia in accelerated phase) (21).

The World Health Organization (WHO) classification of AML identifies distinct categories of this disease on the basis of specific genetic and chromosomal alterations, as well as morphological and immunophenotypical features, that associate with clinical evolution, prognosis and response to therapy (21). Its fifth update (2022) distinguishes 3 main AML subtypes: AML with defining genetic abnormalities, AML defined by differentiation, and myeloid sarcoma (22). Other AML-related neoplasms, namely acute leukemia of ambiguous lineage (ALAL), are also included in the general AML classification (21, 22). If applicable, AML diagnosis is complemented with additional diagnostic qualifiers, particularly in the cases of therapy-related AML, AML progressing from MDS or MDS/MPN

(identified over 3 months before AML diagnosis), and AML with germline predisposition (21). Further main subcategories are presented in Table 1 and thoroughly described in (21) and (22).

Table 1. Fifth edition of the World Health Organization classification of acute myeloid leukemias and AML-related neoplasms. Classifications retrieved from (21) and (22).

Acute myeloid leukemia subtypes and AML-related neoplasms
AML with recurrent genetic abnormalities
Acute promyelocytic leukemia (APL) with t(15;17)(q24.1;q21.2)/ <i>PML::RARA</i> fusion
AML with t(8;21)(q22;q22.1)/ <i>RUNX1::RUNX1T1</i> fusion
AML with inv(16)(p13.1q22) or t(16;16) (p13.1;q22)/ <i>CBFB::MYH11</i> fusion
AML with t(6;9)(p22.3;q34.1)/ <i>DEK::NUP214</i> fusion
AML with t(1;22)(p13.3;q13.1)/ <i>RBM15::MRTFA</i> fusion
AML with t(9;22)(q34.1;q11.2)/ <i>BCR::ABL1</i> fusion
AML with <i>KMT3A</i> rearrangement
AML with <i>MECOM (EVI1)</i> rearrangement
AML with <i>NUP98</i> rearrangement
AML with <i>NPM1</i> mutations
AML with <i>TP53</i> mutations
AML with biallelic or single, in-frame mutations in the bZIP domain of <i>CEBPA</i>
AML with defining myelodysplasia-related gene mutations (<i>ASXL1</i> , <i>BCOR</i> , <i>EZH2</i> , <i>RUNX1</i> , <i>SF3B1</i> , <i>SRSF2</i> , <i>STAG2</i> , <i>U2AF1</i> , and/or <i>ZRSR2</i>)
AML with defining myelodysplasia-related cytogenetic abnormalities
AML with other defined genetic alterations
AML, defined by differentiation
AML with minimal differentiation
AML without maturation
AML with maturation
Acute basophilic leukemia
Acute myelomonocytic leukemia
Acute monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Myeloid sarcoma
Acute leukemia of ambiguous lineage (ALAL)
ALAL, defined by recurrent genetic abnormalities
Mixed-phenotype acute leukemia (MPAL) with t(9;22)(q34.1;q11.2)/ <i>BCR::ABL1</i> fusion
MPAL with <i>KMT2A</i> rearrangement
ALAL with other defined genetic alterations
ALAL, immunophenotypically defined
MPAL, B/myeloid
MPAL, T/myeloid
MPAL, rare type
ALAL, not otherwise specified

Prognostic factors

Due to the intrinsic heterogeneity of the disease, AML outcomes are considerably variable, and there is a continuous effort towards enhancing the forecast of therapy response, relapse-free survival and overall survival, in order to improve patient management. Pretreatment prognostic factors are crucial to guide therapy selection decisions. In terms of patient-related variables, increasing age is known to associate with worse tolerance to intensive chemotherapy, particularly because of a general worsening of performance status and prevalence of additional comorbidities in the older individuals; on the other hand, older age, as well as the occurrence of prior myeloid malignancy, also appear to imply more aggressive molecular phenotypes, which then increase the probability of post-therapy resistance (23). Yet, the molecular landscape, particularly that evaluated during AML diagnosis, is an AML-related, independent, powerful prognostic factor that is especially relevant for the selection of consolidative therapy (5). Indeed, studies correlating genetic and chromosomal abnormalities and prognosis have led to the current definition of three main AML cytogenetic risk groups by the European LeukemiaNet (ELN) organization [Table 2; extensively detailed in (21)]. Nevertheless, attributing one of only three risk categories to all AML cases appears to still have its limitations (2, 23), mainly because as much as 50% of AML cases are, at diagnosis, presenting with normal cytogenetics, while the outcomes of these patients do not fit into one single prognostic category (5).

Table 2. 2022 European LeukemiaNet-defined cytogenetic risk groups and respective cytogenetic and molecular markers. Classifications retrieved from (21).

	Cytogenetic markers	Molecular markers
Favorable risk	t(8;21)(q22;q22.1)/ <i>RUNX1::RUNX1T1</i> fusion inv(16)(p14.1q22) or t(16;16)(p13.1;q22)/ <i>CBFB::MYH11</i> fusion	Mutations in <i>NPM1</i> , without <i>FLT3</i> -ITD Biallelic or single, in-frame mutations in the bZIP domain of <i>CEBPA</i>
Intermediate risk	t(9;11)(p21.3;q23.3)/ <i>MLL3::KMT2A</i> fusion Cytogenetic abnormalities not classifiable as favorable nor adverse	Mutations in <i>NPM1</i> , with <i>FLT3</i> -ITD Wild-type <i>NPM1</i> without <i>FLT3</i> -ITD (without adverse-risk genetic lesions) Molecular abnormalities not classifiable as favorable nor adverse
Adverse risk	t(6;9)(p22.3;q34.1)/ <i>DEK::NUP214</i> fusion t(v;11q23.3)/ <i>KMT2A</i> rearrangement t(9;22)(p34.1;q11.2)/ <i>BCR::ABL1</i> fusion t(8;16)(p11.2;q13.3)/ <i>KAT6A::CREBBP</i> fusion inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/ <i>GATA2, MECOM (EVI1)</i> t(3q26.2;v)/ <i>MECOM (EVI1)</i> rearrangement -5 or del(5q); -7; -17/abn(17p) Complex karyotype Monosomal karyotype	Mutations in <i>ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1</i> , and/or <i>ZRSR2</i> (and absence of favorable-risk-defining markers) Mutations in <i>TP53</i> at a variant allele fraction of at least 10%

Notwithstanding that patient- and AML-associated pretreatment parameters offer valuable information to help guide AML treatment, combining all these data into a model aiming to forecast relapse-free and overall survival is only accurate in up to 80% of cases, indicating further need to input additional prognostic variables (23). In fact, post-treatment factors, notably fulfillment of complete remission (CR) criteria and evolution of MRD during follow-up, are important outcome predictors essential to decide further therapeutic approaches (5).

MRD, determined by flow cytometry or real-time quantitative polymerase chain reaction (RT-qPCR), is evaluated at the ending of a certain therapy for the assessment of response criteria and identification of remission status, and throughout multiple follow-up timepoints to early detect an eventual relapse (5, 21). Several studies have demonstrated that detection of MRD, independently of the methodology used for its assessment, is strongly associated with higher relapse rates and poorer survival (24).

Classically, CR is defined by a relative frequency of bone marrow blasts lower than 5%, absence of circulating blasts, absolute neutrophil and platelet count recovery, as well as no evidence of extramedullary AML (21). Moreover, patients who achieve all CR criteria except neutrophil and platelet recovery may also be regarded as exhibiting CR with incomplete or partial hematological recovery; the morphologic leukemia-free state, on the other hand, only requires achievement of the CR blast clearance metrics (21). Finally, partial remission (PR) is equivalent to CR, with the exception that bone marrow blast percentage is between 5 to 25%, having decreased at least 50% from the pretreatment counts. Considering the aforementioned relevancy of MRD for prognostic evaluation, a novel response category, CR without MRD, is defined by all CR criteria, with the additional confirmation of no MRD detection (21). Patients who exhibit this type of therapy response are less likely to relapse than those achieving other CR categories, but having detectable MRD (24).

Other prognostic factors have been uncovered in several studies, involving specific genetic alterations, epigenetic modifications, immune mediators and therapy response evaluations; however, most are yet to be officially incorporated into AML patient management guidelines (25).

AML treatment

Even though several novel therapies and combinatorial regimens have been recently approved for use in AML, the practical impact on patient management has been limited, primarily because most of these new agents are designed to target a particular molecular

alteration, or are only applicable to patients belonging to a specific AML diagnostic subcategory (26). Therefore, the baseline of therapy selection continues to be the assessment of whether the patient is suitable for intensive chemotherapy. Because of its aggressiveness, this therapy is not adequate for older AML patients or those with poor performance status or significant comorbidities, due to a higher probability of therapy-related mortality (5). Of note, the acute promyelocytic leukemia subtype is an exception of this rule, since a highly effective therapeutic algorithm based on the administration of all-trans retinoic acid and arsenic trioxide is already in practice and leads to survival rates of up to 95% (5, 27).

Induction chemotherapy

Conventional first-line intensive chemotherapy for AML consists of an initial induction therapy aiming to induce complete remission, leading to bone marrow and peripheral blood blast clearance, followed by a post-remission consolidation strategy for the purpose of eliminating any residual AML blasts (28). The standard induction chemotherapy approach, the “7 + 3” regimen, comprises of a 7-day continuous infusion of cytarabine at a daily dose of 100 to 200 mg/m², combined with an anthracycline, mainly daunorubicin (60 to 90 mg/m²) or idarubicin (10 to 12 mg/m²), for the first 3 days of the schedule (23, 28). This therapeutic regiment induces CR in 60 to 80% of AML patients aged 59 years or younger, but only in 40 to 60% of the older individuals (23). Recently approved therapeutic strategies also include the combination of the “7 + 3” regimen with targeted therapy agents, namely *FLT3* inhibitors like midostaurin in *FLT3*-mutated AML, and gemtuzumab ozogamicin, a cytotoxic agent conjugated with anti-CD33 antibodies, for the treatment of CD33-positive favorable or intermediate risk AML. Additionally, CPX-351, a liposome-encapsulated combination of cytarabine and daunorubicin, is approved as monotherapy in alternative to “7 + 3” in patients diagnosed with therapy-related AML or AML with myelodysplasia-related changes (5, 29).

Post-remission therapy

Post-remission treatment in AML includes two main possibilities: consolidation chemotherapy and/or, in selected patients, allogeneic hematopoietic stem cell transplantation (HSCT). Consolidation chemotherapy consists of up to 4 cycles of high-dose cytarabine monotherapy, commonly administered as 3-day cycles of 2 doses of 2 to 3 g/m² per day (every 12 hours) (5, 23). However, recent research has suggested that intermediate doses of cytarabine (1 to 1.5 g/m²) achieve similar outcomes, and high-dose cytarabine is currently only recommended to favorable risk AML patients (5). In an allogeneic HSCT, the AML patient is treated with a conditioning regimen consisting of highly cytotoxic radiation and/or chemotherapy, for the elimination of MRD AML cells and induction of

immunosuppression to facilitate stem cell engraftment. Then, human leukocyte antigen (HLA)-matched donor stem cells are infused in the patient, which reconstitute the hematopoietic landscape, including immune cells. A recovered immune system, then, induces a response against any remaining AML blasts, in the so-called *graft-versus-leukemia* effect; however, donor immune cells also recognize and react against normal host tissue, resulting in *graft-versus-host* disease. This disorder, together with the prevalence of infections during immune recovery, contributes to the therapy-related mortality associated with this treatment (2, 30).

While intensive chemotherapy results, as mentioned, in high rates of CR, a majority of patients eventually relapse, and long-term overall survival ranges between 35 and 45% for patients younger than 60 years of age, and 10 to 15% for the older age group (5). Allogeneic HSCT, on the other hand, offers the best chance of cure; yet, higher risk of therapy-related mortality, as well as scarce HLA-matched donors, challenge the overall benefit of this treatment (31). Thus, selection of patients for further post-induction HSCT or consolidation chemotherapy is based on the assessment of relapse risk, patient performance status and availability of stem cell donors (5). Other variations of HSCT, including autologous HSCT, which uses previously collected patient stem cells as the infused graft, or allogeneic HSCT with reduced-intensity conditioning, that relies on less intensive and toxic radiation/chemotherapy, can also serve as alternatives for, respectively, lower-risk patients or older individuals with relevant comorbidities (6, 23).

Patients unfit for induction chemotherapy

Currently, alternatives for patients who cannot undergo induction chemotherapy are therapy with hypomethylating agents (HMAs) or low-dose cytarabine (LDAC), less intensive alternatives that are better tolerated (23). HMAs, such as azacitidine or decitabine, are thought to revert DNA methylation silencing of tumor suppressor genes in AML blasts, while also activating antileukemic immune cell responses (32). Although only 20 to 30% of patients treated with these agents exhibit any substantial response, HMA therapy is preferred over intensive chemotherapy because of its lower therapy-related mortality, leading to slightly superior outcomes (5). LDAC, on the other hand, is associated with 15 to 25% response rates, but median overall survival only reaches 5 to 6 months (23). In general, both therapeutic approaches lead to equivalent overall survival; nevertheless, LDAC appears to be associated to higher CR rates, whereas HMAs exert superior effects in patients with complex karyotype AML (2, 28). Because of low CR rates and poor survival associated with these agents, the inclusion of unfit patients in clinical trials is advised (23).

Immune dynamics in AML

The immune system and its interaction with cancer

The immune system is defined by a collection of cells, the biological processes they conduct, molecules they secrete, and tissues and organs they are developed in, whose fundamental functions are to provide protection against invading pathogens and toxins, as well as to detect and eliminate transformed host cells that could potentially evolve into cancer (33). These roles are accomplished by the ability of immune cells to specifically discriminate between “self” and abnormal, “non-self” antigens, and then coordinate an effective response aiming to destroy cells harboring the latter (33, 34).

Immune defenses can generally be encompassed into mechanisms of innate or adaptive immunity, which are mostly distinguished by the type of response they trigger. Innate immune responses typically constitute quick reactions upon exposure to abnormal antigens, without the requirement of a prior encounter; the non-specificity of these responses implies an absence of immunological memory potential, and a subsequent exposure to the same antigen results in a response of similar scale (33, 35). They, thus, rely on the recognition of pathogen-associated molecular patterns (PAMPs) conserved throughout a multitude of foreign pathogens and essential to their viability, and damage-associated molecular patterns (DAMPs), commonly expressed by damaged or lysing host cells, by a limited range of pattern recognition receptors (PRRs) encoded in the germline (35). On the other hand, and although first-time triggering of adaptive defenses results in slower effector responses, these are highly specific to the respective antigen, and allow for the generation of long-term immunological memory capable of inducing fast and effective responses upon later exposure to that particular antigen (33). The potential for high specificity arises from the diversity of antigen-specific receptors within the adaptive immune cell pool, which are randomly generated by somatic gene rearrangement during immune cell development (36). Moreover, adaptive immune cells can be induced directly by cells harboring the respective antigen, or via stimulation by activated antigen-presenting cells (APCs), whose functions are to internalize, process and present potential antigens to other immune cells, particularly T lymphocytes (36, 37). Innate and adaptive immune responses are also accomplished by distinct effector cells: innate immunity is orchestrated by natural killer (NK) cells, granulocytes (basophils, neutrophils and eosinophils), macrophages and mast cells, whereas mechanisms of adaptive immunity are mostly carried out by B and T cells (33).

As previously stated, it has been long understood that the immune system can contribute to cancer prevention due to its immunosurveillance function. This process is thought to occur through three possible mechanisms: by detecting and eliminating cancer-inducing viruses, such as the human papillomavirus (HPV), implicated in cervical cancer development; by limiting and resolving tumor-promoting inflammatory responses; and by recognizing and removing transformed host cells, thereby hampering their proliferation and likely subsequent evolution into a clinically detectable cancer (38-40). The latter is of particular significance, since transformed cells often exhibit aberrant antigens that potentially trigger innate and adaptive immune cells. These can be of two main types: tumor-specific antigens, which are abnormal proteins arising from the expression of mutated or viral genes (neoantigens); or tumor-associated antigens, resulting from genes whose expression is typically limited to specific tissues, or from overexpression of unaltered genes (38, 41, 42).

Although several lines of evidence illustrate the importance of the immune system in hindering cancer development [reviewed in (43)], it is also understandable that immunosurveillance is not an infallible process, given the high incidence and mortality associated with cancer (44). In fact, aberrant cells are able to proliferate and evolve into full-blown tumors because they continuously acquire molecular modifications that allow them, not only to escape anticancer immune efforts, but also to subvert the immune system into tolerating or even promoting tumor progression (38). This process, commonly termed cancer immunoediting, occurs throughout cancer evolution and is thought to comprise three main phases: elimination, in which the immune system retains the ability to identify and destroy transformed host cells; equilibrium, which occurs when the immune system fails to eliminate some immune-resistant cells, although their growth remains limited; and escape, in which further mutations allow these aberrant cells to greatly bypass immune control and proliferate, giving rise to a clinically visible tumor (38). Indeed, the acquisition of molecular features that facilitate anticancer immune evasion and incite protumor immune responses is still generally considered to be one of the hallmarks of cancer (45, 46).

Lymphocytes: main immunosurveillance effectors

Numerous studies have demonstrated that the cancer immunoediting process can be manipulated through immune-based therapies aiming to redirect it back into the elimination or equilibrium phases, either by increasing the quantity of certain immune subpopulations, removing the suppressive pressures over the immune system, or optimizing tumor antigen presentation by reverting immune escape mechanisms employed by cancer cells (38, 47,

48). As a result, several approved immunotherapies are currently in use in the clinical setting for treatment of specific types of cancer (49).

In the case of AML, there is only one immunotherapy drug approved for clinical use, gemtuzumab ozogamicin; as previously mentioned, its mechanism of action is based on the targeted destruction of cells binding its anti-CD33 antibody portion, thereby being specifically effective in CD33-positive AML (50). Yet, another therapeutic strategy which avails the immune system to eliminate AML is the transplantation of allogeneic hematopoietic stem cells. Indeed, it remains the intervention offering higher chances of cure, therefore illustrating how an effective immune system can contribute to cancer elimination. As happens with cancer immunosurveillance in general, the immune cells likely to exert a more significant role in post-HSCT residual AML cell elimination are the immune cells arising from the hematopoietic lymphoid lineage, particularly T, B and NK cells (30, 51, 52).

T lymphocytes

T lymphocytes are important coordinators and effectors of the adaptive immune system. Arising from hematopoietic progenitors in the bone marrow, T cells undergo further maturation and selection in the thymus, where, through gene recombination, they acquire expression of a unique T cell receptor (TCR) that recognizes small antigens (53, 54). There are two main subpopulations of T cells: the conventional alpha-beta ($\alpha\beta$) T cells, comprising the vast majority of the circulating T cell pool and whose TCRs include α and β chains, and the rarer gamma-delta ($\gamma\delta$) T cells, with TCRs harboring γ and δ variants (54, 55).

$\alpha\beta$ T cells can be further subdivided into two major populations: the CD8-positive cytotoxic T cells, the main effectors that directly kill abnormal (either virus-infected or mutated) host cells, and CD4-positive helper T cells, whose fundamental function is to coordinate an overall immune response by recruiting and activating other immune cells, such as CD8+ T, B and NK cells (54, 56, 57). Both CD4+ and CD8+ T cells initially emerge out of the thymus as quiescent naïve T cells, until activation by an APC. The latter process generally requires processing and loading of a peptide antigen into molecules of the major histocompatibility complex [MHC, also referred to as HLA in humans] (54, 58). CD4+ T cells are primed by APCs through antigens presented in MHC class II of professional APCs, such as dendritic cells, macrophages and B cells; on the other hand, CD8+ T cells recognize antigen-MHC class I complexes potentially displayed by any nucleated cell (54, 57). However, TCR engagement is insufficient to induce T cell activation, and additional co-stimulatory signaling, such as binding of CD28 in $\alpha\beta$ T cells to CD80 or CD86 expressed by APCs, is necessary (54, 59). Upon activation, these T cells begin to proliferate and differentiate into effector T cells, thereby exerting their immune response roles: CD4+ T

cells release cytokines and orchestrate the response of other immune cells, and CD8⁺ T cells trigger target cell apoptosis through discharge of granules containing perforin and granzymes, while also producing other immune-stimulating cytokines (54). Moreover, CD4⁺ T cells can differentiate into various distinct effector subsets, including T helper (T_H)1, T_H2, T_H9, T_H17, T_H22 and T follicular helper (T_{fh}) cells, depending on the cytokine environment and co-stimulation signaling pattern during activation, and each of these subsets releases a distinct profile of cytokines directing different types of immune response (54, 57). CD8⁺ T cells also differentiate into multiple effector subsets, namely T cytotoxic (T_c)1, T_c2, T_c17 and T_c22, whose cytokine production profiles highly correlate with the equivalent-named helper T cell subpopulations (54).

Following resolution of the primary immune response, approximately 90 to 95% of effector T cells undergo apoptosis, and the remaining cells differentiate into long-lived memory T cells, which typically entail lower antigen thresholds for activation and are capable of faster and more robust responses (60, 61). These cells are then required to scan potential sites of likely secondary antigen encounter, and while some memory T cell populations permanently remain in peripheral tissues (tissue-resident memory T cells), other subsets exert their immunosurveillance by continuous migration. Particularly, effector memory T cells traffic peripheral blood and non-lymphoid organs, whereas central memory T cell circulation is mostly restricted to blood and lymphoid tissues (62, 63). Besides their different localization patterns, these memory T cells also generally exhibit distinct behavior upon secondary stimulation. Effector memory T cells are thought to retain improved effector functions, with quicker and greater secretion of effector cytokines, while central memory T cells appear to proliferate faster following activation (53, 64, 65). Another circulating memory T cell subpopulation includes terminally differentiated effector memory T cells expressing CD45RA (TEMRA T cells). Naïve T cells are characterized by CD45RA expression, which is lost following T cell activation and memory T cell generation. However, TEMRA T cells re-acquire expression of CD45RA and are thought to exhibit impaired functional and proliferative activity, accompanied by increased expression of senescence markers; moreover, these cells seem to accumulate with age (66, 67). How and which effector T cells are selected to survive as memory T cells, how the different memory T cell subsets are originated, as well as whether they can be converted into one another are concepts still remaining to be fully elucidated (62).

Because uncontrolled immune reactions can be detrimental, leading to extensive normal tissue damage, some immune cells are responsible for regulating and limiting the extension of inflammatory damage, promoting homeostasis (68, 69). One of these subpopulations includes the CD4⁺ regulatory T cells (Tregs), defined by expression of the forkhead box protein P3 (FoxP3) transcription factor (68, 70). Beyond their duties in immune

response resolution, Tregs exert the crucial roles of implementing peripheral tolerance, suppressing responses against self-antigens by immune cells that wrongly survived negative selection, and containing reactions against harmless foreign antigens, mitigating the development of autoimmune disorders and allergic reactions, respectively (33, 70, 71). Tregs suppress numerous immune cell types, including CD4⁺ and CD8⁺ effector T cells, and NK and B cells, through the release of immunosuppressive cytokines, such as interleukin (IL)-10, IL-35 and transforming growth factor beta (TGF- β), direct elimination by secretion of perforin and granzymes, and also metabolically, by, for instance, limiting available levels of IL-2 (important for T cell survival) via CD25 (IL-2 receptor) overexpression (70). Another intrinsic mechanism of immune response regulation, particularly that of T cells, is the expression of inhibitory immune checkpoints, consisting of pairs of receptors and their respective ligands, expressed by a myriad of cells, including CD4⁺ and CD8⁺ T cells, APCs and Tregs, that, when bound, result in a signaling cascade that ultimately leads to effector T cell suppression (72, 73). Some of the most studied immune checkpoint molecules include the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) and T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) (73, 74).

Despite belonging to the same CD3-positive T cell population, $\gamma\delta$ T cells exhibit substantial differences from their $\alpha\beta$ counterparts. $\gamma\delta$ T cells are considerably rarer, generally representing only 0.5 to 16% of total peripheral blood T cells; nevertheless, they can extensively proliferate as a response to certain antigens, reaching up to 60% of the circulating T cell pool (75). Moreover, and upon activation, these cells manifest behaviors typical of both innate and adaptive, $\alpha\beta$ T cell-like responses. Indeed, similarly to (adaptive) conventional T cells, $\gamma\delta$ T cells harbor TCRs generated through gene rearrangement in the thymus, leading to TCR variability and subsequent clonal expansion following activation (76); additionally, $\gamma\delta$ T cells appear to develop recall memory to specific antigens and differentiate into memory phenotypes equivalent to those of $\alpha\beta$ T cells (77, 78). Nevertheless, $\gamma\delta$ T cell activation is not dependent on antigen processing or MHC-mediated presentation, nor does it require the CD28 and CD80/CD86 co-stimulation, and results in faster, innate immune cell-like, responses following antigen encounter (79, 80). Furthermore, activation of these cells can also be achieved by stimulation of typically innate cell receptors, such as natural killer cell receptors (NKR) and PRRs recognizing PAMPs or DAMPs (80, 81). Human $\gamma\delta$ T cells can be characterized by the variable (V) region of their TCR δ -chain, with the main subsets being the V δ 1⁺ and V δ 2⁺ T cells; the latter are almost exclusively associated with V γ 9 co-expression, thereby being commonly referred to as the V γ 9V δ 2 T cell subset (82). Briefly, V δ 1⁺ T cells are typically located in peripheral

tissues, where they perform less cytotoxic functions but release several cytokines, such as IL-4 and IL-17. On the other hand, V γ 9V δ 2 T cells comprise the vast majority of circulating $\gamma\delta$ T cells and are associated with recognition of non-peptide antigens (particularly phosphoantigens) and various effector functions, namely potent cytotoxic activity through the release of perforin and granzymes, and production of interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α), as well as induction of target cell apoptosis via expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) (81, 83). These cells can additionally exert antibody-dependent cell-mediated cytotoxicity (ADCC), by which CD16 receptors at their surface recognize antibodies bound to a target cell, triggering the destruction of the latter (82). Interestingly, V γ 9V δ 2 TCR+ $\gamma\delta$ T cells can also function as APCs, illustrating the versatility of this particular subset (82).

B lymphocytes

B lymphocytes are CD19-expressing adaptive immune cells that constitute the main components of the humoral response, in which the main endpoint is the production of antigen-specific immunoglobulins (antibodies) that mediate the effector response (84-86). In adults, B cells are developed and begin to mature in the bone marrow, where, similarly to T cells, the B cell receptor (BCR) – which includes one surface immunoglobulin – is originated through gene recombination mechanisms, giving rise to a high variability of receptors potentially recognizing a multitude of antigens. Furthermore, B cells further mature in the spleen, and, throughout their development, undergo selection to eliminate those recognizing normal self-antigens. After complete maturation, some B cells remain in the spleen, whereas others are released into the peripheral blood and migrate to secondary lymphoid tissues (85, 86). B cell activation by peptide antigens requires assistance by CD4+ T cells: briefly, the process begins with the interaction of an antigen with a BCR, with the former being internalized, processed and presented to CD4+ T cells via MHC class II and associated co-stimulation molecules. Following recognition, the CD4+ T cell becomes activated and expresses additional co-stimulation markers, as well as secretes B cell-triggering cytokines, leading to complete B cell activation. These B cells then further proliferate and differentiate into antibody-secreting plasma cells and, later, memory B cells that confer long-time protection against cells presenting the inciting antigen (84, 87). Antibodies exert effector functions through several mechanisms, such as direct pathogen neutralization, ADCC triggering, complement system activation, and stimulation of other immune cells (85). Besides antibody production and CD4+ T cell stimulation, B cells also exert other immune functions, particularly antigen presentation to CD8+ T cells via MHC class I and immunoregulatory cytokine release (84-86).

Natural killer cells

NK cells, although similarly part of the lymphoid lineage, and as opposed to most T and B cells, mainly operate as effectors of innate immunity (88). Representing about 5 to 20% of the circulating lymphocyte population, these cells are also present in multiple tissues, including skin, lungs, and gut, as well as in the bone marrow, lymph nodes and tonsils (89). These cells, generally characterized by CD56 expression and absence of the CD3 marker, are the most important innate player of the immune defense against transformed and virus-infected host cells (88, 89). Their activation does not require prior antigen encounter nor antigen presentation, and results in quick effector responses, including, not only cytolytic activity against target cells, but also pro-inflammatory cytokine secretion, namely IFN- γ and TNF- α , leading to the stimulation of other immune cells (88, 89). NK cells do not express clonotypic receptors, such as the TCR and BCR in T and B cells, respectively; their responses are, thus, incited by binding of activating surface receptors [such as natural cytotoxicity receptors (NCRs) like natural killer protein 44 (NKp44) 44, NKG2D (natural killer group 2, member D), DNAM-1 (DNAX accessory molecule 1) and PRRs] to non-self, pathogen-derived or stress-induced molecules (88-90). However, NK cell responses are further compensated by engagement of inhibitory receptors, such as NKG2A (natural killer group 2, member A), inhibitory KIRs (killer immunoglobulin-like receptors), as well as immune checkpoint molecules such as CTLA-4, PD-1 and TIM-3; therefore, the relative strength of activating and inhibitory signals determines whether or not NK cell induction occurs (90). One of the most prominent ligands recognized by NK cell inhibitory receptors is the MHC class I complex. Since most somatic, normal cells constitutively express MHC class I, its engagement by inhibitory receptors signals the NK cell not to destroy the bound cell. This also implies that any abnormal cell that downregulates MHC class I expression as a way to evade CD8⁺ T cell-mediated destruction can potentially be targeted by NK cells (89, 90). Cytolytic activity following successful NK cell triggering may occur through different mechanisms: NK cells can eliminate target cells through unloading of granules containing perforin and granzymes, by induction of apoptosis through TRAIL and FasL, or through binding of CD16 to antibodies coating the target cell, consequently triggering ADCC (88, 89). There are two main NK cell subsets: CD56dim CD16⁺ NK cells, the most common NK cell subpopulation in the peripheral blood, exerting extensive cytotoxic activity, while also producing IFN- γ upon activation, and CD56bright CD16⁻ cells, which are more common in the lymph nodes and tonsils, and are typically not much cytotoxic, instead being effective cytokine producers (90, 91).

