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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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1 Zusammenfassung

Invariante natürliche Killer T Zellen (iNKT Zellen), natürliche Killerzellen (NK Zellen) sowie Zytokin-induzierte Killerzellen (CIK Zellen) und ihre Untergruppen sind maßgeblich an der Immunüberwachung beteiligt. Vor kurzem wurde für die Detektion von iNKT Zellen der monoklonale Antikörper „6B11“ entwickelt, welcher die CDR3-Region der invarianten Vα24Jα18-Kette des T-Zell-Rezeptors (TCR) von iNKT Zellen erkennt.

Im ersten Teil meiner Arbeit wurden iNKT, NK und CIK Zellen und deren Untergruppen in peripheren Blutproben von gesunden Spendern (n=9), Patienten mit akuter myeloischer Leukämie (AML; n=23), Patienten mit akuter lymphatischer Leukämie (ALL; n=20) und Patienten mit chronisch lymphatischer Leukämie (CLL; n=21) in akuten Krankheitsphasen untersucht und die Anzahl an iNKT, NK und CIK Zellen mit der Prognose der Patienten korreliert.

Die wichtigsten Ergebnisse waren:

I.1) Im Vergleich zu gesunden mononukleären Zellen (MNC) zeigten MNC von AML, ALL und CLL Patienten (signifikant) geringere Anteile an iNKT Zellen ($6B11^+$/ $6B11^+CD3^+$/ $6B11^+CD161^+$), NK Zellen ($CD3^-CD56^+$/ $CD3^-CD161^+$) und CIK Zellen ($CD3^+CD56^+$/ $CD3^+CD161^+$).

I.2) In AML, ALL und CLL Patienten konnten im Vergleich zu Gesunden (signifikant) höhere Anteile an $6B11^+$ iNKT Zellen in der $CD3^+$ T Zell- (iNKT Zellen vom „T Zell-Typ“) sowie in der $CD161^+$ NK Zell-Fraktion (iNKT Zellen vom „NK Zell-Typ“) gefunden werden.

I.3) Prognostisch günstige AML Untergruppen (Patienten unter 18 Jahren, primärer Krankheitsstatus, kein extramedullärer Befall, Erlangung und Erhaltung einer Vollremission (CR) nach Initialtherapie) korrelierten mit größeren Mengen an iNKT, NK und CIK Zellen. Ähnliche Zusammenhänge konnten auch bei (erwachsenen) ALL und CLL Patienten festgestellt werden.

Im zweiten Teil meiner Arbeit wurden heparinisierte Vollblut- (WB) und MNC-Proben von gesunden Spendern (n=10), Patienten mit AML (n=5) und von einem Patienten mit

Myelodysplastischem Syndrom (MDS; n=1) untersucht. Diese Proben wurden in der Zellkultur mit verschiedenen „Cocktails“ vorbehandelt, die die Konversion von leukämischen Zellen zu Dendritischen Zellen leukämischen Ursprungs (DC_{leu}, Leukämie-Antigenpräsentierende Zellen) induzieren. Als Kontrolle dienten unbehandelte, kultivierte WB- oder MNC-Proben. Sowohl unter Normoxie (mit 21% O_2) als auch unter physiologischen hypoxischen Bedingungen (ca. 10% O_2) wurden iNKT, NK und CIK Zellen und deren Untergruppen quantitativ und qualitativ vor und nach gemischter Lymphozytenkultur (MLC) mit T Zell-angereicherten WB- oder MNC-Proben untersucht. Als „Stimulator-Fraktion“ dienten mit „Cocktails“ vorbehandelte oder nicht vorbehandelte WB- oder MNC-Proben, als Effektorzellen dienten mit T Zellen angereicherte immun-reaktive WB- oder MNC-Proben. Mit einem Zytotoxizitätsassay wurde die blasten-lytische Aktivität von iNKT, NK, CIK und T Zellen nach der MLC untersucht.

Die wichtigsten Ergebnisse waren:

II.1) iNKT, NK und CIK Zellen nahmen nach der MLC unabhängig von den verwendeten „Cocktails“ / „Stimulator-Zellsuspensionen“ (unter dem Einfluss von Interleukin-2 (IL-2)) zu.

II.2) Die Vorbehandlung von WB oder MNC mit „Cocktails“ in der Zellkultur erhöhte die Anzahl von iNKT Zellen und veranlasste außerdem eine Verschiebung der Zusammensetzung der iNKT, NK und CIK Untergruppen nach der MLC, was mit einem verbesserten antileukämischen Potenzial korrelieren könnte.

II.3) Nach der Vorbehandlung mit verschiedenen „Cocktails“ (vor allem Prostaglandin-haltigen) zeigten individuelle WB- oder MNC-Proben variierende, jedoch höhere Zahlen an iNKT und CIK Zellen.

II.4) Die DC, iNKT, NK und CIK Zell Werte nach der MLC waren unter physiologischen Hypoxie- und Normoxie-Bedingungen vergleichbar.

II.5) Die Anzahl an T, iNKT, NK und CIK Zellen nach MLC korrelierte direkt mit antileukämischer, tumorlysierender Aktivität, was für eine Beteiligung dieser Zellen an antileukämischen Reaktionen spricht.

Zusammenfassend konnte gezeigt werden, dass gesunde MNC signifikant höhere Mengen an iNKT, NK und CIK Zellen im Vergleich zu leukämischen AML, ALL und CLL MNC enthalten. Außerdem konnte demonstriert werden, dass Untergruppen von iNKT Zellen in gesunden und leukämischen MNC-Proben unterschiedlich zusammengesetzt sind. Des

Weiteren konnte dargelegt werden, dass die Anzahl an iNKT, NK und CIK Zellen mit der Prognose von AML, ALL und CLL Patienten korreliert. Darüber hinaus konnte gezeigt werden, dass „Cocktail“ behandelte AML Zellen (die zu DC_{leu} umgewandelt wurden) nach MLC mit Effektorzellen (T Zell angereichterte MNC / WB) zu einer Verschiebung der Anzahl und Zusammensetzung von T, iNKT, NK und CIK Zellen führten. Dies korrelierte mit einer verbesserten antileukämischen Aktivität, was für eine Interaktion dieser Zellen spricht.

Das bedeutet, dass die Anzahl an iNKT, NK und CIK Zellen (basierend auf der Detektion mit den Antikörpern 6B11, CD161, CD56 und CD3) regelmäßig bei AML, ALL und CLL Patienten im Rahmen eines diagnostischen Panels gemessen werden sollte, um eine quantitative, qualitative und prognostisch relevante Abschätzung des antileukämischen Potenzials des individuellen Patienten detailliert zu erfassen und um mehr über ihre Rolle in der DC und DC_{leu} vermittelten Immunüberwachung zu lernen.

Die Inhalte dieser Arbeite werden im „Journal of Immunotherapy“ publiziert (derzeit im Druck).

2 Abstract

Invariant natural killer cells (iNKT cells), natural killer cells (NKT cells) and cytokine induced killer cells (CIK cells) and their subsets are important for immune-surveillance. Recently, a new monoclonal antibody '6B11' was developed for iNKT cell detection, which targets the Vα24Jα18 invariant T cell receptor (TCR) in the CDR3-region of iNKT cells.

In the first part of my work iNKT, NK and CIK cells and their subsets were analysed in PB-samples from healthy donors (n=9), patients with acute myeloid leukemia (AML; n=23), patients with acute lymphoid leukemia (ALL; n=20) and patients with chronic lymphoid leukemia (CLL; n=21) in acute disease-stages and findings were correlated with prognosis.

The main results were:

I.1) Compared to healthy 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 MNC from AML, ALL and CLL patients.

I.2) Compared to healthy MNC (significantly) higher proportions of $6B11^+$iNKT cells were found in the $CD3^+$ T cell- ('T cell-like' iNKT cells) and in the $CD161^+$NK cell fraction ('NK cell-like' iNKT cells) in AML, ALL and CLL patients.

I.3) Prognostically favorable AML subgroups (patients younger than 18 years, primary disease-status, no extramedullary disease, achievement and maintenance of complete remission (CR) after induction-chemotherapy) showed higher proportions of iNKT, NK and CIK cells. Comparable correlations were seen in (adult) ALL and CLL patients.

In the second part of my work heparinized whole blood-(WB) and MNC-samples from healthy donors (n=10), AML patients (n=5) and one patient with myelodysplastic syndrome (MDS; n=1) were analysed. Samples were pre-treated with different 'cocktails' in the cell culture, which induce the conversion of leukemic cells to dendritic cells of leukemic origin (DC_{leu}, leukemia-antigen-presenting cells). Not pre-treated, cultured WB- or MNC-samples served as control. iNKT, NK and CIK cells and their subsets were quantitatively and qualitatively analysed before and after mixed-lymphocyte-cultures (MLC) of T cell enriched WB- or MNC-samples under normoxia (21% O_2) as well as under physiological hypoxia (approx. 10% O_2). 'Cocktail' pre-treated or not pre-treated WB- or MNC-samples served as a 'stimulator-suspension', T cell enriched immune-reactive WB- or MNC-samples served as

effector cells. Blast-lytic activity of iNKT, NK, CIK and T cells after MLC was examined using a cytotoxicity (fluorolysis) assay.

The main results were:

II.1) iNKT, NK and CIK cells increased after MLC independent of the applied ‘cocktail’ / ‘stimulator cell suspension’ (under the influence of IL-2).

II.2) Pretreatment of MNC- or WB-samples with ‘cocktails’ in the cell culture increased frequencies of iNKT cells and led to a shift in the composition of iNKT, NK and CIK cell subsets after MLC, what might correlate with an improved antileukemic potential.

II. 3) Individual samples showed varying, however higher iNKT and CIK cell frequencies after pretreatment with different (especially prostaglandin-containing) ‘cocktails’.

II.4) DC, iNKT, NK and CIK cell values after MLC were comparable in physiological hypoxia vs normoxia.

II.5) Frequencies of T, iNKT, NK and CIK cells after MLC correlated directly with antileukemic, blast-lytic activity – pointing to an involvement of these cells in antileukemic reactions.

In summary, healthy MNC presented with significantly higher iNKT, NK and CIK cells compared to leukemic AML, ALL and CLL MNC. Moreover, subtypes of iNKT cells differ in healthy vs leukemic samples. Furthermore, frequencies of iNKT, NK and CIK cells correlated with prognosis of patients with AML, ALL and CLL. In addition, ‘cocktail’ pre-treated AML-blasts (resulting in DC_{leu}) after MLC with effector cells (T cell enriched WB / MNC) led to a shift in T, iNKT, NK and CIK cell counts and compositions. These findings correlated with improved antileukemic activity against AML-blasts - pointing to a cross-talk of these cells.

Proportions of iNKT, NK and CIK-cells (based on detection with 6B11/ CD161/ CD56/ CD3 antibodies) should regularly be evaluated in AML, ALL and CLL diagnosis-panels for quantitative, qualitative and prognostically relevant estimation of individual pts’ antileukemic potential in detail and to learn about their role in DC/DC_{leu} triggered immune-surveillance.

The contents of this work will be published in the “Journal of Immunotherapy” (in press).

3 Introduction

3.1 Acute myeloid, acute lymphoid and chronic lymphoid leukemia

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 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 to 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]

3.2 T, iNKT, NK, CIK cells and DC/ DC_{leu} and their role in immune surveillance

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 immunological 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 MHC-I- or MHC-II- (major-histocompatibility-complex) molecules via antigen presenting cells (APCs).[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 (e.g.: naive (T_{naive}, $CD45RO^{-}$), non-naive ($T_{non\text{-}naïve}$, $CD45RO^{+}$), central-memory (T_{cm}, $CD45RO^{+}CCR7^{+}$), effector/memory ($T_{eff\text{-}em}$,

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-, autoimmune- and antimicrobial responses and tumor surveillance[4]: 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 T cell receptor 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 two 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 Vα24-Jα18 and Vβ11, while ß-chains have a limited variety. On the other hand, 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 cross-regulate of each other.[4]

3.2.1 Origin of iNKT-cells

NKT cells originate from a precursor pool of CD4^{+}CD8^{+} double positive (DP) thymocytes that have to undergo diverse TCR gene rearrangements, are then positively selected by CD1d^{+} DP thymocytes in the thymic cortex, undergo four 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 (DN) and a small population is $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$, but $CD8^+$subsets only exist in humans (not in mice), predominantly in healthy persons with latent EBV infection.[12]

3.2.2 Detection methods of iNKT cells

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 (α-GalCer) or monoclonal antibodies (moAbs) against the Vα24- and Vβ11-chains, what however, can lead to an overestimation of iNKT cells, as non-invariant, non-CD1d restricted $V\alpha24^+$ T cells can also pair with Vß11. Recently iNKT cells were shown to be identified using the moAb 6B11, which recognizes the invariant CDR3 loop of their Vα24Jα18 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 since 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.[14-16] [17,18]

3.3 Stimulation by DC/DC_{leu}

Considering that the antileukemic function of T cells can be stimulated by leukemia-derived DC (DC_{leu})[9,10], our supposion 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 (e.g.: CD13, CD33, CD117) and DC antigens (e.g.: CD80, CD83, CD86).[19,20]

3.4 Influence of hypoxic conditions on immune-reactive cells

Physiological conditions in the stem-cell niche of the BM as well as in the PB are hypoxic with Oxygen (O_2) concentrations between 0.1 to 0.6 % in the BM, 12% in arterial blood and 4-15% in PB.[21,22] Previous studies suggest an influence of the oxygen partial pressure (pO_2) on several pathophysiological mechanisms.[23,24] However, hematopoiesis takes place under physiologically low pO_2/ O_2 concentration and hematopoietic cells are continually exposed to dynamic pO_2 values.[25] Moreover, a recent in vitro study showed that hypoxia might have an enhancing effect on NK cells.[26]

3.5 Objectives of this study

The aim of this study was

1) to test the suitability of different markers and combinations to characterize and quantify iNKT, NK and CIK cells and subsets,

2) to quantify iNKT, NK and CIK subsets in MNC from patients with AML, ALL and CLL compared to healthy controls,

3) to correlate findings with disease entities and prognostic subgroups,

4) to quantify iNKT, NK and CIK subsets as well as T cells under stimulation with DC/ DC_{leu} that are generated from WB or MNC with various ‘cocktails’.

4 Material and Methods

Sample preparations and evaluations, characterization of cells by fluorescence-activated cell-sorting Flow-Cytometer and Cell-Quest-data-acquisition and analysis-software as well as statistical analyses of our results were carried out by me.

Part of the experimental work from the first part of the experiments was performed by cand. Dr. med. Wishnu Sutanto and Dr. med. dent. Thomas Guenther, who have both been working in the laboratory of Prof. Dr. rer. nat. Helga Schmetzer (University Hospital of Munich, Department of Hematopoetic Cell Transplantation, Medical Department 3).

Part of the experimental work from the second part of the experiments was fulfilled by cand. Dr. med. Daniel Christoph Amberger and cand. Dr. rer. biol. hum. Fatemeh Doraneh-Gard, who are working in the laboratory of Prof. Dr. rer. nat. Helga Schmetzer (University Hospital of Munich, Department of Hematopoetic Cell Transplantation, Medical Department 3).

Clinical data of the patients were provided by Dr. med. Joerg Schmohl (Universtiy Hospital of Tuebingen, Department of Hematology and Oncology), Dr. med Schuster (University Hospital of Duesseldorf, Department of Pediatric Hematology, Oncology and Clinical Immunology) and physicians of the University of Munich, Augsburg and Oldenburg.

4.1 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, Berlin, Germany). $CD3^+$ T cells were enriched using the MACS-technology (Milteney Biotech, Bergisch Gladbach, Germany). The purity of $CD3^+$ T cells was on average (Ø) 89% (range 69-98%). Cells were quantified using Neubauer-counting-chambers, used directly or frozen and thawed according to standardized protocols.

4.2 Patients´ characteristics and diagnostics

4.2.1 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 Tubingen, Dusseldorf, Munich, Oldenburg and Augsburg. Cells were obtained by aspirates of peripheral blood (PB) or bone marrow (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, 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.

