

Significance of Frequencies, Compositions, and/or Antileukemic Activity of (DC-stimulated) Invariant NKT, NK and CIK Cells on the Outcome of Patients With AML, ALL and CLL

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Summary: Invariant natural killer T (iNKT)/natural killer (NK)/cytokine-induced killer (CIK) cells are important for immune surveillance. (I) Novel combinations of antibody 6B11 (targeting the V α 24-J α 18-invariant T-cell receptor) with CD4/CD8/CD1d/V α 24 for iNKT subset detection and “T/NK cell-like”-iNKT subsets were defined. Compared with healthy peripheral blood mononuclear cells (MNC) (significantly) lower proportions of iNKT cells (6B11⁺/6B11⁺CD3⁺/6B11⁺CD161⁺), NK cells (CD3⁻CD56⁺/CD3⁻CD161⁺), and CIK cells (CD3⁺CD56⁺/CD3⁺CD161⁺) were found in peripheral blood MNC from acute myeloid (AML)/acute myeloid, lymphoid (ALL)/chronic lymphoid leukemia (CLL) patients in acute disease stages. Subtyping of iNKT cells revealed (significantly) higher proportions of CD3⁺ T cells and CD161⁺ NK cells in AML/ALL/CLL expressing 6B11 compared with healthy MNC. Prognostic evaluations showed higher proportions of iNKT/NK/CIK cells in favorable AML subgroups (younger age, primary, no extramedullary disease, achievement/maintenance of complete remission) or adult ALL and CLL patients. (II) iNKT/NK/CIK cell frequencies increased after (vs. before) mixed lymphocyte cultures of T-cell-enriched immune reactive cells stimulated with MNC/whole blood with or without pretreatment with “cocktails” (dendritic cells generating methods/kits inducing blasts’ conversion to leukemia-derived dendritic cells from AML patients). Individual “cocktails” leading to “highest” iNKT cell frequencies could be defined. Antileukemic blast lytic activity correlated significantly with frequencies of iNKT/NK/CIK cells. In summary healthy MNC show significantly more iNKT/NK/CIK cells compared with AML/ALL/CLL MNC, a shift in the iNKT cell composition is seen in healthy versus leukemic samples and iNKT/NK/CIK cell-proportions in AML/ALL/CLL MNC samples correlate with prognosis. “Cocktail”-treated AML blasts lead to higher iNKT/NK/CIK cell frequencies and samples with antileukemic activity show significantly higher frequencies of iNKT/NK/CIK cells. Proportions of iNKT/NK/CIK cells should regularly be evaluated in AML/ALL/CLL diagnosis panels for quantitative/prognostic estimation of individual patients’ antileukemic potential and their role in dendritic cells/leukemia-derived dendritic cells triggered immune surveillance.

Key Words: iNKT, NK, CIK, DC_{leu}, AML, ALL, CLL
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Acute myeloid (AML), lymphoid (ALL) or chronic lymphoid leukemia (CLL) are clonal diseases with uncontrolled proliferation of myeloid or lymphoid blasts. Prognostic rates of complete remission (CR) and survival depend on grade of anemia, thrombocytopenia, white blood cell expansion and karyotypes, resulting in different therapeutic strategies. Therapy for AML, ALL, and CLL patients with advanced stages consists of chemotherapy and stem cell transplantation (SCT), but the rate of early failures and relapses is still unsatisfying. AML patients can be categorized into 3 risk groups.¹ ALL patients are primarily children with survival rates of about 90% and higher incidences for an assignment to standard risk-groups compared with adult patients with higher tendencies for high risk leukemia.² CLL usually occurs in elderly patients and these are classified in Binet-staging groups: Binet A (early), B (intermediate), and C (advanced stage).³

Effective immune surveillance of patients with hematologic malignancies such as leukemia is mediated by arms of the innate and adaptive immune system. The innate immune system includes macrophages, dendritic cells (DC), and natural killer (NK) cells, which respond quickly to an immunologic threat.⁴ NK cells have the ability to kill tumor cells without activation, arise from CD34⁺ bone marrow (BM) cells and are defined as CD3⁻CD56⁺CD161⁺.⁵ The adaptive immune system includes T and B cells, which mediate tumor immunity by antigen-specific responses and provide long-lasting protection by effector-memory responses. T cells (CD3⁺) express T-cell receptors (TCR) that recognize (peptide)-antigens on blasts, which have to be presented by major histocompatibility complex (MHC)-I or MHC-II molecules via antigen-presenting cells.⁴ A specialty of myeloid blasts is, that they can differentiate to leukemia-derived dendritic cells (DC_{leu}), presenting the complete leukemic antigen repertoire, thereby specifically and efficiently activating an antileukemic T-cell response.^{6–8} T cells occur in various differentiation and functional subsets [eg, naive (T_{naive}, CD45RO⁻), non-naive (T_{non-naive}, CD45RO⁺), central-memory (T_{cm}, CD45RO⁺CCR7⁺), effector/memory (T_{eff-em}, CD45RO⁺CCR7⁻), and regulatory (T_{reg}, CD25⁺CD127^{low})].^{9,10} Furthermore, other cells at the interface of the innate and the adaptive immune system are important mediators in antitumor,

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autoimmune, and antimicrobial responses and tumor surveillance⁴: cytokine-induced killer (CIK) cells have phenotypic and functional features of T and NK cells as they are CD3⁺ CD56⁺ CD161⁺, they are expandable in culture like T cells; however, they do not recognize cells via TCR or MHC molecules as T cells.⁵ NKT cells are a heterogeneous lymphoid population, that bridge innate and adaptive immunity and, in general, share properties of T and NK cells. Similar to NK cells, NKT cells have the ability to react rapidly to antigenic stimulation by quickly secreting large amounts of cytokines and chemokines within minutes to hours.⁴ In addition, they activate DC, NK, and CD4⁺/CD8⁺ T cells, thereby triggering innate and adaptive immune responses.¹¹ Similar to T cells, NKT cells respond via the TCR, recognizing glycolipid antigens presented by the MHC-like molecule CD1d. TCR from NKT cells react with many self and foreign antigens, while the TCR from T cells only reacts with one epitope.⁴ It was shown, that the positive selection of NKT cells is strictly dependent on CD1d during ontogeny in the thymus and therefore CD1d restriction has become the defining characteristic of NKT cells (NK markers are only used to define subpopulations).¹² The NKT cell population consists of many phenotypically and functionally diverse subsets, which are subdivided either by surface markers, TCR, tissue location, antigen recognition or by effector functions. Concerning their heterogeneous TCR rearrangements, NKT cells are divided into 2 main groups: type-I NKT cells are referred to as invariant NKT cells (iNKT cells) as they express a semi-invariant TCR, characterized in humans by V α 24-J α 18 and V β 11, while β -chains have a limited variety. In contrast, NKT cells that do not express this semi-invariant TCR are referred to as type-II NKT cells, which are less well studied than iNKT cells.¹¹ iNKT cells are known for enhancement of tumor immunity, while type-II NKT cells are known for suppression of it, resulting in opposed roles in tumor immunity and crossregulate of each other.⁴

NKT cells originate from a precursor pool of CD4⁺ CD8⁺ double positive thymocytes, that have to undergo diverse TCR gene rearrangements, are then positively selected by CD1d⁺ double positive thymocytes in the thymic cortex, undergo 4 differentiation and maturation steps and then differentiate into mature NKT cells. Expression of CD4 and CD8 allow a subdivision of NKT cells in different subpopulations: a majority is CD4⁺ (90% in mice), the remainder is CD4⁻CD8⁻ double negative and a small population is CD8 α ⁺ and CD8 α β ⁺, but CD8⁺ subsets only exist in humans (not in mice), predominantly in healthy persons with latent Epstein-Barr virus infection.¹²

The evaluation of human iNKT cells is challenging, as their frequency in peripheral blood (PB) is very low. Classically, iNKT cells have been identified using CD1d tetramers loaded with α -galactosylceramide or monoclonal antibodies (moAbs) against the V α 24 and V β 11 chains, what however, can lead to an overestimation of iNKT cells, as noninvariant, non-CD1d restricted V α 24⁺ T cells can also pair with V β 11. Recently iNKT cells were shown to be identified using the moAb 6B11, which recognizes the invariant CDR3 loop of their V α 24J α 18 TCR-rearrangement with high specificity and sensitivity.¹³ CD161 (NKR-P1A) is a C-type lectin receptor and is an important marker for NK and iNKT cell identification as all human NK cells, high proportions of iNKT cells, and T_{eff/em} and T_{cm} express it. Recently it was shown, that CD161⁺ T cell subsets are highly functional during

infections: low frequencies correlate with higher incidence of (viral) infections.^{14–18}

Considering that the antileukemic function of T cells can be stimulated by DC_{leu},^{9,10} our supposition is that the iNKT/NK and CIK cell activity might also be enhanced under DC/DC_{leu} stimulation. DC_{leu} can be generated in vitro by converting myeloid leukemia cells in mononuclear cells (MNC) or whole blood (WB) using DC-generating methods/kits (“cocktails,” containing immune response modifiers in combination with cytokines) and represent as well leukemic (eg, CD13, CD33, CD117) and DC antigens (eg, CD80, CD83, CD86).^{19,20}

Physiological conditions in the stem cell niche of the BM as well as in the PB are hypoxic with Oxygen (O₂) concentrations between 0.1% and 0.6% in the BM, 12% in arterial blood and 4%–15% in PB.^{21,22} Previous studies suggest an influence of the partial pressure of oxygen (pO₂) on several pathophysiological mechanisms.^{23,24} However, hematopoiesis takes place under physiologically low pO₂/O₂-concentration and hematopoietic cells are continually exposed to dynamic pO₂-values.²⁵ Moreover, a recent in vitro study showed that hypoxia might have an enhancing effect on NK cells.²⁶

The aim of this study was (1) to test the suitability of different markers and combinations (Table 1) to characterize and quantify iNKT, NK, and CIK cells/subsets; (2) to quantify iNKT, NK, and CIK subsets in MNC from patients with AML, ALL, and CLL compared with healthy controls; (3) to correlate findings with disease entities and prognostic subgroups; (4) to quantify iNKT, NK, and CIK subsets under stimulation with DC/DC_{leu}, that are generated from WB/MNC with various “cocktails”.

MATERIALS AND METHODS

Sample Collection

After obtaining informed consent, heparinized peripheral WB samples were taken from patients in acute phases of AML, ALL, CLL, and from healthy controls. MNC were prepared from WB samples by density gradient centrifugation using the Ficoll-Hypaque technique (Biocoll separating solution; Biochrom, Berlin, Germany) with a density gradient of 1.077 g/mL. MNC were washed and suspended in phosphate-buffered saline (PBS; Biochrom). CD3⁺ T cells were enriched using the MACS technology (Milteny Biotech, Bergisch Gladbach, Germany). The purity of CD3⁺ T cells was on average (\emptyset) 89% (range: 69%–98%). Cells were quantified using Neubauer counting chambers, used directly or frozen and thawed according to standardized protocols.

Patients' Characteristics and Diagnostics

Patients and Samples Included for Surface Marker Expression Analyses on Thawed Cells

Quantitative and qualitative characterizations of several immune reactive cells were performed with thawed MNC from patients with blast-rich phases of AML (n = 23), ALL (n = 19), and CLL (n = 21). Samples were provided by the University-Hospitals of Tübingen, Düsseldorf, Munich, Oldenburg and Augsburg. Cells were obtained by aspirates of PB or BM, which were anticoagulated with heparin after patients' written informed consent in accordance with the Helsinki protocol and the local Ethic Committee (Pettenkoferstr. 8a, 80336 München,

TABLE 1. Subtypes of T/iNKT/NK/CIK Cells, Blasts and DC as Evaluated by Flow Cytometry

Names of Subgroups	Referred To	Surface Marker	Abbreviation	Explanatory Note/Permise	References
CD3 ⁺ pan-T cells	MNC(WB) or MLC1*	CD3 ⁺	CD3 ⁺ /MNC(WB) or MLC		Schick et al ¹⁰
CD4 ⁺ -coexpressing T cells	MNC(WB) or MLC	CD3 ⁺ CD4 ⁺	CD4 ⁺ /MNC(WB) or MLC	CD4 ⁺ T cells	Schick et al ¹⁰
CD4 ⁺ -coexpressing T cells	CD3 ⁺	CD3 ⁺ CD4 ⁺	CD4 ⁺ /CD3 ⁺	CD4 ⁺ T cells	Schick et al ¹⁰
CD8 ⁺ -coexpressing T cells	MNC(WB) or MLC	CD3 ⁺ CD8 ⁺	CD8 ⁺ /MNC(WB) or MLC	CD8 ⁺ T cells	Schick et al ¹⁰
CD8 ⁺ -coexpressing T cells	CD3 ⁺	CD3 ⁺ CD8 ⁺	CD8 ⁺ /CD3 ⁺	CD8 ⁺ T cells	Schick et al ¹⁰
Naive T cells	MNC(WB) or MLC	CD3 ⁺ CD45RO ⁻	T _{naive} /MNC(WB) or MLC	Unprimed T cells	Vogt et al ⁹
Naive T cells	CD3 ⁺	CD3 ⁺ CD45RO ⁻	T _{naive} /CD3 ⁺	Unprimed T cells	Vogt et al ⁹
Non-naive T cells	MNC(WB) or MLC	CD3 ⁺ CD45RO ⁺	T _{non-naive} /MNC(WB) or MLC	Memory + effector T cells	Vogt et al ⁹
Non-naive T cells	CD3 ⁺	CD3 ⁺ CD45RO ⁺	T _{non-naive} /CD3 ⁺	Memory + effector T cells	Vogt et al ⁹
Central (memory) T cells	MNC(WB) or MLC	CD3 ⁺ CCR7 ⁺ CD45RO ⁺	T _{cm} /MNC(WB) or MLC	Long-term immunity	Vogt et al ⁹
Central (memory) T cells	CD3 ⁺	CD3 ⁺ CCR7 ⁺ CD45RO ⁺	T _{cm} /CD3 ⁺	Long-term immunity	Vogt et al ⁹
Effector (memory) T cells	MNC(WB) or MLC	CD3 ⁺ CCR7 ⁻ CD45RO ⁺	T _{em} -T _{eff} /MNC(WB) or MLC		Vogt et al ⁹
Effector (memory) T cells	CD3 ⁺	CD3 ⁺ CCR7 ⁻ CD45RO ⁺	T _{em} -T _{eff} /CD3 ⁺		Vogt et al ⁹
Proliferating T cells	MNC(WB) or MLC	CD3 ⁺ CD69 ⁺	T _{prol} /MNC(WB) or MLC	proliferating T cells	Schick et al ¹⁰
Proliferating T cells	CD3 ⁺	CD3 ⁺ CD69 ⁺	T _{prol} /CD3 ⁺	proliferating T cells	Schick et al ¹⁰
Viable T cells	MNC(WB) or MLC	CD3 ⁺ 7AAD ⁻	T _{vib} /MNC(WB) or MLC		Schick et al ¹⁰
Viable T cells	CD3 ⁺	CD3 ⁺ 7AAD ⁻	T _{vib} /CD3 ⁺		Schick et al ¹⁰
TCRVα24 ⁺ -coexpressing T cells	MNC(WB) or MLC	CD3 ⁺ Vα24 ⁺	Vα24 ⁺ /MNC(WB) or MLC	Vα24 segment joined with Jα18	Montoya et al ¹³
TCRVα24 ⁺ -coexpressing T cells	CD3 ⁺	CD3 ⁺ Vα24 ⁺	Vα24 ⁺ /CD3 ⁺	Vα24 segment joined with Jα18	Montoya et al ¹³
CD1d ⁻ -coexpressing T cells	MNC(WB) or MLC	CD3 ⁺ CD1d ⁻	CD1d ⁻ /MNC(WB) or MLC	CD1d ⁻ is a MHC class I-like molecule	Matsuda et al ⁵¹
CD1d ⁻ -coexpressing T cells	CD3 ⁺	CD3 ⁺ CD1d ⁻	CD1d ⁻ /CD3 ⁺	CD1d ⁻ is a MHC class I-like molecule	Matsuda et al ⁵¹
CD3 ⁺ CD56 ⁺ CIK cells	MNC(WB) or MLC	CD3 ⁺ CD56 ⁺	CD3 ⁺ CD56 ⁺ /MNC(WB) or MLC	Cells expressing T cell (CD3) and	Pittari et al ⁵
CD3 ⁺ CD161 ⁺ CIK cells	MNC(WB) or MLC	CD3 ⁺ CD161 ⁺	CD3 ⁺ CD161 ⁺ /MNC(WB) or MLC	NK cell (CD56,CD161) markers	
CD3 ⁺ CD161 ⁺ CIK cells	CD3 ⁺	CD3 ⁺ CD161 ⁺	CD3 ⁺ CD161 ⁺ /CD3 ⁺		
CD3 ⁺ CD56 ⁺ NK cells	MNC(WB) or MLC	CD3 ⁺ CD56 ⁺	CD3 ⁺ CD56 ⁺ /MNC(WB) or MLC	Cells expressing NK markers (CD56,	Pittari et al ⁵
CD3 ⁺ CD161 ⁺ NK cells	MNC(WB) or MLC	CD3 ⁺ CD161 ⁺	CD3 ⁺ CD161 ⁺ /MNC(WB) or MLC	CD161), but not expressing T cell	Montoya et al ¹³
CD3 ⁺ CD161 ⁺ NK cells	CD161 ⁺	CD3 ⁺ CD161 ⁺	CD3 ⁺ CD161 ⁺ /CD161 ⁺	markers (CD3)	Montoya et al ¹³
6B11 ⁺ (PE)iNKT cells	MNC(WB) or MLC	6B11 ⁺	6B11 ⁺ (PE)/MNC(WB) or MLC	6B11 recognizes the invariant CDR3	Bienemann et al ¹¹
6B11 ⁺ (FITC)iNKT cells	MNC(WB) or MLC	6B11 ⁺	6B11 ⁺ (FITC)/MNC(WB) or MLC	loop of the TCR α-chain of iNKT cells	Bienemann et al ¹¹
CD3 ⁺ coexpressing 6B11 ⁺ iNKT cells	MNC(WB) or MLC	6B11 ⁺ CD3 ⁺	6B11 ⁺ CD3 ⁺ /MNC(WB) or MLC	6B11 ⁺ CD3 ⁺ iNKT cells	Montoya et al ¹³
CD3 ⁺ coexpressing 6B11 ⁺ iNKT cells	CD3 ⁺	6B11 ⁺ CD3 ⁺	6B11 ⁺ CD3 ⁺ /CD3 ⁺	6B11 ⁺ CD3 ⁺ iNKT cells	Bienemann et al ¹¹
CD4 ⁺ coexpressing 6B11 ⁺ iNKT cells	MNC(WB) or MLC	6B11 ⁺ CD4 ⁺	6B11 ⁺ CD4 ⁺ /MNC(WB) or MLC	6B11 ⁺ CD4 ⁺ iNKT cells	Montoya et al ¹³
CD4 ⁺ coexpressing 6B11 ⁺ iNKT cells	CD4 ⁺	6B11 ⁺ CD4 ⁺	6B11 ⁺ CD4 ⁺ /CD4 ⁺	6B11 ⁺ CD4 ⁺ iNKT cells	Montoya et al ¹³
CD8 ⁺ coexpressing 6B11 ⁺ iNKT cells	MNC(WB) or MLC	6B11 ⁺ CD8 ⁺	6B11 ⁺ CD8 ⁺ /MNC(WB) or MLC	6B11 ⁺ CD8 ⁺ iNKT cells	Montoya et al ¹³
CD8 ⁺ coexpressing 6B11 ⁺ iNKT cells	CD8 ⁺	6B11 ⁺ CD8 ⁺	6B11 ⁺ CD8 ⁺ /CD8 ⁺	6B11 ⁺ CD8 ⁺ iNKT cells	Montoya et al ¹³
CD1d ⁺ coexpressing 6B11 ⁺ iNKT cells	MNC(WB) or MLC	6B11 ⁺ CD1d ⁺	6B11 ⁺ CD1d ⁺ /MNC(WB) or MLC	6B11 ⁺ CD1d ⁺ iNKT cells	Montoya et al ¹³
CD1d ⁺ coexpressing 6B11 ⁺ iNKT cells	6B11 ⁺	6B11 ⁺ CD1d ⁺	6B11 ⁺ CD1d ⁺ /6B11 ⁺	6B11 ⁺ CD1d ⁺ iNKT cells	Montoya et al ¹³
CD161 ⁺ coexpressing 6B11 ⁺ iNKT cells	MNC(WB) or MLC	6B11 ⁺ CD161 ⁺	6B11 ⁺ CD161 ⁺ /MNC(WB) or MLC	6B11 ⁺ CD161 ⁺ iNKT cells	Montoya et al ¹³
CD161 ⁺ coexpressing 6B11 ⁺ iNKT cells	6B11 ⁺	6B11 ⁺ CD161 ⁺	6B11 ⁺ CD161 ⁺ /6B11 ⁺	6B11 ⁺ CD161 ⁺ iNKT cells	Montoya et al ¹³
CD161 ⁺ coexpressing 6B11 ⁺ iNKT cells	CD161 ⁺	6B11 ⁺ CD161 ⁺	6B11 ⁺ CD161 ⁺ /CD161 ⁺	6B11 ⁺ CD161 ⁺ iNKT cells	Montoya et al ¹³
TCRVα24 ⁺ coexpressing 6B11 ⁺ iNKT cells	MNC(WB) or MLC	6B11 ⁺ Vα24 ⁺	6B11 ⁺ Vα24 ⁺ /MNC(WB) or MLC	6B11 ⁺ Vα24 ⁺ iNKT cells	Montoya et al ¹³
TCRVα24 ⁺ coexpressing 6B11 ⁺ iNKT cells	6B11 ⁺	6B11 ⁺ Vα24 ⁺	6B11 ⁺ Vα24 ⁺ /6B11 ⁺	6B11 ⁺ Vα24 ⁺ iNKT cells	Montoya et al ¹³
CD45RO ⁺ coexpressing 6B11 ⁺ iNKT cells	MNC(WB) or MLC	6B11 ⁺ CD45RO ⁺	6B11 ⁺ CD45RO ⁺ /MNC(WB) or MLC	6B11 ⁺ CD45RO ⁺ iNKT cells	Montoya et al ¹³
CD45RO ⁺ coexpressing 6B11 ⁺ iNKT cells	6B11 ⁺	6B11 ⁺ CD45RO ⁺	6B11 ⁺ CD45RO ⁺ /6B11 ⁺	6B11 ⁺ CD45RO ⁺ iNKT cells	Montoya et al ¹³
CD45RO ⁺ 6B11 ⁺ iNKT cells	MNC(WB) or MLC	6B11 ⁺ CD45RO ⁺	6B11 ⁺ CD45RO ⁺ /MNC(WB) or MLC	6B11 ⁺ CD45RO ⁺ iNKT cells	
CD45RO ⁺ 6B11 ⁺ iNKT cells	6B11 ⁺	6B11 ⁺ CD45RO ⁺	6B11 ⁺ CD45RO ⁺ /6B11 ⁺	6B11 ⁺ CD45RO ⁺ iNKT cells	
Blast cells†	MNC(WB) or MLC	bla ⁺	bla ⁺		
Blasts coexpressing CD161	MNC(WB) or MLC	bla ⁺ CD161 ⁺	bla ⁺ CD161 ⁺ /MNC(WB) or MLC		
Blasts coexpressing CD161	bla ⁺	bla ⁺ CD161 ⁺	bla ⁺ CD161 ⁺ /bla ⁺		
Blasts coexpressing 6B11	MNC(WB) or MLC	bla ⁺ 6B11 ⁺	bla ⁺ 6B11 ⁺ /MNC(WB) or MLC		
Blasts coexpressing 6B11	bla ⁺	bla ⁺ 6B11 ⁺	bla ⁺ 6B11 ⁺ /bla ⁺		
DC	Cells	DC ⁺ (CD80, CD86, CD206)	DC ⁺ /cells	≥ 10 % DC ⁺ in cells	Schmetzer et al 2007
Leukemia derived DC	Cells	DC ⁺ bla ⁺	DC _{leu} /cells	≥ 5% DC _{leu} in cells, ≥ 10% DC, ≥ 10% bla	Schmetzer et al 2007
Blasts converted to DC _{leu} in blast fraction	bla ⁺	DC ⁺ bla ⁺	DC _{leu} /bla ⁺	≥ 5% DC _{leu} in cells, ≥ 10% DC, ≥ 10% bla	Schmetzer et al 2007
DC _{leu} in DC fraction	DC	DC ⁺ bla ⁺	DC _{leu} /DC	≥ 5% DC _{leu} in cells, ≥ 10% DC, ≥ 10% bla	Schmetzer et al 2007
Migratory mature DC in DC fraction	Cells	DC ⁺ CCR7 ⁺	DC _{mig} /DC	≥ 10% DC in cells	Grabrucker et al 2010
Viable DC in DC-fraction	Cells	DC ⁺ 7AAD ⁻	DC _{vib} /DC	≥ 10% DC in cells	Grabrucker et al 2010

