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The influence of immune checkpoints on Blinatumomab-mediated effects on primary T cells

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1 Abstract

Relapse and non-responsiveness to standard chemotherapy remain major causes of treatment failure in pediatric B-lineage acute lymphoblastic leukemia and are associated with dismal prognosis. The bi-specific, CD3/CD19 targeting antibody construct Blinatumomab represents a novel immunotherapeutic approach with the ability to re-direct endogenous anti-tumor activity against lymphoblastic cells. Despite encouraging clinical success of Blinatumomab, a significant subset of patients does not respond to treatment and ultimately suffer from relapse. Since the upregulation of inhibitory immune checkpoints on tumor cells represents a highly effective mechanism to undermine endogenous cancer immunosurveillance, further investigation of the immune-suppressive influence of inhibitory immune checkpoints on Blinatumomab-mediated anti-leukemic activity is urgently needed. The present study confirms Blinatumomab as a potent stimulus to enhance anti-leukemic T-cell effector functions *in vitro* including specific target cells lysis, T-cell proliferation and production of pro-inflammatory cytokines. Furthermore, this study provides evidence that the immune checkpoint expression by lymphoblastic cells can control Blinatumomab-mediated anti-leukemic effects *in vitro*. The study outlines PD-L1 upregulation by lymphoblastic cells as a highly effective immune evasive mechanism to diminish Blinatumomab-induced anti-leukemic activity. Taken together, these findings provide further insights into the complex interactions of immune checkpoints on Blinatumomab-mediated effects which will help to develop novel immunotherapeutic strategies against pediatric leukemia. Combinatory approaches with immune checkpoint blocking antibodies may open the possibility to finally achieve the urgently needed improvement of the outcome for children with relapsed and refractory B-lineage ALL.

2 Introduction

2.1 Childhood Acute lymphoblastic leukemia

2.1.1 Epidemiology and classification of childhood ALL

After accidents, childhood cancer represents the leading cause of pediatric mortality. Among various pediatric cancer types, acute lymphoblastic leukemia holds a special position as the most common malignancy with approximately 25% of cancer diagnoses among children younger than 15 years old. A peak in ALL incidence is observed among children aged between 1-4 years old (7.5 cases per 100,000 per year in Germany), whereas the likelihood of developing ALL declines with increasing age (Hehn 2014, Howlader N April 2014).

2.1.2 Pathogenesis

Childhood ALL arises from malignant transformation of hematopoietic cells of the bone marrow. Although the cause leading to malignant transformation is not yet completely understood, genetic alterations appear to be pivotal for the development of pediatric ALL. (Moorman, Ensor et al. 2010).

Even though some aspects on the pathogenesis of pediatric ALL are still not entirely revealed, several predisposing factors have been discovered. Maternal consumption of alcohol during pregnancy may increase the risk for development of childhood leukemia (Menegaux, Ripert et al. 2007). Pre- and postnatal exposure to radiation (e.g. therapeutic x-ray radiation), as well as previous treatment with chemotherapy might have a dismal effect on early age ALL development. Syndromes that go along with genetic disorders like Down syndrome (Lee, Bhansali et al. 2016) and Li Fraumeni Syndrome (Porter, Druley et al. 2017) are highly associated with ALL. Furthermore, rare inherited genetic disorders such as Fanconi anemia (Zhou, He et al. 2017), Bloom syndrome (Amor-Guéret 2006) and ataxia-telangiectasia (Schoenaker, Suarez et al. 2016) represent genetic predispositions for ALL development.

2.1.3 Prognosis of standard risk ALL

Among children with standard risk ALL survival rates have improved significantly over the last decades leading to a five-year survival rate exceeding 90% (Hunger, Lu et al. 2012) (Table 2). This includes pediatric patients aged between 1 to 10 years old with an initial white blood cell count

(WBC) of less than 50,000 at primary diagnosis. The remarkable improvement can particularly be attributed to extensive scientific and clinical research as well as international cooperation in terms of donor registries.

Table 1: Clinical trials on pediatric acute lymphoblastic leukemia.
(Adopted from (Pui and Jeha 2007))

Study Group	Study	Year	Patients	5-y EFS (%)
Berlin-Frankfurt-Münster (Prucker, Attarbaschi et al.)	ALL-BFM-95	1995-2000	2169	79,6*
Children's Oncology Group (COG)	Multiple	2000-2005	7153	90,4
Dana Farber Cancer Institute Consortium	DFCI 95-01	1996-2001	491	82,0
Nordic Society of Pediatric Hematology and Oncology	NOPHO	2002-2007	1023	79,0
St. Jude Children's Research Hospital	TOTXV	2000-2007	498	85,6
United Kingdom Acute Lymphoblastic Leukemia	UKALL	2003-2011	3126	87,2

*6-y EFS used in ALL-BFM-95 study; Abbreviation: EFS, event-free survival

2.1.4 Refractory and relapsed childhood ALL

Standard treatment of pediatric ALL implicates chemotherapy over 2 to 3 years which is basically divided in two major therapeutic parts: Remission induction at the time of diagnosis and post-induction therapy after achieving complete remission. Of those patients who fail to achieve CR, about one half experiences death during induction due to treatment-related side effects, while the other half experiences persistent morphologic leukemia (Prucker, Attarbaschi et al. 2009).

Refractory disease is a rare but highly adverse event in childhood ALL. Affecting 1 - 2% of patients it is associated with a poor 10-year overall survival rate of 32%. Re-induction therapy is attempted with chemotherapeutic agents not initially used for induction treatment. For patients who respond to second-line re-induction therapy and achieve CR, allogenic hematopoietic stem cell transplant (HSCT) is usually pursued (Schrappe, Hunger et al. 2012).

Despite the significant advances in treatment, relapse remains the most frequent cause of treatment failure in childhood ALL, occurring in 15 - 20% of patients. Prognosis of children whose disease recurs is dismal with event-free-survival (EFS) rates of approximately 35%. The majority of ALL relapses occurs during therapy or within the first 2 years after treatment completion and affects mostly the bone marrow or extramedullary sites, such as CNS or testes (Reismuller, Attarbaschi et al. 2009, Locatelli, Schrappe et al. 2012).

The pathogenesis of relapsed ALL relies on the clonal outgrowth of a blast population which could not be entirely eliminated during initial treatment. Most relapses arise either from an ancestral clone with pre-existing functional survival advantages or through acquisition of

additional genetic lesions of the leukemic blast (Figure 2). Due to these secondary genetic alterations drug resistance can be acquired leading to selection and clonal expansion of the relapse-inducing blast population. Additionally, mutated pathways affection B-cell development and cell cycle regulation are frequently found in relapse clones. Consequently, this subpopulation is more likely to evade extinction of standard chemotherapy and is capable of inducing ALL relapse (Mullighan, Phillips et al. 2008, Ma, Edmonson et al. 2015).

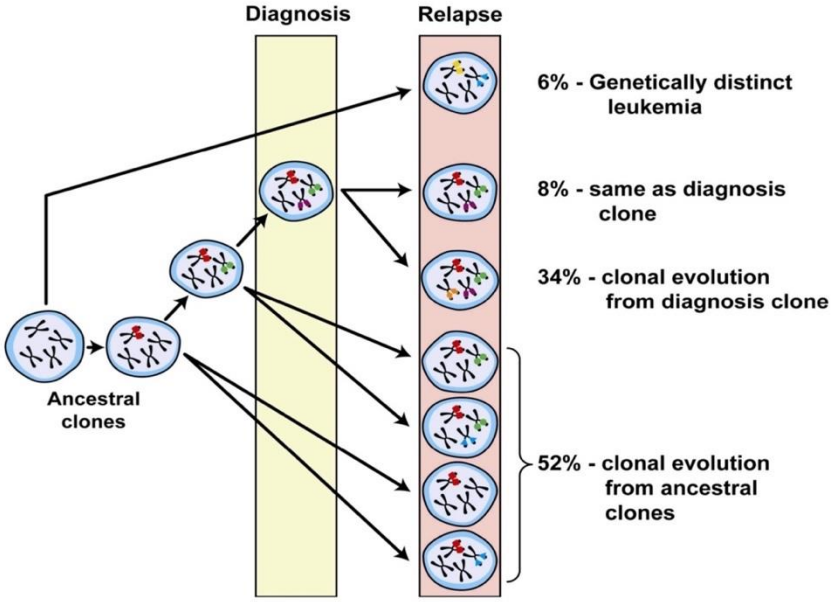


Figure 1: Evolution of relapse in childhood ALL.

Relapse in pediatric ALL arises either from an ancestral clone with pre-existing functional survival advantages or through the acquisition of further genetic mutations of the leukemic blast. The relapse clone acquires various genetic alterations leading to drug resistance and survival advantage. Only few relapse ALL cases represent as a genetically distinct and thus unrelated second leukemia (Mullighan, Phillips et al. 2008).

As current chemotherapeutic regimes fail to increase survival after relapse, there is an urgent need for the development of new therapeutic strategies. Immunotherapy and targeted therapy are novel, highly promising approaches that currently undergo implementation into treatment strategies in childhood ALL.

2.2 Cancer Immunotherapy

Cancer immunotherapy represents a turning point in treatment of malignancies. Improved understanding of the interactions between the immune system and cancer gave rise to novel therapeutic strategies which intend to reinforce the patient's endogenous immune system to erase tumor cells. Groundbreaking efforts have been achieved in treatment of childhood ALL with immune checkpoint blocking agents (Pardoll 2012), adoptive cell therapy with chimeric

antigen receptor (CAR)- transduced T-cells (Sadelain 2015) and treatment with the bi-specific antibody Blinatumomab (Kantarjian, Stein et al. 2017, Stambrook, Maher et al. 2017). Remarkable improvement in outcome of various tumors, as well as acute leukemia, underline the potential of cancer immunotherapy and the necessity of its implication into standard treatment regimes.

2.2.1 Anti-tumor immunity and mechanisms of cancer immune evasion

Further understanding on how the immune system interacts with cancer and hence affects tumor development has provided the basis of cancer immunotherapy.

