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The potential of Prostaglandin E₁ to generate dendritic cells from leukemic whole blood and the stimulating effect on cells of the innate and adaptive immune system

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Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation

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Index of abbreviations

ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
APCs	Antigen-presenting cells
CAR-T-cell-therapy	Chimeric antigen receptor T cell therapy
CIK	Cytokine-induced killer cells
CLL	Chronic lymphoid leukemia
DCs	Dendritic cells
DC _{leu}	Leukemia derived dendritic cells
HSCT	Hematopoietic stem cell transplantation
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good Manufacturing Practice
GvL	Graft vs leukemia
IL-2	Interleukin 2
IL-4	Interleukin 4
iNKT	Invariant natural kill cells
LAA	Leukemic-associated antigens
MHC	Major histocompatibility complex
MDS	Myelodysplastic Syndrome
MLC	Mixed lymphocyte culture
mRNA	Messenger ribonucleic acid
NK cells	Natural killer cells
PBMNCs	Peripheral blood mononuclear cells
PGE ₁	Prostaglandin E1
PGE ₂	Prostaglandin E2
TNF- α	Tumor-necrosis-factor alpha
WB	Whole blood
WT1	Wilms Tumor Antigen 1

List of Publications

In this medical thesis, the following publications are summarized to a cumulative thesis according to the examination rules of the medical faculty of the LMU Munich:

Publication I:

PGE₁-Containing Protocols Generate Mature (Leukemia-Derived) Dendritic Cells Directly from Leukemic Whole Blood.

Amberger DC, Doraneh-Gard F, Gunsilius C, Weinmann M, Möbius S, Kugler C, Rogers N, Böck C, Ködel U, Werner JO, Krämer D, Eiz-Vesper B, Rank A, Schmid C, Schmetzer HM. Int J Mol Sci. 2019 Sep 17;20(18).

Publication II:

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.

Boeck CL, Amberger DC, Doraneh-Gard F, Sutanto W, Guenther T, Schmohl J, Schuster F, Salih H, Babor F, Borkhardt A, Schmetzer H.

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C.L.B. and D.C.A contributed equally

Authors' contributions

Contribution of Daniel Christoph Amberger:

Daniel Christoph Amberger was responsible for the following points in *Publication I and II:*

- Conducting experiments¹ (as described in the Material and Methods parts)
- Statistical analysis
- Drafting the two manuscripts² (including main text, figures, references)
- Coordination of co-authors communication
- Revision of the manuscripts and integration of reviewers' comments towards publication³

All co-authors confirmed with their signature that Daniel Christoph Amberger has the permission to use the two publications for his medical thesis. Furthermore, they confirmed that the publications are not part of other doctoral theses. Daniel Christoph Amberger submitted the list with all signatures separately with this medical thesis.

¹ In publication II together with Corinna L. Böck; C.L.B. and D.C.A are contributed equally

² In publication II together with Corinna L. Böck; C.L.B. and D.C.A are contributed equally

³ In publication II together with Corinna L. Böck; C.L.B. and D.C.A are contributed equally

Introduction

Acute myeloid leukemia (AML) and Myelodysplastic Syndrome (MDS) are clonal diseases characterized by an uncontrolled proliferation and an impaired differentiation of myeloid progenitor cells (blasts). Accumulating blasts replace the physiological hematopoiesis and cause typical symptoms such as anemia, bleeding and infections [1]. Up to now, overall five-year survival rates of about 40% - 60% in younger and 5% in elderly patients (median five-year survival rate 28.3 months) are still disappointing [2-4].

State of the art – current therapy approaches for patients with AML

Depending on age, cytogenetic markers and other risk factors cytarabin ± anthracycline (e.g., 7+3 regime) remains the standard induction therapy approach. As consolidation therapy, high dose chemotherapy with or without following allogenic hematopoietic stem cell transplantation (HSCT) is the therapy standard and has not changed during the last years [5-7]. HSCT is the only potential curative treatment option for younger AML-patients that have an expected relapse rate of > 35 – 40% without treatment [5]. The curative potential of HSCT is driven by the Graft vs leukemia (GvL) effect, mediated by allogenic effector T and natural killer (NK) cells against residual leukemic blasts [8]. Adding Midostaurin, a multitarget tyrosin kinase inhibitor in patients with FMS-like tyrosin kinase 3 (FLT3)-mutation - occurring in 30% of AML-patients - showed a prolonged overall survival [9-12]. Low-dose cytarabin or hypomethylating agents are therapy options for (elderly) patients with low tolerance of intensive induction chemotherapy [13].

Active and passive immunotherapy approaches for patients with AML

Since HSCT and high dose induction chemotherapy is no therapy option especially for unfit or elderly patients and relapse-rates are even high there is a need for new and alternative post remission (immune) therapy approaches, to stabilize the disease and to create long-term survival. Cancer cells, including myeloid blasts can escape the immunosurveillance and therefore different active and passive immunotherapy approaches revolutionized cancer immunotherapy in solid as well as hematological malignancies [7, 14]. Antibody-drug conjugates such as Gemtuzumab ozogamicin, bi-specific antibodies targeting cancer cells and immunocompetent cells simultaneously and antibodies to block immune checkpoints are under development for the treatment of AML. In summary, the potential of these passive

immunotherapy approaches remain still unclear [15, 16]. Furthermore, the chimeric antigen receptor T cell-therapy (CAR-T-cell-therapy) which revolutionized the treatment of relapsed and refractory acute lymphoblastic B cell leukemia is introduced in the AML treatment [17]. First results showed that the therapy with CAR-T-cells leads to an eradication of AML by an anti-tumor activity against myeloid blasts, however therapy is limited by life-threatening adverse events, including neurotoxicity and cytokine release syndromes [18-21].

As an active immunomodulating AML therapy approach, Interleukin 2 (IL-2) a potent T cell and NK cell stimulating and activating cytokine was analyzed with or without a combination of histamine [22]. The administration of whole tumor vaccinations or of leukemic-associated antigens (LAA) induced LAA-specific T cells and prolonged remissions in patients with AML, but the overall responses were variable [22].

Dendritic cell based immunotherapy in the treatment of AML

Antigen presenting cells (APCs) such as dendritic cells (DCs) play a pivotal role in connecting the innate and the adaptive immune system [23, 24]. They internalize and process antigens, present antigen-fragments via major histocompatibility complex (MHC) and form immunological synapses with T cells, resulting in a clonally restricted and potent T cell-activation [25-27].

In general, two different DC-based immunotherapy strategies were developed. On the one hand, monocyte (CD14⁺) derived DCs can be generated in the presence of different response modifiers in cultures *ex vivo*, loaded with LAA by electroporation of messenger ribonucleic acid (mRNA) encoding for example Wilms Tumor Antigen 1 (WT1) [22]. After expensive *ex vivo* manipulation and production of cells under Good Manufacturing Practice (GMP), DCs can be (re-) administrated to patients as a vaccine [28, 29].

On the other hand, leukemic blasts can be converted *ex vivo* to leukemia derived DC (DC_{leu}), presenting the complete leukemic antigen repertoire. It was already shown that the generation of DCs and DC_{leu} is possible from leukemic peripheral blood mononuclear cells (PBMNCs) and directly from leukemic whole blood (WB) [30, 31]. DC_{leu} are characterized by the simultaneous expression of DC-antigens (e.g., CD80, CD86, CD206) and blast-antigens (e.g., CD34, CD65, CD117) [30]. Following Schmetzer et al 2007 a special flow cytometric gating protocol, that enables the detection and quantification of DCs and DC_{leu} in the total cell fraction

(DC_{leu}/cells), in the DC-fraction (DC_{leu}/DC⁺) and in the blast fraction, to count the frequencies of blasts converted to DC_{leu} (DC_{leu}/bla⁺) has been developed [32].

Up to now, different DC/DC_{leu}-generating-protocols that contain combinations of different response modifiers including 1.) Cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or Interleukin 4 (IL-4) that *induce differentiation* of myeloid progenitor cells or 2.) Calcium-Inophore (A23187) as a cytokine free DC/DC_{leu}-generating method 3.) Bacterial or nucleic stimulation with *danger signaling effects* such as Picibanil (OK432) – a lysis product from the streptococcus pyogenes and 4.) Substances *inducing maturation* of DC/DC_{leu} such as Prostaglandin E₂ (PGE₂) or Tumor-nekrosis-factor alpha (TNF- α) have been developed [30, 33].

Stimulation of T cell enriched immunoreactive cells with DC/DC_{leu} *ex vivo* in a mixed lymphocyte culture (MLC) regularly results in (re-) activation of T cells against leukemic blasts [30, 34, 35]. Naïve T cells (T_{naïve}, CD45RO⁻), non-naïve T cells (T_{non-naïve}, CD45RO⁺), central-memory T cells (T_{cm}, CD45RO⁺CCR7⁺), effector (memory) T cells (T_{eff-em}, CD45RO⁺CCR7⁻), proliferating T cells (T_{prol} CD71⁺, CD69⁺) and regulatory T cells (T_{reg}, CD25⁺⁺CD127^{low}) are examples for different T cell subsets and can be stimulated during MLC [36, 37]. Natural kill cells (NK cells) characterized by the expression of CD56⁺CD161⁺CD3⁻ are important mediators of the innate immune system and kill tumor cells directly without previous activation. Cytokine induced killer cells (CIK cells) connect function of T as well as NK cells by the simultaneous expression of CD56⁺CD161⁺CD3⁺ [38]. Invariant natural kill cells (6B11⁺CD3⁺ iNKT cells) are cells on the interface of the innate and adaptive immune system with the opportunity to enhance tumor immunity [39].

Outline of this doctoral thesis

Due to the low five year survival rates and the high rates of relapse in patients with AML there is a need for alternative therapy options such as immunotherapy to maintain stable remissions and/or stable disease. The preferable immunological way to treat patients with AML would be to activate the cellular and/or humoral immune system that can eliminate residual blasts in the bone marrow as well as in the peripheral blood and can create an immunological memory, which avoids relapses. The DC/DC_{leu} concept would be a great tool, which might connect both features. Therefore, I analyzed in this doctoral thesis two new Prostaglandin E₁ (PGE₁) containing DC/DC_{leu}-generating protocols, which might be used *in vivo* to convert myeloid

blasts to DC/DC_{leu}. I generated DCs and DC_{leu} directly from leukemic WB, containing all cellular and soluble factors of the individual patient, to simulate physiological conditions. DCs and DC_{leu} have an impact on many different immunoreactive cells and therefore I analyzed the stimulating effect of DC/DC_{leu} on cells of the innate and the adaptive immune system such as NK cells, CIK cells as well as iNKT cells.

Publication I: PGE₁-containing protocols generate mature (leukemia-derived) dendritic cells directly from leukemic whole blood.

In *publication 1 (Amberger et al. 2019)* of this manuscript, I developed and analyzed two new Prostaglandin E₁ (PGE₁)-containing protocols to generate DCs and DC_{leu} ex vivo from healthy as well as leukemic PBMNCs and directly from WB. The protocol Pici-PGE₁ is a modification of the already established protocol Pici-PGE₂ and contains a combination of GM-CSF, IL-4, Picibanil and PGE₁. Furthermore, I analyzed the minimalized Kit M, containing GM-CSF and PGE₁ to generate DCs and DC_{leu} directly from leukemic WB. In summary, I could show that the PGE₁ containing protocol Pici-PGE₁ is suitable to generate significantly higher amounts of DCs and DC_{leu} from healthy as well as leukemic PBMNCs compared to control and comparable amounts as the already established protocol Pici-PGE₂. According to the defined cut-off value, even more cases with sufficient DC-generation after DC/DC_{leu}-generation were found with Pici-PGE₁ compared to Pici-PGE₂. Moreover, I generated DC/DC_{leu} directly from healthy and leukemic WB to simulate physiological conditions. With Kits, containing different combinations of response-modifiers (e.g., Kit M: GM-CSF and PGE₁, Kit K: GM-CSF and PGE₂, Kit I: GM-CSF and Picibanil) it was possible to generate significant more DCs and DC_{leu} from leukemic WB compared to control. Proliferation of blast was not induced by Kits. The average amounts of generated DCs and DC_{leu}-subgroups were comparable with all three Kits. The PGE₁ containing Kit M generated significantly higher amounts of mature DCs that are characterized by the expression of the lymph-node-homing-receptor CCR7 when compared to the PGE₂-containing Kit K. After stimulating T cell enriched immunoreactive cells with the generated DCs and DC_{leu} in a mixed lymphocyte culture (MLC) the activation status of various T cell subsets increased compared to control. Furthermore, I could show that PGE₁-containing protocols also increased the anti-leukemic-activity especially after 24 hours, pointing to an induction of leukemia specific immunoreactive cells against leukemic blasts. In addition, Kits influenced cytokine-release-profiles during DC/DC_{leu}-cultures with higher amounts of inflammatory cytokines as well antitumor response related cytokines after culture.

Publication II: 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.