Diagnosis and classification of **AML** patients (**Table 1**) was based on the 'French-American-British" (FAB) classification: minimally differentiated AML (M0: n=1), AML without

Table 1: Uncultured AML samples studied for proportions and co-expression 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 SCT [1]	Resp. to ind. chemo.
P1050	32/f	s/M1	dgn.	PB	**117,33,**13	78	46,XX	n.d.	CR	n.d.
P1053	25/m	p/M1	rez.	PB	**117,34,**33,56	65	n.d.	n.d.	NCR	n.d.
P1056	27/m	p/M4	dgn.	PB	**117,34,**33,13,15	53	46,XY	n.d.	n.d.	CR
P1057	32/m	p/M5	dgn.	PB	**117,34,**33,13,14,15,38,2	39	inv(16)pq, +22	favorable	n.d.	n.d.
P1058	71/m	p/M1	dgn.	PB	**117,34,**33,13,15,38,45,71,w65	91	+11	intermediate	n.d.	n.d.
P1059	66/m	p/M4	dgn.	PB	**117,34,**33,13,7	84	del(5)qq, add(17)p, add(21)q, der(9)	adverse	n.d.	CR
P1060	81/f	s/n.d.	dgn.	PB	**117,34,**33,13	97	n.d.	n.d.	n.d.	n.d.
P1061	62/f	p/M0	rel. a. SCT	PB	**117,34,**33,13	96	46,XX	intermediate	NCR	n.d.
P1062	41/f	p/M4	dgn.	PB	**117,34,**33,13,14,15,71	61	t(6;9)pq, add(21), -7, del(8), del(15), +mar	adverse	n.d.	NCR
P1063	26/m	p/M5	rel. a. SCT	PB	**117,34,**2	86	n.d.	n.d.	n.d.	n.d.
P1066	60/m	s/M2	dgn.	PB	**117,34,**33,65	74	46,XY	adverse	n.d.	n.d.
P1067	27/m	p/M4eo	dgn.	PB	**117,34,**33,13,15,65	41	inv(16)pq, +8	favorable	n.d.	CR
P1068	76/m	p/M2	dgn.	PB	**117,34,**33,13	39	n.d.	intermediate	n.d.	n.d.
P1069	33/m	p/M4eo	dgn.	PB	**117,34,**33,13,15	61	inv(16)pq, t(12;17)pq	favorable	n.d.	CR
P1071	71/f	s/M1	dgn.	PB	**117,34,**33,13,15	60	+4	intermediate	n.d.	CR
P1072	58/m	p/M2	pers.	PB	**117,34,**33,13	70	-4,-5,-7,-11,-12,-14,-16,-21,+mar	adverse	n.d.	NCR
P1073	23/m	p/M5	dgn.	PB	**117,34,**33,13,15,7,65	18	+8,+13	intermediate	n.d.	n.d.
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	n.d.	n.d.
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	n.d.	CR
P1085	71/f	p/M2	dgn.	PB	**117,33,**56	96	46,XX	intermediate	n.d.	n.d.

f=female; m=male; n.d.=no data; PB=peripheral blood; dgn =first diagnosis; pers.=persisting disease; rel.a.SCT = relapse after stem cell transplantation; ind. chemo. = induction chemotherapy; [1]complete remission (CR) achieved or not achieved (NCR) after treatment with SCT; [2]at dgn.; bold letter= antibodies used for (co)expression analyses

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). Pts presented with pAML (n=18) or with sAML (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 five patients, no further data were available.

The subtypes of the 19 **ALL** (**Table 2**) patients were immune cytologically classified according to the European Group for Immunophenotyping of Leukemias (EGIL) 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 pALL (n=5) or with sALL (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.

Table 2: Uncultured ALL samples 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	Risk Score (GMALL)	Resp. to SCT [1]	Resp. to ind. chemo.
	P1106	**24**/f	p/BII+My	dgn.	PB	**34,19,**33,133,13	81	46,XX	**high risk**	n.d.	**CR**
	P1107	**31**/m	s/BII+My	dgn.	PB	**34,19,**33,10,24	36	t(9;11)qq	**highest risk**	n.d.	**CR**
	P1108	**32**/m	s/TIII	dgn.	PB	**5,2,**1a,15,cy3	98	46,XY	**standard**	n.d.	**CR**
	P1109	**21**/m	p/BII+My	dgn.	PB	**34,19,**33,13,10,38	85	46,XY	**standard**	n.d.	**CR**
	P1110	**50**/f	p/BIII+My	dgn.	PB	**34,133,**33	59	t(9;22)qq, der(22)	**highest risk**	n.d.	**CR**
	P1111	**26**/m	s/BII	rel. a. SCT	PB	**34,19,**10	64	del(11q)	n.d.	n.d.	n.d.
	P1112	**37**/m	s/BII	rel. a. SCT	PB	**34,19,**10	17	n.d.	n.d.	n.d.	n.d.
	P1113	**64**/f	/B	rez./pers.	PB	**34,19,**15		n.d.	n.d.	n.d.	n.d.
	P1114	**22**/m	p/BIII	dgn.	PB	**19,20,**34,38	97	46,XY	**highest risk**	n.d.	**CR**
ALL	P1115	**45**/m	p/TIII	dgn.	PB	**5,7,**38,71	99	46,XY	**standard**	n.d.	**CR**
	P1120	**20**/m	s/TII	dgn.	PB	**19,5,**34,20,3	28	46,XY	n.d.	**CR**	n.d.
	P1121	**25**/f	s/BI	dgn.	PB	**34,19,** 33	32	n.d.	n.d.	n.d.	n.d.
	P1122	**23**/m	s/TIII	dgn.	PB	**4,8,**1,2,5,7	86	46,XY			
	P1129	**11**/m	s/BII	dgn.	BM	**34,19,**10,22	85	46,XY	n.d.	n.d.	**CR**
	P1132	**12**/m	s/BIII	dgn.	BM	**19,10,**22	84	n.d.	n.d.	n.d.	**CR**
	P1133	**3**/f	s/BII	dgn.	BM	**34,19,**10,22	55	46,XX	n.d.	n.d.	**CR**
	P1135	**17**/m	s/TI	dgn.	BM	**34,7,**4,5,10,13,33	98	46,XY	n.d.	n.d.	**CR**
	P1136	**5**/f	s/TIII	dgn.	PB	**34,7,**1a,2,3,5,10	82	46,XX	n.d.	n.d.	**CR**
	P1137	**3**/f	s/BII	dgn.	BM	**34,10,**19,22	71	46,XX	n.d.	n.d.	**CR**
	P1146	**8**/f	s/TIV	dgn.	PB	**7,3,**1,34	98	46,XX	n.d.	n.d.	**CR**

f=female; m=male; n.d.=no data; PB=peripheral blood; dgn =first diagnosis; pers.=persisting disease; rel.a.SCT = relapse after stem cell transplantation; ind. chemo. = induction chemotherapy; [1]complete remission (CR) achieved or not achieved (NCR) after treatment with SCT; bold letter= antibodies used for (co)expression analyses

All **CLL** patients (**Table 3**) 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).

Table 3: Uncultured CLL samples 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	Risk Score (BINET)	Resp. to SCT [1]	Need for initial. th.
	P1088	**44**/f	p/B-CLL	pers.	PB	**5,19,**20	98	n.d.	**A**	n.d.	**yes**
	P1089	**54**/m	p/B-CLL	pers.	PB	**5,19**	95	n.d.	**B**	n.d.	**yes**
	P1090	**43**/m	p/B-CLL	dgn.	PB	**5,19,**kappa	95	add(1)q, del(9)q,del(1	**A**	n.d.	**yes**
	P1091	**68**/m	p/B-CLL	pers.	PB	**5,19,**20	96	del(13)q	**A**	n.d.	**no**
	P1092	**66**/m	p/B-CLL	pers.	PB	**5,19,**20	71	n.d.	**B**	n.d.	**no**
	P1093	**51**/f	p/B-CLL	pers.	PB	**5,23,**kappa	94	n.d.	**A**	n.d.	**no**
	P1094	**67**/m	p/B-CLL	pers.	PB	**5,19,**kappa	91	n.d.	**A**	n.d.	**no**
	P1095	**65**/f	p/B-CLL	pers.	PB	**5,19**	88	del(13)q	**A**	n.d.	**yes**
	P1096	**64**/m	p/B-CLL	pers.	PB	**5,19,**lambda	95	n.d.	**A**	n.d.	**no**
	P1097	**72**/f	p/B-CLL	pers.	PB	**5,19,**20,22,kappa	97	n.d.	**A**	n.d.	**yes**
CLL	P1098	**60**/f	p/B-CLL	pers.	PB	**5,19,**kappa	93	n.d.	**B**	n.d.	**no**
	P1099	**67**/m	p/B-CLL	dgn.	PB	**5,19,**20,23,lambda	89	n.d.	**A**	n.d.	**no**
	P1100	**36**/m	p/B-CLL	pers.	PB	**5,19,**lambda	91	del(17)p, der(11)q	**B**	n.d.	**yes**
	P1101	**52**/m	p/B-CLL	pers.	PB	**5,19,**20	96	n.d.	**A**	n.d.	**yes**
	P1102	**45**/f	p/B-CLL	pers.	PB	**5,19**	91	del(13)q	**A**	n.d.	**yes**
	P1103	**67**/f	p/B-CLL	pers.	PB	**5,19,**20	87	del(17)q	**A**	n.d.	**yes**
	P1104	**66**/f	p/B-CLL	pers.	PB	**5,19,**kappa	94	n.d.	**A**	n.d.	**yes**
	P1116	**66**/f	p/B-CLL	pers.	PB	**5,19,**kappa	96	t(8;13)qq	**A**	n.d.	**yes**
	P1117	**33**/m	p/B-CLL	pers.	PB	**5,19,**23,kappa	40	del(13)q	**B**	n.d.	**yes**
	P1118	**60**/f	p/B-CLL	pers.	PB	**5,19,**20,22,23,kappa	32	del(11)q	**C**	n.d.	**yes**
	P1119	**55**/f	p/B-CLL	pers.	PB	**5,19,**20,22,23,38,kappa	57	n.d.	**B**	**CR**	**yes**

f=female; m=male; n.d.=no data; PB=peripheral blood; dgn =first diagnosis; pers.=persisting disease; rel.a.SCT = relapse after stem cell transplantation; th. = therapy; [1]complete remission (CR) achieved or not achieved (NCR) after treatment with SCT; [2]at dgn.; bold letter= antibodies used for (co)expression analyses

4.2.2 Cell-lines included for surface marker analyses

The following cell lines (**Table 4**) 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 (ATCC) and cultured according to manufacturer's instructions.

Table 4: Cell lines 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	n.d.	**33,**13,2,3,4,5,6,7,34

f=female; m=male; n.d.=no data; PB=peripheral blood; bold letter= antibodies used for (co)expression analyses

4.2.3 AML patients and samples included for culture-experiments

Cellular composition of immune reactive cells in MNC or WB samples from 5 AML patients, 1 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 (**Table 5**).

Table 5: 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	n.d.
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	n.d.
P1434	61/f	s/n.d.	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)

f=female; m=male; n.d.=no data; PB=peripheral blood; dgn. = first diagnosis; rez. = relapse; FAB = "French-American-British" classification; bold letter= antibodies used for (co)expression analyses

Cell cultures were either performed under 'normoxic conditions' (37°C, 5% CO_2 and 21% O_2). 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% CO_2 and with either varying O_2-concentrations between 0-17% during the incubation time in some cases or with a defined O_2-concentration of 6 or 10%) using an InVivo400 working station (Ruskinn Technology, Bridgend, United Kingdom).

4.3 DC generation from isolated MNC or WB

DC/ DC_{leu} were generated from 4-5x10^6 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, 'Pici1' or 'Pici2' (**Table 6**).

Table 6: Overview of the different DC-generating methods/Kits ('cocktails')

'Picibanil 1' ('Pici1')	GM-CSF IL-4 OK-432 PGE_2	**GM-CSF:** induction of myeloid (DC-) differentiation	7-10 days
'Picibanil 2' ('Pici2')	GM-CSF IL-4 OK-432 PGE_1	**IL-4:** induction of DC-differentiation **Picibanil (OK-432):** lysis product from streptococcus pyogenes; stimulates DC-differentiation	7-10 days
Kit-D (D)	GM-CSF OK-432 PGE_2		7-10 days
Kit-I (I)	GM-CSF OK-432	**PGE_2**: increases CCR7-expression and enhances migration	7-10 days
Kit-K (K)	GM-CSF PGE_2		7-10 days
Kit-M (M)	GM-CSF PGE_1	**PGE_1:** effects are comparable to PGE_2	7-10 days

GM-CSF granulocyte-macrophage-colony-stimulating factor; IL-4 interleukin 4; OK-432 Picibanil; PGE_2 prostaglandin E_2; PGE_1 prostaglandin E_1; references [19,20,29]

Therefore, cells were pipetted into 12-multiwell-tissue-culture-plates (ThermoFisher Scientific, Darmstadt, Germany) and were diluted in 2ml 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.[20,28] 500µl 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 six different DC-generating methods: 'Pici1', 'Pici2', Kit-D, -I, -K and -M (**Table 6**). 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 severed 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'.[20,28]

4.3.1 'Picibanil 1' ('Pici 1')

DC were generated from MNC or WB with the DC-generating protocol 'Pici1'-containing 500U/ml granulocyte macrophage colony stimulation factor (GM-CSF, Sanofi-Aventis, Frankfurt, Germany) and 250U/ml Interleukin-4 (IL-4) (PeproTech, Berlin, Germany). After 6-7 days, 10µg/ml Picibanil (OK 432) - a lysis product from Streptococcus pyogenes which has unspecific immune modulatory effects (Chugai Pharmaceutical Co., Kajiwara, Japan) and 1µg/ml Prostaglandin E_2 (PGE_2) (PeproTech, Berlin, Germany) were added.[19,29] After 7-10 days of incubation cells were harvested and used for further experiments.

4.3.2 'Picibanil 2' ('Pici 2')

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 PGE_2 by PGE_1 (PeproTech, Berlin, Germany).

4.3.3 Kit-D

The generation of DC from MNC or WB with Kit-D was performed using 800U/ml GM-CSF, 10µg/ml Picibanil and 1µg/ml PGE_2 [20]. After 2-3 days, the same amounts of cytokines were

added and after in total 7-10 days of incubation cells were harvested and used for subsequent experiments.

4.3.4 Kit-I

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

4.3.5 Kit-K

Kit-K consisted of 800U/ml GM-CSF and 1µg/ml PGE_2 [20]. Incubations were performed in analogy to Kit-D.

4.3.6 Kit-M

For the generation of DC with Kit-M, 800U/ml GM-CSF and 1µg/ml PGE_1 were added to the culture [20]. Incubations were performed in analogy to Kit-D.

4.4 Mixed-lymphocyte-culture (MLC) of T cell enriched immune reactive cells with 'cocktail' pretreated or not pretreated stimulator cell suspensions from MNC or WB

Immune reactive cells were enriched with $1x10^6$ positively selected $CD3^+$ T cells (effector cells) from AML patients or healthy controls and co-cultured in 24-multiwell-tissue-culture-plates (ThermoFisher Scientific, Darmstadt, Germany) with a stimulator cell suspension containing $2.5x10^5$ DC/ DC_{leu} ($MLC^{*'MNC\text{-}DC'}$ or $MLC^{*'WB\text{-}DC'}$ which were generated with different 'cocktails'. The same setting, but with a stimulator cell suspension without pretreatment with 'cocktails' ($MLC^{*'MNC'}$ or $MLC^{*'WB'}$) severed as a control. In one parallel tested case the stimulator cell suspensions were irradiated with 33Gy to inactivate residual immune reactive cells (e.g. iNKT/ NK and CIK cells). The total volume of the cell culture was adjusted to 1ml

with RPMI-1640 medium (Biochrom, Berlin, Germany) containing 1% Penicillin (Biochrom, Berlin, Germany) and 50U/ml IL-2 (PeproTech, Berlin, Germany). The MLC$^{*\text{'MNC'}}$ or MLC$^{*\text{'MNC-DC'}}$ further contained 15% human serum (Healthcare Europe GmbH, Vienna, Austria). After 2-3 days 50U/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 and WB fractions were quantified by flow cytometry (**Table 7**).

4.5 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, Darmstadt, Germany ([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[e], 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 manufacturer's instructions. To stain cells (MNC or WB) with moAbs they were resuspended in PBS (Biochrom, Berlin, Germany), containing 5-10% fetal calf-serum (FCS, Biochrome, Berlin, Germany) to avoid unspecific bindings and were incubated for 15 minutes in the dark at room-temperature. Afterwards cells were washed, centrifuged and resuspended in 100-200µl PBS. At least 5000 events were evaluated with a fluorescence-activated cell-sorting Flow-Cytometer (FACSCalibur^{TM}) and Cell-Quest-data-acquisition and analysis-

software (Becton Dickson, Heidelberg, Germany). Isotype controls were conducted according to manufacturer's instructions.

For the analysis and quantification of T, iNKT, NK and CIK cells/ subtypes were quantified in the total cell-fraction (e.g: $CD3^+$ cells) or in the subpopulations (e.g: $6B11^+CD3^+$). According to their expression profile we quantified proportions of immune reactive cells as given in **Table 7-10**: T cells: $CD3^+$, $CD8^+$, $CD4^+$, $V\alpha24^+$, $CD1d^+$ T-cells, T_{naive}, $T_{non\text{-}naïve}$, T_{cm} or $T_{eff\text{-}em}$.[9,10,13,14] CIK cells: $CD3^+CD56^+$ or $CD3^+CD161^+$ cells[5]. NK cells: $CD3^-CD56^+$ or $CD3^-CD161^+$ cells.[5,13] ($6B11^+$) iNKT cells: $6B11^+CD3^+$, $6B11^+CD161^+$, $6B11^+V\alpha24^+$, $6B11^+CD4^+$, $6B11^+CD8^+$, $6B11^+CD1d^+$, $6B11^+CD45RO^+$ or $6B11^+CD45RO^-$ iNKT cells[11,13].