Immune defense and evasion in AML

AML cells, as with cancer in general, have been shown to employ various strategies to escape the anti-tumoral effector functions of immune cells, particularly of lymphocytes (Figure 2). For instance, AML cells harbor impaired mechanisms of antigen processing and presentation, including downregulation of MHC expression (92). This decreased display of MHC, especially of class II molecules, is particularly observed in patients relapsing after HSCT (92-95). Moreover, AML is also associated with increased expression of immune checkpoint molecules and their ligands, leading to a disruption of T and NK cell activation mechanisms. In particular, overexpression of CTLA-4, programmed cell death ligand 1 (PD-L1, ligand of PD-1), galectin-9 (ligand of TIM-3), and CD112 and CD155 (ligands of TIGIT), has been documented in AML blasts and is typically associated with poorer outcomes (92, 96-106). High expression of CTLA-4 in Tregs of AML patients has also been reported (107, 108). Additionally, AML blasts produce and release increased quantities of reactive oxygen and nitrogen species, as well as of two enzymes, indoleamine 2,3-dioxygenase 1 (IDO-1) and arginase II, whose reactions result in a decrease in, respectively, tryptophan and arginine, all leading to an inhibition of T cell expansion; moreover, IDO-1 is also known to contribute to the peripheral conversion of CD4⁺ T cells into Tregs in AML, leading to higher numbers of these cells in AML patients (109-113). AML appears to evade NK and $\gamma\delta$ T cell-based responses through induction of activating receptor downregulation, including NKG2D and DNAM-1, in these immune cells, and of NKG2D and NCR-specific ligands on AML blasts (92, 99, 106, 114-117). AML patients have also been shown to exhibit low surface expression of NCRs in NK cells, consequently leading to ineffective cytotoxic functions of these cells (118, 119).

Immune evasion mechanisms by AML cells have been further evidenced by the observation of immune cell functional impairments in AML. T cells have been found to exhibit defective capability to form immune synapses with AML cells, as well as aberrant activation-associated gene expression profiles (120). Moreover, these cells, including their CD4⁺, CD8⁺ and/or $\gamma\delta$ subtypes, typically present with impaired effector cytokine secretion, and are often associated with exhausted and senescent phenotypes (101, 116, 121, 122). Other abnormal immune functional behaviors include reduced proliferation capability by V γ 9V δ 2 T cells and hampered cytokine synthesis by NK cells (114, 123).

All these multiple immune dysregulations also translate into distinct immune cell counts, when comparing AML patients with healthy individuals. Indeed, some studies reported lower frequencies of T, B, NK and $\gamma\delta$ T cells, and higher levels of Tregs, in AML patients, in relation to controls (99, 100, 107, 108, 116, 121, 124, 125); however, other research suggests the opposite results (114, 120, 126).

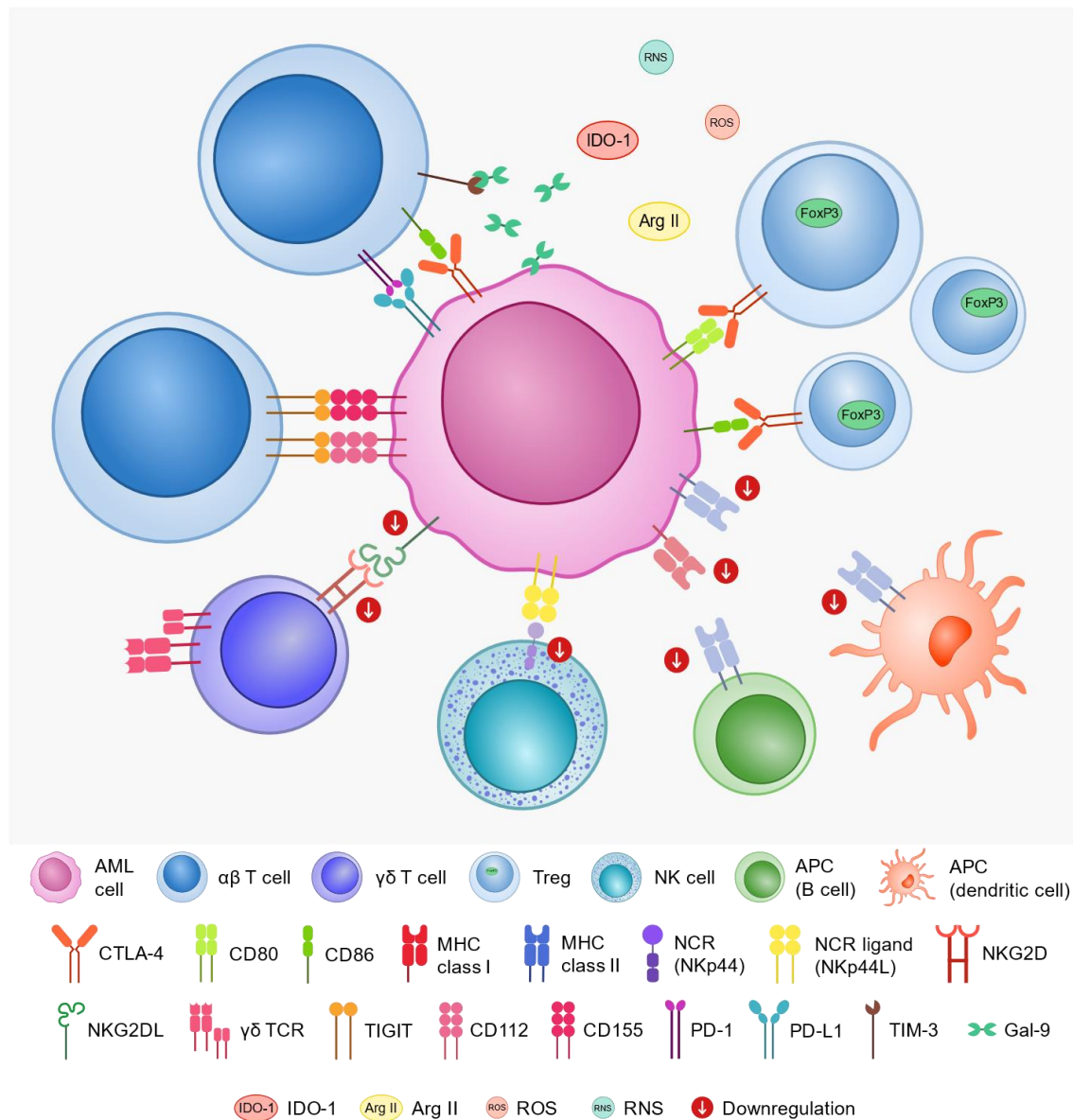


Figure 2. Mechanisms of immune evasion in AML. Treg, regulatory T cell; NK, natural killer, APC, antigen-presenting cell, CTLA-4, cytotoxic T lymphocyte-associated antigen 4; MHC, major histocompatibility complex; NCR, natural cytotoxicity receptor; NKp44, natural killer protein 44; NKp44L, NKp44 ligand; NKG2D, natural killer group 2, member D; NKG2DL, NKG2D ligand; TCR, T cell receptor; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domain; PD-1, programmed cell death protein 1; PD-L1, PD-1 ligand; TIM-3, T cell immunoglobulin and mucin domain-containing protein 3; Gal-9, galectin-9; IDO-1, indoleamine 2,3-dioxygenase 1; Arg II, arginase II; ROS, reactive oxygen species; RNS, reactive nitrogen species.

In spite of the observed immune dysfunction, data from some studies suggests that immune defense mechanisms are not entirely shut down in AML, given that increased immune counts in AML patients appear to confer protection from disease progression, resulting in improved outcomes (116, 127). However, other studies indicate that lower counts of the same cells are instead present in patients achieving complete remission, and are associated with extended remission, relapse-free and overall survival times (115, 128).

On the other hand, because Tregs are considered to be immunosuppressive, higher counts of these cells have been typically associated with worse outcomes (100, 107, 124). Interestingly, higher levels of Tregs in HSCT grafts have also been found to associate with superior survival and reduced mortality, as well as with lowered risk of acute graft-*versus*-host disease, with the latter likely explaining the benefits of higher immunosuppressive cell counts in this setting, through suppression of graft immune cell responses against normal host cells (129).

Overall, studies assessing the functional status and prognostic value of different immune subpopulations still present inconsistent results, likely a reflection of the heterogeneity of AML and patient-intrinsic features, as well as of variability in study design. Moreover, most evaluate the immune landscape in AML at one timepoint, commonly at diagnosis, and do not take into consideration the dynamics of clonal evolution and therapeutic regimens that may affect immune-AML interactions and, consequently, disease outcome.

Aims of the study

AIMS OF THE STUDY

Despite extensive research data suggesting the potential of reverting immune dysregulations in AML via immunotherapeutic strategies [reviewed in (50)], various AML-intrinsic factors contribute to the current difficulty in conceiving effective immunotherapies against this malignancy, including: **i)** low mutational burden and, consequently, decreased immunogenicity; **ii)** unavailability of AML-specific antigens essential to its survival; and **iii)** clonal heterogeneity and evolution, associated with the development of resistance (50, 130). Furthermore, therapy-related toxicity and technical challenges also compromise immunotherapy development (50). Nevertheless, chemotherapy is considerably effective in eliminating the bulk of AML blasts, and the problem resides in the remaining, chemotherapy-resistant AML clones that later proliferate and constitute the AML relapse. Chemotherapy has generally been found to contribute to anti-cancer immune responses, by, for instance, inducing expression of stress signals recognizable by the immune system at the cancer cell surface (131). Taking this evidence into consideration, we hypothesize that there may be a benefit in inciting the immune system to eliminate residual AML blasts, specifically at post-chemotherapy administration stages, in which there is a low AML burden, potentially improved immune recognition due to chemotherapy effects, and before further clonal evolution ensues, perhaps introducing additional immune evasion mechanisms.

However, it is known that various chemotherapeutic drugs can also induce immune depletion, particularly of lymphocytes, that, not only hinders the anti-infection roles of the immune system, but also potentially impairs anti-cancer immune responses (132). Therefore, in order to consider the potential of developing therapeutic strategies to improve anti-leukemic immune responses following chemotherapy administration, it is necessary to evaluate the status of the immune system throughout chemotherapy recovery. Moreover, if immune responses involving specific immune subpopulations at these timepoints are relevant and contribute to AML cell elimination, then an improvement in survival should be observed in patients exhibiting higher frequencies of those cells.

To assess these hypotheses, we conducted a pilot study, in which we aimed to characterize the degree of immune depletion of AML patients following induction, after consolidation, and during post-chemotherapy recovery, and evaluate the association between immune cell counts and patient clinical features and outcomes. To do so:

1. Absolute cell counts of lymphocytes, B, NK, and T cells (including CD4+ and CD8+ T cells and their respective differentiation subsets, as well as regulatory T cells) of AML patients at each timepoint were determined by flow cytometry and compared to those of a cohort of healthy controls.

2. Associations between immune subset frequencies and patient response to first-line induction chemotherapy were evaluated.
3. Uni- and multivariable overall and relapse-free survival analyses were performed for each of the defined immune subpopulations.

Methodology

METHODOLOGY

Patient and healthy cohorts

A total of 91 patients with a suspected diagnosis of AML were recruited from March of 2015 to December 2017 at the University Hospital Center of São João (UHCSJ), Porto, Portugal. Eligible patients were then selected based on the fulfillment of the following three main inclusion criteria: **1)** being 18 years or older; **2)** having a confirmed diagnosis of *de novo* or secondary AML, acute leukemia of ambiguous lineage (including MPAL) or other high-risk myelodysplastic/myeloproliferative neoplasm (characterized by bone marrow blast percentages above 5% and/or complex karyotypes); and **3)** having received standard intensive chemotherapy. Additionally, any patients for which none of the obtained samples fitted relevant post-therapy timepoints were retrospectively excluded from this study. After applying these criteria, the final patient cohort consisted of 31 patients. Peripheral blood samples from 6 healthy volunteers were also collected, processed and stored in September 2020. Biological sample and clinical data collection was approved by the Ethics Committee of UHCSJ, and, as established by the Declaration of Helsinki, all donors provided written informed consent. A description of pertinent patient and healthy cohort data is provided in the Results chapter.

Sample collection and storage

The initial peripheral blood samples of our patients cohort were collected together with the aliquots obtained for diagnostic assessment. Similarly, and whenever possible, the following samples were collected as patients were subjected to sampling for therapy response assessment purposes. In general, evaluation (and, therefore, sample collection) during intensive chemotherapy was conducted after induction, after consolidation, and approximately 3 months following chemotherapy completion; these timepoints correspond, respectively, to roughly 1 to 2 months, 3 to 4 months and 6 to 7 months after beginning of treatment.

Since long-term cryopreservation prior to flow cytometry analysis was intended, peripheral blood samples from both patient and healthy donors were collected into BD Vacutainer cell preparation tubes with sodium citrate (BD Biosciences) for further peripheral blood mononuclear cell (PBMC) isolation. To separate PBMCs from the remaining whole blood components, cell preparation tubes were centrifuged at room temperature for 20

minutes at 1600 RCF (relative centrifugal force) and PBMC-containing buffy coats were removed into separate tubes. PBMCs were washed twice by resuspending the cells in saline solution (0.9% NaCl, Braun), following sedimentation by centrifugation at 300 RCF for 15 minutes, at room temperature. After removal of supernatant following the last washing step, cells were resuspended in freezing medium [fetal bovine serum (FBS), Gibco + 10% dimethyl sulfoxide, Sigma-Aldrich], aliquoted into 2 mL cryogenic vials, transferred into Mr. Frosty freezing containers (Nalgene) and kept at -80 °C overnight. The following day, cells were removed from the freezing containers; then, because the diagnostic samples were originally intended to also be used in *in vivo* studies, these vials were preferentially stored in liquid nitrogen to maximize cell viability, whereas the remaining samples, including those of healthy controls, were kept at -80 °C.

Flow cytometry

Protocol optimization

Prior to sample analysis, a previously established immunophenotyping protocol was further optimized to identify the main immune subpopulations of interest in a more efficient and accurate manner. From the initial protocol, two test tubes were prepared for all PBMC aliquots: the first tube was designed to identify the main immune cell populations – T, B and NK cells –, as well as a subset of $\gamma\delta$ T cells (panel 1), whereas the second tube allowed for the discrimination of CD4⁺ and CD8⁺ T cell subsets (panel 2). Additionally, a third, regulatory T cell quantification tube (panel 3) was prepared in some samples which exhibited high post-thawing cell counts and viability and/or had multiple frozen aliquots available. The main modifications in the original sample preparation protocol were the inclusion of the CD45 marker for a more accurate identification of the lymphocyte population, and an optimization of the thawing procedure and washing steps to minimize cell loss. Moreover, single-stained cell aliquots used as compensation controls were also replaced by compensation beads, in order to maximize the number of cells included in the test tubes.

Sample preparation

PBMC samples were quickly thawed by placing the cryovials in a heat block previously set at 37 °C. When only a small ice fragment was visible, cells were transferred into 15 mL conical centrifuge tubes and diluted by slowly adding cell culture medium [RPMI (Roswell Park Memorial Institute) 1640 (Gibco) supplemented with 10% FBS and 1X penicillin/streptomycin (Gibco)] with 50 µg/mL DNase I (Roche Diagnostics). Then, cells were centrifuged at room temperature for 10 minutes at 500 RCF, after which cells were again washed with culture medium and collected via the same centrifugation settings. In order to assess cell viability and estimate the total number of viable cells, a small aliquot of each sample was stained with trypan blue and both live and dead cells were counted using a Neubauer hemocytometer (Marienfeld Superior) placed under a microscope (Leica). Whenever cell viability was below 60%, the aliquots were discarded and, if available, another vial from the same patient/control sample was thawed. Likewise, if the total cell number was insufficient to include a minimum of 2×10^5 cells per each main flow cytometry test tube, more aliquots were thawed and pooled with the initial sample; if this was not possible, only the tube for panel 1 was prepared. As previously mentioned, quantification of regulatory T cells was only performed on samples with sufficient cells for all three test tubes.

After cell quantifications, cells were washed with 2.5% FBS in PBS (phosphate-buffered saline) (Braun) and centrifuged at room temperature for 5 minutes at 400 RCF. Then, 2×10^5 to 1×10^6 cells per test were added into individual wells in a 96-well V-bottom plate, which were then incubated with Zombie NIR™ Fixable Viability dye (Biolegend) for the exclusion of dead cells for 25 minutes at room temperature and in the dark. Then, for surface staining, the appropriate antibody cocktail volume was added to the respective test well, followed by a room temperature, 30-minute incubation in the dark. Between staining incubations, cells were washed with 2.5% FBS in PBS. The antibodies used for each flow cytometry test are listed in Tables 3 to 5.

Table 3. List of antibodies used in the identification of T, B, NK, and $\gamma\delta$ T cells by flow cytometry (panel 1).

Marker	Fluorochrome	Clone	Manufacturer
CD45	PE-Cy7	HI30	Biolegend
CD3	VioGreen	BW264/56	Miltenyi Biotec
CD19	APC	HIB19	Biolegend
CD56	PerCP-Cy5.5	HCD56	Biolegend
CD16	eFluor 450	eBioCB16	eBioscience
Pan $\gamma\delta$ TCR	PE	B1.1	eBioscience
V γ 9V δ 2 TCR	FITC	IMMU 360	Beckman Coulter

Table 4. List of antibodies used in the identification of CD4+ and CD8+ T cell subsets by flow cytometry (panel 2).

Marker	Fluorochrome	Clone	Manufacturer
CD45	PE-Cy7	HI30	Biolegend
CD3	VioGreen	BW264/56	Miltenyi Biotec
CD4	PerCP-Cy5.5	RPA-T4	eBioscience
CD8	eFluor 450	RPA-T8	eBioscience
CD45RA	APC	HI100	Biolegend
CD27	FITC	O323	Biolegend
CD62L	PE	DREG-56	BD Biosciences

Table 5. List of antibodies used in the identification of regulatory T cells by flow cytometry (panel 3).

Marker	Fluorochrome	Clone	Manufacturer
CD45	PE-Cy7	HI30	Biolegend
CD3	VioGreen	BW264/56	Miltenyi Biotec
CD4	eFluor 450	RPA-T4	eBioscience
CD25	APC	BC96	BD Biosciences
CD27	FITC	O323	Biolegend
CD127	PE	hIL-7R-M21	BD Biosciences
FoxP3	PerCP-Cy5.5	PCH101	eBioscience

For intracellular FoxP3 staining, the respective cells were prepared using a FoxP3 staining buffer kit (eBioscience) according to the manufacturer's instructions. Briefly, cells were treated with previously diluted fixation/permeabilization solution for 50 minutes at 4 °C in the dark, washed with permeabilization buffer and stained with FoxP3 antibody for 30 minutes (4 °C, in the dark). Following antibody incubation, cells were washed with permeabilization buffer and then with PBS.

Finally, after all preparation steps, samples were kept in 1% paraformaldehyde (Sigma-Aldrich) in PBS and stored in the dark at 4 °C until acquisition (up to 48 hours after sample preparation).

In parallel with the test tubes, one fluorescence minus one (FMO) tube per experiment was prepared for lower-frequency antigens in each antibody panel (CD16, CD19, CD56, $\gamma\delta$ TCR and V γ 9V δ 2 TCR for panel 1, CD27 and CD45RA for panel 2, and CD25, CD27, CD127 and FoxP3 for panel 3), in which cells were stained with all panel antibodies except that for which the FMO was intended. Moreover, whenever cell numbers allowed, an additional well was included for each sample to serve as an unstained control.

For single-color compensation controls of antibody-binding fluorochromes, UltraComp eBeads (Invitrogen) were suspended in PBS (3 drops per 400 μ L of PBS) and 25 μ L were distributed by 96-plate U-bottom plate wells (one per compensation control). Then, the predetermined volume of each antibody was added to the respective well and

beads incubated for 30 minutes in the dark and at 4 °C, after which PBS was added to the well and beads separated via a 5-minute, 400 RCF centrifugation at room temperature. On the other hand, the viability dye compensation controls were prepared using the ArC Amine Reactive Compensation Bead Kit (Invitrogen) – 2 drops of the reactive beads were diluted in 750 µL of PBS; then, 50 µL of this suspension were added into a 96-well U-bottom plate well, followed by 1 µL of the Zombie viability dye. Beads were incubated for 30 minutes at room temperature and in the dark, and then washed with PBS. Negative beads were also diluted (2 drops per 750 µL of PBS) and 50 µL added into another well. To emulate any effects of cell fixation on the antibody-fluorochrome complexes, all beads were also kept in 1% PFA and stored at 4 °C in the dark until acquisition.

Data acquisition and analysis

Flow cytometry data acquisition was performed using a BD FACSCanto II flow cytometer (BD Biosciences), together with the BD FACSDiva software, version 6 (BD Biosciences). Data was then further analyzed with the FlowJo software, version 10 (FlowJo, LLC).

Antibody panel 1 was designed to identify the major lymphocyte subpopulations, namely T, B and NK cells, as well as $\gamma\delta$ T cells; additionally, this panel allowed for the quantification of the main NK and $\gamma\delta$ T cell subsets. However, upon analyzing the acquired data, identification of the $\gamma\delta$ T cell population was not possible. The $\gamma\delta$ T cells are, as its name implies, a subset of T cells; therefore, expression of the $\gamma\delta$ TCR should be exclusive to T cells, yet our samples exhibited $\gamma\delta$ TCR expression in both CD3⁺ and CD3⁻ lymphocytes, as exemplified in Figure 3A. Moreover, there were virtually no $\gamma\delta$ TCR⁺ T cells in the $\gamma\delta$ TCR FMO samples (Figure 3B), which seemingly rules out the hypothesis that this $\gamma\delta$ TCR positivity was at least partially due to the detection of fluorescence spillover emitted from other fluorochromes, and leads us to hypothesize that the problem may have originated from unspecific binding of the $\gamma\delta$ TCR antibody. Nevertheless, determination of the V γ 9V δ 2 subset percentage within the overall T cell population was still possible and these results were included in the statistical analysis.

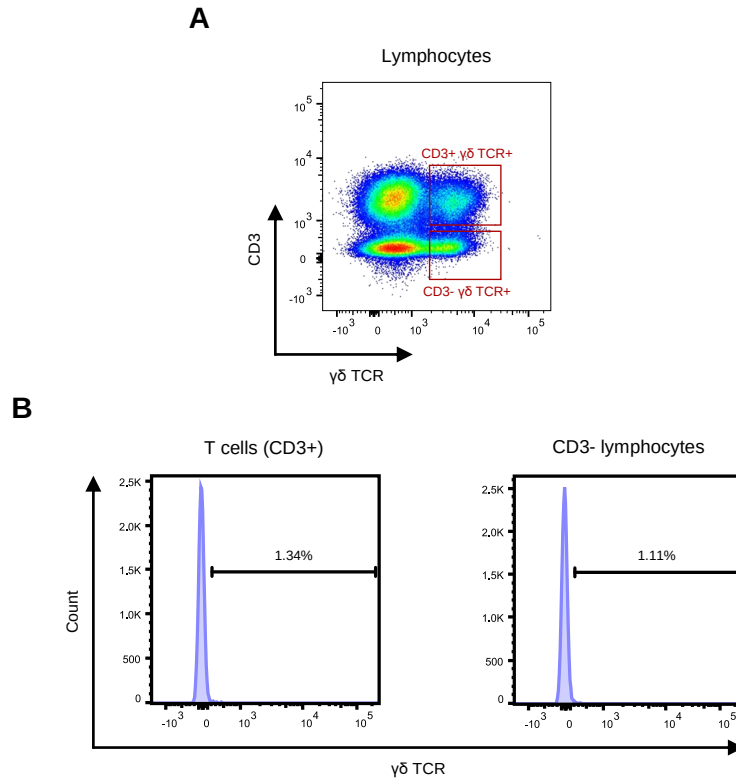
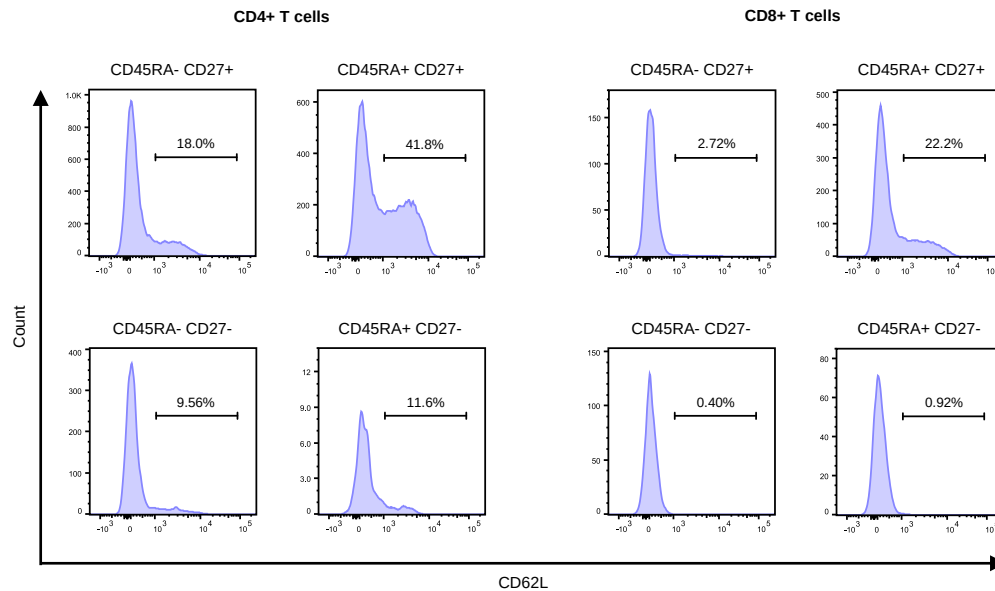


Figure 3. Rationale for the exclusion of $\gamma\delta$ TCR expression from panel 1 flow cytometry analysis. **A.** Nonspecific detection of $\gamma\delta$ TCR+ cells in the CD3- lymphocyte population of an exemplificative AML patient peripheral blood sample. **B.** Absence of PE detection in the T cell and CD3- lymphocyte subpopulations in a FMO sample.

Panel 2, on the other hand, was developed to determine the relative frequencies of distinct T cell subsets. Originally, CD4+ and CD8+ T cells were to be identified and, within each of these populations, four different subsets would be quantified – naïve, central memory, effector memory and TEMRA T cells – on the basis of the expression of CD27, CD45RA and CD62L. Unexpectedly, although some CD62L expression was observed in the healthy donor samples (Figure 4A), CD62L+ cells were particularly scarce in patient samples, as demonstrated in Figure 4B. Because of the discrepancy between both sample types, we suggest that this may be a result of loss of CD62L expression due to long-term cryopreservation. In fact, a 2003 study compared freshly drawn whole peripheral blood with cryopreserved PBMCs, and demonstrated that there is a substantial loss of CD62L expression, evidenced by a decrease in the percentages of total CD62L+ T cells, as well as in CD4+ and CD8+ naïve and central memory subsets in the frozen samples (133). Although this effect has not yet been correlated with cryopreservation duration, we hypothesize that both patient and healthy donor samples may have decreased CD62L+ expression, but, because patient PBMCs were kept at -80 °C from roughly 3 to 5 years, whereas healthy donor cells were analyzed only 1 to 2 months after cryopreservation, lymphocytes in patient

samples may have exhibited a more pronounced loss of CD62L expression. Given the impossibility of reliably assessing CD62L+ and CD62L- cell percentages, we decided to exclude this marker from the analysis, and consider four CD4+ and CD8+ T cell subsets: CD45RA+ CD27+, CD45RA- CD27+, CD45RA- CD27- and CD45RA+ CD27- cells.

A



B

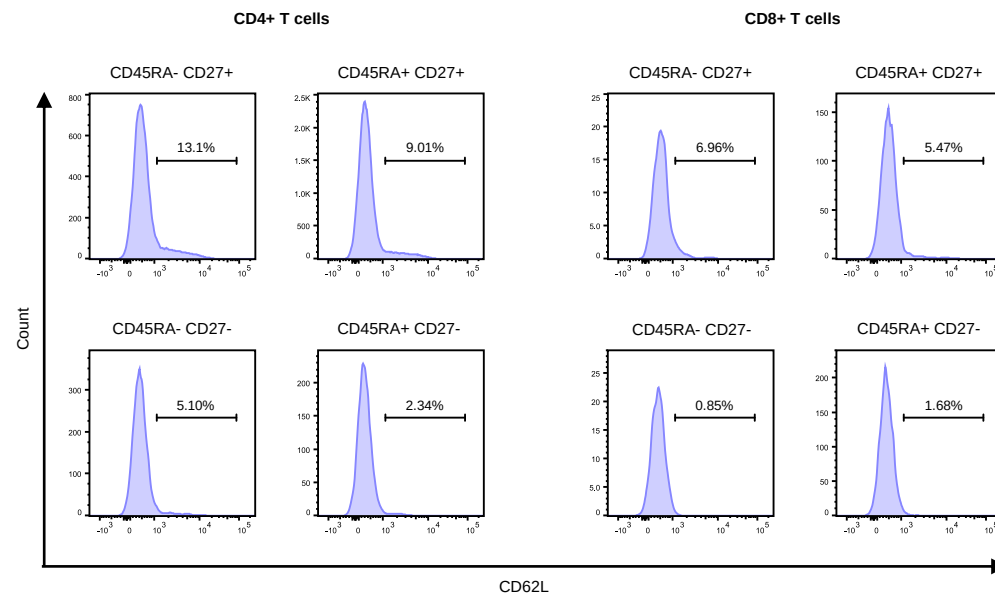


Figure 4. Rationale for the exclusion of CD62L expression from panel 2 flow cytometry analysis. **A.** Detection of CD62L expression in the various CD4+ and CD8+ T cell subpopulations of a healthy donor sample. **B.** Example demonstrating decreased detection of CD62L+ cells in different CD4+ and CD8+ T cell subsets in AML patient samples.

A description of the final immune cell phenotypes identified by each panel, as well as the gating strategies applied in the FlowJo analysis are described in Tables 6 to 8 and Figures 5 to 7, respectively.

Table 6. Immune cell subsets identified using panel 1 and respective phenotypes.

Immune subset	Phenotype
Lymphocytes	SSCdim CD45bright
B cells	SSCdim CD45bright CD3- CD19+
NK cells	SSCdim CD45bright CD3- CD19- CD56+
CD56bright CD16- NK cells	SSCdim CD45bright CD3- CD19- CD56+ CD56bright CD16-
CD56dim CD16+ NK cells	SSCdim CD45bright CD3- CD19- CD56+ CD56dim CD16+
T cells	SSCdim CD45bright CD3+
Vy9V62 TCR+ T cells	SSCdim CD45bright CD3+ Vy9V62 TCR+

Table 7. Immune cell subsets identified using panel 2 and respective phenotypes.