Surface marker combinations as well as T/iNKT/NK/CIK cell and DC subtypes after flow cytometric staining with fluorochrome-labeled antibodies are given.

*Total proportions of cell subsets in uncultured MNC/WB or in MLC (after culture with T cells and IL-2).

†Cells expressing blast markers: patients' individually selected blast-markers with the highest expression (Table 2: blast phenotype).

CIK indicates cytokine-induced killer; DC, dendritic cells; iNKT, invariant natural killer T; NK, natural killer; MLC, mixed lymphocyte cultures; MNC, mononuclear cells; WB, whole blood.

Ludwigs-Maximilians-University Hospital in Munich; Vote-No 339-05). The mean age of AML patients was 50 (range: 23–81) years, of ALL patients 22 (range: 3–50) years and of CLL patients 57 (range: 33–72) years. The female to male ratio of AML patients was 1:1.9, of ALL patients was 1:1.7, and of CLL patients was 1:0.9 (Table 2A).

Diagnosis and classification of AML patients was based on the “French American British” classification: minimally differentiated AML (M0: n = 1), AML without maturation (M1: n = 6), AML with granulocytic maturation (M2: n = 6), acute myelomonocytic leukemia (M4: n = 4), acute myelomonocytic leukemia together with BM eosinophilia (M4eo: n = 2), acute monocytic leukemia (M5: n = 3). Patients presented with primary AML (n = 18) or with secondary AML (n = 5). Patients stages were: first diagnosis (n = 20), relapse before (n = 1) or after SCT (n = 2). Patients were classified in cytogenetic risk groups based on the National Comprehensive Cancer Network (NCCN) guidelines as “favorable” (n = 3), “intermediate” (n = 11), or “adverse risk” (n = 4); for 5 patients no further data were available.

The subtypes of the 19 ALL patients were immune cytologically classified according to the European Group of Immunophenotyping of Leukemias classification: pro B-ALL (BI: n = 1), c ALL (BII: n = 5), pre B-ALL (BIII: n = 2), My⁺ c ALL (BII + My: n = 3), My⁺ pre B-ALL (BIII + My: n = 1), pro T-ALL (TI: n = 1), pre T-ALL (TII: n = 1), cortical T ALL (TIII: n = 4), mature T ALL (TIV: n = 1). Patients presented with primary ALL (n = 5) or with secondary ALL (n = 14). Stages of samples were: diagnosis before SCT (n = 17) or at relapse after SCT (n = 2). Risk stratification of adult ALL was based on the Study Group for Adult Acute Lymphoblastic Leukemia (GMALL) as “standard” (n = 3), “high” (n = 1) or “highest risk” (n = 3); for 12 patients no further data were available.

All CLL patients were classified as pB-CLL (n = 21) at diagnosis (n = 2) or with persisting disease (n = 19). Risk stratification was based on the Binet-classification: Binet A (n = 14), Binet B (n = 6) or Binet C (n = 1).

Cell Lines Included for Surface Marker Analyses

The following cell lines were studied for surface marker profiles: HL-60 (AML-M2), OCI-AML2 (AML-M4), Mono-Mac 6 (AML-M5), THP-1 (AML-M5), MOLM (AML-M5a), RAMOS (B-ALL-L3), RAJI (B-ALL-L3) and JURKAT (T-ALL). Cell lines were purchased from the American Type Culture Collection and were cultured according to the manufacturer’s instructions (Table 2A).

AML Patients and Samples Included for Culture Experiments

Cellular composition of immune reactive cells in MNC or WB samples from 5 AML patients, 1 myelodysplastic syndrome (MDS) patient and from 10 healthy volunteers were studied before or after culture with/without DC/DC_{leu}-generating strategies or mixed lymphocyte culture (MLC) with (T) cells from the patients. Details about patient and sample characteristics are given in Table 2B.

Cell cultures were either performed under “normoxic conditions” (37°C, 5% CO₂ and 21% O₂). Further we studied the influence of hypoxia on the composition and function of different immune reactive cells and cultured samples in parallel under “hypoxic conditions” (37°C, 5%

CO₂ and with either varying O₂-concentrations between 0% and 17% during the incubation time in some cases or with a defined O₂-concentration of 6% or 10%) using an InVivo400 working station (Ruskin Technology, Bridgend, United Kingdom).

DC Generation From Isolated MNC or WB

DC/DC_{leu} were generated from 4 to 5 × 10⁶ isolated MNC from healthy volunteers or AML/MDS patients in blast-rich stages of the disease as described previously by others or us^{19,27} using Kit-D, Picibanil 1 (“Pici1”) or “Pici2” (Table 3) (D.C. Amberger, personal written communication). Therefore, cells were pipetted into 12-multiwell tissue culture plates (ThermoFisher Scientific, Darmstadt, Germany) and were diluted in 2 mL serum-free X-Vivo15-medium (Lonza, Basel, Swiss).

Moreover, DC/DC_{leu} were generated from WB (presenting the physiological cellular and soluble composition of the individual samples) obtained from AML/MDS patients in blast-rich stages of the disease or from healthy volunteers.^{20,29} A total of 500 μL WB was pipetted in 12-multiwell plates and diluted 1:2 in X-Vivo15-medium to imitate the physiological conditions. DC were generated from WB using 6 different DC-generating methods: “Pici1,” “Pici2,” Kit-D, Kit-I, Kit-K, and Kit-M (Table 3). A patent was written to save the idea of Kit-compositions (102014014993.5, German Patent Office), but no financial conflicts of interest have to be declared. In the subsequent chapters we summarize all DC-generating methods and Kits under the term “cocktails.” WB/MNC cultures without added response modifiers served as a control. All substances used for the DC generation are approved for human treatment.

DC-subtypes were quantified as described in the chapter “Cell-characterization by flow cytometry”.^{19,20,29}

“Pici1”

DC were generated from MNC or WB with the DC-generating protocol “Pici1” containing 500 U/mL granulocyte macrophage colony-stimulating factor (GM-CSF, Sanofi-Aventis, Frankfurt, Germany) and 250 U/mL interleukin 4 (IL-4) (PeproTech, Berlin, Germany). After 6–7 days, 10 μg/mL Picibanil (OK 432)—a lysis product from *Streptococcus pyogenes* which has unspecific immune modulatory effects (Chugai Pharmaceutical Co., Kajiwara, Japan) and 1 μg/mL prostaglandin E₂ (PGE₂) (PeproTech) were added.^{19,28} After 7–10 days of incubation cells were harvested and used for further experiments.

“Pici2”

DC were generated from MNC or WB with the “Pici2”-DC-generating protocol—a protocol developed by our group, with the same composition as given above for “Pici1,” however substituting PGE₂ by PGE₁ (PeproTech; D.C. Amberger, personal communication).

Kit-D

The generation of DC from MNC or WB with Kit-D was performed using 800 U/mL GM-CSF, 10 μg/mL Picibanil and 1 μg/mL PGE₂.²⁰ After 2–3 days the same amounts of cytokines were added and after 7–10 days of incubation cells were harvested and used for subsequent experiments.

TABLE 2. Patients' and Cell-lines' Characteristics are Presented

(A) Uncured AML, ALL, CLL Samples and Cellines Studied for Proportions and Coexpression of iNKT, NK, and CIK cells										
Pat.#	Age at Dgn./Sex	Subtype	Stage	Cell Source	Blast Phenotype (CD)	Blasts %	Cytogenetic Marker at Dgn.	Risk score (NCCN)	Resp. to SCT1	Resp. to Induction Chemotherapy (CLL: need for initial therapy)
P1050	32/F	s/M1	Dgn.	PB	117,33,13	78	46,XX	ND	CR	ND
P1053	25/M	p/M1	Rez.	PB	117,34,33,56	65	ND	ND	NCR	ND
P1056	27/M	p/M4	Dgn.	PB	117,34,33,13,15	53	46,XY	ND	ND	CR
P1057	32/M	p/M5	Dgn.	PB	117,34,33,13,14,15,38,2	39	inv(16)q,+22	Favorable	ND	ND
P1058	71/M	p/M1	Dgn.	PB	117,34,33,13,15,38,45,71,w65	91	+11	Intermediate	ND	ND
P1059	66/M	p/M4	Dgn.	PB	117,34,33,13,7	84	del(5)q, add(17)p, add(21)q, der(9)	Adverse	ND	CR
P1060	81/F	s/n.d.	Dgn.	PB	117,34,33,13	97	ND	ND	ND	ND
P1061	62/F	p/M0	Rel. a. SCT	PB	117,34,33,13	96	46,XX	Intermediate	NCR	ND
P1062	41/F	p/M4	Dgn.	PB	117,34,33,13,14,15,71	61	t(6;9)q, add(21), -7, del(8), del(15), +mar	Adverse	ND	NCR
P1063	26/M	p/M5	Rel. a. SCT	PB	117,34,2	86	ND	ND	ND	ND
P1066	60/M	s/M2	Dgn.	PB	117,34,33,65	74	46,XY	Adverse	ND	ND
AML P1067	27/M	p/M4eo	Dgn.	PB	117,34,33,13,15,65	41	inv(16)q,+8	Favorable	ND	CR
P1068	76/M	p/M2	Dgn.	PB	117,34,33,13	39	ND	Intermediate	ND	ND
P1069	33/M	p/M4eo	Dgn.	PB	117,34,33,13,15	61	inv(16)q, t(12;17)q	Favorable	ND	CR
P1071	71/F	s/M1	Dgn.	PB	117,34,33,13,15	60	+4	Intermediate	ND	CR
P1072	58/M	p/M2	Pers.	PB	117,34,33,13	70	-4,-5,-7,-11,-12,-14,-16,-21,+mar	Adverse	ND	NCR
P1073	23/M	p/M5	Dgn.	PB	117,34,33,13,15,7,65	18	+8,+13	Intermediate	ND	ND
P1076	47/F	s/M1	Dgn.	PB	117,34,33,13,14	99	+8	Intermediate	CR	CR
P1077	41/F	p/M1	Dgn.	PB	117,34,33,13,15,7	71	del(12)p	Intermediate	CR	CR
P1078	73/M	p/M2	Dgn.	PB	117,34	84	46,XY	Intermediate	ND	ND
P1083	41/M	p/M2	Dgn.	PB	117,133,33,13,65	43	46,XY	Intermediate	CR	CR
P1084	55/M	p/M4	Dgn.	PB	117,33,13,71	84	46,XY	Intermediate	ND	CR
P1085	71/F	p/M2	Dgn.	PB	117,33,56	96	46,XX	Intermediate	ND	ND
P1106	24/F	p/BII+My	Dgn.	PB	34,19,33,133,13	81	46,XX	High risk	ND	CR
P1107	31/M	s/BII+My	Dgn.	PB	34,19,33,10,24	36	t(9;11)q	Highest risk	ND	CR
P1108	32/M	s/TII	Dgn.	PB	5,2,1a,15,cy3	98	46,XY	Standard	ND	CR
P1109	21/M	p/BII+My	Dgn.	PB	34,19,33,13,10,38	85	46,XY	Standard	ND	CR
P1110	50/F	p/BIII+My	Dgn.	PB	34,133,33	59	t(9;22)q, der(22)	Highest risk	ND	CR
P1111	26/M	s/BII	Rel. a. SCT	PB	34,19,10	64	del(11)q	ND	ND	ND
P1112	37/M	s/BII	Rel. a. SCT	PB	34,19,10	17	ND	ND	ND	ND
P1113	64/F	/B	Rez./pers.	PB	34,19,15	ND	ND	ND	ND	ND
P1114	22/M	p/BIII	Dgn.	PB	19,20,34,38	97	46,XY	Highest risk	ND	CR
ALL P1115	45/M	p/TII	Dgn.	PB	5,7,38,71	99	46,XY	Standard	ND	CR
P1120	20/M	s/TI	Dgn.	PB	19,5,34,20,3	28	46,XY	ND	CR	ND
P1121	25/F	s/BI	Dgn.	PB	34,19, 33	32	ND	ND	ND	ND
P1122	23/M	s/TII	Dgn.	PB	4,8,1,2,5,7	86	46,XY	ND	ND	CR
P1129	11/M	s/BII	Dgn.	BM	34,19,10,22	85	46,XY	ND	ND	CR
P1132	12/M	s/BIII	Dgn.	BM	19,10,22	84	ND	ND	ND	CR
P1133	3/F	s/BII	Dgn.	BM	34,19,10,22	55	46,XX	ND	ND	CR
P1135	17/M	s/TI	Dgn.	BM	34,7,4,5,10,13,33	98	46,XY	ND	ND	CR
P1136	5/F	s/TII	Dgn.	PB	34,7,1a,2,3,5,10	82	46,XX	ND	ND	CR
P1137	3/F	s/BII	Dgn.	BM	34,10,19,22	71	46,XX	ND	ND	CR
P1146	8/F	s/TIV	Dgn.	PB	7,3,1,34	98	46,XX	ND	ND	CR
P1088	44/F	p/B-CLL	Pers.	PB	5,19,20	98	ND	A	ND	Yes
P1089	54/M	p/B-CLL	Pers.	PB	5,19	95	ND	B	ND	Yes
P1090	43/M	p/B-CLL	Dgn.	PB	5,19,kappa	95	add(1)q, del(9)q, del(11)q	A	ND	Yes
P1091	68/M	p/B-CLL	Pers.	PB	5,19,20	96	del(13)q	A	ND	No
P1092	66/M	p/B-CLL	Pers.	PB	5,19,20	71	ND	B	ND	No
P1093	51/F	p/B-CLL	Pers.	PB	5,23,kappa	94	ND	A	ND	No
P1094	67/M	p/B-CLL	Pers.	PB	5,19,kappa	91	ND	A	ND	No
P1095	65/F	p/B-CLL	Pers.	PB	5,19	88	del(13)q	A	ND	Yes
P1096	64/M	p/B-CLL	Pers.	PB	5,19,lambda	95	ND	A	ND	No
P1097	72/F	p/B-CLL	Pers.	PB	5,19,20,22,kappa	97	ND	A	ND	Yes
CLL P1098	60/F	p/B-CLL	Pers.	PB	5,19,kappa	93	ND	B	ND	No
P1099	67/M	p/B-CLL	Dgn.	PB	5,19,20,23,lambda	89	ND	A	ND	No
P1100	36/M	p/B-CLL	Pers.	PB	5,19,lambda	91	del(17)p, der(11)q	B	ND	Yes
P1101	52/M	p/B-CLL	Pers.	PB	5,19,20	96	ND	A	ND	Yes
P1102	45/F	p/B-CLL	Pers.	PB	5,19	91	del(13)q	A	ND	Yes
P1103	67/F	p/B-CLL	Pers.	PB	5,19,20	87	del(17)q	A	ND	Yes
P1104	66/F	p/B-CLL	Pers.	PB	5,19,kappa	94	ND	A	ND	Yes
P1116	66/F	p/B-CLL	Pers.	PB	5,19,kappa	96	t(8;13)q	A	ND	Yes
P1117	33/M	p/B-CLL	Pers.	PB	5,19,23,kappa	40	del(13)q	B	ND	Yes
P1118	60/F	p/B-CLL	Pers.	PB	5,19,20,22,23,kappa	32	del(11)q	C	ND	Yes
P1119	55/F	p/B-CLL	Pers.	PB	5,19,20,22,23,38,kappa	57	ND	B	CR	Yes
Cellines and their origin										
Name	Age /Sex	Subtype	Cell type	Cell Source	Blast phenotype (CD)					
HL-60	35/F	FAB M2	AML	PB	33,13,15					
OCI-AML2	65/M	FAB M4	AML	PB	13,14,15,33,4					
THP-1	1/M	FAB M5	AML	PB	33,13,14,15					
Mono-Mac-6	64/M	FAB M5	AML	PB	13,14,15,33,68					
MOLM-13	20/M	FAB M5a	AML	PB	33,13,15,4					
RAMOS	3/M	B-lymphoblastic	Burkitt lymphoma	Ascitic fluid	19,10,20,37,38,80					
RAJI	11/M	B-lymphoblastic	Burkitt lymphoma	Left maxilla	20,10,13,19,37					
JURKAT	14/M	my ⁺ T-linear	T-ALL	ND	33,13,2,3,4,5,6,7,34					
(B) AML-samples used for culture experiments in hypoxia vs. normoxia										
Pat.#	Age at dgn./sex	Subtype (FAB)	Stage	Cell Source	Blast phenotype	Blasts %	Cytogenetic marker at dgn.			
P1424	37/F	p/M4	Rez.	PB	117,13,33,45	30	46,XX			
P1426	61/F	p/M5	Dgn.	PB	34,117,13,33,64	40	ND			
AML P1430	79/M	p/M5/M6	Dgn.	PB	34,13,33,117	70	46,XY			
P1433	59/M	p/MDS (RAEB-II)	Dgn.	PB	34,13,15	18	ND			
P1434	61/F	s/ND	Dgn.	PB	34,117,64,56,33,13,7	61	46,XX, t(3;8)			
P1439	61/F	s/M5	Dgn.	PB	34,117,13,33	9	inv(16)			

AML/ALL/CLL subtypes, age, sex, stages of the disease, proportions of blasts, blast phenotypes evaluated by flow cytometry are given.