As shown in *publication II (Boeck et al. 2017)* DC/DC_{leu}, probably contribute to stimulate and activate cells from the innate as well as adaptive immune system. In the first part of this publication, we analyzed the amounts of NK cells, CIK cells as well as iNKT cells in patients with AML, acute lymphoid leukemia (ALL) and chronic lymphoid leukemia (CLL) in active stages of the disease and compared results to healthy control groups. In summary, we found lower amounts of NK cells, CIK cells as well as iNKT cells in patients with AML, ALL and CLL compared to the healthy control groups. Furthermore, we could show that the amounts of these immunoreactive cells influenced the prognosis of patients with leukemia. For example, higher amounts of CD3⁺CD56⁺ CIK cells were associated with significantly higher complete remission rates after chemotherapy as well as lower amounts of extramedullary foci in patients with AML. In the second part of the publication, we stimulated T cell enriched immunoreactive cells in MLC with DC/DC_{leu} generated with different DC/DC_{leu}-generating-protocols from leukemic and healthy WB and analyzed compositions of NK cells, CIK cells as well as iNKT cells. DCs and DC_{leu} stimulated iNKT cells but not CIK or NK cells during MLC compared to control. For each individual patient, we defined the best DC/DC_{leu}-generating protocol with highest amounts of iNKT cells. Furthermore, we could correlate amounts of generated iNKT cells in MLC with the anti-leukemic activity and found that high amounts of iNKT cells correlated with a higher anti-leukemic activity.

Conclusion

In summary, I can conclude that PGE₁ is suitable to generate and to mature monocyte and leukemic derived DCs from healthy and leukemic PBMCs as well as WB. GM-CSF and PGE₁ are drugs that are approved for human treatment and so I conclude that, the PGE₁-containing Kit M might qualify to convert (residual) myeloid blasts to DCs and DC_{leu} in vivo after the application of the Kit to AML-patients. Furthermore, the DC/DC_{leu}-generating protocol Pici-PGE₁ might qualify for the ex vivo production of DCs from healthy monocytes, which can be used for an adaptive cell transfer. Therefore, ongoing research trials with animals and or humans have to be performed. Furthermore, we could demonstrate that amounts of iNKT cells, CIK cells as well as NK cells are influenced in patients with leukemia compared to the healthy control group. The amounts of these cells correlate with the treatment outcome and the prognosis of

the patients. We could show evidence that DC/DC_{leu} generated with different protocols stimulate iNKT cells in MLC. The quantitative analysis of these cells should be included in diagnosis panels for quantitative prognostic estimations of patients with leukemia.

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Summary

Dendritic cells (DCs) and leukemia-derived DC (DC_{leu}) are potent stimulators of various immunoreactive cells and play a pivotal role on the interface of the innate as well as adaptive immune system. Different working-groups developed various strategies to utilize DCs or DC_{leu} as a treatment tool for patients with acute myeloid leukemia (AML).

In this doctoral thesis, I developed two new Prostaglandin E_1 (PGE_1) containing protocols (Pici- PGE_1 , Kit M) to generate DC/ DC_{leu} ex vivo from leukemic peripheral blood mononuclear cells (PBMNCs) or directly from leukemic whole blood (WB) containing all soluble and cellular factors of the individual patient. With Pici- PGE_1 DC/ DC_{leu} generation improved significantly from leukemic as well as healthy PBMNCs compared to the control group. Furthermore, sufficient DC-generation from leukemic PBMNCs was superior compared to the already established protocol Pici- PGE_2 . To simulate physiological conditions, I generated DCs and DC_{leu} directly from leukemic and healthy WB with Kits containing a combination of at least two response modifiers. Stimulation of T cell enriched immunoreactive cells with the generated DC/ DC_{leu} in a mixed lymphocyte culture increased the activation status of T cells as well as their anti-leukemic activity. This finding, points to an induction of a specific anti-leukemic immunity ex vivo.

Furthermore, we analyzed the composition of natural killer cells (NK cells), cytokine-induced killer cells (CIK cells) as well as invariant natural killer cells (iNKT cells) in patients with AML, acute lymphoid leukemia (ALL) as well as chronic lymphoid leukemia (CLL) which are key players in the innate and adaptive immune system. Decreased amounts of NK cells, CIK cells as well as iNKT cells in the PBMNCs fractions of patients with leukemia compared to the healthy control group characterized the main findings. We correlated the amounts of these immunoreactive cells with the prognosis and the treatment outcome. Moreover, we provided evidence that generated DC/ DC_{leu} from leukemic WB (especially with DC/ DC_{leu} -generating protocols containing prostaglandins) might stimulate iNKT cells. In addition, we could demonstrate that amounts of iNKT influence the anti-leukemic activity in a positive way.

In conclusion, PGE_1 is suitable to generate DC/ DC_{leu} directly from leukemic WB ex vivo. These generated antigen-presenting cells reliably (re-) activate cells of the innate as well as adaptive immune system and improve the overall ex vivo anti-leukemic activity. All Kit substances are

approved for human treatment and so these Kits might be used in vivo to convert myeloid blast to DCs and DC_{leu}. These cells might induce an anti-leukemic immunity in patients with AML. To improve the DC/DC_{leu} therapy either with adaptive transfer or even with in vivo generation of DC/DC_{leu} for patients with AML ongoing in vivo research trials with animals and or humans have to be performed.

Zusammenfassung

Dendritische Zellen und dendritische Zellen leukämischen Ursprungs sind potente Stimulatoren von verschiedensten immunreaktiven Zellen und spielen eine Schlüsselrolle an der Schnittstelle zwischen dem angeboren und erworbenen Immunsystem. Verschiedene Forschungsgruppen haben bereits diverse Strategien entwickelt, um dendritische Zellen oder dendritische Zellen leukämischen Ursprungs als Therapieoption für Patienten mit akuter myeloischer Leukämie zu verwenden.

In dieser Doktorarbeit entwickelte ich zwei neue Prostaglandin E₁ (PGE₁) enthaltende Protokolle (Pici-PGE₁, Kit M) für die ex vivo Generierung von dendritischen Zellen und dendritischen Zellen leukämischen Ursprungs von isolierten leukämischen mononuklearen Zellen oder direkt im leukämischen Vollblut. Die Generierung von dendritischen Zellen und dendritischen Zellen leukämischen Ursprungs war mit dem neuem Protokoll Pici-PGE₁ sowohl von leukämischen als auch von gesunden mononuklearen Zellen möglich. Pici-PGE₁ war dem bereits etablierten Protokoll Pici-PGE₂ bezüglich des Prozentsatzes an erfolgreicher Generierung von dendritischen Zellen deutlich überlegen. Da Vollblut alle zellulären sowie löslichen Komponenten der individuellen Patienten beinhaltet und daher eine physiologische Situation widerspiegelt, generierten wir dendritische Zellen und dendritische Zellen leukämischen Ursprungs mit verschiedenen Kits direkt in leukämischen und gesunden Vollblutproben. Kits bestehen aus einer Kombination von zumindest zwei klinisch zugelassenen Wirkstoffen zur Behandlung von unterschiedlichen Krankheiten. Die Generierung von dendritischen Zellen war im Vergleich zur Kontrolle möglich. Die Stimulation von mit T-Zellen angereicherten immunreaktiven Zellen mit diesen generierten dendritischen Zellen und dendritischen Zellen leukämischen Ursprungs verbesserte den Aktivitätsstatus sowie die antileukämische Aktivität. Dies unterstreicht eine spezifische Induktion von einer antileukämischen Immunität ex-vivo.

Des Weiteren analysierten wir die Zusammensetzung von natürlichen Killerzellen, Zytokin-induzierten-Zellen sowie invarianten natürlichen Killerzellen in Patienten mit akuter myeloischer Leukämie, akuter lymphatischer Leukämie sowie chronisch lymphatischer Leukämie. Diese Zellen haben Schlüsselfunktionen im angeboren sowie im erworbenen Immunsystem. Das wichtigste Ergebnis dieser Arbeit ist, dass deutlich geringere Mengen an natürlichen Killerzellen, Zytokin-induzierten-Zellen sowie invarianten natürlichen Killerzellen in Patienten

mit Leukämie, verglichen zu gesunden Kontrollgruppen, nachweisbar waren. Wir korrelierten die Menge dieser immunreaktiven Zellen mit der Prognose sowie dem Therapieerfolg der Patienten. Außerdem konnten wir demonstrieren, dass die generierten dendritischen Zellen (insbesondere mit Prostaglandinen) eine stimulierende Wirkung auf invariante natürliche Killerzellen haben und dadurch die anti-leukämische Immunantwort verbessern können. Zusätzlich konnten wir zeigen, dass die Menge von invarianten natürlichen Killerzellen die anti-leukämische Aktivität in einem positiven Sinn beeinflusst.

Mit Prostaglandin E₁ enthaltenden Protokollen können dendritische Zellen und dendritische Zellen leukämischen Ursprungs direkt aus Blasten in leukämischem Vollblut generiert werden. Diese antigenpräsentierenden Zellen aktivieren zuverlässig Zellen des angeborenen als auch des erworbenen Immunsystems und verstärken die allgemeine anti-leukämische Immunantwort. Alle Kit Wirkstoffe sind für die Behandlung von unterschiedlichen Krankheiten zugelassen und könnten in-vivo für die Konvertierung von leukämischen Blasten zu dendritischen Zellen und dendritischen Zellen leukämischen Ursprungs verwendet werden. Studien an Tieren sowie Menschen sind in Zukunft notwendig, um die Therapie mit dendritischen Zellen für Patienten mit akuter myeloischer Leukämie zu verbessern.



Article

PGE₁-Containing Protocols Generate Mature (Leukemia-Derived) Dendritic Cells Directly from Leukemic Whole Blood

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Abstract: Dendritic cells (DCs) and leukemia-derived DC (DC_{leu}) are potent stimulators of various immunoreactive cells and they play a pivotal role in the (re-) activation of the immune system. As a potential treatment tool for patients with acute myeloid leukemia, we developed and analyzed two new PGE₁-containing protocols (Pici-PGE₁, Kit M) to generate DC/DC_{leu} ex vivo from leukemic peripheral blood mononuclear cells (PBMCs) or directly from leukemic whole blood (WB) to simulate physiological conditions. Pici-PGE₁ generated significantly higher amounts of DCs from leukemic and healthy PBMCs when compared to control and comparable amounts as the already established protocol Pici-PGE₂. The proportions of sufficient DC-generation were even higher after DC/DC_{leu}-generation with Pici-PGE₁. With Kits, it was possible to generate DCs and DC_{leu} directly from leukemic and healthy WB without induction of blast proliferation. The average amounts of generated DCs and DC_{leu}-subgroups were comparable with all Kits. The PGE₁ containing Kit M generated significantly higher amounts of mature DCs when compared to the PGE₂-containing Kit K and increased the anti-leukemic-activity. In summary PGE₁-containing protocols were suitable for generating DC/DC_{leu} from PBMCs as well as from WB, which reliably (re-) activated immunoreactive cells, improved the overall ex vivo anti-leukemic activity, and influenced cytokine-release-profiles.

Keywords: PGE₁; AML; leukemia-derived dendritic cells; immunotherapy; dendritic cells

1. Introduction

Acute myeloid leukemia (AML) is a clonal disease that is characterized by an uncontrolled proliferation and an impaired differentiation of myeloid progenitor cells (blasts) with overall five-year-survival rates of about 28.3 % [1,2]. In the last few decades, the therapy with antigen presenting cells (APCs), such as dendritic cells (DCs) revolutionized immunotherapy of AML [3–5].

APCs play a pivotal role in connecting the innate and the adaptive immune system with the properties to migrate into different tissues and activate different immune reactive cells. They internalize and process antigens, present antigen-fragments via major histocompatibility complex (MHC), and form immunological synapses with T cells, resulting in a clonally restricted and potent T cell-activation [6–10].

Two different DC-based immunotherapy strategies have been developed. Monocyte (CD14⁺) derived DCs can be generated with different response modifiers in cultures and they are loaded with leukemic-associated-antigens (LAA) by the electroporation of messenger ribonucleic acid (mRNA) or by peptide pulsing [11–13]. After expensive ex vivo manipulation and the production of cells under Good Manufacturing Practice (GMP), DCs can be (re-) administrated to patients as a vaccine [14,15].

Moreover, leukemic blasts can be converted ex vivo directly to leukemia derived DC (DC_{leu}), presenting the whole leukemic antigen repertoire. DC_{leu} simultaneously express DC-antigens and -individual patients' blast markers (blast-antigens) [16].

DCs and DC_{leu} can be generated ex vivo from leukemic peripheral blood mononuclear cells (PBMCs) or whole blood (WB) without the induction of blast proliferation [17–19]. DC/DC_{leu}-generating-protocols contain combinations of different response modifiers, including (1) cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or Interleukin 4 (IL-4) that induce differentiation of myeloid progenitor cells; or (2) Calcium-Ionophore (A23187) as a cytokine free DC/DC_{leu}-generating method; (3) bacterial or nucleic stimulation with danger signaling effects, such as Picibanil (OK432), a lysis product from the streptococcus pyogenes or Polyinosinic:polycytidylic acid (poly I:C); and, (4) substances inducing maturation of DC/DC_{leu}, such as Prostaglandin E₂ (PGE₂) or Tumor-necrosis-factor alpha (TNF- α) [17,20]. Furthermore, Interleukin 1 beta (IL-1 β), Interleukin 6 (IL-6), Interferon gamma (IFN- γ), Interferon alpha (IFN- α), and Fms-related tyrosine kinase 3 ligand (FLT3-L) were used and analyzed in different protocols to generate DCs and/or DC_{leu} from leukemic or healthy PBMCs [17,20–24].