In theory, cancer cells are highly immunogenic transformed cells expressing various tumor-specific neoantigens and tumor-associated antigens (Schumacher and Schreiber 2015). These antigens trigger the priming of naive T cells to effector T cells, which are subsequently capable of executing specific anti-tumor activity. Effector T cells fall into two large classes, expressing either the co-receptor CD8 or CD4 on their surface. $CD8^+$ cytotoxic T cells eliminate their target cells directly by releasing granzymes, perforin and granulysin. Activated $CD4^+$ T cells rather orchestrate specific immune functions by secreting cytokines. The main functional subsets of $CD4^+$ T cells are defined on the bases of the different cytokines they secrete. Th_1 cells produce pro-inflammatory cytokines (INFγ and TNFα) which further stimulate T-cell differentiation and hence increase anti-tumor activity. In contrast, Th_2 cytokines, such as IL-10 and IL-4, dampen immune responses. $CD8^+$ cytotoxic T cells are also capable of producing INFγ and TNFα upon stimulation (Mosmann and Sad 1996).

While anti-tumor immunity represents a highly effective mechanism to eliminate early-stage malignancies, it may also shape tumor immunogenicity. The cancer immunoediting hypothesis implies that the immune system also promotes tumor progression by selecting tumor clones that are most fit to survive. These clones are able to generate immunosuppressive conditions through production of immunosuppressive cytokines or the expression of inhibitory immune checkpoints, such as PDL-1. Furthermore, tumor cells are able to recruit inhibitory regulatory T-cells (T_{Reg} cells) (Francisco, Salinas et al. 2009). T_{Reg} cells inhibit the activity of tumor-specific T cells by either releasing immunosuppressive cytokines (IL-10, IL-4) or expressing co-inhibitory immune checkpoints (CTLA-4, PD‑1, PD‑L1) (Schreiber, Old et al. 2011).

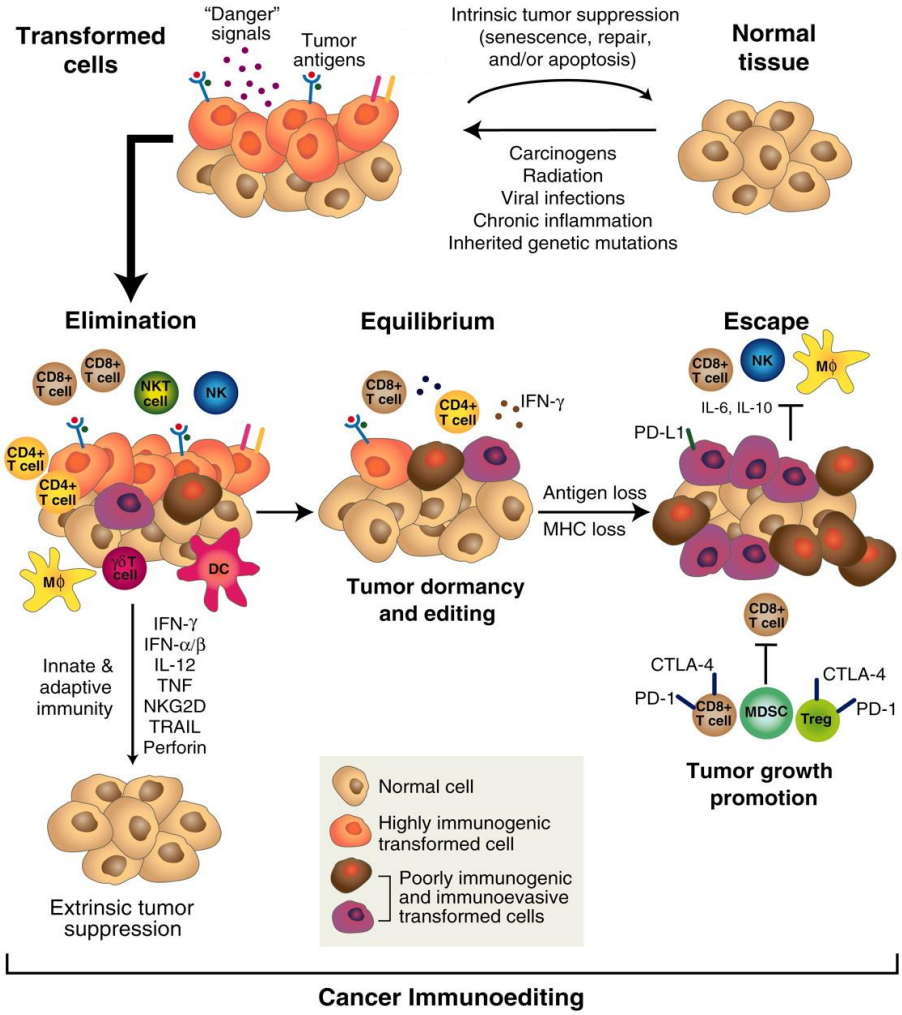


Figure 2: Cancer immunoediting

Cancer immunoediting consists of three consecutive phases: *Elimination*: Various components of both, the innate (NK cells, natural killer cells; Mϕ, macrophages; DC, dendritic cells) and adoptive ($CD4^+$ and $CD8^+$ T cells) immune system initially counteract tumor development. Elimination phase can either result in extrinsic tumor suppression or can process into equilibrium phase. *Equilibrium*: Tumor outgrowth is controlled by the adoptive immune system. Selective pressure triggered by anti-tumorous activity may give rise to the expansion of an immunoevasive tumor cell population. *Escape*: Tumor cells that circumvent anti-tumor immune response (II) are no longer recognized by the adoptive immune system (antigen loss, MHC loss), (II) become insensitive to immune effector mechanisms, or (III) establish an immunosuppressive state through production of immunosuppressive cytokines (IL6, IL-10) or recruiting inhibitory immune cells (MDSCs, T_{Reg} cells) expressing inhibitory immune checkpoints (PD-1).
(Adapted from (Schreiber, Old et al. 2011).

2.2.2 Immune checkpoints

Intensity and quality of a physiological immune reaction can be regulated by co-stimulatory and co-inhibitory signals, also referred to as immune checkpoints. These immune checkpoints are pivotal for the regulation of various immune responses, including peripheral tolerance, autoimmunity, infections, and notably anti-tumor immunity. The balance between positive and

negative signaling determines T-cell function. Simultaneous activation of the T-cell receptor (TCR) by antigen presentation via major histocompatibility complexes (MHC) class I/II and ligation of co-stimulatory immune checkpoints (CD28, CD80/CD86) initiate T-cell activation. Both mechanisms are essential to trigger T-cell activity. Consequently, TCR signaling without co-stimulation leads to anergy. In contrast, the expression of co-inhibitory immune checkpoints (CTLA-4, PD‑1, BTLA, LAG-3, TIM-3, CD200R) is crucial to attenuate T-cell effector function and is frequently misemployed by cancer cells to circumvent anti-tumor immunity (Pardoll 2012).

During the process of cancer immunoediting, tumor cells develop various mechanisms to undermine immunosurveillance (Swann and Smyth 2007). Downregulation of stimulatory and overexpression of inhibitory immune checkpoints by tumor cells represents one of the most effective approaches to circumvent endogenous anti-tumor activity. Signaling via co-inhibitory immune checkpoints results in a successive decrease and loss of T-cell proliferation, cytokine secretion and tumor-specific cytotoxicity. Various co-inhibitory molecules are identified as being hijacked by tumor cells to downregulate anti-tumor immune responses (Table 4).

Table 2: Co-inhibitory immune checkpoints

Receptor		Ligand		
Name	Cellular expression	Name	Cellular expression	Function
CTLA-4	Activated T, T_{Reg}	**CD80/CD86**	T, B, DCs, macrophages	Inhibition
PD-1	Activated T and B, NKT, monocytes myeloid cells	**PD‑L1**	Activated T, B, DCs, macrophages, monocytes, non-lymphoid tissues	Inhibition
		PD-L2	DCs, monocytes	Inhibition
BTLA	T, B, DCs, myeloid cells	**HVEM**	T, B, DCs, NK, myeloid cells and non-lymphoid tissues	Inhibition
LAG-3	Activated T, T_{Reg}, B, DCs, NK	**MHC-II**	Activated T, B, DCs, macrophages, monocytes, endothelium	Inhibition
Tim-3	Th1 $CD4^+$ T, $CD8^+$ T, DCs, NK, monocytes, epithelium	**Galectin-9**	$CD4^+$ T, T_{Reg}, DCs, fibroblast, granulocytes, endothelium	Inhibition
CD200R	Activated T, B, NK, DCs, mast cells, myeloid cells, neutrophils	**CD200**	Activated T, DCs, thymocytes, endothelium, non-lymphoid tissue	Inhibition

Abbreviations: CTLA-4, Cytotoxic T-lymphocyte-associated Protein; PD-1, programmed death-1; PD-L programmed death ligand; BTLA, B and T lymphocyte attenuator; LAG-3, Lymphocyte-activation protein 3; Tim3, T-cell immunoglobulin and mucin domain 3; T, T cell; B, B cell, T_{Reg}, T regulatory cell; DCs, dendritic cells; NK, NK cells; NKT, NKT cells.

Programmed death-1 (PD‑1) represents a co-inhibitory immune checkpoint of particular interest. PD‑1 (and CTLA-4) are members of B7/CD28 family. PD-1 is expressed in effector $CD4^+$ and $CD8^+$ T cells, NK cells, B cells and monocytes. In resting T cells, PD‑1 is not expressed. The physiological PD‑1 function is to dampen immune responses and maintain immunological

homeostasis. Signaling of PD-1 is induced by binding its ligands, PD-L1 and PD-L2, and initiated to downregulate T-cell activity. PD-L1 is found on non-lymphoid tissue and on various cancer types as well as on leukemic blasts (Chen, Liu et al. 2008). Upregulation of PD-L1 on tumor cells negatively influences the anti-tumor immune response by dampening the activity of PD-1^+ tumor-reactive T cells. Furthermore, PD-L1 converts naive $CD4^+$ T cells to T_{Reg} cells. The recruitment of T_{Reg} cells contributes to the development of an immunosuppressive tumor environment (Francisco, Salinas et al. 2009). Chronic antigen penetration and persistent IFNγ exposure induce a constant PD-1 expression which marks a state of exhaustion. T-cell exhaustion is characterized by genetic alterations that lead to changes in cytokine pathways and an upregulation of different co-inhibitory immune checkpoints, including PD-1, Tim3, LAG3. PD-1 blockade can restore anti-tumor activity of exhausted T cells, leading to the assumption that T-cell exhaustion is at least partly reversible (Keir, Butte et al. 2008, Hino, Kabashima et al. 2010, Sakuishi, Apetoh et al. 2010, Norde, Hobo et al. 2012).