Table 7: Subtypes of T cells as evaluated by flow cytometry

Names of subgroups	referred to	Surface marker	Abbreviation	Explanatory note/premise
$CD3^+$ pan-T-cells	MNC(WB) or MLC[1]	$CD3^+$	$CD3^+$/MNC(WB) or MLC	
$CD4^+$-coexpressing T-cells	MNC(WB) or MLC	$CD3^+CD4^+$	$CD4^+$/MNC(WB) or MLC	$CD4^+$ T-cells
$CD4^+$-coexpressing T-cells	$CD3^+$	$CD3^+CD4^+$	$CD4^+/CD3^+$	$CD4^+$ T-cells
$CD8^+$-coexpressing T-cells	MNC(WB) or MLC	$CD3^+CD8^+$	$CD8^+$/MNC(WB) or MLC	$CD8^+$ T-cells
$CD8^+$-coexpressing T-cells	$CD3^+$	$CD3^+CD8^+$	$CD8^+/CD3^+$	$CD8^+$ T-cells
naive T-cells	MNC(WB) or MLC	$CD3^+CD45RO^-$	T_{naive}/MNC(WB) or MLC	Unprimed T-cells
naive T-cells	$CD3^+$	$CD3^+CD45RO^-$	$T_{naive}/CD3^+$	Unprimed T-cells
non-naive T-cells	MNC(WB) or MLC	$CD3^+CD45RO^+$	$T_{non\text{-}naive}$/MNC(WB) or MLC	Memory + effector T-cells
non-naive T-cells	$CD3^+$	$CD3^+CD45RO^+$	$T_{non\text{-}naive}/CD3^+$	Memory + effector T-cells
central (memory) T-cells	MNC(WB) or MLC	$CD3^+CCR7^+CD45RO^+$	T_{cm}/MNC(WB) or MLC	Long-term immunity
central (memory) T-cells	$CD3^+$	$CD3^+CCR7^+CD45RO^+$	$T_{cm}/CD3^+$	Long-term immunity
effector (memory) T-cells	MNC(WB) or MLC	$CD3^+CCR7^-CD45RO^+$	T_{ems}-T_{effs}/MNC(WB) or MLC	
effector (memory) T-cells	$CD3^+$	$CD3^+CCR7^-CD45RO^+$	T_{ems}-$T_{effs}/CD3^+$	
proliferating T-cells	MNC(WB) or MLC	$CD3^+CD69^+$	T_{prol}/MNC(WB) or MLC	proliferating T-cells
proliferating T-cells	$CD3^+$	$CD3^+CD69^+$	$T_{prol}/CD3^+$	proliferating T-cells
Viable T-cells	MNC(WB) or MLC	$CD3^+7AAD^-$	T_{via}/MNC(WB) or MLC	
Viable T-cells	$CD3^+$	$CD3^+7AAD^-$	$T_{via}/CD3^+$	
$TCRV\alpha24^+$-coexpressing T-cells	MNC(WB) or MLC	$CD3^+V\alpha24^+$	$V\alpha24^+$/MNC(WB) or MLC	Vα24 segment joined with Jα18
$TCRV\alpha24^+$-coexpressing T-cells	$CD3^+$	$CD3^+V\alpha24^+$	$V\alpha24^+/CD3^+$	Vα24 segment joined with Jα18
$CD1d^+$-coexpressing T-cells	MNC(WB) or MLC	$CD3^+CD1d^+$	$CD1d^+$/MNC(WB) or MLC	$CD1d^+$ is a MHC class I-like molecule
$CD1d^+$-coexpressing T-cells	$CD3^+$	$CD3^+CD1d^+$	$CD1d^+/CD3^+$	$CD1d^+$ is a MHC class I-like molecule

[1]total proportions of cell-subsets in uncultured MNC, WB or in MLC (after culture with T cells and IL-2)

Table 8: Subtypes of CIK cells as evaluated by flow cytometry

Names of subgroups	referred to	Surface marker	Abbreviation	Explanatory note/premise
$CD3^+CD56^+$ CIK-cells	MNC(WB) or MLC	$CD3^+CD56^+$	$CD3^+CD56^+$/MNC(WB) or MLC	Cells expressing T-cell (CD3) and
$CD3^+CD161^+$ CIK-cells	MNC(WB) or MLC	$CD3^+CD161^+$	$CD3^+CD161^+$/MNC(WB) or MLC	NK-cell (CD56,CD161) markers
$CD3^+$ $CD161^+$ CIK-cells	$CD3^+$	$CD3^+CD161^+$	$CD3^+CD161^+/CD3^+$	

Table 9: Subtypes of NK cells as evaluated by flow cytometry

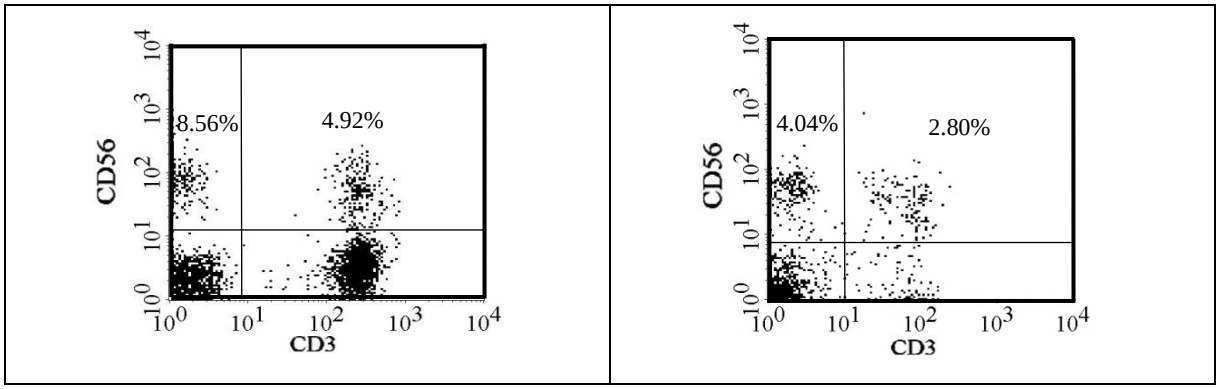
Names of subgroups	referred to	Surface marker	Abbreviation	Explanatory note/premise
$CD3^-CD56^+$ NK-cells	MNC(WB) or MLC	$CD3^-CD56^+$	$CD3^-CD56^+$/MNC(WB) or MLC	Cells expressing NK markers (CD56, CD161), but not expressing T-cell markers (CD3)
$CD3^-CD161^+$ NK-cells	MNC(WB) or MLC	$CD3^-CD161^+$	$CD3^-CD161^+$/MNC(WB) or MLC	
$CD3^-CD161^+$ NK-cells	$CD161^+$	$CD3^-CD161^+$	$CD3^-CD161^+/CD161^+$	

Table 10: Subtypes of iNKT cells as evaluated by flow cytometry

Names of subgroups	referred to	Surface marker	Abbreviation	Explanatory note/premise
$6B11^+$(PE) iNKT-cells	MNC(WB) or MLC	$6B11^+$	$6B11^+$(PE)/MNC(WB) or MLC	6B11 recognizes the invariant CDR3 loop of the TCR α-chain of iNKT cells
$6B11^+$(FITC) iNKT-cells	MNC(WB) or MLC	$6B11^+$	$6B11^+$(FITC)/MNC(WB) or MLC	
$CD3^+$ coexpressing $6B11^+$ iNKT-cells	MNC(WB) or MLC	$6B11^+CD3^+$	$6B11^+CD3^+$/MNC(WB) or MLC	$6B11^+CD3^+$ iNKT-cells
$CD3^+$ coexpressing $6B11^+$ iNKT-cells	$CD3^+$	$6B11^+CD3^+$	$6B11^+CD3^+/CD3^+$	$6B11^+CD3^+$ iNKT-cells
$CD4^+$ coexpressing $6B11^+$ iNKT-cells	MNC(WB) or MLC	$6B11^+CD4^+$	$6B11^+CD4^+$/MNC(WB) or MLC	$6B11^+CD4^+$ iNKT-cells
$CD4^+$ coexpressing $6B11^+$ iNKT-cells	$CD4^+$	$6B11^+CD4^+$	$6B11^+CD4+/CD4+$	$6B11^+CD4^+$ iNKT-cells
$CD8^+$ coexpressing $6B11^+$ iNKT-cells	MNC(WB) or MLC	$6B11^+CD8^+$	$6B11^+CD8^+$/MNC(WB) or MLC	$6B11^+CD8^+$ iNKT-cells
$CD8^+$ coexpressing $6B11^+$ iNKT-cells	$CD8^+$	$6B11^+CD8^+$	$6B11^+CD8^+/CD8^+$	$6B11^+CD8^+$ iNKT-cells
$CD1d^+$ coexpressing $6B11^+$ iNKT-cells	MNC(WB) or MLC	$6B11^+CD1d^+$	$6B11^+CD1d^+$/MNC(WB) or MLC	$6B11^+CD1d^+$ iNKT-cells
$CD1d^+$ coexpressing $6B11^+$ iNKT-cells	$6B11^+$	$6B11^+CD1d^+$	$6B11^+CD1d^+/6B11^+$	$6B11^+CD1d^+$ iNKT-cells
$CD161^+$ coexpressing $6B11^+$ iNKT-cells	MNC(WB) or MLC	$6B11^+CD161^+$	$6B11^+CD161^+$/MNC(WB) or MLC	$6B11^+CD161^+$ iNKT-cells
$CD161^+$ coexpressing $6B11^+$ iNKT-cells	$6B11^+$	$6B11^+CD161^+$	$6B11^+CD161^+/6B11^+$	$6B11^+CD161^+$ iNKT-cells
$CD161^+$ coexpressing $6B11^+$ iNKT-cells	$CD161^+$	$6B11^+CD161^+$	$6B11^+CD161^+/CD161^+$	$6B11^+CD161^+$ iNKT-cells
TCRV$\alpha24^+$ coexpressing $6B11^+$ iNKT-cells	MNC(WB) or MLC	$6B11^+V\alpha24^+$	$6B11^+V\alpha24^+$/MNC(WB) or MLC	$6B11^+V\alpha24^+$ iNKT-cells
TCRV$\alpha24^+$ coexpressing $6B11^+$ iNKT-cells	$6B11^+$	$6B11^+V\alpha24^+$	$6B11^+V\alpha24^+/6B11^+$	$6B11^+V\alpha24^+$ iNKT-cells
$CD45RO^+$ coexpressing $6B11^+$ iNKT-cells	MNC(WB) or MLC	$6B11^+CD45RO^+$	$6B11^+CD45RO^+$/MNC(WB) or MLC	$6B11^+CD45RO^+$ iNKT-cells
$CD45RO^+$ coexpressing $6B11^+$ iNKT-cells	$6B11^+$	$6B11^+CD45RO^+$	$6B11^+CD45RO^+/6B11^+$	$6B11^+CD45RO^+$ iNKT-cells
$CD45RO^-6B11^+$ 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

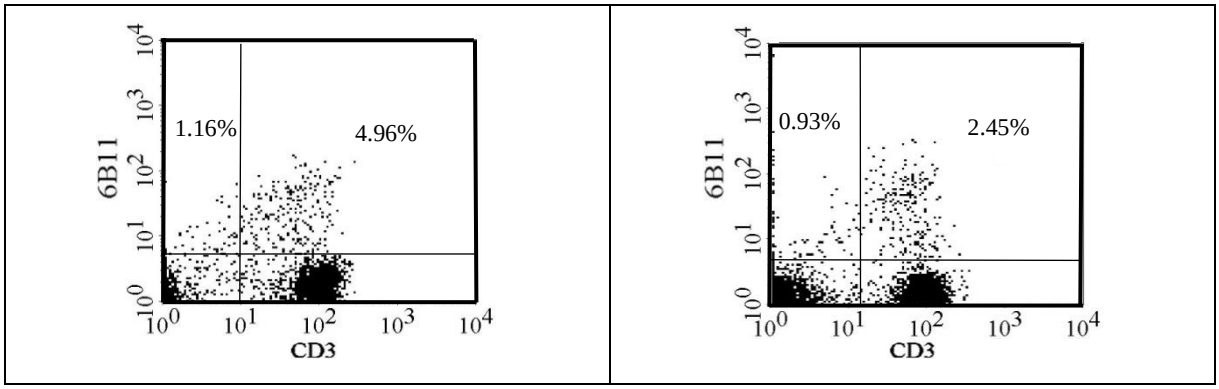
Exemplarily, dot plots iNKT, NK and CIK cells (**Figure 1+2**) and the gating strategy of iNKT cells and subsets are given (**Figure 3**).

Figure 1: Dot plots of NK and CIK cells in healthy- (left side) and AML-MNC (right side)



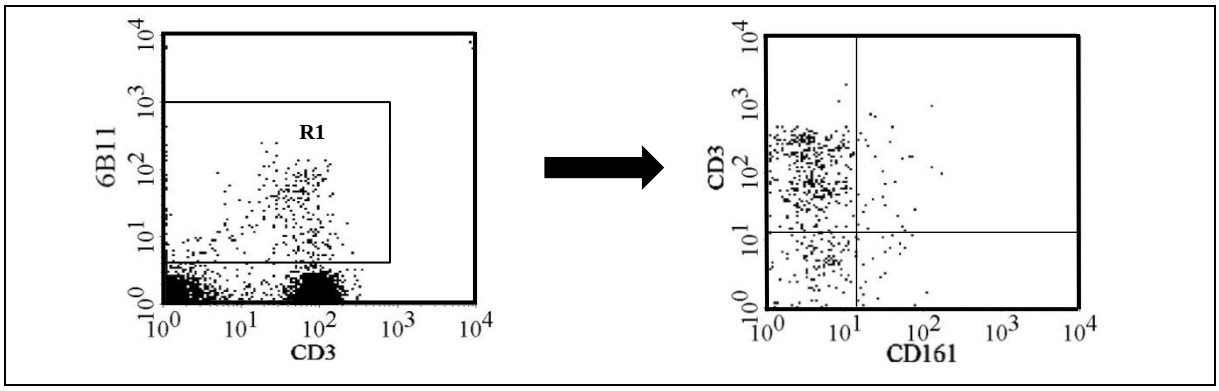
Legend: Dot plots of various frequencies of NK ($CD3^-CD56^+$) and CIK cells ($CD3^+CD56^+$) in healthy or AML MNC are given.

Figure 2: Dot plots of iNKT cells in healthy- (left side) and AML-MNC (right side)



Legend: Dot plots of various frequencies of iNKT cells ($6B11^+CD3^+$ and $6B11^+CD3^-$) in healthy or AML MNC are given.

Figure 3: Gating strategy of iNKT cells and subsets in AML-MNC



Legend: Gating strategy of $6B11^+$ iNKT cells and characterization of corresponding dot plots of iNKT cells subsets ($6B11^+CD3^+CD161^+$ and $6B11^+CD3^-CD161^+$) in healthy and AML MNC are given. R1 = Gate surrounding all $6B11^+$cells in the AML-MNC-fraction.

For the analysis and quantification of DC/ DC_{leu} subtypes in the total- or in subtype-cell-fractions 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 ≥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 (co-expressing 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 11**) [6,8]. For this purpose, cells were stained with patient-specific 'blast'-staining antibodies (e.g. CD15, CD34, CD65 and CD117) according to diagnostic reports before culture in combination with 'DC'-staining

antibodies (e.g. CD80, CD83, CD86, CD206 and CD209), which were not expressed before culture.

Table 11: Subtypes of DC as evaluated by flow cytometry

Names of subgroups	referred to	Surface marker	Abbreviation	Explanatory note/premise
DC	cells	DC^+ (CD80, CD86, CD206)	DC/cells	≥ 10 % DC in cells
leukemia derived DC	cells	DC^+bla^+	DC_{leu}/cells	≥ 5% DC_{leu} in cells, ≥ 10% DC, ≥ 10% bla
blasts converted to DC_{leu} in blast fraction	bla^+	DC^+bla^+	DC_{leu}/bla^+	≥ 5% DC_{leu} in cells, ≥ 10% DC, ≥ 10% bla
DC_{leu} in DC fraction	DC	DC^+bla^+	DC_{leu}/DC	≥ 5% DC_{leu} in cells, ≥ 10% DC, ≥ 10% bla
migratory mature DC in DC fraction	cells	DC^+CCR7^+	DC_{mig}/DC	≥ 10% DC in cells
viable DC in DC-fraction	cells	DC^+7AAD^-	DC_{via}/DC	≥ 10% DC in cells

To analyze whether 6B11 or CD161 is aberrantly expressed on blasts or not (**Table 12**), we evaluated eight different leukemic cell lines (HL-60, OCI-AML2, THP-1, Mono-Mac-6, MOLM-13, RAMOS, RAJI, JURKAT).

Table 12: Subtypes of blasts as evaluated by flow cytometry

Names of subgroups	referred to	Surface marker	Abbreviation
Blast cells [1]	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^+

[1]cells expressing blast markers: patients' individually selected blast-markers with the highest expression (see Table 1-5: blast phenotype)

4.6 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 co-cultured 1:1 with thawed blast-containing target cells (T) for 3h and 24h at 37°C, with 21% O_2 and 5% CO_2. As a control effector- 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 3h and 24h and resuspended in PBS containing 7AAD (Becton Dickson, Heidelberg, Germany) and a defined number of Fluorospheres beads (Beckman

Coulter, Krefeld, Germany). 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, Heidelberg, Germany) 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.

4.7 Statistical Methods

Data were presented as mean +/- standard-deviation. Statistic comparisons for two 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, Washington, USA). Differences were considered as 'not significant' (n.s.) 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.

5 Results

5.1 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 and CLL samples are shown in **Table 13+14**. 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 (**Table 13**), we compared frequencies and compositions of iNKT, NK and 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.