The stimulation of T cell enriched immunoreactive cells with DC/DC_{leu} ex vivo in a mixed lymphocyte culture (MLC) regularly results in the (re-) activation of T cells against leukemic blasts, although depending on the DC/DC_{leu} protocol used for the generation of DC/DC_{leu} [17,25,26]. We could already show that proliferating T cells (T_{prol} CD71⁺, CD69⁺), non-naïve T cells (T_{non-naïve}, CD45RO⁺), regulatory T cells (T_{reg}, CD25⁺⁺CD127^{low}), β -integrin⁺ T cells and T cells with effector function, such as central-memory T cells (T_{cm}, CD45RO⁺CCR7⁺), effector (memory) T cells (T_{eff-em}, CD45RO⁺CCR7⁻) increase, while naïve T cells (T_{naïve}, CD45RO⁻) decrease during MLC [27,28]. DC/DC_{leu} probably contribute to stimulating and activating cells from the innate immune system and cells on the interface of the innate and the adaptive immune system, such as natural killer cells, invariant natural killer cells, or cytokine induced killer cells [29].

Soluble factors (e.g., cytokines) are involved in the leukemogenesis, as well as in anti-leukemic immunoreactions, thereby influencing the persistence or elimination of AML-cells and patients' treatment outcome [30]. Monocyte chemotactic protein 1 (MCP-1, also known as CC-chemokine ligand 2, CCL-2) is an inflammatory cytokine, with antitumor activity [31]. Interleukin 17A (IL17-A) is classified as an anti-tumor response related cytokine and Interleukin 10 (IL-10) is characterized as an anti-inflammatory cytokine [32–35].

Prostaglandins are responsible and involved in different physiological functions, such as inflammation, regulation of renal-blood circulation, induction of fever, and the protection of the gastric mucosa from gastric acid [36–39]. PGE₂ and Prostaglandin E₁ (PGE₁) are arachidonic acid derivatives, which are synthesized via the cyclooxygenase 1 and 2 pathway (COX1 and COX2) [40]. The biochemical differences between PGE₁ and PGE₂ are caused by different amounts of double bonds

in the side chain [41]. PGE₂ (e.g., Dinoprost) is approved by the US Food and Drug Administration (FDA) for the induction of labor in cases with medical or obstetrical indication. Drugs that contain PGE₁ are approved for the risk reduction of gastrointestinal ulcers during the treatment with nonsteroidal anti-inflammatory drugs (NSAID) (e.g., Misoprostol) and to treat erectile dysfunction (e.g., Alprostadil). Furthermore, PGE₁ is used to treat peripheral arterial disease and to maintain the patency of the ductus arteriosus in patients with ductal-dependent cardiac lesions [42–44]. PGE₁ was also analyzed in a combination with heparin to prevent liver veno-occlusive disease (VOD), which is a life-threatening obliteration of hepatic venules, in patients with AML after bone marrow transplantation (BMT) [45,46].

To improve the DC/DC_{leu}-treatment of patients with AML, we have developed minimalized Kits, containing combinations of at least two response modifiers. Following our hypotheses Kits should be able to convert leukemic blasts directly to DC/DC_{leu} in WB cultures. With respect to clinical applications this could mean, that patients could be directly treated with Kits, thereby inducing DC/DC_{leu}-generation in vivo, which would render an adoptive transfer of ex vivo generated DC/DC_{leu} unnecessary.

The aim of this study was

- (1) to develop and to functionally evaluate a new PGE₁-containing DC/DC_{leu} generating protocol to produce DCs and DC_{leu} from healthy and leukemic PBMCs;
- (2) to develop and to functionally evaluate an immunomodulatory Kit M (containing GM-CSF and PGE₁) to produce DCs and DC_{leu} directly from healthy and leukemic WB, thereby simulating in vivo conditions;
- (3) to deduce an optimized protocol for the ex vivo generation of DC/DC_{leu} which might be used for an adoptive cell transfer; and,
- (4) to deduce immunomodulatory Kits that might be able to convert myeloid leukemic blasts in vivo to DC/DC_{leu}.

2. Results

2.1. Prolog

In the first part of this manuscript, we present a new PGE₁-containing protocol (Pici-PGE₁) for the generation of DC/DC_{leu} from healthy and leukemic PBMCs.

In the second part, we simulated physiological conditions and generated DC/DC_{leu} with the DC/DC_{leu}-generating protocols Pici-PGE₁ and Pici-PGE₂ and immunomodulatory Kits directly from healthy and leukemic WB. The compositions of Pici and Kits are shown in Table 1. We correlated data with the DC/DC_{leu} stimulatory potential of T cell enriched immunoreactive cells and with the potential to generate anti-leukemia directed T cells as well as with cytokine-release-profiles.

Table 1. Compositions of DC/DC_{leu}-generating protocols.

DC/DC _{leu} -Generating Protocols	Composition	Concentration	Sources of DC/DC _{leu}	Mode of Action	Culture Time	Reference
Picibanil-PGE ₁ (Pici-PGE ₁)	GM-CSF IL-4 OK-432 PGE ₁	500 U/mL 250 U/mL 10 µg/mL 1 µg/mL	PBMC WB	GM-CSF: induction of myeloid (DC-) differentiation	7–10 days	
Picibanil-PGE ₂ (Pici-PGE ₂)	GM-CSF IL-4 OK-432 PGE ₂	500 U/mL 250 U/mL 10 µg/mL 1 µg/mL	PBMC WB	IL-4: induction of DC-differentiation Picibanil (OK-432): lysis product from streptococcus pyogenes; stimulates DC-differentiation	7–10 days	[17,20]
Kit M [#]	GM-CSF PGE ₁	800 U/mL 10 µg/mL	WB	PGE ₂ : increases CCR7-expression and enhances DC-migration	7–10 days	[47]
Kit K [#]	GM-CSF PGE ₂	800 U/mL 1 µg/mL	WB	PGE ₁ : effects are comparable to PGE ₂	7–10 days	[47]
Kit I [#]	GM-CSF OK-432	800 U/mL 1 µg/mL	WB		7–10 days	[47]

DC dendritic cells; DC_{leu} dendritic cells of leukemic origin; GM-CSF granulocyte macrophage colony stimulating factor; IL-4 interleukin 4; OK-432 picibanil; PGE₂ prostaglandin E₂; PGE₁ prostaglandin E₁; PBMC peripheral blood mononuclear cells, WB whole blood; [#] 15 801 987.7-1118 European Patent.

2.2. DC/DC_{leu}-Generation from Healthy and Leukemic PBMCs

2.2.1. Significantly Higher Amounts of DCs Generated from Healthy and Leukemic PBMCs with Pici-PGE1 and Pici-PGE2 Compared to Controls

With Pici-PGE1 and Pici-PGE2 we generated on average significantly*** higher amounts of DCs from healthy PBMCs as compared to controls ($n = 9$) (Pici-PGE1: $17.4 \pm 4.7\%$ DC⁺/PBMC, $p < 0.00003$; Pici-PGE2: $15.6 \pm 5.1\%$ DC⁺/PBMC, $p < 0.0003$; control: $6.0 \pm 2.2\%$ DC⁺/PBMC). Although differences were not significant, we found, on average, higher amounts of DC⁺/PBMC after the stimulation of healthy PBMCs with Pici-PGE1 when compared to Pici-PGE2. No significant differences were found in amounts of DC_{mig}/DC⁺ with Pici-PGE1 and Pici-PGE2 (26.8 vs. 25.1% DC_{mig}/DC⁺, $p < 0.77$) (Figure 1A).

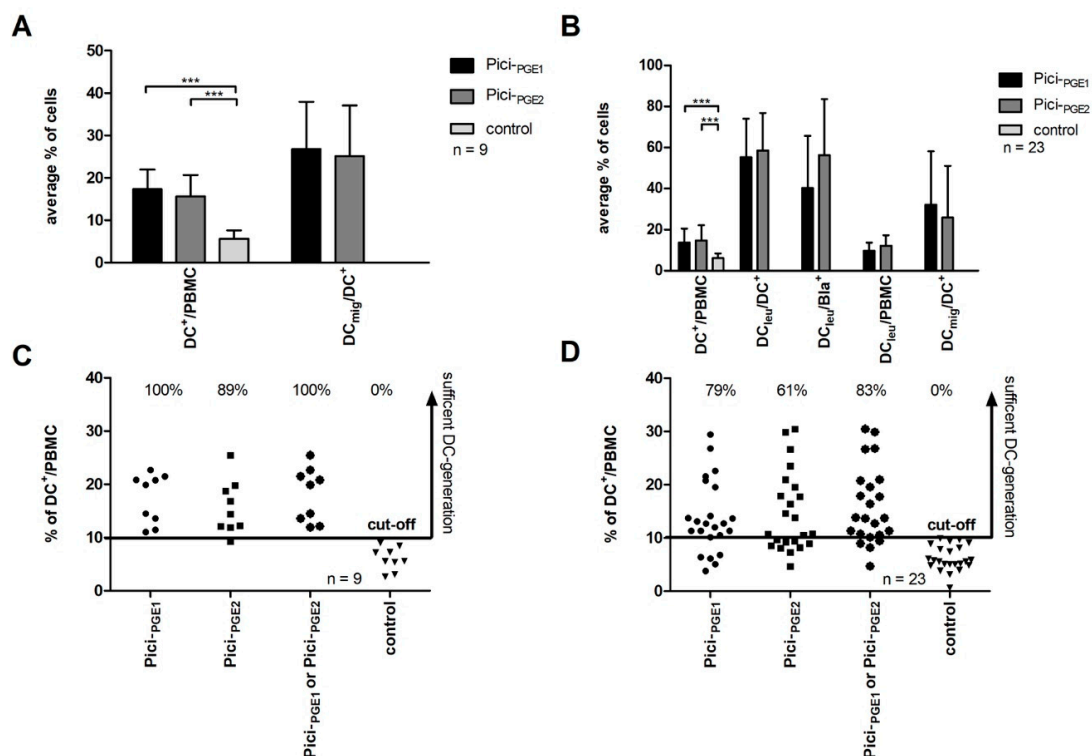


Figure 1. DC/DC_{leu}-generation from healthy (left side) and leukemic peripheral blood mononuclear cells (PBMCs) (right side). (A) shows the average amounts \pm standard deviation of generated dendritic cells (DCs) in the PBMC-fraction and mature DCs in the DC-fraction [CD197⁺DC⁺, (DC_{mig}/DC⁺)] from healthy PBMCs with Pici-PGE1, Pici-PGE2 and control without added cytokines. (B) presents the average amounts \pm standard deviation of generated DCs in the PBMC-fraction, DC_{leu}-subgroups [including DC_{leu} in the DC-fraction (DC_{leu}/DC⁺), DC_{leu} in the blast-fraction (to quantify amounts of leukemic blasts converted to DC_{leu}) (DC_{leu}/Bla⁺), DC_{leu} in the PBMC-fraction (DC_{leu}/PBMC)] and DC_{mig} in the DC-fraction (DC_{mig}/DC⁺) from leukemic PBMCs with Pici-PGE1, Pici-PGE2 and control without added cytokines. (C) and (D) show the percentages of sufficient DC-generation from healthy (C) and leukemic (D) PBMCs with Pici-PGE1, Pici-PGE2, Pici-PGE1 or Pici-PGE2 and control without added response modifiers according to cut-off-values ($\geq 10\%$ DC⁺/PBMC). Each dot (●●●▼) represents DC-proportions generated from each individual healthy volunteer or AML-patient. DCs dendritic cells; DC_{leu} leukemic derived dendritic cells; PBMCs peripheral blood mononuclear cells. The differences were considered as significant*** with p values < 0.005 .

We generated DCs and DC_{leu} from leukemic PBMCs and found, on average, significantly*** higher amounts of DC⁺/PBMC after culture with Pici-PGE1 and Pici-PGE2 compared to controls ($n = 23$) (Pici-PGE1: $13.7 \pm 6.8\%$ DC⁺/PBMC, $p < 0.00003$; Pici-PGE2: $14.7 \pm 7.5\%$ DC⁺/PBMC, $p < 0.00002$, control $6.1 \pm 2.3\%$ DC⁺/PBMC). No significant differences in the amounts of DC⁺/PBMC were found

between Pici-PGE₁ and Pici-PGE₂ ($p < 0.65$). We found (not significantly) higher amounts of DC_{mig}/DC⁺ after culture with Pici-PGE₁ compared to Pici-PGE₂ (32.1 vs. 25.9% DC_{mig}/DC⁺, $p < 0.35$) (Figure 1B). Moreover, we could show that subtype (primary or secondary AML) and stage of the AML did not have an impact on the generation of DCs and DC_{leu} from leukemic PBMCs with Pici-PGE₁ or Pici-PGE₂ (data not shown).

In summary, we conclude that DCs and DC_{mig} can be generated with Pici-PGE₁ and Pici-PGE₂ in comparable amounts from healthy and leukemic PBMCs.

2.2.2. Efficiency of Sufficient DC-Generation is Higher with Pici-PGE₁ Compared to Pici-PGE₂ from Leukemic PBMCs

In healthy and leukemic control groups, we found, in every given case, less than 10% DC⁺/PBMC. Therefore, we defined a cut-off value of $\geq 10\%$ DC⁺/PBMC as a successful DC-generation from healthy and leukemic PBMCs. According to this cut-off value a successful DC-generation from healthy PBMCs was possible in 100% of cases (nine of nine cases) with Pici-PGE₁ and in 89% of cases (eight of nine cases) with Pici-PGE₂ (Figure 1C).