Immune subset	Phenotype
Lymphocytes	SSCdim CD45bright
T cells	SSCdim CD45bright CD3+
CD4+ T cells	SSCdim CD45bright CD3+ CD4+
CD4+ CD45RA+ CD27+ T cells	SSCdim CD45bright CD3+ CD4+ CD45RA+ CD27+
CD4+ CD45RA- CD27+ T cells	SSCdim CD45bright CD3+ CD4+ CD45RA- CD27+
CD4+ CD45RA- CD27- T cells	SSCdim CD45bright CD3+ CD4+ CD45RA- CD27-
CD4+ CD45RA+ CD27- T cells	SSCdim CD45bright CD3+ CD4+ CD45RA+ CD27-
CD8+ T cells	SSCdim CD45bright CD3+ CD8+
CD8+ CD45RA+ CD27+ T cells	SSCdim CD45bright CD3+ CD8+ CD45RA+ CD27+
CD8+ CD45RA- CD27+ T cells	SSCdim CD45bright CD3+ CD8+ CD45RA- CD27+
CD8+ CD45RA- CD27- T cells	SSCdim CD45bright CD3+ CD8+ CD45RA- CD27-
CD8+ CD45RA+ CD27- T cells	SSCdim CD45bright CD3+ CD8+ CD45RA+ CD27-

Table 8. Immune cell subsets identified using panel 3 and respective phenotypes.

Immune subset	Phenotype
Lymphocytes	SSCdim CD45bright
T cells	SSCdim CD45bright CD3+
CD4+ T cells	SSCdim CD45bright CD3+ CD4+
Regulatory T cells	SSCdim CD45bright CD3+ CD4+ CD25+ CD27+ CD127- FoxP3+

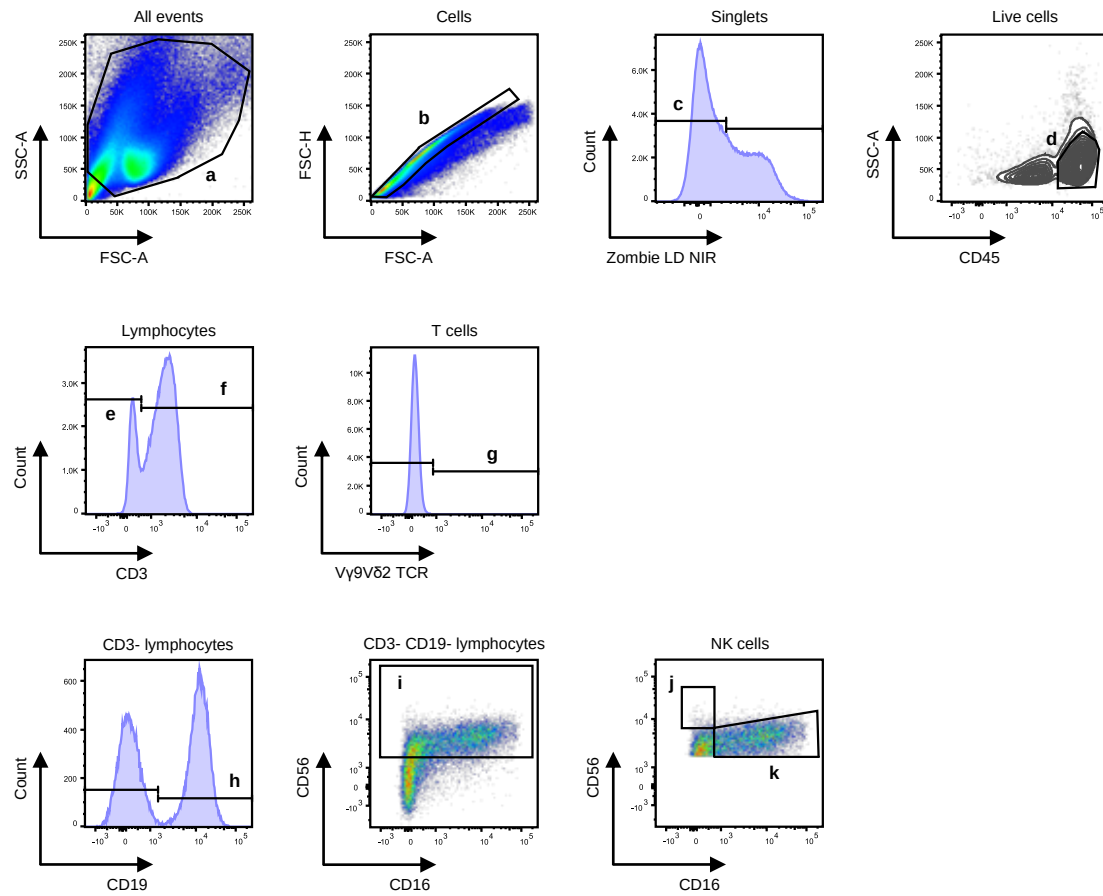


Figure 5. Panel 1 gating strategy. **a.** Cells, **b.** Singlets, **c.** Live cells, **d.** Lymphocytes, **e.** CD3- lymphocytes, **f.** T cells, **g.** Vγ9Vδ2 T cells, **h.** B cells, **i.** NK cells, **j.** CD56bright CD16- NK cells, **k.** CD56 dim CD16+ NK cells.

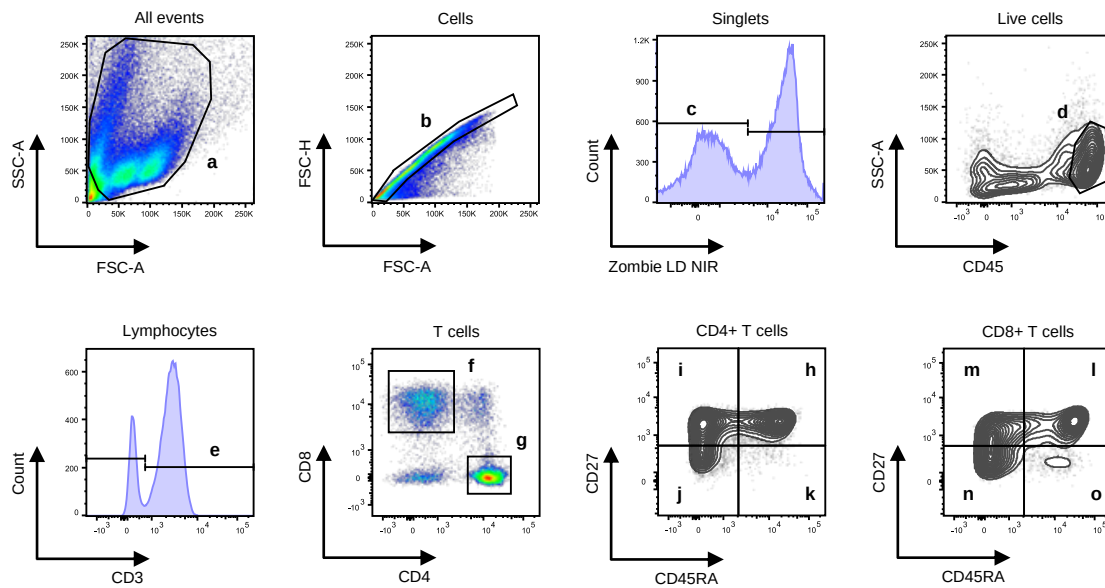


Figure 6. Panel 2 gating strategy. **a.** Cells, **b.** Singlets, **c.** Live cells, **d.** Lymphocytes, **e.** T cells, **f.** CD8+ T cells, **g.** CD4+ T cells, **h.** CD4+ CD45RA+ CD27+ T cells, **i.** CD4+ CD45RA- CD27+ T cells, **j.** CD4+ CD45RA- CD27- T cells, **k.** CD4+ CD45RA+ CD27- T cells, **l.** CD8+ CD45RA+ CD27- T cells, **m.** CD8+ CD45RA- CD27+ T cells, **n.** CD8+ CD45RA- CD27- T cells, **o.** CD8+ CD45RA+ CD27- T cells.

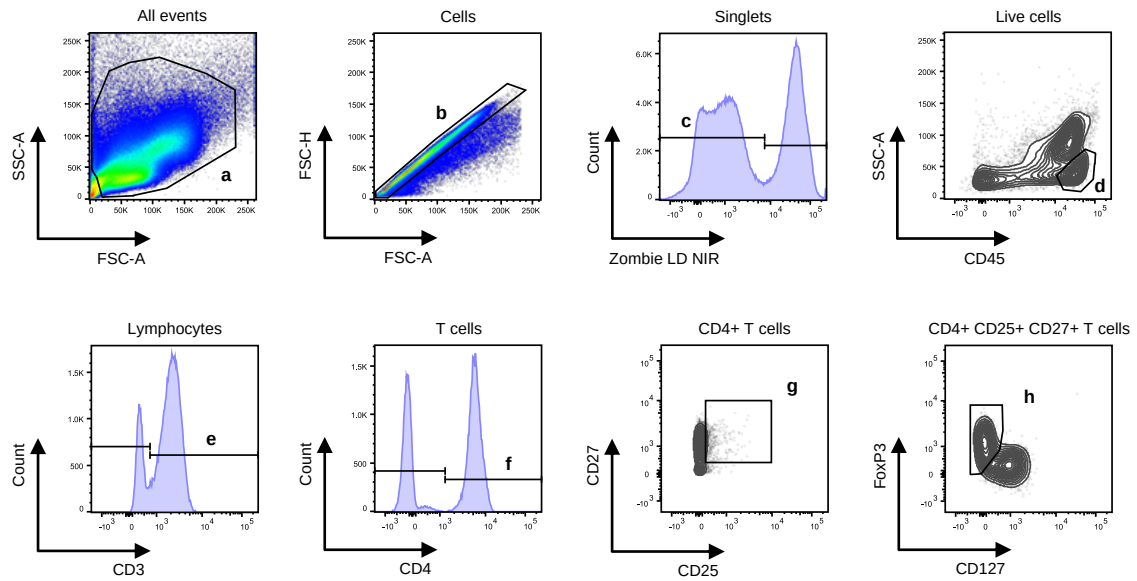


Figure 7. Panel 3 gating strategy. **a.** Cells, **b.** Singlets, **c.** Live cells, **d.** Lymphocytes, **e.** T cells, **f.** CD4+ T cells, **g.** CD4+ CD25+ CD27+ T cells, **h.** Regulatory T cells.

The output of all FlowJo analyses consisted in the determination of the percentage of each immune subset within the general lymphocyte population. However, since absolute lymphocyte counts are not consistent between samples, equal percentages of a specific subpopulation may not translate into similar absolute cell counts per unit of peripheral blood volume. Furthermore, because PBMCs were isolated and cryopreserved in all samples prior to any flow cytometry analysis, absolute lymphocyte count determination was not possible. Nevertheless, and as aforementioned, all patient specimens were collected together with peripheral blood samples drawn for clinical evaluation; therefore, an absolute lymphocyte count assessment performed by the UHCSJ flow cytometry laboratory was available for each patient sample. To estimate the absolute counts of each immune subset, we obtained absolute lymphocyte frequencies corresponding to each patient sample from clinical history charts and multiplied each value by its respective subpopulation percentages obtained in the FlowJo analysis. Absolute lymphocyte frequency determination was not, however, available for the healthy individuals; thus, to perform statistical comparisons between patients and controls, we searched the literature to find a representative healthy cohort for which mean and median absolute lymphocyte counts were provided. A 2020 study by Fonseca, Pereira et al. reported both mean and median lymphocyte counts of a cohort comprised of 30 healthy Portuguese adults with a median age of 47 years (134). Absolute immune subset counts were, then, estimated by multiplying the reported median lymphocyte frequency by each percentage obtained in the analysis of our healthy cohort.

Statistical analysis

All statistical procedures were performed by the SPSS Statistics software, version 28 (IBM) and graphical representations constructed using the GraphPad Prism software, version 8 (GraphPad Software, Inc).

Immune cell counts of patients collected at each of the selected timepoints were compared with those of healthy donors. Then, immune cell counts of AML patients were compared according to their response to induction chemotherapy. Additionally, overall and relapse-free survival analyses were performed. It is important to note that statistical analysis of regulatory T cell counts was only performed by comparing healthy donor results with patient samples collected after induction chemotherapy, since sample size was insufficient to conduct further evaluation.

The threshold of significance was considered to be $\alpha < 0.05$ for all statistical analyses. With the exception of survival analysis, statistical test selection was preceded by an assessment of the approximation to the normal distribution by each of the groups under study using the Shapiro-Wilk test. Obtaining a significant result for at least one of the groups implied the selection of a non-parametric test; on the other hand, if all tests resulted in a *p*-value equal to or above 0.05, the comparison would be performed using a parametric test. Moreover, if one of the groups was comprised of only two samples, the non-parametric test was automatically selected.

When comparing cell frequencies of two groups, an independent samples *t*-test (parametric) or a Mann-Whitney U test (non-parametric) were selected. When a *t*-test was performed, it was preceded by a Levene's test to evaluate the fulfillment of the homogeneity of variances assumption, and if the results were statistically significant, the Welch's version of the *t*-test was presented. When comparing absolute lymphocyte counts of patients *versus* healthy controls, and since no lymphocyte counts were available for the healthy individuals, we considered the mean absolute lymphocyte frequency described in Fonseca, Pereira et al., 2020 (134) as a reference value and performed one-sample *t*-tests to compare both groups (parametric tests were selected for all analyses because the Shapiro-Wilk tests performed on the patient samples were consistently non-significant).

Furthermore, comparison by response to induction chemotherapy was performed using one-way analysis of variance (ANOVA) if a parametric test was selected, or, in alternative, using the Kruskal-Wallis test. When an overall significant result was achieved in the one-way ANOVA or Kruskal-Wallis tests, pairwise comparisons were computed using, respectively, an independent samples *t*-test or a Mann-Whitney U test, both adjusted for the use of multiple tests by a Bonferroni correction.

Overall survival was defined as the time elapsed between the beginning of first-line therapy and death or, alternatively, last follow-up, whereas relapse-free survival consisted of the time span from remission achievement until the occurrence of relapse or last follow-up. For both analyses, two approaches were presented. The first consisted in the application of the univariate Kaplan-Meier method and the Logrank test; using this method, results related to each immune subpopulation had to be categorized in two groups, yet, because this is a pilot study, no reference cutoff values were available. To determine the best-performing cutoff value, three different procedures were tested: **i)** using the population median frequency as threshold; **ii)** using healthy cohort-derived values (median counts and minimum values observed); and **iii)** using the receiver operating characteristic (ROC) curve analysis. The latter is a statistical approach that selects the cutoff value that best predicts the occurrence of a binary outcome variable – in this case, death and relapse. Using this approach, Youden's indexes were calculated for each proposed cutoff, and the value with highest Youden's index was selected, as previously described (135). Once all four thresholds for each subpopulation were obtained, patients were categorized into two groups on the basis of each cutoff value, and, whenever possible, four Logrank tests were ran. For each subpopulation, the Logrank test that rendered the lowest *p*-value was presented as the best-performing result obtained by the Kaplan-Meier method. The second approach was the application of the Cox proportional hazards method, which not only does not require the dichotomization of the main predictor (immune cell counts), but also allows for the inclusion of multiple other covariates. Using this method, three models were tested: a model comprised by the immune subpopulation of interest, a model including a cell population as well as age, and a model including the immune counts, age and also ELN cytogenetic risk group. It should be noted that, in order for each tested model to be valid, the assumption of proportional hazards has to be met. To evaluate this requirement, every model under study was also ran in parallel with the inclusion of interaction terms between a time variable and each of the covariates; if any interaction term was found to be significant, then the assumption would be violated and the model deemed invalid.

Results

RESULTS

A. Cohort characterization

Samples from a total of 31 patients diagnosed with AML or other clinically equivalent myeloid neoplasms (hereinafter collectively referred to as “AML”) were included in this study, as well as of 6 healthy donors that served as controls. A characterization of the most relevant patient clinical variables is presented in Table 9. Of note, 80.65% of the AML patients fulfilled complete remission criteria, almost half experienced at least one relapse, and 64.52% eventually died within the duration of the study.

It is important to acknowledge that, out of the available healthy donor data (Table 10), median age was found to be statistically different than that of patients ($p = 0.004$, Mann-Whitney U test).

Table 9. Demographic and clinical characteristics of the AML patient cohort. N/A, not available.

Patient cohort (n = 31)	
Age, median (range), years	57 (26 – 69)
Age < 60 years, n (%)	19 (61.29)
Age ≥ 60 years, n (%)	12 (38.71)
Sex, n (%)	
Female	12 (38.71)
Male	19 (61.29)
Diagnosis (WHO classification), n (%)	
Acute myeloid leukemia (AML)	27 (87.10)
De novo AML	25 (80.65)
AML with t(8;21)(q22;q22.1)/RUNX1::RUNX1T1 fusion	3 (9.68)
AML with inv(16)(p13.1q22)/CBFB::MYH11 fusion	1 (3.23)
AML with myelodysplasia-related changes	4 (12.90)
AML, defined by differentiation (formerly AML, not otherwise specified)	17 (54.84)
Secondary AML	2 (6.45)
Acute leukemia of ambiguous lineage (ALAL)	1 (3.23)
Mixed-phenotype acute leukemia (MPAL), B/myeloid	1 (3.23)
Myelodysplastic syndrome (MDS)	3 (9.68)
MDS with excess blasts	3 (9.68)
ELN cytogenetic risk, n (%)	
Favorable	8 (25.81)
Intermediate	12 (38.71)
Adverse	8 (25.81)
N/A	3 (9.68)
First-line chemotherapy response, n (%)	
Complete remission (CR)	25 (80.65)
Partial remission (PR)	2 (6.45)
Progression (Prog)	4 (12.90)

Table 9 (cont.). Demographic and clinical characteristics of the AML patient cohort.

Patient cohort (n = 31)	
Hematopoietic stem cell transplant (HSCT), n (%)	
Yes	15 (48.39)
Autologous	4 (12.90)
Allogeneic	11 (35.48)
No	16 (51.61)
First relapse, n (%)	
Yes	15 (48.39)
Post-first-line therapy	9 (29.03)
Post-second-line therapy	1 (3.23)
Post-HSCT	5 (16.13)
Progression	2 (6.45)
No	14 (45.16)
Death, n (%)	
Yes	20 (64.52)
No	11 (35.48)

Table 10. Characteristics of the healthy donor cohort.

Healthy donor cohort (n = 6)	
Age, median (range), years	28 (26 – 54)
Sex, n (%)	
Female	5 (83.33)
Male	1 (16.67)

B. Patient samples collected after first-line induction chemotherapy

1. Comparing immune cell counts of AML patients with healthy controls

When comparing cell counts of AML samples acquired after first-line induction chemotherapy with healthy controls, statistically significant differences were found in most immune subsets (13 out of the 18 analyzed subpopulations, Table 11, Figure 8). However, our results suggest that, after induction chemotherapy, CD4⁺ CD45RA⁻ CD27⁻ and CD45RA⁺ CD27⁻ T cells, as well as CD8⁺ CD45RA⁻ CD27⁺, CD45RA⁻ CD27⁻ and CD45RA⁺ CD27⁻ T cell levels in patients were equivalent to those of healthy individuals. Furthermore, AML patients displayed significantly lower frequencies of all other immune cell populations, comparatively to healthy controls.

Table 11. Immune subset level comparisons between AML patients after first-line induction CT and healthy controls. Statistical analyses were performed using one-sample *t*-tests, independent samples *t*-tests or Mann-Whitney U tests. Statistically significant results ($p < 0.05$) are highlighted in bold. N/A, not applicable. **a)** For total lymphocyte comparisons, the healthy control sample was represented by the mean absolute lymphocyte count of 2.102×10^9 cells/L of peripheral blood reported by Fonseca, Pereira et al., 2020 (134).

Cell population	Group	<i>n</i>	Median	Statistical test	<i>p</i> -value
Lymphocytes	Healthy controls ^{a)}	N/A	N/A	One-sample <i>t</i> -test	< 0.001
	AML patients	25	7.200×10^8		
B cells	Healthy controls	6	1.854×10^8	Mann-Whitney U test	< 0.001
	AML patients	19	2.530×10^6		
NK cells	Healthy controls	6	1.629×10^8	Mann-Whitney U test	< 0.001
	AML patients	19	3.744×10^7		
CD56bright CD16- NK cells	Healthy controls	6	6.574×10^6	Mann-Whitney U test	< 0.001
	AML patients	19	1.330×10^6		
CD56dim CD16+ NK cells	Healthy controls	6	1.243×10^8	Mann-Whitney U test	< 0.001
	AML patients	19	1.973×10^7		
T cells	Healthy controls	6	1.610×10^9	Independent samples <i>t</i> -test	< 0.001
	AML patients	19	6.430×10^8		
V γ 9V δ 2 TCR+ T cells	Healthy controls	6	2.244×10^7	Mann-Whitney U test	0.043
	AML patients	19	8.584×10^6		
CD4+ T cells	Healthy controls	6	1.083×10^9	Independent samples <i>t</i> -test	< 0.001
	AML patients	19	3.721×10^8		
CD4+ CD45RA+ CD27+ T cells	Healthy controls	6	4.425×10^8	Independent samples <i>t</i> -test	< 0.001
	AML patients	19	1.380×10^8		
CD4+ CD45RA- CD27+ T cells	Healthy controls	6	4.268×10^8	Mann-Whitney U test	< 0.001
	AML patients	19	9.112×10^7		
CD4+ CD45RA- CD27- T cells	Healthy controls	6	1.506×10^8	Independent samples <i>t</i> -test	0.566
	AML patients	19	1.024×10^8		
CD4+ CD45RA+ CD27- T cells	Healthy controls	6	6.053×10^6	Mann-Whitney U test	0.092
	AML patients	19	2.180×10^7		
CD8+ T cells	Healthy controls	6	3.413×10^8	Mann-Whitney U test	0.036
	AML patients	19	1.896×10^8		
CD8+ CD45RA+ CD27+ T cells	Healthy controls	6	1.731×10^8	Mann-Whitney U test	< 0.001
	AML patients	19	3.758×10^7		
CD8+ CD45RA- CD27+ T cells	Healthy controls	6	4.905×10^7	Mann-Whitney U test	0.092
	AML patients	19	2.498×10^7		
CD8+ CD45RA- CD27- T cells	Healthy controls	6	3.173×10^7	Mann-Whitney U test	0.598
	AML patients	19	3.152×10^7		
CD8+ CD45RA+ CD27- T cells	Healthy controls	6	4.748×10^7	Mann-Whitney U test	0.975
	AML patients	19	5.393×10^7		
Regulatory T cells	Healthy controls	6	2.244×10^7	Independent samples <i>t</i> -test	0.025
	AML patients	6	1.155×10^7		

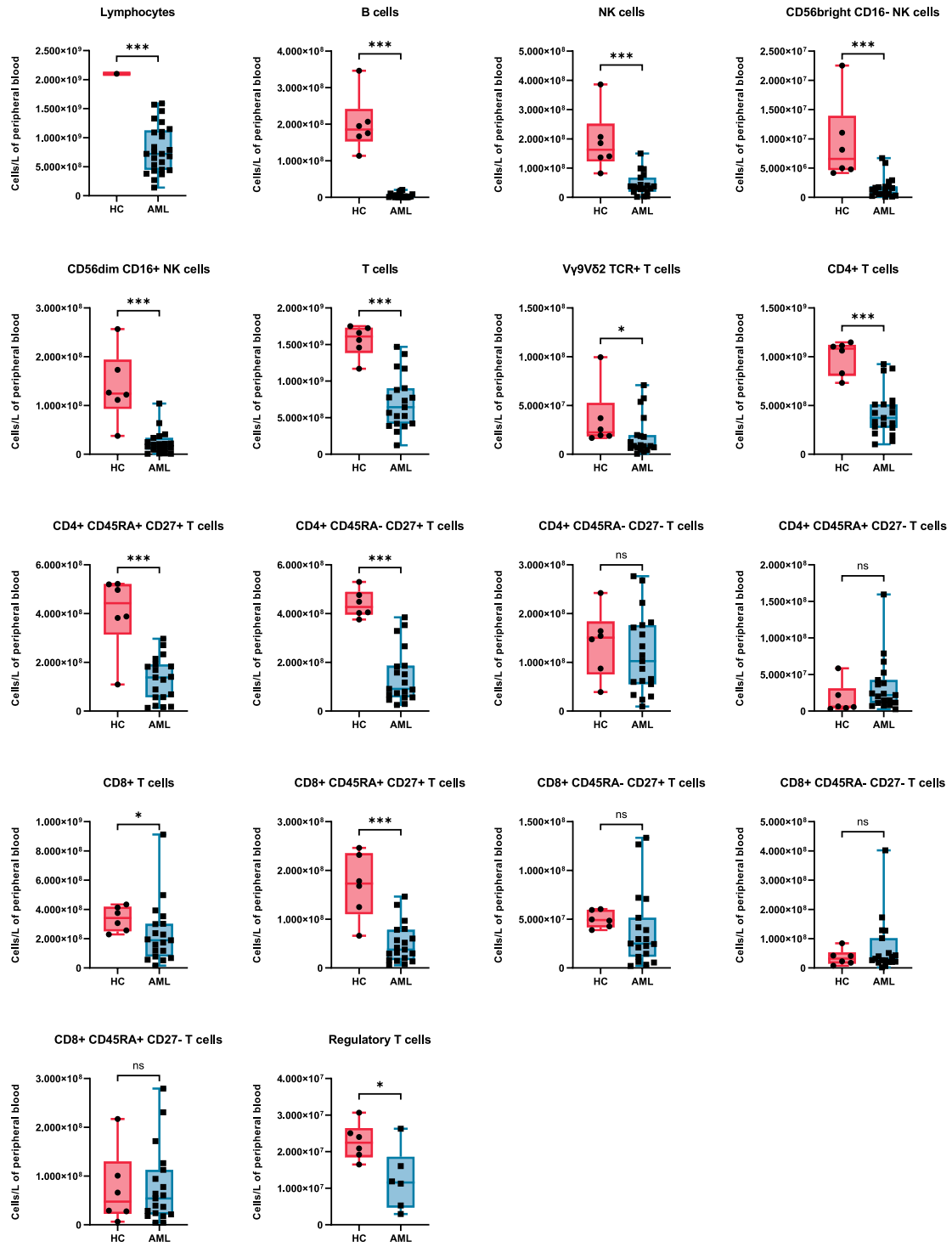


Figure 8. Immune cell levels of AML patients after first-line induction CT and of healthy controls. Data are presented in boxplots depicting median cell counts, interquartile range (boxes), and minimum and maximum values (whiskers). Statistical *p*-values for the comparisons between groups are presented as *, *p* < 0.05; **, *p* < 0.01; and ***, *p* < 0.001; ns, not significant. HC, healthy controls; AML, acute myeloid leukemia patients.

2. Comparing immune cell counts of AML patients by their response to first-line induction chemotherapy

The statistical analysis of the data suggests that different types of response to first-line induction chemotherapy (complete remission, partial remission or progression) were not associated with varying levels of any of the studied immune subpopulations (Table 12, Figure 9).

Table 12. Immune subset level comparisons between AML patients by their response to first line induction CT in samples collected after first-line induction CT. Statistical analyses were performed using Kruskal-Wallis tests.

Cell population	Group	n	Median	Statistical test	p-value
Lymphocytes	Complete remission	20	7.200×10^8	Kruskal-Wallis test	0.918
	Partial remission	2	7.300×10^8		
	Progression	3	7.600×10^8		
B cells	Complete remission	15	2.530×10^6	Kruskal-Wallis test	0.820
	Partial remission	2	7.989×10^6		
	Progression	2	2.383×10^6		
NK cells	Complete remission	15	3.060×10^7	Kruskal-Wallis test	0.149
	Partial remission	2	9.367×10^7		
	Progression	2	6.836×10^7		
CD56bright CD16- NK cells	Complete remission	15	1.020×10^6	Kruskal-Wallis test	0.564
	Partial remission	2	1.234×10^6		
	Progression	2	1.803×10^6		
CD56dim CD16+ NK cells	Complete remission	15	1.278×10^7	Kruskal-Wallis test	0.117
	Partial remission	2	6.439×10^7		
	Progression	2	2.747×10^7		
T cells	Complete remission	15	6.430×10^8	Kruskal-Wallis test	0.780
	Partial remission	2	5.780×10^8		
	Progression	2	6.044×10^8		
V γ 9V δ 2 TCR+ T cells	Complete remission	15	8.584×10^6	Kruskal-Wallis test	0.991
	Partial remission	2	2.996×10^7		
	Progression	2	1.303×10^7		
CD4+ T cells	Complete remission	15	4.238×10^8	Kruskal-Wallis test	0.630
	Partial remission	2	2.945×10^8		
	Progression	2	3.709×10^8		
CD4+ CD45RA+ CD27+ T cells	Complete remission	15	1.685×10^8	Kruskal-Wallis test	0.057
	Partial remission	2	1.456×10^7		
	Progression	2	9.850×10^7		
CD4+ CD45RA- CD27+ T cells	Complete remission	15	9.112×10^7	Kruskal-Wallis test	0.761
	Partial remission	2	9.231×10^7		
	Progression	2	1.771×10^8		
CD4+ CD45RA- CD27- T cells	Complete remission	15	8.666×10^7	Kruskal-Wallis test	0.385
	Partial remission	2	1.766×10^8		
	Progression	2	8.307×10^7		
CD4+ CD45RA+ CD27- T cells	Complete remission	15	2.418×10^7	Kruskal-Wallis test	0.198
	Partial remission	2	1.111×10^7		
	Progression	2	1.215×10^7		
CD8+ T cells	Complete remission	15	1.896×10^8	Kruskal-Wallis test	0.835
	Partial remission	2	1.781×10^8		
	Progression	2	1.528×10^8		
CD8+ CD45RA+ CD27+ T cells	Complete remission	15	4.158×10^7	Kruskal-Wallis test	0.157
	Partial remission	2	1.075×10^7		
	Progression	2	3.616×10^7		
CD8+ CD45RA- CD27+ T cells	Complete remission	15	2.498×10^7	Kruskal-Wallis test	0.723
	Partial remission	2	1.789×10^7		
	Progression	2	4.671×10^7		
CD8+ CD45RA- CD27- T cells	Complete remission	15	3.152×10^7	Kruskal-Wallis test	0.788
	Partial remission	2	7.704×10^7		
	Progression	2	3.582×10^7		
CD8+ CD45RA+ CD27- T cells	Complete remission	15	5.393×10^7	Kruskal-Wallis test	0.645
	Partial remission	2	7.229×10^7		
	Progression	2	3.407×10^7		

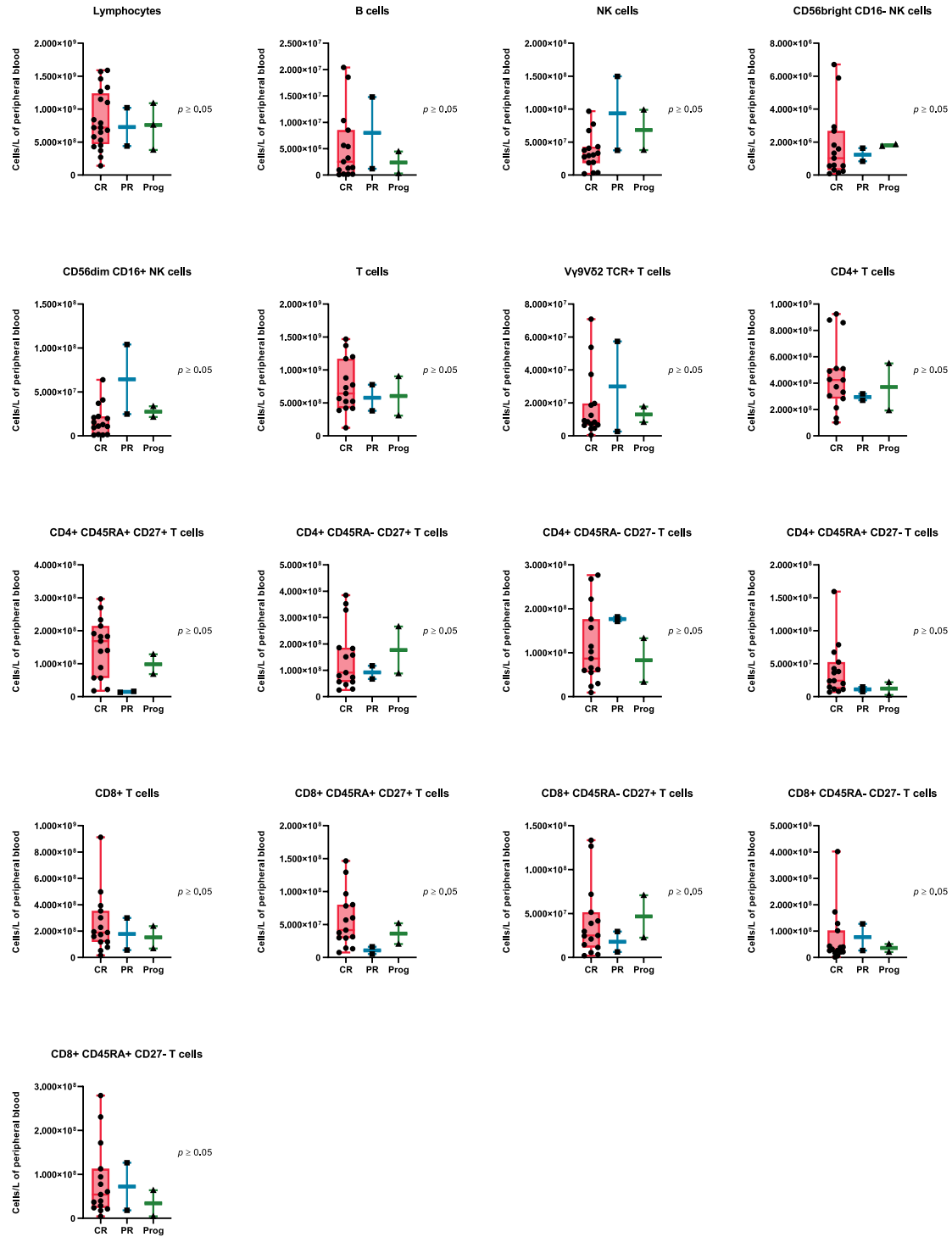


Figure 9. Immune cell levels of AML patients after first-line induction CT by response to first-line induction CT. Data are presented in boxplots depicting median cell counts, interquartile range (boxes), and minimum and maximum values (whiskers). Statistical p -values for the overall comparisons between groups are presented on the right side of the respective plot. CR, complete remission; PR, partial remission; Prog, progression.