Table 13: Uncultured AML, ALL, CLL and healthy samples

	Cell type	**Ø % (MNC)**	**range % (MNC)**
AML	Blasts (myeloid blasts)	69	18 - 99
	T-cells ($CD3^+$)	6	1 - 33
	B-cells ($CD19^+$)	6	1 - 33
	Monocytes ($CD14^+$)	8	2 - 18
ALL	Blasts (B- or T-linear blasts)	71	17 - 99
	T-cells[1] ($CD3^+$)	14	5 - 33
	B-cells[2] ($CD19^+$)	8	3 - 11
	Monocytes ($CD14^+$)	5	1 - 29
CLL	Blasts (B-linear blasts)	85	32 - 98
	T-cells ($CD3^+$)	11	1 - 60
	Monocytes ($CD14^+$)	4	1 - 25
Healthy	T-cells ($CD3^+$)	41	24 - 64
	B-cells ($CD19^+$)	24	14 - 32
	Monocytes ($CD14^+$)	3	1 - 6

[1]only quantified in B-linear ALL; [2]only quantified in T-linear ALL

In our second approach (**Table 14**), we analysed 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 14: AML and healthy samples used for culture experiments

	Cell type	Ø % (WB/MNC)	range % (WB/MNC)
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
Healthy	T-cells ($CD3^+$)	18 / 35	14-21 / 9-48
	B-cells ($CD19^+$)	3 / 7	1-4 / 3-12
	Monocytes ($CD14^+$)	6 / 7	5-8 / 3-12

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 two 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 to 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 to 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 to leukemic samples (AML: 2.47%±3.12% vs 0.75%%±1.10%, $p<0.081$; ALL: 2.47%±3.12% vs 0.74%±0.65%, $p<0.083$; CLL: 2.47%±3.12% vs 0.78%±0.47%, $p<0.0827$). We decided for better comparibility to present only data obtained with 6B11-PE.

We evaluated eight 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%±0.02% or 2.67%±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% ± 0.01% or 0.35%± 0.01%; ALL: 0.55%± 0.01% or 0.66%± 0.01%; CLL: 0.17%± 0.00% or 1.60%± 0.01%.

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

5.2 Monoclonal antibodies 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, Vα24, CD1d, CD45RO, CD4 and CD8. We defined iNKT cells detected with 6B11 in combination with T cell markers (CD3, Vα24, 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%±1.10% vs 0.74%±0.65% vs 0.78%±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%±1.15% vs 0.61%±0.42% vs 0.46%±0.47%), but the percentages were comparable to results with 6B11 in combination with CD1d ($6B11^{+}CD1d^{+}$/MNC, 0.66%±1.16% vs 0.66%±0.96% vs 0.48%±0.64%). Proportions of $6B11^{+}CD161^{+}$/MNC iNKT cells were even smaller in AML, ALL and CLL patients (0.14%±0.25% vs 0.18%±0.16% vs 0.22%±0.26%), while $6B11^{+}$ Vα24 $^{+}$/MNC could only detect very few frequencies of iNKT cells (0.09%±0.10% vs 0.08%±0.18% vs 0.03%±0.03%). We could show higher frequencies of $6B11^{+}CD4^{+}$ MNC vs $6B11^{+}$ $CD8^{+}$/MNC iNKT cells detected in AML, ALL and CLL patients ($CD4^{+}$ iNKT cells: 0.18%±0.20% vs 0.32%±0.33% vs 0.25%±0.12%; $CD8^{+}$ iNKT cells:

0.03%±0.04% vs 0.07%±0.09% vs 0.06%±0.05%). Moreover, we could show that the majority of 6B11^{+}iNKT cells express CD45RO in AML, ALL and CLL patients (88.58%±14,43% vs 79.75%±30.00% vs 64.58%±29.53%).

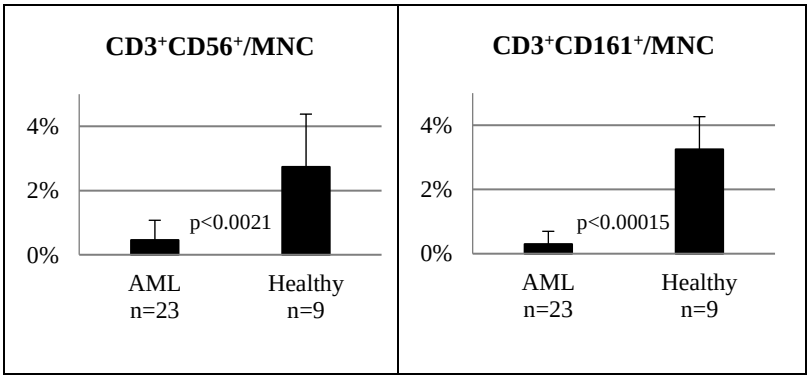
In conclusion, frequencies of iNKT cells and their subsets can be detected with 6B11 alone (PE-labeled clone preferred) or in combination with CD3, CD161, CD1d or Vα24. The combination of 6B11 with CD4, CD8 and CD45RO can be used for further subset-analyses of iNKT cells.

5.3 Numbers and compositions of iNKT, NK and CIK cells from AML patients compared to healthy probands and their correlations with the prognosis

5.3.1 AML patients show significantly lower proportions of iNKT, NK and CIK cells in MNC compared to healthy MNC

We found significantly*** lower frequencies of CD3^{+}CD56^{+} CIK cells in MNC of AML patients compared to healthy MNC (0.46%±0.62% vs 2.74%±1.64%, p<0.0021) and significantly*** lower percentages of CD3^{+}CD161^{+} CIK cells (0.30%±0.40% vs 3.25%±1.02%, p<0.000152, **Figure 4**).

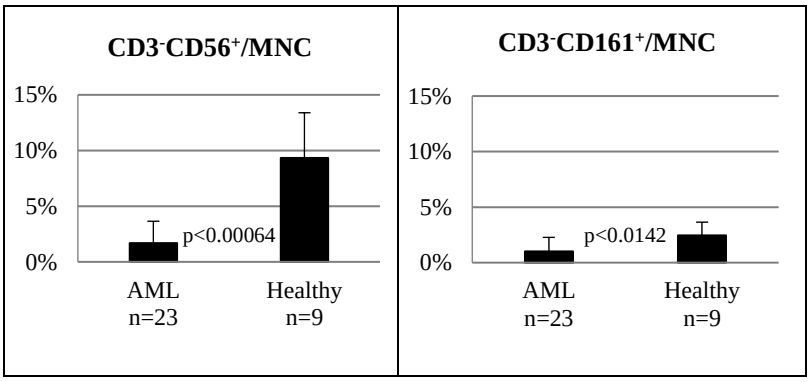
Figure 4: CIK cell frequencies in healthy- versus AML-MNC



Legend: Frequencies of CIK cells and their subsets (CD3^{+}CD56^{+}/MNC and CD3^{+}CD161^{+}/MNC) in healthy vs AML MNC are given.

Additionally, we could show significantly*** lower proportions of $CD3^-CD56^+$ NK cells (1.68%±1.97% vs 9.33%±4.06%, p<0.00064) and significantly** lower proportions of $CD3^-CD161^+$ NK cells in MNC of AML patients compared to healthy MNC (1.01%±1.26% vs 2.44%±1.20%, p<0.0142, **Figure 5**).

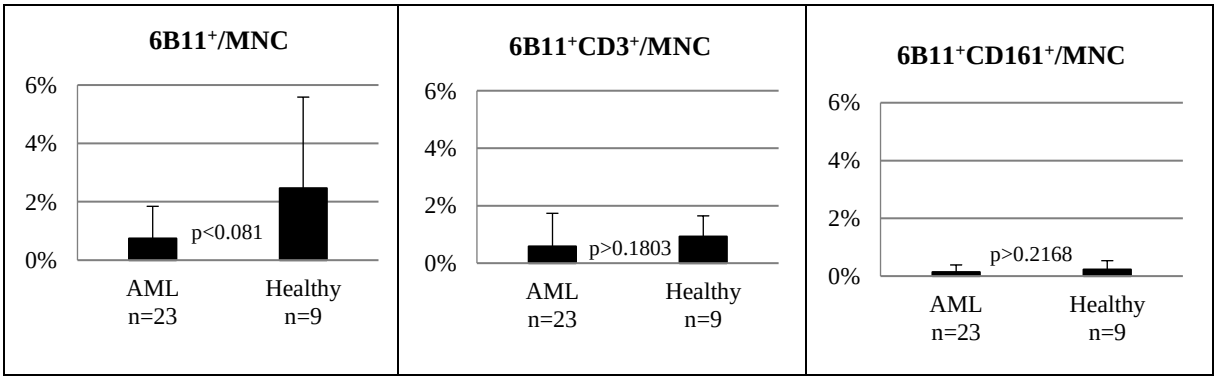
Figure 5: NK cell frequencies in healthy- versus AML-MNC



Legend: Frequencies of NK cells and their subsets ($CD3^-CD56^+$/MNC and $CD3^-CD161^+$/MNC) in healthy vs AML MNC are given.

We could demonstrate significantly* lower percentages of $6B11^+$ iNKT cells (0.75%±1.10% vs 2.47%±3.12%, p<0.081) and (n.s.) lower proportions of $6B11^+CD3^+$ and $6B11^+CD161^+$iNKT-cells in MNC of AML patients compared to healthy MNC (**Figure 6**).

Figure 6: iNKT cell frequencies in healthy- versus AML-MNC



Legend: Frequencies of iNKT cells and their subsets ($6B11^+$/MNC, $6B11^+CD3^+$/MNC and $6B11^+CD161^+$/MNC) in healthy vs AML MNC are given.

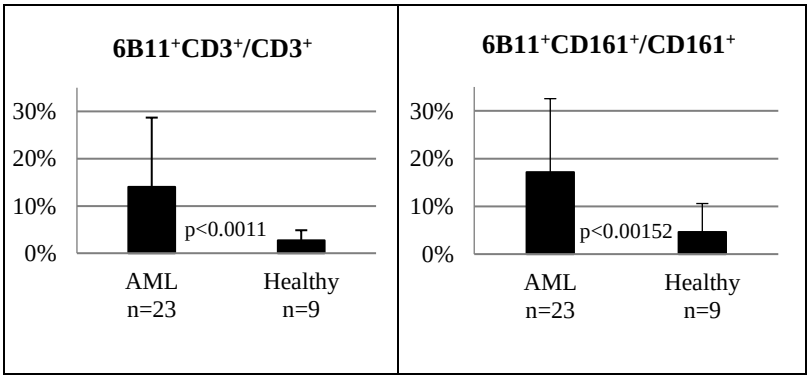
Moreover, $6B11^+CD8^+$ and $6B11^+V\alpha24^+$ iNKT cells were found in lower proportions in MNC from AML patients vs healthy MNC (data not shown (d.n.s.)).

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 MNC from AML patients than in healthy MNC.

5.3.2 Significantly higher proportions of T and NK cells express 6B11 in AML patients compared to healthy probands

We found significantly*** increased proportions of $CD3^+$ T cells expressing 6B11 in AML patients compared to healthy controls (14.03%±14.66% vs 2.69%±2.17%, p<0.00111, **Figure 7**).

Figure 7: iNKT cell frequencies in the NK and T cell fraction in healthy- versus AML-MNC



Legend: Frequencies of iNKT cell subsets in the T cell fraction ($6B11^+CD3^+/CD3^+$) and in the NK cell fraction ($6B11^+CD161^+/CD161^+$) in healthy vs AML MNC are given.

Moreover, we found significantly** higher frequencies of $CD4^+$ (8.36%±15.22% vs 0.31%±0.38%, p<0.0123) and $CD161^+$ cells expressing 6B11 (17.19%±15.36% vs 4.62%±5.97%, p<0.0152, **Figure 7**) and significantly* increased frequencies of $CD8^+$ cells expressing 6B11 in MNC from AML patients vs healthy controls (1.89%±3.55% vs 0.59%±1.12%, p<0.0723). We did not find significant differences of $CD3^+CD161^+$ CIK cells

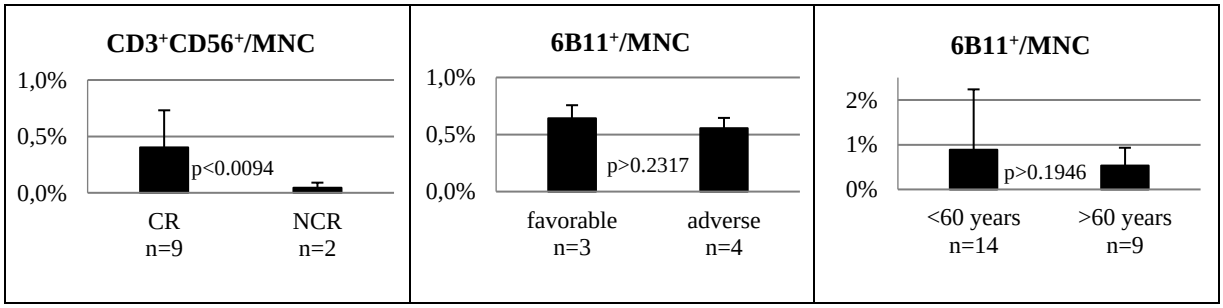
in the $CD3^+$ T cell fraction of AML patients compared to $CD3^+CD161^+$ CIK cells in the healthy T cell fraction (d.n.s.).

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

5.3.3 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% ± 0.33% vs 0.05% ± 0.05%, p<0.0094, **Figure 8**, left side), whereas proportions of NK or iNKT cells were not different in MNC fractions of patients who achieved vs not achieved a CR (d.n.s).

Figure 8: Prognostic relevance of CIK and iNKT cells for AML patients - 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)



Legend: Relevance of CIK and iNKT cells for prognosis of AML patients. Frequencies of CIK cells ($CD3^+CD56^+$/MNC) in patients with CR vs NCR after induction chemotherapy and iNKT cells ($6B11^+$/MNC) in patients with favorable vs adverse risk score (NCCN) and <60 vs >60 years are given.

AML patients with favorable vs adverse NCCN risk score presented with (n.s) higher values of $6B11^+$ iNKT cells in the MNC-fraction (0.64%± 0.11% vs 0.56%± 0.09%; **Figure 8**, middle

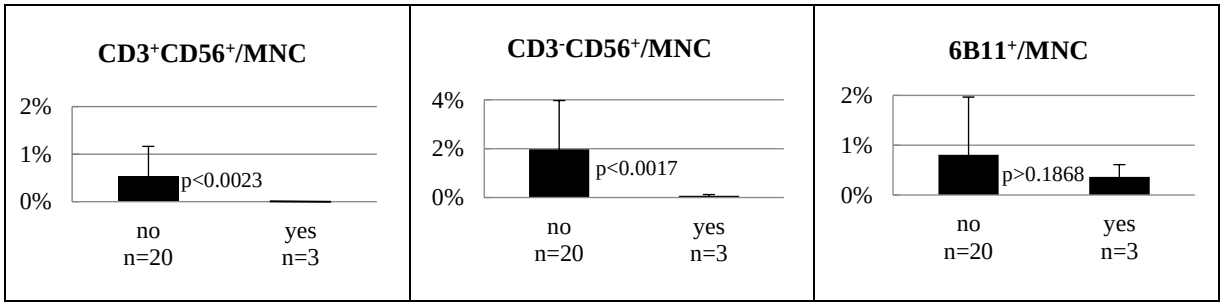
row). Comparable results were found for $6B11^+Va24^+$- and $6B11^+CD161^+$ iNKT cel proportions in the MNC-fraction of AML patients with favorable vs adverse NCCN risk score (d.n.s).

AML patients younger than 60 years presented with (n.s) higher frequencies of $6B11^+$iNKT cells in the MNC-fraction compared to AML patients older than 60 years (0.89%±1.35% vs 0.53%±0.40%, **Figure 8**, 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 vs older than 60 years (d.n.s). No differences were found for proportions of NK or CIK cells in the groups compared (d.n.s).

AML patients with primary vs secondary AML presented with (n.s) 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 (d.n.s).

AML patients without extramedullary foci (n=20) compared to 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, **Figure 9** left side).

Figure 9: Prognostic relevance of CIK, NK and iNKT cells for AML patients - extramedullary versus no extramedullary foci



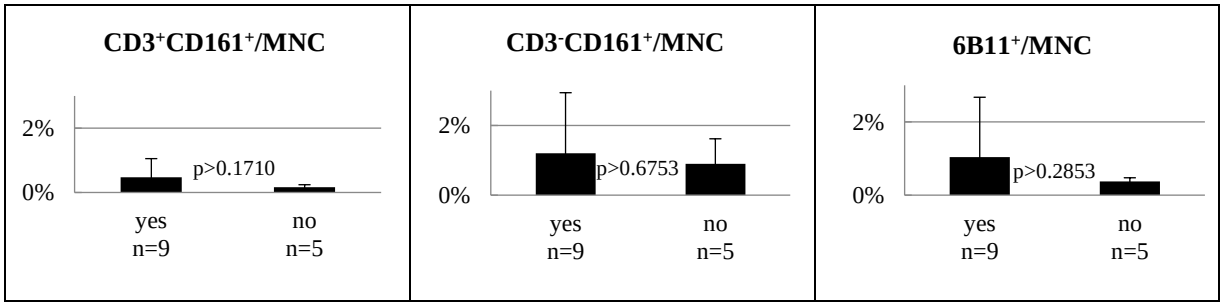
Legend: Relevance of CIK, NK and iNKT cells for prognosis of AML patients. Frequencies of CIK ($CD3^+CD56^+$/MNC), NK ($CD3^-CD56^+$/MNC) and iNKT cells ($6B11^+$/MNC) in patients with extramedullary vs no extramedullary foci are given.