¹Complete remission (CR) achieved or not achieved (NCR) after treatment with SCT.

Bold letters antibodies used for (co)expression analyses.

ALL indicates acute lymphoid leukemia; AML, acute myeloid leukemia; BM, bone marrow; CIK, cytokine-induced killer; CLL, chronic lymphoid leukemia; Dgn., first diagnosis; F, female; FAB, French American British classification; iNKT, invariant natural killer T; M, male; NCCN, National Comprehensive Cancer Network; ND, no data; NK, natural killer; Pat.#, patient's number; PB, peripheral blood; Pers., persisting disease; Rel., relapse; Rel.a.SCT, relapse after stem cell transplantation.

TABLE 3. Overview of the Different DC-generating Methods/Kits (“Cocktails”)^{19,20,28}

“Cocktails”	Composition	Mode of Action	Culture Time (d)
“Picibanil 1” (Pici1)	GM-CSF IL-4 OK-432 PGE ₂	GM-CSF: induction of myeloid (DC) differentiation	7–10
“Picibanil 2” (Pici2)*	GM-CSF IL-4 OK-432 PGE ₁	IL-4: induction of DC-differentiation Picibanil (OK-432): lysis product from <i>Streptococcus pyogenes</i> ; stimulates DC-differentiation	7–10
Kit-D	GM-CSF OK-432 PGE ₂		7–10
Kit-I	GM-CSF	PGE ₂ : increases CCR7-expression and enhances migration	7–10
Kit-K	OK-432 GM-CSF PGE ₂		7–10
Kit-M	GM-CSF PGE ₁	PGE ₁ : effects are comparable with PGE ₂	7–10

*Personal communication D.C. Amberger.

DC indicates dendritic cells; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-4, interleukin 4; OK-432, Picibanil; PGE₁, prostaglandin E₁; PGE₂, prostaglandin E₂.

Kit-I

DC were generated with Kit-I using 800 U/mL GM-CSF and 10 µg/mL Picibanil.²⁰ Incubations were performed in analogy to Kit-D.

Kit-K

Kit-K consisted of 800 U/mL GM-CSF and 1 µg/mL PGE₂.²⁰ Incubations were performed in analogy to Kit-D.

Kit-M

For the generation of DC with Kit-M, 800 U/mL GM-CSF and 1 µg/mL PGE₁ were added to the culture.²⁰ Incubations were performed in analogy to Kit-D.

MLC of T-Cell–Enriched Immune Reactive Cells With “Cocktails” Pretreated or Not Pretreated Stimulator Cell Suspensions From MNC or WB

Immune reactive cells were enriched with 1 × 10⁶ positively selected CD3⁺ T cells (effector cells) from AML patients or healthy controls and cocultured in 24-multiwell tissue culture plates (ThermoFisher Scientific) with a stimulator cell suspension containing 2.5 × 10⁵ DC/DC_{leu} (MLC*^{MNC-DC} or MLC*^{WB-DC}) which were generated with different “cocktails.” The same setting, but with a stimulator cell suspension without pretreatment with “cocktails” (MLC*^{MNC} or MLC*^{WB}) served as a control. In 1 parallel tested case the stimulator cell suspensions were irradiated with 33 Gy to inactivate residual

immune reactive cells (eg, iNKT/NK and CIK cells). The total volume of the cell culture was adjusted to 1 mL with RPMI-1640 medium (Biochrom) containing 1% Penicillin (Biochrom) and 50 U/mL IL-2 (PeproTech). The MLC*^{MNC} or MLC*^{MNC-DC} further contained 15% human serum (Healthcare Europe GmbH, Vienna, Austria). After 2–3 days 50 U/mL IL-2 were added to the WB and MNC cultures. Half medium exchange for MNC cultures was carried out every 2–3 days. Cells were harvested after 6–9 days and were used for the cytotoxicity-fluorolysis assay as described below.

Before and after culture different cell subsets in the MNC-fraction and WB-fraction were quantified by flow cytometry (Table 1).

Enzyme-linked Immunosorbent Assay (ELISA)

Cell culture supernatants after AML-WB-DC culture stimulated with different “cocktails” (“Pici1,” “Pici2,” Kit-D, Kit-I, Kit-K, and Kit-M) and after MLC*^{WB-DC} (n = 4) were analyzed for IL-10, IL-17, Monocyte Chemoattractant Protein-1 (MCP-1) and interferon gamma (IFN-γ) secretion using the human IL-10, IL-17A, MCP-1, and IFN-γ immunoassay kits (DRG Instruments GmbH, Marburg, Germany). Moreover, WB-DC cultures without added response modifiers and MLC*^{WB} served as a control. The samples were evaluated with a Tristar LB941 ELISA reader (Berthold company, Bad Wildbach, Germany) and the concentration of the 4 different cytokines evaluated using the corresponding standard curve.

Cell Characterization by Flow Cytometry

Flow cytometric analyses were carried out to evaluate and quantify amounts, subsets and phenotypes of leukemic cells, B, T, iNKT, NK, and CIK cells, monocytes and DC in the MNC and WB fractions before and after different cultures. Panels with several moAbs labeled with fluorescein isothiocyanat (FITC), phycoerythrin (PE), tandem Cy7-PE conjugation (Cy7-PE), or allophycocyanin (APC) were used. Antibodies were provided by Beckman Coulter, Krefeld, Germany (a); Becton Dickinson, Heidelberg, Germany (b); Biozol, Eching, Germany (c); Caltag, Darmstadt, Germany (d); Bioscience, Heidelberg, Germany (e); Miltenyi Biotech, Bergisch Gladbach, Germany (f); and ThermoFisher Scientific (g). FITC-conjugated moAbs against CD3^a, CD8^b, CD33^a, CD34^a, CD45RO^a, CD83^a, CD161^b, 6B11^c, and Vα24^a were used. We used PE-conjugated moAbs for CD1d^e, CD3^a, CD4^b, CD34^a, CD80^a, CD83^a, CD117^a, CD206^a, and 6B11^b. MoAbs against CD3^a, CD4^a, CD5^a, CD14^b, CD15^b, CD19^a, CD34^a, CD80^b, CD117^a, and CD197^b were labeled with Cy7-PE. As APC-labeled moAbs we used CD1a^a, CD3^a, CD4^{a,b}, CD5^a, CD7^c, CD8^b, CD10^a, CD14^a, CD15^b, CD19^a, CD20^a, CD33^a, CD34^{a,d}, CD45RO^d, CD56^a, CD69^b, CD86^g, CD117^a, CD133^f, CD206^b, and CD209^b. To detect dead cells 7AAD^b was used.

Erythrocytes in WB samples were lysed using Lysing-Buffer (BD, Heidelberg, Germany) according to the manufacturer’s instructions. To stain cells (MNC or WB) with moAbs they were resuspended in PBS (Biochrom), containing 5%–10% fetal calf-serum (Biochrome) to avoid unspecific bindings and were incubated for 15 minutes in the dark at room temperature. Afterwards cells were washed, centrifuged, and resuspended in 100–200 µL PBS. At least 5000 events were evaluated with a fluorescence-activated cell-sorting Flow Cytometer (FACSCalibur) and

Cell-Quest data acquisition and analysis software (Becton Dickson, Heidelberg, Germany). Isotype controls were conducted according to the manufacturer's instructions.

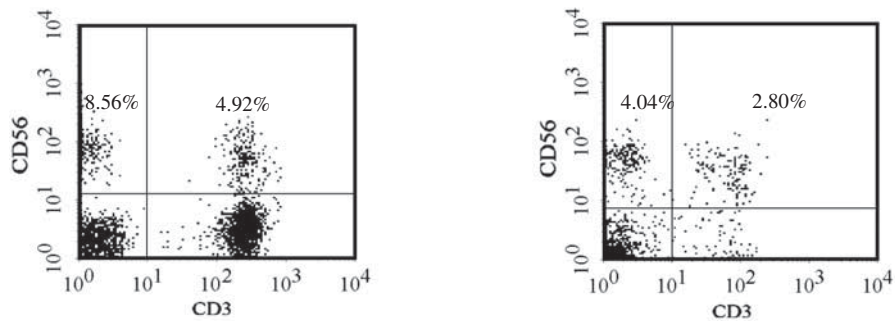
For the analysis and quantification of T, iNKT, NK, and CIK cells/subtypes were quantified in the total cell-fraction (eg, CD3⁺ cells) or in the subpopulations (eg, 6B11⁺CD3⁺). According to their expression profile we quantified proportions of immune reactive cells as given in Table 1: T cells: CD3⁺, CD8⁺, CD4⁺, V α 24⁺, CD1d⁺ T-cells, T_{naive}, T_{non-naive}, T_{cm} or T_{eff-em}.^{9,10,13} CIK cells: CD3⁺CD56⁺ or CD3⁺CD161⁺ cells. NK cells: CD3⁻CD56⁺ or CD3⁻CD161⁺ cells.^{5,13} (6B11⁺) iNKT cells: 6B11⁺CD3⁺, 6B11⁺CD161⁺, 6B11⁺V α 24⁺, 6B11⁺CD4⁺, 6B11⁺CD8⁺, 6B11⁺CD1d⁺, 6B11⁺CD45RO⁺ or 6B11⁺CD45RO⁻ iNKT cells. Exemplarily,

dot plots iNKT, NK, and CIK cells and the gating strategy of iNKT cells/subsets are given in Figure 1. Moreover, aberrant expressions of CD161 and 6B11 on blasts were studied (Table 1).¹³

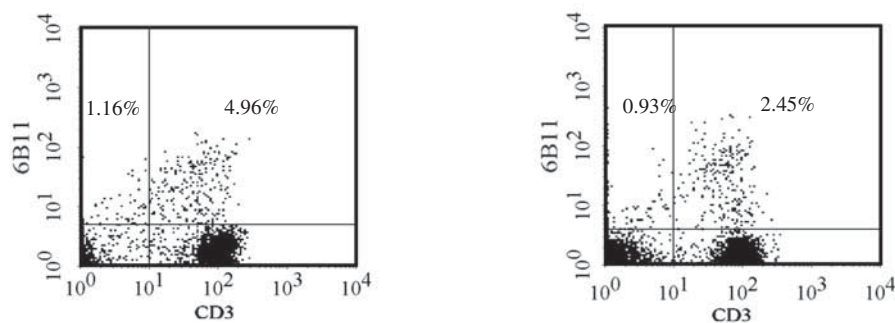
For the analysis and quantification of DC/DC_{leu} subtypes in the total cell fraction or in subtype cell fraction after DC culture in the MNC or WB fraction (DC_{leu}/cells) we used a refined gating strategy^{6,8}: subgroup analyses were conducted only in cases with $\geq 10\%$ DC. DC_{leu} were quantified in the total fraction (DC_{leu}/cells) in the DC fraction (DC_{leu}/DC) or in the blast fraction, to quantify the amount of blasts converted to DC_{leu} (DC_{leu}/bla), mature DC (coexpressing the migration marker CCR7) and viable DC (negative for 7AAD) were quantified in the DC fraction after culture (DC_{mig}/DC; DC_{via}/DC, Table 1,

1.1. Dot plots of NK-, CIK-, iNKT-cells in healthy or AML-MNC

A NK-and CIK-cells in healthy-(left side) or AML-MNC (right side)



B iNKT-cells in healthy-(left side) or AML-MNC (right side)



1.2. Gating of iNKT-subsets in AML-MNC

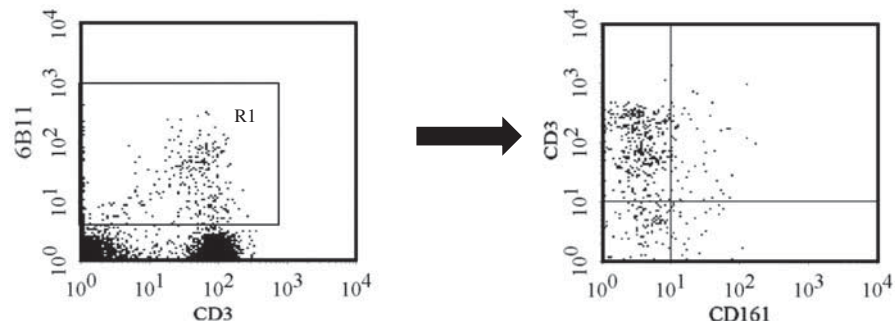


FIGURE 1. Gating strategy and corresponding dot plots of CIK, NK and iNKT cells in healthy or AML MNC are given. 1.1. Various frequencies of NK (CD3⁻CD56⁺) and CIK cells (CD3⁺CD56⁺) in healthy or AML MNC are given (A). Various frequencies of iNKT cells (6B11⁺CD3⁺ and 6B11⁺CD3⁻) in healthy or AML MNC are given (B). 1.2. Gating of 6B11⁺ cells and characterization of corresponding subsets (6B11⁺CD3⁺CD161⁺, 6B11⁺CD3⁻CD161⁺) are given. ALL indicates acute lymphoid leukemia; AML, acute myeloid leukemia; CIK, cytokine-induced killer; MNC, mononuclear cells; NK, natural killer; iNKT, invariant natural killer T. R1 = gate surrounding all 6B11⁺ cells in the AML-MNC fraction.

lower part). For this purpose, cells were stained with patient-specific “blast”-staining antibodies (eg, CD15, CD34, CD65, and CD117) according to diagnostic reports before culture in combination with “DC”-staining antibodies (eg, CD80, CD83, CD86, CD206, and CD209), which were not expressed before culture.

Cytotoxicity (Fluorolysis) Assay

To analyze the blast lytic activity of T-cell-enriched immune reactive cells after MLC with “cocktails”-pretreated or not pretreated stimulator cell suspensions from MNC or WB a fluorolysis assay was performed.¹⁹ Therefore, effector cells (E) were cocultured 1:1 with thawed blast-containing target cells (T) for 3 and 24 hours at 37°C, with 21% O₂ and 5% CO₂. As a control effector cells and target cells were cultured for the same time separately and mingled on ice shortly before the flow cytometric analyses were carried out. Before culture, target cells were stained for 15 minutes with FITC, PE or APC-conjugated blast, monocyte or T cell-specific target cell antibodies. To evaluate viable cells and the lytic activity of effector cells, the cultures were harvested after 3 and 24 hours and resuspended in PBS containing 7AAD (Becton Dickson) and a defined number of Fluorospheres beads (Beckman Coulter). For analyses, a refined gating was used.⁸ Therefore, viable target cells were gated in a FSC/7AAD-gate. With a FACS Calibur Flow Cytometer and a Cell-Quest software (Becton Dickson) cells were analyzed. The lytic activity was calculated and defined as the difference in the percentage of viable target blasts before and after the effector cell contact.

Statistical Methods

Data were presented as mean ± SD. Statistic comparisons for 2 groups were performed using the *t* test and Mann-Whitney-Wilcoxon test. The statistical analysis was done with Microsoft Excel 2010 or 2013 (Microsoft, Redmond, WA) and JMP10.0 statistical software (SAS Institute, Cary, NY). Differences were considered as “not significant” (ns) with *P*-values >0.1, as “borderline significant” (significant*) with *P*-values between 0.1 and 0.05, as “significant” (significant**) with *P*-values between 0.05 and 0.005 and as “highly significant” (significant***) with *P*-values <0.005.

RESULTS

Prolog

During the development of acute and chronic leukemia B, T lymphocytes and monocytes are displaced by increasing blast cell-populations. In our samples between 8% and 99% of blast cells and varying proportions of the remaining hematopoietic cells were detectable. Details of the cellular composition of AML/ALL/CLL samples are shown in Table 4. Samples with blasts, that aberrantly expressed CD3, CD4, CD8, CD14 or CD19, were excluded from quantification analyses for the respective lineage markers.

In our first approach, we compared frequencies and compositions of iNKT, NK, CIK cells as well as T cell subtypes in (unstimulated) MNC from healthy donors with AML, ALL and CLL patients and correlated findings with prognosis of these patients. Moreover, we studied potential (aberrant) coexpressions of iNKT markers on leukemic cell lines. In our second approach we analyzed proportions of AML blasts and immune reactive cells (with a special focus on iNKT, NK, and CIK cells) and their expression profiles after MLC.

TABLE 4. Cellular Composition of AML/ALL/CLL and Healthy Samples

	Cell Type	Ø (MNC) (%)	Range (MNC) (%)
4.1. AML/ALL/CLL and healthy samples used for surface marker expression analyses			
AML	Blasts (myeloid blasts)	69	18–99
	T cells (CD3 ⁺)	6	1–33
	B cells (CD19 ⁺)	6	1–33
	Monocytes (CD14 ⁺)	8	2–18
	Blasts (B or T lineage blasts)	71	17–99
ALL	T cells (CD3 ⁺)*	14	5–33
	B cells (CD19 ⁺)†	8	3–11
	Monocytes (CD14 ⁺)	5	1–29
	Blasts (B lineage blasts)	85	32–98
CLL	T cells (CD3 ⁺)	11	1–60
	Monocytes (CD14 ⁺)	4	1–25
	T cells (CD3 ⁺)	41	24–64
Healthy	B cells (CD19 ⁺)	24	14–32
	Monocytes (CD14 ⁺)	3	1–6
	Cell type	Ø (WB/MNC) (%)	Range (WB/MNC) (%)
4.2. AML and healthy samples used for culture experiments			
AML	Blasts	28/17	8–60/15–18
	T cells (CD3 ⁺)	21/11	2–54/5–17
	B cells (CD19 ⁺)	1/5	0–2/2–8
	Monocytes (CD14 ⁺)	11/7	0–32/3–13
	T cells (CD3 ⁺)	18/35	14–21/9–48
Healthy	B cells (CD19 ⁺)	3/7	1–4/3–12
	Monocytes (CD14 ⁺)	6/7	5–8/3–12

*Only quantified in B lineage ALL.

†Only quantified in T lineage ALL.

ALL indicates acute lymphoid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphoid leukemia; MNC, mononuclear cells; WB, whole blood; Ø, mean.

The 6B11-antibody, targeting the invariant CDR3 loop of the Vα24Jα18 TCR, is regarded as a specific antibody to detect iNKT cells. In our experiments, we used 2 different antibodies: 6B11 (clone 6B11) labeled with PE (6B11-PE, delivered by BD) and 6B11 (clone 6B11) labeled with FITC (6B11-FITC, delivered by Biozol). First comparative analyses with these markers in MNC from AML, ALL, CLL patients revealed significantly* higher proportions of iNKT cells detected with the 6B11-FITC compared with 6B11-PE (AML: 1.52% ± 1.74% vs. 0.75% ± 1.10%, *P* < 0.0652; ALL: 1.24% ± 1.52% vs. 0.74% ± 0.64%, *P* < 0.103; CLL: 0.92% ± 0.87% vs. 0.78% ± 0.47%, *P* < 0.299). However, proportions of iNKT cells detected with 6B11-FITC were significantly** lower in healthy samples compared with leukemic samples (AML: 0.40% ± 0.26% vs. 1.52% ± 1.74%, *P* < 0.01073; ALL: 0.40% ± 0.26% vs. 1.24% ± 1.52%, *P* < 0.01622; CLL: 0.40% ± 0.26% vs. 0.92% ± 0.87%, *P* < 0.02267).

while iNKT cells detected with 6B11-PE were significantly* higher in healthy samples compared with leukemic samples (AML: $2.47\% \pm 3.12\%$ vs. $0.75\% \pm 1.10\%$, $P < 0.081$; ALL: $2.47\% \pm 3.12\%$ vs. $0.74\% \pm 0.65\%$, $P < 0.083$; CLL: $2.47\% \pm 3.12\%$ vs. $0.78\% \pm 0.47\%$, $P < 0.0827$). We decided for better comparability to present only data obtained with 6B11-PE.