3. Comparing overall survival of AML patients with higher or lower frequencies of immune populations

a. Method 1: Kaplan-Meier analysis

After choosing the best-performing cutoff selection methodology (with lowest p -value), significant results were obtained in the analysis of lymphocytes ($p = 0.027$), CD4+ T cells ($p = 0.011$), CD4+ CD45RA- CD27+, CD4+ CD45RA- CD27- and CD4+ CD45RA+ CD27- T cell subsets (respectively $p = 0.011$, $p = 0.043$ and $p = 0.031$), and CD8+ CD45RA+ CD27+ T cells ($p = 0.039$) (Table 13, Figure 10). In all these results, median overall survival at the post-induction chemotherapy timepoint was found to be significantly increased in cell frequencies equal to or above the respective threshold, in comparison with cell counts below these values.

Table 13. Best-performing Kaplan-Meier results for overall survival of AML patients categorized by their immune subset levels after first-line induction CT. Statistically significant results ($p < 0.05$) are highlighted in bold. ROC, receiver operating characteristic; HC, healthy control. **a)** Median survival was not reached (undefined), since, at the end of the follow-up period, overall survival of the patients included in the group was higher than 50%.

Cell population	Group	Total n	Events	Censored	Median survival, days	p -value
Lymphocytes	Cell counts < ROC cutoff	19	14	5	704	0.027
	Cell counts $\geq$ ROC cutoff	6	1	5	a)	
B cells	Cell counts < ROC cutoff	6	3	3	1966	0.300
	Cell counts $\geq$ ROC cutoff	13	10	3	895	
NK cells	Cell counts < ROC cutoff	15	9	6	1121	0.131
	Cell counts $\geq$ ROC cutoff	4	4	0	617	
CD56bright CD16- NK cells	Cell counts < ROC cutoff	11	6	5	949	0.464
	Cell counts $\geq$ ROC cutoff	8	7	1	895	
CD56dim CD16+ NK cells	Cell counts < HC minimum	16	10	6	949	0.374
	Cell counts $\geq$ HC minimum	3	3	0	895	
T cells	Cell counts < HC minimum	15	12	3	704	0.070
	Cell counts $\geq$ HC minimum	4	1	3	a)	
V γ 9V δ 2 TCR+ T cells	Cell counts < ROC cutoff	9	5	4	1966	0.329
	Cell counts $\geq$ ROC cutoff	10	8	2	704	
CD4+ T cells	Cell counts < ROC cutoff	5	5	0	254	0.011
	Cell counts $\geq$ ROC cutoff	14	8	6	1009	
CD4+ CD45RA+ CD27+ T cells	Cell counts < ROC cutoff	12	10	2	617	0.079
	Cell counts $\geq$ ROC cutoff	7	3	4	a)	
CD4+ CD45RA- CD27+ T cells	Cell counts < ROC cutoff	6	6	0	254	0.011
	Cell counts $\geq$ ROC cutoff	13	7	6	1966	
CD4+ CD45RA- CD27- T cells	Cell counts < HC minimum	4	4	0	168	0.043
	Cell counts $\geq$ HC minimum	15	9	6	1009	
CD4+ CD45RA+ CD27- T cells	Cell counts < ROC cutoff	5	5	0	329	0.031
	Cell counts $\geq$ ROC cutoff	14	8	6	1009	
CD8+ T cells	Cell counts < ROC cutoff	7	7	0	895	0.110
	Cell counts $\geq$ ROC cutoff	12	6	6	1009	
CD8+ CD45RA+ CD27+ T cells	Cell counts < HC minimum	14	12	2	704	0.039
	Cell counts $\geq$ HC minimum	5	1	4	a)	
CD8+ CD45RA- CD27+ T cells	Cell counts < ROC cutoff	8	8	0	895	0.052
	Cell counts $\geq$ ROC cutoff	11	5	6	a)	
CD8+ CD45RA- CD27- T cells	Cell counts < ROC cutoff	7	7	0	895	0.101
	Cell counts $\geq$ ROC cutoff	12	6	6	949	
CD8+ CD45RA+ CD27- T cells	Cell counts < ROC cutoff	9	8	1	895	0.179
	Cell counts $\geq$ ROC cutoff	10	5	5	1009	

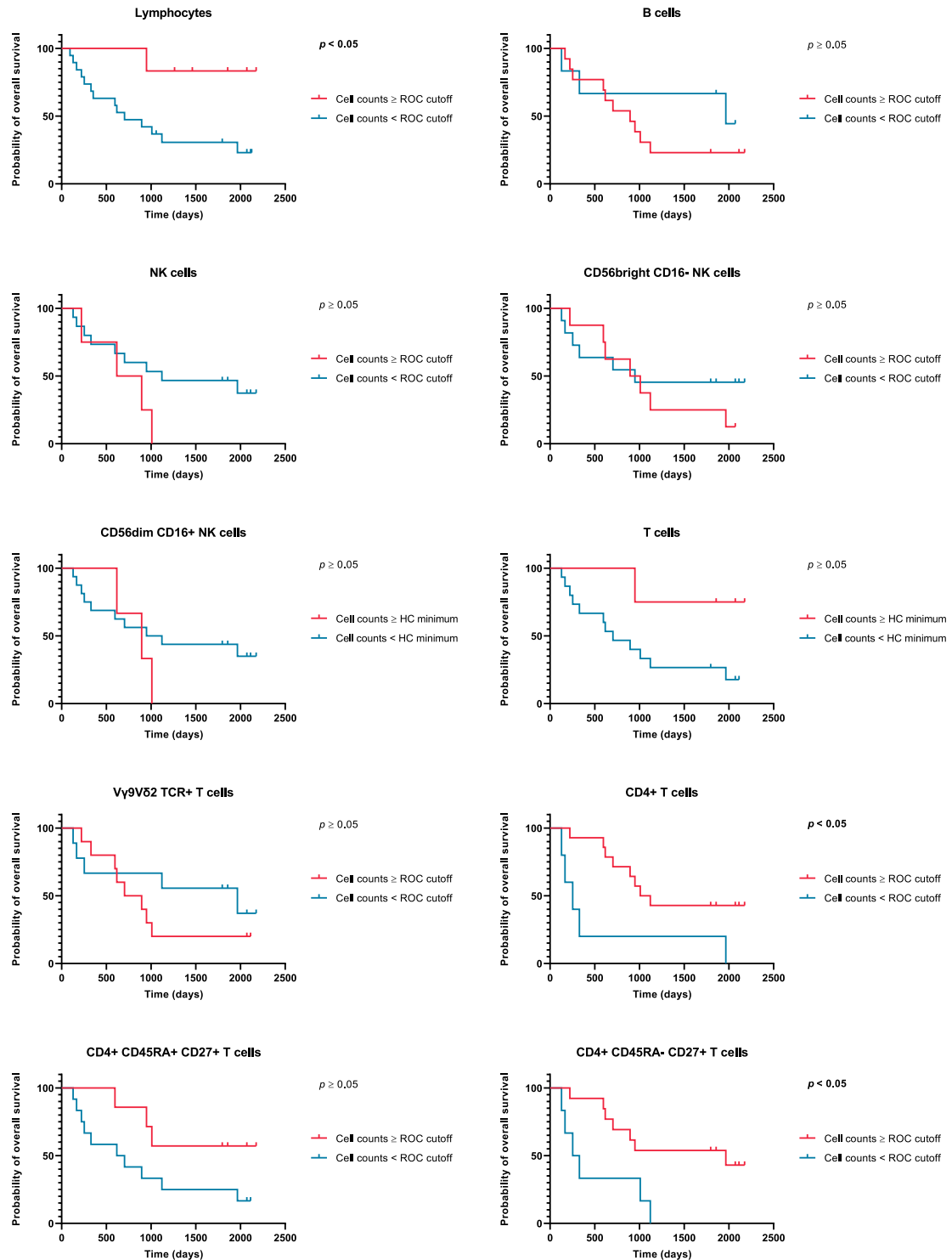


Figure 10. Kaplan-Meier overall survival curves of AML patients categorized by their immune subset levels after first-line induction CT. Statistical p -values for the Logrank tests are presented on the right side of the respective plot. ROC, receiver operating characteristic; HC, healthy control.

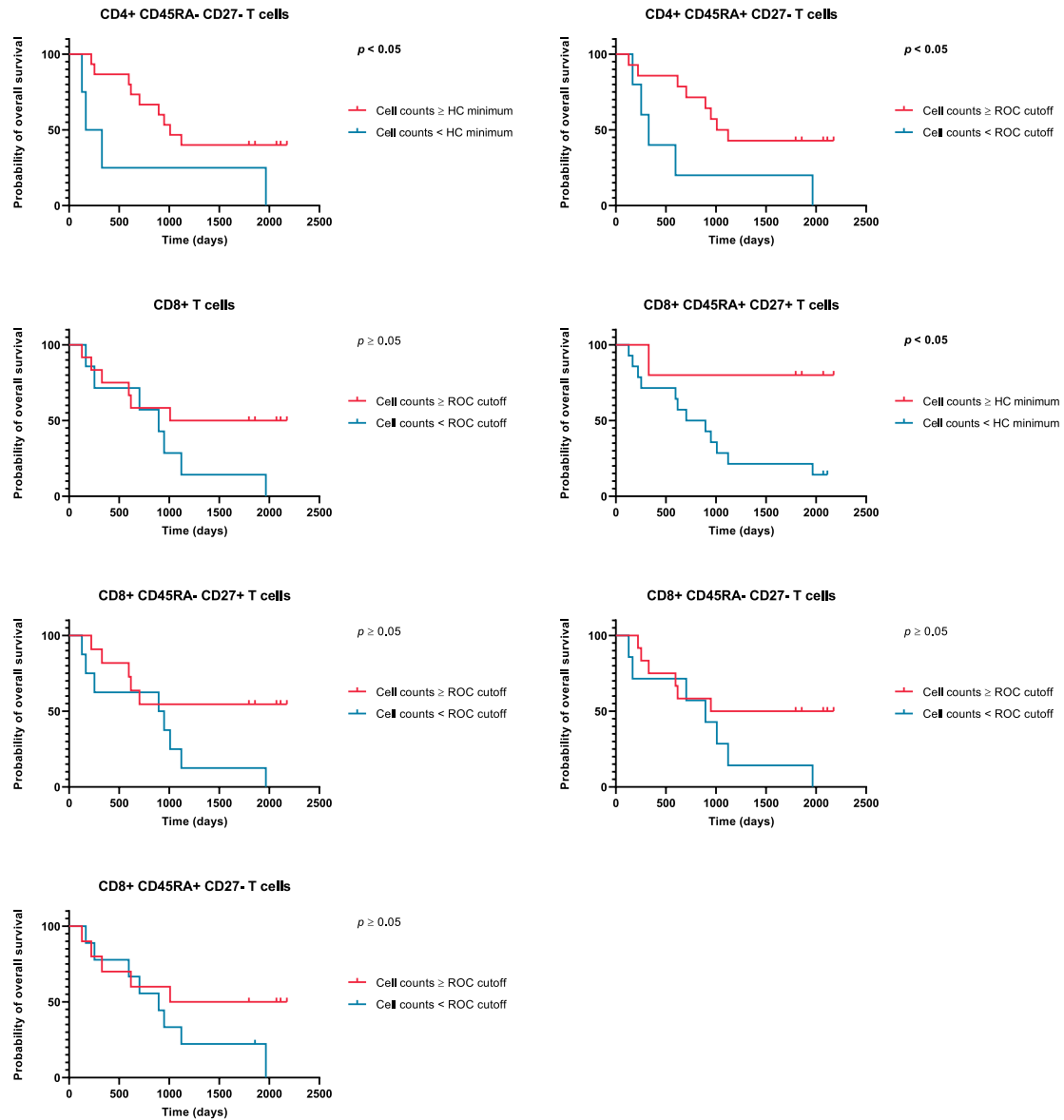


Figure 10 (cont.). Kaplan-Meier overall survival curves of AML patients categorized by their immune subset levels after first-line induction CT.

b. Method 2: Cox regression analysis

When applying the Cox regression strategy to the overall survival analysis, findings were considerably more restricted than those yielded by the previous analytical method. In fact, no population was found to be statistically significant in the univariate analysis (Table 14). Moreover, out of the cell subpopulations highlighted in the Kaplan-Meier findings, and when adjusting the data for age, only the lymphocyte cell counts sustained significance ($p = 0.035$; $HR = 0.998$, 95% CI: 0.997 – 1.000), although the CD4+ CD45RA- CD27- T cell

covariate p -value was close to the significance threshold ($p = 0.056$). Including the ELN cytogenetic risk stratification in the multivariate analysis did not generate any other significant results.

Table 14. Cox regression results for overall survival of AML patients, with models including immune cell counts assessed after first-line induction CT. Statistically significant results ($p < 0.05$) are highlighted in bold. ELN, European LeukemiaNet; HR, hazard ratio; CI, confidence interval. **a)** Although p -value is presented, the tested model is not valid, as the assumption of proportional hazards was not met.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	p -value	HR (95% CI)
Lymphocytes	Immune cell count	25	15	10	3.811	0.051	0.999 (0.997 – 1.000)
	Immune cell count	25	15	10	4.447	0.035	0.998 (0.997 – 1.000)
	Age				2.570	0.109	1.040 (0.991 – 1.092)
	Immune cell count				2.672	0.102	0.998 (0.997 – 1.000)
	Age				0.944	0.331	1.026 (0.974 – 1.081)
	ELN cytogenetic risk	22	12	10			
	Favorable				3.115	0.077	Reference
B cells	Intermediate				0.232	0.630	1.543 (0.265 – 8.993)
	Adverse				2.432	0.119	3.887 (0.706 – 21.411)
	Immune cell count	19	13	6	0.015	0.903	0.995 (0.911 – 1.085)
	Immune cell count	19	13	6	0.338	0.561	0.972 (0.882 – 1.070)
	Age				3.901	0.048	1.056 (1.000 – 1.115)
	Immune cell count				0.131	0.717	0.978 (0.865 – 1.105)
	Age				3.347	0.067	1.048 (0.997 – 1.101)
NK cells	ELN cytogenetic risk	17	11	6			
	Favorable				1.637	0.441	Reference
	Intermediate				1.167	0.280	2.631 (0.455 – 15.211)
	Adverse				1.551	0.213	3.027 (0.530 – 17.298)
	Immune cell count	19	13	6	0.155	0.694	1.003 (0.987 – 1.020)
	Immune cell count	19	13	6	0.193	0.660	0.996 (0.978 – 1.014)
	Age				3.640	0.056	1.059 (0.998 – 1.123)
CD56bright CD16- NK cells	Immune cell count				1.090	0.296	0.985 (0.958 – 1.013)
	Age				3.648	0.056	1.063 (0.998 – 1.133)
	ELN cytogenetic risk	17	11	6			
	Favorable				2.173	0.337	Reference
	Intermediate				1.989	0.158	3.540 (0.611 – 20.520)
	Adverse				1.405	0.236	2.831 (0.506 – 15.829)
	Immune cell count	19	13	6	0.013	0.909	0.983 (0.727 – 1.328)
CD56dim CD16+ NK cells	Immune cell count	19	13	6	0.360	0.549	0.919 (0.697 – 1.211)
	Age				3.745	0.053	1.059 (0.999 – 1.122)
	Immune cell count				0.621	0.431	0.862 (0.595 – 1.248)
	Age				3.256	0.071	1.055 (0.995 – 1.118)
	ELN cytogenetic risk	17	11	6			
	Favorable				2.466	0.291	Reference
	Intermediate				2.131	0.144	4.004 (0.622 – 25.795)
T cells	Adverse				1.644	0.200	3.039 (0.556 – 16.615)
	Immune cell count	19	13	6	0.482	0.487	1.008 (0.986 – 1.031)
	Immune cell count	19	13	6	0.008	0.929	0.999 (0.974 – 1.024)
	Age				3.292	0.070	1.055 (0.996 – 1.117)
	Immune cell count				0.170	0.680	0.989 (0.938 – 1.043)
	Age				2.868	0.090	1.053 (0.992 – 1.117)
	ELN cytogenetic risk	17	11	6			
T cells	Favorable				1.802	0.406	Reference
	Intermediate				1.459	0.227	2.877 (0.518 – 15.978)
	Adverse				1.453	0.228	2.953 (0.508 – 17.174)
	Immune cell count	19	13	6	2.782	0.095	0.999 (0.997 – 1.000)
	Immune cell count	19	13	6	2.272	0.132	0.999 (0.997 – 1.000)
	Age				3.086	0.079	1.049 (0.995 – 1.106)
	Immune cell count				1.659	0.198	0.998 (0.996 – 1.001)
T cells	Age				2.775	0.096	1.048 (0.992 – 1.106)
	ELN cytogenetic risk	17	11	6			
	Favorable				0.756	0.685	Reference
	Intermediate				0.655	0.418	2.060 (0.358 – 11.863)
	Adverse				0.612	0.434	2.054 (0.338 – 12.459)

Table 14 (cont.). Cox regression results for overall survival of AML patients, with models including immune cell counts assessed after first-line induction CT.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	<i>p</i> -value	HR (95% CI)
V γ 9V δ 2 TCR+ T cells	Immune cell count	19	13	6	0.149	0.700	1.005 (0.978 – 1.033)
	Immune cell count	19	13	6	0.214	0.644	0.993 (0.966 – 1.022)
	Age				3.764	0.052	1.059 (0.999 – 1.121)
	Immune cell count	17	11	6	0.179	0.672 ^{a)}	0.991 (0.949 – 1.034)
	Age				3.289	0.070	1.050 (0.996 – 1.108)
	ELN cytogenetic risk						
	Favorable				1.783	0.410 ^{a)}	Reference
CD4+ T cells	Intermediate				0.953	0.329	2.450 (0.406 – 14.804)
	Adverse				1.771	0.183 ^{a)}	3.155 (0.581 – 17.136)
	Immune cell count	19	13	6	1.972	0.160	0.998 (0.995 – 1.001)
	Immune cell count	19	13	6	2.825	0.093	0.997 (0.993 – 1.001)
	Age				4.744	0.029	1.060 (1.006 – 1.116)
	Immune cell count	17	11	6	1.783	0.182	0.997 (0.993 – 1.001)
	Age				4.242	0.039	1.059 (1.003 – 1.119)
CD4+ CD45RA+ CD27+ T cells	ELN cytogenetic risk						
	Favorable				0.292	0.864	Reference
	Intermediate				0.291	0.589	1.634 (0.275 – 9.720)
	Adverse				0.153	0.696	1.460 (0.219 – 9.720)
	Immune cell count	19	13	6	0.918	0.338	0.997 (0.991 – 1.003)
	Immune cell count	19	13	6	0.917	0.338	0.997 (0.990 – 1.003)
	Age				3.580	0.058	1.053 (0.998 – 1.112)
CD4+ CD45RA- CD27+ T cells	Immune cell count	17	11	6	0.276	0.599	0.998 (0.990 – 1.006)
	Age				3.145	0.076	1.047 (0.995 – 1.102)
	ELN cytogenetic risk						
	Favorable				1.729	0.421	Reference
	Intermediate				1.433	0.231	2.844 (0.514 – 15.744)
	Adverse				1.409	0.235	2.887 (0.501 – 16.630)
	Immune cell count	19	13	6	1.098	0.295	0.997 (0.991 – 1.003)
CD4+ CD45RA- CD27- T cells	Immune cell count	19	13	6	1.057	0.304	0.997 (0.991 – 1.003)
	Age				3.497	0.061	1.050 (0.998 – 1.105)
	Immune cell count	17	11	6	0.001	0.979	1.000 (0.991 – 1.009)
	Age				3.176	0.075	1.046 (0.995 – 1.100)
	ELN cytogenetic risk						
	Favorable				1.006	0.605	Reference
	Intermediate				0.658	0.417	2.771 (0.236 – 32.539)
CD4+ CD45RA+ CD27- T cells	Adverse				1.003	0.316	3.283 (0.321 – 33.603)
	Immune cell count	19	13	6	2.511	0.113	0.993 (0.985 – 1.002)
	Immune cell count	19	13	6	3.666	0.056	0.991 (0.982 – 1.000)
	Age				4.862	0.027	1.058 (1.006 – 1.113)
	Immune cell count	17	11	6	3.212	0.073	0.988 (0.975 – 1.001)
	Age				4.898	0.027	1.060 (1.007 – 1.115)
	ELN cytogenetic risk						
CD4+ CD45RA- CD27+ T cells	Favorable				0.190	0.909	Reference
	Intermediate				0.027	0.870	0.842 (0.107 – 6.645)
	Adverse				0.023	0.880	1.164 (0.162 – 8.388)
	Immune cell count	19	13	6	0.020	0.887 ^{a)}	0.999 (0.984 – 1.014)
	Immune cell count	19	13	6	2.099	0.147 ^{a)}	0.983 (0.962 – 1.006)
	Age				5.802	0.016	1.085 (1.015 – 1.159)
	Immune cell count	17	11	6	2.735	0.098	0.982 (0.961 – 1.003)
CD8+ T cells	Age				5.661	0.017	1.090 (1.015 – 1.170)
	ELN cytogenetic risk						
	Favorable				2.951	0.229	Reference
	Intermediate				2.913	0.088	4.704 (0.795 – 27.846)
	Adverse				1.528	0.216	2.910 (0.535 – 15.822)
	Immune cell count	19	13	6	2.657	0.103	0.996 (0.992 – 1.001)
	Immune cell count	19	13	6	1.469	0.226	0.997 (0.993 – 1.002)
CD8+ CD45RA+ CD27+ T cells	Age				1.882	0.170	1.040 (0.983 – 1.100)
	Immune cell count	17	11	6	2.351	0.125	0.995 (0.989 – 1.001)
	Age				1.541	0.215	1.038 (0.979 – 1.101)
	ELN cytogenetic risk						
	Favorable				2.594	0.273	Reference
	Intermediate				1.575	0.210	3.214 (0.519 – 19.912)
	Adverse				2.470	0.116	3.903 (0.714 – 21.327)
CD8+ CD45RA- CD27+ T cells	Immune cell count	19	13	6	3.228	0.072	0.982 (0.963 – 1.002)
	Immune cell count	19	13	6	1.721	0.190	0.986 (0.965 – 1.007)
	Age				1.510	0.219	1.035 (0.980 – 1.093)
	Immune cell count	17	11	6	1.869	0.172	0.983 (0.958 – 1.008)
	Age				1.089	0.297	1.028 (0.976 – 1.084)
	ELN cytogenetic risk						
	Favorable				2.164	0.339	Reference
	Intermediate				1.174	0.279	2.593 (0.463 – 14.533)
	Adverse				2.142	0.143	3.515 (0.653 – 18.928)

Table 14 (cont.). Cox regression results for overall survival of AML patients, with models including immune cell counts assessed after first-line induction CT.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	<i>p</i> -value	HR (95% CI)
CD8+ CD45RA- CD27+ T cells	Immune cell count	19	13	6	2.587	0.108	0.981 (0.958 – 1.004)
	Immune cell count	19	13	6	0.709	0.400	0.989 (0.964 – 1.015)
	Age				1.137	0.286	1.035 (0.971 – 1.103)
	Immune cell count	17	11	6	1.030	0.310	0.982 (0.948 – 1.017)
	Age				0.280	0.597	1.018 (0.953 – 1.088)
	ELN cytogenetic risk						
	Favorable				2.365	0.306	Reference
CD8+ CD45RA- CD27- T cells	Intermediate				0.752	0.386	2.172 (0.376 – 12.540)
	Adverse				2.361	0.124	3.833 (0.691 – 21.269)
	Immune cell count	19	13	6	2.148	0.143	0.990 (0.978 – 1.003)
	Immune cell count	19	13	6	0.969	0.325	0.993 (0.979 – 1.007)
	Age				1.327	0.249	1.035 (0.976 – 1.098)
	Immune cell count	17	11	6	1.048	0.306	0.987 (0.963 – 1.012)
	Age				0.308	0.579	1.018 (0.956 – 1.083)
CD8+ CD45RA+ CD27- T cells	ELN cytogenetic risk						
	Favorable				1.883	0.390	Reference
	Intermediate				0.302	0.583	1.709 (0.252 – 11.567)
	Adverse				1.75	0.186	3.167 (0.574 – 17.478)
	Immune cell count	19	13	6	0.560	0.454	0.997 (0.989 – 1.005)
	Immune cell count	19	13	6	0.562	0.453	0.997 (0.989 – 1.005)
	Age				3.572	0.059	1.056 (0.998 – 1.117)
CD8+ CD45RA+ CD27+ T cells	Immune cell count	17	11	6	2.242	0.134	0.993 (0.984 – 1.002)
	Age				3.503	0.061	1.060 (0.997 – 1.127)
	ELN cytogenetic risk						
	Favorable				3.461	0.177	Reference
	Intermediate				2.860	0.091	4.931 (0.776 – 31.339)
	Adverse				2.807	0.094	4.354 (0.779 – 24.346)

4. Comparing relapse-free survival of AML patients with higher or lower frequencies of immune populations

a. Method 1: Kaplan-Meier analysis

When comparing the selected Logrank test results of the various patient categorization approaches, significantly separated relapse-free survival curves were identified for eight cell populations (Table 15, Figure 11). These include lymphocytes ($p = 0.013$), NK ($p = 0.001$) and T cells ($p = 0.022$), CD4+ CD45RA- CD27+ ($p < 0.001$), CD4+ CD45RA- CD27- ($p = 0.002$) and CD4+ CD45RA+ CD27- ($p = 0.014$) T cell subsets, as well as CD8+ CD45RA+ CD27+ and CD8+ CD45RA- CD27- T cells ($p = 0.047$ and $p = 0.001$, respectively). In a similar manner as to what was obtained for the overall survival analysis, patients with higher frequencies of these immune cells exhibit higher relapse-free survival probabilities than patients with lower cell counts.

Table 15. Best-performing Kaplan-Meier results for relapse-free survival of AML patients categorized by their immune subset levels after first-line induction CT. Statistically significant results ($p < 0.05$) are highlighted in bold. ROC, receiver operating characteristic; HC, healthy control. **a)** Median survival was not reached (undefined), since, at the end of the follow-up period, relapse-free survival of the patients included in the group was higher than 50%.