2.2.3 Immunotherapeutic approaches for childhood acute lymphoblastic leukemia

Advanced understanding of complex interactions between immune checkpoints and their ligands, as well as the knowledge that cancer frequently misemploys immunological mechanisms to circumvent anti-tumor immunity, created new targets for treatment of pediatric ALL. Cancer immunotherapeutic approaches can be systematized along the backbone of immune modulation (inhibition of co-inhibitory immune checkpoints), cellular approaches (adoptive transfer of genetically engineered chimeric antigen receptor-modified T cells) and humoral approaches (monoclonal antibodies). Furthermore, tumor vaccines and the application of oncolytic viruses aim to enhance anti-tumor immune responses but have not yet found relevance in treatment of childhood ALL (Patel, Kaufman et al. 2017).

2.2.3.1 Immune checkpoint blockade

Monoclonal antibodies selectively occupying the recognition domain of co-inhibitory immune checkpoints subsequently interfere receptor-ligand interactions. Blocking the inhibitory signaling, which is frequently misemployed by tumor cells, results in the reinforcement of T-cell anti-tumor activity. Therapeutic immune checkpoint blockade of PD-1/PD-L1 and CTLA-4 has shown promising results in both solid and hematological malignancies (Seidel, Otsuka et al. 2018). Therapeutic agents targeting PD-1/PD-L1 (Nivolumab, Pembrolizumab) and CTLA-4

(Ipilimumab) have demonstrated remarkable results in non-small cell lung cancer and advanced melanoma and are now approved for clinical application (Brahmer, Reckamp et al. 2015, Postow, Chesney et al. 2015). Encouraging data was also shown in studies on PD-1 blockade in adult hematologic malignancies (Jelinek, Mihalyova et al. 2017). For patients with refractory or relapsed Hodgkin's lymphoma treated with nivolumab response rates of 87% was achieved (Ansell, Lesokhin et al. 2015).

These highly encouraging data for adult malignancies legitimate further investigations on the therapeutic efficacy of CTLA-4 and PD-1/PD-L1 blockade in pediatric ALL. Although immune checkpoint blockade has proven its potential in various adult cancer types, there is only limited data available for pediatric ALL. Future investigations should particularly intend to improve the outcome of refractory and relapsed disease in childhood ALL.

2.3 Blinatumomab, the Bi-specific T-cell engager

Progress in antibody technology has generated a novel method in cancer immunotherapy to engage the endogenous immune system against cancer. Bi-specific T-cell engager (BiTE®) basically reinforces cytotoxic T cells to recognize tumor-specific antigens and consequently enhances endogenous anti-tumor activity.

2.3.1 Mechanism of action

Blinatumomab represents the most clinically advanced bi-specific T-cell engager. The monoclonal antibody construct consists of two single-chain variable fragments (scFv), each binding to CD3ε and CD19, joined by a flexible, non-immunogenic linker of 25 amino acids. CD3ε is a constant part of the TCR on T cells, including $CD8^+$, $CD4^+$ and T_{Reg} cells (Offner, Hofmeister et al. 2006). CD19 is expressed throughout most phases of healthy B-cell development, as well as on 95% of leukemic blasts of B-linage ALL (Raponi, De Propris et al. 2011). Compared to traditional antibodies, for instance IgG with a size of 150kDa, Blinatumomab is a small protein of 55kDa. The small size of Blinatumomab and the ability of bivalent antibody recognition enables Blinatumomab to draw $CD19^+$ leukemic blasts into close proximity of $CD3^+$ T cells. This tight binding forms an immunological synapse and induces a strong stimulus of the engaged T cells. Subsequent T-cell activation occurs independently of TCR/MHC class I interaction and antigen presentation (Haas, Krinner et al. 2009). These frequently misemployed tumor escape mechanisms can therefore be circumvented (see 2.2.1). Initiation of anti-tumor activity upon Blinatumomab-mediated linkage of T cells and leukemic blasts leads to direct

target cell killing, secretion of pro-inflammatory cytokines (TNFα, IFNγ) and polyclonal T-cell expansion. Tumor cell elimination is accomplished by release of granzymes and perforin which either induce cell lysis directly or activate apoptotic pathways (Gruen, Bommert et al. 2004). The high efficacy of Blinatumomab is based on a higher affinity of the anti-CD19 scFv to the anti-CD3ε domain so that a single T-cell can eliminate multiple leukemic $CD19^+$ blast T cells (Gruen, Bommert et al. 2004, Hoffmann, Hofmeister et al. 2005).

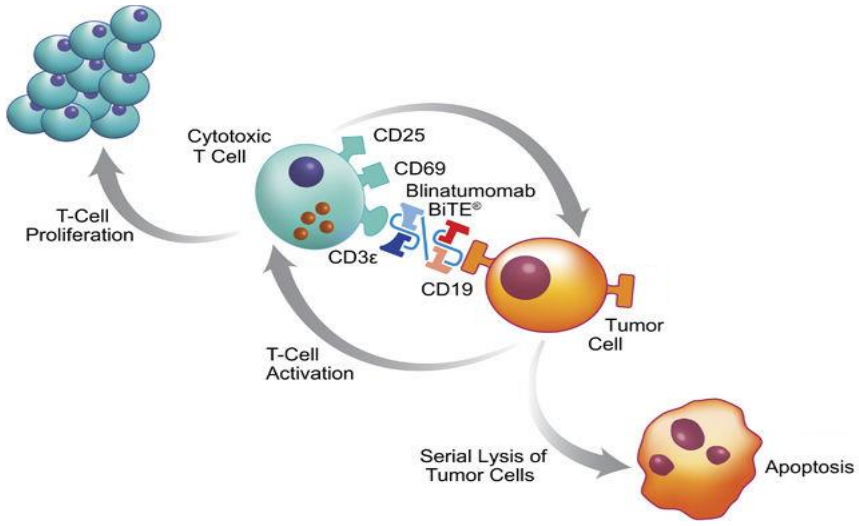


Figure 3: Blinatumomab-mediated target cells elimination.
Bivalent binding capacity of Blinatumomab draws $CD3^+$ T cells into close proximity to $CD19^+$ leukemic blats. Subsequent activation of T cells initiates target cells lysis, T-cell proliferation and apoptosis of $CD19^+$ cells. (Adapted from (Aldoss, Bargou et al. 2017).

2.3.2 Approval and clinical application

Current research provides encouraging results that led to the integration of Blinatumomab to clinical application: Blinatumomab recently gained approval for treatment of Philadelphia chromosome negative, refractory or relapses ALL in adults and children by the US Food and Drug Administration (FDA) and for adults by the European Medicines Agency (Gokbuget, Kneba et al. , Jen, Xu et al. 2018). One treatment cycle consists of 4 weeks of continuous intravenous application of Blinatumomab, followed by a therapy-free interval of 2 weeks. For the first cycle, successive step-dosing is recommended to reduce severe side effects. Therapy cycles can be repeated up to 4 times. Transient and reversible changes in laboratory parameters and lymphocyte kinetics are frequently observed during Blinatumomab administration. After an initial decline, T cells start to expand after a few days, reaching T-cell levels even higher than at treatment initiation. The T-cell recovery is mainly attributed to increased proliferation of $CD4^+$ and $CD8^+$ effector memory cells (Klinger, Brandl et al. 2012). Upon Blinatumomab treatment, healthy B cells are depleted as well. The resulting

hypogammaglobulinemia is mainly caused by decreased levels of IgG and IgA, while IgM levels recover rapidly. (Zugmaier, Topp et al. 2014). Furthermore, Blinatumomab-mediated T-cell activation results in a phenotypic change of T-cell surface expression with an upregulation of CD69, CD25, HLA-DR. Release of cytokines occurs at the beginning of the first cycle and, except of IL-10, could not be observed in subsequent cycles (von Stackelberg, Locatelli et al. 2016, Nagele, Kratzer et al. 2017).

2.3.3 Safety and Blinatumomab-induced adverse events

Evaluation of safety and management of possible adverse events (AEs) are crucial to consider for the integration of Blinatumomab in clinical assignment. The short half-life time of Blinatumomab of approximately 2 hours represents a major safety feature, as it allows a quick dose-adjustment in case of side effects. However, cytokine release syndrome (CRS) and neurotoxicity represent the most severe and dose-limiting side effects.

The risk of developing CRS seems to correlate with level of tumor burden, as patients with high $CD19^+$ blast cell count at baseline develop CRS more frequently (Teachey, Rheingold et al. 2013). Blinatumomab-mediated neurotoxic events include convulsions, tremor and encephalopathy. These symptoms might be contributed to disturbance of the blood-liquor barrier due to local inflammation and $CD19^+$ expression on cells of the CNS. Occurrence of neurologic AEs was observed less frequently in children compared to adults treated with Blinatumomab (von Stackelberg, Locatelli et al. 2016). Further frequently observed AEs include electrolytes disturbances, blood chemistry changes, edema infections and influence-like symptoms, such as pyrexia, headache or nausea. The most common sever or life-threatening AEs that require hospitalization (grade ≥ 3) are fever in neutropenia, cytopenia and hypokalemia (Topp, Gokbuget et al. 2015).

2.3.4 Efficacy of Blinatumomab for refractory/relapsed B-linage ALL

Several extensive clinical trials have currently demonstrated the therapeutic relevance of Blinatumomab, especially its potential for patients with refractory or relapsed (r/r) ALL. Those patients face particularly poor prognosis and allogenic HSCT remains the only curative treatment option. Several clinical trials have been performed to evaluate the anti-leukemic effect and safety of Blinatumomab in adult, pediatric and high risk (Ph^+) patients with r/r B-lineage ALL (Table 5).