We evaluated 8 different leukemic cell lines (HL-60, OCI-AML2, THP-1, Mono-Mac-6, MOLM-13, RAMOS, RAJI, JURKAT) to analyze if 6B11 or CD161 is aberrantly expressed on blasts. We could demonstrate that neither CD161 nor 6B11 were (aberrantly) expressed on blasts of leukemic myeloid and B lineage cell lines with a mean coexpression on blasts of $2.59\% \pm 0.02\%$ or $2.67\% \pm 0.03\%$. However, $8.38\% 6B11^+ bla^+$ and $10.03\% CD161^+ bla^+$ cells were found in the T-linear Jurkat cell line. Moreover, we could demonstrate that neither CD161 nor 6B11 were aberrantly expressed on leukemic blasts obtained from patients with AML, ALL or CLL. On average, coexpression of CD161 and 6B11 on blasts was: AML: $0.23\% \pm 0.01\%$ or $0.35\% \pm 0.01\%$; ALL: $0.55\% \pm 0.01\%$ or $0.66\% \pm 0.01\%$; CLL: $0.17\% \pm 0.00\%$ or $1.60\% \pm 0.01\%$.

Therefore, analyzing iNKT cells with 6B11-PE and NK/CIK cells with CD161 represent specific results as 6B11 and CD161 antibodies do not show unpecific bindings on blasts, although combinations of (fluorochrome)-labeled antibodies have to be tested thoroughly.

moAbs and their Combinations for iNKT, NK, and CIK Cell/Subtype Analyses

For iNKT cell detection in healthy and AML, ALL, and CLL samples, we used moAb 6B11 alone or in combination with CD3, CD161, V α 24, CD1d, CD45RO, CD4, and CD8. We defined iNKT cells detected with 6B11 in combination with T cell markers (CD3, V α 24, CD1d, CD45RO, CD4, and CD8) as “T cell-like” iNKT cells and iNKT cells detected with 6B11 in combination with NK cell markers (CD161) as “NK cell-like” iNKT cells. The frequencies of iNKT cells detected with 6B11 alone were similar in AML, ALL, and CLL patients ($0.75\% \pm 1.10\%$ vs. $0.74\% \pm 0.65\%$ vs. $0.78\% \pm 0.47\%$). Frequencies of iNKT cells detected with 6B11 in combination with CD3 ($6B11^+ CD3^+ /MNC$) were slightly lower in AML, ALL, and CLL patients ($0.58\% \pm 1.15\%$ vs. $0.61\% \pm 0.42\%$ vs. $0.46\% \pm 0.47\%$), but the percentages were comparable to results with 6B11 in combination with CD1d ($6B11^+ CD1d^+ /MNC$, $0.66\% \pm 1.16\%$ vs. $0.66\% \pm 0.96\%$ vs. $0.48\% \pm 0.64\%$). Proportions of $6B11^+ CD161^+ /MNC$ iNKT cells were even smaller in AML, ALL, and CLL patients ($0.14\% \pm 0.25\%$ vs. $0.18\% \pm 0.16\%$ vs. $0.22\% \pm 0.26\%$), while $6B11^+ V\alpha 24^+ /MNC$ could only detect very few frequencies of iNKT cells ($0.09\% \pm 0.10\%$ vs. $0.08\% \pm 0.18\%$ vs. $0.03\% \pm 0.03\%$). We could show higher frequencies of $6B11^+ CD4^+ /MNC$ versus $6B11^+ CD8^+ /MNC$ iNKT cells detected in AML, ALL, and CLL patients ($CD4^+ iNKT$ cells: $0.18\% \pm 0.20\%$ vs. $0.32\% \pm 0.33\%$ vs. $0.25\% \pm 0.12\%$; $CD8^+ iNKT$ cells: $0.03\% \pm 0.04\%$ vs. $0.07\% \pm 0.09\%$ vs. $0.06\% \pm 0.05\%$). Moreover, we could show that the majority of $6B11^+ iNKT$ cells express CD45RO in AML, ALL, and CLL patients ($88.58\% \pm 14.43\%$ vs. $79.75\% \pm 30.00\%$ vs. $64.58\% \pm 29.53\%$).

In conclusion, frequencies of iNKT cells and their subsets can be detected with 6B11 alone (PE-labeled clone preferred) or in combination with CD3, CD161, CD1d or V α 24. The combination of 6B11 with CD4, CD8, and CD45RO can be used for further subset-analyses of iNKT cells.

Frequencies and Compositions of iNKT, NK, and CIK Cells From AML Patients Compared With Healthy Controls and their Correlation With Prognosis

AML Patients Show Significantly Lower Proportions of iNKT, NK, and CIK Cells in MNC Compared With Healthy MNC

We found significantly*** lower frequencies of $CD3^+ CD56^+$ CIK cells in MNC of AML patients compared with healthy MNC ($0.46\% \pm 0.62\%$ vs. $2.74\% \pm 1.64\%$, $P < 0.0021$) and significantly*** lower percentages of $CD3^+ CD161^+$ CIK cells ($0.30\% \pm 0.40\%$ vs. $3.25\% \pm 1.02\%$, $P < 0.000152$, Fig. 2A). In addition, we could show significantly*** lower proportions of $CD3^- CD56^+$ NK cells ($1.68\% \pm 1.97\%$ vs. $9.33\% \pm 4.06\%$, $P < 0.00064$) and significantly** lower proportions of $CD3^- CD161^+$ NK cells in MNC of AML patients compared with healthy MNC ($1.01\% \pm 1.26\%$ vs. $2.44\% \pm 1.20\%$, $P < 0.0142$, Fig. 2B). We could demonstrate significantly* lower percentages of $6B11^+ iNKT$ cells ($0.75\% \pm 1.10\%$ vs. $2.47\% \pm 3.12\%$, $P < 0.081$) and (ns) lower proportions of $6B11^+ CD3^+$ and $6B11^+ CD161^+ iNKT$ cells in MNC of AML patients compared with healthy MNC (Fig. 2C). Moreover, $6B11^+ CD8^+$ and $6B11^+ V\alpha 24^+ iNKT$ cells were found in lower proportions in MNC from AML patients versus healthy MNC [data not shown (dns)].

In summary frequencies of $6B11^+ /6B11^+ CD3^+ /6B11^+ CD161^+ /6B11^+ CD8^+ /6B11^+ V\alpha 24^+ iNKT$, $CD3^- CD56^+ /CD3^- CD161^+ NK$, and $CD3^+ CD56^+ /CD3^+ CD161^+ CIK$ cells/subsets were (significantly) lower in MNC from AML patients than in healthy MNC.

(Significantly) Higher Proportions of T and NK Cells Express 6B11 in AML Patients Compared With Healthy Controls

We found significantly*** increased proportions of $CD3^+$ T cells expressing 6B11 in AML patients compared with healthy controls ($14.03\% \pm 14.66\%$ vs. $2.69\% \pm 2.17\%$, $P < 0.00111$, Fig. 2D). Moreover, we found significantly** higher frequencies of $CD4^+$ ($8.36\% \pm 15.22\%$ vs. $0.31\% \pm 0.38\%$, $P < 0.0123$) and $CD161^+$ cells expressing 6B11 ($17.19\% \pm 15.36\%$ vs. $4.62\% \pm 5.97\%$, $P < 0.0152$, Fig. 2D) and significantly* increased frequencies of $CD8^+$ cells expressing 6B11 in MNC of AML patients versus healthy controls ($1.89\% \pm 3.55\%$ vs. $0.59\% \pm 1.12\%$, $P < 0.0723$). We did not find significant differences of $CD3^+ CD161^+ CIK$ cells in the $CD3^+$ T cell fraction of AML patients compared with $CD3^+ CD161^+ CIK$ cells in the healthy T cell fraction (dns).

In summary a shift to higher proportions of ($CD3^+ /CD4^+ /CD8^+$) T cells and ($CD161^+$) NK cells coexpressing 6B11 was found in AML patients compared with healthy samples. No differences of $CD3^+ CD161^+ CIK$ cells in the $CD3^+$ T-cell fraction were observed.

AML Patients With Prognostically Favorable Subgroups are Characterized by Higher Proportions of iNKT, NK, and CIK Cells

AML patients who achieved CR after induction chemotherapy were characterized by significantly** higher frequencies of $CD3^+ CD56^+$ CIK cells in MNC-fractions than AML patients with no complete remission (NCR; $0.4\% \pm 0.33\%$ vs. $0.05\% \pm 0.05\%$, $P < 0.0094$, Fig. 5.1A, left side), whereas proportions of NK or iNKT cells were not different in MNC fractions of patients who achieved versus not achieved a CR (dns). AML-patients with

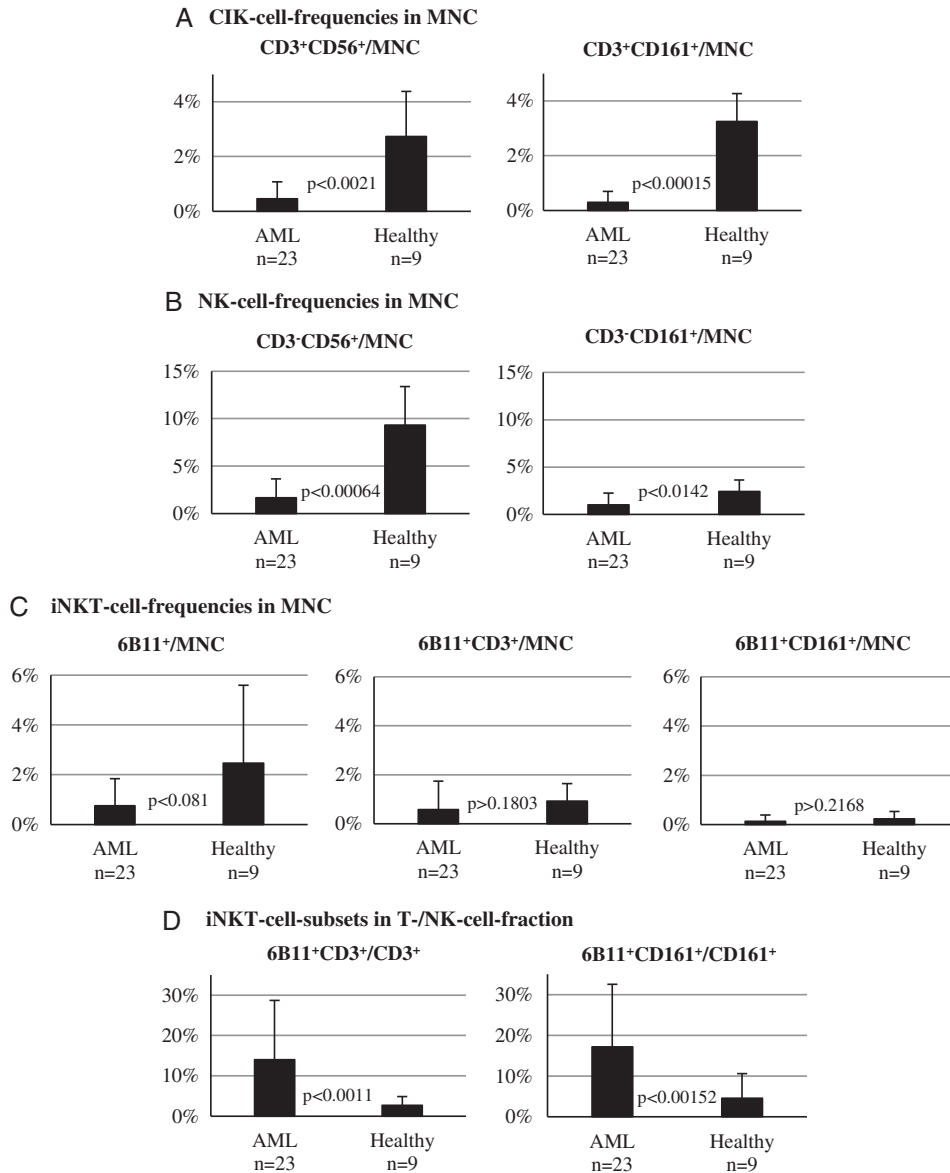


FIGURE 2. Frequencies of CIK, NK and iNKT cells and their subsets in healthy versus AML MNC are given. Percentages of CIK cells (CD3⁺CD56⁺/MNC and CD3⁺CD161⁺/MNC) (A), NK cells (CD3⁻CD56⁺/MNC and CD3⁻CD161⁺/MNC) (B), iNKT cells in MNC (6B11⁺, 6B11⁺CD3⁺/MNC, 6B11⁺CD161⁺/MNC) (C), and iNKT cell subsets in the T cell fraction (6B11⁺CD3⁺/CD3⁺) and in the NK cell fraction (6B11⁺CD161⁺/CD161⁺) (D) are given. ALL indicates acute lymphoid leukemia; AML, acute myeloid leukemia; CIK, cytokine-induced killer; MNC, mononuclear cells; NK, natural killer; iNKT, invariant natural killer T.

favorable versus adverse NCCN risk-score presented with (ns) higher values of 6B11⁺-iNKT cells in the MNC-fraction (0.64% ± 0.11% vs. 0.56% ± 0.09%; Fig. 5.1A, middle row). Comparable results were found for 6B11⁺ Va24⁺ and 6B11⁺CD161⁺ iNKT cell-proportions in the MNC-fraction of AML-patients with favorable versus adverse NCCN risk-score (dns).

AML-patients younger than 60 years presented with (ns) higher frequencies of 6B11⁺ iNKT cells in the MNC-fraction compared with AML-patients older than 60 years (0.89% ± 1.35% vs. 0.53% ± 0.40%, Fig. 5.1A, right side). Comparable results were found for 6B11⁺CD3⁺ and 6B11⁺CD161⁺ iNKT cell proportions in the MNC-fraction

of AML-patients younger than 60 years versus older than 60 years (dns). No differences were found for proportions of NK or CIK cells in the groups compared (dns).

AML-patients with primary versus secondary AML presented with (ns) higher frequencies of 6B11⁺ and 6B11⁺CD3⁺ iNKT cells in the MNC-fraction (0.78% ± 1.21% vs. 0.62% ± 0.46%; 0.66% ± 1.29% vs. 0.32% ± 0.23%). No differences were found for proportions of NK or CIK cells in the groups compared (dns).

AML-patients without extramedullary foci (n = 20) compared with those with extramedullary foci (n = 3) presented with significantly*** increased proportions of CD3⁺CD56⁺ CIK cells in the MNC-fraction (0.53% ± 0.63% vs. 0.00% ± 0%,

$P < 0.0023$; Fig. 5.1B left side). Moreover, the percentages of $CD3^-CD56^+$ NK cells was significantly*** higher in cases without compared with patients with extramedullary foci ($1.97\% \pm 0.21\%$ vs. $0.08\% \pm 0.04\%$, $P < 0.0017$, Fig. 5.1B, middle row) Furthermore we found (ns) higher frequencies of $6B11^+$ iNKT cells in the MNC fraction of ALL-patients without versus with extramedullary foci ($0.81\% \pm 1.16\%$ vs. $0.37\% \pm 0.24\%$; Fig. 5.1B right side). Comparable results were found for $6B11^+CD3^+$ and $6B11^+CD161^+$ iNKT cell-proportions in the MNC-fraction in the groups compared (dns).

AML-patients who stayed in stable CR versus no stable CR (NCR) showed (ns) higher frequencies of $CD3^+CD161^+$ CIK cells and $CD3^-CD161^+$ NK cells in the MNC-fraction ($0.47\% \pm 0.58\%$ vs. $0.16\% \pm 0.08\%$; $1.21\% \pm 1.73\%$ vs. $0.90\% \pm 0.72\%$; Fig. 5.1C left side, middle row). Moreover, we found (ns) higher percentages of $6B11^+$ iNKT cells in the MNC-fraction of ALL-patients who stayed in stable CR versus NCR ($1.04\% \pm 1.63\%$ vs. $0.37\% \pm 0.10\%$; Fig. 5.1C, right side). Comparable results were found for $6B11^+CD3^+$ and $6B11^+CD161^+$ iNKT cell proportions in the MNC fraction in the groups compared (dns).

In summary in ALL-patients (significantly) higher proportions of $6B11^+$ iNKT cells correlated with favorable NCCN risk-score, younger age, primary ALL, no extramedullary foci and stable CR. Similar correlations were found for $6B11^+CD3^+/6B11^+CD161^+$ and $6B11^+V\alpha24^+$ iNKT-subsets. High frequencies of $CD3^-CD56^+$ NK cells and $CD3^+CD56^+$ CIK cells NK and CIK cells correlated significantly with no extramedullary foci. Moreover, higher proportions of $CD3^-CD161^+$ NK cells and $CD3^+CD161^+$ CIK cells correlated with stable CR in ALL-patients and $CD3^+CD56^+$ CIK cells correlated with the achievement of CR after induction chemotherapy in ALL-patients.

Frequencies and Subtypes of iNKT, NK, and CIK Cells From ALL-Patients Compared With Healthy Controls and their Correlation With Prognosis

ALL-Patients Show Significantly Lower Proportions of iNKT, NK, and CIK Cells in MNC Compared With Healthy MNC

We found significantly** lower frequencies of $CD3^+CD56^+$ CIK cells in MNC of ALL-patients compared with healthy MNC ($1.36\% \pm 1.63\%$ vs. $2.74\% \pm 1.64\%$, $P < 0.0395$) and significantly*** lower percentages of $CD3^+CD161^+$ CIK cells ($0.94\% \pm 1.45\%$ vs. $3.25\% \pm 1.02\%$, $P < 0.00039$, Fig. 3A). Moreover, we found significantly*** lower proportions of $CD3^-CD56^+$ NK cells ($2.68\% \pm 2.35\%$ vs. $9.33\% \pm 4.06\%$, $P < 0.00163$) and significantly*** lower proportions of $CD3^-CD161^+$ NK cells in MNC of ALL-patients versus healthy MNC ($0.52\% \pm 0.92\%$ vs. $2.44\% \pm 1.20\%$, $P < 0.00344$, Fig. 3B). We could show significantly* lower frequencies of $6B11^+$ iNKT cells in MNC of ALL-patients versus healthy MNC ($0.74\% \pm 0.65\%$ vs. $2.47\% \pm 3.12\%$, $P < 0.083$, Fig. 3C). Moreover, $6B11^+CD3^+/6B11^+CD8^+/6B11^+CD161^+$ and $6B11^+V\alpha24^+$ iNKT cells were found in (ns) lower proportions in MNC from ALL-patients versus healthy MNC (dns).

In summary frequencies of $6B11^+/6B11^+CD3^+/6B11^+CD161^+/6B11^+CD8^+/6B11^+V\alpha24^+$ iNKT, $CD3^-CD56^+/CD3^-CD161^+$ NK, and $CD3^+CD56^+/CD3^+CD161^+$ CIK cells/subsets were (significantly) lower in ALL-MNC than in healthy MNC.

(Significantly) Higher Proportions of T and NK Cells Express 6B11 in ALL-Patients Compared With Healthy Controls

We found significantly* lower percentages of $CD3^+CD161^+$ CIK cells in the $CD3^+$ T-cell fraction of ALL-patients compared with $CD3^+CD161^+$ CIK cells in the healthy $CD3^+$ T-cell fraction ($5.25\% \pm 7.17\%$ vs. $8.92\% \pm 2.82\%$, $P < 0.053$), but significantly** higher proportions of $CD3^+$ T cells expressing 6B11 in ALL-patients compared with healthy controls ($6.03\% \pm 5.25\%$ vs. $2.69\% \pm 2.17\%$, $P < 0.0102$, Fig. 3D). Furthermore, we found significantly** higher frequencies of $CD4^+$ ($7.27\% \pm 11.04\%$ vs. $0.31\% \pm 0.38\%$, $P < 0.00745$) and $CD161^+$ cells expressing 6B11 ($15.63\% \pm 16.46\%$ vs. $4.62\% \pm 5.97\%$, $P < 0.0057$, Fig. 3D) and significantly* higher percentages of $CD8^+$ cells expressing 6B11 in ALL patients versus healthy controls ($1.28\% \pm 1.44\%$ vs. $0.59\% \pm 1.12\%$, $P < 0.081$).

In summary a shift to higher proportions of ($CD3^+/CD4^+/CD8^+$) T cells and ($CD161^+$) NK cells coexpressing 6B11 was found in ALL-patients compared with healthy samples. However, unlike in ALL-patients, lower frequencies of $CD3^+CD161^+$ CIK cells were found in the $CD3^+$ T-cell fraction of ALL-patients versus in the healthy $CD3^+$ T-cell fraction.