A sufficient DC-generation from leukemic PBMCs was possible in 79% of cases (18 of 23 cases) with Pici-PGE₁ and in 61% of cases (14 of 23 cases) with Pici-PGE₂. In 83% of cases, a sufficient DC-generation was possible with Pici-PGE₁ or Pici-PGE₂ (19 of 23 cases) (Figure 1D). In all cases with successful DC-generation, the amounts of DC_{leu} were comparable with Pici-PGE₁ ($n = 18$) and Pici-PGE₂ ($n = 14$). With Pici-PGE₁ we generated on average $55.3 \pm 18.8\%$ DC_{leu}/DC⁺ and Pici-PGE₂ $58.5 \pm 18.2\%$ DC_{leu}/DC⁺. The average amounts of blasts converted to DC_{leu} (DC_{leu}/Bla⁺) were $40.3 \pm 25.4\%$ DC_{leu}/Bla⁺ with Pici-PGE₁ and $56.3 \pm 27.2\%$ DC_{leu}/Bla⁺ with Pici-PGE₂. $9.7 \pm 4.0\%$ DC_{leu}/PBMC could be generated with Pici-PGE₁ and $12.2 \pm 5.1\%$ DC_{leu}/PBMC with Pici-PGE₂ (Figure 1B).

In summary, the efficiencies of a sufficient DC-generation from leukemic PBMCs are higher with Pici-PGE₁ as compared to Pici-PGE₂ and comparable to healthy PBMCs. In four cases, no sufficient DC-generation was possible with both protocols.

2.2.3. Pici-PGE₁ and Pici-PGE₂ Do Not Induce Blast Proliferation During DC/DC_{leu}-Culture from Leukemic PBMCs

After DC/DC_{leu}-culture from leukemic PBMCs, we found on average comparable amounts of proliferating blasts that were not converted to DC_{leu} (Bla_{prol-CD71}) with Pici-PGE₁ or Pici-PGE₂ as compared to control: Pici-PGE₁: $26.8 \pm 19.9\%$, $p < 0.29$; Pici-PGE₂: $25.6 \pm 16.8\%$, $p < 0.39$; control: $21.3 \pm 14.7\%$. Comparable distributions were found for Bla_{prol-Ipo-38} (data not shown).

We conclude that neither Pici-PGE₁ nor Pici-PGE₂ induce proliferation of blasts not converted to DC_{leu}.

2.3. DC/DC_{leu}-Generation from Healthy and Leukemic WB

2.3.1. Comparable DC-Amounts can be Generated with Immunomodulatory Kits and Picis

We compared the DC⁺/WB values generated with Kit M, Kit K, and Kit I (Kits $n = 27$) and DC⁺/WB values that were generated with Pici-PGE₁ and Pici-PGE₂ from leukemic WB (Picis $n = 18$).

Amounts of generated DC⁺/WB from leukemic WB-samples were not significantly different in both groups (Kits: $9.3 \pm 3.4\%$ DC⁺/WB vs. Picis: $8.8 \pm 3.7\%$ DC⁺/WB, $p < 0.62$). Comparable results were found after the DC-generation from healthy WB (data not shown). Moreover, we found comparable amounts of DC_{leu}/DC⁺, DC_{leu}/Bla⁺, and DC_{leu}/WB with Kits as compared to Picis (DC_{leu}/DC⁺: $62.7 \pm 25.30\%$ vs. $60.6 \pm 20.8\%$; DC_{leu}/Bla⁺: $21.3 \pm 7.4\%$ vs. $20.3 \pm 9.1\%$; DC_{leu}/WB: $8.1 \pm 3.0\%$ vs. $7.2 \pm 2.6\%$) (Figure 2A).

We summarize that DC/DC_{leu}-generation (including subgroups) is possible in comparable amounts with Kits from leukemic and healthy WB when compared to Picis.

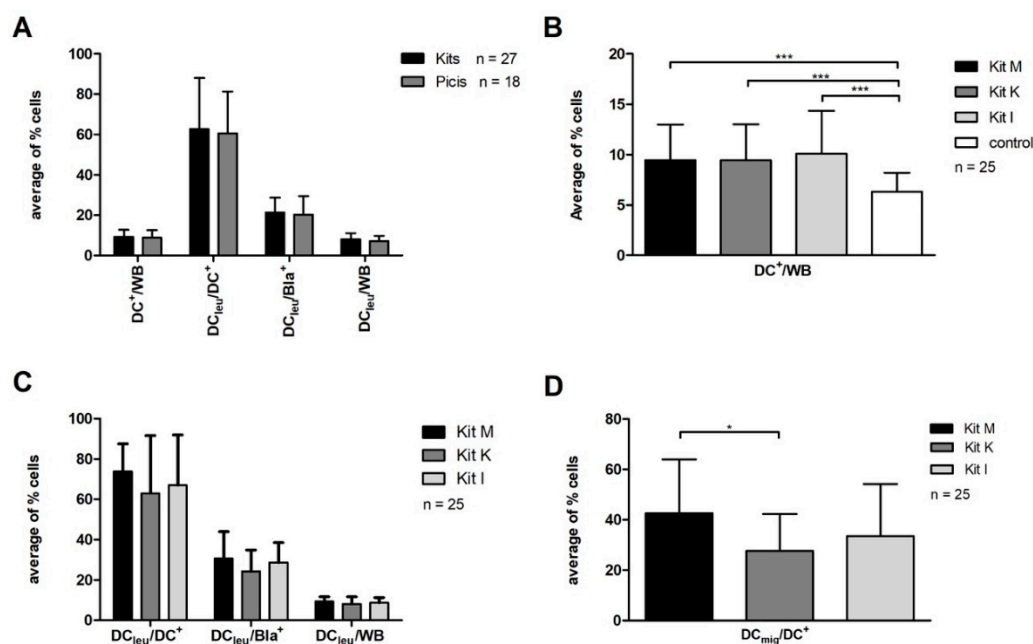


Figure 2. DC/DC_{leu}-generation from leukemic whole blood (WB). (A) shows average amounts \pm standard deviation of DC- and DC_{leu}-proportions [including DC_{leu}-subgroups: DC_{leu} in the DC-fraction (DC_{leu}/DC⁺), DC_{leu} in the blast-fraction (to quantify amounts of leukemic blasts converted to DC_{leu}) (DC_{leu}/Bla⁺) and DC_{leu} in the WB-fraction (DC_{leu}/WB)] from leukemic WB with Kits (including Kit M, Kit K, Kit I) compared to protocols Picis (including Pici-PGE₁, Pici-PGE₂). (B) shows average amounts \pm standard deviation of generated DCs with Kit M, Kit K and Kit I compared to control. (C) presents average amounts \pm standard deviation of generated DC_{leu} subgroups [including DC_{leu}-subgroups DC_{leu} in the DC-fraction (DC_{leu}/DC⁺), DC_{leu} in the blast-fraction (to quantify amounts of leukemic blasts converted to DC_{leu}) (DC_{leu}/Bla⁺) and DC_{leu} in the WB-fraction (DC_{leu}/WB)] with Kit M, Kit K and Kit I. (D) shows average amounts \pm standard deviation of DC_{mig}/DC⁺ generated with Kit M, Kit K and Kit I. DC_{mig} are characterized by the expression of CCR7. DCs dendritic cells; DC_{leu} leukemic derived dendritic cells; WB whole blood. The differences were considered as significant*, with *p* values between 0.1 and 0.05 and as significant*** with *p* values <0.005.

2.3.2. Significantly Higher Amounts of DC⁺/WB Generated from Healthy and Leukemic WB with Immunomodulatory Kit M, Kit K and Kit I Compared to Control

We generated DCs from leukemic WB and found, on average, significantly*** higher amounts of DC⁺/WB after WB-DC/DC_{leu}-cultures with Kit M, Kit K, and Kit I as compared to control (*n* = 25) (Kit M: $9.5 \pm 3.6\%$ DC⁺/WB, *p* < 0.0004; Kit K: $9.5 \pm 3.6\%$ DC⁺/WB, *p* < 0.0004; Kit I: $10.1 \pm 4.4\%$ DC⁺/WB, *p* < 0.0003; control: $6.3 \pm 1.9\%$ DC⁺/WB) (Figure 2B). Comparable distributions were found after DC-cultures from healthy WB (*n* = 9, data not shown). In the comparison of Kit M, Kit K, and Kit I we found, on average, comparable amounts of generated DCs in the WB-fraction from healthy and leukemic WB. In DC_{leu}-subgroups, including DC_{leu}/Bla⁺, DC_{leu}/DC⁺, as well as DC_{leu}/WB no significant differences were found (Figure 2C).

With Kit M, significantly* higher amounts of DC_{mig}/DC⁺ could be generated when compared to Kit K (*n* = 25): ($42.6 \pm 21.5\%$ DC_{mig}/DC⁺ vs. $27.6 \pm 14.7\%$ DC_{mig}/DC⁺; *p* < 0.09). In the comparison of Kit M and Kit I, no significant differences were found (Kit I: $33.5 \pm 20.7\%$ DC_{mig}/DC⁺; *p* < 0.35). (Figure 2D).

In summary, significantly higher amounts of DC⁺/WB can be generated with immunomodulatory Kits from healthy and leukemic WB as compared to control. The amounts of generated DC and DC_{leu}-subgroups are comparable with all three Kits, with the exception of significantly higher frequencies of DC_{mig}/DC⁺ after DC/DC_{leu}-culture with Kit M as compared to Kit K.

2.3.3. Kits Do Not Induce Blast-Proliferation Compared to Control

We found comparable amounts of proliferating blasts that were not converted to DC_{leu} (Bla_{prol-CD71}) in the WB-fraction after culture of leukemic WB with Kit M, Kit K, Kit I, and control ($n = 24$): Kit M: $8.5 \pm 13.0\%$, $p < 0.48$; Kit K: $8.4 \pm 11.0\%$, $p < 0.45$; Kit I: $9.9 \pm 10.7\%$, $p < 0.18$ and control: $6.5 \pm 6.1\%$. Comparable distributions were found for Bla_{prol-Ipo-38} (data not shown).

In summary, Kits do not induce blast proliferation during DC/DC_{leu}-culture from leukemic WB when compared to control.

2.4. Stimulatory Effect of DC/DC_{leu} (Generated with Kits from Leukemic WB) on T Cell Enriched Immunoreactive Cells in MLC and the Corresponding Blast-Lysis Activity

2.4.1. Comparison of T Cell Amounts, Phenotypes, and Blast Lysis Activity in Uncultured Cells, after MLC^{WB-DC Kits} and after MLC^{WB}

As shown above, DC- and DC_{leu}-generation was possible from healthy and leukemic WB with Kits. Here we studied the (potential) stimulating effect of generated DCs and DC_{leu} on T cell enriched immunoreactive cells in MLC. Therefore, we compared T cell compositions in uncultured cells ($n = 11$) (uncultured cells) with those after stimulation in MLC^{WB-DC-Kit M}, MLC^{WB-DC-Kit K}, and MLC^{WB-DC-Kit I} ($n = 24$) (MLC^{WB-DC Kits}) and after stimulation in MLC^{WB} ($n = 11$) (MLC^{WB}) as control.

In general, we found a significantly higher activation status of immunoreactive T cells after MLC^{WB-DC Kits} as compared to MLC^{WB} and uncultured T cells. The main findings were characterized by increased amounts of CD3⁺CD8⁺/CD3⁺ and the corresponding decrease of CD3⁺CD4⁺/CD3⁺ after MLC^{WB-DC Kits} when compared to MLC^{WB}. We found after MLC^{WB-DC Kits} as well as after MLC^{WB} increased amounts of proliferating T cells when compared to uncultured cells, and also a shift from naive to non-naïve T cell subsets. Detailed results are shown in Table 2A and in Figure 3A. Since IL-2 was added to all MLC-experiments (including MLC^{WB}), amounts of T cell subsets also increased after MLC^{WB}.

Furthermore, we pooled all the results obtained with the cytotoxicity assay after MLC^{WB-DC Kit} (including MLC^{WB-DC Kit M}, MLC^{WB-DC Kit K}, MLC^{WB-DC Kit I}) and compared the results to MLC^{WB} after 3 or 24 h ($n = 24$) of incubation of effector with target cells. Anti-leukemic activity was defined as lysis of blast-target-cells obtained either after 3 or 24 h. Moreover, we analyzed the anti-leukemic activity in each individual patient ($n = 13$).

After MLC^{WB-DC} lysis of blasts target cells could be improved in 75.0% of cases (18 of 24 cases) as compared to MLC^{WB} after 3 or 24 h.

Furthermore, in 92.3% of patients (12 of 13 patients) we could select at least one MLC^{WB-DC Kit} in each individual patient, which improved the blasts lysis after 3 h or 24 h when compared to MLC^{WB} (Figure 3B).

In summary, we regularly demonstrate an increase of T cells' anti-leukemic activity after stimulation with DC/DC_{leu} generated with Kits from leukemic WB.

2.4.2. Comparison of T Cell Amounts, Phenotypes and Blast Lytic Activity after MLC^{WB-DC Kit M} and MLC^{WB-DC Kit K}

DC/DC_{leu}-generation was possible with Kits from leukemic WB and the stimulation of T cell enriched immunoreactive cells with these DC/DC_{leu} resulted in T cell activation. The premise for this analysis was that MLC^{WB-DC Kit M} and MLC^{WB-DC Kit K} ($n = 6$) were performed in parallel in patients with the corresponding leukemic WB-samples.