The German Multicenter Study Group for Adult ALL (GMALL) performed a phase II study in adult patients with persistent or relapsed MRD^+ B-cell ALL showed an induction of MRD negativity in 80% of cases within 4 cycles of Blinatumomab. (Topp, Gokbuget et al. 2012). Independently, a European

MRD trial (Hoelzer, Walewski et al.) could confirm these findings. Out of 116 adult ALL patients with persistent or relapsed MRD^+ 80% achieved MRD response after one cycle of Blinatumomab (Nicola Goekbuget 2014).

An international, multicenter trial (NCT01466179) on Blinatumomab treatment of r/r B-lineage ALL included 189 adult patients with negative prognostic factors, such as early relapse (<12 months), high blast T cell count at baseline, failure to previous allogenic HSCT and patients receiving Blinatumomab as second or later salvage. CR or CR with partial hematologic recovery (CRh) occurred in 43% of patients after 2 cycles of Blinatumomab treatment (Topp, Gokbuget et al. 2015). Barlev et al. performed an estimation study on this patient cohort to predict long-term survival of patients treated with Blinatumomab beyond the observed study period. At month 60, the estimated survival of patients receiving Blinatumomab was more than double compared to patients treated with standard chemotherapy (12.6% vs 5.4%). Blinatumomab treatment enhanced the mean overall survival of 39.8 months to 76.1 months (Barlev, Lin et al. 2017).

The TOWER trial (NCT02013167), as an extensive, randomized controlled, phase III study compared Blinatumomab efficacy (271 patients) with standard chemotherapy (134 patients). The observed Blinatumomab treated cohort showed significantly superior results concerning the median overall survival (7.7 vs 4.0 months), complete remission with full, partial or incomplete hematologic recovery (44% vs 25%), estimated event free survival (31% vs 12%) and median duration of remission (7.3 vs 4.6 months). Furthermore, standard chemotherapy lead more often to severe AEs (grade ≥ 3) than Blinatumomab (92% vs 87%) (Kantarjian, Stein et al. 2017).

To extend the knowledge of Blinatumomab effect on relevant ALL risk groups, the phase II ALCANTARA study investigated Blinatumomab effects on Ph^+ disease. The trial could provide evidence of Blinatumomab having the same efficacy on Ph^- than on Ph^+ r/r ALL in adults (Martinelli, Boissel et al. 2017).

The only study (NCT01471782, Phase I/II) evaluating the anti-leukemic efficacy and safety of Blinatumomab on children available at present, was carried out with 70 children with r/r B-cell ALL. Pediatric patients were selected for unfavorable characteristics based on tumor load, prior relapses, previous allogenic HSCT and cytogenetic risk factors. After two cycles of Blinatumomab, 30% of children with refractory ALL and 48% with relapsed but not refractory disease achieved CR. Collectively, 39% of children attained CR and 20% of this group became MRD negative (von Stackelberg, Locatelli et al. 2016).

Table 3: Clinical trials on the efficacy and safety of Blinatumomab
(adopted form (Aldoss, Bargou et al. 2017, Queudeville, Handgretinger et al. 2017))

Clinical trial, study design	ALL characteristics	N	Age	Prior alloHSCT (%)	CR/CRh (%)	MRD response (%)	Adverse events
Phase III[1] **Randomized controlled (Blinatumomab vs chemotherapy)** **Multicenter, international (TOWER)**	r/r Ph^- B-ALL	405	Adult	35	44	33	CRS > II°=5% Neurotoxicity > II°=9%
Phase II[2,3] **Single arm** **Multicenter, international**	r/r Ph^- B-ALL	189	Adult	34	43	33	CRS > II°=2% Neurotoxicity > II°=11%
Phase I/II[4] **Single arm** **Multicenter, international**	r/r Ph^- B-ALL	70	Pediatric	57	39	20	CRS > II°=6% Neurotoxicity > II°=0
Phase I/II[5] **Single arm** **Multicenter in Germany (GMALL group)**	r/r Ph^- B-ALL	36	Adult	42	69	61	CRS > II°=6% Neurotoxicity > II°=14%
Phase II[6,7,8] **Single arm** **Multicenter in Germany (GMALL group)**	Ph^+/Ph^- B-ALL with CR but persisting/relapse MRD	21	Adult	0	N/A	80	CRS > II°=0 Neurotoxicity > II°=1%

Abbreviations: alloHSCT, allogenic hematopoietic stem cell transplantation; CR, complete remission; CRh, complete remission with partial hematological recovery; MRD minimal residual disease; r/r, refractory/relapsed; Ph, Philadelphia chromosome; B-ALL, B-cell linage acute lymphoblastic leukemia; CRS, cytokine release syndrome. References: [1](Kantarjian, Stein et al. 2017) [2](Topp, Gokbuget et al. 2015) [3](Barlev, Lin et al. 2017) [4](von Stackelberg, Locatelli et al. 2016) [5](Topp, Gokbuget et al. 2014) [6](Topp, Gokbuget et al. 2012) [7](Topp, Kufer et al. 2011) [8](Gokbuget, Zugmaier et al. 2017)

2.3.5 Escape mechanisms after Blinatumomab treatment

Since the introduction of novel CD19 specific immunotherapies, including CD19 targeting chimeric antigen receptor (CAR) modified T cells as well as Blinatumomab, the development of new specific tumor escape mechanisms has been observed. Immunologic plasticity of leukemic blast T cells give rise to resistance to Blinatumomab treatment.

Relapses after Blinatumomab treatment occur either as $CD19^+$ relapse, where the phenotype did not change throughout treatment, or as $CD19^-$ relapse where persistent leukemic blast T cells subsequently lost their CD19 expression. Approximately 30% of Blinatumomab relapses are based on CD19 loss (Topp, Gokbuget et al. 2012, Topp, Gokbuget et al. 2014). This novel cancer escape mechanism is exclusively associated with CD19 targeted therapies which accentuates cancer as a heterogeneous disease which is perpetually able to adopt to changing therapies. Sotillo et al. could prove that genetic alterations and alternative splicing which are responsible for the disappearance of the CD19 epitope are found constantly in samples after CART19 treatment (Sotillo, Barrett et al. 2015). Furthermore, $CD19^-$ relapse might be explained by the presence of a preexisting $CD19^-$ clone in the tumor bulk before treatment initiation. CD19-specific selection by constitutive Blinatumomab-mediated cytotoxicity and subsequent expansion of a $CD19^-$ population may give rise to recurrent disease (Grupp, Kalos et al. 2013, Nagel, Bartels et al. 2017). Braig et al. could discover a disturbed CD19 membrane trafficking as an escape mechanism to Blinatumomab therapy. In addition, the group analyzed the phenotype of $CD19^-$ relapsed patient samples. Flow cytometric analyzes showed an identic cellular phenotype to the primary diagnosis except the lack of CD19 expression. These findings disagree with the hypothesis of a Blinatumomab-driven selection for a preexisting $CD19^-$ clone (Braig, Brandt et al. 2017). An alternative option for $CD19^-$ escape represents a phenotypic switch from lymphoblastic to monoblastic leukemia. This phenomenon could be observed in murine models and was also found in patient samples after treatment with CART19 or Blinatumomab. Gardner et al. observed the leukemic linage switch following CART19 treatment in patients with rearrangement of the mixed linage leukemia (MLL). (Gardner, Wu et al. 2016, Jacoby, Nguyen et al. 2016). A case report of a 3-month old infant with chemotherapy-refractory MLL suggests that the selective pressure of Blinatumomab treatment triggered the phenotypic switch from lymphoid to myeloid linage leukemia (Rayes, McMasters et al. 2016).

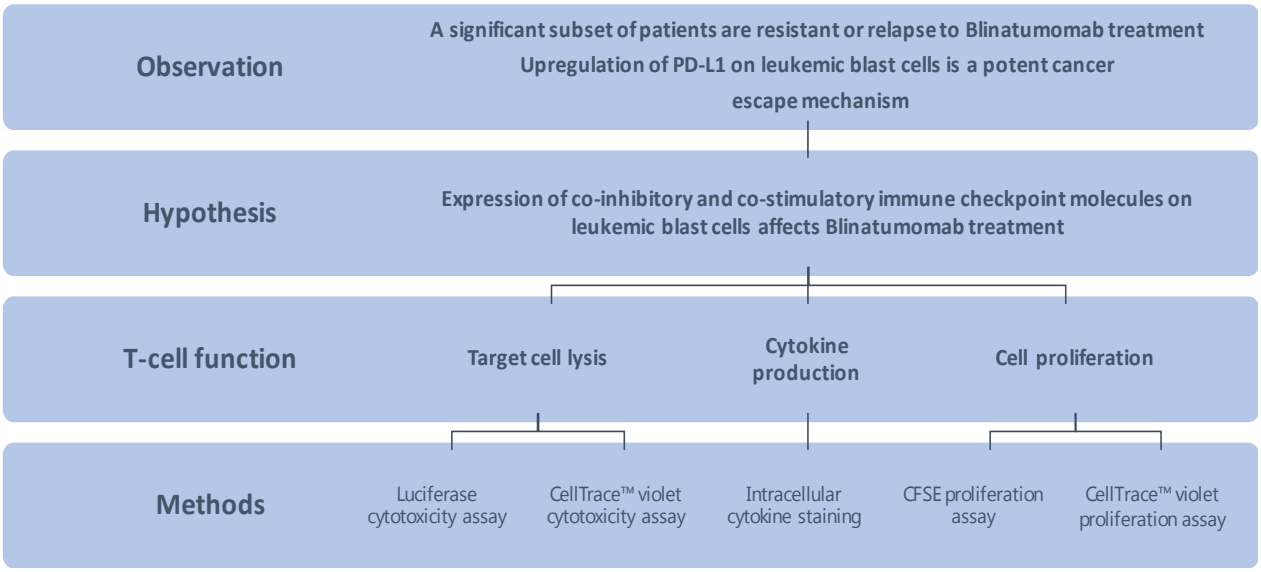
Despite the loss of CD19 expression on leukemic blasts, the upregulation of co-inhibitory immune checkpoints is consequently observed in the context to Blinatumomab treatment. Subklewe et al. showed that a BiTE construct targeting CD33/CD3 (AMG 330) in myeloid leukemia (AML) favored the upregulation of PD-L1 on leukemic blasts. Persistent inflammatory conditions driven by the BiTE apparently led to the development of an escape strategy particularly triggered by the overexpression of PD-L1. PD-1/PD-L1 blockade attenuated T-cell inhibition by re-enhanced target cells lysis and might therefore be clinically useful to outweigh this escape mechanism (Krupka, Kufer et al. 2016). In the setting of r/r B-lineage ALL the same group observed an increased PD-L1 expression of an adult patient primary refractory to Blinatumomab treatment. This finding further suggests PD-1/PD-L1 as a potent escape mechanism to Blinatumomab (Kohnke, Krupka et al. 2015). However, studies especially in the setting of r/r childhood ALL that provide further insights in the interaction between co-inhibitory immune checkpoints and Blinatumomab are very limited.