Adult ALL-Patients With Prognostically Favorable Subgroups are Characterized by Higher Proportions of iNKT and NK Cells

The ALL-patients' cohort included children and adults. As only 1 child relapsed only adult ALL-patients (treated with GMALL-therapy) were included in prognostic analyses.

Adult ALL-patients who achieved a CR after GMALL-induction chemotherapy were characterized by (ns) higher frequencies of $CD3^-CD161^+$ NK cells in MNC fractions than ALL-patients who achieved without CR ($1.09\% \pm 1.48\%$ vs. $0.18\% \pm 0.11\%$, dns). Moreover, we found (ns) higher percentages of $6B11^+$, $6B11^+CD3^+$, and $6B11^+V\alpha24^+$ iNKT cells in the MNC-fraction of ALL-patients who achieved CR versus NCR ($0.95\% \pm 1.02\%$ vs. $0.65\% \pm 0.37\%$; $0.61\% \pm 0.34\%$ vs. $0.58\% \pm 0.26\%$; $0.16\% \pm 0.30\%$ vs. $0.03\% \pm 0.05\%$, dns). No differences were found for CIK cells.

Adult ALL-patients without extramedullary foci compared with those with extramedullary foci presented with (ns) higher proportions of $6B11^+$, $6B11^+CD3^+$, and $6B11^+V\alpha24^+$ iNKT cells in the MNC fraction ($1.12\% \pm 1.07\%$ vs. $0.54\% \pm 0.30\%$; $0.63\% \pm 0.38\%$ vs. $0.56\% \pm 0.21\%$; $0.20 \pm 0.32\%$ vs. $0.02\% \pm 0.02\%$, dns). No differences were found for NK and CIK cells.

Although only few data were available we can demonstrate, that higher proportions of $6B11^+/6B11^+CD3^+/6B11^+V\alpha24^+$ iNKT cells correlate with adult ALL-patients who achieved CR and without extramedullary foci. Moreover, $CD3^-CD161^+$ NK cells correlated with adult ALL-patients who achieved CR. no correlations were found for CIK cells.

Frequencies and Subtypes of iNKT, NK, and CIK Cells From CLL Patients Compared With Healthy Controls and Correlations With Prognosis

CLL-Patients Show Significantly Lower Proportions of iNKT, NK, and CIK Cells in MNC Compared With Healthy MNC

We found significantly*** lower frequencies of $CD3^+CD56^+$ CIK cells in MNC of CLL-patients compared with healthy MNC ($0.67\% \pm 0.73\%$ vs. $2.74\% \pm 1.64\%$,

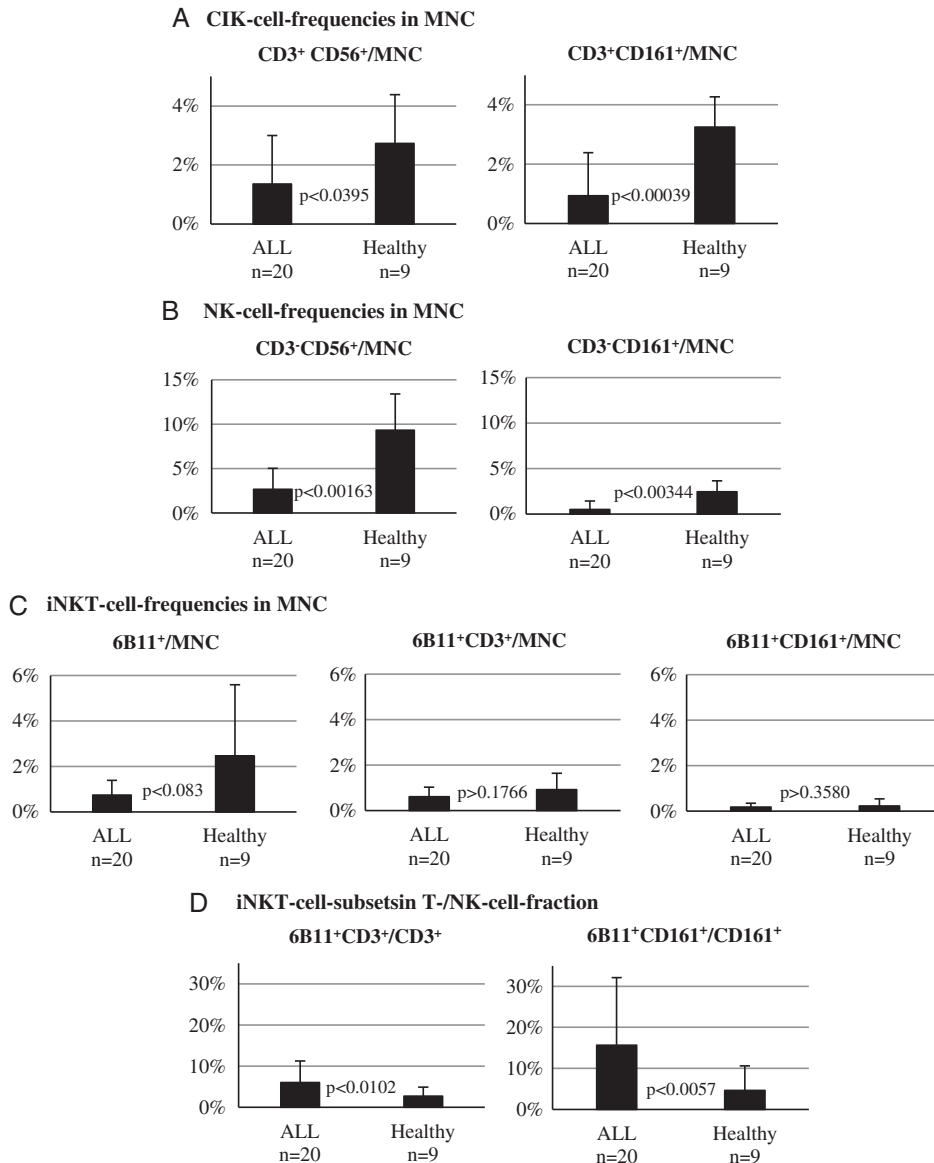


FIGURE 3. Frequencies of CIK, NK and iNKT cells and their subsets in healthy versus ALL MNC are given. Percentages of CIK cells (CD3⁺CD56⁺/MNC and CD3⁺CD161⁺/MNC) (A), NK cells (CD3⁻CD56⁺/MNC and CD3⁻CD161⁺/MNC) (B), iNKT cells in MNC (6B11⁺, 6B11⁺CD3⁺/MNC, 6B11⁺CD161⁺/MNC) (C), and iNKT cell subsets in the T cell fraction (6B11⁺CD3⁺/CD3⁺) and in the NK cell fraction (6B11⁺CD161⁺/CD161⁺) (D) are given. ALL indicates acute lymphoid leukemia; CIK, cytokine-induced killer; MNC, mononuclear cells; NK, natural killer; iNKT, invariant natural killer T.

$P < 0.0036$) and significantly*** lower frequencies of CD3⁺CD161⁺ CIK cells ($0.67\% \pm 0.84\%$ vs. $3.25\% \pm 1.02\%$, $P < 0.000168$, Fig. 4A). Moreover, we found significantly*** lower proportions of CD3⁻CD56⁺ NK cells ($3.27\% \pm 3.26\%$ vs. $9.33\% \pm 4.06\%$, $P < 0.00243$) and significantly*** lower proportions of CD3⁻CD161⁺ NK cells in MNC of CLL-patients versus healthy MNC ($0.45\% \pm 0.48\%$ vs. $2.44\% \pm 1.20\%$, $P < 0.00302$, Fig. 4B). Moreover, we found significant* lower frequencies of 6B11⁺ ($0.78\% \pm 0.47\%$ vs. $2.47\% \pm 3.12\%$, $P < 0.0828$), 6B11⁺CD3⁺ ($0.46\% \pm 0.47\%$ vs. $0.93\% \pm 0.72\%$, $P < 0.072$, Fig. 4C) and 6B11⁺V α 24⁺ iNKT cells in MNC of CLL patients versus healthy MNC ($0.03\% \pm 0.03\%$ vs. $0.16\% \pm 0.27\%$, $P < 0.09522$). Moreover, lower proportions of

6B11⁺CD8⁺ and 6B11⁺CD161⁺ iNKT cells were found in MNC from CLL patients versus healthy MNC (dns).

In summary frequencies of 6B11⁺/6B11⁺CD3⁺/6B11⁺CD161⁺/6B11⁺CD8⁺/6B11⁺V α 24⁺ iNKT, CD3⁻CD56⁺/CD3⁻CD161⁺ NK and CD3⁺CD56⁺/CD3⁺CD161⁺ CIK cells/subsets were (significantly) lower in CLL MNC than in healthy MNC.

Significantly Higher Proportions of T and NK Cells Express 6B11 in CLL-Patients Compared With Healthy Controls

We found significantly*** higher frequencies of CD3⁺ T cells expressing 6B11 in CLL-patients compared with

healthy controls ($11.27\% \pm 11.83\%$ vs. $2.69\% \pm 2.17\%$, $P < 0.00384$, Fig. 4D). Furthermore, we found significantly** higher frequencies of for $CD4^+$ cells expressing 6B11 ($7.14\% \pm 5.90\%$ vs. $0.31\% \pm 0.38\%$, $P < 0.00908$) and significantly*** higher frequencies of $CD161^+$ cells expressing 6B11 in CLL-patients versus healthy controls ($23.55\% \pm 20.47\%$ vs. $4.62\% \pm 5.97\%$, $P < 0.00043$, Fig. 4D). We did not find significant differences in the numbers of $CD3^+CD161^+$ CIK cells in the $CD3^+$ T cell fraction and $CD8^+$ T cells expressing 6B11 (dns).

In summary a shift to higher proportions of ($CD3^+ / CD4^+$) T cells and ($CD161^+$) NK cells coexpressing 6B11 was found in CLL-patients compared with healthy samples. Like in AML patients, but unlike in ALL patients, no differences were found for CIK cells in the $CD3^+$ T-cell

fraction. Unlike AML and ALL patients no differences were found for $CD8^+$ T cells expressing 6B11.

CLL-Patients With Prognostically Favorable Subgroups are Characterized by Higher Proportions of iNKT, NK, and CIK Cells

CLL-patients with no need versus need for therapy presented with significantly* higher proportions of $CD3^+CD56^+$ CIK cells in the MNC fraction ($1.07\% \pm 1.03\%$ vs. $0.43\% \pm 0.28\%$, $P < 0.093$; Fig. 5.2A, left side). We found (ns) higher frequencies of $6B11^+$ iNKT cells in the MNC fraction of CLL patients with no need versus need for therapy ($0.81\% \pm 0.50\%$ vs. $0.73\% \pm 0.40\%$; Fig. 5.2A, middle row). Comparable results were found for $6B11^+CD3^+$ and $6B11^+V\alpha24^+$ iNKT cell proportions in the MNC fraction

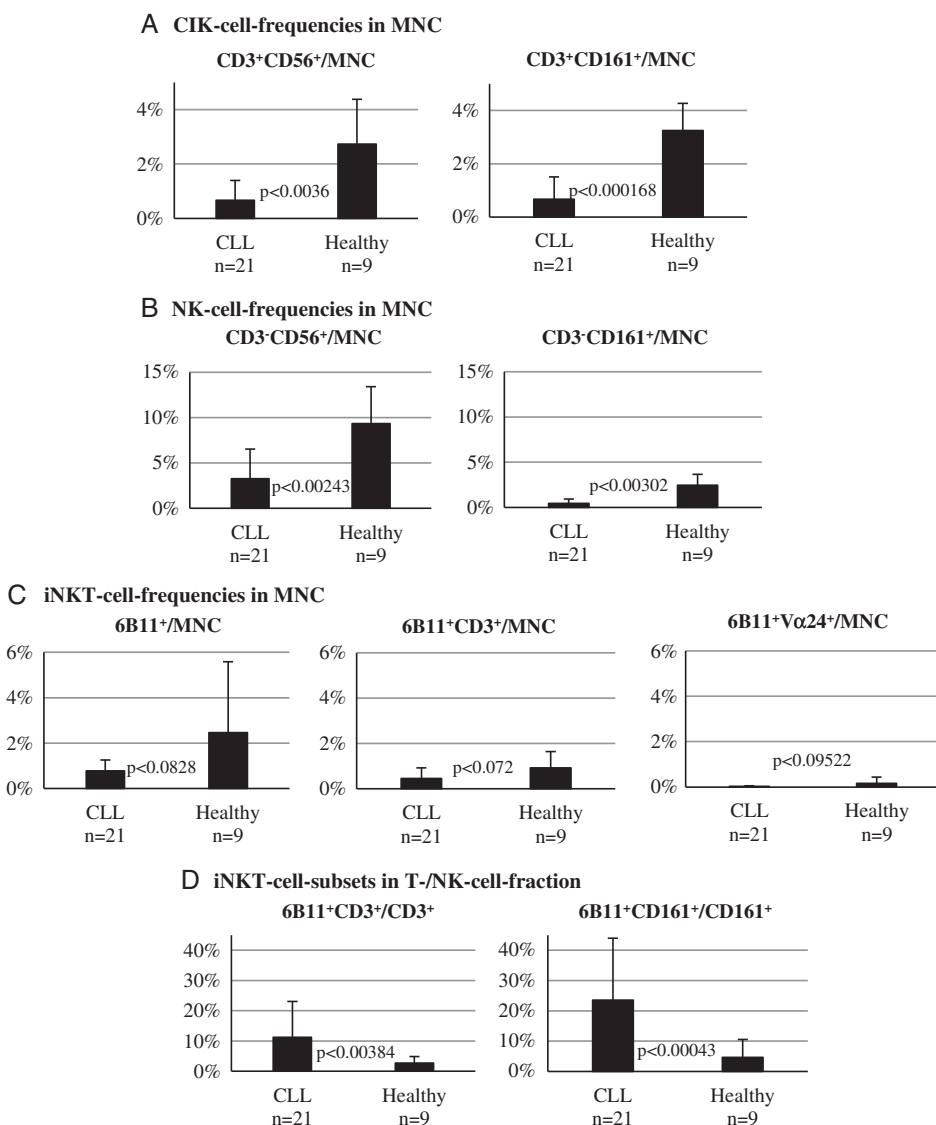


FIGURE 4. Frequencies of CIK, NK, and iNKT cells and their subsets in healthy versus CLL MNC are given. Percentages of CIK cells ($CD3^+CD56^+/MNC$ and $CD3^+CD161^+/MNC$) (A), NK cells ($CD3^-CD56^+/MNC$ and $CD3^-CD161^+/MNC$) (B), iNKT cells in MNC ($6B11^+$, $6B11^+CD3^+/MNC$, $6B11^+V\alpha24^+/MNC$) (C), and iNKT cell subsets in the T-cell fraction ($6B11^+CD3^+/CD3^+$) and in the NK cell fraction ($6B11^+CD161^+/CD161^+$) (D) are given. CIK indicates cytokine-induced killer; CLL, chronic lymphoid leukemia; MNC, mononuclear cells; NK, natural killer; iNKT, invariant natural killer T.

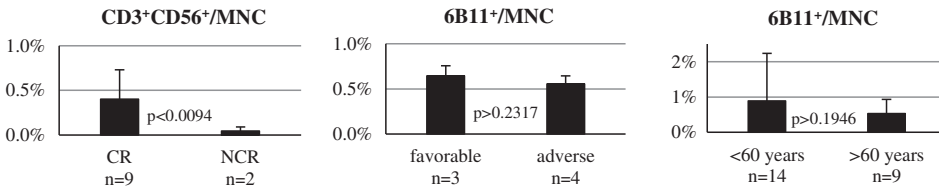
of CLL patients with no need versus need for therapy (dns). No differences were found for NK cells.

CLL-patients younger versus older than 60 years presented with significantly* higher frequencies of 6B11⁺ and 6B11⁺Vα24⁺ iNKT cells in the MNC fraction (0.87% ± 0.55% vs. 0.72% ± 0.40%; 0.04% ± 0.03% vs. 0.01% ± 0.01%; *P* < 0.0711, Fig. 5.2A, right side).

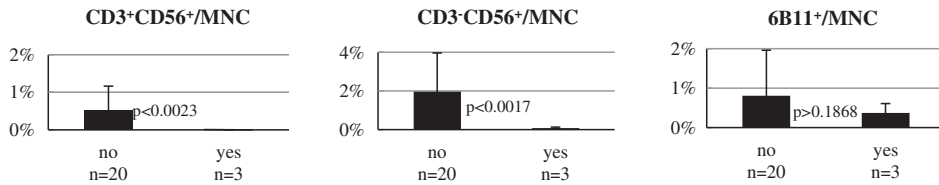
CLL-patients with stable versus no stable disease (NCR, relapse or death with disease) presented with (ns) higher percentages of CD3⁺CD56⁺ CIK cells and CD3⁻CD56⁺ NK cells in MNC fraction (0.70% ± 0.79% vs. 0.47% ± 0.23%; 3.40% ± 3.33% vs. 3.50% ± 2.64%, Fig. 5.2B, left side; middle row). Moreover, we could demonstrate significantly** higher frequencies of 6B11⁺ (0.82% ± 0.49%

5.1. Prognostic relevance for AML-pts

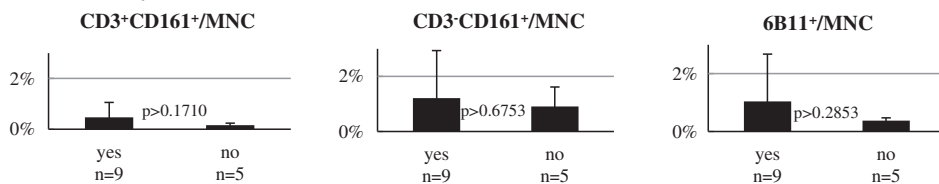
A Response to chemotherapy (CR vs NCR, left side), allocation to favorable vs adverse risk score (NCCN, middle row), allocation to age groups (<60 vs > 60 years, right side)



B Extramedullary vs no extramedullary foci

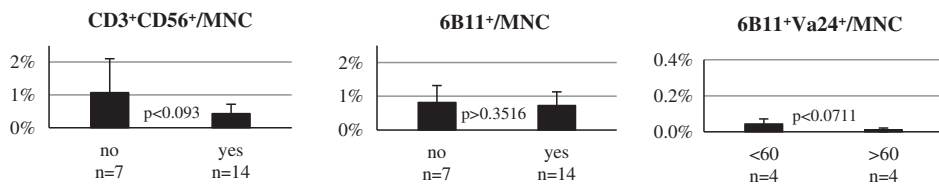


C Stable CR (yes or no)



5.2. Prognostic relevance for CLL-pts

A Need for therapy (yes or no, left side + middle row), allocation to age groups (<60 vs >60 years, right side)



B Stable disease (yes or no)

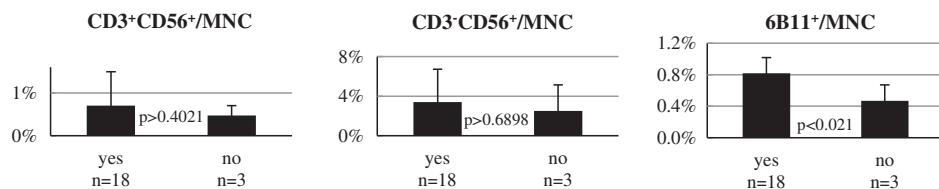


FIGURE 5. Relevance of CIK, NK, and iNKT cells and their subsets for prognosis of AML and CLL patients. 5.1. AML patients: Frequencies of CIK cells in patients with CR versus NCR after induction chemotherapy, iNKT cells in patients with favorable versus adverse risk score (NCCN) and younger than 60 versus older than 60 years (A), CIK, NK, and iNKT cells in patients with extramedullary versus no extramedullary foci (B), and CIK, NK, and iNKT cells in patients with stable versus no stable disease are given (C). 5.2. CLL patients: frequencies of CIK and iNKT cells in patients with no need versus need for therapy, iNKT cells in patients younger than 60 versus older than 60 years (A) and CIK, NK, and iNKT cells in patients with stable versus no stable disease are given (B). AML indicates acute myeloid leukemia; CIK, cytokine-induced killer; CLL, chronic lymphoid leukemia; CR, complete remission; iNKT, invariant natural killer T; MNC, mononuclear cells; NCCN, National Comprehensive Cancer Network; NCR, no complete remission; NK, natural killer; pts, patients.

vs. $0.47\% \pm 0.06\%$, $P < 0.021$, Fig. 5.2B) and significantly* higher frequencies of $6B11^+ CD3^+ iNKT$ cells in the MNC fraction of CLL patients with stable CR versus NCR ($0.49\% \pm 0.48\%$ vs. $0.17\% \pm 0.09\%$, $P < 0.0597$ right side).