Moreover, the percentages of $CD3^-CD56^+$ NK cells were significantly*** higher in cases without compared to patients with extramedullary foci (1.97% ± 0.21% vs 0.08% ± 0.04%; p<0.0017,

Figure 9 middle row). Furthermore, we found (n.s) higher frequencies of $6B11^+$ iNKT cells in the MNC fraction of AML patients without vs with extramedullary foci (0.81%± 1.16% vs 0.37%±0.24%, **Figure 9** right side). Comparable results were found for $6B11^+CD3^+$ and $6B11^+CD161^+$iNKT cell proportions in the MNC-fraction in the groups compared (d.n.s).

AML patients who stayed in stable CR vs no stable CR (NCR) showed (n.s) higher frequencies of $CD3^+CD161^+$ CIK cells and $CD3^-CD161^+$NK cells in the MNC-fraction (0.47%±0.58% vs 0.16%±0.08%; 1.21%±1.73% vs 0.90%±072%, **Figure 10** left side and middle row).

Figure 10: Prognostic relevance of CIK, NK and iNKT cells for AML patients - stable CR (yes or no)



Legend: Relevance of CIK, NK and iNKT cells for prognosis of AML patients. Frequencies of CIK ($CD3^+CD56^+$/MNC), NK ($CD3^-CD56^+$/MNC) and iNKT cells ($6B11^+$/MNC) in patients with stable vs no stable disease are given.

Moreover, we found (n.s) higher percentages of $6B11^+$iNKT cells in the MNC-fraction of AML patients who stayed in stable CR vs NCR (1.04%±1.63% vs 0.37%±0.10%, **Figure 10** right side). Comparable results were found for $6B11^+CD3^+$ and $6B11^+CD161^+$ iNKT cell proportions in the MNC fraction in the groups compared (d.n.s).

In summary, in AML patients (significantly) higher proportions of $6B11^+$iNKT cells correlated with favorable NCCN risk score, younger age, primary AML, 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 correlated significantly with no extramedullary foci. Moreover, higher proportions of $CD3^-CD161^+$NK cells and $CD3^+CD161^+$CIK cells correlated with stable CR in AML patients and $CD3^+CD56^+$

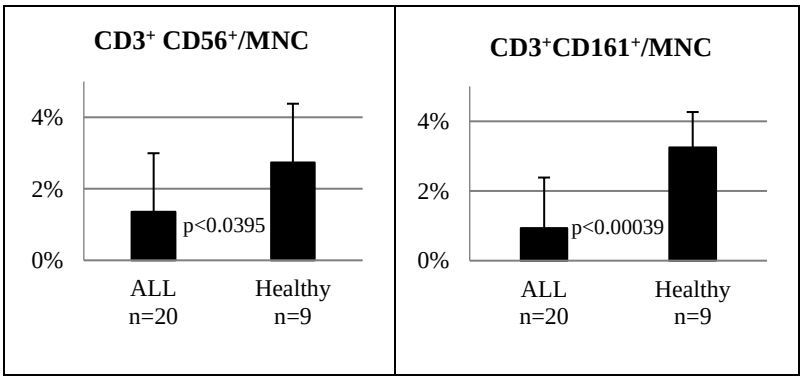
CIK cells correlated with the achievement of CR after induction chemotherapy in AML patients.

5.4 Numbers and compositions of iNKT, NK and CIK cells from ALL patients compared to healthy probands and their correlations with the prognosis

5.4.1 ALL patients show significantly lower proportions of iNKT, NK and CIK cells in MNC compared to healthy MNC

We found significantly** lower frequencies of $CD3^+CD56^+$CIK cells in MNC of ALL patients compared to healthy MNC (1.36%±1.63% vs 2.74%±1.64%, $p<0.0395$) and significantly*** lower percentages of $CD3^+CD161^+$CIK cells (0.94%±1.45% vs 3.25%±1.02%, $p<0.00039$, **Figure 11**).

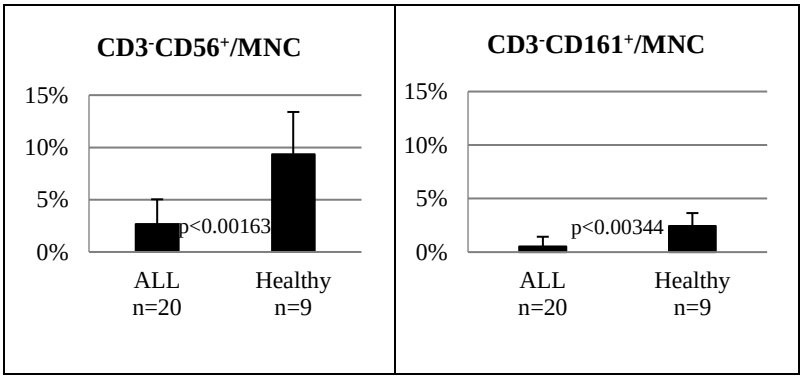
Figure 11: CIK cell frequencies in healthy- versus ALL-MNC



Legend: Frequencies of CIK cells and their subsets ($CD3^+CD56^+$/MNC and $CD3^+CD161^+$/MNC) in healthy vs ALL MNC are given.

Moreover, we found significantly*** lower proportions of $CD3^-CD56^+$NK cells (2.68%±2.35% vs 9.33%±4.06% $p<0.00163$) and significantly*** lower proportions of $CD3^-CD161^+$NK cells in MNC from ALL patients vs healthy MNC (0.52%±0.92% vs 2.44%±1.20%, $p<0.00344$, **Figure 12**).

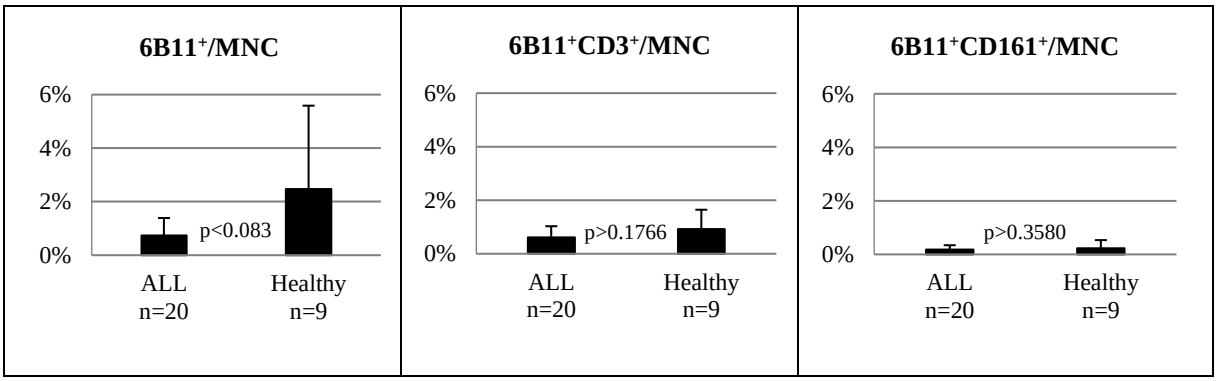
Figure 12: NK cell frequencies in healthy- versus ALL-MNC



Legend: Frequencies of NK cells and their subsets ($CD3^-CD56^+$/MNC and $CD3^-CD161^+$/MNC) in healthy vs ALL MNC are given.

We could show significantly* lower frequencies of $6B11^+$iNKT cells in MNC from ALL patients vs healthy MNC (0.74%±0.65% vs 2.47%±3.12%, p<0.083, **Figure 13**).

Figure 13: iNKT cell frequencies in healthy- versus ALL-MNC



Legend: Frequencies of iNKT cells and their subsets ($6B11^+$/MNC, $6B11^+CD3^+$/MNC and $6B11^+CD161^+$/MNC) in healthy vs ALL MNC are given.

Moreover, $6B11^+CD3^+$/ $6B11^+CD8^+$/ $6B11^+CD161^+$ and $6B11^+V\alpha24^+$iNKT cells were found in (n.s) lower proportions in MNC from ALL patients vs healthy MNC (d.n.s).

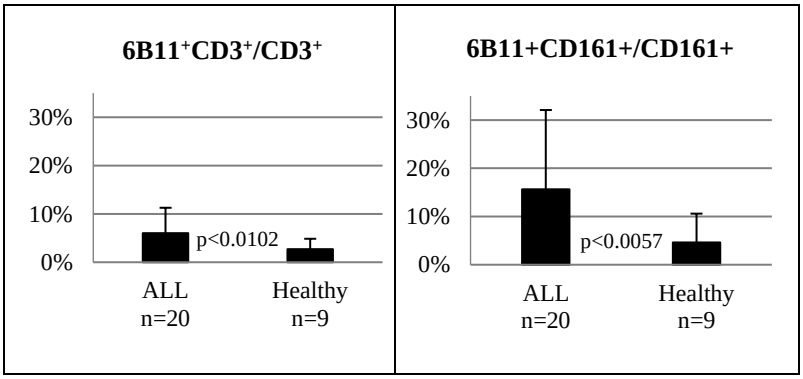
In summary frequencies of $6B11^+$/ $6B11^+CD3^+$/ $6B11^+CD161^+$/ $6B11^+CD8^+$/ $6B11^+V\alpha24^+$ iNKT, $CD3^-CD56^+$/ $CD3^-CD161^+$ NK and $CD3^+CD56^+$/ $CD3^+CD161^+$ CIK cells/ subsets were (significantly) lower in ALL-MNC than in healthy MNC.

5.4.2 Significantly higher proportions of T and NK cells express 6B11 in ALL patients compared to healthy probands

We found significantly* *lower* percentages of $CD3^+CD161^+$ CIK cells in the $CD3^+$ T cell fraction of ALL-patients compared to $CD3^+CD161^+$CIK cells in the healthy $CD3^+$ T cell fraction (5.25%±7.17% vs 8.92%±2.82%, p<0.053), but significantly** higher proportions of $CD3^+$ T cells expressing 6B11 in ALL patients compared to healthy controls (6.03%±5.25% vs 2.69%±2.17%, p<0.0102, **Figure 14**).

Furthermore, we found significantly** higher frequencies of $CD4^+$ (7.27%±11.04% vs 0.31%±0.38%, p<0.00745) and $CD161^+$ cells expressing 6B11 (15.63%±16.46% vs 4.62%±5.97%, p<0.0057, **Figure 14**) and significantly* higher percentages of $CD8^+$cells expressing 6B11 in ALL patients vs healthy controls (1.28%±1.44% vs 0.59%±1.12%, p<0.081).

Figure 14: iNKT cell frequencies in the NK and T cell fraction in healthy- versus ALL-MNC



Legend: Frequencies of iNKT cell subsets in the T cell fraction ($6B11^+CD3^+/CD3^+$) and in the NK cell fraction ($6B11^+CD161^+/CD161^+$) in healthy vs ALL MNC are given.

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 to healthy samples. However, unlike in AML -patients, lower frequencies of $CD3^+CD161^+$CIK cells were found in the $CD3^+$T cell fraction of ALL patients vs in the healthy $CD3^+$ T cell fraction.

5.4.3 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. Since only one 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 (n.s) higher frequencies of $CD3^{-}CD161^{+}$NK cells in MNC-fractions than ALL patients without achieved CR (1.09% ± 1.48% vs 0.18% ± 0.11%, d.n.s). Moreover, we found (n.s) higher percentages of $6B11^{+}$, $6B11^{+}CD3^{+}$ and $6B11^{+}V\alpha24^{+}$ iNKT cells in the MNC-fraction of ALL patients who achieved CR vs NCR (0.95%±1.02% vs 0.65%±0.37%; 0.61%±0.34% vs 0.58%±0.26%; 0.16%±0.30% vs 0.03%±0.05%, d.n.s). No differences were found for CIK cells.

Adult ALL patients without extramedullary foci compared to those with extramedullary foci presented with (n.s) higher proportions of $6B11^{+}$, $6B11^{+}CD3^{+}$ and $6B11^{+}V\alpha24^{+}$ iNKT cells in the MNC fraction (1.12%±1.07% vs 0.54%± 0.30%; 0.63%±0.38% vs 0.56%±0.21%; 0.20±0.32% vs 0.02%±0.02%, d.n.s). 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.

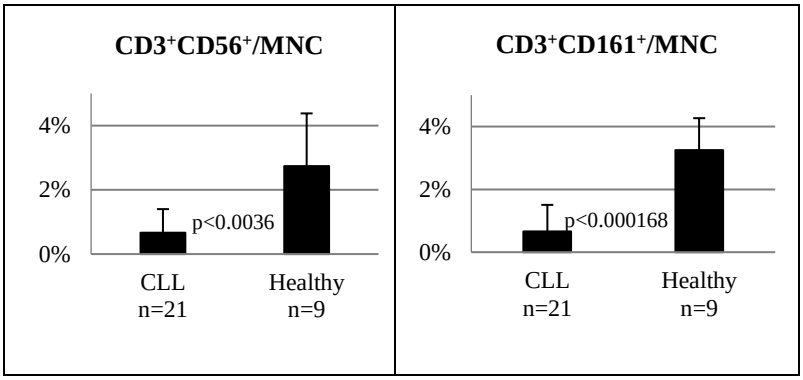
5.5 Numbers and compositions of iNKT, NK and CIK cells from CLL patients compared to healthy probands and their correlations with the prognosis

5.5.1 CLL patients show significantly lower proportions of iNKT, NK and CIK cells in MNC compared to healthy MNC

We found significantly*** lower frequencies of $CD3^{+}CD56^{+}$ CIK cells in MNC of CLL patients compared to healthy MNC (0.67%±0.73% vs 2.74%±1.64%, $p<0.0036$) and significantly*** lower frequencies of $CD3^{+}CD161^{+}$ CIK cells (0.67%±0.84% vs 3.25%±1.02%, $p<0.000168$,

Figure 15).

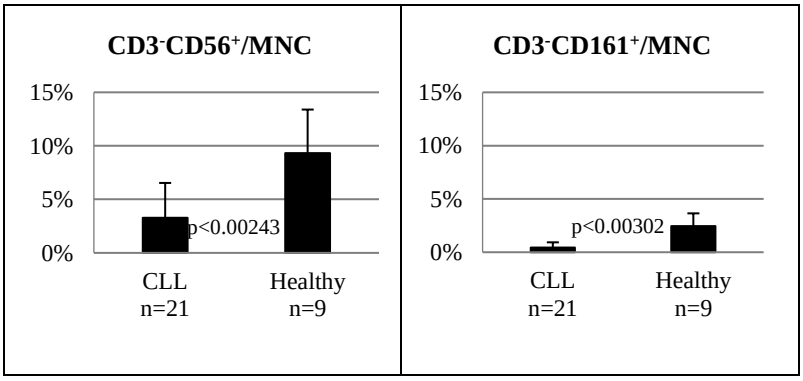
Figure 15: CIK cell frequencies in healthy- versus CLL-MNC



Legend: Frequencies of CIK cells and their subsets ($CD3^+CD56^+$/MNC and $CD3^+CD161^+$/MNC) in healthy vs CLL MNC are given.

Moreover, we found significantly*** lower proportions of $CD3^-CD56^+$ NK cells (3.27%±3.26% vs 9.33%±4.06%, p<0.00243) and significantly*** lower proportions of $CD3^-CD161^+$ NK cells in MNC of CLL patients vs healthy MNC (0.45%±0.48% vs 2.44%±1.20%, p<0.00302, **Figure 16**).

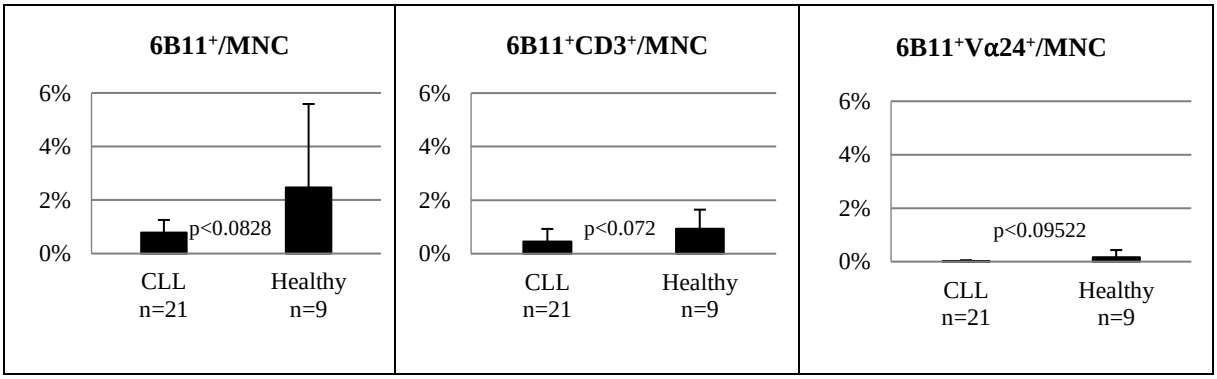
Figure 16: NK cell frequencies in healthy- versus CLL-MNC



Legend: Frequencies of NK cells and their subsets ($CD3^-CD56^+$/MNC and $CD3^-CD161^+$/MNC) in healthy vs CLL MNC are given.