As shown in Figure 3C and Table 2B, we found, on average, comparable amounts of T cells (subsets), including CD3⁺CD8⁺/CD3⁺, CD3⁺CD4⁺/CD3⁺ cells and CD3⁺CD71⁺/CD3⁺ cells after MLC^{WB-DC Kit M} as compared to MLC^{WB-DC Kit K} from leukemic WB. Furthermore, no significant differences were found for the non-naïve or naïve T cells as well as CD3⁺CD45RO⁺CCR7⁻/CD3⁺ and CD3⁺CD45RO⁺CCR7⁺/CD3⁺

after $MLC^{WB-DC\ Kit\ M}$ and $MLC^{WB-DC\ Kit\ K}$ (data not shown). Comparable distributions were found after $MLC^{WB-DC\ Kit\ I}$.

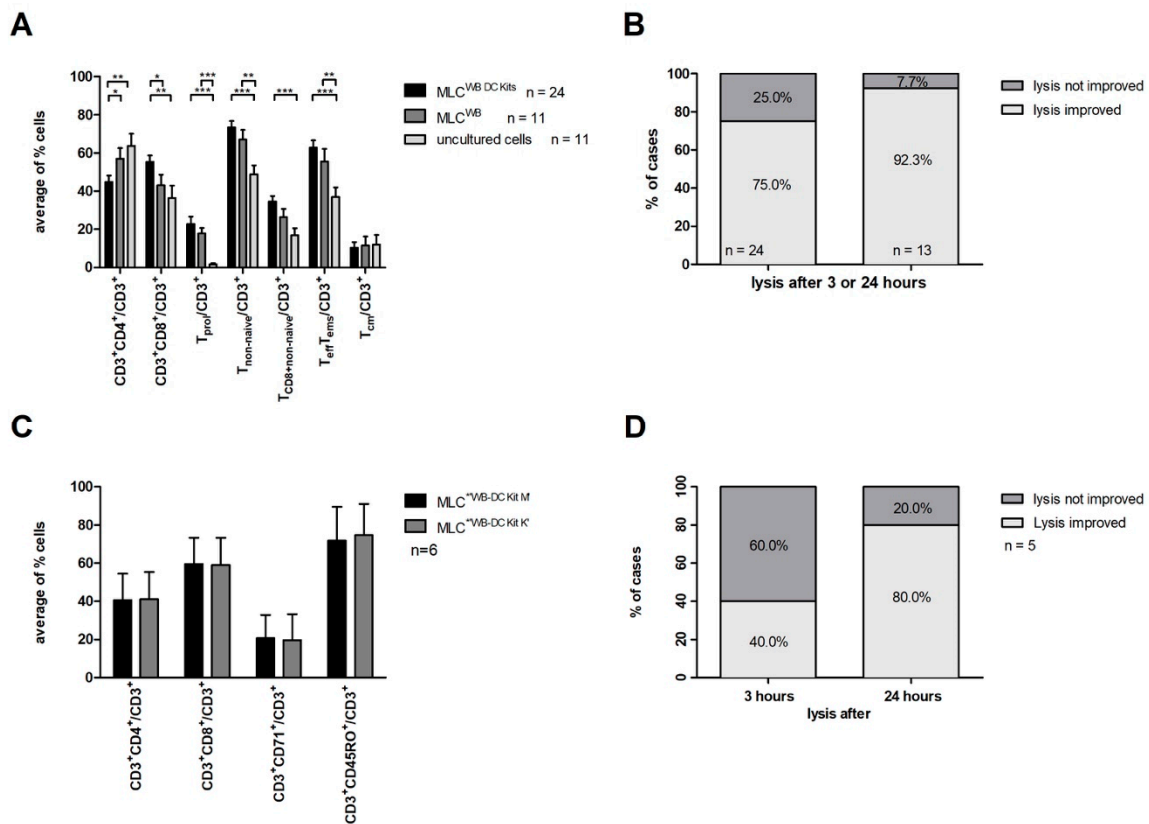


Figure 3. Stimulatory effect of DC/DC_{leu} generated with Kits from leukemic WB on T cell enriched immunoreactive cells in MLC and the corresponding blast-lysis activity. (A) shows average amounts ± standard deviation of T cell subsets after stimulation of T cell enriched immunoreactive cells with DC/DC_{leu} generated with Kits from leukemic WB ($MLC^{WB-DC\ Kits}$), after stimulation of T cell enriched immunoreactive cells with a WB cell suspension not pretreated with Kits (MLC^{WB}) and in uncultured cells. Cells were analyzed by flow cytometry and referred to the CD3⁺ cell fraction. (B) shows the improvement of blast-lysis-activity compared to controls after 3 or 24 h of co-culture of target and effector cells (left column) and of cases with improved blast lysis with at least one $MLC^{WB-DC\ Kit}$ proportions compared to MLC^{WB} in each individual patient (right column). (C) shows average amounts ± standard deviation of different T cell subsets after $MLC^{WB-DC\ Kit\ M}$ and $MLC^{WB-DC\ Kit\ K}$. (D) shows percentage of cases with improved blast-lysis after $MLC^{WB-DC\ Kit\ M}$ compared to $MLC^{WB-DC\ Kit\ K}$ after 3 h (left column) and after 24 h (right column). MLC mixed lymphocyte culture; WB whole blood. The differences were considered as significant*, with *p* values between 0.1 and 0.05, as significant** with *p* values between 0.05 and 0.005 and as significant*** with *p* values <0.005.

After 3 h of incubation of DC/DC_{leu} stimulated effector cells with blast target cells we found an improved lysis after $MLC^{WB-DC\ Kit\ M}$ in 40% of cases (two of five cases) when compared to $MLC^{WB-DC\ Kit\ K}$. Blast lysis was improved in 80% of cases (four of five cases) after 24 h after $MLC^{WB-DC\ Kit\ M}$ compared to $MLC^{WB-DC\ Kit\ K}$ (Figure 3D).

We conclude that DC/DC_{leu} in a MLC^{WB-DC} have an impact on the amounts of different T cell subsets after stimulation as compared to MLC^{WB} and uncultured cells, however T cell compositions after $MLC^{WB-DC\ Kits}$ were comparable with Kits used to generate DC/DC_{leu}. Furthermore, we conclude that blast-lysis was improved in all cases (except one) after $MLC^{WB-DC\ Kit\ M}$ when compared to $MLC^{WB-DC\ Kit\ K}$ after 24 h.

Table 2. Results of T cell compositions after MLC^{WB}-DC Kits, MLC^{WB}, or in uncultured cells.

(A)					
MLC ^{WB} -DC Kits	MLC ^{WB}	Uncultured Cells	<i>p</i> -Values		
% of Cells in the Corresponding Subgroup			MLC ^{WB} -DC Kits vs. MLC ^{WB}	MLC ^{WB} -DC Kits vs. Uncultured Cells	MLC ^{WB} vs. Uncultured Cells
CD3 ⁺ CD4 ⁺ /CD3 ⁺	44.7 ± 16.1	57.0 ± 18.6	63.6 ± 21.4	<0.02**	<0.44
CD3 ⁺ CD8 ⁺ /CD3 ⁺	55.3 ± 16.1	43.0 ± 18.6	36.4 ± 21.4	<0.02**	<0.44
CD3 ⁺ CD71 ⁺ /CD3 ⁺	22.8 ± 18.3	17.8 ± 9.2	1.1 ± 0.6	<0.00001***	<0.00001***
CD3 ⁺ CD69 ⁺ /CD3 ⁺	23.0 ± 16.7	18.4 ± 15.5	3.1 ± 4.4	<0.00001***	<0.009***
CD3 ⁺ CD45RO ⁺ /CD3 ⁺	73.5 ± 15.9	67.0 ± 16.9	48.8 ± 15.6	<0.0003***	<0.02**
CD3 ⁺ CD8 ⁺ CD45RO ⁺ /CD3 ⁺	34.6 ± 13.4	26.4 ± 14.6	16.8 ± 12.4	<0.001***	<0.14
CD3 ⁺ CD4 ⁺ CD45RO ⁺ /CD3 ⁺	38.9 ± 17.3	40.7 ± 16.1	32.0 ± 12.5	<0.19	<0.17
CD3 ⁺ CD45RO ⁻ (including subsets)	26.2 ± 15.6	32.7 ± 16.9	51.1 ± 15.5	<0.0003***	<0.02**
CD3 ⁺ CCR7 ⁻ CD45RO ⁺ /CD3 ⁺	62.8 ± 18.9	55.5 ± 21.8	36.8 ± 16.6	<0.0005***	<0.04**
CD3 ⁺ CCR7 ⁺ CD45RO ⁺ /CD3 ⁺	10.3 ± 14.0	11.5 ± 15.5	11.9 ± 16.7	<0.79	<0.95
(B)					
			MLC ^{WB} -DC Kit M	MLC ^{WB} -DC Kit K	
			T Cell Subtypes		
CD3 ⁺ CD8 ⁺ /CD3 ⁺			59.4 ± 13.9	59.0 ± 14.3	
CD3 ⁺ CD4 ⁺ /CD3 ⁺			40.6 ± 13.9	41.0 ± 14.3	
			Proliferating T Cells		
CD3 ⁺ CD71 ⁺ /CD3 ⁺			20.7 ± 12.1	19.7 ± 13.5	
			Non-Naïve or Naïve T Cells		
CD3 ⁺ CD45RO ⁺ /CD3 ⁺			71.8 ± 17.7	74.6 ± 16.4	

MLC mixed lymphocyte culture; MLC^{WB} mixed lymphocyte culture with WB not preincubated with DC/DC_{leu}-generating protocols; MLC^{WB}-DC mixed lymphocyte culture with WB preincubated with DC/DC_{leu}-generating protocols, highest average values are presented in bold figures.

2.5. Cytokine-Release-Profiles in Serum and after WB DC/DC_{leu}-Culture

Cytokine releases by blasts, DCs, as well as by immunoreactive cells are known to influence immunological as well as immune-escape-reactions. Therefore, we correlated cytokine releases in DC/DC_{leu}-culture-supernatants as well as in serum.

2.5.1. Significantly Higher Concentrations of the Inflammatory Cytokine and Antitumor Response Related Cytokine Found after WB-DC/DC_{leu}-Culture with Kits Compared to Serum

We studied the cytokine release levels after WB-DC/DC_{leu}-culture with Kits and in serum. Therefore, we pooled all of the results after WB-DC/DC_{leu}-culture with Kits (Kit M, Kit K and Kit I) ($n = 27$) from leukemic WB and compared it to cytokine concentrations in serum ($n = 8$).

We found significantly*** higher median concentrations of MCP-1 (CCL-2) after WB-DC/DC_{leu}-culture with Kits as compared to serum [5.5 ng/mL (range: 1.0–11.4) vs. 0.2 ng/mL (range: 0.1–2.5), $p < 0.000001$]. Furthermore, we found significantly*** higher median concentrations of the anti-inflammatory cytokine IL-10 after DC/DC_{leu}-culture [9.2 pg/mL (range: 2.9–46.1) vs. 4.1 pg/mL (range: 0.3–4.1), $p < 0.01$]. No significant differences were found for the cytokine IL-17A ($p < 0.6$). Comparable results were found after DC-generation from healthy WB (data not shown) (Figure 4A).

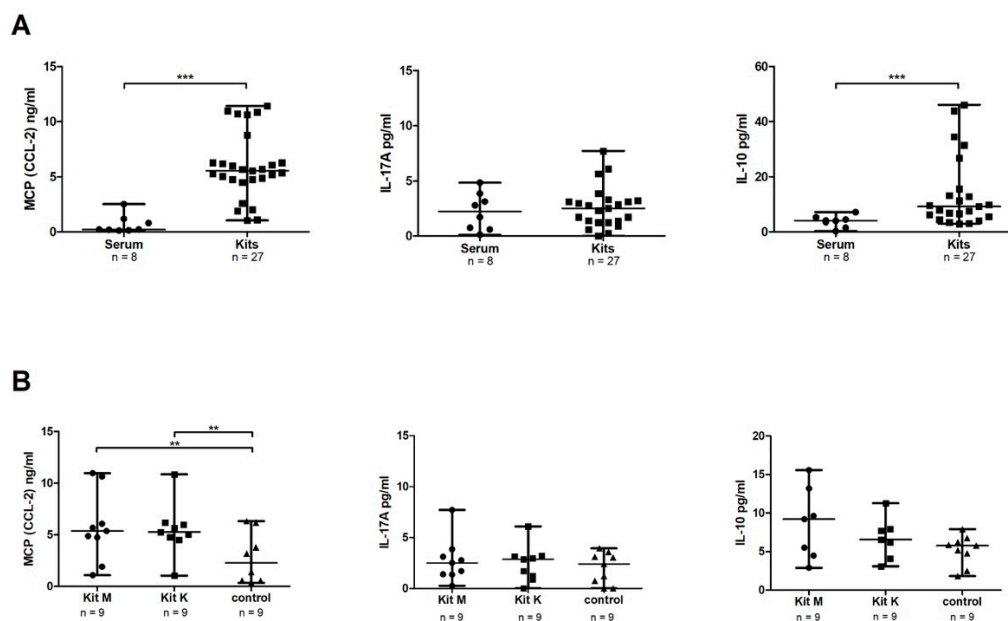


Figure 4. Cytokine-release-profiles after DC/DC_{leu}-culture from leukemic WB. (A) shows concentrations of MCP-1 (CCL-2) (ng/mL), IL-17A (pg/mL) and IL-10 (pg/mL) in serum and after DC/DC_{leu}-culture from leukemic WB with Kits (including Kit M, Kit K, and Kit I). (B) shows the comparison of concentrations of MCP-1 (CCL-2), IL-17A, and IL-10 after DC/DC_{leu}-culture from leukemic WB with Kit M, Kit K and control without added cytokines. All cytokines were measured with ELISA. MCP-1 Monocyte chemotactic protein 1; CCL-2 CC-chemokine ligand 2; IL17-A Interleukin 17A; IL-10 Interleukin 10. The differences were considered as significant** with p values between 0.05 and 0.005 and as significant*** with p values < 0.005 .