Cell population	Group	Total <i>n</i>	Event <i>s</i>	Censored	Median survival, days	<i>p</i> -value
Lymphocytes	Cell counts < ROC cutoff	17	13	4	408	0.013
	Cell counts ≥ ROC cutoff	6	1	5	a)	
B cells	Cell counts < ROC cutoff	6	3	3	905	0.198
	Cell counts ≥ ROC cutoff	12	10	2	408	
NK cells	Cell counts < ROC cutoff	3	3	0	116	0.001
	Cell counts ≥ ROC cutoff	15	10	5	881	
CD56bright CD16- NK cells	Cell counts < HC minimum	16	11	5	643	0.305
	Cell counts ≥ HC minimum	2	2	0	184	
CD56dim CD16+ NK cells	Cell counts < ROC cutoff	15	10	5	643	0.519
	Cell counts ≥ ROC cutoff	3	3	0	650	
T cells	Cell counts < ROC cutoff	14	12	2	395	0.022
	Cell counts ≥ ROC cutoff	4	1	3	a)	
Vγ9Vδ2 TCR+ T cells	Cell counts < ROC cutoff	12	9	3	223	0.314
	Cell counts ≥ ROC cutoff	6	4	2	881	
CD4+ T cells	Cell counts < ROC cutoff	15	12	3	408	0.095
	Cell counts ≥ ROC cutoff	3	1	2	a)	
CD4+ CD45RA+ CD27+ T cells	Cell counts < ROC cutoff	11	9	2	395	0.195
	Cell counts ≥ ROC cutoff	7	4	3	949	
CD4+ CD45RA- CD27+ T cells	Cell counts < ROC cutoff	6	6	0	184	< 0.001
	Cell counts ≥ ROC cutoff	12	7	5	905	
CD4+ CD45RA- CD27- T cells	Cell counts < ROC cutoff	8	8	0	184	0.002
	Cell counts ≥ ROC cutoff	10	5	5	949	
CD4+ CD45RA+ CD27- T cells	Cell counts < ROC cutoff	5	5	0	208	0.014
	Cell counts ≥ ROC cutoff	13	8	5	881	
CD8+ T cells	Cell counts < ROC cutoff	6	6	0	208	0.069
	Cell counts ≥ ROC cutoff	12	7	5	650	
CD8+ CD45RA+ CD27+ T cells	Cell counts < ROC cutoff	6	6	0	208	0.047
	Cell counts ≥ ROC cutoff	12	7	5	650	
CD8+ CD45RA- CD27+ T cells	Cell counts < ROC cutoff	11	10	1	643	0.100
	Cell counts ≥ ROC cutoff	7	3	4	a)	
CD8+ CD45RA- CD27- T cells	Cell counts < HC minimum	2	2	0	95	0.001
	Cell counts ≥ HC minimum	16	11	5	650	
CD8+ CD45RA+ CD27- T cells	Cell counts < ROC cutoff	5	5	0	208	0.064
	Cell counts ≥ ROC cutoff	13	8	5	650	

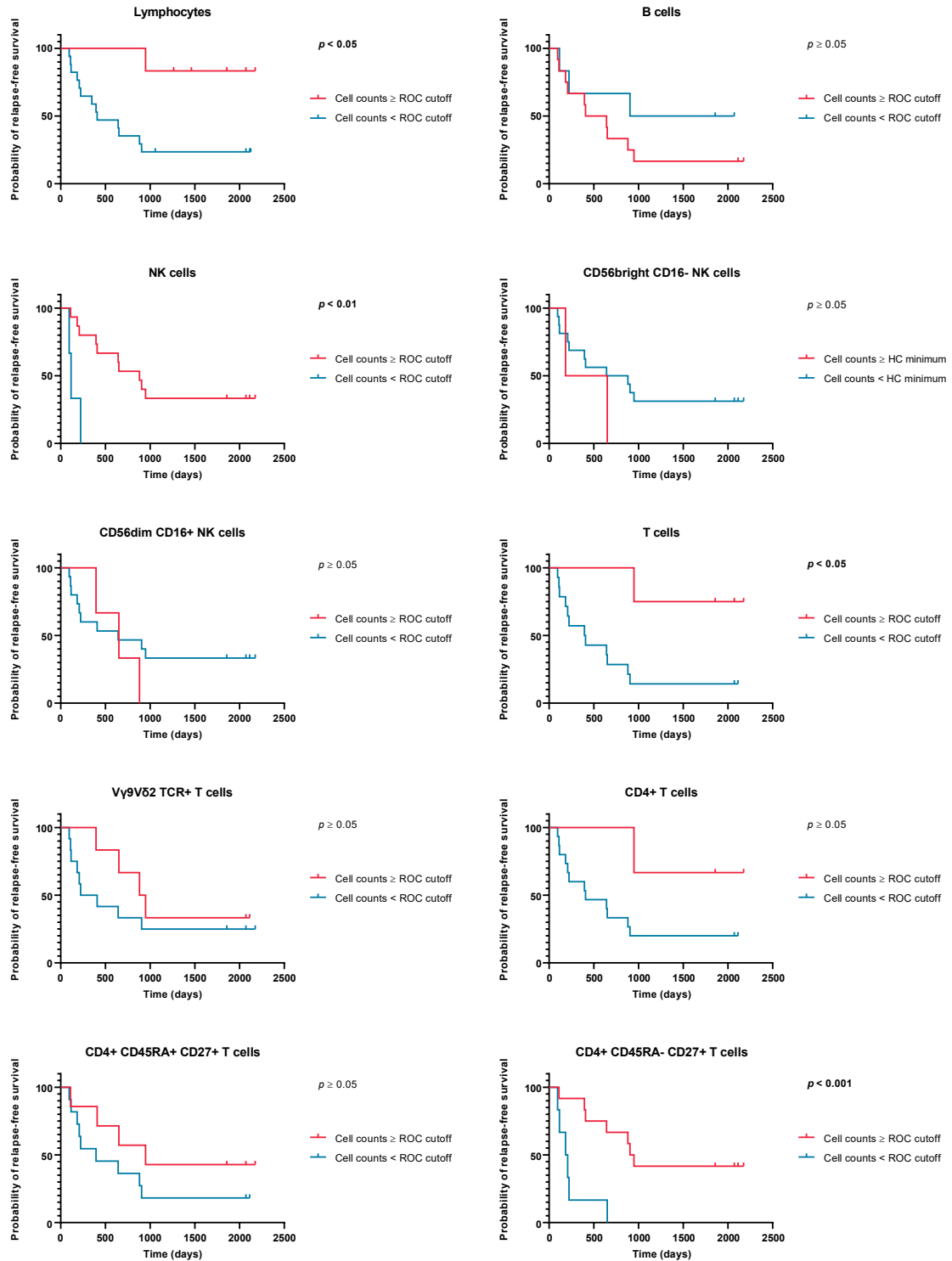


Figure 11. Kaplan-Meier relapse-free survival curves of AML patients categorized by their immune subset levels after first-line induction CT. Statistical p -values for the Logrank tests are presented on the right side of the respective plot. ROC, receiver operating characteristic; HC, healthy control.

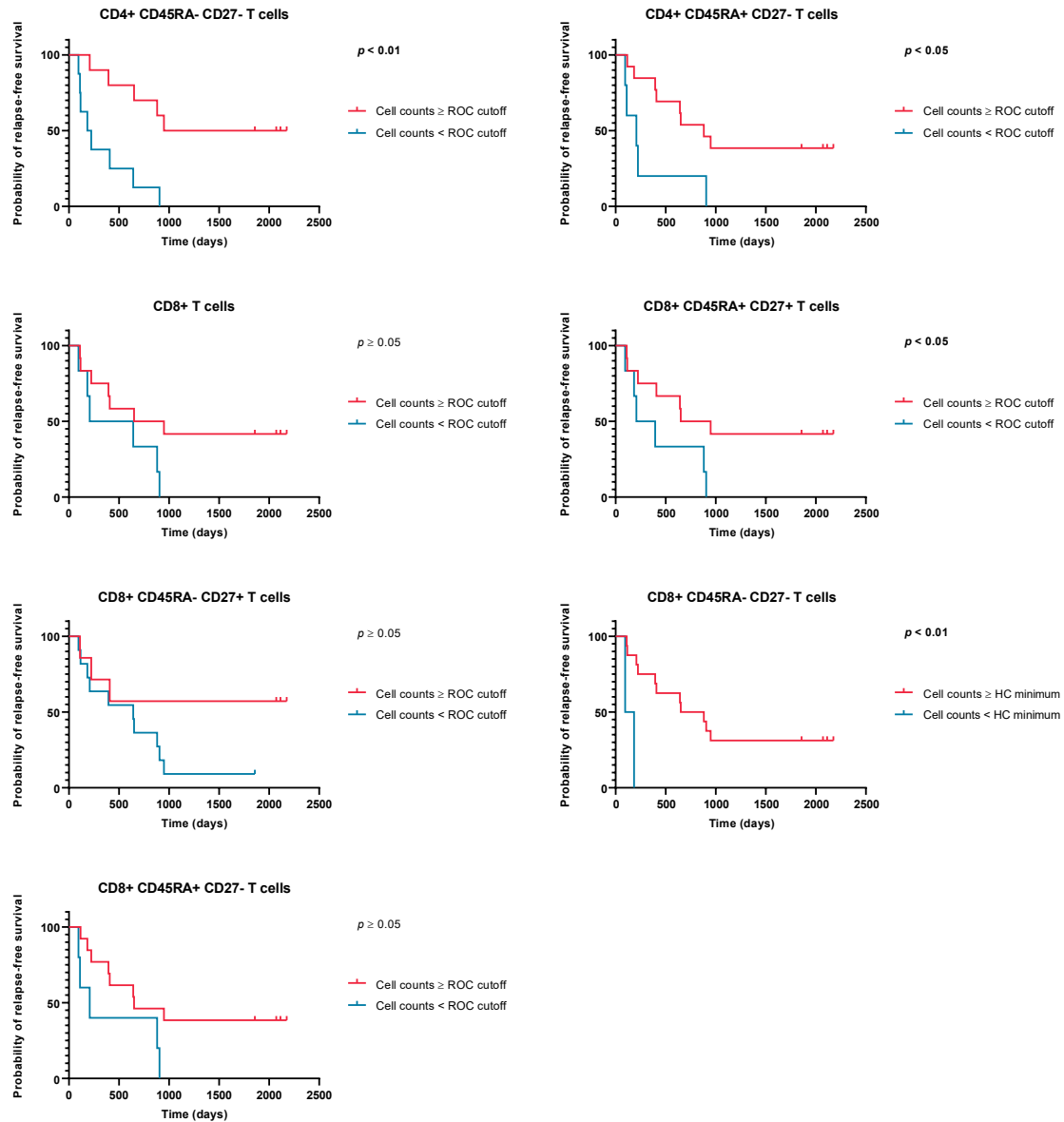


Figure 11 (cont.). Kaplan-Meier relapse-free survival curves of AML patients categorized by their immune subset levels after first-line induction CT.

b. Method 2: Cox regression analysis

The effect of each immune cell count obtained after induction chemotherapy on relapse-free survival time was also assessed through the Cox regression method. Only the results for the CD4+ CD45RA- CD27- T cell population and its respective parent subsets (CD4+ T cells, T cells and lymphocytes) were found to be statistically significant at a univariate level and/or after controlling for age. Moreover, adjusting for age generally led to an improvement of the p -values for the aforementioned immune subsets. Strikingly, only

the CD4+ CD45RA- CD27- T cell count covariate maintained statistical significance when controlling for both age and ELN cytogenetic risk, albeit with the highest covariate p -value of the three models ($p = 0.033$, as opposed to $p = 0.016$ for the univariate analysis and $p = 0.005$ for the model including age). Although the NK cell analysis also resulted in a significant p -value when controlling for age, statistical tests may not be robust because the proportional hazards assumption required for Cox regression application was violated. Main statistical data is described in Table 16.

Table 16. Cox regression results for relapse-free survival of AML patients, with models including immune cell counts assessed after first-line induction CT. Statistically significant results ($p < 0.05$) are highlighted in bold. ELN, European LeukemiaNet; HR, hazard ratio; CI, confidence interval. **a)** Although p -value is presented, the tested model is not valid, as the assumption of proportional hazards was not met.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	p -value	HR (95% CI)
Lymphocytes	Immune cell count	23	14	9	5.350	0.021	0.998 (0.997 – 1.000)
	Immune cell count	23	14	9	7.157	0.007	0.997 (0.996 – 0.999)
	Age				4.078	0.043	1.059 (1.002 – 1.120)
	Immune cell count	20	11	9	3.381	0.066	0.998 (0.996 – 1.000)
	Age				1.891	0.169	1.044 (0.982 – 1.110)
	ELN cytogenetic risk						
	Favorable				1.431	0.489	Reference
B cells	Intermediate				1.126	0.289	2.550 (0.452 – 14.370)
	Adverse				1.286	0.257	2.870 (0.464 – 17.751)
	Immune cell count	18	13	5	0.145	0.703	1.016 (0.937 – 1.102)
	Immune cell count	18	13	5	0.038	0.846	0.991 (0.906 – 1.084)
	Age				1.963	0.161	1.037 (0.986 – 1.091)
	Immune cell count	16	11	5	0.803	0.370	1.048 (0.946 – 1.161)
	Age				2.224	0.136	1.038 (0.988 – 1.089)
NK cells	ELN cytogenetic risk						
	Favorable				5.772	0.056	Reference
	Intermediate				5.771	0.016	10.389 (1.539 – 70.145)
	Adverse				2.910	0.088	5.264 (0.781 – 35.481)
	Immune cell count	18	13	5	0.126	0.723 ^{a)}	0.996 (0.977 – 1.016)
	Immune cell count	18	13	5	0.756	0.385 ^{a)}	0.991 (0.971 – 1.012)
	Age				2.555	0.110	1.044 (0.990 – 1.100)
CD56bright CD16- NK cells	Immune cell count	16	11	5	4.636	0.031 ^{a)}	0.952 (0.911 – 0.996)
	Age				5.246	0.022	1.091 (1.013 – 1.176)
	ELN cytogenetic risk						
	Favorable				7.998	0.018	Reference
	Intermediate				7.738	0.005	29.238 (2.710 – 315.392)
	Adverse				1.870	0.171	3.598 (0.574 – 22.550)
	Immune cell count	18	13	5	0.172	0.679	1.068 (0.783 – 1.457)
CD56dim CD16+ NK cells	Immune cell count	18	13	5	0.068	0.795	1.039 (0.780 – 1.384)
	Age				1.902	0.168	1.035 (0.986 – 1.086)
	Immune cell count	16	11	5	1.768	0.184	0.764 (0.514 – 1.136)
	Age				3.242	0.072	1.053 (0.995 – 1.113)
	ELN cytogenetic risk						
	Favorable				7.109	0.029	Reference
	Intermediate				7.082	0.008	15.258 (2.050 – 113.546)
CD56dim CD16+ NK cells	Adverse				1.929	0.165	3.437 (0.602 – 19.619)
	Immune cell count	18	13	5	0.016	0.898 ^{a)}	1.002 (0.978 – 1.026)
	Immune cell count	18	13	5	0.219	0.640 ^{a)}	0.994 (0.967 – 1.021)
	Age				2.157	0.142	1.040 (0.987 – 1.097)
	Immune cell count	16	11	5	1.515	0.218	0.956 (0.891 – 1.027)
	Age				3.209	0.073	1.061 (0.994 – 1.133)
	ELN cytogenetic risk						
	Favorable				5.613	0.060	Reference
	Intermediate				5.589	0.018	12.313 (1.536 – 98.692)
	Adverse				1.579	0.209	3.088 (0.532 – 17.925)

Table 16 (cont.). Cox regression results for relapse-free survival of AML patients, with models including immune cell counts assessed after first-line induction CT.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	<i>p</i> -value	HR (95% CI)
T cells	Immune cell count	18	13	5	4.245	0.039	0.998 (0.996 – 1.000)
	Immune cell count	18	13	5	4.280	0.039	0.998 (0.996 – 1.000)
	Age				2.185	0.139	1.037 (0.988 – 1.089)
	Immune cell count	16	11	5	1.600	0.206	0.998 (0.996 – 1.001)
	Age				2.479	0.115	1.043 (0.990 – 1.099)
	ELN cytogenetic risk						
	Favorable				3.348	0.188	Reference
Vγ9Vδ2 TCR+ T cells	Intermediate				3.049	0.081	5.127 (0.819 – 32.111)
	Adverse				0.654	0.419	2.186 (0.328 – 14.558)
	Immune cell count	18	13	5	0.150	0.698	0.995 (0.968 – 1.022)
	Immune cell count	18	13	5	1.434	0.231	0.982 (0.953 – 1.012)
	Age				3.204	0.073	1.051 (0.995 – 1.110)
	Immune cell count	16	11	5	0.324	0.569	0.987 (0.942 – 1.033)
	Age				2.796	0.095	1.044 (0.993 – 1.099)
CD4+ T cells	ELN cytogenetic risk						
	Favorable				4.246	0.120	Reference
	Intermediate				4.234	0.040	6.878 (1.096 – 43.165)
	Adverse				2.191	0.139	3.736 (0.652 – 21.397)
	Immune cell count	18	13	5	2.742	0.098	0.998 (0.995 – 1.000)
	Immune cell count	18	13	5	4.127	0.042	0.996 (0.993 – 1.000)
	Age				4.066	0.044	1.053 (1.001 – 1.107)
CD4+ CD45RA+ CD27+ T cells	Immune cell count	16	11	5	1.616	0.204	0.997 (0.993 – 1.002)
	Age				3.596	0.058	1.053 (0.998 – 1.112)
	ELN cytogenetic risk						
	Favorable				3.136	0.208	Reference
	Intermediate				2.236	0.135	4.170 (0.642 – 27.088)
	Adverse				0.184	0.668	1.558 (0.206 – 11.782)
	Immune cell count	18	13	5	0.180	0.671	0.999 (0.993 – 1.005)
CD4+ CD45RA- CD27+ T cells	Immune cell count	18	13	5	0.298	0.585	0.998 (0.992 – 1.004)
	Age				2.100	0.147	1.036 (0.987 – 1.088)
	Immune cell count	16	11	5	0.023	0.881	1.001 (0.993 – 1.008)
	Age				2.513	0.113	1.039 (0.991 – 1.088)
	ELN cytogenetic risk						
	Favorable				5.161	0.076	Reference
	Intermediate				5.132	0.023	8.254 (1.329 – 51.255)
CD4+ CD45RA- CD27- T cells	Adverse				2.132	0.144	4.095 (0.617 – 27.174)
	Immune cell count	18	13	5	2.529	0.112	0.995 (0.988 – 1.001)
	Immune cell count	18	13	5	3.479	0.062	0.994 (0.987 – 1.000)
	Age				3.028	0.082	1.042 (0.995 – 1.092)
	Immune cell count	16	11	5	0.007	0.936	1.000 (0.989 – 1.011)
	Age				2.500	0.114	1.039 (0.991 – 1.089)
	ELN cytogenetic risk						
CD4+ CD45RA- CD27- T cells	Favorable				2.290	0.318	Reference
	Intermediate				1.878	0.171	7.324 (0.424 – 126.372)
	Adverse				0.801	0.371	3.533 (0.223 – 56.034)
	Immune cell count	18	13	5	5.766	0.016	0.989 (0.980 – 0.998)
	Immune cell count	18	13	5	7.809	0.005	0.983 (0.971 – 0.995)
	Age				5.618	0.018	1.062 (1.010 – 1.115)
	Immune cell count	16	11	5	4.559	0.033	0.980 (0.962 – 0.998)
CD4+ CD45RA+ CD27- T cells	Age				5.335	0.021	1.076 (1.011 – 1.144)
	ELN cytogenetic risk						
	Favorable				1.551	0.461	Reference
	Intermediate				0.524	0.469	2.113 (0.279 – 16.016)
	Adverse				0.025	0.874	0.837 (0.093 – 7.549)
	Immune cell count	18	13	5	0.005	0.944	0.999 (0.984 – 1.015)
	Immune cell count	18	13	5	0.911	0.340	0.990 (0.969 – 1.011)
CD4+ CD45RA+ CD27- T cells	Age				3.042	0.081	1.051 (0.994 – 1.111)
	Immune cell count	16	11	5	2.896	0.089	0.983 (0.965 – 1.003)
	Age				5.364	0.021^{a)}	1.078 (1.012 – 1.149)
	ELN cytogenetic risk						
	Favorable				7.368	0.025	Reference
	Intermediate				7.229	0.007	13.590 (2.028 – 91.055)
	Adverse				2.077	0.150	3.587 (0.631 – 20.372)
CD8+ T cells	Immune cell count	18	13	5	2.815	0.093	0.996 (0.992 – 1.001)
	Immune cell count	18	13	5	2.093	0.148	0.996 (0.992 – 1.001)
	Age				0.433	0.510	1.017 (0.968 – 1.068)
	Immune cell count	16	11	5	1.944	0.163	0.996 (0.991 – 1.002)
	Age				1.522	0.217 ^{a)}	1.034 (0.980 – 1.092)
	ELN cytogenetic risk						
	Favorable				5.176	0.075	Reference
CD8+ T cells	Intermediate				5.048	0.025	8.655 (1.317 – 56.865)
	Adverse				2.992	0.084	4.643 (0.815 – 26.438)

Table 16 (cont.). Cox regression results for relapse-free survival of AML patients, with models including immune cell counts assessed after first-line induction CT.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	<i>p</i> -value	HR (95% CI)
CD8+ CD45RA+ CD27+ T cells	Immune cell count	18	13	5	2.556	0.110	0.985 (0.967 – 1.003)
	Immune cell count	18	13	5	1.588	0.208	0.987 (0.968 – 1.007)
	Age				0.581	0.446	1.019 (0.971 – 1.070)
	Immune cell count	16	11	5	0.512	0.474	0.992 (0.972 – 1.013)
	Age				1.330	0.249	1.030 (0.979 – 1.084)
	ELN cytogenetic risk						
	Favorable				4.493	0.106	Reference
CD8+ CD45RA- CD27+ T cells	Intermediate				4.465	0.035	6.974 (1.151 – 42.245)
	Adverse				2.336	0.126	3.824 (0.685 – 21.363)
	Immune cell count	18	13	5	2.511	0.113	0.977 (0.950 – 1.005)
	Immune cell count	18	13	5	1.645	0.200	0.979 (0.949 – 1.011)
	Age				0.061	0.805	1.007 (0.953 – 1.064)
	Immune cell count	16	11	5	0.663	0.416	0.986 (0.952 – 1.021)
	Age				0.314	0.575	1.018 (0.956 – 1.084)
CD8+ CD45RA- CD27- T cells	ELN cytogenetic risk						
	Favorable				3.937	0.140	Reference
	Intermediate				3.583	0.058	6.034 (0.938 – 38.809)
	Adverse				2.588	0.108	4.204 (0.731 – 24.189)
	Immune cell count	18	13	5	2.521	0.112	0.987 (0.971 – 1.003)
	Immune cell count	18	13	5	2.146	0.143	0.987 (0.971 – 1.004)
	Age				0.084	0.772	1.007 (0.958 – 1.059)
CD8+ CD45RA+ CD27- T cells	Immune cell count	16	11	5	0.927	0.336	0.986 (0.958 – 1.015)
	Age				0.293	0.588	1.015 (0.961 – 1.073)
	ELN cytogenetic risk						
	Favorable				2.826	0.243	Reference
	Intermediate				2.473	0.116	4.811 (0.679 – 34.076)
	Adverse				2.176	0.140	3.718 (0.650 – 21.281)
	Immune cell count	18	13	5	0.755	0.385	0.996 (0.988 – 1.005)
CD8+ CD45RA+ CD27- T cells	Immune cell count	18	13	5	0.478	0.489	0.997 (0.989 – 1.005)
	Age				1.754	0.185	1.034 (0.984 – 1.086)
	Immune cell count	16	11	5	2.501	0.114	0.993 (0.985 – 1.002)
	Age				3.393	0.065 ^{a)}	1.055 (0.997 – 1.117)
	ELN cytogenetic risk						
	Favorable				7.041	0.030	Reference
	Intermediate				7.016	0.008	14.786 (2.015 – 108.515)
	Adverse				3.56	0.059	5.556 (0.936 – 32.990)

C. Patient samples collected after first-line consolidation chemotherapy

1. Comparing immune cell counts of AML patients with healthy controls

After first-line consolidation chemotherapy, the immune landscape of AML patients was generally distinct from that of healthy donor individuals, with 12 out of the 17 analyses rendering *p*-values below the significance threshold. With the exception of five immune populations, namely CD56bright CD16- NK cells and the CD45RA- CD27- and CD45RA+ CD27- subsets of both CD4+ and CD8+ T cells, results suggested that AML patients had lower cell frequencies than healthy individuals. All results are described in Table 17 and represented in Figure 12.

Table 17. Immune subset level comparisons between AML patients after first-line consolidation CT and healthy controls. Statistical analyses were performed using one-sample *t*-tests, independent samples *t*-tests or Mann-Whitney U tests. Statistically significant results ($p < 0.05$) are highlighted in bold. N/A, not applicable. **a)** For total lymphocyte comparisons, the healthy control sample was represented by the mean absolute lymphocyte count of 2.102×10^9 cells/L of peripheral blood reported by Fonseca, Pereira et al., 2020 (134).

Cell population	Group	<i>n</i>	Median	Statistical test	<i>p</i> -value
Lymphocytes	Healthy controls ^{a)}	N/A	N/A	One-sample <i>t</i> -test	< 0.001
	AML patients	18	7.100×10^8		
B cells	Healthy controls	6	1.854×10^8	Mann-Whitney U test	0.002
	AML patients	17	1.826×10^6		
NK cells	Healthy controls	6	1.629×10^8	Independent samples <i>t</i> -test	0.016
	AML patients	17	3.636×10^7		
CD56bright CD16- NK cells	Healthy controls	6	6.574×10^6	Mann-Whitney U test	0.062
	AML patients	17	2.555×10^6		
CD56dim CD16+ NK cells	Healthy controls	6	1.243×10^8	Independent samples <i>t</i> -test	0.008
	AML patients	17	1.051×10^7		
T cells	Healthy controls	6	1.610×10^9	Independent samples <i>t</i> -test	< 0.001
	AML patients	17	6.338×10^8		
V γ 9V δ 2 TCR+ T cells	Healthy controls	6	2.244×10^7	Mann-Whitney U test	0.013
	AML patients	17	4.140×10^6		
CD4+ T cells	Healthy controls	6	1.083×10^9	Independent samples <i>t</i> -test	< 0.001
	AML patients	16	4.332×10^8		
CD4+ CD45RA+ CD27+ T cells	Healthy controls	6	4.425×10^8	Independent samples <i>t</i> -test	< 0.001
	AML patients	16	1.609×10^8		
CD4+ CD45RA- CD27+ T cells	Healthy controls	6	4.268×10^8	Independent samples <i>t</i> -test	< 0.001
	AML patients	16	1.506×10^8		
CD4+ CD45RA- CD27- T cells	Healthy controls	6	1.506×10^8	Mann-Whitney U test	0.083
	AML patients	16	7.954×10^7		
CD4+ CD45RA+ CD27- T cells	Healthy controls	6	6.053×10^6	Mann-Whitney U test	0.154
	AML patients	16	2.543×10^7		
CD8+ T cells	Healthy controls	6	3.413×10^8	Mann-Whitney U test	0.017
	AML patients	16	1.449×10^8		
CD8+ CD45RA+ CD27+ T cells	Healthy controls	6	1.731×10^8	Mann-Whitney U test	0.003
	AML patients	16	3.234×10^7		
CD8+ CD45RA- CD27+ T cells	Healthy controls	6	4.905×10^7	Mann-Whitney U test	0.008
	AML patients	16	1.165×10^7		
CD8+ CD45RA- CD27- T cells	Healthy controls	6	3.173×10^7	Mann-Whitney U test	0.590
	AML patients	16	1.798×10^7		
CD8+ CD45RA+ CD27- T cells	Healthy controls	6	4.748×10^7	Mann-Whitney U test	0.590
	AML patients	16	3.175×10^7		

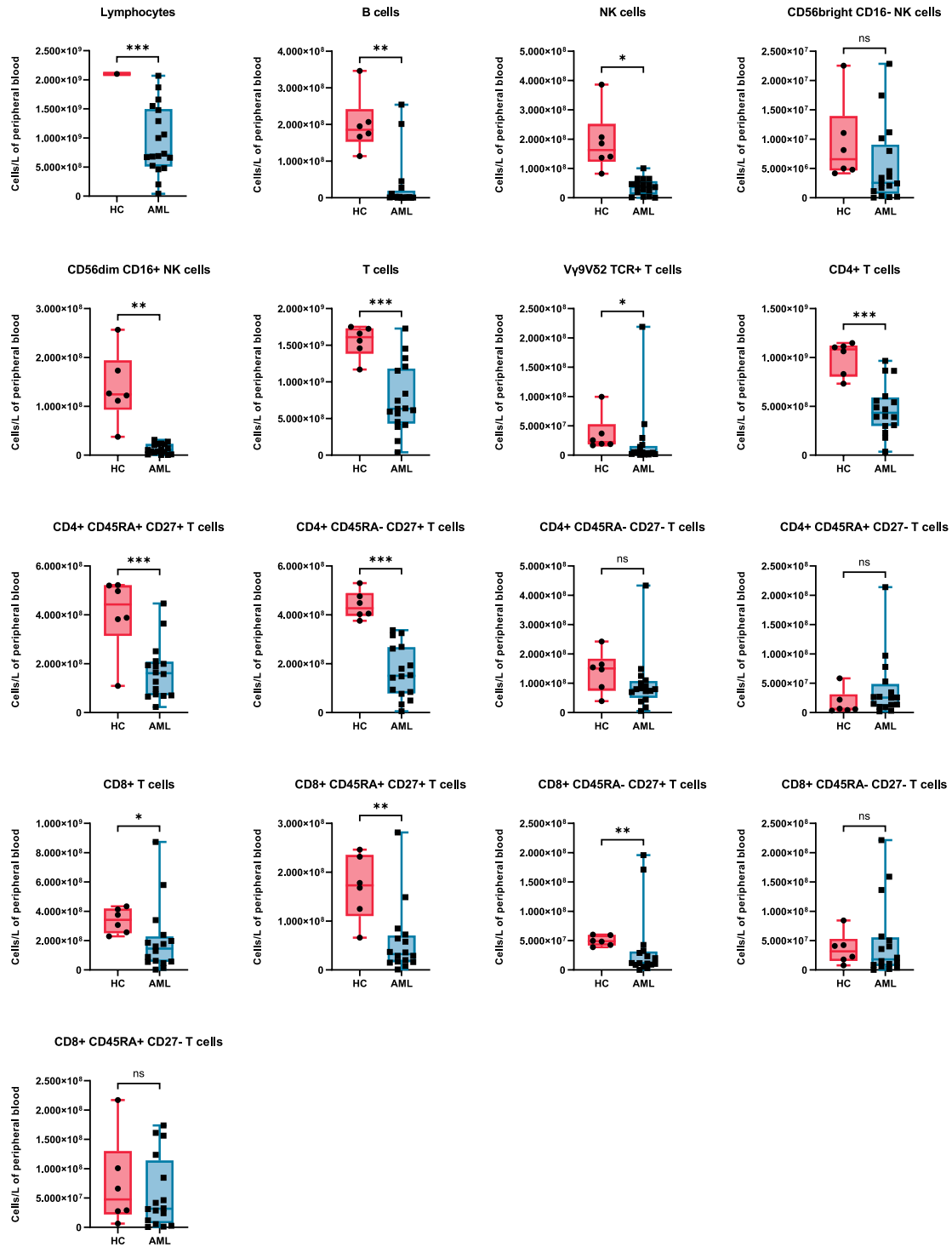


Figure 12. Immune cell levels of AML patients after first-line consolidation CT and of healthy controls. Data are presented in boxplots depicting median cell counts, interquartile range (boxes), and minimum and maximum values (whiskers). Statistical *p*-values for the comparisons between groups are presented as *, *p* < 0.05; **, *p* < 0.01; and ***, *p* < 0.001; ns, not significant. HC, healthy controls; AML, acute myeloid leukemia patients.