Moreover, we found significant* lower frequencies of $6B11^+$ (0.78%±0.47% vs 2.47%±3.12%, p<0.0828), $6B11^+CD3^+$ (0.46%±0.47% vs 0.93%±0.72%, p<0.072, **Figure 17**) and $6B11^+V\alpha24^+$ iNKT cells in MNC from CLL patients vs healthy MNC (0.03%±0.03% vs 0.16%±0.27%, p<0.09522). Moreover, lower proportions of $6B11^+CD8^+$ and $6B11^+CD161^+$ iNKT cells were found in MNC from CLL patients vs healthy MNC (d.n.s).

Figure 17: iNKT cell frequencies in healthy- versus CLL-MNC



Legend: Frequencies of iNKT cells and their subsets ($6B11^+$/MNC, $6B11^+CD3^+$/MNC and $6B11^+CD161^+$/MNC) in healthy vs CLL MNC are given.

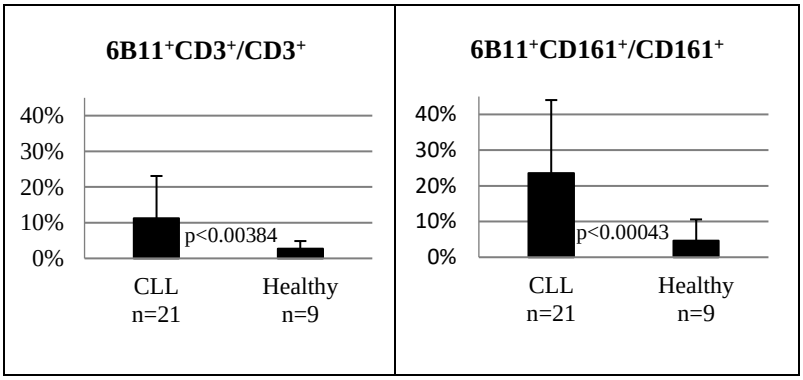
In summary frequencies of $6B11^+$/ $6B11^+CD3^+$/ $6B11^+CD161^+$/ $6B11^+CD8^+$/ $6B11^+V\alpha24^+$ iNKT, $CD3^-CD56^+$/ $CD3^-CD161^+$ NK and $CD3^+CD56^+$/ $CD3^+CD161^+$ CIK cells/ subsets were (significantly) lower in CLL MNC than in healthy MNC.

5.5.2 Significantly higher proportions of T and NK cells express 6B11 in CLL patients compared to healthy probands

We found significantly*** higher frequencies of $CD3^+$T-cells expressing 6B11 in CLL patients compared to healthy controls (11.27%±11.83% vs 2.69%±2.17%, p<0.00384, **Figure 18**). Furthermore, we found significantly** higher frequencies of for $CD4^+$ cells expressing 6B11 (7.14%±5.90% vs 0.31%±0.38%, p<0.00908) and significantly*** higher frequencies of $CD161^+$ cells expressing 6B11 in CLL patients vs healthy controls (23.55%±20.47% vs

4.62%±5.97%, p<0.00043, Figure 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 (d.n.s).

Figure 18: iNKT cell frequencies in the NK and T cell fraction in healthy- versus CLL-MNC



Legend: Frequencies of iNKT cell subsets in the T cell fraction ($6B11^+CD3^+/CD3^+$) and in the NK cell fraction ($6B11^+CD161^+/CD161^+$) in healthy vs CLL MNC are given.

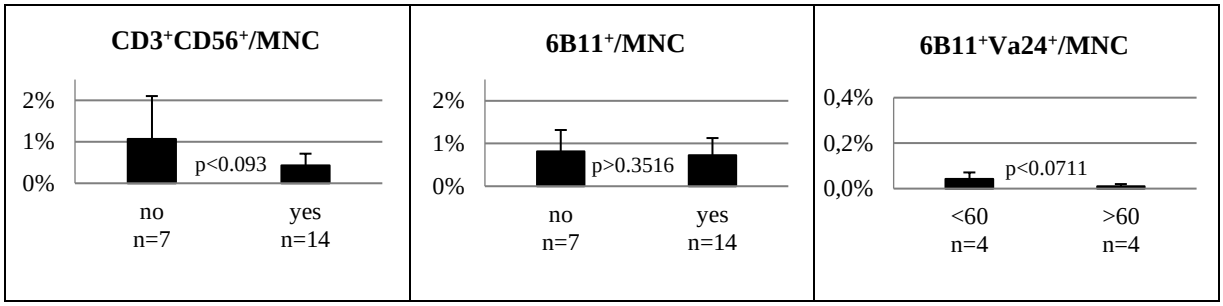
In summary, a shift to higher proportions of ($CD3^+/CD4^+$) T cells and ($CD161^+$) NK cells coexpressing 6B11 was found in CLL patients compared to healthy samples. Like in AML patients, but unlike in ALL patients, no differences were found for CIK cells in the $CD3^+$ T cell fraction. Unlike AML and ALL patients no differences were found for $CD8^+$ T cells expressing 6B11.

5.5.3 CLL patients with prognostically favorable subgroups are characterized by higher proportions of iNKT, NK and CIK cells

CLL patients with no need vs need for therapy presented with significantly* higher proportions of $CD3^+CD56^+$ CIK cells in the MNC-fraction (1.07%±1.03% vs 0.43% ± 0.28%, p<0.093, **Figure 19** left side). We found (n.s) higher frequencies of $6B11^+$ iNKT cells in the MNC-fraction of CLL patients with no need vs need for therapy (0.81%±0.50% vs 0.73%±0.40%, **Figure 19** middle row). Comparable results were found for $6B11^+CD3^+$ and $6B11^+V\alpha24^+$ iNKT cell proportions in the MNC-fraction of CLL patients with no need vs need for therapy (d.n.s). No differences were found for NK cells. CLL patients younger vs 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 072%±0.40%; 0.04%±0.03% vs 0.01%±0.01%, p<0.0711, **Figure 19** right side).

Figure 19: Prognostic relevance of CIK, NK and iNKT cells for CLL patients - need for therapy (yes or no, left side and middle row), allocation to age groups (<60 versus >60 years, right side)

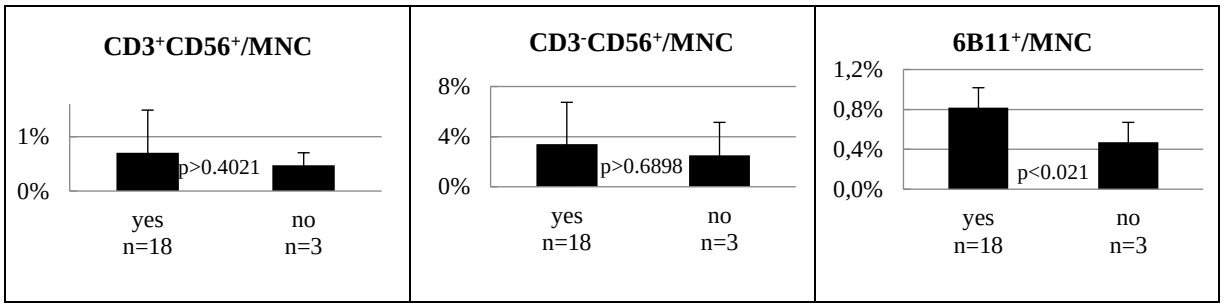


Legend: Relevance of CIK and iNKT cells for prognosis of CLL patients. Frequencies of CIK (CD3$^+$CD56$^+$/MNC) and iNKT cells (6B11$^+$/MNC) in patients with no need vs need for therapy and iNKT cells (6B11$^+$Vα24$^+$/MNC) in patients <60 vs >60 years are given.

CLL patients with stable vs no stable disease (NCR, relapse or death with disease) presented with (n.s) 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%, **Figure 20** left side and middle row). Moreover, we could demonstrate significantly** higher frequencies of 6B11$^+$ (0.82%±0.49% vs 0.47%±0.06%, p<0.021) and significantly* higher frequencies of 6B11$^+$CD3$^+$ iNKT cells in the MNC-fraction of CLL patients with stable CR vs NCR (0.49%±0.48% vs 0.17%±0.09%, p<0.0597, **Figure 20** 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$^+$Vα24$^+$ 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.

Figure 20: Prognostic relevance of CIK, NK and iNKT cells for CLL patients - stable disease (yes or no)



Legend: Relevance of CIK and iNKT cells for prognosis of CLL patients. Frequencies of CIK ($CD3^+CD56^+$/MNC), NK ($CD3^-CD56^+$/MNC) and iNKT cells ($6B11^+$/MNC) in patients with stable versus no stable disease are given.

5.6 iNKT, NK and CIK cells and their subtypes increase under the influence of IL-2 and after pre-stimulation 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 analysed in cases the amount of DC were ≥10%) and achieved the following results:

'MNC-healthy': With 'Kit D' we generated Ø 10.79%±2.07%, with 'Pici 1' 15.42%±4.97% and with 'Pici 2' 15.33%±6.09% DC in MNC.

'WB-healthy': With 'Kit D' we generated Ø 7.44%±1.28%, with 'Kit I' 11.05%±6.82%, with 'Kit K' 8.62%±2.00%, with 'Kit M' 8.08%±3.38%, with 'Pici 1' 10.50%±2.62% and with 'Pici 2' 9.48%±1.85% DC in WB.

'MNC-AML': With 'Kit D' we generated Ø 36.51%±7.33% DC and 6.70%±0.58% DC_{leu}, with 'Pici 1' 23.53%±0.00% DC and 12.63%±0.00% DC_{leu} and with 'Pici 2' 19.39%±7.39 DC and 10,20%±0,00 DC_{leu} in MNC.

'**WB-AML'**: With 'Kit D' we generated Ø 6.85%±1.10% DC, with 'Kit I' 13.18%±7.43% DC and 19.95%±0.00% DC_{leu}, with 'Kit K' 10.48%±3.77% DC and 8.11%±2.38% DC_{leu}, with 'Kit

M' 9.92%±3.93% DC and 13.62%±0.00% DC_{leu}, with 'Pici 1' 10.16%±0.00% DC and 8.98%±0.00% DC_{leu} and with 'Pici 2' 12.88%±0.00% DC and 11.39%±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 Ø 6.28%±2.09% DC.

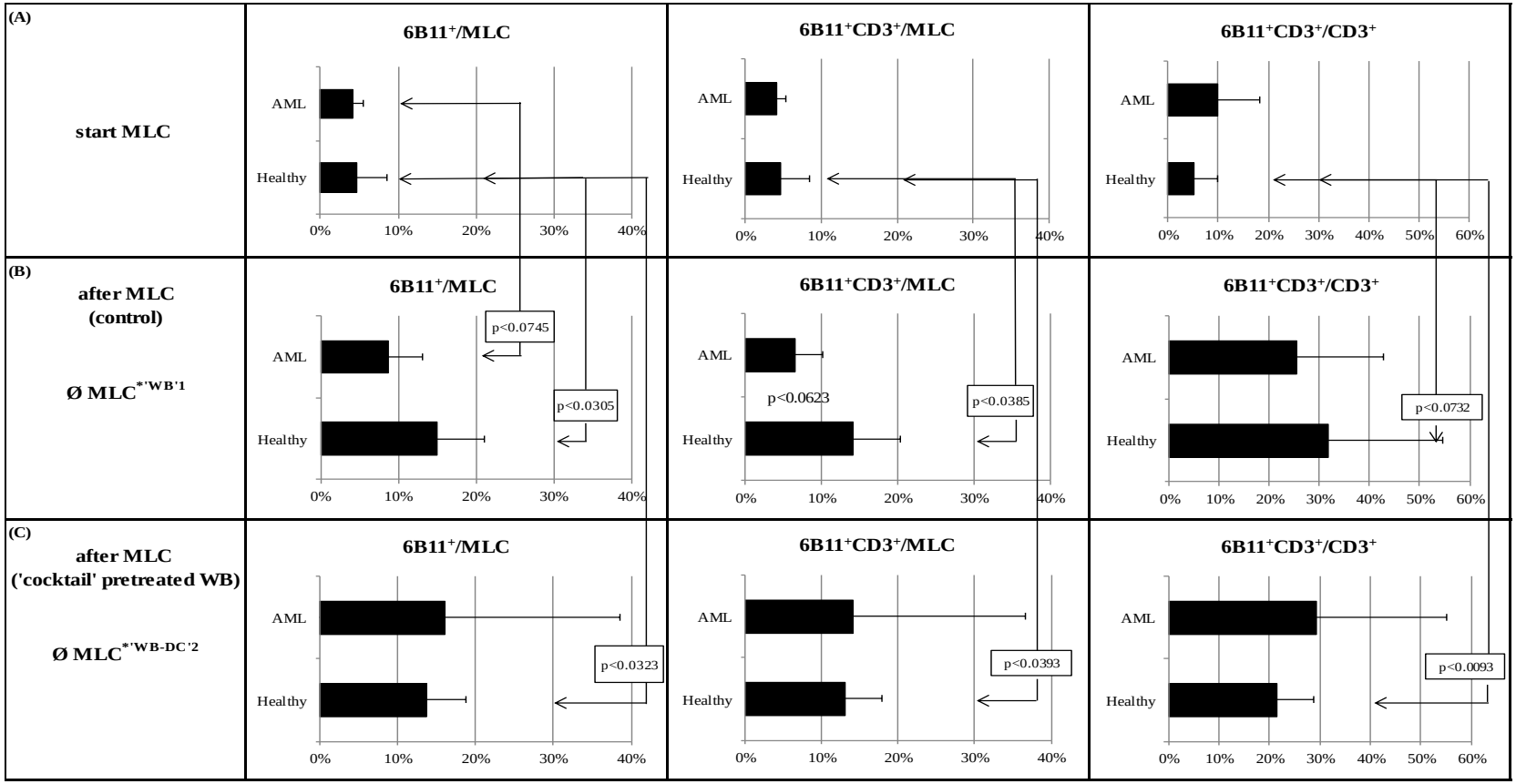
Here we studied, whether iNKT/ NK/ CIK cells (in MNC or WB samples) could contribute to improve the overall-antileukemic activity after stimulation with DC/ DC_{leu}. Therefore, we stimulated in MLC (prepared with MNC or WB) T cell enriched immune reactive cells with a stimulator cell suspension containing DC/ DC_{leu} ($MLC^{*'MNC\text{-}DC'}$ or $MLC^{*'WB\text{-}DC'}$) 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 analysed 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.

5.6.1 In MLC of healthy and AML patients ($MLC^{*'WB\text{-}DC'}$ 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) vs healthy WB samples (n=5; **Figure 21A**). 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%±1.32% vs 8.63%±4.40%, $p<0.0745^*$; healthy $6B11^+$/MLC: 4.68%±3.92% vs 12.34%±8.10%, $p<0.0305^{**}$; healthy $6B11^+CD3^+$/MLC: 4.60%±3.90% vs 12.11%±8.10%, $p<0.0385^{**}$; healthy $6B11^+CD3^+/CD3^+$: 5.17%±4.63% vs 31.77%±22.85%, $p<0.0732^*$; **Figure 21A vs 21B**) and start of MLC versus after $MLC^{*'WB\text{-}DC'}$ (healthy $6B11^+$/MLC: 4.68%±3.92% vs 13.67%±5.01%, $p<0.0323^{**}$; healthy $6B11^+CD3^+$/MLC: 4.60%±3.90% vs 13.03%±4.91%, $p<0.0393^{**}$; $6B11^+CD3^+/CD3^+$: 5.17%±4.63% vs 21.51%±7.38%, $p<0.0093^{**}$, **Figure 21A vs 21C**).

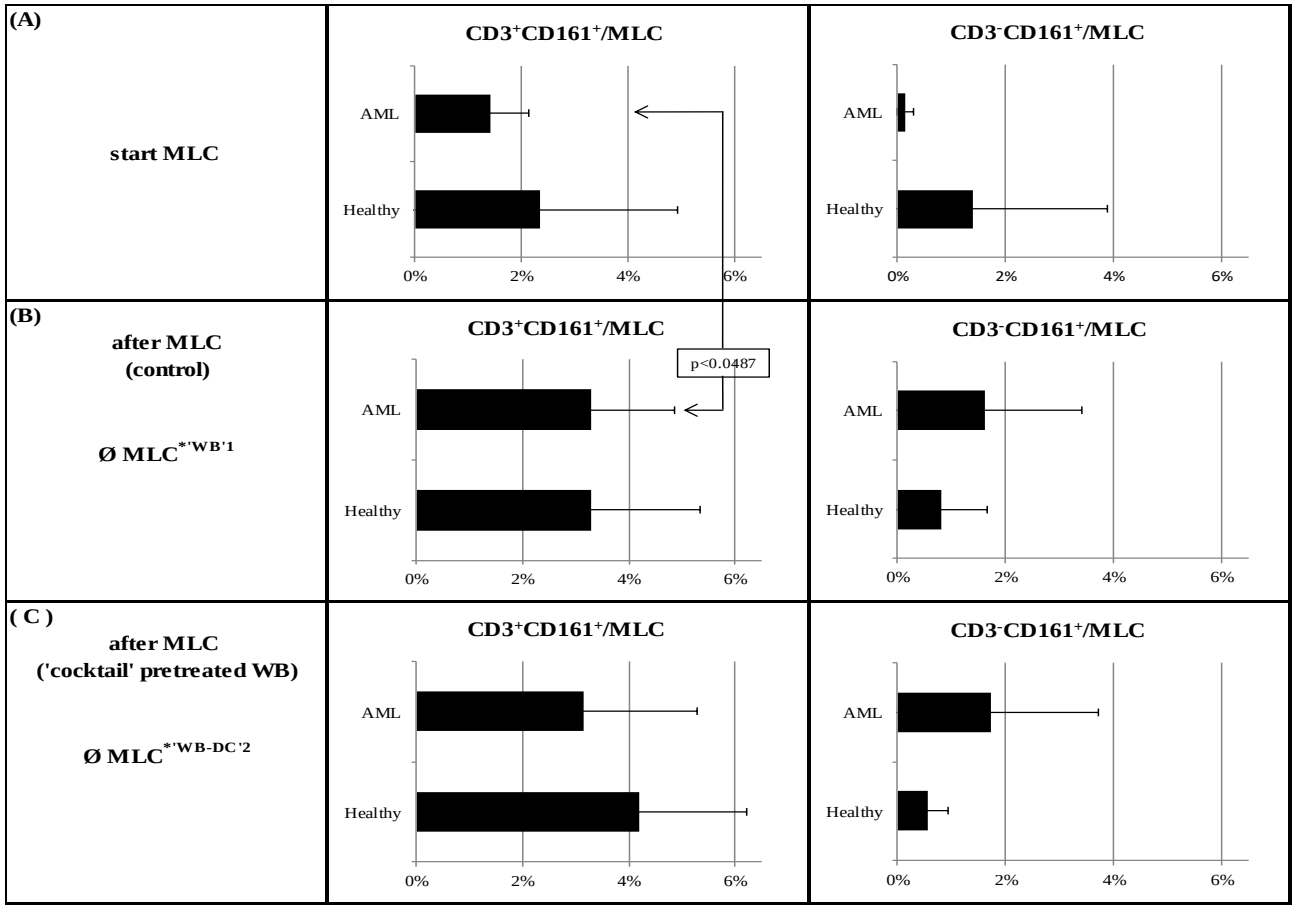
Pooling all results after $MLC^{*'WB\text{-}DC'}$ compared to $MLC^{*'WB'}$, an astonishing finding was that cellular compositions in healthy samples were comparable in both settings (**Figure 21B vs 21C**). This could be due to the general influence of IL-2 in MLC.