In summary (significantly) higher proportions of $6B11^+ iNKT$ cells correlated with no need for therapy, CLL patients younger than 60 years and stable CR. Comparable correlations were found for $6B11^+ CD3^+ / 6B11^+ V\alpha 24^+ iNKT$ subsets. $CD3^- CD56^+ NK$ and $CD3^+ CD56^+ CIK$ cells regularly correlated with stable CR, while only $CD3^- CD56^+ NK$ cells correlated with no need for therapy.

iNKT, NK, and CIK Cells and their Subtypes Increase Under the Influence of IL-2 and After Prestimulation With DC/DC_{leu}

It is well known, that DC/DC_{leu} are professional stimulators of T cells, thereby giving rise to antileukemic active effector cells after MLC (containing IL-2).

We generated DC/DC_{leu} from healthy or leukemic MNC or WB (DC_{leu} were only analyzed in cases where the amount of DC were $\geq 10\%$) and achieved the following results.

“MNC-Healthy”

With “Kit-D” we generated $\emptyset 10.79\% \pm 2.07\%$, with “Pici 1” $15.42\% \pm 4.97\%$ and with “Pici 2” $15.33\% \pm 6.09\%$ DC in MNC.

“WB-Healthy”

With “Kit-D” we generated $\emptyset 7.44\% \pm 1.28\%$, with “Kit-I” $11.05\% \pm 6.82\%$, with “Kit-K” $8.62\% \pm 2.00\%$, with “Kit-M” $8.08\% \pm 3.38\%$, with “Pici 1” $10.50\% \pm 2.62\%$ and with “Pici 2” $9.48\% \pm 1.85\%$ DC in WB.

“MNC-AML”

With “Kit-D” we generated $\emptyset 36.51\% \pm 7.33\%$ DC and $6.70\% \pm 0.58\%$ DC_{leu}, with “Pici 1” $23.53\% \pm 0.00\%$ DC and $12.63\% \pm 0.00\%$ DC_{leu} and with “Pici 2” $19.39\% \pm 7.39\%$ DC and $10.20\% \pm 0.00\%$ DC_{leu} in MNC.

“WB-AML”

With “Kit-D” we generated $\emptyset 6.85\% \pm 1.10\%$ DC, with “Kit-I” $13.18\% \pm 7.43\%$ DC and $19.95\% \pm 0.00\%$ DC_{leu}, with “Kit-K” $10.48\% \pm 3.77\%$ DC and $8.11\% \pm 2.38\%$ DC_{leu}, with “Kit-M” $9.92\% \pm 3.93\%$ DC and $13.62\% \pm 0.00\%$ DC_{leu}, with “Pici 1” $10.16\% \pm 0.00\%$ DC and $8.98\% \pm 0.00\%$ DC_{leu} and with “Pici 2” $12.88\% \pm 0.00\%$ DC and $11.39\% \pm 0.00\%$ DC_{leu} in WB.

Pooling all results from the controls without added response modifiers (“MNC-healthy,” “WB-healthy,” “MNC-AML,” “WB-AML”) we found $\emptyset 6.28\% \pm 2.09\%$ DC.

Here we studied, whether iNKT/NK/CIK cells (in MNC or WB samples) could contribute to improve the overall antileukemic activity after stimulation with DC/DC_{leu}. Therefore, we stimulated in MLC (prepared with MNC or WB) T-cell-enriched immune reactive cells with a stimulator cell suspension containing DC/DC_{leu} (MLC*^{MNC-DC} or MLC*^{WB-DC}) which were generated with different “cocktails.” The same setting, but with a stimulator cell suspension without pretreatment with “cocktails” (MLC*^{MNC} or MLC*^{WB}) served as a

control. First, we quantified these cells before and after MLC with added T cells and IL-2. In a second step, we analyzed the effect of different “cocktail”-generated DC/DC_{leu} on the composition of immune reactive cells after stimulation. Further, we correlated our findings with antileukemic reactivity in a context with iNKT, NK, CIK cells, and T-cell subsets.

In MLC of Healthy and AML-Patients (MLC*^{WB-DC} or only MLC*^{WB}) Proportions of iNKT/NK and CIK Cells Increase in the Presence of IL-2

At start of MLC we found lower total frequencies of iNKT cells and a shift to higher proportions of T/NK cells coexpressing 6B11 in AML patients ($n = 6$) versus healthy WB samples ($n = 5$; Fig. 6A). In a next step we quantified these cells after MLC and found that iNKT cells significantly(*) increased in both AML and healthy WB-samples after MLC: at start of MLC versus after MLC*^{WB} (AML $6B11^+ / MLC$: $4.18\% \pm 1.32\%$ vs. $8.63\% \pm 4.40\%$, $P < 0.0745^*$; healthy $6B11^+ / MLC$: $4.68\% \pm 3.92\%$ vs. $12.34\% \pm 8.10\%$, $P < 0.0305^{**}$; healthy $6B11^+ CD3^+ / MLC$: $4.60\% \pm 3.90\%$ vs. $12.11\% \pm 8.10\%$, $P < 0.0385^{**}$; healthy $6B11^+ CD3^+ / CD3^+$: $5.17\% \pm 4.63\%$ vs. $31.77\% \pm 22.85\%$, $P < 0.0732^*$; Figs. 6A, B) and start of MLC versus after MLC*^{WB-DC} (healthy $6B11^+ / MLC$: $4.68\% \pm 3.92\%$ vs. $13.67\% \pm 5.01\%$, $P < 0.0323^{**}$; healthy $6B11^+ CD3^+ / MLC$: $4.60\% \pm 3.90\%$ vs. $13.03\% \pm 4.91\%$, $P < 0.0393^{**}$; $6B11^+ CD3^+ / CD3^+$: $5.17\% \pm 4.63\%$ vs. $21.51\% \pm 7.38\%$, $P < 0.0093^{**}$, Fig.s 6A, C).

Pooling all results after MLC*^{WB-DC} compared with MLC*^{WB}, an astonishing finding was, that cellular compositions in healthy samples were comparable in both settings (Figs. 6B, C). This could be due to the general influence of IL-2 in MLC. However, in AML-cases the proportions of iNKT cells increased after MLC*^{WB-DC} compared with MLC*^{WB} (Figs. 6B, C), although differences were not significant. Comparable results were found with MNC as cell sources (dns).

At start of MLC we found lower total frequencies of CIK and NK cells in AML ($n = 6$) versus healthy WB-samples ($n = 5$) (Fig. 7A). In a next step we quantified these cells after MLC and found that CIK and NK cells (significantly) increased in both AML and healthy WB-samples after MLC: at start of MLC versus after MLC*^{WB} (AML $CD3^+ CD161^+ / MLC$: $1.41\% \pm 0.74\%$ vs. $3.28\% \pm 1.59\%$, $P < 0.0487^{**}$; healthy $CD3^+ CD161^+ / MLC$: $2.35\% \pm 2.59\%$ vs. $3.30\% \pm 2.04\%$; AML $CD3^- CD161^+ / MLC$: $0.15\% \pm 0.15\%$ vs. $1.62\% \pm 1.81\%$; healthy $CD3^- CD161^+ / MLC$: $1.41\% \pm 2.48\%$ vs. $0.80\% \pm 0.86\%$; Figs. 7A, B) and start of MLC versus after MLC*^{WB-DC} (AML $CD3^+ CD161^+ / MLC$: $1.41\% \pm 0.74\%$ vs. $3.15\% \pm 2.13\%$; healthy $CD3^+ CD161^+ / MLC$: $2.35\% \pm 2.59\%$ vs. $4.20\% \pm 2.02\%$; AML $CD3^- CD161^+ / MLC$: $0.15\% \pm 0.15\%$ vs. $1.73\% \pm 1.98\%$; healthy $CD3^- CD161^+ / MLC$: AML $1.41\% \pm 2.48\%$ vs. $0.57\% \pm 0.37\%$; Figs. 7A, C).

Pooling all results after MLC*^{WB-DC} compared with MLC*^{WB}, an astonishing finding was, that cellular compositions in healthy samples were comparable in both settings (Figs. 7B, C). This could be due to the general influence of IL-2 in MLC.

Moreover, we determined values of IL-10, IL-17A, IFN- γ and MCP-1 after MLC*^{WB-DC}, MLC*^{WB}, WB-DC culture and WB-DC control in the supernatant of samples from AML patients ($n = 4$) using ELISA. An increased cytokine release was shown for IL-17A, MCP-1

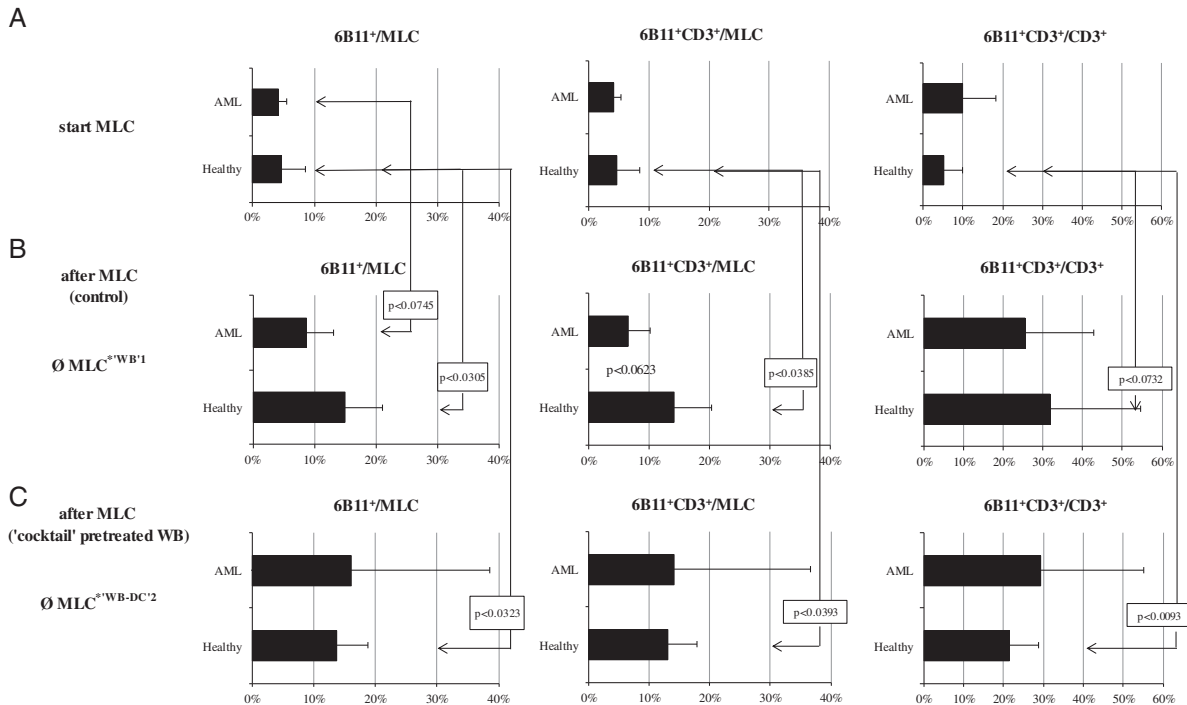


FIGURE 6. Frequencies of iNKT cells before (A) or after (B, C) MLC (WB) are given. T cell-enriched immune reactive cells were stimulated with a stimulator cell suspension without pretreatment of WB (MLC*^{WB}1 (control)) (B) or with a stimulator cell suspension pretreated with “cocktails” (MLC*^{WB-DC2}) (C). ¹MLC*^{WB} T-cell enriched immune-reactive cells were stimulated with a stimulator cell suspension without pretreatment of WB with “cocktails.” ²MLC*^{WB-DC} T-cell-enriched immune-reactive cells were stimulated with a stimulator cell suspension pretreated with “cocktails,” “cocktails” = all DC-generating methods/Kits. DC indicates dendritic cells; iNKT, invariant natural killer T; MLC, mixed lymphocyte cultures; WB, whole blood.

and IL-10 in WB-DC supernatants compared with WB-DC control—what correlated with higher DC counts compared with control. An increased release of the chemoattractant MCP-1 was found after MLC*^{WB-DC} with all DC-generating methods/Kits compared with MLC*^{WB}. An increased antitumor and anti-inflammatory cytokine profile (IL-17A and IL-10) was found after MLC*^{WB-DC} especially with Kit-M pretreated WB (dns) compared with MLC*^{WB}.

In summary iNKT as well as CIK and NK cells increased after MLC independent of the stimulator cell suspension, what might be explained by a general iNKT, NK, and CIK cells inducing effect by IL-2. AML samples treated with “cocktails” and used as stimulator cells seemed to increase iNKT but not CIK and NK cell counts compared with not pretreated controls, pointing to an “iNKT-inducing influence” of these different “cocktails.”

Highest iNKT Frequencies After MLC Could be Found in Cases With DC Generation Conducted With Prostaglandin-containing “Cocktails”

Studying the influence of different DC/DC_{leu}, generated with “cocktails,” on the frequencies of 6B11⁺ iNKT cells after MLC showed, that in MNC/WB samples iNKT cell proportions increased in general during the MLC. For each individual patient we defined the best stimulator cell source resulting in the “highest” (+ + +) 6B11⁺ iNKT cell frequencies after MLC (Ø6B11⁺ /WB: 24.91% ± 19.61%, Ø6B11⁺ /MNC: 10.98% ± 7.05%); in “high” (+ +) 6B11⁺ iNKT cell frequencies (only defined for WB, Ø6B11⁺ /WB: 13.56% ± 6.59); with a

“good” (+) frequency of 6B11⁺ iNKT cells (Ø6B11⁺ /WB: 8.06% ± 2.77%; Ø6B11⁺ /MNC: 5.88% ± 4.42%) and with “no increase” (–) or even in a decrease of iNKT cells (Ø6B11⁺ /WB: 3.94% ± 2.45%, Ø6B11⁺ /MNC: 0.93% ± 0.21%). Frequencies were applied for healthy and AML samples.

Similar distributions were found for 6B11⁺ CD161⁺ and 6B11⁺ CD3⁺ iNKT cells. We could show that especially in cases in that the DC generation was performed with prostaglandin-containing (PGE₁, PGE₂) “cocktails” the percentages of iNKT cells increased the most (Kit-D, Kit-K, Kit-M, “Pici1,” “Pici2,” Fig. 8.1) Moreover, we found that in almost every given patient (except P1433) we could select at least one of several “cocktails,” that increased iNKT proportions—pointing to an effect of a certain pretreatment with “cocktails” on the frequencies of iNKT cells in healthy as well as of AML samples. Effects seen in MNC and WB samples from healthy and AML samples were comparable (Figs. 8.1A, B). Comparable effects were found for CIK cells (dns).

Moreover, we correlated percentages of 6B11⁺ / 6B11⁺ CD3⁺ / 6B11⁺ CD161⁺ iNKT cells (defined as “highest,” “high,” “good,” and “no increase”) after MLC in MNC (Fig. 7.1A, healthy “MNC”) for each individual sample with the frequencies of DC after culture of healthy MNC (n = 8) with different “cocktails.” We found that samples with the “highest” (Ø6B11⁺ /MNC: 10.98% ± 7.05%) frequencies of iNKT cells showed (although not significant) higher percentages of DC after the DC-generating process compared with MLC with “no increase” (Ø6B11⁺ /MNC: 0.93% ± 0.21%) or even a

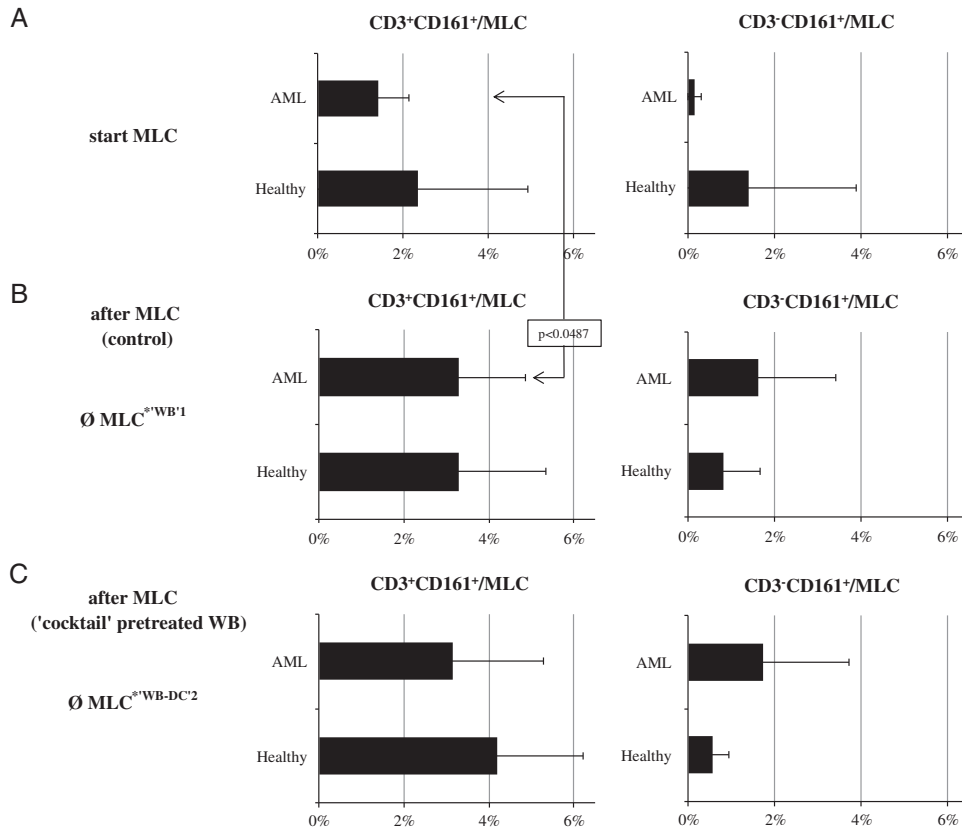


FIGURE 7. Frequencies of CIK/NK cells before (A) or after (B, C) MLC (WB) are given. T cell-enriched immune reactive cells were stimulated with a stimulator cell suspension without pretreatment of WB (MLC^{WB-1} (control)) (B) or with a stimulator cell suspension pretreated with “cocktails” (MLC^{WB-DC²}) (C). ¹MLC^{WB} T-cell enriched immune reactive cells were stimulated with a stimulator cell suspension without pretreatment of WB with “cocktails.” ²MLC^{WB-DC} T-cell enriched immune reactive cells were stimulated with a stimulator cell suspension pretreated with “cocktails.” “Cocktails” = all DC-generating methods/Kits. CIK indicates cytokine-induced killer; DC, dendritic cells; MLC, mixed lymphocyte cultures; WB, whole blood.

decrease of iNKT cells (Ø DC-counts: 12.87% ± 5.44% vs. 9.11% ± 8.81%; Fig. 8.2). Those correlations were not found for MNC of the 2 AML-patients we have analyzed (dns). In a next step, we correlated these values with DC/DC-subtype values obtained from 5 healthy and 6 AML-WB samples and did not find correlations between DC/DC subtype counts and iNKT cells after MLC (dns).

In summary, differences in iNKT cell compositions after MLC could be found in individual samples (healthy or AML) after pretreatment with different “cocktails.” Highest percentages were found with prostaglandin-containing methods. Together, high frequencies of DC correlated with “highest” frequencies of iNKT cells after MLC in MNC of healthy controls, while lower percentages of DC correlated with “no increase” or even a decrease of iNKT cells after MLC in MNC of healthy controls—however, these correlations were not found for the 2 AML patients analyzed.

Physiological Hypoxia Does Not Influence Frequencies and Compositions of iNKT, NK, and CIK Cells Compared With Normoxic Conditions

Under physiological conditions the O₂-concentration in PB is lower than the normoxic 21%. With some preliminary experiments, we wanted to work out results under physiologically most adapted conditions in order to

draw first conclusions for the functional relevance on immune-reactive cells: physiological low O₂ concentrations could possibly influence the conversion of blasts to DC_{leu} and in consequence the composition and reactivity of immune-reactive cells. Here we studied whether hypoxic (compared with normoxic) conditions influence the composition of iNKT/NK/CIK cells after MLC (in WB samples). Results included in this chapter were cultured in 6% or 10% O₂ or with varying O₂-concentrations between 0% and 17%. For our evaluations, all results were pooled. In a first step, we quantified DC-(subtype) proportions in healthy WB samples (n = 4) with or without stimulation with 3 “cocktails” (Kit-I, Kit-K or Kit-M). We could show that DC proportions [overall and mature DC (DC_{mig})] were not significantly different in cases cultured under normoxic versus hypoxic conditions (dns).