2.4 Aim of the study

Blinatumomab has impressively demonstrated its potential in treatment of pediatric r/r B-lineage ALL and has drastically changed the outcome of children with otherwise extremely poor prognosis. Despite the outstanding clinical response achieved by the CD19 targeted immunotherapies, a significant subset of patients does not respond to treatment and therefore ultimately relapses. Current investigations have identified several escape mechanisms responsible for considerable treatment failure. Nevertheless, the mechanisms that give rise to the development of leukemic escape strategies remain unclear. Since therapeutic blockade of immune checkpoints has proven remarkable clinical success, combinatorial approaches of Blinatumomab and PD-1/PD-L1 blockade may open the possibility to further improve the outcome of children with r/r B-lineage ALL.

The aim of this study is to *ex vivo* investigate the influence of co-stimulatory (CD80) and co-inhibitory (PD-L1) immune checkpoints on Blinatumomab-mediated anti-leukemic effects on primary T cells regarding target cell lysis, T-cell proliferation and cytokine production.

Table 4: Overview of the performed experiments on this study



3 Material

3.1 Cell lines

Description	Growth medium
Raji Wildtype	RPMI + 10% FCS + 1% Pen/Strep + 2mM L-Glutamin
Raji PD-L1	RPMI + 10% FCS + 1% Pen/Strep + 2mM L-Glutamin
Nalm6 Wildtype	RPMI + 10% FCS + 1% Pen/Strep + 2mM L-Glutamin
Nalm6 PD-L1	RPMI + 10% FCS + 1% Pen/Strep + 2mM L-Glutamin
Nalm6 CD80	RPMI + 10% FCS + 1% Pen/Strep + 2mM L-Glutamin

As a model for acute lymphoblastic leukemia Nalm6 and Raji cell lines were used. Nalm6 cell line represents a B cell precursor ALL which was established from peripheral blood of a male patient with relapsed ALL. Raji cell line was established from a patient with Burkitt lymphoma and was used as a model of mature B-cell acute lymphoblastic leukemia. All cell lines were transduced with firefly luciferase and green fluorescent protein (GFP).

Raji WT, Raji PD-L1, Nalm6 WT, Nalm6 PD-L1 and Nalm6 CD80 cell lines were kindly provided by Judith Feucht and Michel Sadelain from Center for Cell Engineering, Memorial Sloan Kettering Cancer Center, New York. Flow cytometric analyses on the checkpoint expression of lymphoblastic cell lines was assessed by Dana Stenger.

Table 5: Checkpoint expression of lymphoblastic cell lines.

	Inhibitory immune checkpoints				**Stimulatory** immune checkpoints					
	PD-L1	CTLA-4	TIM-3	LAG-3	HVEM	CD80	CD86	CD27	CD40	CD70
Raji WT				n.a.						
Raji PD-L1				n.a.					n.a.	
Nalm6 WT										
Nalm6 CD80				n.a.					n.a.	
Nalm6 PD-L1				n.a.					n.a.	

3.2 Medium and serum for cell culture

Description	Manufacturer
Albiomin® 5% Infusionslösung Humanalbumin	Biotest Pharma GmbH, Dreieich, Germany
Fetal Bovine Serum	Sigma-Aldrich CHEMIE GmbH, Steinheim Germany
Human AB-Serum	Universitätsklinikum Ulm, Ulm, Germany (provided by Prof. Dr. med. Ramin Lofti)
L-Glutamin 200mM	Biochrom GmBH, Berlin Germany
Penicilin/Streptomycin	Gibco©, Life Technologies, Darmstadt, Germany
TexMACS™ Medium	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
TexMACS™GMP Medium	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
VLE RPMI 1640 Medium	Biochrom GmbH, Berlin, Germany

3.3 Chemicals and reagents

Description	Manufacturer
Biocoll Separating Solution	Biochrom GmbH, Berlin, Germany
Brefeldin A	Sigma-Aldrich CHEMIE GmbH, Steinheim, Germany
CellTrace™Violet	Invitrogen, Thermo Fisher Scientific, Life Technologies Cooperation, Eugene, Oregon, USA
CFDA SE	Molecular Probes, Life Technologies, Eugene, USA
D-Luciferin	SynChem, Illionois, USA
Dimethylsulfoxid	Honeywell, Seelze, Germany
Staphylococcal enterotoxin B	Sigma-Aldrich CHEMIE GmbH, Steinheim, Germany
Triton X 100	Sigma-Aldrich CHEMIE GmbH, Steinheim Germany
Tween® 20	Carl Roth Gmbh + Co. KG, Karlsruhe, Germany
QIAzol Lysis Reagent	QIAGEN GmbH, Hilden, Germany

3.4 Puffers

Description	Manufacturer
CliniMACS® PBS/EDTA Buffer	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Dulbeccos Phosphate Buffer Saline	Gibco®, Life Techonologies, Darmstadt, Germany
HEPES-Buffer (1M)	Biochrom GmbH, Berlin, Germany

3.5 Solutions

Description	Contents
D-Luciferin Solution	0,5 mM D-Luciferin 0,3 mM Coenzym A 33 mM DTT 0,6 mM ATP 1 mM (MgCO3)4Mg(OH)2 5 mM MgSO4 20mM Tricine 0,1mM EDTA
Lysis Buffer	100µl HEPES-Buffer (1M) 1µl Tween® 20
Staining Buffer	45ml CliniMACS® PBS/EDTA Buffer 5ml Albiomin® 5%

3.6 Reagent kits

Description	Manufacturer
CellTrace™ VioleT-cell proliferation Kit	Invitrogen, Thermo Fisher Scientific, Life Technologies Cooperation, Eugene, USA
Fix and Perm® Cell Permeabilization Kit	Life Technologies, Frederick, USA
MACS® Comp Bead Kit anti mouse	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
MACS® Comp Bead Kit anti REA	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Vybrant® CFDA SE Cell Tracer Kit	Molecular Probes, Life Technologies, Eugene, USA

3.7 Antibodies and viability dyes

Fluorochrome	Antigen	Clone	Manufacturer
7AAD	Viability	-	Biolegend, San Diego, USA
APC	CD62L	145/15	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
APC	CD8	RPA-T8	BD Biosciences, Franklin Lakes, USA
APC	CTLA-4	L3D10	BD Biosciences, Franklin Lakes, USA
APC eFluor780	Viability	SK1	eBioscience, Thermo Fisher Scientific, Life Technologies Cooperation, Eugene, Oregon, USA
APC Vio770	CD3	-	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
APC Vio770	CD8	BW135/80	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
BB515	CD62L	DREG-56	BD Biosciences, Franklin Lakes, USA
BUV395	CD3	SK7	BD Biosciences, Franklin Lakes, USA
BV421	PD-1	29E.2A3	Biolegend, San Diego, USA
BV650	CD4	SK3	BD Biosciences, Franklin Lakes, USA
PE	CD45RO	UCHL1	Biolegend, San Diego, USA
PE Cy-7	CD3	UCHT1	Biolegend, San Diego, USA
PE	IFNY	45-15	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
PE	IL-10	JES3-9D7	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
PE	IL-4	7A3-3	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
PE	TNFα	cA2	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
PE Vio615	CD56	REA196	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
PerCP Cy5.5	CD8	SK1	Biolegend, San Diego, USA
PE-Vio 770	CD45RO	UCHL1	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
PI	Viability	-	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Vio Blue	CD4	REA623	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Vio Blue	CD8	REA734	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
VioBright ™ FITC	CD4	VIT4	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Vio Green	CD4	REA623	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Viobility™ 405/520	Viability	-	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

3.8 Consumables

Description	Manufacturer
Cell culture flasks	T25, T75, T175, Sarstedt, Nürnbrecht, Germany
Compress	Compress 10x10cm, NOBA Verbandmittel Danz GmbH u. Co. KG, Wetter, Germany
Cover slip	Menzel-Gläser 20x20mm, Gerhard Menzel B.V & Co. KG, Braunschweig, Germany
Cryo tubes	Cryo Pure Gefäß 1,8ml, Sarstedt AG u. Co., Nümbrecht, Germany
Germicide	Ethanol 80% verg. MEK/Bitrex CLN GmbH, Niederhummel, Germany Bacillol®AF, Paul Harmann AG, Heidenheim, Germany
Gloves	Vaso® Nitril blue, B. Braun Melsungen AG, Melsungen, Germany
Multiwell plates for cell culture, 48-Well	Cellstar® Greiner Labortechnik, Kremsmünste, Austria
Multiwell plates for cell culture, 6-Well	Costar® Corning Incorporated, New York, USA
Multiwell plates for cell culture, 96-Well	Nunclon™ Delta Surface, Thermo Fisher Scientific, Roskilde, Denmark
Pipette attachment	Glas pasteur pipette 230mm, BRAND GmbH + Co, Wertheim, Germany
Pipette tips	10µl, 20µl, 100µl, 1000µl, Sartedt, Nürnbrecht, Germany 0,1-2,5µl, 2-100µl, 2-200µl, 100-1000µl, Biosphere® Plus, Sarstedt, Nürnbrecht, Germany
Reaction tube	15ml, 50ml Falcon®, Corning Science, Tamaulipas, México 15ml, Sarstedt, Nürnbrecht, Germany 50ml, Orange Scientific, Braine-l'Alleud, Belgium 1,5ml, 2ml, Eppendorf Safe Lock Tubes, Eppendorf, Hamburg Germany
Round bottom tube with integrated cell strainer	5ml Polystyrene Round Bottom Tube, Falcon®, Corning Science, Taumaulipas, México