2. Comparing immune cell counts of AML patients by their response to first-line induction chemotherapy

Similarly to the post-induction results, patients who responded differently to induction chemotherapy still had equivalent post-consolidation circulating levels of each of the evaluated cell populations (Table 18, Figure 13).

Table 18. Immune subset level comparisons between AML patients by their response to first line induction CT in samples collected after first-line consolidation CT. Statistical analyses were performed using Mann-Whitney U tests.

Cell population	Group	n	Median	Statistical test	p-value
Lymphocytes	Complete remission	15	7.300×10^8	Mann-Whitney U test	0.618
	Partial remission	2	7.700×10^8		
	Progression	1	N/A		
B cells	Complete remission	14	2.019×10^6	Mann-Whitney U test	0.600
	Partial remission	2	1.012×10^8		
	Progression	1	N/A		
NK cells	Complete remission	14	3.910×10^7	Mann-Whitney U test	1.000
	Partial remission	2	4.017×10^7		
	Progression	1	N/A		
CD56bright CD16- NK cells	Complete remission	14	3.078×10^6	Mann-Whitney U test	0.700
	Partial remission	2	2.552×10^6		
	Progression	1	N/A		
CD56dim CD16+ NK cells	Complete remission	14	9.083×10^6	Mann-Whitney U test	0.150
	Partial remission	2	2.342×10^7		
	Progression	1	N/A		
T cells	Complete remission	14	6.357×10^8	Mann-Whitney U test	0.600
	Partial remission	2	5.775×10^8		
	Progression	1	N/A		
V γ 9V δ 2 TCR+ T cells	Complete remission	14	4.384×10^6	Mann-Whitney U test	1.000
	Partial remission	2	8.682×10^6		
	Progression	1	N/A		
CD4+ T cells	Complete remission	13	4.683×10^8	Mann-Whitney U test	0.933
	Partial remission	2	4.314×10^8		
	Progression	1	N/A		
CD4+ CD45RA+ CD27+ T cells	Complete remission	13	1.897×10^8	Mann-Whitney U test	0.229
	Partial remission	2	8.301×10^7		
	Progression	1	N/A		
CD4+ CD45RA- CD27+ T cells	Complete remission	13	1.481×10^8	Mann-Whitney U test	0.305
	Partial remission	2	2.451×10^8		
	Progression	1	N/A		
CD4+ CD45RA- CD27- T cells	Complete remission	13	7.956×10^7	Mann-Whitney U test	0.933
	Partial remission	2	9.158×10^7		
	Progression	1	N/A		
CD4+ CD45RA+ CD27- T cells	Complete remission	13	2.645×10^7	Mann-Whitney U test	0.171
	Partial remission	2	1.147×10^7		
	Progression	1	N/A		
CD8+ T cells	Complete remission	13	1.767×10^8	Mann-Whitney U test	0.381
	Partial remission	2	9.177×10^7		
	Progression	1	N/A		
CD8+ CD45RA+ CD27+ T cells	Complete remission	13	3.692×10^7	Mann-Whitney U test	0.381
	Partial remission	2	2.251×10^7		
	Progression	1	N/A		
CD8+ CD45RA- CD27+ T cells	Complete remission	13	1.183×10^7	Mann-Whitney U test	0.686
	Partial remission	2	2.190×10^7		
	Progression	1	N/A		
CD8+ CD45RA- CD27- T cells	Complete remission	13	2.093×10^7	Mann-Whitney U test	1.000
	Partial remission	2	2.532×10^7		
	Progression	1	N/A		
CD8+ CD45RA+ CD27- T cells	Complete remission	13	4.170×10^7	Mann-Whitney U test	0.571
	Partial remission	2	2.213×10^7		
	Progression	1	N/A		

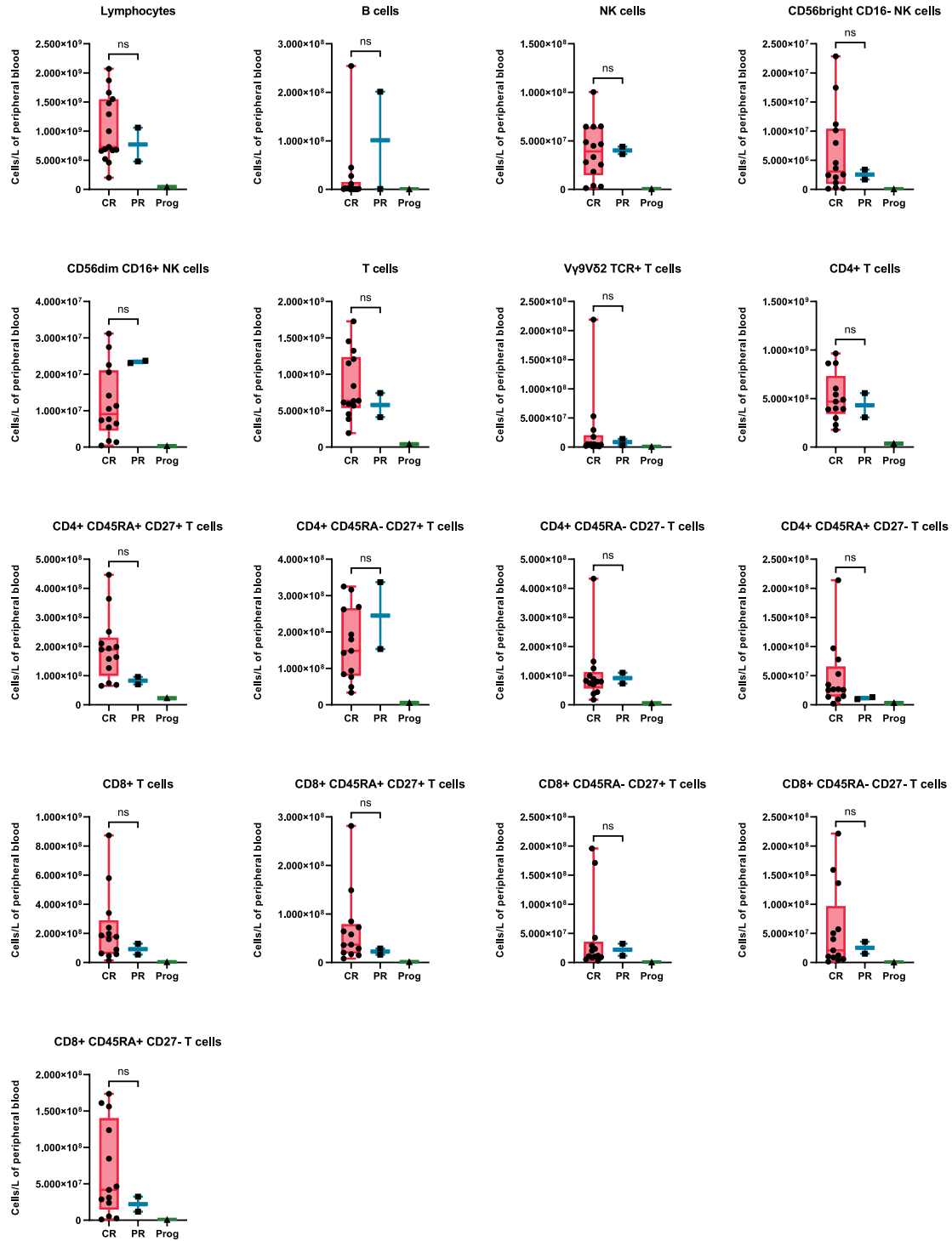


Figure 13. Immune cell levels of AML patients after first-line consolidation CT by response to first-line induction CT. Data are presented in boxplots depicting median cell counts, interquartile range (boxes), and minimum and maximum values (whiskers). Statistical p -values for the comparisons between groups are presented as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$; ns, not significant. CR, complete remission; PR, partial remission; Prog, progression.

3. Comparing overall survival of AML patients with higher or lower frequencies of immune populations

a. Method 1: Kaplan-Meier analysis

The selected results suggest that patients with post-consolidation cell levels of CD4+ T cells and its CD45RA+ CD27+ and CD45RA+ CD27- subsets equal to or above the respective optimal cutoff values are associated with better overall survival than patients belonging to the lower strata, as evidenced by the data presented in Table 19 and depicted in Figure 14. Although still above 0.05, *p*-values obtained in B and NK cell analyses were particularly close to the significance threshold.

Table 19. Best-performing Kaplan-Meier results for overall survival of AML patients categorized by their immune subset levels after first-line consolidation CT. Statistically significant results ($p < 0.05$) are highlighted in bold. ROC, receiver operating characteristic; HC, healthy control. **a)** Median survival was not reached (undefined), since, at the end of the follow-up period, overall survival of the patients included in the group was higher than 50%.

Cell population	Group	Total <i>n</i>	Events	Censored	Median survival, days	<i>p</i> - value
Lymphocytes	Cell counts < ROC cutoff	15	14	1	544	0.122
	Cell counts ≥ ROC cutoff	3	1	2	a)	
B cells	Cell counts < ROC cutoff	14	11	3	597	0.066
	Cell counts ≥ ROC cutoff	3	3	0	187	
NK cells	Cell counts < ROC cutoff	13	12	1	480	0.063
	Cell counts ≥ ROC cutoff	4	2	2	1009	
CD56bright CD16- NK cells	Cell counts < HC median	12	11	1	480	0.125
	Cell counts ≥ HC median	5	3	2	1121	
CD56dim CD16+ NK cells	Cell counts < ROC cutoff	11	10	1	480	0.123
	Cell counts ≥ ROC cutoff	6	4	2	949	
T cells	Cell counts < HC minimum	13	12	1	544	0.136
	Cell counts ≥ HC minimum	4	2	2	949	
Vγ9Vδ2 TCR+ T cells	Cell counts < HC minimum	13	11	2	544	0.442
	Cell counts ≥ HC minimum	4	3	1	949	
CD4+ T cells	Cell counts < ROC cutoff	6	6	0	297	0.001
	Cell counts ≥ ROC cutoff	10	7	3	949	
CD4+ CD45RA+ CD27+ T cells	Cell counts < ROC cutoff	7	7	0	480	0.031
	Cell counts ≥ ROC cutoff	9	6	3	1009	
CD4+ CD45RA- CD27+ T cells	Cell counts < ROC cutoff	12	11	1	544	0.221
	Cell counts ≥ ROC cutoff	4	2	2	617	
CD4+ CD45RA- CD27- T cells	Cell counts < ROC cutoff	14	12	2	544	0.252
	Cell counts ≥ ROC cutoff	2	1	1	949	
CD4+ CD45RA+ CD27- T cells	Cell counts < ROC cutoff	5	5	0	480	0.033
	Cell counts ≥ ROC cutoff	11	8	3	949	
CD8+ T cells	Cell counts < HC minimum	12	11	1	544	0.104
	Cell counts ≥ HC minimum	4	2	2	1009	
CD8+ CD45RA+ CD27+ T cells	Cell counts < HC minimum	12	11	1	597	0.399
	Cell counts ≥ HC minimum	4	2	2	168	
CD8+ CD45RA- CD27+ T cells	Cell counts < HC minimum	13	12	1	597	0.150
	Cell counts ≥ HC minimum	3	1	2	a)	
CD8+ CD45RA- CD27- T cells	Cell counts < ROC cutoff	13	12	1	597	0.115
	Cell counts ≥ ROC cutoff	3	1	2	a)	
CD8+ CD45RA+ CD27- T cells	Cell counts < ROC cutoff	12	11	1	544	0.104
	Cell counts ≥ ROC cutoff	4	2	2	1009	

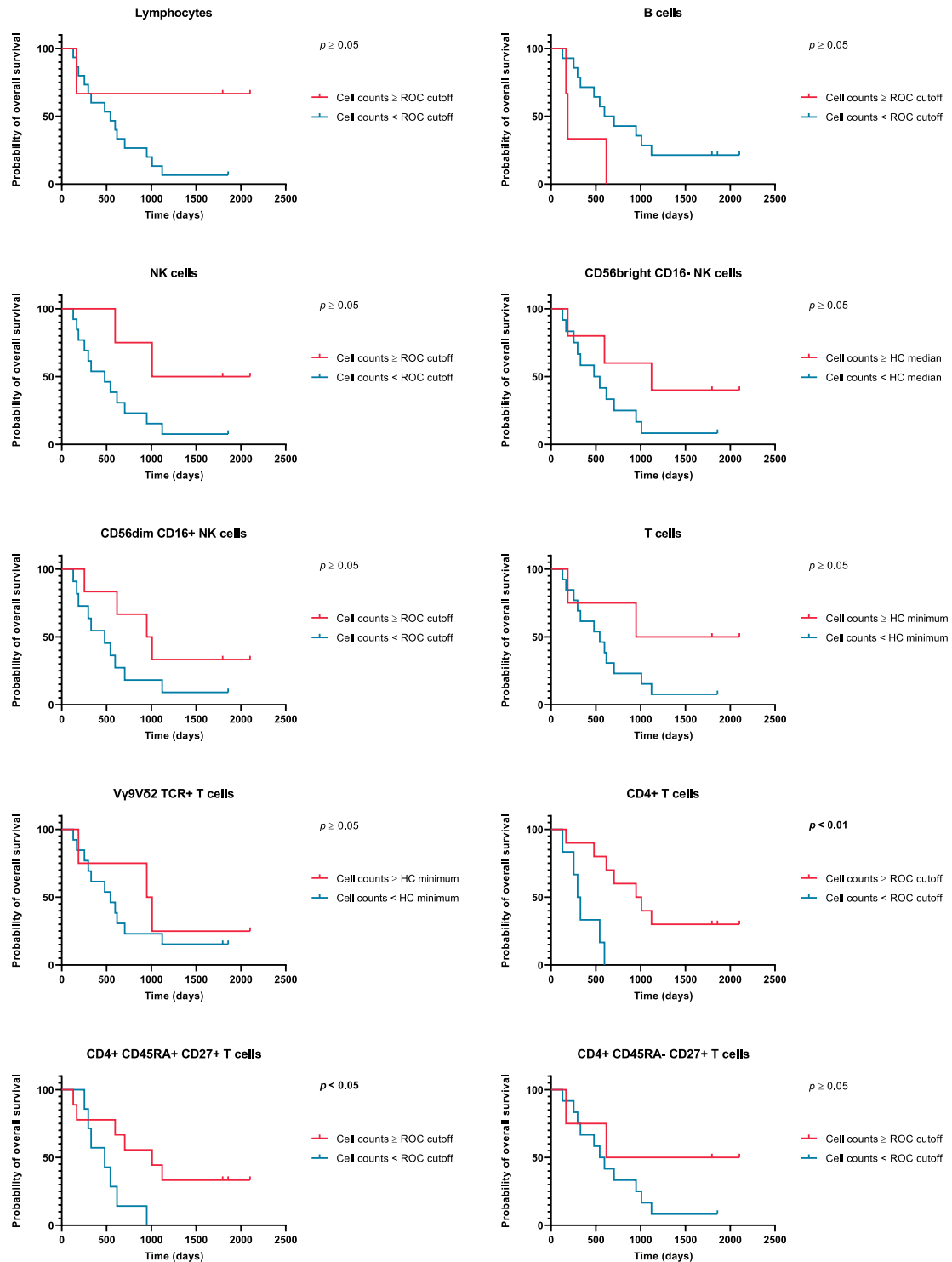


Figure 14. Kaplan-Meier overall survival curves of AML patients categorized by their immune subset levels after first-line consolidation CT. Statistical p -values for the Logrank tests are presented on the right side of the respective plot. ROC, receiver operating characteristic; HC, healthy control.

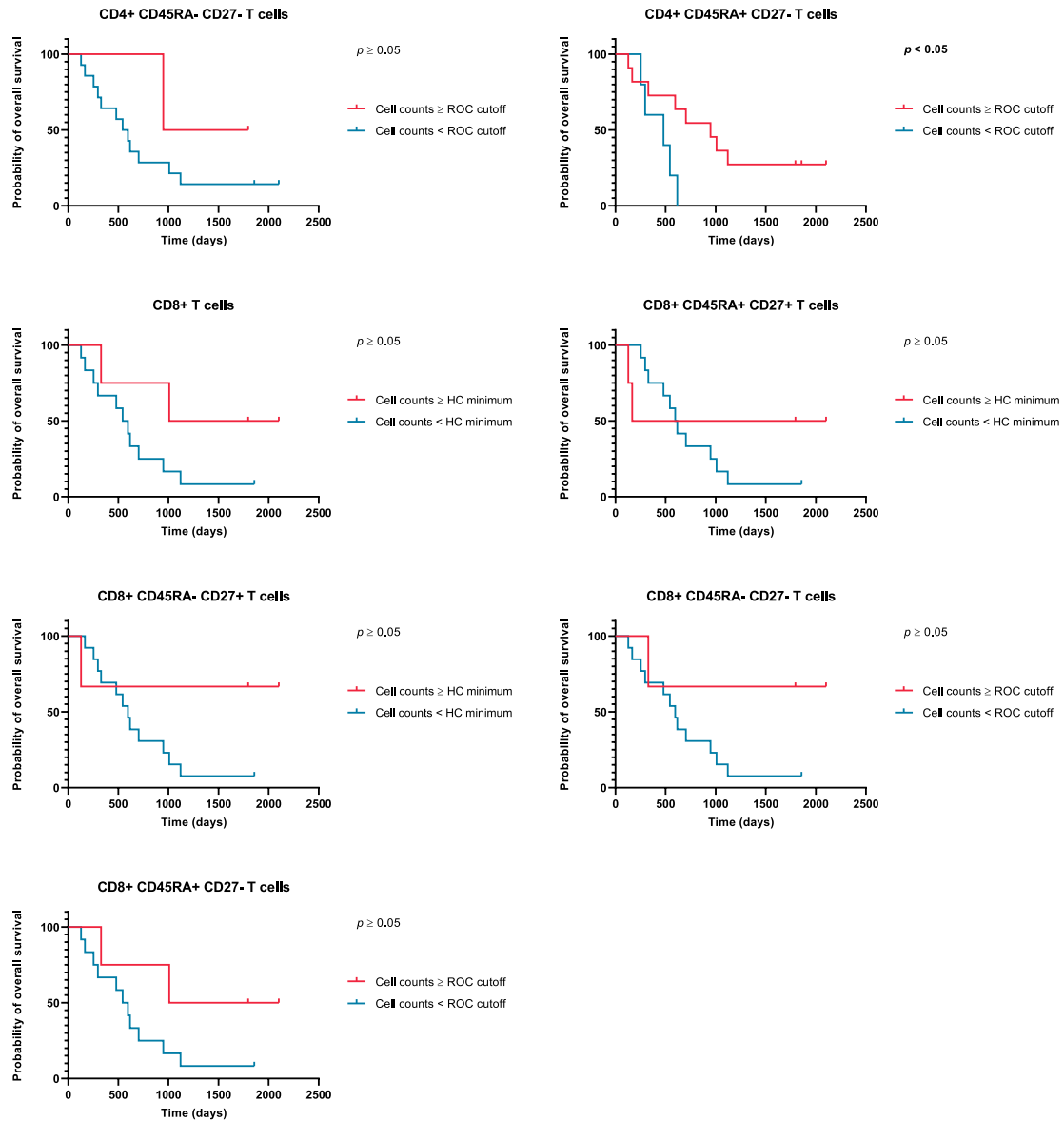


Figure 14 (cont.). Kaplan-Meier overall survival curves of AML patients categorized by their immune subset levels after first-line consolidation CT.

b. Method 2: Cox regression analysis

While the previous methodology yielded some statistically significant results, the Cox regression method suggested there was no correlation between any post-consolidation immune subset levels and overall survival, even when controlling for age and both age and cytogenetic risk. Detailed results are described in Table 20.

Table 20. Cox regression results for overall survival of AML patients, with models including immune cell counts assessed after first-line consolidation CT. Statistically significant results ($p < 0.05$) are highlighted in bold. ELN, European LeukemiaNet; HR, hazard ratio; CI, confidence interval. **a)** Although p -value is presented, the tested model is not valid, as the assumption of proportional hazards was not met.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	<i>p</i> -value	HR (95% CI)
Lymphocytes	Immune cell count	18	15	3	0.956	0.328	1.000 (0.999 – 1.000)
	Immune cell count	18	15	3	1.151	0.283	0.999 (0.998 – 1.000)
	Age				1.010	0.315	0.976 (0.932 – 1.023)
	Immune cell count	16	13	3	1.066	0.302	0.999 (0.998 – 1.001)
	Age				1.867	0.172	0.965 (0.918 – 1.015)
	ELN cytogenetic risk						
	Favorable				3.951	0.139	Reference
B cells	Intermediate	15	12	3	0.661	0.416	1.974 (0.383 – 10.171)
	Adverse				3.574	0.059	6.148 (0.935 – 40.409)
	Immune cell count				1.424	0.233	1.005 (0.997 – 1.012)
	Immune cell count	17	14	3	1.753	0.185	1.005 (0.997 – 1.013)
	Age	17	14	3	0.501	0.479	0.981 (0.929 – 1.035)
	Immune cell count	15	12	3	1.512	0.219	1.007 (0.996 – 1.019)
	Age				0.808	0.369	0.974 (0.919 – 1.032)
NK cells	ELN cytogenetic risk						
	Favorable				2.914	0.233	Reference
	Intermediate				0.111	0.739	1.316 (0.261 – 6.642)
	Adverse				2.356	0.125	4.534 (0.658 – 31.243)
	Immune cell count	17	14	3	2.086	0.149	0.987 (0.969 – 1.005)
	Immune cell count	17	14	3	2.349	0.125	0.985 (0.967 – 1.004)
	Age				0.494	0.482	0.980 (0.928 – 1.036)
CD56bright CD16- NK cells	Immune cell count	15	12	3	2.060	0.151	0.980 (0.954 – 1.007)
	Age				1.584	0.208	0.959 (0.898 – 1.024)
	ELN cytogenetic risk						
	Favorable				3.493	0.174	Reference
	Intermediate				1.248	0.264	2.901 (0.448 – 18.805)
	Adverse				3.471	0.062	6.565 (0.907 – 47.536)
	Immune cell count	17	14	3	0.493	0.482	0.969 (0.888 – 1.058)
CD56dim CD16+ NK cells	Immune cell count	17	14	3	0.661	0.416	0.961 (0.872 – 1.058)
	Age	17	14	3	0.408	0.523	0.982 (0.929 – 1.038)
	Immune cell count	15	12	3	0.810	0.368	0.884 (0.676 – 1.157)
	Age				1.594	0.207	0.945 (0.867 – 1.031)
	ELN cytogenetic risk						
	Favorable				3.896	0.143	Reference
	Intermediate				0.825	0.364	2.598 (0.331 – 20.411)
T cells	Adverse				3.664	0.056	6.664 (0.956 – 46.476)
	Immune cell count	17	14	3	0.659	0.417	0.979 (0.931 – 1.030)
	Immune cell count	17	14	3	0.614	0.433	0.980 (0.931 – 1.031)
	Age				0.130	0.718	0.990 (0.940 – 1.044)
	Immune cell count	15	12	3	0.129	0.719	0.987 (0.918 – 1.061)
	Age				0.723	0.395	0.976 (0.921 – 1.033)
	ELN cytogenetic risk						
V γ 9V δ 2 TCR+ T cells	Favorable				3.003	0.223	Reference
	Intermediate				0.254	0.615	1.559 (0.277 – 8.766)
	Adverse				2.820	0.093	5.077 (0.762 – 33.819)
	Immune cell count	17	14	3	2.375	0.123	0.999 (0.998 – 1.000)
	Immune cell count	17	14	3	2.423	0.120	0.999 (0.998 – 1.000)
	Age				0.252	0.616	0.987 (0.939 – 1.038)
	Immune cell count	15	12	3	2.146	0.143	0.999 (0.997 – 1.000)
V γ 9V δ 2 TCR+ T cells	Age				0.861	0.353	0.975 (0.925 – 1.028)
	ELN cytogenetic risk						
	Favorable				3.886	0.143	Reference
	Intermediate				0.545	0.460	1.866 (0.356 – 9.783)
	Adverse				3.413	0.065	6.272 (0.894 – 44.002)
	Immune cell count	17	14	3	1.299	0.254	1.007 (0.995 – 1.019)
	Immune cell count	17	14	3	1.127	0.288	1.007 (0.994 – 1.019)
V γ 9V δ 2 TCR+ T cells	Age				0.089	0.765	0.992 (0.940 – 1.046)
	Immune cell count	15	12	3	2.028	0.154	1.009 (0.997 – 1.021)
	Age				0.749	0.387	0.974 (0.919 – 1.033)
	ELN cytogenetic risk						
	Favorable				4.935	0.085	Reference
	Intermediate				0.108	0.742	1.317 (0.255 – 6.795)
	Adverse				3.893	0.048	6.657 (1.013 – 43.758)

Table 20 (cont.). Cox regression results for overall survival of AML patients, with models including immune cell counts assessed after first-line consolidation CT.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	<i>p</i> -value	HR (95% CI)
CD4+ T cells	Immune cell count	16	13	3	2.339	0.126	0.998 (0.995 – 1.001)
	Immune cell count	16	13	3	2.335	0.127	0.998 (0.994 – 1.001)
	Age				0.017	0.898	1.004 (0.947 – 1.065)
	Immune cell count	14	11	3	1.333	0.248	0.998 (0.995 – 1.001)
	Age				0.145	0.703	0.988 (0.929 – 1.051)
	ELN cytogenetic risk						
	Favorable				3.350	0.187	Reference
CD4+ CD45RA+ CD27+ T cells	Intermediate				0.056	0.813	1.221 (0.233 – 6.397)
	Adverse				2.357	0.125	4.537 (0.658 – 31.269)
	Immune cell count	16	13	3	1.651	0.199	0.995 (0.988 – 1.003)
	Immune cell count	16	13	3	1.744	0.187	0.995 (0.987 – 1.002)
	Age				0.114	0.735	0.990 (0.932 – 1.051)
	Immune cell count	14	11	3	1.626	0.202	0.995 (0.987 – 1.003)
	Age				0.443	0.506	0.980 (0.922 – 1.041)
CD4+ CD45RA- CD27+ T cells	ELN cytogenetic risk						
	Favorable				4.733	0.094	Reference
	Intermediate				0.339	0.561	1.658 (0.302 – 9.097)
	Adverse				3.877	0.049	6.717 (1.009 – 44.719)
	Immune cell count	16	13	3	2.286	0.131	0.995 (0.989 – 1.001)
	Immune cell count	16	13	3	2.293	0.130	0.995 (0.989 – 1.001)
	Age				0.011	0.916	0.997 (0.944 – 1.053)
CD4+ CD45RA- CD27- T cells	Immune cell count	14	11	3	1.470	0.225	0.996 (0.988 – 1.003)
	Age				0.389	0.533	0.982 (0.927 – 1.04)
	ELN cytogenetic risk						
	Favorable				3.326	0.190	Reference
	Intermediate				0.010	0.922	1.088 (0.202 – 5.867)
	Adverse				2.059	0.151	4.154 (0.594 – 29.059)
	Immune cell count	16	13	3	0.117	0.733	0.999 (0.993 – 1.005)
CD4+ CD45RA- CD27- T cells	Immune cell count	16	13	3	0.116	0.734	0.999 (0.993 – 1.005)
	Age				< 0.001	0.996	1.000 (0.944 – 1.059)
	Immune cell count	14	11	3	0.002	0.965	1.000 (0.993 – 1.007)
	Age				0.400	0.527	0.980 (0.922 – 1.042)
	ELN cytogenetic risk						
	Favorable				3.899	0.142	Reference
	Intermediate				0.044	0.833	1.207 (0.209 – 6.965)
CD4+ CD45RA+ CD27- T cells	Adverse				2.669	0.102	5.261 (0.718 – 38.559)
	Immune cell count	16	13	3	0.095	0.758	0.998 (0.988 – 1.009)
	Immune cell count	16	13	3	0.106	0.745	0.998 (0.987 – 1.009)
	Age				0.011	0.916	1.003 (0.942 – 1.069)
	Immune cell count	14	11	3	0.014	0.905 ^{a)}	0.999 (0.986 – 1.013)
	Age				0.219	0.640	0.983 (0.913 – 1.057)
	ELN cytogenetic risk						
CD8+ T cells	Favorable				3.744	0.154	Reference
	Intermediate				0.066	0.797	1.246 (0.233 – 6.649)
	Adverse				2.957	0.086	5.272 (0.793 – 35.06)
	Immune cell count	16	13	3	2.610	0.106	0.997 (0.993 – 1.001)
	Immune cell count	16	13	3	2.756	0.097	0.997 (0.993 – 1.001)
	Age				0.168	0.682	0.989 (0.937 – 1.044)
	Immune cell count	14	11	3	3.212	0.073	0.996 (0.992 – 1.000)
CD8+ CD45RA+ CD27+ T cells	Age				1.016	0.313	0.971 (0.917 – 1.028)
	ELN cytogenetic risk						
	Favorable				4.064	0.131	Reference
	Intermediate				0.965	0.326	2.433 (0.413 – 14.347)
	Adverse				3.904	0.048	8.725 (1.018 – 74.812)
	Immune cell count	16	13	3	2.337	0.126	0.988 (0.973 – 1.003)
	Immune cell count	16	13	3	2.532	0.112	0.987 (0.972 – 1.003)
CD8+ CD45RA- CD27+ T cells	Age				0.504	0.478	0.980 (0.927 – 1.036)
	Immune cell count	14	11	3	2.465	0.116	0.987 (0.970 – 1.003)
	Age				0.961	0.327	0.972 (0.919 – 1.029)
	ELN cytogenetic risk						
	Favorable				3.186	0.203	Reference
	Intermediate				1.006	0.316	2.464 (0.423 – 14.353)
	Adverse				3.149	0.076	6.025 (0.829 – 43.788)

Table 20 (cont.). Cox regression results for overall survival of AML patients, with models including immune cell counts assessed after first-line consolidation CT.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	<i>p</i> -value	HR (95% CI)
CD8+ CD45RA- CD27+ T cells	Immune cell count	16	13	3	2.249	0.134	0.985 (0.965 – 1.005)
	Immune cell count	16	13	3	2.393	0.122	0.984 (0.964 – 1.004)
	Age				0.342	0.559	0.984 (0.933 – 1.038)
	Immune cell count	14	11	3	2.401	0.121	0.984 (0.964 – 1.004)
	Age				1.066	0.302	0.971 (0.919 – 1.027)
	ELN cytogenetic risk						
	Favorable				3.637	0.162	Reference
CD8+ CD45RA- CD27- T cells	Intermediate				0.759	0.384	2.110 (0.394 – 11.308)
	Adverse				3.443	0.064	6.451 (0.900 – 46.229)
	Immune cell count	16	13	3	1.718	0.190	0.993 (0.982 – 1.004)
	Immune cell count	16	13	3	1.951	0.162	0.992 (0.982 – 1.003)
	Age				0.250	0.617	0.986 (0.935 – 1.041)
	Immune cell count	14	11	3	2.660	0.103	0.991 (0.980 – 1.002)
	Age				1.596	0.207	0.963 (0.908 – 1.021)
CD8+ CD45RA+ CD27- T cells	ELN cytogenetic risk						
	Favorable				4.768	0.092	Reference
	Intermediate				0.388	0.534	1.707 (0.317 – 9.184)
	Adverse				4.183	0.041	8.977 (1.096 – 73.544)
	Immune cell count	16	13	3	2.191	0.139	0.992 (0.982 – 1.003)
	Immune cell count	16	13	3	2.176	0.140	0.992 (0.982 – 1.003)
	Age				< 0.001	0.988	1.000 (0.946 – 1.058)
CD8+ CD45RA+ CD27+ T cells	Immune cell count	14	11	3	2.431	0.119	0.991 (0.980 – 1.002)
	Age				0.396	0.529	0.981 (0.922 – 1.042)
	ELN cytogenetic risk						
	Favorable				3.906	0.142	Reference
	Intermediate				0.704	0.402	2.167 (0.356 – 13.203)
	Adverse				3.715	0.054	7.958 (0.966 – 65.590)

4. Comparing relapse-free survival of AML patients with higher or lower frequencies of immune populations

a. Method 1: Kaplan-Meier analysis

Selected Logrank test results indicated that patients with higher or lower post-consolidation cell counts of each of the 17 analyzed populations have statistically equivalent relapse-free survival times (Table 21, Figure 15).