In a next step, we quantified iNKT/NK/CIK cell proportions after MLC^{WB-DC} and MLC^{WB} under hypoxic versus normoxic conditions. Pooling all results after MLC^{WB-DC} we found comparable proportions of iNKT/NK/CIK cells after MLC under normoxic versus hypoxic conditions (dns).

In summary low O₂ culture concentrations do not seem to have neither a significant effect on the generation of DC nor on the amount and composition of iNKT, NK, and CIK cells after MLC.

8.1. Frequencies of iNKT-cells (subsets) after MLC

A 6B11 ⁺ /6B11 ⁺ CD3 ⁺ /6B11 ⁺ CD161 ⁺ -iNKT-cells after MLC ^{*MNC-DC} and MLC ^{*MNC}							
Healthy 'MNC'	Kit-D	'Pici1'	'Pici2'	control			
P1418	+	+++	-	+			
P1421	+	+	+++	+			
P1422	+	+++	+	+			
P1425	+++			+			
P1428	+		+++	+			
P1429		+++	-	-			
P1436		+++	+	+++			
P1438	+++	+	+	+			
AML 'MNC'	Kit-D	'Pici1'	'Pici2'	control			
P1424	-		-	+++			
P1426	+++	+	-	+			
B 6B11 ⁺ /6B11 ⁺ CD3 ⁺ /6B11 ⁺ CD161 ⁺ -iNKT-cells after MLC ^{*WB-DC} and MLC ^{*WB}							
Healthy 'WB'	Kit-D	Kit-I	Kit-K	Kit-M	'Pici1'	'Pici2'	control
P1420	+++	+	+	++	+	+	++
P1428	+	+	+++	++	+	++	++
P1429	++	+	+	+	++	+	+++
P1436				+	+++	+	+
P1438	-	-	-	+++	++	-	+
AML 'WB'	Kit-D	Kit-I	Kit-K	Kit-M	'Pici1'	'Pici2'	control
P1424	+++	-	-	-	-	-	-
P1426	++		+	+	+	+	+++
P1430	+	+	+++				++
P1433		-					-
P1434		+++	+++	++			+
P1439				+++			++

8.2. iNKT-cells after MLC correlate with DC-values from healthy MNC

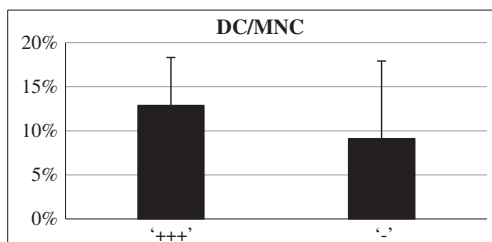


FIGURE 8. Frequencies of iNKT cells (6B11⁺ or 6B11⁺CD3⁺ or 6B11⁺CD161⁺ iNKT cells) after MLC (MNC and WB) are given. T cell-enriched immune reactive cells were stimulated with a stimulator cell suspension without pretreatment of MNC/WB [MLC^{*MNC/WB}1 (control)] (B) or with a stimulator cell suspension pretreated with “cocktails” (MLC^{*MNC/WB-DC}2) (C). AML indicates acute myeloid leukemia; DC, dendritic cells; iNKT, invariant natural killer T; MLC, mixed lymphocyte cultures; MNC, mononuclear cells; WB, whole blood. MNC/WB: “+++” “highest” counts of iNKT cells; “++” “high” counts of iNKT cells, “+” “good” counts of iNKT cells, “-” “no” increase or decrease of iNKT cells.

iNKT, NK, CIK Cells Contribute to Antileukemic Activity

As shown above iNKT cell proportions increase after MLC^{*WB-DC} or MLC^{*MNC-DC}. Here we studied, whether iNKT/NK/CIK cells could contribute to improve the overall-antileukemic activity after MLC. In a first step, we quantified these cells and their subtypes in individual AML-WB samples (precultured with various “cocktails”) after MLC (with added T cells and IL-2). In the next step, we correlated frequencies of iNKT, NK, CIK cells with the antileukemic activity [blast-lysis evaluated with a cytotoxicity (fluorolysis) assay] of the individual WB samples from AML patients.

We found that cases with versus without an antileukemic activity (“lysis” vs. “nonlysis”) were characterized by significantly*** increased frequencies of 6B11⁺ iNKT cells in the total MLC (7.25% ± 4.21% vs. 2.60% ± 0.96%, *P* < 0.0022) and significantly** higher frequencies of 6B11⁺CD161⁺/6B11⁺CD3⁺ iNKT cells in MLC (3.67% ± 3.55% vs. 1.06% ± 0.29%, *P* < 0.0083; 5.10% ± 2.57% vs. 2.12% ± 1.04%, *P* < 0.0232, Fig. 9A). However, the subtype-compositions of iNKT and CIK cell subsets were comparable in the 2 groups compared (6B11⁺CD161⁺/CD161⁺: 52.63% ± 24.14% vs. 51.34% ± 9.10%; 6B11⁺CD3⁺/CD3⁺: 18.45% ± 15.34% vs. 17.25% ± 9.79%; CD3⁺CD161⁺/CD3⁺: 15.08% ± 14.03% vs. 14.88% ± 5.34%,

Fig. 9B). Moreover, significantly* higher proportions of CD3⁺CD161⁺ CIK cells in MLC (3.26% ± 2.35% vs. 1.85% ± 0.48%, *P* < 0.0509) and significantly*** increased frequencies of CD3⁻CD161⁺ NK cells in MLC were found in cases with versus without an antileukemic activity (2.07% ± 2.13% vs. 0.21% ± 0.13%, *P* < 0.0023, Fig. 9C). However, significantly* higher proportions of proliferating T cells (T_{prol}: CD3⁺CD69⁺/MLC) and significantly** higher frequencies of central memory T cells (T_{cm}: CD3⁺CD45RO⁺CCR7⁺/MLC) were found in cases with versus without an antileukemic activity (T_{prol}: 17.56% ± 7.86 vs. 15.29% ± 2.61%, *P* < 0.0825, T_{cm}: 9.35% ± 10.47% vs. 2.32% ± 1.5%, *P* < 0.01451, Fig. 9D). No significant differences were found in the composition of naive/non-naive T cells in the groups compared (dns).

Moreover, we assorted our samples according to their antileukemic activity (“lysis” vs. “nonlysis”) and evaluated predictive cutoff values for NK/CIK/iNKT cells after MLC*^{“WB-DC”} and MLC*^{“WB”}. A total of 100% of samples

with >4% 6B11⁺ iNKT cells, with > 4% 6B11⁺CD3⁺ iNKT cells and with >1.5% 6B11⁺CD161⁺ iNKT cells after MLC*^{“WB-DC”} and MLC*^{“WB”} showed antileukemic activity (lysis). Furthermore, 100% of samples with >2.3% CD3⁺CD161⁺ CIK cells and with >1.9% CD3⁺CD56⁺ CIK cells after MLC*^{“WB-DC”} and MLC*^{“WB”} showed antileukemic activity. Moreover, 89%–100% of samples with >0.4% CD3⁻CD161⁺ NK cells and with >1.3% CD3⁻CD56⁺ NK cells after MLC*^{“WB-DC”} and MLC*^{“WB”} showed antileukemic activity.

Further, we analyzed whether the addition of “cocktails” to cultures improves the antileukemic activity after MLC compared with controls. Samples with more lysis compared with controls, were defined as improved blast-lysis, samples with less lysis compared with controls as “not improved” blast-lysis. Samples with improved blast-lysis showed significantly* higher 6B11⁺ iNKT cell frequencies compared with samples with not improved

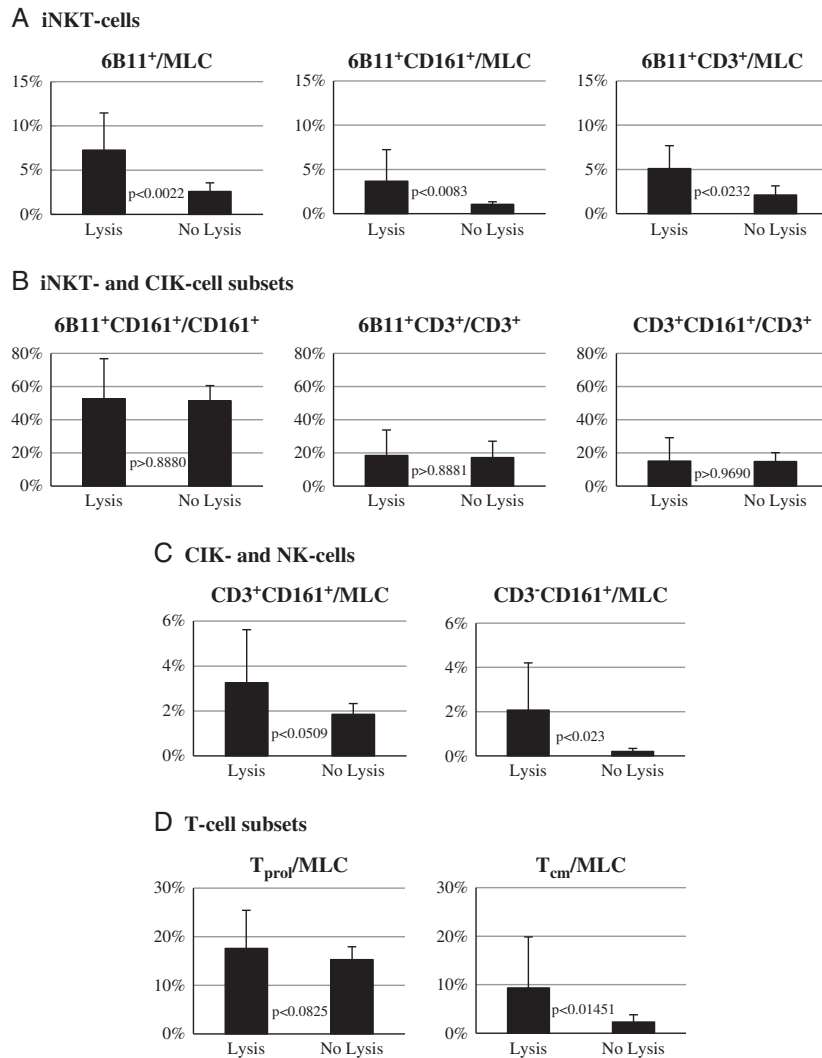


FIGURE 9. Frequencies of iNKT cells (A), CIK cells (B), NK cells (C), and T cell subsets (D) after MLC (MNC/WB) are given. T cell-enriched immune reactive cells were stimulated with a stimulator cell suspension without pretreatment of MNC/WB [MLC*^{“MNC/WB”}1 (control)] or with a stimulator cell suspension pretreated with “cocktails” (MLC*^{“MNC/WB-DC”}2) were compared in cases with/without blast lysis. Results were obtained with a cytotoxicity (fluorolysis) assay. CIK indicates cytokine-induced killer; iNKT, invariant natural killer T; MLC, mixed lymphocyte cultures; NK, natural killer; T_{prol}, proliferating T cells (CD69⁺CD3⁺), T_{cm}, central-memory T cells (CD45RO⁺CCR7⁺).

blast-lysis ($7.94\% \pm 4.12\%$ vs. $4.39\% \pm 3.12\%$, $P < 0.0921$). Moreover, samples with improved blast-lysis showed (ns) higher frequencies of $6B11^+CD161^+$ and $6B11^+CD3^+$ iNKT cells compared with samples with not improved blast-lysis ($4.82\% \pm 3.49\%$ vs. $1.80\% \pm 2.90\%$; $5.25\% \pm 2.13\%$ vs. $3.50\% \pm 1.84\%$). Furthermore, samples with “improved” blast-lysis showed (ns) higher frequencies of $CD3^+CD161^+$ CIK cells and $CD3^-CD161^+$ NK cells compared with samples with not improved blast-lysis ($3.99\% \pm 2.51\%$ vs. $2.10\% \pm 1.83\%$; $2.73\% \pm 1.95\%$ vs. $0.95\% \pm 1.87\%$).

Furthermore, we correlated findings of ELISA of supernatants of MLC^{*WB-DC} and MLC^{*WB} of samples from AML patients ($n = 4$) with antileukemic activity (“lysis” vs. “no lysis”). We could show that cases with achieved “lysis” compared with “no lysis” after MLC^{*WB-DC}/MLC^{*WB} were characterized by higher release of antitumor and anti-inflammatory cytokines (IL-17A and IL-10), what correlated with higher iNKT, NK, and CIK cell frequencies.

With one exemplary experiment, we could show that a radiation of stimulator-cells did not reduce proportions of T/iNKT/NK and CIK cells; however, reduced the overall blast-lysis compared with unirradiated settings. This could point to a role of iNKT/NK and CIK cells beside T cells in antileukemic reactions (dns).

In summary in cases with antileukemic blast-lytic activity after MLC not only a T cell stimulation was induced, followed by a creation of memory T cells, but also that iNKT, NK, CIK cell proportions are significantly increased—pointing to an involvement of these cells in antileukemic reactions. Moreover, the presence of increased proportions (above cutoff values) of iNKT as well as of CIK and NK cells after MLC might correlate with successful blast-lysis.

DISCUSSION

Role of T, iNKT, NK, and CIK Cells in Tumor-immune Surveillance

T, iNKT, NK, and CIK cells and their subsets are important mediators of immune responses: T cells are known to be activated by tumor or leukemia antigen-presenting DC and—as shown exemplarily by our DC/DC_{leu} ex vivo strategies—their antitumor functionality can be improved by DC_{leu} - T_{eff-em} enable—in case of a secondary challenge—a very quick and immediate secondary immune response, while T_{reg} are able to downregulate immune responses—important to inhibit autoaggressive immune reactions—but also in a context to downregulate antitumor responses.^{9,10} In contrast to T cells, iNKT, NK, and CIK cells react quickly to an immunologic threat by secreting cytokines and chemokines^{4,30} and kill tumor cells without prior activation and, unlike T and iNKT cells, NK and CIK cells do not recognize target cells by a TCR.⁵ iNKT cells can produce a variety of chemokines and cytokines like $IFN-\gamma$, IL-4/IL-2/IL-12, tumor necrosis factor (TNF)- α , transforming growth factor- β , and GM-CSF that in consequence activate different cells like DC, NK cells, $CD4^+/CD8^+$ T cells, but also T_{reg} .^{31,32} A previous study could show that all subsets of iNKT cells produce almost the same amounts of $IFN-\gamma$ and TNF- α , while IL-4 is mainly produced by $PB-CD4^+$ iNKT cells.¹³ Moreover, iNKT cells show a strong cytolytic potential and can directly kill tumor cells as they express granzyme-B, Perforin, and FasL.^{4,31} Therefore, it might be promising to

use or even to increase the antileukemic potential of NK, CIK cells and especially iNKT cells to enhance the patients’ anti-tumor immune response.

Methods to Detect iNKT, NK, and CIK Cells

The identification and quantification of iNKT cells (in healthy blood samples) is known to be difficult due to their low frequency as well as missing marker (combinations). Previous methods using CD1d tetramers or the combination of $V\alpha 24/V\beta 11$ -antibodies did not yield the necessary specific results.¹³ Therefore, the first aim of our study was to develop a highly specific iNKT cell detection panel for the quantification of iNKT cells/subsets in healthy, but also to be used for leukemia patients. Furthermore, we compared strategies and markers to detect and quantify NK and CIK cells and their subsets in healthy, but especially in leukemic patients. A previous study performed with healthy samples could show that a new moAb (clone 6B11-PE) can recognize the invariant CDR3 loop of the $V\alpha 24J\alpha 18$ -TCR rearrangement on the cell surface and can be used to specifically characterize iNKT cells in combination with anti-CD3.¹³ In our current study, we also used 6B11-PE, but also 6B11-FITC (purchased from different companies) to characterize and define iNKT cells. In previous studies,^{11,13} only 6B11-PE was used for analysis, but not 6B11-FITC. We found (significantly) higher proportions of iNKT cells detected with 6B11-FITC compared with 6B11-PE in AML, ALL, CLL patients. The proportions of iNKT cells detected with 6B11-FITC compared with 6B11-PE were (significantly) lower in healthy samples. Moreover, 6B11-FITC-stainings seemed to yield varying results in several stainings, whereas 6B11-PE-stainings showed stable and precise results for iNKT detection. It is well known, that moAbs can react and bind differently to their targets—depending not only on the selected clones, but also on the purchasing companies, the fluorochromes used or the combination with partner moAbs in a panel.³³ In order to exclude these variations, we focused mainly on 6B11-PE results in our setting.

Coexpression analyses of 6B11 with myeloid (eg, CD33, CD34, CD117) or lymphoid (eg, CD1a, CD5, CD7, CD10, CD19, CD20) markers on blasts from malignant myeloid and lymphoid cell lines showed, that 6B11 was not expressed on myeloid or B-lymphoid blasts. However, we found a low expression of 6B11 on T-linear cells of the Jurkat cell line. As we do not know, whether this cell line has a $V\alpha 24/V\beta 11$ -rearrangement, typical for iNKT cells we cannot decide whether this expression is “aberrant” on this cell line or specific. In general, our results show, that the 6B11-PE-marker, but not 6B11-FITC, can be regarded as a very specific marker to detect iNKT cells without cross-reactivity or aberrant expression on leukemic cells. Previous studies did not analyze whether 6B11 is aberrantly expressed on leukemic blasts and if 6B11-FITC is specific for iNKT cells analyses. Thereby, we can confirm preliminary findings of other groups, that the detection of iNKT cells with 6B11 alone or in combination with subtype markers is very precise, as 6B11 specifically recognizes the CDR3 loop of the invariant TCR of CD1d-restricted iNKT cells.¹³

It is known, that the iNKT cell population consists of different subsets with diverse phenotypic and functional characteristics that can be subdivided according to their expression of surface molecules, receptors, effector functionality or tissue localization.⁴ In a former study it was shown that 6B11 could be used in combination with further

T (CD27, CD28, CD45RA, CD45RO) and NK cell markers (CD16, CD56, CD161) to analyze iNKT subsets.¹³ Moreover, iNKT cells were characterized by the combination of CD1d and CD3 or the combination of V α 24 and V β 11. The percentage of iNKT cells expressing CD4 or CD8 was evaluated in a region comprising 6B11⁺CD3⁺ iNKT cells. Like the previous study, we used 6B11 in combination with CD3, CD45RO, and CD161. Moreover we included the combinations of 6B11 with CD1d, V α 24, CD4 or with CD8. This means that we evaluated the expression of T cell (CD1d, CD3, CD45RO, V α 24) and NK cell markers (CD161) on 6B11⁺ iNKT cells and divided them into “T cell-like” and “NK cell-like” iNKT subsets. We are the first group, that defined “T cell-like” iNKT subsets as CD3⁺6B11⁺, CD4⁺6B11⁺, CD8⁺6B11⁺, V α 24⁺6B11⁺, CD1d⁺6B11⁺, CD45RO⁺6B11⁺, and CD45RO⁻6B11⁺ iNKT cells.

Classically, iNKT cells have been identified with CD1d-tetramers loaded with α -galactosylceramide, but this method could lead to an overestimation of iNKT cells as some T cell subsets also express CD1d.¹³ This study did not find significant differences in 6B11⁺ iNKT cells compared with CD3⁺6B11⁺ iNKT cells or CD3⁺CD1d⁺ iNKT cells in healthy samples. Although we did not combine CD1d with CD3, but with 6B11, we could also show that frequencies of 6B11⁺CD3⁺ and 6B11⁺CD1d⁺ iNKT cells were comparable in healthy samples. We could show in addition that frequencies of 6B11⁺CD3⁺ and 6B11⁺CD1d⁺ iNKT cells were similar in AML, ALL, and CLL samples. Unlike the previous study,¹³ we detected higher numbers of 6B11⁺ iNKT cells compared with 6B11⁺CD3⁺ and 6B11⁺CD1d⁺ iNKT cells in healthy samples as well as in AML, ALL, and CLL samples.