Skin germicide	Cutasept ®F, BODE Chemie GmbH, Hamburg, Germany Sterilium® classic pure, BODE Chemie GmbH, Hamburg, Germany
Stripette	2ml, 25ml, Costar® Stripette®, Corning Incorporated, New York, USA 5ml, 10ml, Serological pipette, Sarstedt, Nürnbrecht, Germany

3.9 Equipment and software

Description	Manufacturer
Autoclave	VX-150, Sytec, Linden, Germany DX-65, Sytec, Linden, Germany
Centrifuge	Multifuge™ X3, Heraeus, Hanau, Germany Mikrozentrifuge Fresco™ 17, Heraeus, Hanau, Germany
Counting chamber	Neubauer Counting chamber, 0,1mm, Paul Marienfeld GmbH & Co. KG, Lauda Königshofen, Germany
Flow cytometer	BD LSRFortessa™ cell analyzer, BD, Franklin Lakes, USA BD FACSAria™ III sorter, BD, Franklin Lakes, USA MACSQuant® Analyzer 10, Miltenyi Biotec, Bergisch Gladbach, Germany
Freezer	Refrigerator Comfort No Frost, Liebherr, Biberach an der Riß, Germany Freezer (-20°C) Premium No Frost, Liebherr, Biberach an der Riß, Germany Freezer (-86°C) HERAfreeze™ HFU T Serie, Heraeus, Hanau, Germany Kryotank MVE 600 Serie, Chart, Luxemburg
Incubator	HERAcell® CO_2-Incubator, VWR International GmbH, Darmstadt, Germany
Microscope	Axiovert 25, Zeiss, Oberkochen, Germany Axiovert 200M, Zeiss, Oberkochen, Germany

Pipette (electric)	Easypet® 3, Eppendorf, Hamburg, Germany
Pipette (manual)	2,5µl, 20µl, 200µl, 1000µl Eppendof Research®, Eppendorf, Hamburg, Germany
Rack	Kisker Biotech GmbH & Co. KG, Steinfurt, Germany
Software	Microsoft Office 2010, Redmond WA, USA GraphPad PRISM 7.0c, La Jolla, USA FLUOstar Optima Software 1.30-0, BMG Labtech GmbH, Offenburg, Germany FlowJo™ 10.3, USA MACSQuantify™ Software 2.8, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany BD FACSDiva 8.0.1 Software, BD, Franklin Lakes, USA
Spectrometer	FLUOstar Optima, BMG Labtech GmbH, Offenburg, Germany
Sterile work bench	Uniflow KR-130, Uniequip, Planegg, Germany
Vacuum pump	Vacuumsytem BVC 21 NT, Vacuubrand GmbH + Co GmbH, Wertheim, Deutschland
Vortex Genie 3	IKA®-Werke GmbH & CO. KG, Staufen, Germany
Water bath	GFL 1083, Gesellschaft für Labortechnik mbH, Burgwedel, Germany

4 Methods

4.1 Cell culture conditions

The leukemia blast T cell lines Nalm6 and Raji as well as PBMCs isolated from human donors were cultured with Roswell Park Memorial Institute growth medium (RPMI 1640) using standard conditions at 37°C in a 5% CO_2 incubator. The growth medium was supplemented with 10% Fetal Bovine Serum (FBS), 1% Penicillin / Streptomycin and 2mM L-Glutamine. Cells were split every 2-3 days cells reaching a maximum confluency of 80%. All cell lines were tested mycoplasma negative before use.

4.2 Isolation and cryo-preservation of PBMCs

Peripheral Blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. Blood samples were obtained from 3 healthy individuals (female and male) after informed consent. A maximum volume of 100 ml per blood donation was not exceeded. Ethic approval was obtained from the local Research Ethics Committee and the study was performed in accordance with the Declarations of Helsinki.

Ethylenediaminetetraacetic acid (EDTA) blood was diluted (1:1) with Dulbecco's Phosphate Buffer Saline (PBS) and carefully coated onto a layer of Ficoll (Biocoll Separating Solution, Biochrom GmbH). Centrifugation was performed for 30 min at 800 g without acceleration and deceleration. Mononuclear cells located at the interspace between serum and the Ficoll layer were transferred to a fresh collection tube and were washed twice with PBS.

Cells were frozen in Human Serum Albumin (HSA) supplemented with 10% Dimethylsulfoxid (DMSO) at the recommended controlling rate of 1°C in an isoprooanol contained container. Cells were placed into a box containing Isopropanol and immediately stored at -80°C. For storage, longer than 24 hours cells were transferred to liquid nitrogen.

For cell thawing RPMI 1640 growth medium was prepared by being pre-warmed to 37°. Cells from the -80°C freezer or the liquid nitrogen tank were rapidly placed in a 37°C water bath until until they were about 75% thawed. Cells were diluted 1:30 with growth medium RPMI 1640 medium followed by two washing steps with PBS.

4.3 Flow cytometry

4.3.1 Extracellular staining

Cells were diluted to 1 x 10^6 cells/100 µl staining buffer (CliniMACS PBS/EDTA + 0,5% HSA). Fluorescent-labelled antibodies targeting extracellular surface structures, as well as the respective viability dye, were prepared to a master mix. Antibodies were titrated for optimal staining conditions (2.2 Antibodies and viability dyes). Incubation was performed for 10 minutes on ice, shielded from light and cells were washed with 2 ml CliniMACS PBS/EDTA + 0,5% HSA to absorb any unbound dye. Cells were re-suspended with 300 µl staining buffer and stored on ice until flow cytometric measurement was performed.

4.3.2 Intracellular staining

Intracellular staining was performed by using the Fix and Perm® Cell Permeabilization Kit (Life Technologies) according to the manufacturer's instructions. Samples were incubated with reagent A for 15 minutes at room temperature shielded from light. After performing a washing step with PBS, reagent B was added with intracellular antibodies. Cells were incubated for 30 minutes at room temperature shielded from light.

4.3.3 Measurement and evaluation by flow cytometry

Fluorescence-activated cell scanning was performed using BD LSRFortessa™ cell analyzer and MACSQuant® Analyzer 10. Prior to use, devices were calibrated according to manufacturer's instructions. Stained cells were strained directly before cytometric measurement.

Flow cytometric data was evaluated by FlowJo™ 10.3.

4.4 Cytotoxicity assay

4.4.1 Luciferase cytotoxicity assay

Cytotoxic activity was analyzed by Luciferase- and CellTrace™ Violet cytotoxicity assays.

For Luciferase-based cytotoxicity assay, PBMCs were thawed and re-suspended with pre-warmed TexMACS medium. Luciferase transduced target cells (Raji, Nalm6 carrying a Luciferase reporter plasmid) were re-suspended with TexMACs. PBMCs, as effector cells, and target cells were pooled in an E/T ratio of 10/1 before being seeded on a 96-well plate. Blinatumomab was added in a concentration of 500 pg/ml. Co-cultures were incubated under standard conditions for 24 hours (37°C, 5% CO_2).

Target cells cultured without addition of PBMCs and Blinatumomab served as negative control. Incubating target cells with lysis buffer (Hepes Buffer + 1% Tween20) were used for positive control. A dilution row with target cells and a blank control served as technical controls.

After 24 hours of co-cultivation D-Luciferin was added. Measurement of luminescence intensity was performed by FLUOstar Optima spectrometer 0, 5 and 10 minutes after the substrate addition.

Target cell killing of the luciferase cytotoxicity assay was calculated by the mean luminescence intensity of the viable target cells stimulated with Blinatumomab compared to the mean luminescence of viable cells in the unstimulated target only control, according to the following formula:

$$\%\ \text{target cells lysis} = \left[\ 1 - \frac{\text{luminescence of viable target cells in sample}}{\text{luminescence of viable target cells in unstimulated control}}\ \right] \times 100$$

In some cases, more living cells were found in the target only control than in the unstimulated control, resulting formally in a negative target cells lysis. Since there was no significant difference between both controls, negative target cells lysis was defined as 0%.

4.4.2 CellTrace™ Violet cytotoxicity assay

After thawing, PBMCs were incubated in human AB serum for 2 hours at 37°C, 5% CO_2. For CellTrace™ Violet staining of target cells (Raji, Nalm6), stock solution was diluted with PBS to a working concentration of 1 µM. The cell pellet was re-suspended in CTV staining solution and

incubated for 5 minutes at 37°C, 5% CO_2. TexMACS medium and human AB serum were added. After two washing steps with PBS, target cells were re-suspended in TexMACS. Co-culture was prepared in an E/T ration of 10/1 and cells were seeded to a 96-well plate. Blinatumomab was added in a concentration of 500 pg/ml. Co-cultures were incubated at 37°C, 5% CO_2 for 24 hours.

Controls were performed by preparing target cells only without addition of effector cells and Blinatumomab and maximum lysed cells served as a positive control. For cell lysis, target cells were incubated with Hepes buffer + 1% Tween20 for 15 minutes on ice.

Flow cytometric analysis was performed using MACSQuant® Analyzer 10. To access cell viability, viability dye was used as described above. Gates were set on lymphocytes (FSC vs. SSC), single cells (FSC-H vs. FSC-A), viable cells (PI vs. PE-A) and viable target cells (CellTrace® Violet vs. FSC).

Target cells lysis was calculated according to the following formula:

$$\%\ \text{target cells lysis} = \left[\ 1 - \frac{\text{number of viable target cells in sample}}{\text{number of viable target cells in unstimulated control}}\ \right] \times 100$$

4.4.3 Comparison of cytotoxicity assays

To evaluate the comparability between Luciferase and CellTrace™ Violet cytotoxicity assay the measurement and method precision was determined. Therefore, the variance coefficient (CV) was calculated according to the following formula:

$$\text{VC} = \frac{\text{standard deviation s (X)}}{\text{mean } \bar{x}\ \text{(X)}} = \frac{\sqrt{\text{variance (X)}}}{\bar{x}}$$

4.5 Proliferation assays

4.5.1 CellTrace™ Violet proliferation assay

Proliferation was analyzed using CellTrace Violet™ Cell Proliferation Kit or carboxyfluorecsein diacetate succinimidyl ester (CFSE). CellTrace Violet™ Cell Proliferation Kit was performed according to the manufacturer's instructions.