Table 21. Best-performing Kaplan-Meier results for relapse-free survival of AML patients categorized by their immune subset levels after first-line consolidation CT. ROC, receiver operating characteristic; HC, healthy control; pop., population.

Cell population	Group	Total <i>n</i>	Events	Censored	Median survival, days	<i>p</i> - value
Lymphocytes	Cell counts < ROC cutoff	3	2	1	544	0.248
	Cell counts ≥ ROC cutoff	15	4	1	223	
B cells	Cell counts < HC minimum	15	13	2	297	0.227
	Cell counts ≥ HC minimum	2	2	0	95	
NK cells	Cell counts < ROC cutoff	4	3	1	544	0.231
	Cell counts ≥ ROC cutoff	13	12	1	223	
CD56bright CD16- NK cells	Cell counts < ROC cutoff	4	3	1	544	0.231
	Cell counts ≥ ROC cutoff	13	12	1	223	
CD56dim CD16+ NK cells	Cell counts < ROC cutoff	3	2	1	544	0.278
	Cell counts ≥ ROC cutoff	14	13	1	223	
T cells	Cell counts < HC minimum	13	12	1	291	0.259
	Cell counts ≥ HC minimum	4	3	1	408	
Vγ9Vδ2 TCR+ T cells	Cell counts < HC minimum	13	12	1	291	0.137
	Cell counts ≥ HC minimum	4	3	1	650	
CD4+ T cells	Cell counts < pop. median	8	7	1	223	0.348
	Cell counts ≥ pop. median	8	7	1	408	
CD4+ CD45RA+ CD27+ T cells	Cell counts < HC minimum	6	6	0	297	0.671
	Cell counts ≥ HC minimum	10	8	2	291	
CD4+ CD45RA- CD27+ T cells	Cell counts < ROC cutoff	13	11	2	297	0.312
	Cell counts ≥ ROC cutoff	3	3	0	395	
CD4+ CD45RA- CD27- T cells	Cell counts < ROC cutoff	3	2	1	544	0.314
	Cell counts ≥ ROC cutoff	13	12	1	291	
CD4+ CD45RA+ CD27- T cells	Cell counts < pop. median	8	7	1	291	0.459
	Cell counts ≥ pop. median	8	7	1	408	
CD8+ T cells	Cell counts < HC median	14	13	1	291	0.222
	Cell counts ≥ HC median	2	1	1	408	
CD8+ CD45RA+ CD27+ T cells	Cell counts < ROC cutoff	2	2	0	297	0.843
	Cell counts ≥ ROC cutoff	14	12	2	291	
CD8+ CD45RA- CD27+ T cells	Cell counts < HC median	14	13	1	291	0.222
	Cell counts ≥ HC median	2	1	1	408	
CD8+ CD45RA- CD27- T cells	Cell counts < ROC cutoff	3	2	1	544	0.314
	Cell counts ≥ ROC cutoff	13	12	1	291	
CD8+ CD45RA+ CD27- T cells	Cell counts < HC median	11	10	1	291	0.137
	Cell counts ≥ HC median	5	4	1	650	

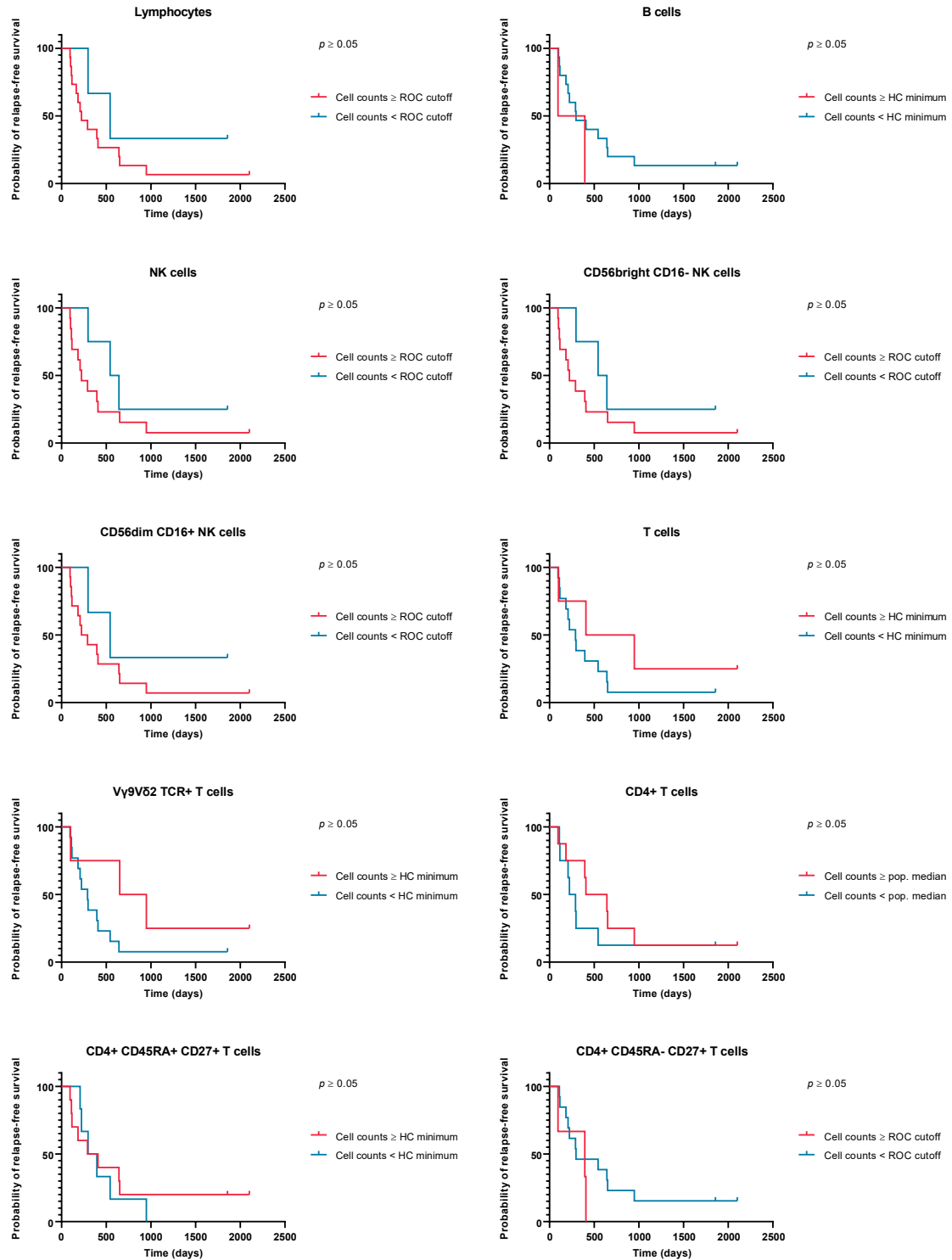


Figure 15. Kaplan-Meier relapse-free survival curves of AML patients categorized by their immune subset levels after first-line consolidation CT. Statistical p -values for the Logrank tests are presented on the right side of the respective plot. ROC, receiver operating characteristic; HC, healthy control; pop., population.

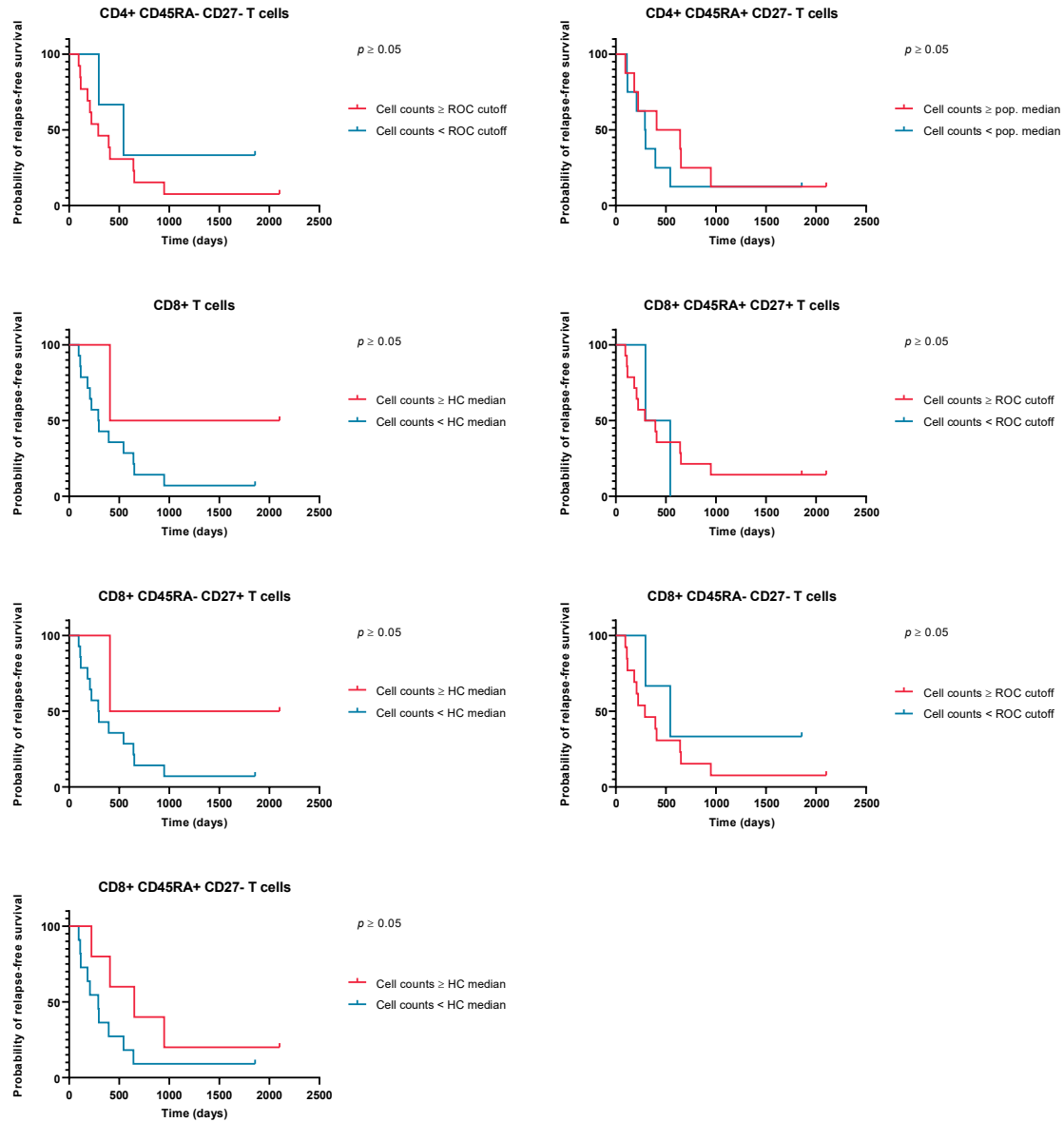


Figure 15 (cont.). Kaplan-Meier relapse-free survival curves of AML patients categorized by their immune subset levels after first-line consolidation CT.

b. Method 2: Cox regression analysis

Performing the Cox regression method allowed for the identification of post-consolidation B cell frequency as a statistically relevant covariate when modelling relapse-free survival. Even though the univariate analysis rendered a non-significant result ($p = 0.109$, Table 22), including the age covariate in the model diluted its confounding effect and highlighted the statistical significance of B cell counts ($p = 0.032$; HR = 1.009, 95% CI: 1.001 – 1.018).

Table 22. Cox regression results for relapse-free survival of AML patients, with models including immune cell counts assessed after first-line consolidation CT. Statistically significant results ($p < 0.05$) are highlighted in bold. ELN, European LeukemiaNet; HR, hazard ratio; CI, confidence interval.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	<i>p</i> -value	HR (95% CI)
Lymphocytes	Immune cell count	18	16	2	0.014	0.905	1.000 (0.999 – 1.001)
	Immune cell count	18	16	2	0.220	0.639	1.000 (0.999 – 1.001)
	Age				3.965	0.046	0.947 (0.897 – 0.999)
	Immune cell count				0.962	0.327	1.000 (0.999 – 1.000)
	Age				7.809	0.005	0.898 (0.833 – 0.968)
	ELN cytogenetic risk	16	14	2			
	Favorable				6.739	0.034	Reference
B cells	Intermediate				3.075	0.080	4.884 (0.830 – 28.756)
	Adverse				6.696	0.010	16.674 (1.979 – 140.488)
	Immune cell count	17	15	2	2.567	0.109	1.006 (0.999 – 1.013)
	Immune cell count	17	15	2	4.607	0.032	1.009 (1.001 – 1.018)
	Age				4.366	0.037	0.935 (0.878 – 0.996)
	Immune cell count				2.384	0.123	1.024 (0.994 – 1.056)
	Age				5.739	0.017	0.900 (0.826 – 0.981)
NK cells	ELN cytogenetic risk	15	13	2			
	Favorable				4.995	0.082	Reference
	Intermediate				1.685	0.194	3.051 (0.566 – 16.438)
	Adverse				4.932	0.026	12.062 (1.340 – 108.593)
	Immune cell count	17	15	2	0.252	0.616	0.996 (0.980 – 1.012)
	Immune cell count	17	15	2	0.318	0.573	0.995 (0.979 – 1.012)
	Age				2.673	0.102	0.953 (0.900 – 1.010)
CD56bright CD16- NK cells	Immune cell count				1.823	0.177	0.984 (0.961 – 1.007)
	Age				6.919	0.009	0.891 (0.817 – 0.971)
	ELN cytogenetic risk	15	13	2			
	Favorable				6.286	0.043	Reference
	Intermediate				3.557	0.059	7.600 (0.924 – 62.544)
	Adverse				6.281	0.012	20.377 (1.929 – 215.267)
	Immune cell count	17	15	2	2.356	0.125	1.073 (0.981 – 1.174)
CD56bright CD16+ NK cells	Immune cell count	17	15	2	1.357	0.244	1.061 (0.960 – 1.173)
	Age				1.648	0.199	0.962 (0.908 – 1.020)
	Immune cell count				0.164	0.686	0.955 (0.763 – 1.195)
	Age				4.778	0.029	0.897 (0.814 – 0.989)
	ELN cytogenetic risk	15	13	2			
	Favorable				6.237	0.044	Reference
	Intermediate				1.975	0.160	4.454 (0.555 – 35.756)
CD56dim CD16+ NK cells	Adverse				6.075	0.014	14.691 (1.734 – 124.468)
	Immune cell count	17	15	2	0.286	0.593	0.988 (0.945 – 1.033)
	Immune cell count	17	15	2	0.063	0.801	0.994 (0.948 – 1.042)
	Age				2.363	0.124	0.955 (0.901 – 1.013)
	Immune cell count				0.032	0.858	1.006 (0.938 – 1.079)
	Age				6.225	0.013	0.908 (0.842 – 0.980)
	ELN cytogenetic risk	15	13	2			
T cells	Favorable				6.100	0.047	Reference
	Intermediate				1.796	0.180	3.252 (0.580 – 18.245)
	Adverse				6.071	0.014	13.721 (1.709 – 110.201)
	Immune cell count	17	15	2	0.505	0.477	1.000 (0.998 – 1.001)
	Immune cell count	17	15	2	0.637	0.425	1.000 (0.998 – 1.001)
	Age				2.770	0.096	0.953 (0.900 – 1.009)
	Immune cell count				0.961	0.327	0.999 (0.998 – 1.001)
Vy9Vδ2 TCR+ T cells	Age				6.750	0.009	0.904 (0.838 – 0.976)
	ELN cytogenetic risk	15	13	2			
	Favorable				6.330	0.042	Reference
	Intermediate				2.804	0.094	4.461 (0.775 – 25.684)
	Adverse				6.283	0.012	15.409 (1.816 – 130.773)
	Immune cell count	17	15	2	0.844	0.358	1.007 (0.992 – 1.021)
	Immune cell count	17	15	2	0.647	0.421	1.005 (0.992 – 1.019)
Vy9Vδ2 TCR+ T cells	Age				2.467	0.116	0.954 (0.900 – 1.012)
	Immune cell count				1.213	0.271	1.007 (0.994 – 1.021)
	Age				5.721	0.017	0.908 (0.839 – 0.983)
	ELN cytogenetic risk	15	13	2			
	Favorable				6.694	0.035	Reference
	Intermediate				1.894	0.169	3.191 (0.611 – 16.658)
	Adverse				6.497	0.011	14.939 (1.868 – 119.484)

Table 22 (cont.). Cox regression results for relapse-free survival of AML patients, with models including immune cell counts assessed after first-line consolidation CT.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	<i>p</i> -value	HR (95% CI)
CD4+ T cells	Immune cell count	16	14	2	0.334	0.563	0.999 (0.997 – 1.002)
	Immune cell count	16	14	2	0.257	0.612	0.999 (0.997 – 1.002)
	Age				1.395	0.238	0.963 (0.904 – 1.025)
	Immune cell count				0.001	0.974	1.000 (0.997 – 1.003)
	Age				4.996	0.025	0.912 (0.842 – 0.989)
	ELN cytogenetic risk	14	12	2			
	Favorable				5.901	0.052	Reference
CD4+ CD45RA+ CD27+ T cells	Intermediate				1.760	0.185	3.108 (0.582 – 16.600)
	Adverse				5.739	0.017	13.447 (1.604 – 112.718)
	Immune cell count	16	14	2	0.139	0.710	1.001 (0.995 – 1.007)
	Immune cell count	16	14	2	0.027	0.869	1.000 (0.995 – 1.006)
	Age				1.340	0.247	0.963 (0.904 – 1.026)
	Immune cell count				0.001	0.970	1.000 (0.994 – 1.006)
	Age				5.002	0.025	0.912 (0.842 – 0.989)
CD4+ CD45RA- CD27+ T cells	ELN cytogenetic risk	14	12	2			
	Favorable				6.165	0.046	Reference
	Intermediate				1.717	0.190	3.126 (0.568 – 17.194)
	Adverse				5.934	0.015	13.547 (1.664 – 110.286)
	Immune cell count	16	14	2	0.364	0.546	0.998 (0.993 – 1.004)
	Immune cell count	16	14	2	0.553	0.457	0.998 (0.992 – 1.003)
	Age				1.681	0.195	0.959 (0.901 – 1.021)
CD4+ CD45RA- CD27- T cells	Immune cell count				0.445	0.505	0.998 (0.991 – 1.004)
	Age				5.241	0.022	0.910 (0.839 – 0.987)
	ELN cytogenetic risk	14	12	2			
	Favorable				5.315	0.070	Reference
	Intermediate				1.805	0.179	3.211 (0.585 – 17.619)
	Adverse				5.211	0.022	12.060 (1.422 – 102.278)
	Immune cell count	16	14	2	0.242	0.623	0.999 (0.994 – 1.004)
CD4+ CD45RA- CD27- T cells	Immune cell count	16	14	2	0.158	0.691	0.999 (0.994 – 1.004)
	Age				1.366	0.243	0.964 (0.905 – 1.025)
	Immune cell count				0.211	0.646	1.002 (0.995 – 1.009)
	Age				5.133	0.023	0.911 (0.840 – 0.987)
	ELN cytogenetic risk	14	12	2			
	Favorable				6.219	0.045	Reference
	Intermediate				1.854	0.173	3.621 (0.568 – 23.079)
CD4+ CD45RA+ CD27- T cells	Adverse				5.878	0.015	15.749 (1.696 – 146.264)
	Immune cell count	16	14	2	0.435	0.510	0.997 (0.986 – 1.007)
	Immune cell count	16	14	2	0.051	0.822	0.999 (0.988 – 1.010)
	Age				0.987	0.320	0.966 (0.901 – 1.035)
	Immune cell count				0.241	0.624	1.003 (0.990 – 1.016)
	Age				4.815	0.028	0.903 (0.825 – 0.989)
	ELN cytogenetic risk	14	12	2			
CD8+ T cells	Favorable				6.309	0.043	Reference
	Intermediate				1.687	0.194	3.048 (0.567 – 16.380)
	Adverse				6.098	0.014	14.563 (1.738 – 122.043)
	Immune cell count	16	14	2	0.567	0.452	0.999 (0.996 – 1.002)
	Immune cell count	16	14	2	1.043	0.307	0.999 (0.996 – 1.001)
	Age				2.129	0.144	0.955 (0.898 – 1.016)
	Immune cell count				2.659	0.103	0.998 (0.995 – 1.000)
CD8+ CD45RA+ CD27+ T cells	Age				7.015	0.008	0.889 (0.816 – 0.970)
	ELN cytogenetic risk	14	12	2			
	Favorable				7.265	0.026	Reference
	Intermediate				3.737	0.053	6.712 (0.974 – 46.263)
	Adverse				7.231	0.007	26.067 (2.421 – 280.678)
	Immune cell count	16	14	2	0.314	0.575	0.998 (0.989 – 1.006)
	Immune cell count	16	14	2	0.855	0.355	0.996 (0.987 – 1.005)
CD8+ CD45RA- CD27+ T cells	Age				2.232	0.135	0.953 (0.894 – 1.015)
	Immune cell count				1.248	0.264	0.995 (0.985 – 1.004)
	Age				6.141	0.013	0.900 (0.828 – 0.978)
	ELN cytogenetic risk	14	12	2			
	Favorable				6.264	0.044	Reference
	Intermediate				2.891	0.089	4.909 (0.784 – 30.732)
	Adverse				6.261	0.012	15.357 (1.808 – 130.473)

Table 22 (cont.). Cox regression results for relapse-free survival of AML patients, with models including immune cell counts assessed after first-line consolidation CT.

Cell population	Model (covariates)	Total <i>n</i>	Events	Censored	Covariates		
					Wald's χ^2	<i>p</i> -value	HR (95% CI)
CD8+ CD45RA- CD27+ T cells	Immune cell count	16	14	2	0.903	0.342	0.995 (0.985 – 1.005)
	Immune cell count	16	14	2	1.438	0.230	0.994 (0.983 – 1.004)
	Age	16	14	2	2.321	0.128	0.954 (0.897 – 1.014)
	Immune cell count	14	12	2	2.853	0.091	0.990 (0.979 – 1.002)
	Age				6.786	0.009	0.894 (0.821 – 0.973)
	ELN cytogenetic risk						
	Favorable				6.798	0.033	Reference
	Intermediate				3.888	0.049	7.014 (1.012 – 48.628)
	Adverse				6.796	0.009	20.54 (2.117 – 199.269)
CD8+ CD45RA- CD27- T cells	Immune cell count	16	14	2	0.223	0.637	0.998 (0.989 – 1.007)
	Immune cell count	16	14	2	0.800	0.371	0.996 (0.987 – 1.005)
	Age	16	14	2	2.255	0.133	0.953 (0.896 – 1.015)
	Immune cell count	14	12	2	3.060	0.080	0.991 (0.980 – 1.001)
	Age				7.573	0.006	0.876 (0.797 – 0.963)
	ELN cytogenetic risk						
	Favorable				7.768	0.021	Reference
	Intermediate				3.604	0.058	6.559 (0.941 – 45.727)
	Adverse				7.629	0.006	38.342 (2.883 – 509.902)
CD8+ CD45RA+ CD27- T cells	Immune cell count	16	14	2	0.585	0.445	0.997 (0.988 – 1.005)
	Immune cell count	16	14	2	0.578	0.447	0.997 (0.988 – 1.005)
	Age	16	14	2	1.474	0.225	0.963 (0.905 – 1.024)
	Immune cell count	14	12	2	2.114	0.146	0.992 (0.982 – 1.003)
	Age				6.166	0.013	0.896 (0.822 – 0.977)
	ELN cytogenetic risk						
	Favorable				6.944	0.031	Reference
	Intermediate				3.249	0.071	5.803 (0.858 – 39.272)
	Adverse				6.859	0.009	27.484 (0.302 – 328.126)

D. Patient samples collected during post-first-line chemotherapy recovery (off-treatment)

1. Comparing immune cell counts of AML patients with healthy controls

As reported in Table 23, after chemotherapy completion, AML patients still had significantly lower levels of 11 out of the 17 immune subsets, in comparison with healthy individuals. While results indicate similar frequencies of CD56bright CD16- NK cells, V γ 9V δ 2 TCR+ T cells and all four CD27- T cell subsets between AML cases and healthy controls, absolute counts of the main immune populations (lymphocytes, B, T, and both CD4+ and CD8+ T cells), as well as CD56dim CD16+ NK and all CD27+ T cell subsets were significantly distinct. Graphical representations of the data are included in Figure 16.

Table 23. Immune subset level comparisons between AML patients during first-line CT recovery (off-treatment) and healthy controls. Statistical analyses were performed using one-sample *t*-tests, independent samples *t*-tests or Mann-Whitney U tests. Statistically significant results ($p < 0.05$) are highlighted in bold. N/A, not applicable. **a)** For total lymphocyte comparisons, the healthy control sample was represented by the mean absolute lymphocyte count of 2.102×10^9 cells/L of peripheral blood reported by Fonseca, Pereira et al., 2020 (134).

Cell population	Group	<i>n</i>	Median	Statistical test	<i>p</i> -value
Lymphocytes	Healthy controls ^{a)}	N/A	N/A	One-sample <i>t</i> -test	< 0.001
	AML patients	10	8.900×10^8		
B cells	Healthy controls	6	1.854×10^8	Mann-Whitney U test	< 0.001
	AML patients	10	1.498×10^7		
NK cells	Healthy controls	6	1.629×10^8	Independent samples <i>t</i> -test	0.007
	AML patients	10	6.532×10^7		
CD56bright CD16- NK cells	Healthy controls	6	6.574×10^6	Mann-Whitney U test	0.713
	AML patients	10	1.392×10^7		
CD56dim CD16+ NK cells	Healthy controls	6	1.243×10^8	Mann-Whitney U test	< 0.001
	AML patients	10	1.637×10^7		
T cells	Healthy controls	6	1.610×10^9	Independent samples <i>t</i> -test	< 0.001
	AML patients	10	4.779×10^8		
V γ 9V δ 2 TCR+ T cells	Healthy controls	6	2.244×10^7	Mann-Whitney U test	0.147
	AML patients	10	1.332×10^7		
CD4+ T cells	Healthy controls	6	1.083×10^9	Independent samples <i>t</i> -test	< 0.001
	AML patients	10	3.073×10^8		
CD4+ CD45RA+ CD27+ T cells	Healthy controls	6	4.425×10^8	Independent samples <i>t</i> -test	< 0.001
	AML patients	10	7.877×10^7		
CD4+ CD45RA- CD27+ T cells	Healthy controls	6	4.268×10^8	Mann-Whitney U test	0.002
	AML patients	10	1.007×10^8		
CD4+ CD45RA- CD27- T cells	Healthy controls	6	1.506×10^8	Independent samples <i>t</i> -test	0.108
	AML patients	10	7.827×10^7		
CD4+ CD45RA+ CD27- T cells	Healthy controls	6	6.053×10^6	Mann-Whitney U test	0.713
	AML patients	10	1.580×10^7		
CD8+ T cells	Healthy controls	6	3.413×10^8	Independent samples <i>t</i> -test	0.036
	AML patients	10	2.075×10^8		
CD8+ CD45RA+ CD27+ T cells	Healthy controls	6	1.731×10^8	Mann-Whitney U test	0.003
	AML patients	10	2.684×10^7		
CD8+ CD45RA- CD27+ T cells	Healthy controls	6	4.905×10^7	Independent samples <i>t</i> -test	0.005
	AML patients	10	2.272×10^7		
CD8+ CD45RA- CD27- T cells	Healthy controls	6	3.173×10^7	Independent samples <i>t</i> -test	0.375
	AML patients	10	4.330×10^7		
CD8+ CD45RA+ CD27- T cells	Healthy controls	6	4.748×10^7	Mann-Whitney U test	0.958
	AML patients	10	4.027×10^7		

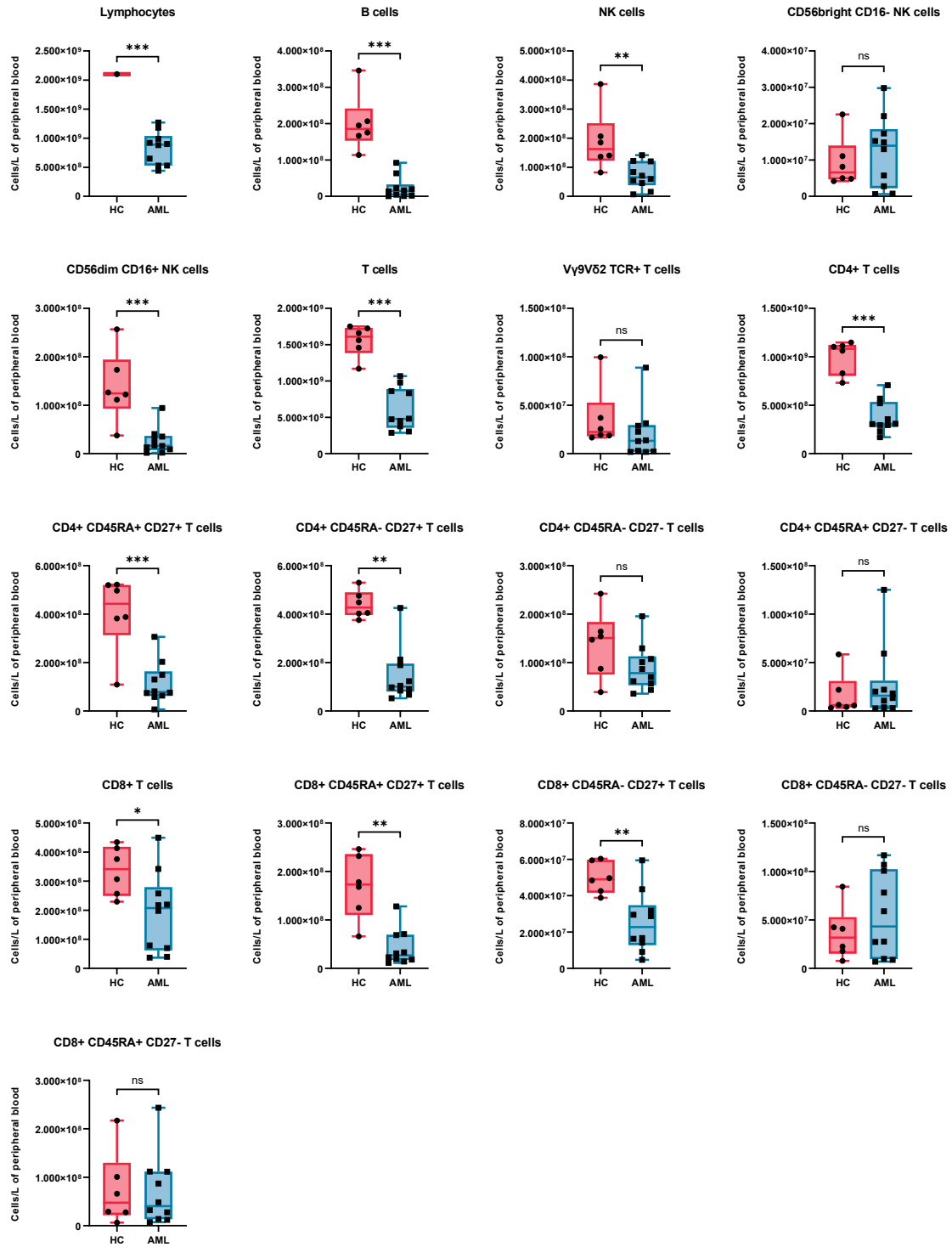


Figure 16. Immune cell levels of AML patients during first-line CT recovery (off-treatment) and of healthy controls. Data are presented in boxplots depicting median cell counts, interquartile range (boxes), and minimum and maximum values (whiskers). Statistical p -values for the comparisons between groups are presented as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$; ns, not significant. HC, healthy controls; AML, acute myeloid leukemia patients.