Using CD3-/V α 24-antibodies, we could demonstrate specific results and detected lower frequencies of V α 24⁺6B11⁺ iNKT cells compared with CD3⁺6B11⁺ iNKT subsets in healthy as well as in AML, ALL, and CLL patients. These findings are consistent with a previous study, which found 3 times more CD3⁺6B11⁺ iNKT cells compared with CD3⁺V α 24⁺V β 11⁺ iNKT cells in healthy adult and children's samples.¹¹ However, this group combined V α 24 with CD3 and V β 11, while we combined V α 24 with 6B11. Unlike these results, another group found similar proportions of CD3⁺6B11⁺ iNKT cells and V α 24⁺V β 11⁺ iNKT cells in healthy controls.¹³ However, this group used the combination of V α 24 and V β 11 without combining it with CD3. These differences may be explained by the fact, that different combinations of V α 24 with CD3, V β 11 or 6B11 were used for iNKT cell detection. Furthermore, the results of our group and Bienemann may be explained by the fact that the invariant α -chain of the TCR sometimes binds with other β -chains than V β 11, which could lead to an underestimation of iNKT cells.¹¹

Both type I iNKT cells and type II iNKT cells, are divided into CD4⁺, CD4⁻CD8⁻, CD8 $\alpha\alpha$ ⁺, and CD8 $\alpha\beta$ ⁺ iNKT cells, with higher proportions being CD4⁺, lower proportions being CD4⁻CD8⁻ and only a few cells CD8 $\alpha\alpha$ ⁺ or CD8 $\alpha\beta$ ⁺, which could only be found in humans but not in mice.⁴ Unlike previous studies, we combined 6B11 with CD4 and CD8 for subtype analyses. We can confirm that the majority of 6B11⁺ iNKT cells coexpresses CD4⁺ and the minority CD8⁺ in healthy samples. We can add as a new finding in addition, that higher proportions of 6B11⁺CD4⁺ iNKT cells and lower proportions of 6B11⁺CD8⁺ iNKT cells were also found

in AML, ALL and CLL patients. A previous study suggested that CD4⁺ iNKT cells produce both Th1 and Th2 cytokines, whereas CD4⁻ iNKT cells tend to produce mainly Th1 cytokines.³¹ However, another study found that CD4⁺, CD4⁻, and CD8⁺ iNKT cells produced similar amounts of IFN- γ and TNF- α , but CD4⁺ iNKT cells produced significantly more IL-4.¹³ In our patients' cohort, we had not further analyzed the functional role of CD4 and CD8 expressions on iNKT cells. However, we suggest to combine 6B11 with CD4 and CD8 to analyze the differences between 6B11⁺CD4⁺, 6B11⁺CD4⁻, 6B11⁺CD8⁺, and 6B11⁺CD8⁻ iNKT cells.

Consistent to a previous study we found that the majority of iNKT cells is CD45RO⁺6B11⁺, while the minority is CD45RO⁻6B11⁺, what could be interpreted as iNKT cells with a memory-effector phenotype.¹³

We defined “NK-like” iNKT cells as CD161⁺6B11⁺ iNKT cells and found lower proportions of CD161⁺6B11⁺ iNKT cells compared with 6B11⁺ iNKT cells and 6B11⁺CD3⁺ iNKT cells (“T-like” iNKT cells). A previous study showed different NK cell markers expressed by iNKT cells and demonstrated that the majority of iNKT cells expressed CD161, while only a minority expressed CD56 and CD16. Furthermore, this study showed that CD161 was expressed on significantly higher frequencies of CD8⁺ or double negative iNKT cells compared with CD4⁺ iNKT cells. CD161 (KLRB1) is a C-type lectin receptor expressed on human NK cells, but also on iNKT and T cell subsets—especially on subgroups found in liver and gut.^{13–15,18} In humans, the CD161 receptor interacts with its ligand LLT1 and suppresses the cytotoxic potential of NK cells. Moreover, it was shown that an infection with cytomegalovirus leads to decreased expression of CD161 on NK cells.³⁴

In conclusion, we suggest that the most precise detection of iNKT cells should be based on 6B11-PE alone or in combination with CD3 and CD161-moAbs. Moreover, we found new detection methods for iNKT cells/subsets using 6B11 in combination with CD1d, V α 24, CD4 or CD8. Furthermore, we could demonstrate that 6B11 can be used to analyze iNKT cells of leukemia patients, knowing that there is no aberrant expression of 6B11 on blasts. Our results confirm the heterogeneity of iNKT cells, as we could show that there are various “T cell-like” and “NK cell-like” iNKT subsets. This highlights that the iNKT cell population consists of several phenotypically and potentially functionally different subsets with diverse surface markers. A previous study could demonstrate that neonatal CD4⁺, adult CD4⁺, and adult CD4⁻ iNKT cells are phenotypically diverse and show different effector/memory T-cell markers and NK cell markers. Neonatal CD4⁺ iNKT cells show a more naive phenotype and can be modified more easily in their capacity to acquire Th1-like or Th2-like functions compared with adult iNKT cells, which are mainly resistant to functional reprogramming, but show higher cytotoxic functions.³⁵

To detect NK and CIK cells, we used recommended combinations of CD3 with CD161 or CD56 moAbs. NK cells were defined as CD3⁻CD56⁺ or CD3⁻CD161⁺ cells and CIK cells were defined as CD3⁺CD56⁺ or CD3⁺CD161⁺ cells. Both combinations detect similar amounts of NK and CIK cells in healthy controls. However, CD56 is known to be aberrantly expressed on blasts of certain AML-subtypes and correlates with a worse prognosis.³⁶ In these cases, NK cells cannot be evaluated with a CD56-

marker. We recommend using the CD161-moAb to quantify NK cells in cases with aberrant expression of CD56. Therefore, CD161 can be regarded as a better NK and CIK cell marker for leukemic patients with aberrant CD56 expression.

In conclusion, we suggest that NK and CIK cells can always be detected with the combination of CD3 with CD161-moAbs and with CD3 and CD56-moAbs in cases without aberrant expression of CD56. The potential functional differences in these subsets, however, should be evaluated.

iNKT, NK, and CIK Cells in AML, ALL, and CLL Patients Compared With Healthy Samples

The present study shows that AML, ALL as well as CLL patients' MNC are characterized by (significantly) lower frequencies of CIK cells ($CD3^+CD56^+$ or $CD161^+$), NK cells ($CD3^-CD56^+$ or $CD161^+$) and iNKT cells ($6B11^+$, $6B11^+CD3^+$ or $CD8^+$ or $CD161^+$ or $V\alpha 24^+$) compared with healthy donors. This could be confirmed by another group that demonstrated significantly lower median values of NK cells, NKT, and iNKT cells in AML patients compared with healthy donors' PB (cells/ μ L: 303.47 vs. 101.54; 55.86 vs. 21.85; 0.515 vs. 0.0814).⁷ However, this group did not compare values of CIK cells in AML patients versus healthy and did not consider different NK and iNKT subsets: NK and NKT cells were detected with CD3 and CD16/56 and iNKT cells with CD3 in combination with $V\alpha 24$ and $V\beta 11$.⁷ In general, these findings can be expected due to the displacement of immune-reactive cells by uncontrolled proliferating and expanding blasts.

In summary, we could show that a high blast-load in AML, ALL or CLL patients directly correlated with low frequencies of iNKT, NK, and CIK cells. Therefore, the majority of iNKT, NK, and CIK cells is displaced by blasts in AML, ALL, and CLL patients.

An interesting finding was, that—although proportions of iNKT, NK, and CIK cells were comparable in leukemic patients, their subtype composition was different in leukemic patients compared with healthy donors showing a shift to (significantly) higher frequencies of T and NK cells coexpressing 6B11 in AML, ALL, and CLL patients compared with healthy donors. This might suggest that certain T/NK-derived iNKT cells in leukemic patients might either be subtypes with downregulated antileukemic functionality or could be special subsets that could be triggered in antileukemic reactivity.

In summary, we present the new finding that proportions of iNKT, NK, and CIK cells were significantly lower in AML, ALL, and CLL patients and compared with healthy donors. Their subset compositions as well as iNKT subsets were comparable in AML, ALL, and CLL patients. We can demonstrate in addition to findings in the literature a shift to higher proportions of T and NK cells expressing 6B11 in AML, ALL, and CLL patients compared with healthy donors, what might point to comparable mechanisms in leukemic diseases, that favor the extension of subsets in these diseases and what might help to develop new iNKT, NK, and CIK cell-based immune therapeutic strategies for leukemia patients.

Prognostic Relevance of iNKT, NK, and CIK Cells in AML, ALL, and CLL Patients

A previous study could show, that the overall number of iNKT cells in AML patients is an important prognostic factor as iNKT cell proportions >0.2 cells/ μ L are associated with favorable survival.⁷ Furthermore, this study could demonstrate, that a lower number of iNKT cells in PB in combination with higher proportions of $CD4^+$ and $CD8^+$ T cells in BM at diagnosis is characterized by a lower overall survival (OS) in AML patients. Another study could demonstrate that a high expression of NKp30/NKp46 on $CD16^+$ NK cells in elderly AML patients correlated with leukemia-free and better OS.³⁷ Moreover, previous studies pointed out that CIK cells (especially $CD3^+CD56^+$) show strong cytotoxic potential, for example against leukemia,^{4,30} but did not correlate the frequencies of CIK cells with survival or favorable prognosis for leukemia patients. With our results we confirm these findings and can add in addition, that higher percentages of iNKT, NK, and CIK cells correlate with the allocation of patients to diverse prognostically more favorable subgroups not only in AML, but also in ALL and CLL.

We could show, that higher frequencies of iNKT cells in leukemic patients correlate with prognostically better subsets in AML, adult ALL, and CLL patients. AML patients with favorable (vs. adverse) NCCN risk score showed higher percentages of $6B11^+$, $6B11^+CD161^+$, and $6B11^+Va24^+$ iNKT cells. Moreover, AML and CLL patients younger (vs. older) than 60 years showed higher numbers of $6B11^+$, $6B11^+CD161^+$, $6B11^+CD3^+$, and $6B11^+Va24^+$ iNKT cells. AML patients with primary (vs. secondary) AML showed higher frequencies of $6B11^+$ and $6B11^+CD3^+$ iNKT cells. Furthermore, AML and adult ALL patients without (vs. with) extramedullary foci showed higher frequencies of $6B11^+$, $6B11^+CD3^+$, $6B11^+CD161^+$, and $6B11^+Va24^+$ iNKT cells. Moreover, adult ALL patients achieving CR after GMALL induction chemotherapy (vs. NCR) and CLL patients with no need (vs. need) for therapy showed higher frequencies of $6B11^+$, $6B11^+CD3^+$, and $6B11^+Va24^+$ iNKT cells. AML and CLL patients with stable CR (vs. no stable CR: relapse or death by disease) showed (significantly) higher percentages of $6B11^+$, $6B11^+CD3^+$, and $6B11^+CD161^+$ iNKT cells, however, 1 patient had died from pneumonia. This data can confirm previous findings^{7,37,38} that low frequencies of iNKT cells can be correlated with worse prognosis and worse OS. In contrast to previous studies and our results, another study found that lower frequencies of NK and NK like T cells correlated with higher survival in AML patients, while higher frequencies were correlated with poor survival.^{7,37,38}

Moreover, we could show, that higher frequencies of NK cells in leukemia patients correlate with prognostically better subsets in AML, adult ALL, and CLL patients. AML patients without (vs. with) extramedullary foci showed (significantly) higher frequencies of $CD3^-CD56^+$ NK cells. Moreover, adult ALL patients, who had achieved CR after GMALL induction chemotherapy, showed higher numbers of $CD3^+CD161^+$ NK cells. Furthermore, AML and CLL patients, who stayed in stable CR (vs. no stable CR) showed higher amounts of $CD3^-CD161^+$ and $CD3^+CD56^+$ NK cells. Consistent to previous studies,^{7,37,38} our data show that higher percentages of NK cells

can be correlated with better prognosis and stabilization of disease. In contrast to a previous study, we could not correlate low frequencies of NK cells with better survival.^{7,37,38}

We can add in addition, that higher frequencies of CIK cells in leukemia patients correlate with prognostically better subgroups in AML and CLL patients. AML patients who achieved CR (vs. NCR) and CLL patients with no need for initial therapy (vs. need for initial therapy) showed (significantly) more CIK cells. Moreover, AML as well as ALL patients without extramedullary foci presented with significantly more CIK cells. Furthermore, AML and CLL patients, who stayed in stable CR (vs. no stable CR), showed higher numbers of CD3⁺CD161⁺ and CD3⁺CD56⁺ CIK cells. Consistent to previous studies,^{4,30} these data might point to an antileukemic reactivity of CIK cells—leading to stabilized disease.

We conclude that higher frequencies of iNKT, NK as well as CIK cells can be regarded as a favorable prognostic factor for AML, adult ALL, and CLL patients. For the future, we recommend to perform subtype-analyses of iNKT, NK as well as CIK cells in more detail to be able to allocate defined subtypes to prognostic groups. Moreover, we recommend multifactorial statistical analyses to work out the role of cellular partners involved in the mediation of anti-leukemic reactions and better prognosis for individual pts in more detail.

iNKT, NK, CIK Cells Contribute to Antitumor and Antileukemic Activity

A previous study has shown, that iNKT cells are important mediators in tumor-protection, as reduced frequencies of iNKT cells were correlated with a variety of cancers and increased frequencies of iNKT cells were related with favorable response to therapy.³⁹ Another study showed, that NK and CIK cells have cytotoxic potential against leukemia and other cancers.^{30,40–42}

In the current study, we could show in general, that higher proportions of iNKT, NK, and CIK cells and higher frequencies of proliferating T cells and T_{cm} correlated with antileukemic activity (blast-lysis). Moreover, we could show (although only with one exemplary experiment up to now), that radiation of stimulator-cells did not reduce proportions of T/iNKT/CIK and NK cells, but reduce the overall blast lysis of (stimulator cell activated) effector cell-mediated antileukemic activity compared with unirradiated settings—what could be explained by a functional knockout of these T/iNKT/CIK and NK cells and therefore point to their antileukemic contribution. Consistent, a previous study has shown that in knockout experiments in mice absence of iNKT cells was correlated with tumor-growth and poor survival, while a transfer of iNKT cells into mice could reduce tumor growth.³⁹ All of these results emphasize the relevance of iNKT/CIK and NK cells in antileukemic reactions. More studies have to be performed to work out the specific role of these cell fractions in the mediation of antileukemic reactions. To further analyze the function of different iNKT/CIK or NK cells and their subtypes we recommend performing, for example blocking experiments knocking out certain cellular subtypes and correlating results with antileukemic function.

With an attempt to evaluate the predictivity of iNKT/CIK and NK cell frequencies we defined cutoff values: we could show that cases presenting with higher values of iNKT (subtypes) or CIK or NK cells were characterized by

a higher chance to belong to the group with “antileukemic activity.”

In summary, we suggest to define and applicate cut-off values for iNKT/CIK and NK cells and their subtypes for leukemia-pts in the future to enable a refined estimation of prognosis.

Induction of iNKT, NK, and CIK Cells After Stimulation With DC/DC_{leu}

It was already shown, that a crosstalk between NK cells and DC improves antitumor reactions. Recently, details about the mechanisms behind the increase of antitumor-reactivity (eg, pathogen-associated molecular patterns or cytokines) have been detected.^{39,43} Moreover cytokines, cellular factors, antibodies promote NK cell activations, and DC-NK crosstalk establishing a micro-environment which enables antitumor reactions.^{44,45} Moreover, a crosstalk between CIK cells and DC has been reported recently, demonstrating an influence of a DC stimulation on the phenotype as well as antileukemic cytotoxicity of CIK cells.⁴⁶ Recently a crosstalk between DC and iNKT cells has been shown (mediated, eg, by chemokine receptor-expression), leading to an amplification of antitumor-immune reactions.^{4,47}

With our data, we contribute that iNKT cells not only respond to a stimulus with IL-2, but in addition are significantly expanded in cases with previous treatment of AML-or healthy MNC/WB with “cocktails.” Comparable phenomena were found for CIK cells. These finding might point to an induction of these cell-populations by DC/DC_{leu} stimulation.

Moreover, we can add important new data to a previous study that highlighted 3 possible mechanisms of antitumor-reactivity mediated by iNKT cells: indirect cytotoxicity, direct cytotoxicity and modulation of the tumor microenvironment. “Indirect cytotoxicity” means that iNKT cells and DC stimulate each other by TCR/CD1d and CD40/CD154 interactions leading to a release of cytokines (e.g. IL-10, IL-17A, IFN- γ , and IL-12) and increased antitumor activity of other effector cells.^{39,43} Mature DCs are known to produce IL-12 stimulating iNKT cells to increase IFN- γ release coactivating NK cells and their production of IFN- γ ,³⁹ what could in return lead to a release of pro-inflammatory cytokines by DC.⁴³ Our preliminary data obtained with some cases in part confirm these findings: increased release of cytokines (IL-17A, IL-10) correlated with achieved “lysis”/antitumor activity and with higher iNKT, NK and CIK cell frequencies—pointing to a cytokine-mediated antitumor activity.³⁹ Moreover, we could show that “highest” iNKT cell frequencies were correlated with higher DC—pointing to a coactivation between iNKT cells and DC.

Recently it was shown, that soluble as well as cellular components of the immune-system are involved in a “crosstalk” with NK/iNKT cells leading to a “cross-priming” in infectious diseases or cancer: for example, IL-21 improved cytotoxicity and increased IFN- γ production of NK cells against breast cancer cells,⁴⁸ TH17 frequencies directly correlated with iNKT frequencies and good prognosis in CLL⁴⁹ and iNKT cells were shown to be involved in cross-talk between DC and CD8⁺ cells—at least in infectious diseases.⁵⁰ These findings emphasize the importance to study these kinds of cross-talks in detail in future studies.

We can add in addition that the highest 6B11⁺ iNKT and CIK values were found after culture in prostaglandin-containing “cocktails.” This could point to a special effect of these “cocktails” on the allocation and recruitment of iNKT and CIK cells—resulting in an improved antileukemic activity—up to now an effect of Prostaglandin E₂ on the maturation of DC has been shown.^{19,28} Alternatively, it might be discussed, that “only” the 6B11 or CD56-antigens are upregulated on T/NK cells, however without expansion of functionally reactive iNKT/CIK cell populations.

Physiologic Culture-conditions (Hypoxia, WB) Do Not Have an Effect on the Generation of DC Nor on the Frequencies and Composition of iNKT, NK or CIK Cells

Preliminary experiments with MNC/WB cultured under hypoxic versus normoxic conditions did not yield an effect on the generation of DC nor on the frequencies and composition of iNKT, NK, and CIK cells after MLC. Although more experiments have to be performed we can assume, that working under normoxic conditions yields “physiological” data.

CONCLUSIONS

In conclusion, we recommend to regularly evaluate proportions of iNKT/NK/CIK cells and include specific markers in diagnosis panels (based on 6B11/CD161/CD56/CD3 antibodies) in AML/ALL/CLL for quantitative, qualitative, and prognostically relevant estimation of individual patients antileukemic potential. Detection of iNKT cells should be based on 6B11-(PE)-staining alone or in combination with (especially) CD3 and CD161; NK and CIK cell detection should be based on the combination of CD3 with CD161 or with CD56 (in cases without aberrant expression of CD56). As we could demonstrate a shift to higher proportions of T and NK cells expressing 6B11 in AML, ALL, and CLL patients compared with healthy donors, we suggest that mechanisms behind these subtype extensions have to be studied in detail. These findings might be considered in the development of iNKT-based immunotherapies.

Considering that higher frequencies of iNKT, NK, and CIK cells correlate with prognostically better subgroups and with antileukemic activity (blast lysis) in AML, adult ALL, and CLL patients, high amounts of iNKT, NK, and CIK cells can be regarded as a favorable prognostic factor in leukemic patients.

Moreover, proportions of iNKT and CIK cells increase under stimulation of MLC with IL-2 and particularly after culture with (prostaglandin-containing) DC/DC_{leu}-inducing “cocktails”. Cytokine profiling revealed a correlation of release of antitumor and anti-inflammatory cytokines with higher frequencies of iNKT, NK, and CIK cells after MLC, what correlated with improved antileukemic cytotoxicity. These findings not only point to a cross-talk between soluble factors and these immune reactive cells and DC, but in addition a correlation with an improved antileukemic reactivity. In conclusion, “cocktails” not only activate antileukemic T cells, but also iNKT and CIK cells and improve the antileukemic activity. Inclusion of prostaglandin-containing DC/DC_{leu}-generating methods might be especially promising in the recruitment of antileukemic active immune reactive cells.

Although the quantification of these small cell populations (using selected markers and special gating-

strategies in flow cytometric settings) has to be performed from experienced groups and the contribution of these cell populations to antileukemic reactions should not be underestimated.

Novel immunotherapeutic protocols in the treatment of pts with leukemia should be designed, that include the quantification of iNKT, NK, and CIK cells and their subtypes in diagnostic panels. Moreover, the role of iNKT, NK, and CIK cells as well as soluble factors in a cross-talk in DC/DC_{leu}-triggered antileukemic reactions in AML patients should be studied in detail and probably be included in immunotherapeutic protocols against AML.

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