Figure 21: Frequencies of iNKT cells in MLC (WB) at the start of MLC (A), after MLC$^{*'WB'1}$ (control) (B) and after MLC$^{*'WB\text{-}DC'2}$ (C) are given.



Legend: 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 with 'cocktails' (*^{1}MLC$^{*'WB'}$*) or with a stimulator-cell-suspension *pretreated* with 'cocktails' (^{2}MLC$^{*\ 'WB\text{-}DC'}$). 'Cocktails' = all DC-generating methods/Kits.

Figure 22: Frequencies of CIK/NK cells in MLC (WB) at the start of MLC (A), after MLC$^{*'WB'1}$ (control) (B) and after MLC$^{*'WB\text{-}DC'2}$ (C) are given.



Legend: Frequencies of CIK and 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 with 'cocktails' (*^{1}MLC$^{*'WB'}$*) or with a stimulator-cell-suspension *pretreated* with 'cocktails' (^{2}MLC$^{*\ 'WB\text{-}DC'}$). 'Cocktails' = all DC-generating methods/Kits

However, in AML cases the proportions of iNKT cells increased after MLC$^{*\text{'WB-DC'}}$ compared to MLC$^{*\text{'WB'}}$ (**Figure 21B vs 21C**). Differences were not significant. Comparable results were found for MNC (d.n.s.)

At start of MLC we found lower total frequencies of CIK and NK cells in AML (n=6) vs healthy WB-samples (n=5) (**Figure 22A**).

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$^{*\text{'WB'}}$(AML $CD3^+CD161^+$/MLC: 1.41%±0.74% vs 3.28%±1.59%, $p<0.0487^{**}$; healthy $CD3^+CD161^+$/MLC: 2.35%±2.59% vs 3.30%±2.04%; AML $CD3^-CD161^+$/MLC: 0.15%±0.15% vs 1.62%±1.81%; healthy $CD3^-CD161^+$/MLC: 1.41%±2.48% vs 0.80%±0.86%; **Figure 22A vs 22B**) and start of MLC versus after MLC$^{*\text{'WB-DC'}}$ (AML $CD3^+CD161^+$/MLC: 1.41%±0.74% vs 3.15%±2.13%; healthy $CD3^+CD161^+$/MLC: 2.35%±2.59% vs 4.20%±2.02%; AML $CD3^-CD161^+$/MLC: 0.15%±0.15% vs 1.73%±1.98%; healthy $CD3^-CD161^+$/MLC: AML 1.41%±2.48% vs 0.57%±0.37%; **Figure 22A vs 22C**).

Pooling all results after MLC$^{*\text{'WB-DC'}}$ compared to MLC$^{*\text{'WB'}}$, an astonishing finding was, that cellular compositions in healthy samples were comparable in both settings (**Figure 22B vs 22C**). This could be due to the general influence of IL-2 in MLC.

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 frequencies compared to not pretreated controls, pointing to an 'iNKT-inducing influence' of these different 'cocktails'.

5.6.2 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.

Figure 23: Frequencies of iNKT cells (6B11$^+$ or 6B11$^+$CD3$^+$ or 6B11$^+$CD161$^+$ iNKT cells) after MLC$^{*\text{'MNC-DC'}}$ and MLC$^{*\text{'MNC'}}$ are given.

Healthy 'MNC'	**Kit-D**	**'Pici1'**	**'Pici2'**	**control**
P1418	+	+++	-	+
P1421	+	+	+++	+
P1422	+	+++	+	+
P1425	+++			+
P1428	+		+++	+
P1429		+++	-	-
P1436		+++	+	+++
P1438	+++	+	+	+
AML 'MNC'	**Kit-D**	**'Pici1'**	**'Pici2'**	**control**
P1424	-		-	+++
P1426	+++	+	-	+

Legend: Frequencies of iNKT cells (6B11+ or 6B11+CD3+ or 6B11+CD161+) after MLC (MNC) are given. T cell enriched immune reactive cells were stimulated with a stimulator cell suspension *without pretreatment* of MNC with 'cocktails' (*^{1}MLC$^{*\text{'MNC'}}$*) or with a stimulator-cell-suspension *pretreated* with 'cocktails' (^{2}MLC$^{*\text{ 'MNC-DC'}}$). '+++' = *'highest'* counts of iNKT-cells; '++' = *'high'* counts of iNKT-cells, '+' = *'good'* counts of iNKT-cells, '-' = *'no'* increase or decrease of iNKT-cells

We could show that especially in cases in that the DC generation was performed with prostaglandin-containing (PGE_1, PGE_2) 'cocktails' the percentages of iNKT cells increased the most (Kit-D, Kit-K, Kit-M, 'Pici1', 'Pici2', **Figure 23**).

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 (**Figure 23 vs 24**). Comparable effects were found for CIK cells (d.n.s.).

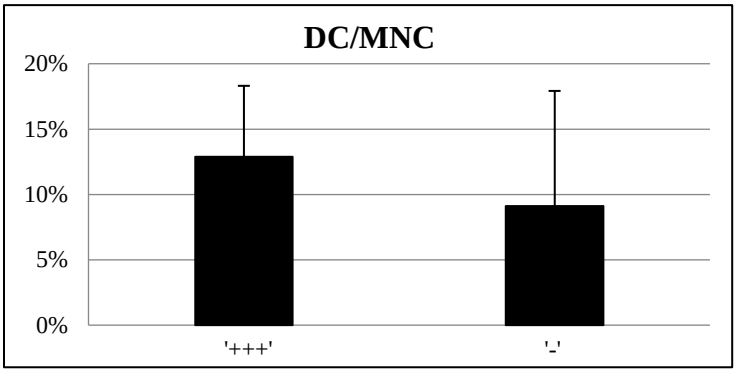
Figure 24: Frequencies of iNKT cells (6B11$^+$ or 6B11$^+$CD3$^+$ or 6B11$^+$CD161$^+$ iNKT cells) after MLC$^{*\text{'WB-DC'}}$ and MLC$^{*\text{'WB'}}$ are given.

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				+++			++

Legend: Frequencies of iNKT cells (6B11+ or 6B11+CD3+ or 6B11+CD161+) after MLC (WB) are given. T cell enriched immune reactive cells were stimulated with a stimulator cell suspension *without pretreatment* of MNC with 'cocktails' (*^{1}MLC$^{*\text{'WB'}}$*) or with a stimulator-cell-suspension *pretreated* with 'cocktails' (^{2}MLC$^{*\text{ 'WB-DC'}}$). '+++' = *'highest'* counts of iNKT-cells; '++' = *'high'* counts of iNKT-cells, '+' = *'good'* counts of iNKT-cells, '-' = *'no'* increase or decrease of iNKT-cells

Moreover, we correlated percentages of 6B11$^+$/ 6B11$^+$CD3$^+$/ 6B11$^+$CD161$^+$ iNKT cells (defined as 'highest', 'high', 'good' and 'no increase') after MLC in MNC (**Figure 23**: 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 to MLC with 'no increase' (Ø6B11$^+$ /MNC: 0.93%±0.21%) or even a decrease of iNKT cells (Ø DC-counts: 12.87%±5.44% vs 9.11%±8.81%; **Figure 25**).

Figure 25: Frequencies of iNKT cells (6B11$^+$ or 6B11$^+$CD3$^+$ or 6B11$^+$CD161$^+$ iNKT cells) after MLC$^{*\text{'MNC-DC'}}$ and MLC$^{*\text{'MNC'}}$ correlate with DC-values from healthy MNC



Legend: Frequencies of DC values in healthy MNC are given. Frequencies are correlated with '+++' = *'highest'* counts of iNKT-cells and '-' = *'no'* increase or decrease of iNKT-cells (see Figure 23+24).

Those correlations were not found for MNC of the two AML-patients we have analysed (d.n.s). 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 frequencies and iNKT cells after MLC (d.n.s).

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 two AML-patients analysed.

5.6.3 Physiological hypoxia does not influence amounts and compositions of iNKT, NK and CIK cells compared to normoxic conditions

Under physiological conditions the O_2-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 O_2 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 to 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% O_2 or with varying O_2-concentrations between 0-17%. 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 three '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 vs hypoxic conditions (d.n.s.).

In a next step, we quantified iNKT, NK and CIK cell proportions after $MLC^{*'WB\text{-}DC'}$ and $MLC^{*'WB'}$ under hypoxic vs normoxic conditions. Pooling all results after $MLC^{*'WB\text{-}DC'}$ we found comparable proportions of iNKT, NK and CIK cells after MLC under normoxic vs hypoxic conditions (d.n.s.).

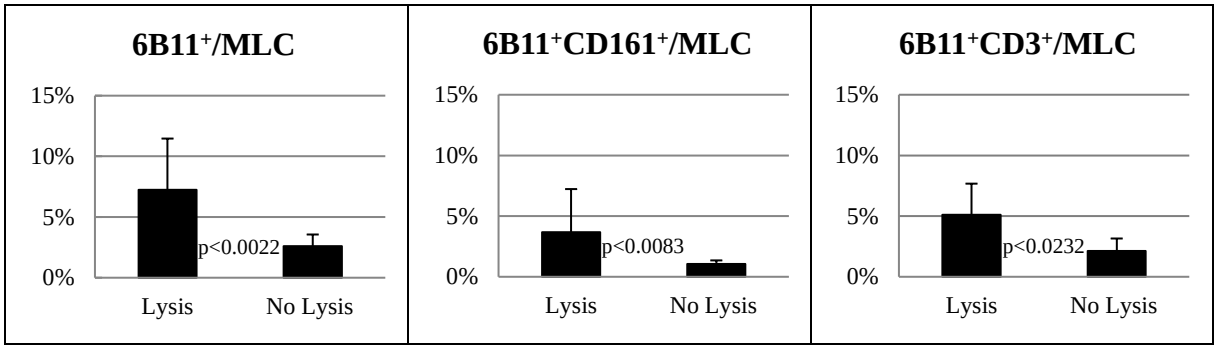
In summary, low O_2-culture-concentrations do not seem to have neither a significant impact on the generation of DC nor on the amount and composition of iNKT, NK and CIK cells after MLC.

5.6.4 iNKT, NK and CIK cells contribute to antileukemic activity

As shown above iNKT cell proportions increase after $MLC^{*'WB\text{-}DC'}$ or $MLC^{*'MNC\text{-}DC'}$. Here we studied, whether iNKT, NK and 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 AM WB samples (precultured with various 'cocktails') after MLC (with added T cells and IL-2). In a 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 vs without an antileukemic activity ('lysis' vs 'non-lysis') 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%, $p<0.0232$, **Figure 26**).

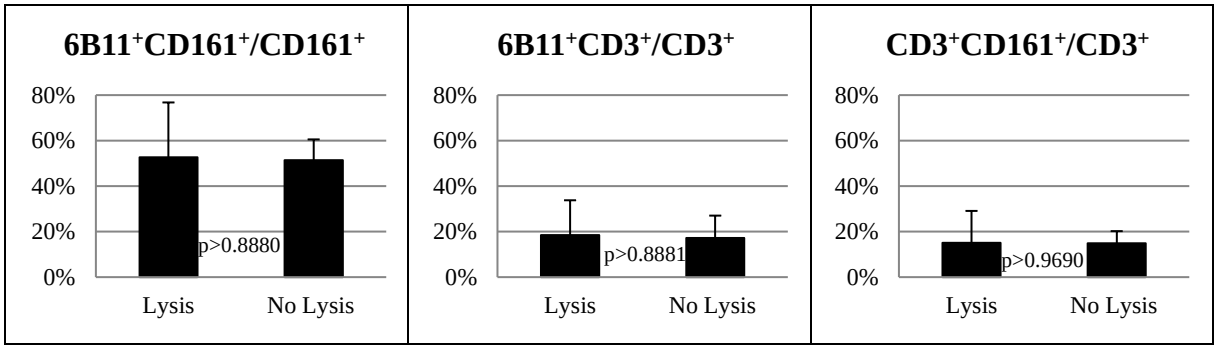
Figure 26: iNKT cell frequencies – lysis versus no lysis



Legend: Frequencies of iNKT cells after MLC (MNC/WB) are given. T cell enriched immune-reactive cells with stimulated with a stimulator cell suspension *without pretreatment* of MNC with 'cocktails' (*[1]MLC*$^{*\text{'MNC/WB'}}$) or with a stimulator-cell-suspension *pretreated* with 'cocktails' ([2]MLC$^{*\text{ 'MNC/WB-DC'}}$) were compared in cases with and without blast lysis. Results were obtained with a cytotoxicity (fluorolysis) assay.

However, the subtype compositions of iNKT and CIK cell subsets were comparable in the two 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%, **Figure 27**).

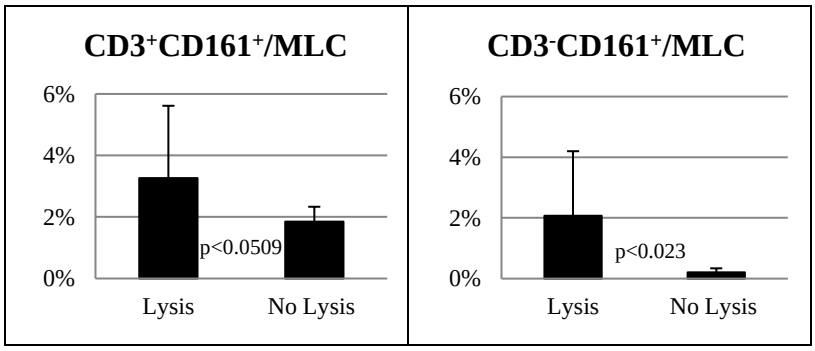
Figure 27: Frequencies of iNKT and CIK cell subsets – lysis versus no lysis



Legend: Frequencies of iNKT and CIK cells subsets after MLC (MNC/WB) are given. *MLC*$^{*\text{'MNC/WB'}}$ or MLC$^{*\text{ 'MNC/WB-DC'}}$ were compared in cases with and without blast lysis. Results were obtained with a cytotoxicity (fluorolysis) assay.

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 vs without an antileukemic activity (2.07%±2.13% vs 0.21%±0.13%, p<0.0023, **Figure 28**).

Figure 28: CIK and NK cell frequencies – lysis versus no lysis



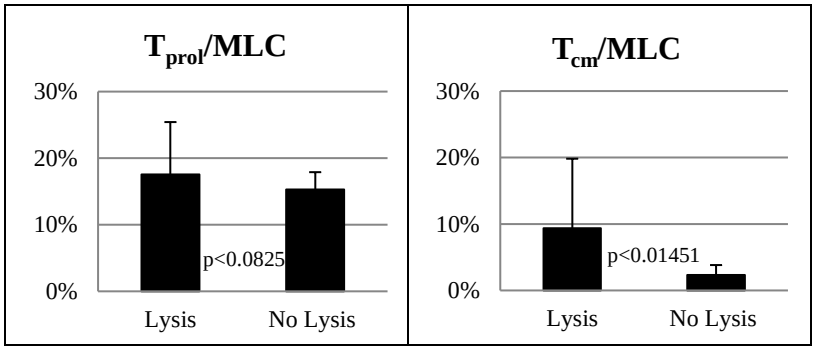
Legend: Frequencies of CIK and NK cells after MLC (MNC/WB) are given. *MLC*$^{*\text{'MNC/WB'}}$ or MLC$^{*\text{ 'MNC/WB-DC'}}$ were compared in cases with and without blast lysis. Results were obtained with a cytotoxicity (fluorolysis) assay.