In brief, PBMCs were thawed and washed twice with pre-warmed RPMI growth medium followed by a washing step with PBS. Cell pellet was re-suspended in CellTrace™ Violet staining

solution (final concentration 5 mM) and incubated for 20 minutes, shielded from light (37°C, 5% CO_2). FCS was added to absorb any unbound dye. Stained PBMCs were washed twice with PBS and re-suspended in full RPMI growth medium. Target cells (Nalm6 $CD80^+$, Nalm6 $PD\text{-}L1^+$, Nalm6 WT) were washed once with PBS and re-suspended in RPMI. Effector and target cells were co-cultured in an E/T ratio of 10/1 in a 48-well plate under standard conditions (37°C, 5% CO_2) for 5 days. Subsequently Blinatumomab was added in a concentration of 500 pg/ml. Cells stimulated with 10 µg/ml Staphylococcal Enterotoxin B (SEB) served as a positive control.

Flow cytometric analysis was performed after 0, 3 and 5 days of incubation. Remaining cells were transferred to fresh tubes. Extracellular staining was performed as described above with fluorescent-labelled antibodies targeting CD3, CD4 and CD8. Viability was determined using 7AAD staining.

Flow cytometric data was analyzed by gating on lymphocytes (FSC vs. SSC), single cells (FSC-H vs. FSC-A), effector cells (GFP vs. CellTrace® Violet), living cells (SSC vs. 7AAD) and T cells (SSC vs. CD3). T-cell subpopulations were separately analyzed by CD4 and CD8 staining (CD4 vs. CD8). To evaluate T-cell proliferation the final gate was set on CellTrace™ Violet positive cells. Cell proliferation was analyzed by FlowJo 10.3. proliferation tool.

T-cell proliferation was described by percentage of divided T cells among all T cells defining the fraction of the origin population that divided at least once.

4.5.2 CFSE-proliferation assay

Cell staining with Carboxyfluorescein succinimidyl ester (CFSE) was performed using Vybrant® CFDA SE Cell Tracer Kit Vybrant® CFDA SE Cell Tracer Kit was used according to the manufacturer's instructions.

CFSE stock was diluted with appropriate amount of PBS to achieve a 1,7 µM staining solution. PBMCs were thawed and washed twice and re-suspended in pre-warmed PBS. Cells were incubated with CFSE staining solution for exactly 9 minutes to avoid CFSE mediated cytotoxicity. Heat-inactivated FCS was added followed by two washing steps. Stained PBMCs were diluted in RPMI.

Target cells (Nalm6 WT, Nalm6 $CD80^+$, Nalm6 $PD\text{-}L1^+$) were diluted in RPMI. PBMCs and target cells were pooled in an E/T ratio of 10/1 and were seeded to a 48-well plate. Co-cultures were

incubated under standard conditions (37°C, 5% CO_2) for 5 days. Blinatumomab was added in a concentration of 500 pg/ml. As positive control 10 µl Staphylococcal Enterotoxin B (SEB) was added. Unstimulated PBMCs served as negative control.

On day 0, day 3 and day 5 of co-culture flow cytometric analysis was performed. Cells were harvested by transferring them in fresh tubes. Extracellular staining was performed with fluorescent-labelled antibodies targeting CD14, CD4 and CD8 as described above. To access cell viability, APC eFluor780 was used as viability dye.

For analyzing flow cytometric data, gates were set on lymphocytes (FSC vs. SSC), single cells (FSC-H vs. FSC-A) and living CD14 negative cells (SSC vs. APC eFluor). T-cell proliferation was detected by gating on $CD4^+$ and $CD8^+$ subpopulations (CD4 vs. CD8) and on CFSE positive cells (SSC vs. CFSE).

4.6 Intracellular cytokine staining

For detection of cytokine production, flow cytometry-based intracellular cytokine staining (ICS) was performed.

Avoiding any mechanical stimulation PBMCs were thawed carefully in 50 ml pre-warmed RPMI and were re-suspended to a concentration of 1 x 10^6/ml with TexMACS medium. Target cells (Nalm6 $CD80^+$, Nalm6 $PD\text{-}L1^{+,}$ Nalm6 WT) were diluted to a concentration of 1 x 10^5/ml with TexMACS. Effector and target cells were co-cultured in an E/T ratio of 10/1 in a 6-well plate. Co-cultures were stimulated with 500 pg/ml Blinatumomab. Incubation was performed under standard conditions (37°C, 5% CO_2) for 24 hours. Co-cultures stimulated with 10 µg/ml Staphylococcal Enterotoxin B (SEB) served as positive controls, whereas cells without addition of any stimulation reagent served as negative.

24 hours *post* stimulation Brefeldin A was added in a concentration of 10 µg/ml. After incubation for 4 hours (37°C, 5% CO_2), cold PBS was added to stop the exocytosis inhibition mediated by Brefeldin A. Extracellular staining was performed as described above. In brief, cells were incubated for 10 minutes on ice, shielded from light with fluorescent-labelled antibodies targeting CD3, CD4, CD8, CD56, CD45RO, CD62L and ViobilityDye® for viability staining. IL-4, IL-10, IFNγ, TNFα were stained intracellularly as described above.

Samples were analyzed by flow cytometric measurement. Gates were set on lymphocytes (FSC vs. SSC), single cells (FSC-H vs. FSC-A), viable cells (SSC vs. ViobilityDye®) and CD56 negative T cells (CD3 vs. CD56) to exclude NK cells. T cells were divided in $CD4^+$ and $CD8^+$ subpopulations (CD4 vs. CD8), as well as memory T cells and naive T cells (CD45RO vs. CD62L). Cytokine production was detected by gating on CD3 and IL-4, IL-10, IFNγ, TNFα positive populations. Gate for cytokine positive cells was adjusted referring to FMO and unstimulated control.

4.7 Fluorescence activated cell sorting and cryo-conservation of sorted effector cells

Fluorescence activated cell sorting has been performed with FACS-Aria III cell sorting system with FACS-Diva software (BD Bioscience).

Primary human PBMCs were thawed in pre-warmed RPMI as previously described and co-cultured with target cells stably transduced with GFP (Nalm6 WT, Nalm6 $CD80^+$, Nalm6 $PD\text{-}L1^+$, Raji WT, Raji $PD\text{-}L1^+$). Co-culture was prepared in an E/T ration of 10/1 and cells were seeded to a 6-well plate. Blinatumomab was added in a concentration of 500 pg/ml. Co-cultures were incubated under standard conditions at 37°C, 5% CO_2.

After 72 hours of incubation cells were harvested, re-suspended in PBS containing 2% FCS and analyzed. The population of interest has been identified through light scatter properties (lymphocyte population) and then gated based on GFP negative expression. The sorting on GFP negative T cells has been conducted using a 70µm nozzle. Sorted cells were collected in collection tubes containing PBS with 2% FCS.

For cryo-conservation of sorted cells, T cells were either frozen as cell pellet or were re-suspended with 500 µl QIAzol lysis reagent before freezing. Cells were directly placed at -80°C and were transferred to liquid nitrogen after 24 hours.

4.8 Statistical analysis

Standard statistical evaluation on mean, standard deviation and P value was assessed using Microsoft Excel 2010 and GraphPad PRISM 8.0. Statistically significant differences between two means were evaluated by using the unpaired *t* test. Assumption that the data of two populations have the same variances was evaluated by performing the *F* test. Differences with P values < 0,05 were considered as statistically significant.

5 Results

T-cell effector function is crucial for the initiation and maintenance of an endogenous anti-leukemic activity. Overexpression of inhibitory immune checkpoints on leukemic blast T cells represents a potent mechanism to escape cancer immunosurveillance (Pardoll 2012). Even though Blinatumomab improves the outcome of pediatric r/r B-lineage ALL, a significant subset of patients ultimately relapses under Blinatumomab treatment. In this study we investigated the influence of immune checkpoints on Blinatumomab-mediated effects by analyzing target cells lysis, cell proliferation and cytokine production of primary human T cells.

5.1 Influence of immune checkpoints on Blinatumomab-mediated cytotoxicity of primary T cells

5.1.1 Blinatumomab-mediated cytotoxicity of primary T cells

To assess the Blinatumomab-mediated CD19 targeting cell lysis, luciferase and flow cytometry-based CellTrace Violet™ (CTV) cytotoxicity assays were performed on co-cultures of primary human PBMCs with lymphoblastic cell lines, Raji and Nalm6, with and without Blinatumomab addition.

Blinatumomab stimulation significantly increased the cytotoxic activity of T cells against Nalm6 cells within 24 hours detected by flow cytometry-based CTV cytotoxicity assay. Mediated by Blinatumomab, target cells lysis increased from 10.33% (±2.50%) in the untreated control to 47.73% (±6.92%) ($P = 0.0005$; Figure 4A and B).

Moreover, Blinatumomab-mediated cytotoxic capacity significantly correlated with the ratio of target to effector cells (T/E). Target cells lysis was significantly reduced by decreasing the T/E ratio analyzed by luciferase cytotoxicity assay. Cytotoxic activity of T cells co-cultured with Raji cells in a T/E ratio of 1/10 was 46.85% (±9.89%) compared to 18.22% (±1.60%) and 10.54% (±6.59%) for T/E ratios of 1/5 and 1/1 respectively ($P = 0.0488$ and $P = 0.0448$; Figure 4C).