2. Comparing immune cell counts of AML patients categorized on the basis of diagnostic and therapy outcome variables

The statistical approach performed in data from post-induction and post-consolidation chemotherapy samples was also applied to analyze post-chemotherapy completion samples; however, several comparisons were unable to be carried out due to sample size limitations. Nevertheless, none of the applied statistical tests yielded a significant p -value (data not shown).

Discussion

DISCUSSION

The basis of AML treatment in patients with favorable fitness is still intensive chemotherapy. However, while this strategy allows for the elimination of AML blasts to an appreciable extent, it similarly induces severe immune depletion, and, ultimately, most patients relapse (136, 137). After induction chemotherapy, and at a stage in which the bulk of AML blasts has been depleted, reconstitution of the immune system begins, and it is believed that the recovering immune cells may, in some cases, be capable to partially eliminate residual AML cells that would, eventually, contribute to the establishment of relapse (138). In fact, an association between higher lymphocyte counts and improved outcomes has already been described in this context (139), and, although some studies have also characterized the immune repertoire at this stage (137, 138, 140-142), the contribution of each cell subtype to prolonged survival is currently not understood.

It is important to note that because, as reported in the Methodology chapter, staining for CD62L was not possible due to technical constraints, the biological and clinical interpretation of the CD4⁺ and CD8⁺ T cell subsets results, as well as their comparison with those published in the literature, must be carried out cautiously. CD45RA is generally considered to accurately distinguish between naïve/TEMRA (CD45RA⁺) and memory (CD45RA⁻) T cells (143), and CCR7 or CD62L expression is commonly evaluated in combination with CD45RA to help discern naïve, central memory, effector memory and TEMRA T cells (144-146). CD27, a costimulation receptor involved in T cell activation, is also frequently used to characterize the activation status of these four subpopulations (143). Nonetheless, while CD27 identification as an equivalent of CCR7 or CD62L to distinguish naïve and TEMRA cells within the CD45RA⁺ populations has been generally accepted (147, 148), its use as a unique identifier of central memory and effector memory T cells in CD45RA⁻ T cells remains controversial. Indeed, while some studies report the use of CD27 as a replacement of CCR7 or CD62L to separate the four subsets (149, 150), there is CD27 expression variability within the effector memory T cell population (151). This implies that restricting the identification of this subset using the CD45RA⁻ CD27⁻ phenotype inherently disregards a portion of effector memory T cells that express CD27, which would be wrongly included in the CD45RA⁻ CD27⁺ central memory subpopulation. Taking these into consideration, results regarding CD45RA⁺ CD27⁺ and CD27⁺ CD27⁻ will be interpreted, respectively, as naïve T and TEMRA cells. On the other hand, interpretation of results for CD45RA⁻ CD27⁺ and CD45RA⁻ CD27⁻ T cells will be, whenever possible, based on research performed using these exact phenotypes, but, because other definitions are more

commonly reported in the literature, these subsets will also be considered to be central memory-like and effector memory-like, respectively.

The results of this study suggest that, throughout intensive chemotherapy and some months after its completion, AML patients remain in a general immunocompromised state, with the overall lymphocyte population continuously present in levels significantly lower than in the healthy cohort, as happened with the majority of the analyzed subsets. To compare the results between the three first-line chemotherapy timepoints for each of the subpopulations, one would need to apply a statistical test for use with paired (dependent) samples, as, for each patient, the frequencies of a specific immune subset at one timepoint are not independent of the results of the previous ones. Because samples collected for two or three of the timepoints were only available in a few patients, paired comparisons were not carried out. Nevertheless, to interpret the evolution of the immune landscape throughout the therapy course, one can consider the results of the AML versus HC comparisons and verify if the results (significant or non-significant) were consistent at the three moments.

Besides the lymphocytes, and within all subpopulations, only CD4⁺ and CD8⁺ CD45RA⁻ CD27⁻ T cells and TEMRA T cells were consistently present in levels comparable to those of the healthy donors throughout the three measured timepoints. Moreover, CD56bright CD16⁻ NK cells were recovered by the end of consolidation chemotherapy, and V γ 9V δ 2 TCR⁺ T cell counts became stabilized by 3 months off-treatment. Interestingly, while there was no significant difference in the peripheral blood CD8⁺ CD45RA⁻ CD27⁺ T cell counts between the healthy donors and the post-induction chemotherapy samples, the levels of this subpopulation were found to be lower than those of healthy individuals in the following two timepoints. These differences may be explained by an eventual discrepancy in duration, intensity and composition of the therapy regimens; however, as previously mentioned, variability caused by the intrinsic features of the patients and the disease itself may have also contributed to this effect.

Lymphocyte recovery after lymphopenia can occur via two biological pathways: thymopoiesis, which produces naïve T cells and whose contribution becomes substantially limited with age, and homeostatic proliferation, a mechanism by which remaining peripheral lymphocytes are stimulated by cytokines to divide, thereby rebalancing the immune system (152). Kanakry et al. analyzed the mechanisms of lymphocyte recovery after lymphopenia induced by a different chemotherapy regimen in AML, and demonstrated that, although AML patients are typically older, thymopoiesis contributes to the initial step of lymphocyte recovery independently of age. However, an extensive percentage of recovered lymphocytes are derived via homeostatic proliferation (153). Some studies show that, upon cytokine-stimulated homeostatic proliferation, effector memory T and TEMRA cells can be

preferentially generated (154, 155). This may justify the quicker recovery of CD45RA-CD27- (effector memory-like) T cells and TEMRA cells at each timepoint; nevertheless, these mechanisms are not yet fully understood, and further studies to confirm this hypothesis will be required.

In regard to the levels of Tregs, cellular mediators recruited by AML blasts to promote immunosuppression (113), analysis was only performed at the timepoint following induction chemotherapy, and suggested that AML patients had significantly lower numbers than the healthy donors. Studies have shown that Treg levels of AML patients at diagnosis are typically increased, in comparison with healthy controls, both in peripheral blood (107, 141, 156) and in bone marrow (157). However, post-treatment analyses are not as consistent, with a paper describing higher Treg counts during post-intensive chemotherapy cytopenia than healthy donors (140), and another reporting the opposite result, comparable to what was obtained in our study (156). Although the disparity in these reports may be explained through differences in, for instance, the exact Treg phenotype, sample collection timepoint or origin (peripheral blood or bone marrow), or patient variability, the problem may also reside in the form of presenting flow cytometry results. All these studies presented Treg counts as percentages, which, as previously mentioned, do not account for differences in total lymphocyte frequencies. In fact, repeating the same analysis for the comparison of patients and healthy donors, but instead using Treg percentages within CD4+ T cells, leads to a non-significant result, with median Treg relative frequency of AML patients even being slightly higher than healthy controls (data not shown), hence why it is recommended that flow cytometry results are presented as absolute cell counts per unit of blood or bone marrow volume (158).

Immune cell frequencies were also compared by type of response to first-line induction chemotherapy; however, no significant results were found at any of the timepoints. Since, out of the overall 31 AML patients treated with first-line chemotherapy, only 2 and 4 achieved partial remission and progressed, respectively, the statistical tests applied were most likely not sufficiently powerful to detect any potential difference, and a broader study will be needed to evaluate this association. Nevertheless, induction chemotherapy is, at most cases and particularly in young individuals, capable to induce complete remission (136); therefore, it is possible that, in reality, achieving remission after chemotherapy is independent of the immune landscape of the patients.

For the overall and relapse-free survival analyses, two distinct methods were performed: the univariate Kaplan-Meier analysis, coupled with the Logrank test, and the Cox proportional hazards method (Cox regression), which was both conducted at a

univariate level, as well as with the inclusion of relevant covariates. Both methods were presented because each provides different information. The Logrank test compares the survival of 2 or more categories of a predictive variable. Since the predictive values under study were, in this case, immune subset counts, these continuous variables had to be categorized into 2 groups prior to application of the method. The cutoff value that was selected per each cell subtype was the one that provided the lowest p -value, corresponding to the best degree of separation between the survival curves. Yet, in some analyses, the cutoff that yielded the most successful p -value separated the patients in an unbalanced manner, with one of the groups comprising of a very limited sample size. This implies that, in these situations, and when significant p -values were found, the majority of patients was integrated into one group, within which no difference in survival was considered, and that better or worse survival was restricted to patients who exhibited extreme cell counts. Frequently, this translated into a non-significant p -value in the corresponding univariate Cox regression. The Cox regression method, on the contrary, does not demand categorization of continuous variables, and also grants the possibility of including other covariates that may influence the effect of the main predictor on survival. However, this strategy relies on greater sample sizes when the number of included covariates is increased. Consequently, given the low sample size of our patient cohort, the statistical tests that include covariates lose power and increase the chance of type II statistical errors (159).

Thus, notwithstanding the significant Logrank test results providing justification for further, larger-scale research to clarify the prognostic value of these subsets, interpretation of the results will be restricted to the subpopulations that yielded significant p -values in the Cox proportional hazards analyses.

Regarding overall survival analysis in samples collected after induction chemotherapy, and using the Kaplan-Meier method, statistically significant differences were found for lymphocytes, CD4+ T cells, CD4+ CD45RA- CD27+, CD4+ CD45RA- CD27- and CD4+ TEMRA cells, as well as for CD8+ naïve T cells, with higher immune subset counts being associated with improved OS. When repeating the analysis using Cox regression, univariate analysis yielded no results. Yet, when adjusting for age, lymphocytes were found to be significant, with CD4+ CD45RA- CD27- T cells being very close to the threshold of significance ($p = 0.056$). In the relapse-free survival, AML patients with higher lymphocytes, NK and T cells, CD4+ CD45RA- CD27+, CD4+ CD45RA- CD27- and CD4+ TEMRA cells, and CD8+ CD45RA- CD27- T cells were found to have better RFS using the Logrank test, whereas Cox regression generated significant results for lymphocytes, T and CD4+ CD45RA- CD27- T cells (univariate), lymphocytes, T, CD4+ T and CD4+ CD45RA- CD27-

T cells (adjusted for age), and CD4⁺ CD45RA⁻ CD27⁻ T cells (adjusting for age and ELN cytogenetic risk).

Considering all these results, the CD4⁺ CD45RA⁻ CD27⁻ T cell subpopulation, which, as established, can also be taken as CD4⁺ effector memory-like, consistently appears to be associated with better prognosis when present in higher absolute frequencies following induction chemotherapy. Indeed, the best result for OS suggests that, although not significant, an increase of 1×10^6 CD4⁺ CD45RA⁻ CD27⁻ T cells per L of peripheral blood after induction chemotherapy leads to a 0.9% decrease in risk of dying at a certain point in time, after adjusting for age. Similarly, for every 1×10^6 cells/L increase in this cell subset, the risk of relapse or death at any particular time, after adjusting for patient age at diagnosis, decreases 1.7%. The disparity between OS and RFS may be justified by the fact that, whereas from first-line chemotherapy until relapse there was mostly no other anti-leukemic treatment influencing AML evolution, that was not the case for the duration of time until death or last follow-up. Particularly, a considerable percentage of patients (48.39%) underwent HSCT, which intrinsically provides a “reset” of both the immune and AML landscapes, thereby mostly overwriting any advantage rendered by increased CD4⁺ CD45RA⁻ CD27⁻ T cells following induction chemotherapy.

Some hypotheses attempting to explain the relevance of this immune subpopulation, and specifically after induction chemotherapy, can be formulated. Firstly, T cell activity against AML blasts appears to be potentiated by chemotherapy, which may disrupt the mechanisms of AML-mediated immunosuppression, at least partially due to a decrease in AML cell burden (138, 160, 161). Yet, another mechanism triggered by chemotherapy that seems to further enhance T cell activation is immunogenic cell death (ICD) (Figure 17) (160). In fact, several chemotherapeutic drugs, including anthracyclines, often used in standard first-line chemotherapy in AML, eliminate cancer cells primarily by interfering with DNA synthesis, triggering a signaling cascade that leads to the upregulation of various stress-associated cell signals (131). This also results in the surface exposure of endoplasmic reticulum proteins, such as calreticulin (CRT); because these molecules are typically confined to the intracellular space, their externalization can be promptly recognized as a DAMP by PRRs (for example, CD91) in APCs (131, 162). APCs, then, become activated, incorporate and process antigens of dying and stressed cancer cells, and present them to T lymphocytes, thus triggering an adaptive immune response (131, 162). Fucikova et al. demonstrated that AML blasts often upregulate DAMP exposure upon chemotherapy treatment, which correlates with increased peripheral blood counts of immune subsets, including, interestingly, CD4⁺ effector memory T cells, as well as NK and CD8⁺ effector memory T cells (163). Importantly, AML patients with higher frequencies of CRT⁺ cells exhibited improved OS and RFS, with a lower *p*-value observed in the latter (163). This

work, while possibly explaining the contribution of CD4⁺ effector memory-like T cells towards better outcomes, also suggests a role of NK and CD8⁺ effector memory T cells in CTR-mediated immune responses. In our study, although neither of the equivalent subsets was significant in the Cox regression analysis, the Logrank test signaled these two subpopulations as impactful in RFS. Therefore, further research with a wider sample size may similarly solidify the role of these cells in the elimination of residual AML after induction chemotherapy.

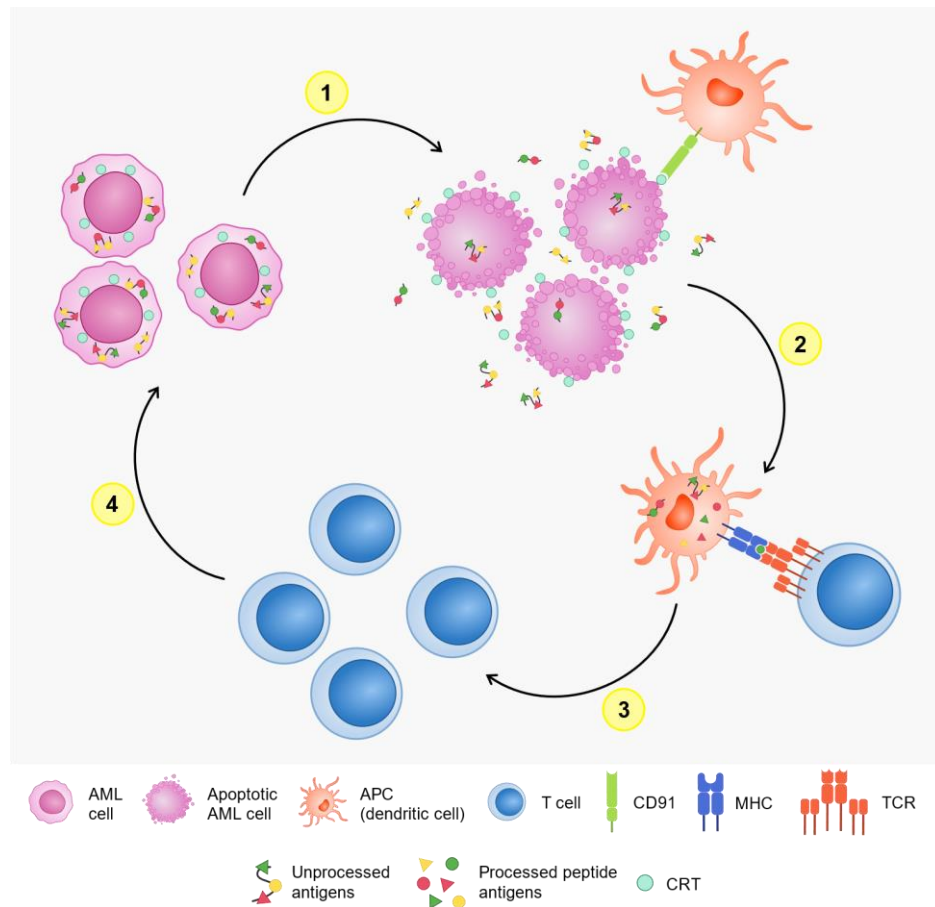


Figure 17. Immunogenic cell death and subsequent T cell-mediated elimination of AML cells after first-line chemotherapy. **1.** Cytotoxic effects of chemotherapy, including of anthracyclines, lead to surface expression of calreticulin on dying, apoptotic AML blasts, which is promptly recognized by pattern recognition receptors, such as CD91, in antigen-presenting cells; **2.** antigen-presenting cells then become activated, and incorporate, process and present antigens from dying AML cells to T cells; **3.** T cells are also, thereby, activated, proliferate, and **4.** exert their effector functions in chemotherapy-resistant AML blasts, leading to their elimination and further hampering AML progression. APC, antigen presenting cell; MHC, major histocompatibility complex; TCR, T cell receptor; CRT, calreticulin.

Surprisingly, CD4⁺ CD45RA⁻ CD27⁻ T cell counts in AML patients after consolidation or 3 months after the end of chemotherapy do not seem to impact OS nor RFS, indicating that the effect of this subpopulation is only relevant after induction chemotherapy. This fact

may be explained by the (re)establishment of immune evasion strategies by the AML blasts, due to clonal evolution of the residual, induction chemotherapy-resistant AML cells. Within the most common immunosuppressive mechanisms deployed by AML blasts [reviewed in (96, 164)], some immune evasion strategies specifically targeting CD4⁺ T cells may develop between the end of induction and the end of consolidation, including MHC class II downregulation, which hinders self-antigen presentation by AML blasts to CD4⁺ T cells, and class II-associated invariant chain peptide (CLIP) overexpression (Figure 18) (164, 165). Briefly, CLIP is a small molecule that temporarily binds the MHC class II complex within specialized lysosomes in antigen presenting cells, before being replaced by the antigen to be displayed by the cell through the action of HLA-DM (human leukocyte antigen-DM). If HLA-DM does not remove CLIP, then the antigen is not presented and CLIP becomes over expressed at the cell surface (166). In AML, patients with lower frequencies of CLIP⁺ blasts (impaired antigen presentation) at diagnosis (167) or during post-chemotherapy follow-up (168) exhibited improved disease-free survival. Of note, an in vitro study has shown that, when co-culturing CLIP⁻ AML blasts with CD4⁺ T cells, higher generation of CD4⁺ CD45RA⁻ CD27⁻ T cells was observed, in comparison with CLIP⁺ co-cultures; moreover, CD4⁺ CD45RA⁻ CD27⁻ T cells in CLIP⁻ co-cultures exhibited better AML-specific responses (166). These results suggest that post-induction AML cells may be primarily CLIP⁻, whereas CLIP⁺ AML could predominate from after consolidation and onwards.

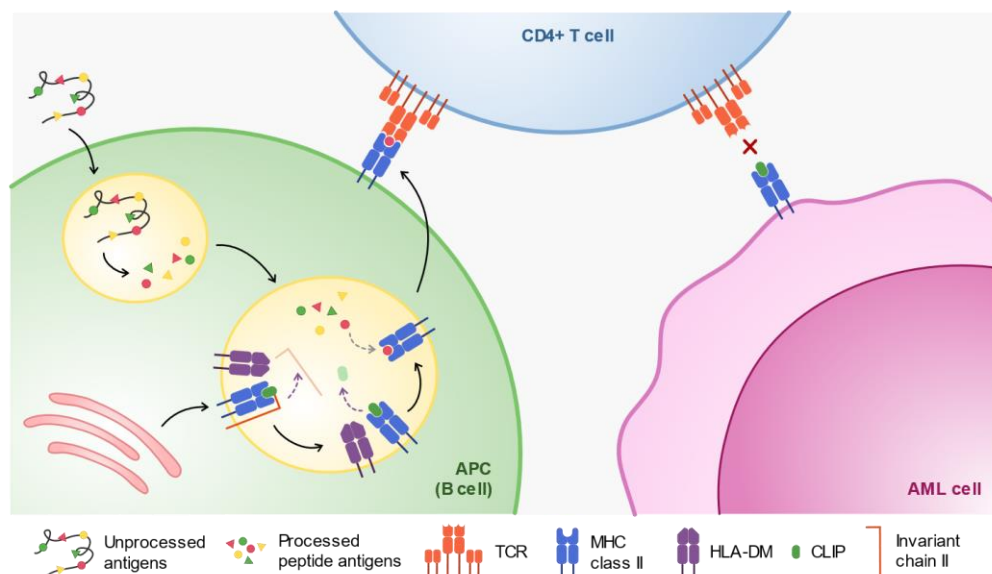


Figure 18. Normal CLIP removal and antigen presentation, and CLIP overexpression as a possible mechanism of AML immune evasion from CD4⁺ T cells after first-line induction chemotherapy. APC, antigen-presenting cell; TCR, T cell receptor, MHC, major histocompatibility complex; HLA-DM, human leukocyte antigen-DM; CLIP, class II-associated invariant chain peptide.

Nevertheless, if these hypotheses were to be confirmed, the exact mechanism by which CD4⁺ CD45RA⁻ CD27⁻ T cells accumulate after immunogenic cell death-mediated activation would still need to be clarified. CD4⁺ T cells are typically associated with their role in mediating the activation, recruitment and memory generation of other immune effectors, such as CD8⁺ T cells (169). Yet, the preliminary results of this project do not demonstrate a clear benefit for any CD8⁺ T cell subset. Alternatively, CD4⁺ CD45RA⁻ CD27⁻ T cells could, upon recall, release IFN- γ and trigger the effector roles of anticancer M1 macrophages (170), and bone marrow frequencies of this immune population were even found to predict better overall and event-free survival (171, 172). A subset of CD4⁺ T cells can also, upon activation following MHC class II recognition, directly eliminate cancer cells through the secretion of granzyme B and perforin (170, 173). Interestingly, these cells, conveniently named cytotoxic CD4⁺ T cells, are considered to be effector memory (and, therefore, antigen-experienced) cells that exhibit a downregulated expression of CD27 and CD28 (173). Thus, these cells may actually be a subpopulation of CD4⁺ CD45RA⁻ CD27⁻ T cells and be at least partially responsible for the improved survival of AML patients presenting with higher counts following induction chemotherapy.

The post-consolidation survival analyses using the Kaplan-Meier method rendered CD4⁺ T cells, CD4⁺ CD45RA⁺ CD27⁺ and CD4⁺ CD45RA⁺ CD27⁻ as being statistically significant in OS, with higher counts suggesting better survival. However, these results were not sustained when applying the Cox regression method. RFS analysis, on the other hand, did not yield any significant results through the Logrank test, whereas the Cox regression allowed for the identification of B cells, with higher counts being associated with worse prognosis. This last result was particularly unexpected, since, in spite of not being as well explored as T cells in the context of cancer and, in particular, of AML, it is generally understood that B cells participate in anti-cancer responses, since they present tumor antigens to CD4⁺ and CD8⁺ T cells (174). However, upon analyzing the previous plots for B cells quantified after consolidation, there are two patients whose values are clear outliers, with quantifications far greater than those of the remaining patients. Considering that these two extreme values could be influencing the overall *p*-value, a separate analysis was performed after exclusion of those two outliers, and the *p*-value became non-significant (data not shown). This disparity in *p*-values with only the inclusion/exclusion of 2 samples illustrates how Cox regression requires high sample sizes to achieve reliable and robust results. Clarification of which of the conclusions is more accurate will, thereby, require the study of a larger sample.

No significant results were found in the survival analyses for samples collected 3 months after chemotherapy completion.

Because of its nature, there were several limitations associated with this study, the main being the restricted patient sample size. Firstly, low sample sizes have limited the number of covariates potentially affecting survival that could be included in the survival models. Furthermore, the inclusion of age, and particularly of both age and ELN cytogenetic risk into these models has already impacted the power of the analysis, as evidenced by the increasing range of the 95% confidence intervals for the multivariate hazard ratios. Additionally, the lack of paired samples for each therapy did not allow for comparisons between timepoints to be made, and inferences regarding the evolution of immune cell counts through different stages of treatment based on comparisons with the healthy controls may not be accurate. Another limitation was the fact that treatment with intensive chemotherapy was not homogeneous between patients due to variations, not only in the administered agents and therapy schemes, but also in the number of treatment cycles within each timepoint. Ideally, patients treated with distinct therapy regimens would be analyzed separately. As mentioned throughout the interpretation of results, it was also not possible to obtain a healthy control sample that matched the age of the AML cohort, and because the immune repertoire varies with age, some statistically significant findings in AML patient versus healthy donor comparisons may be attributed to this difference, rather than a consequence of the dynamics of immune cell recovery. Other technical constraints, besides the aforementioned loss of CD62L upon cryopreservation, namely low lymphocyte counts per frozen PBMC vial and low cell viability of the AML samples, may have limited the quantification of cells expressing rarer markers, such as V γ 9V δ 2 TCR or FoxP3. However, this problem was generalized across AML samples, and thereby should not have particularly impacted any specific group of patients.

Given the limitations of this project, it should be followed by a larger-scale study, not only to confirm the obtained significant results, correct less accurate methodologies and clarify some inconsistencies, but also to answer some questions that were not addressed. An ideal study would go as follows: firstly, both bone marrow and peripheral blood AML samples would be collected, since some immune-AML interactions occurring within the bone marrow may not be reflected in peripheral blood immune frequency variations. Moreover, AML samples would be collected after each therapeutic intervention, including after each intensive chemotherapy cycle, and periodically throughout follow-up. Additionally, diagnostic samples would also be collected, to serve as a baseline for the pre-treatment conditions. Information regarding the occurrence of infections, which also may influence immune cell frequencies, as well as of prophylactic or therapeutic interventions to mitigate those infections should be collected and taken into consideration during statistical analysis. A higher number of healthy donor peripheral blood and, if possible, non-AML bone marrow samples would also be acquired, and the age of these individuals would be more

diversified to match the ages of the AML patients. Samples would preferably be processed and analyzed without cryopreservation, in order to avoid variability in the expression of some markers, and also to limit the number of sample-processing steps that implicitly lead to cell loss. Besides the ones included in this study, other immune markers would be analyzed, in order to quantify the expression of immune checkpoint and costimulatory molecules, activating and inhibitory receptors, and other targets of interest, such as MHC class II and CLIP, in lymphocytes and/or AML blasts. In parallel with immune subset frequency quantification, functional assays would be performed to characterize the CD4⁺ effector T cell subsets upon stimulation, as well as to identify AML-specific T cells. Finally, it would also be interesting to analyze the genetics underlying the clonal evolution of AML throughout the collected samples, and correlate emerging or disappearing clones with the dynamics of the immune landscape.

Concluding remarks

CONCLUDING REMARKS

AML typically develops multiple mechanisms of evasion from immunosurveillance, and efforts aiming to sensitize AML blasts to the effector functions of the immune system still face critical challenges, with no new immunotherapy for AML surpassing the clinical trial stage in recent years. Yet, it is known that the immune system can be effective in eliminating AML, as evidenced by the *graft-versus-leukemia* effect in patients who underwent hematopoietic stem cell transplantation; moreover, conventional intensive chemotherapy may stimulate immune responses via induction of immunogenic cell death. We hypothesized that the timepoints pertaining to post-chemotherapy immune recovery could be associated with improved immune responses in some patients, as a result of reduced AML bulk and chemotherapy-induced sensitization of AML cells. In summary, our results suggest that, following induction and consolidation chemotherapy administration, AML patients still remain in a general immunodepletion state, with a recovery of some immune subpopulations throughout the three analyzed timepoints; nevertheless, some subpopulations, including CD4⁺ CD45RA⁻ CD27⁻ (effector memory-like) T cells, were consistently present in levels equivalent to normal controls. Interestingly, higher levels of this subpopulation after induction chemotherapy were further associated with superior outcomes, suggesting that, at that stage, these cells may be involved in residual AML cell elimination that contributes to an extension of relapse-free and overall survival. However, the limitations of this study may have diluted the impact of other immune subsets on prognosis, and also did not allow for further exploration of immune effector functions or AML-mediated immunosuppression assessment at these timepoints.

While this study did not provide definite answers to our initial questions, it certainly provided sufficient preliminary results that justify further, wider-scale research on how the multiple interactions between AML, its therapy regimens and the immune system evolve throughout disease progression, and how they affect AML patient prognosis. Clearer and more robust results may not only help guide patient management, but also uncover novel mechanisms of immune defense against AML, and/or of immune evasion by AML, potentially targetable by immunotherapy.

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