Furthermore, we compared the cytokine concentrations in supernatants after DC/DC_{leu}-cultures from leukemic WB with Kits and with Picis. In both groups, comparable concentrations of MCP-1 (CCL-2), IL-17A, and IL-10 were found (data not shown).

2.5.2. Significantly Higher Concentrations of MCP-1 (CCL-2) Found after DC/DC_{leu}-Culture with Kit M and Kit K Compared to Control

We compared cytokine secretion after DC/DC_{leu}-culture of leukemic WB with Kits when compared to control without added response modifiers. We found significantly** higher median concentrations of MCP-1 (CCL-2) after DC/DC_{leu}-culture with Kit M and Kit K as compared to control ($n = 9$) [Kit M: 5.4 ng/mL (range: 1.1–11.0), $p < 0.06$; Kit K: 5.3 ng/mL (range: 1.0–10.9), $p < 0.04$; control: 2.3 ng/mL (range: 0.4–6.3)]. For the cytokines IL-17A and IL-10, no significant differences were found as compared to controls (Figure 4B). Comparable results were found for the Kit I (data not shown).

We conclude that the addition of Kits to leukemic WB influences the cytokine release profiles when compared to control. Kit M and Kit K produce comparable cytokine-release-profiles.

3. Discussion

3.1. DC and DC_{leu} Based Immunotherapy for Patients with AML

DCs play a crucial role in the (re-) activation of the immune system and in linking the innate and adaptive immune system. These professional APCs have the ability to migrate into different tissues and to induce an immunological memory. During the last decades, different strategies have been developed to utilize DCs as a treatment tool for patients with AML. DCs can be generated ex vivo from CD14⁺ monocytes, loaded with different LAAs or tumor antigens, and they can be re-administrated to the patient as vaccine. It was already reported that vaccination with ex vivo generated monocyte-derived DCs increased the amounts of leukemia specific T cells in AML-patients and stabilized complete remission [15,48,49]. Unfortunately, the production of monocyte-derived DCs ex vivo is time-consuming, expensive, has to be performed under GMP-conditions, and the cell production is limited by the selection of LAA [11,14,15,50].

On the other hand, we and others could already show that clonal leukemic blasts can be regularly converted to DC_{leu} with different DC/DC_{leu}-generating protocols, independent from age, FAB-classification, mutation or hematopoietic stem cell transplantation (HSCT) status, mutations of the disease, and FAB classification [17,51]. The clonal leukemic origin of DC_{leu} was already confirmed with fluorescence in situ hybridization (FISH) analysis [52].

3.2. The PGE₁-Containing Protocol Pici-PGE₁ is More Reliable to Generate DCs in Sufficient Amounts from Healthy and Leukemic PBMCs Compared to the PGE₂-Containing Protocol Pici-PGE₂

It was already shown that PGE₂ produced by the enzyme cyclooxygenase 2 (COX-2) has a crucial role in tumor genesis in colorectal cancers in vivo [53,54]. It was reported that PGE₂ influences the apoptosis, induces the angiogenesis, and activates the proliferation of cancer cells [55–57]. Whereas, in highly metastatic melanoma cells, it was shown that PGE₁ promotes anti-tumor effects, such as the inhibition of invasion and cell growth [58]. Moreover, PGE₁ increased the differentiation of tumor cells and decreased the expression levels of metalloproteinase (MMP) 2 and 9 [59]. Furthermore, it was shown that PGE₁ increased the concentrations of Cis-Platin in cancers cells after treating rats with peritoneal carcinomatosis [60]. Transferring these findings into the AML treatment, we hypothesize that PGE₁ could influence anti-cancer reactions in a positive way. Therefore, we analyzed the potential of PGE₁-containing DC/DC_{leu}-generating protocols to produce DCs and DC_{leu} from healthy and leukemic PBMCs as well as from WB. In the past few years it was shown, that it is possible to generate mature DCs and DC_{leu} from leukemic and healthy PBMCs with the PGE₂-containing protocol Pici-PGE₂ [17,20]. We could confirm this and we add, in addition, that the new protocol Pici-PGE₁ produced comparable amounts of DCs and DC_{leu} with a higher efficiency of sufficient DC

generation from healthy and leukemic PBMCs when compared to Pici-PGE₂. These results suggest, that PGE₁ mediates the maturation of DCs and DC_{leu} with comparable efficiency as PGE₂, but might be also involved in the differentiation of myeloid progenitor cells and the production of DC/DC_{leu}. This effect might be explained by the different mode of action of PGE₁ and PGE₂ on prostaglandin receptors (EP receptors) with different down streaming effects due to their different biochemical reactivities [61]. The generation of DC/DC_{leu} was possible with both protocols, independent of patients' FAB-classification, stage (first diagnosis vs. relapse), or status (primary vs. secondary AML) of the disease. Moreover, we could confirm that neither Pici-PGE₁ nor Pici-PGE₂ induced the proliferation of non-converted blasts during DC/DC_{leu}-cultures [19]. In four AML-cases, no sufficient DC/DC_{leu}-generation was possible from leukemic PBMCs with Pici-PGE₂ and Pici-PGE₁, which might be due to various expressions of cytokine-receptors (e.g., GM-CSF receptors, stem cell-receptors) or prostaglandin-receptors (EP-receptors) on leukemic blasts [62,63]. These patients did not receive chemotherapy before conducting DC/DC_{leu} culture experiments, however spontaneous apoptosis or cell death of blood cells has to be discussed. Technical limitations were excluded.

3.3. Successful Generation of DCs and DC_{leu} from Healthy and Leukemic WB Cultures

We established a WB-model to generate DC/DC_{leu} from leukemic WB containing all soluble and cellular factors of the individual patient to simulate physiological conditions. According to our hypothesis, DCs and DC_{leu} could also be generated in vivo by modulating myeloid blasts in their natural microenvironment after administrating a combination of different response modifiers (e.g., Kit M, Kit K, Kit I) to the patient. Therefore, well known DC/DC_{leu} generating protocols (e.g., Pici-PGE₁, Pici-PGE₂) were applied in WB settings and results that were obtained with Kit M, Kit K, or/and Kit I compared: our results show that DCs and DC_{leu} (including subgroups) could be generated in comparable amounts with Kits and Pici directly from WB [17,20]. Additionally, DC_{leu} subgroups did not significantly differ. We conclude that DCs and DC_{leu} can be generated with Kits from leukemic WB ex vivo. The fact that significantly more DC/DC_{leu} could be generated with Kit M, Kit K, and Kit I when compared to control without added response modifiers points to the fact that the combination of two response modifiers is necessary to generate DCs and DC_{leu} in sufficient amounts directly from leukemic WB: the induction of hematopoietic differentiation is induced by GM-CSF, danger signaling, and/or maturation signaling of DC/DC_{leu} is caused by PGE₁, PGE₂, or Picibanil. When compared to PBMC—cultures, response modifiers, such as IL-4 and other/unknown cytokines, are physiological components of the microenvironment in WB, and therefore they have not to be added or included in DC/DC_{leu}-generating protocols [35]. The average amounts of generated DCs (including DC_{leu} subgroups) were comparable with all three Kits, although amounts of matured DCs were significantly higher after WB-treatment with the PGE₁-containing Kit M as compared to the PGE₂-containing Kit K. This finding might also be explained by the different mode of action of PGE₁ and PGE₂ on EP receptors with different down streaming effects [61]. Especially, for the in vivo use of DC/DC_{leu}, the expression of the lymph-node-homing-receptor CCR7 (marker for mature DCs) is crucial for the migratory capacity of DCs and DC_{leu} to the lymph node, where they activate T cells and other immunoreactive cells and induce anti-tumor/anti-leukemic activity [64–66]. Comparable results were already found under hypoxic conditions [67]. The proliferation of blasts (not converted to DC_{leu}) was not induced with Kits during DC/DC_{leu}-cultures. Therefore, we conclude that Kits might be safe tools for an in vivo treatment.

We could already show that, after the stimulation of T cell, enriched immunoreactive cells with DCs and DC_{leu} generated from leukemic PBMCs composition of T cells could be shifted and influenced in a positive way and that anti-leukemic activity could be induced [17,25,27,28]. T cells directly kill cancer-cells via the Granzyme B and Perforin pathway and/or indirectly through the secretion of IFN- γ or tumor-necrosis-factor-alpha (TNF- α). We found after MLC^{WB DC-Kits} a higher activation status with increased amounts of proliferating T cells, as well as a shift from naive to non-naïve T cell subsets when compared to cells before culture. When compared to MLC^{WB}, amounts of CD3⁺CD8⁺ significantly increased. After MLC^{WB} higher amounts of different T cell subsets could also be found and they can be explained by IL-2 added to all MLC, which is a potent endogenous T cell and NK cell activating cytokine [68]. The induction of an immunological memory can be postulated due to the DC concept. We found comparable amounts of central memory T cells after MLC^{WB-Kits}, MLC^{WB} and in uncultured cells. However, we found significantly more effector memory T cells after MLC^{WB-Kits}, pointing to an induction of these specialized memory cells, which can enter different tissues to initiate inflammation and cytotoxicity [69]. We speculate that, in our setting, the incubation time to produce memory T cells after DC/DC_{leu}-stimulation is too short, therefore only effector memory T cells are produced. The induction of memory T cells is crucial for the induction of long term remission in patients with AML.

The composition of Kits to produce DC/DC_{leu}, used for the stimulation of T cell enriched immunoreactive cells has no impact on the average amounts and phenotypes of T cell subsets after MLC, but on the anti-leukemic activity. The most important result of our study was that we could demonstrate that anti-leukemic activity could be improved after MLC^{WB-DC-Kits} compared to MLC^{WB}. Increased production of leukemia-specific cells after MLC^{WB-DC-Kits} as compared to MLC^{WB} was shown in the proof of concept-assays. Extended leukemia-specific evaluations are part of our ongoing research. These results suggest that DC/DC_{leu} generated with Kits from leukemic WB induce the immune system and can activate specific anti-leukemic activity against leukemic blasts after MLC. Furthermore, we could show that the anti-leukemic activity is comparable and it might be superior after MLC DC/DC_{leu} generated with the PGE₁-containing Kit M when compared to the PGE₂-containing Kit K, especially after 24 h of simultaneous incubation of the effector and target cells. This finding might be explained by the fact that PGE₂ could induce the expression of indoleamin 2,3-dioxygenase-1 (IDO1), an immunoregulatory enzyme that activates immunosuppressive T_{reg}, but does not impair the antigen presentation capacity of DCs [70–74].

Moreover, during WB DC/DC_{leu}-cultures with Kits as well as Picis cytokine-release-profiles were influenced compared to serum as well as compared to control. We can conclude that Kits induce the production of anti-tumor response related cytokines as well as inflammatory cytokines during DC/DC_{leu} cultures, and therefore improve anti-leukemic reactivity of T cells after stimulation with Kit treated WB.

4. Material and Methods

4.1. Sample Collection

After obtaining written informed consent, in accordance with the Helsinki protocol and the local Ethic Committee (Pettenkoferstraße 8a, 80336 Munich, Germany, Ludwigs-Maximilians-University-Hospital in Munich; VoteNo 33905), the heparinized peripheral WB samples were taken from patients in acute phases of AML and from healthy volunteers. The University-Hospitals of Oldenburg, Tuebingen, Munich and Augsburg provided samples. Anticoagulation was performed with Lithium-heparin-tubes (7.5 mL, Sarstedt, Nuernberg, Germany) containing standardized concentrations of Heparin.

PBMCs were isolated from WB-samples by density gradient centrifugation while using the Ficoll-Hypaque-Technique (Biocoll-Separating-solution, Biochrom, Berlin, Germany) with a density gradient of 1.077 g/mL. PBMCs were washed and then suspended in phosphate-buffered saline (PBS, Biochrom, Berlin, Germany). CD3⁺T cells were enriched while using the MACS-technology (Milteney Biotech, Bergisch Gladbach, Germany). The purity of the viable T cells was, on average, 87.9% (range 77.4–96.1%). The viable cells were quantified while using Trypan Blue (Biochrom, Berlin, Germany) and they were counted with Neubauer-counting-chambers. PBMCs were directly used to set up DC/DC_{leu}-cultures. T cells and the remaining PBMCs were used for subsequent experiments, therefore cells were frozen at –80 °C (using DMSO) and then thawed according to standardized protocols. Furthermore, the serum was frozen for subsequent ELISA-experiments.

4.2. Patients' Characteristics and Diagnostics

DC/DC_{leu} were generated from PBMC- and WB-samples that were obtained from AML patients ($n = 29$) and healthy volunteers ($n = 10$). The average age of AML patients was 58.6 years (range 21–79) and of healthy volunteers 28.1 years (range 20–56). The female to male ratio of AML patients was 1:1.2 and of healthy 1:0.4. The ages of healthy volunteers and AML patients were not age-matched, since no direct comparisons of those two groups were needed.