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 vs 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, **Figure 29**). No significant differences were found in the composition of naïve/non-naïve T cells in the groups compared (d.n.s.).).

Moreover, we assorted our samples according to their antileukemic activity ('lysis' vs 'non-lysis') and evaluated predictive cut-off values for NK/ CIK/ iNKT cells after MLC$^{*\text{'WB-DC'}}$ and MLC$^{*\text{'WB'}}$. 100% of samples with >4% $6B11^+$ iNKT cells, with > 4% $6B11^+CD3^+$ iNKT cells and with >1.5% $6B11^+CD161^+$ iNKT cells after MLC$^{*\text{'WB-DC'}}$ and MLC$^{*\text{'WB'}}$ 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$^{*\text{'WB-DC'}}$ and MLC$^{*\text{'WB'}}$ showed antileukemic activity. Moreover, 89-100% of samples with >0.4% $CD3^-CD161^+$ NK cells and

with >1.3% $CD3^{-}CD56^{+}$ NK cells after $MLC^{*\text{'WB-DC'}}$ and $MLC^{*\text{'WB'}}$ showed antileukemic activity.

Figure 29: Frequencies of T cell subsets - lysis versus no lysis



Legend: Frequencies of T cell subsets after MLC (MNC/WB) are given. $MLC^{*\text{'MNC/WB'}}$ or MLC^{*} $^{\text{'MNC/WB-DC'}}$ were compared in cases with and without blast lysis. Results were obtained with a cytotoxicity (fluorolysis) assay. T_{prol} proliferating T-cells ($CD69^{+}CD3^{+}$), T_{cm} central-memory T-cells ($CD45RO^{+}CCR7^{+}$).

Further, we analysed whether the addition of 'cocktails' to cultures improves the antileukemic activity after MLC compared to controls. Samples with more lysis compared to controls, were defined as improved blast-lysis, samples with less lysis compared to controls as 'not improved' blast-lysis. Samples with improved blast-lysis showed significantly* higher $6B11^{+}$ iNKT cell frequencies compared to samples with not improved blast-lysis (7.94%±4.12% vs 4.39%±3.12%; p<0.0921). Moreover, samples with improved blast-lysis showed (n.s.) higher frequencies of $6B11^{+}CD161^{+}$- and $6B11^{+}CD3^{+}$ iNKT cells compared to samples with not improved blast-lysis (4.82%±3.49% vs 1.80%±2.90%; 5.25%±2.13% vs 3.50%±1.84%). Furthermore, samples with 'improved' blast-lysis showed (n.s.) higher frequencies of $CD3^{+}CD161^{+}$ CIK cells and $CD3^{-}CD161^{+}$ NK cells compared to samples with not improved blast-lysis (3.99%±2.51% vs 2.10%±1.83%; 2.73%±1.95% vs 0.95%±1.87%).

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 to un-irradiated settings. This could point to a role of iNKT/ NK and CIK cells beside T cells in antileukemic reactions (d.n.s.).

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 cut-off values) of iNKT as well as of CIK and NK cells after MLC might correlate with successful blast-lysis.

6 Discussion

6.1 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 anti-tumor functionality can be improved by $DC_{leu.}$[9]. 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 immunological 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 don't recognize target cells by a TCR [5]. iNKT cells can produce a variety of chemokines and cytokines like IFN-γ, IL-4/-2/-12, TNF-α, TGF-ß 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-γ 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.

6.2 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 Vα24-/Vβ11-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 and 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 Vα24Jα18-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 to 6B11-PE in AML, ALL, CLL patients. The proportions of iNKT cells detected with 6B11-FITC compared to 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.

Co-expression analyses of 6B11 with myeloid-(e.g.: CD33, CD34, CD117) or lymphoid (e.g.: 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. Since we do not know, whether this cell line has a Vα24-/Vβ11-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 analyse 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 analyse iNKT subsets.[13] Moreover, iNKT cells were characterized by the combination of CD1d and CD3 or the combination of Vα24 and Vβ11. 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, CD45R0 and CD161. Moreover, we included the combinations of 6B11 with CD1d, Vα24, CD4 or with CD8. This means that we evaluated the expression of T cell (CD1d, CD3, CD45RO, Vα24) 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 $CD3^+6B11^+$-, $CD4^+6B11^+$-, $CD8^+6B11^+$-, $V\alpha24^+6B11^+$-, $CD1d^+6B11^+$-, $CD45RO^+6B11^+$- and $CD45RO^-6B11^+$ iNKT cells.

Classically, iNKT cells have been identified with CD1d-tetramers loaded with α-GalCer, 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 to $CD3^+6B11^+$ 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 to $6B11^+CD3^+$ and $6B11^+CD1d^+$ iNKT cells in healthy samples as well as in AML, ALL and CLL samples.

Using CD3-/Vα24-antibodies, we could demonstrate specific results and detected lower frequencies of $V\alpha24^+6B11^+$ iNKT cells compared to $CD3^+6B11^+$ iNKT subsets in healthy as well as in AML, ALL and CLL patients. These findings are consistent with a previous study, which found three times more $CD3^+6B11^+$ iNKT cells compared to $CD3^+V\alpha24^+V\beta11^+$ iNKT cells in healthy adult and children's samples.[11] However, this group combined Vα24 with CD3 and Vβ11, while we combined Vα24 with 6B11. Unlike these results, another group found similar proportions of $CD3^+6B11^+$ iNKT cells and $V\alpha24^+V\beta11^+$ iNKT cells in healthy controls.[13] However, this group used the combination of Vα24 and Vβ11 without combining it with CD3. These differences may be explained by the fact, that different combinations of Vα24 with CD3, Vβ11 or 6B11 were used for iNKT cell detection. Furthermore, the results or our group and Bienemann may be explained by the fact that the invariant α-chain of the TCR sometimes binds with other ß-chains than Vβ11, 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 CD8αα+ 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 analysed the functional role of CD4 and CD8 expressions on iNKT cells. However, we suggest to combine 6B11 with CD4 and CD8 to analyse the diffences 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 to $6B11^+$ 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 (DN) iNKT cells compared to $CD4^+$ 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 (CMV) 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, Va24, CD4 or CD8. Furthermore, we could demonstrate that 6B11 can be used to analyse 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 naïve phenotype and can be modified more easily in their capacity to acquire Th1- or Th2-like functions compared to 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.

6.3 iNKT, NK and CIK cells in AML, ALL and CLL patients compared to 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 $-V\alpha24^+$) compared to 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 to healthy donors' PB (cells/μL: 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 vs 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 Vα24 and Vβ11. [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 are 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 to healthy donors showing a shift to (significantly) higher frequencies of T and NK cells co-expressing 6B11 in AML, ALL and CLL patients compared to 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 to 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 to 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.

6.4 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/µL 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, e.g. 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 (versus adverse) NCCN risk score showed higher percentages of $6B11^+$-, $6B11^+CD161^+$- and $6B11^+Va24^+$ iNKT cells. Moreover, AML and CLL patients younger (versus older) than 60 years showed higher numbers of $6B11^+$-, $6B11^+CD161^+$-, $6B11^+CD3^+$- and $6B11^+Va24^+$ iNKT cells. AML patients with primary (versus secondary) AML showed higher frequencies of $6B11^+$- and $6B11^+CD3^+$ iNKT cells. Furthermore, AML and adult ALL patients without (versus 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 (versus NCR) and CLL patients with no need (versus need) for therapy showed higher frequencies of $6B11^+$-, $6B11^+CD3^+$- and $6B11^+Va24^+$ iNKT cells. AML and CLL patients with stable CR (versus no stable CR: relapse or death by disease) showed (significantly) higher percentages of $6B11^+$-, $6B11^+CD3^+$- and $6B11^+CD161^+$ iNKT cells, however, one 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 overall survival. 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 (versus 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 (versus 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 (versus NCR) and CLL patients with no need for initial therapy (versus 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 (versus 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.

6.5 iNKT, NK and CIK cells contribute to anti-tumor and anti-leukemic 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 to unradiated 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 analyse the function of different iNKT, CIK or NK cells and their subtypes we recommend performing e.g. 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 cut-off 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

6.6 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 (e.g. 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 microenvironment 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 e.g. by chemokine receptor-expression), leading to an amplification of anti-tumor-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 three possible mechanisms of antitumor-reactivity mediated by iNKT cells: indirect cytotoxicity, direct cytotoxicity and modulation of the tumor microenvironment. 'Indirect cytotoxicity' says that iNKT cells and DC stimulate each other by TCR/CD1d and CD40/CD154 interactions leading

to a release of cytokines and increased antitumor activity of other effector cells.[39,43] Our data might support this theory as we could show that 'highest' iNKT cell frequencies were correlated with higher DC – pointing to a co-activation between iNKT cells and DC.

We can add 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 E_2 on the maturation of DC has been shown.[19,29] Alternatively, it might be discussed, that 'only' the 6B11- or CD56-antigens are upregulated on T and NK cells, however without expansion of functionally reactive iNKT and CIK cell populations.

6.7 Physiologic culture conditions (hypoxia, WB) do not have an impact on the generation of DC nor on the amount and composition of iNKT, NK or CIK cells

Preliminary experiments with MNC/ WB cultured under hypoxic vs normoxic conditions did not yield an impact 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.

7 Conclusions

In conclusion, we recommend to regularly evaluate proportions of iNKT, NK and CIK cells and include specific markers in diagnosis-panels (based on 6B11/ CD161/ CD56/ CD3-antibodies) in AML, ALL and 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 to 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'. These findings not only point to a cross-talk between these immune reactive cells with 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 in DC/ DC_{leu}-triggered antileukemic

reactions in AML patients should be studied in detail and probably be included in immunotherapeutic protocols against AML.

8 References

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9 List of tables

10 List of figures

11 Abbreviations

α-GalCer	α-galactosylceramide
ALL	acute lymphoid leukemia
AML	acute myeloid leukemia
APC	allophycocyanin
ATTC	American Type Culture Collection
BI	pro-B-acute lymphoid leukemia
BII	c-acute lymphoid leukemia
BIII	pre-B- acute lymphoid leukemia
$BII^{+}My^{+}$	My^{+}c- acute lymphoid leukemia
$BIII^{+}My^{+}$	My^{+}pre- acute lymphoid leukemia
B-CLL	B-cell-type, chronic lymphatic leukemia
BM	bone marrow
CD	Cluster of differentiation
CIK cells	cytokine-induced killer cells
CLL	chronic lymphatic leukemia
'Cocktails'	DC-generating methods/Kits, containing immune-response-modifiers in combination with cytokines
CR	complete remission
Cy7-PE	Cy7-PE conjugation
D	day
DC	dendritic cells
DC_{leu}	leukemia-derived dendritic cells
dgn.	first diagnosis
DP	$CD4^{+}CD8^{+}$double-positive-thymocytes
DN	$CD4^{-}CD8^{-}$double-negative-thymocytes
EBV	Eppstein-Barr virus
EGIL	European Group for Immunophenotyping of Leukemias
FAB	French-American-British-classification
FACS	fluorescent-activated cell-sorting
FCS	foetal calf serum
FITC	fluorescein isothyocianate
GMALL	Study Group for Adult Acute Lymphoblastic Leukemia

GM-CSF	granulocyten-macrophage-colony-stimulating factor
I	Kit-I
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-12	Interleukin-12
K	Kit-K
M	Kit-M
M0	minimally differentiated AML
M1	AML without maturation
M2	AML with granulocytic maturation
M4	acute myelomonocytic leukemia
M4eo	acute myelomonocytic leukemia together with BM-eosinophilia
M5	acute monocytic leukemia
MACS	magnetic associated cell sorting
MDS	myelodysplastic syndrome
MHC-I	major-histocompatibility-complex-I-molecules
MHC-II	major-histocompatibility-complex-II-molecules
MNC	mononuclear cells
MLC	mixed lymphocyte culture
moAb	monoclonal antibody
moAbs	monoclonal antibodies
NCCN	National Comprehensive Cancer Network
NCR	no complete remission
NK cells	natural killer cells
NKT cells	natural killer T cells
iNKT cells	invariant natural killer T cells
p	primary
PB	peripheral blood
PBS	phosphate-buffered saline
PE	phyoerythrin
PGE_1	prostaglandin E1
PGE_2	prostaglandin E2
‚Pici1‘	‚Picibanil 1‘

‚Pici2‘	‘Picibanil 2’
pO_2	oxygen-partial-pressure
RPMI	Roswell Memorial Park Institute
rel.	relapse
rel.a.SCT	relapse after stem cell transplantation
s	secondary
SCT	stem cell transplantation
TI	pro-T-acute lymphoid leukemia
TII	pre-T-acute lymphoid leukemia
TIII	cortical-T-acute lymphoid leukemia
TIV	mature-T-acute lymphoid leukemia
T_{cm}	central-memory T cells
TCR	T cell receptor
T_{eff-em}	effector/ memory T cells
T_{naive}	naive T cells
$T_{non-naive}$	non-naive T cells
TNF-α	tumor necrosis factor α
T_{reg}	regulatory T cells
WB	whole blood

12 List of publications

12.1 Original studies

C. L. Boeck, D. C. Amberger, F. Doraneh-Gard, W. Sutanto, T. Guenther, J. Schmohl, F. Schuster, H. Salih, F. Babor, A. Borkhardt, H. Schmetzer: 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. *J Immunother. 2017 Jul/Aug;40(6):224-248. doi: 10.1097/CJI.0000000000000171*.

D. C. Amberger, F. Doraneh-Gard, C. L. Boeck, C. Plett, C. Gunsilius[1], C. Kugler[1], J. O. Werner, D. Kraemer, B. Ismann, A. Rank, C. Schmid, H. Schmetzer: A new method to generate mature (leukemia-derived) dendritic cells that improve antileukemic T cell reactivity from mononuclear cells or whole blood from healthy volunteers or patients with AML. *Article in preparation (2018).*

F. Doraneh-Gard, D. C. Amberger, C. L. Boeck, C. Plett, C. Gunsilius, C.Kugler, J. O. Werner, J. Schmohl, D. Kraemer, B. Ismann, A. Rank, C. Schmid, H. Schmetzer: Influence of physiological hypoxia on whole blood (WB) from healthy donors and AML-pts: the (leukemic) antigen-presentation on blasts, on leukemia-derived dendritic cells (DC_{leu}) after stimulation with 'DC-generating cocktails' as well as the antileukemic functions of DC/ DC_{leu}-stimulated immune reative cells. *Article in preparation (2018).*

12.2 Congress contributions

C. L. Boeck, D. C. Amberger, F. Doraneh-Gard, W. Sutanto, T. Guenther, J. Schmohl, F. Schuster, H. Salih, F. Babor, A. Borkhardt, H. Schmetzer: Significance of frequencies, compositions and/ or antileukemic activity of (DC-stimulated) invariant NKT, NK and CIK cells is predictive for outcome of patients with AML, ALL and CLL. *Abstract and poster presentation; ITOC 4 2017- Journal for ImmunoTherapy of Cancer 2017, 5 (1), 13 (2017).*

C. L. Boeck, D. C. Amberger, F. Doraneh-Gard, W. Sutanto, T. Guenther, J. Schmohl, F. Schuster, H. Salih, F. Babor, A. Borkhardt, H. Schmetzer: Number, composition and/ or antileukemic activity of (DC-stimulated) invariant NKT, NK and CIK cells is predictive for

outcome of patients with AML, ALL and CLL. *Abstract and poster presentation; EBMT 2017: www.ebmt2017.org/poster-abstracts; Physician Poster Abstracts: B222.*

D. C. Amberger, F. Doraneh-Gard, C. L. Boeck, C. Plett, C. Gunsilius[1], C. Kugler[1], J. O. Werner, D. Kraemer, B. Ismann, A. Rank, C. Schmid, H. Schmetzer: A new method to generate mature (leukemia-derived) dendritic cells that improve antileukemic T cell reactivity from mononuclear cells or whole blood from healthy volunteers or patients with AML. *Abstract and poster presentation; ITOC 4 2017- Journal for ImmunoTherapy of Cancer 2017, 5 (1), 4-5 (2017).*

D. C. Amberger, F. Doraneh-Gard, C. L. Boeck, C. Plett, C. Gunsilius[1], C. Kugler[1], J. O. Werner, D. Kraemer, B. Ismann, A. Rank, C. Schmid, H. Schmetzer: A new method to generate mature (leukemia-derived) dendritic cells that improve antileukemic T cell reactivity from mononuclear cells or whole blood from healthy volunteers or patients with AML. *Abstract and poster presentation; EBMT 2017: www.ebmt2017.org/poster-abstracts; Physician Poster Abstracts: A121.*

C. L. Boeck, D. C. Amberger, F. Doraneh-Gard, W. Sutanto, T. Guenther, J. Schmohl, F. Schuster, H. Salih, F. Babor, A. Borkhardt, H. Schmetzer: Significance of frequencies, compositions and/ or antileukemic activity of (DC-stimulated) invariant NKT, NK and CIK cells is predictive for outcome of patients with AML, ALL and CLL. *Poster presentation and abstract for 19th scientific symposium of Med 3, University of Munich (2017).*

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14 Eidesstaatliche Versicherung

Ich, **Corinna Lesley Böck**, erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

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

selbstständig verfasst, mich außer den angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annährend übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorliegende Dissertation nicht in gleicher oder ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, den 18.06.2018 Corinna Lesley Böck

Ort, Datum Unterschrift des Doktoranden