The diagnosis and classification of AML patients was based on the French-American-British (FAB) classification: AML without maturation (M1: $n = 2$), AML with granulocytic maturation (M2: $n = 1$), acute myelomonocytic leukemia (M4: $n = 6$), and acute monocytic leukemia (M5: $n = 6$). No FAB-classification was available in 14 AML cases. Patients presented with primary AML [pAML ($n = 17$)] or with secondary AML [sAML ($n = 12$)]. Patients' stages were: first diagnosis ($n = 20$), relapse ($n = 2$) or relapse after HSCT ($n = 4$), and persisting disease ($n = 3$). Table 3 gives patients' characteristics.

The cellular composition of AML- and healthy-peripheral-WB-samples as well as of AML- and healthy-PBMCs-samples are shown in Table 4. In cases with the aberrant expression of T-, B-, or monocytoïd-antigens or CD56 on leukemic blasts, proportions of the corresponding cells were not included in the analysis.

Table 3. Characteristics of AML-patients and healthy volunteers included in this study are presented.

Stage	Pat. #	Age at dgn.	Sex	Subtype		Blast Phenotype (CD)	Blasts in PB %	Conducted DC/DC _{leu} -Cultures	Conducted Experiments
				FAB	FAB				
AML	P1419	64	f	p/M1		34, 117, 33, 15, 13	93	PBMC	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1426	61	f	s/M5		34, 117, 64, 33, 13	40	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1430	79	m	p/M5		34, 117, 33, 13	70	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1432	34	m	p/M5		34, 64, 33, 13	81	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1434	61	f	s/n.d.		34, 117, 64, 56, 33, 13, 7	61	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1439	61	f	s/M5		34, 117, 33, 13	17	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1441	60	m	s/M4		117, 65, 64, 33, 13, 14,	81	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1442	73	f	s/M4		117, 138, 61, 33	14	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1443	64	m	p/n.d.		34, 117, 33, 13	50	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1447	21	m	p/M5		56, 33, 45	65	WB	CTX
	P1449	78	m	s/n.d.		15, 65, 64, 45, 4	62	WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1452	44	m	p/n.d.		34, 117, 45, 33, 13	55	PBMC, WB	CTX
	P1453	54	m	p/M4		15, 64, 56, 33, 14	52	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC
	P1454	60	f	s/n.d.		34, 117, 61, 20	33	WB	
	P1459	54	m	p/M4		56, 64, 38, 33, 11c, 11b	14	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1460	78	f	p/M4		15, 34, 117	68	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1462	49	f	p/M5		34, 56, 64, 45, 33, 13	60	WB	
P1466	47	f	p/n.d.		34, 117, 33, 15, 13	15	PBMC		
P1471	40	m	p/M1		34, 117, 33, 13	69	PBMC, WB		
P1472	33	f	p/M2		117, 34, 15, 13	30	PBMC, WB		
Relapse Before or after HSCT	P1474	70	m	p/n.d.		117, 34, 56, 33	80	PBMC, WB	
	P1475	77	m	s/n.d.		117, 34, 33, 13	20	PBMC, WB	
	P1455	63	m	s/n.d.		34, 117, 13	12	WB	
	P1463	60	f	s/n.d.		34, 56, 33, 13, 2	8	PBMC, WB	CTX
	P1469	49	m	p/M4		34, 117, 65, 33, 13	94	PBMC	
	P1470	67	m	p/n.d.		56, 117, 34, 33, 13,	9	PBMC, WB	
Persisting Disease	P1464	72	m	s/n.d.		34, 117, 71, 20	44	PBMC, WB	MLC ^{WB} , MLC ^{WB} -DC, CTX
	P1467	59	f	s/n.d.		34, 117, 33, 15, 13	30	PBMC, WB	
	P1468	66	m	p/n.d.		117, 56, 34, 33	75	PBMC, WB	
Healthy	P1418	22	m					PBMC, WB	
	P1420	26	f					PBMC, WB	
	P1421	27	f					PBMC	
	P1422	20	f					PBMC, WB	
	P1428	56	f					PBMC, WB	
	P1429	22	f					WB	
	P1436	25	m					PBMC, WB	
	P1438	31	f					PBMC, WB	
	P1445	27	f					PBMC, WB	
	P1446	25	m					PBMC, WB	

Pat.# Patient's number; f female; m male; p primary AML; s secondary AML; FAB French-American-British classification; PB peripheral blood; n.d. no data; dgn. diagnosis; PBMC peripheral blood mononuclear cells; WB whole blood; HSCT hematopoietic stem cell transplantation; MLC^{WB} mixed lymphocyte culture with WB not preincubated with DC/DC_{leu}-generating protocols; MLC^{WB}-DC mixed lymphocyte culture with WB preincubated with DC/DC_{leu}-generating protocols; CTX cytotoxicity (fluorolysis) assay; bold blasts markers used to detect DC_{leu} at the day of harvest

Table 4. Cellular composition of Acute myeloid leukemia (AML) and healthy samples.

	Cell Type	Average of Cells in WB/PBMCs (%)	Range of Cells in WB/PBMCs (%)
AML	Leukemic blasts *	46.2/45.3	8.0–81.0/8.0–93.0
	CD3+T cells	9.9/6.3	1.5–23.3/0.4–24.0
	CD19+B cells	2.8/2.4	0.1–8.4/0.2–7.6
	CD56+CD3-NK-cells	4.8/1.9	0.4–9.8/0.2–6.3
	CD14+monocytes	3.4/1.8	0.1–11.5/0.1–5.7
Healthy	CD14+monocytes	5.6/7.0	4.4–8.5/2.6–12.7
	CD3+T cells	19.4/36.3	13.6–26.3/21.1–46.7
	CD56+CD3-NK-cells	3.4/4.8	2.3–6.9/3.5–6.6
	CD19+B cells	2.3/6.0	0.8–4.8/1.7–11.8

* expression of CD15⁺, CD33⁺, CD34⁺, CD65⁺ and/or CD117⁺; AML acute myeloid leukemia; WB whole blood; PBMCs peripheral blood mononuclear cells.

4.3. DC/DC_{leu}-Generation from Isolated PBMCs

DC/DC_{leu} were generated from 3–4 × 10⁶ isolated healthy and leukemic PBMCs with the DC/DC_{leu}-generating protocols Pici-PGE₁ and Pici-PGE₂ [17,20]. Therefore, cells were pipetted into 12-multiwell-tissue-culture-plates (ThermoFisher Scientific, Darmstadt, Germany) and they were diluted in 2 mL serum-free X-Vivo-15-medium (Lonza, Basel, Swiss). Cytokines were added, as described below. Half medium exchange was carried out after 3–4 cell culture days. A culture without added response modifiers served as a control.

4.4. DC/DC_{leu}-Generation from WB

DC/DC_{leu} were generated from healthy and leukemic WB (presenting the physiological cellular and soluble composition of the individual samples) with the DC/DC_{leu}-generating protocols Pici-PGE₂, Pici-PGE₁, Kit M, Kit K and Kit I [47]. Therefore, 500 µL WB were pipetted in 12-multiwell-plates and then diluted 1:2 in X-Vivo-15-medium (Lonza, Basel, Swiss) to imitate the physiological conditions. Response modifiers and immune-modulating factors were added to cultures, as described below. A culture without added response modifiers served as a control. All of the response modifiers used for the DC/DC_{leu}-generation are approved for human treatment. Table 1 provides the compositions of DC/DC_{leu}-generating protocols.

All of the cell-culture-experiments were conducted at standard laboratory conditions comprising 37°C, 21% O₂ and 5% CO₂. At the day of harvest, cell culture supernatants were collected from DC/DC_{leu}-cultures and MLC-cultures and frozen, according to the standardized protocols for subsequent ELISA-experiments.

4.4.1. Picibanil-PGE₁ (Pici-PGE₁)

DC/DC_{leu} were generated from PBMCs and WB with the DC/DC_{leu}-generating protocol Pici-PGE₁-containing 500 U/mL granulocyte-macrophage colony-stimulation factor (GM-CSF, Sanofi-Aventis, Frankfurt, Germany) and 250 U/mL Interleukin-4 (IL-4) (PeproTech, Berlin, Germany). After 6–7 days, 10 µg/mL Picibanil (OK 432), a lysis product from *Streptococcus pyogenes* that has unspecific immune modulatory effects (Chugai Pharmaceutical Co., Kajiwarra, Japan) and 1 µg/mL Prostaglandin E₁ (PGE₁) (PeproTech, Berlin, Germany) were added. After 7–10 days of incubation, the cells were harvested and used for subsequent experiments.

4.4.2. Picibanil-PGE₂ (Pici-PGE₂)

DC/DC_{leu} were generated from PBMCs and WB with the Pici-PGE₂ DC/DC_{leu}-generating protocol, with the same composition, as given above, for Pici-PGE₁, however substituting PGE₁ by PGE₂ (PeproTech, Berlin, Germany) [17,20].

4.4.3. Kit M:

The generation of DC/DC_{leu} from WB with Kit M was performed while using 800 U/mL GM-CSF and 1 µg/mL PGE₁ [47]. 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.

4.4.4. Kit K:

Kit K consisted of 800 U/mL GM-CSF and 1 µg/mL PGE₂ and it was used to generate DC/DC_{leu} from WB [47]. Incubations were performed in analogy to Kit M.

4.4.5. Kit I:

DC/DC_{leu} were generated with Kit I from WB using 800 U/mL GM-CSF and 10 µg/mL Picibanil [47]. The incubations were performed in analogy to Kit M.

4.5. Cell-Characterization by Flow Cytometry

Flow cytometric analyses were carried out to evaluate and quantify the amounts and phenotypes of leukemic blasts, T cell subsets, B cells, monocytes and DC/DC_{leu} subsets in the PBMC- and WB-fractions before and after different cultures. Panels with several monoclonal antibodies (moAbs) labeled with Fluorescein isothiocyanat (FITC), phycoerythrin (PE), tandem Cy7-PE conjugation (Cy7-PE), or allophycocyanin (APC) were used. The antibodies were provided by Beckman Coulter, Krefeld, Germany (^a), Becton Dickinson, Heidelberg, Germany (^b), Miltenyi Biotec, Bergisch Gladbach, Germany (^c), Thermo Fisher, Darmstadt, Germany (^d) and Santa Cruz Biotechnology, Heidelberg, Germany (^e). FITC-conjugated moAbs against CD3^a, CD15^a, CD33^a, CD34^a, CD45RO^a, CD65^a, CD71^a, CD83^a, and IPO-38^e were used. To detect CD3^a, CD4^a, CD19^a, CD33^a, CD34^a, CD56^a, CD80^b, CD83^a, CD117^a, and CD206^a PE-conjugated moAbs were used. MoAbs against CD3^a, CD4^a, CD14^b, CD15^b, CD19^a, CD33^a, CD34^a, CD56^a, CD65^c, CD80^b, CD117^a, and CD197^b were labeled with Cy7-PE. APC-labeled moAbs against CD3^a, CD4^b, CD14^a, CD15^b, CD34^a, CD45RO^d, CD56^a, CD65^c, CD69^b, CD83^b, CD86^g, CD117^a, CD206^b, and CD209^b were used. 7AAD^b was used to detect dead cells. To stain intracellular antigens (e.g., IPO-38) the FIX & PERM[®] Cell Fixation and Cell Permeabilization Kit (ThermoFisher Scientific, Darmstadt, Germany) was used.

Erythrocytes in WB samples were lysed while using Lysing-Buffer (BD, Heidelberg, Germany), according to the manufacturer's instructions. To stain cells with moAbs, they were resuspended in PBS (Biochrom, Berlin, Germany), containing 10% fetal calf-serum (FCS, Biochrome, Berlin, Germany) to avoid unspecific bindings and they were incubated for 15 min. in the dark at room temperature. Afterwards, the cells were washed, centrifuged, and resuspended in 100–200 µL PBS. At least 5000 events were evaluated with a fluorescence-activated cell sorting Flow-Cytometer (FACSCalibur[™]) and Cell-Quest-data-acquisition and analysis software (Becton Dickson, Heidelberg, Germany). The isotype controls were conducted according to manufacturer's instructions.

Table 5. Subtypes of DC/DC_{Ieu} and T cells as evaluated by flow cytometry.

Names of Subgroups	Referred to	Surface Marker	Abbreviation	Explanatory Note Premise for Analysis	Reference
leukemic blasts	cells (PBMC, WB)	CD15, CD34, CD65, CD117	Bla ⁺ /cells [PBMC, WB]		[16]
dendritic cells	cells (PBMC, WB)	CD80, CD83, CD86, CD206, CD209	DC ⁺ /cells [PBMC, WB]		[16]
DC _{Ieu} in DC fraction	DC ⁺	DC ⁺ Bla ⁺	DC _{Ieu} /DC	≥ 10% DC ⁺ in cells	[16]
blasts converted to DC _{Ieu}	Bla ⁺	DC ⁺ Bla ⁺	DC _{Ieu} /Bla ⁺	≥ 10% DC ⁺ in cells	[16]
leukemia derived DC	cells (PBMC, WB)	DC ⁺ Bla ⁺	DC _{Ieu} /cells [PBMC, WB]	≥ 10% DC ⁺ in cells	[16]
migratory mature DC in DC fraction	DC ⁺	DC ⁺ CCR7 ⁺	DC _{CCR7} /DC	≥ 10% DC ⁺ in cells	[25]
proliferating blasts	cells (PBMC, WB)	Bla ⁺ DC ⁻ CD71 ⁺	Bla ⁺ DC ⁻ CD71 ⁺ /cells [PBMC, WB]		[19]
proliferating blasts	cells (PBMC, WB)	Bla ⁺ DC ⁻ IPO-38 ⁺	Bla ⁺ DC ⁻ IPO38/cells [PBMC, WB]		[19]
CD3 ⁺ pan T cells	gated cells	CD3 ⁺	CD3 ⁺ /cells		[27]
CD4 ⁺ coexpressing T cells	CD3 ⁺	CD3 ⁺ CD4 ⁺	CD4 ⁺ CD3 ⁺ /CD3 ⁺	CD4 ⁺ T cells	[27]
CD8 ⁺ coexpressing T cells	CD3 ⁺	CD3 ⁺ CD8 ⁺	CD8 ⁺ CD3 ⁺ /CD3 ⁺	CD8 ⁺ T cells	[27]
early proliferating T cells	CD3 ⁺	CD3 ⁺ CD69 ⁺	T _{prol} /CD3 ⁺	proliferating T cells	[27]
proliferating T cells	CD3 ⁺	CD3 ⁺ CD71 ⁺	T _{prol} /CD3 ⁺	proliferating T cells	[27]
naive T cells	CD3 ⁺	CD3 ⁺ CD45RO ⁻	T _{naive} /CD3 ⁺	Unprimed T cells	[28]
naive CD4 ⁺ T cells	CD3 ⁺	CD3 ⁺ CD4 ⁺ CD45RO ⁻	T _{CD4-naive} /CD3 ⁺	Unprimed CD4 ⁺ T cells	[28]
naive CD8 ⁺ T cells	CD3 ⁺	CD3 ⁺ CD8 ⁺ CD45RO ⁻	T _{CD8-naive} /CD3 ⁺	Unprimed CD8 ⁺ T cells	[28]
non-naive T cells	CD3 ⁺	CD3 ⁺ CD45RO ⁺	T _{non-naive} /CD3 ⁺	Memory + effector T cells	[28]
non-naive CD4 ⁺ T cells	CD3 ⁺	CD3 ⁺ CD4 ⁺ CD45RO ⁺	T _{CD4-non-naive} /CD3 ⁺	Memory + effector CD4 ⁺ T cells	[28]
non-naive CD8 ⁺ T cells	CD3 ⁺	CD3 ⁺ CD8 ⁺ CD45RO ⁺	T _{CD8-non-naive} /CD3 ⁺	Memory + effector CD8 ⁺ T cells	[28]
central (memory) T cells	CD3 ⁺	CD3 ⁺ CCR7 ⁺ CD45RO ⁺	T _{cm} /CD3 ⁺	Memory + effector CD8 ⁺ T cells	[28]
effector (memory) T cells	CD3 ⁺	CD3 ⁺ CCR7 ⁻ CD45RO ⁺	T _{eff} /CD3 ⁺	Long-term immunity	[28]

Surface marker combinations for the analysis of DC and DC_{Ieu} (including subsets) and T cell subtypes after flow cytometric staining with fluorochrome-labelled-antibodies are given. Cells were analyzed before and after different cultures.

To quantify generated DC_{leu}, the cells were stained with patient-specific blast-staining antibodies (e.g., CD15, CD34, CD65, and CD117), according to diagnostic reports in combination with DC-staining antibodies (e.g., CD80, CD83, CD86, CD206, and CD209), which were not expressed on blasts before culture. For the analysis and quantification of DCs and DC_{leu} in the total- or in subtype-cell fractions after DC/DC_{leu}-cultures, we used a refined gating strategy [16,17]. DC/DC_{leu} subgroup analyses were only conducted in cases with $\geq 10\%$ DCs in the PBMC- and WB-fractions (premise for analysis). DC_{leu} were quantified in the total cell fraction (DC_{leu}/PBMC or WB), 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⁺). The workflow and FACS analysis of DC/DC_{leu}-generation with Pici-PGE₁ as compared to control is exemplarily illustrated in Figure 5. The amount of mature DCs [DC co-expressing the migration marker CCR7 (CD197)] in the DC fraction after culture (DC_{mig}/DC⁺) was quantified in the cases with $\geq 10\%$ DC⁺/cells. A refined gating strategy was used to detect non-converted proliferating blasts in the PBMC- or WB-fractions (Bla_{prol}/PBMC or WB) after DC/DC_{leu}-culture [19]. The proliferating blasts were characterized by the co-expression of CD71 or IPO-38 without the co-expression of DC-markers. (Table 5).

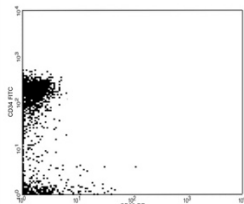
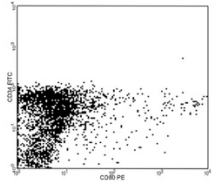
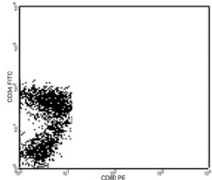
Methods	Cells	FACS before culture	Cytokines added		FACS after culture
			Day 0	Day 6-7	
Pici-PGE ₁	Culture of 3-4 × 10 ⁶ PBMCs in 2 ml X-Vivo-15 - medium		500 U/ml GM-CSF 250 U/ml IL-4	10 μl/ml Picibanil (OK 432) 1 μg/ml PGE ₁	
Control			No response modifiers added	No response modifiers added	

Figure 5. Workflow and FACS analysis of DC/DC_{leu} generated with Pici-PGE₁ compared to control from leukemic PBMCs in a case of AML. 3–4 × 10⁶ PBMCs were cultured in 12-multiwell-tissue-culture-plates and diluted in 2 mL serum-free X-vivo-15-medium. For the generation of DC/DC_{leu} with Pici-PGE₁ 500 U/mL GM-CSF and 250 U/mL IL-4 were added on day 0. After 6–7 days, 10 μg/mL Picibanil a lysis product from *Streptococcus pyogenes*, which has unspecific immune modulatory effects and 1 μg/mL PGE₁ were added. No response modifiers were added to the control culture. Cells were harvested after 7–10 days of incubation. Half medium exchange was carried out after 3–4 days. x-axis: CD80; y-axis: CD34.

4.6. Mixed-Lymphocyte-Culture (MLC) of T Cell-Enriched Immunoreactive Cells with WB-Stimulator-Cell-Suspensions, Preincubated or Not Preincubated with DC/DC_{leu}-Generating Protocols

1 × 10⁶ CD3⁺T cells (effector cells) from AML patients were co-cultured in 24-multiwell-tissue-culture-plates (ThermoFisher Scientific, Darmstadt, Germany) with a stimulator cell suspension containing approximately 2.5 × 10⁵ DC/DC_{leu} (MLC^{WB-DC}), which were generated with different DC/DC_{leu}-generating protocols from leukemic WB or PBMCs. A MLC of T cell enriched immunoreactive cells with a stimulator cell suspension without preincubation with different DC/DC_{leu}-generating protocols (MLC^{WB}) served as a control. The total volume of the cell culture was adjusted to 1 mL with RPMI-1640 medium (Biochrom, Berlin, Germany) containing 1% Penicillin (Biochrom, Berlin, Germany) and 50 U/mL Interleukin 2 (IL-2, PeproTech, Berlin, Germany). After 2–3 days, 50 U/mL IL-2 was added to all cultures. PBMC-cultures contained 15% human serum (Healthcare Europa GmbH, Vienna, Austria). The cells were harvested after 6–7 days and they were used for cytotoxicity-fluorolysis-assay,

as described below. Before and after culture different T cell subsets in MLC were quantified by flow cytometry (Table 5).

4.7. Cytotoxicity (Fluorolysis) Assay

A Fluorolysis assay was performed to analyze the blast lytic activity of T cell-enriched immunoreactive cells after MLC^{WB-DC} and MLC^{WB} [25]. Therefore, effector cells were co-cultured 1:1 with thawed blast-containing target cells for 3 and 24 h in standard laboratory conditions, as described above. As a control effector- and target-cells were cultured separately for the same time and mingled on ice shortly before flow cytometric analyses were carried out. Before culture, the target-cells were stained for 15 min. with FITC-, PE-, or APC-conjugated blast specific target cell antibodies. To evaluate viable cells and the lytic activity of effector cells, the cultures were harvested after 3 h and 24 h and then resuspended in PBS containing 7AAD (Becton Dickson, Heidelberg, Germany) and a defined number of Fluorosphere beads (Beckman Coulter, Krefeld, Germany). A refined gating was used to analyse blast lytic activity (anti-leukemic activity) [25]. Therefore, viable target cells were gated in a Forward Scatter (FSC) 7AAD⁻ gate. The cells were analyzed with a fluorescence-activated cell sorting Flow-Cytometer (FACSCaliburTM) and Cell-Quest-data-acquisition and analysis software (Becton Dickson, Heidelberg, Germany). The lytic activity of effector cells was calculated and defined as the difference in the percentage of viable target cells in the culture with co-cultured effector and target cells (for 3 h and 24 h) as compared to control.

4.8. Enzyme-Linked Immunosorbent Assay (ELISA)

Serum and WB-DC/DC_{leu}-culture supernatants were analyzed for the concentration of Interleukin 10 (IL-10), Interleukin-17A (IL-17A), and Monocyte Chemoattractant Protein-1 (MCP-1, also known as CC-chemokine ligand 2, CCL-2) while using the human IL-10 (detection limit: 1.0 pg/mL), IL-17A (detection limit: 0.5 pg/mL) and MCP-1 (detection limit: 2.3 pg/mL) immunoassay kits (DRG Instruments GmbH, Marburg, Germany). The samples were evaluated with a Tristar LB941 ELISA reader (Berthold Company, Bad Wildbach, Germany) and the concentrations of the different cytokines were evaluated with the corresponding standard curve.

4.9. Statistical Methods

Data were presented as mean ± standard-deviations. The cytokine-concentrations were presented as median and the corresponding range. Statistical comparisons of two groups were performed while using the two-tailed t test (in cases with data normally distributed) and the Mann-Whitney-Wilcoxon-Test (in cases with data not normally distributed). Statistical analyses were performed with Microsoft Excel 2013[®] (Microsoft, Redmond, Washington, USA) and SSPS Statistic 24 software[©] (IBM, Armonk, USA). The differences were considered as 'not significant' in cases 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. The figures were created with GraphPad Prism7[©] (GraphPad Software, California, USA).

5. Conclusion: DC/DC_{leu} Based Treatment Protocols for AML-Patients

We developed a DC/DC_{leu}-generating protocol Pici-PGE₁ and demonstrated (by replacing PGE₂ with PGE₁) that the efficiency of sufficient DC/DC_{leu} generation is superior from leukemic PBMCs. Furthermore, the PGE₁-containing Kit M is a reliable tool for generating ex vivo mature DC/DC_{leu} from healthy and leukemic WB, containing the complete patient individual soluble and cellular microenvironment. These 'new PGE₁ generated' DC/DC_{leu} reliably (re-) activate immunoreactive cells, improve the overall ex vivo anti-leukemic activity, and influence cytokine-release-profiles compared to the PGE₂-containing Kit K.

We conclude that

- (1) PGE₁-containing protocols qualify to generate and to mature monocyte derived DCs from healthy or even patients' PBMCs ex vivo that could, in consequence, be manipulated (e.g., pulsed with LAA) and (re-) administered to the patients as a vaccine.
- (2) PGE₁-containing protocols qualify to produce ex vivo DC_{leu} from leukemic PBMCs. These DC/DC_{leu} could be (re-) administered to the patient in the course of an adaptive cell transfer.
- (3) GM-CSF, PGE₁, and Picibanil are drugs that are approved for human treatment, and so we conclude that, for example, the PGE₁-containing Kit M qualify to convert (residual) myeloid blasts to DCs and DC_{leu} in vivo after the application of Kits to AML-patients. This could contribute to stabilize remission or the disease by presentation of the complete leukemic antigen repertoire to T cells and other immunoreactive cells independent of mutation or transplantation status, cytogenetic markers, FAB-classification, as well as sex or age of the patients.
- (4) In vivo trials with PGE₁-containing Kits (in animals and humans with AML) have to be performed to study safety, the efficiency of DC/DC_{leu}-generation, the mediation of anti-leukemic reactions, and the establishment of immunological effects in vivo.

6. Patent

HMS is the inventor of the European Patent 15 801 987.7-1118 'Use of immunomodulatory Kits for immunotherapeutic treatment of patients with myeloid leukemia's'. No financial conflicts of interest have to be declared.

Author Contributions: D.C.A. conducted cell-culture experiments (including DC/DC_{leu}-cultures, MLC- and CTX-experiments) and all FACS- and statistical-analysis. F.D.-G., C.G., M.W., C.B., S.M., C.K., N.R. performed DC/DC_{leu}-culture-, MLC- and CTX-experiments, which were analyzed by D.C.A. D.C.A. and C.G. conducted ELISA-experiments. U.K. was responsible for the measurement of ELISA-experiments. J.-O.W., D.K., A.R., C.S. provided leukemic samples and corresponding patients' diagnostic reports. B.E.-V. supported functionality assays. H.M.S. designed the study and was responsible for the funding. D.C.A. and H.M.S. drafted this manuscript.

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Publication II:

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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