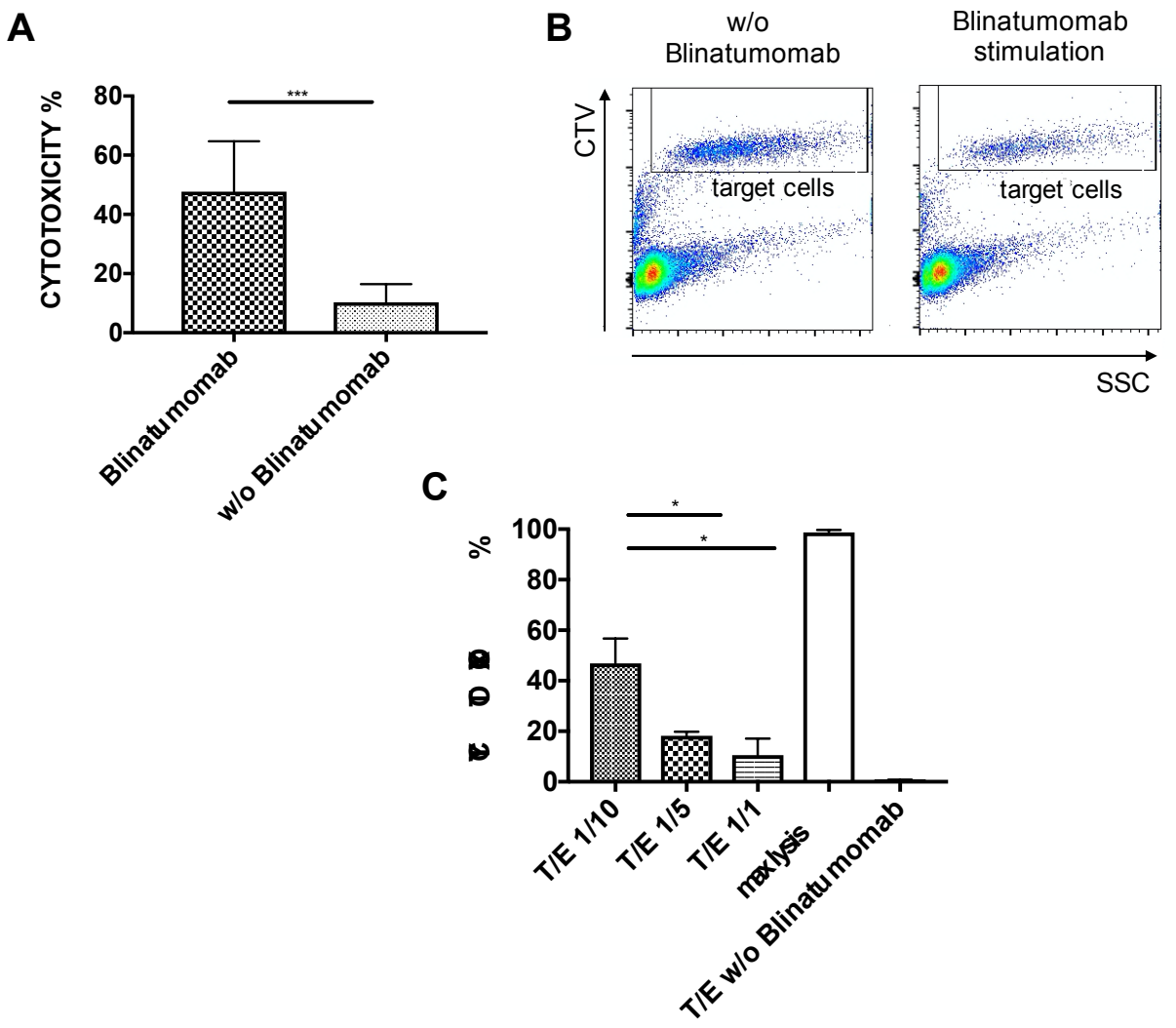


Figure 4: Blinatumomab-mediated cytotoxicity of primary T cells against leukemic blast cell lines.

Primary PBMCs from healthy human donors were co-cultured with Nalm6 and Raji cell lines with and without addition of 500 pg/ml Blinatumomab for 24 hours. Flow cytometric analysis of CellTrace Violet™ cytotoxicity assay was performed on PBMCs in the presence of Nalm6 cells as target cells (A). Representative flow cytometric density plots of viable Raji cells from a co-culture with PBMCs stimulated with Blinatumomab (B). Luciferase cytotoxicity assay analyses were performed on PBMCs co-cultured in different T/E ratios with Raji cells (C). Cytotoxicity was calculated by the number of viable target cells stimulated with Blinatumomab compared to the viable target cells in the unstimulated control. Bars represent mean values ± S.D. of three healthy individuals. For each donor, three experiments each in technical triplicates were performed. * $P \leq 0.05$, *** $P \leq 0.001$.

5.1.2 Influence of immune checkpoints on Blinatumomab-mediated cytotoxicity of primary T cells

In order to further assess the influence of immune checkpoints on Blinatumomab-induced target cell killing, primary human PMBCs were co-cultured with lymphoblastic cell lines stably transfected with the stimulatory CD80 or inhibitory PD-L1 immune checkpoint. Flow cytometry-based CTV cytotoxicity assays were performed on primary human PBMCs in the presence of Nalm6 cells as target cells stably transfected with either CD80 or PD-L1 and with Raji cells stably transfected with PD-L1. Blinatumomab stimulation on co-cultures was performed for 24 hours.

A non-significant trend of decreased cytotoxic capacity of T cells co-cultured with PD-L1 transduced Nalm6 cells was shown. Blinatumomab-mediated cytotoxicity of T cells against Nalm6 cells stably transfected with PD-L1 was 37.87% (±7.38%) compared to 48.45% (±10.28) in the controls (Figure 5A).

However, expression of PD-L1 on Raji cells significantly decreased Blinatumomab-mediated cytotoxic activity of T cells. Target cells lysis against Raji cells stably transfected with PD-L1 of 29.6% (±6.68%) compared to 57.09% (±4.49%) in the wildtype controls was shown (P = 0.0007; Figure 5B).

Blinatumomab stimulation of Nalm6 cells stably transfected with CD80 significantly increased the cytotoxic capacity of T cells from 48.45% to 70.70% (±5.27) (P = 0.0063; Figure 5A).

Comparison of T-cell cytotoxicity mediated by Blinatumomab against Raji WT and Nalm6 WT showed a non-significant difference in target cell lysis. Cytotoxic activity of T cells against Raji WT cells was 57.09% (±4.49%) versus 48.45% (±10.28) against Nalm6 cells (Figure 5A and B).

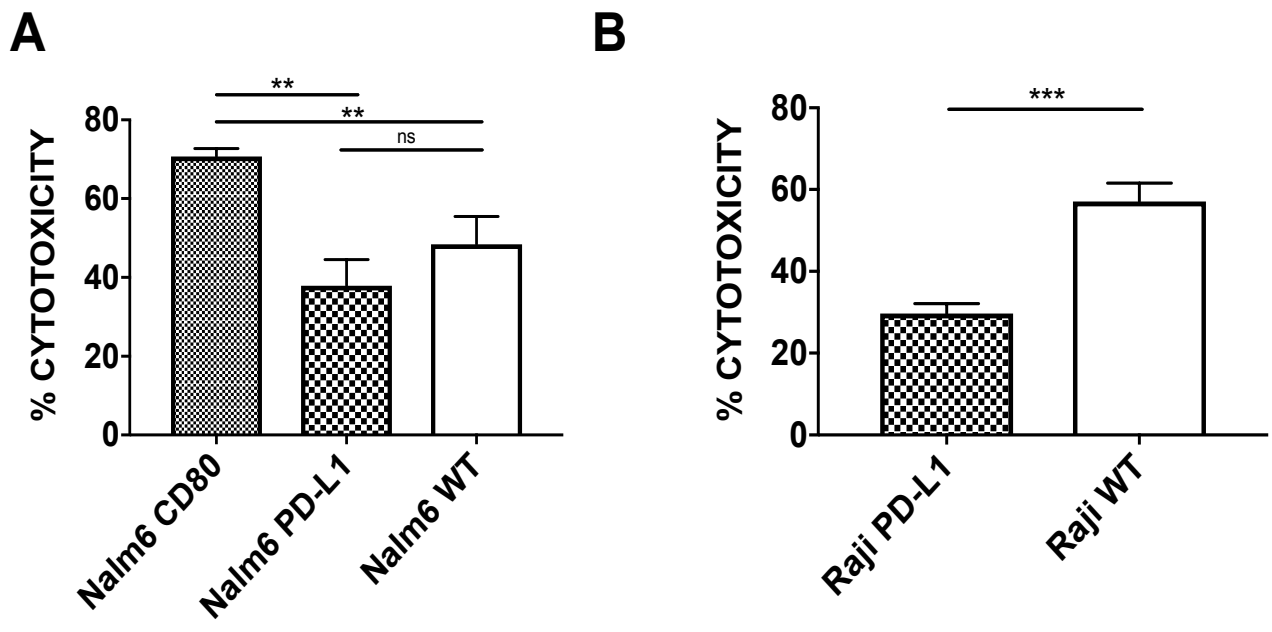


Figure 5: Immune checkpoint dependent T-cell cytotoxicity against lymphoblastic target cells mediated by Blinatumomab.

Primary PBMCs from healthy donors were co-cultured with lymphoblastic Nalm6 and Raji cell lines in an effector/target ratio of 10/1 and stimulated with Blinatumomab. After 24h of co-culture flow cytometry-based CTV cytotoxicity assay was performed. Analysis of Blinatumomab-mediated cytotoxicity of T cells against Nalm6 cells stably transfected with CD80 or PD-L1 (A) and Raji cell lines stably transfected with PD-L1 (B). Bars represent mean values ± S.D. of 3 healthy donors. For each donor 2 independent experiments were performed in technical triplicates. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

5.2 Influence of immune checkpoints on Blinatumomab-mediated proliferation of primary T cells

5.2.1 Blinatumomab-mediated proliferation of primary T cells

In addition to T-cell cytotoxicity against lymphoblastic cell lines, the evaluation of Blinatumomab-exerted effect on T-cell proliferation is highly relevant since it controls the effector to target ratio and consequently affects target cells lysis upon Blinatumomab stimulation (Figure 4). To assess the Blinatumomab-mediated influence on T-cell proliferation, flow cytometry-based CellTrace Violet™ cell proliferation assays were performed on Blinatumomab-stimulated primary PBMCs co-cultured with Nalm6 cells for 5 days.

Blinatumomab stimulation significantly increased T-cell expansion in the presence of Nalm6 cells resulting in a T-cell proliferation of 21.90% (±1.98%) as compared with a proliferation rate of 0.92% (±0.80) in the unstimulated controls ($P < 0.0001$; Figure 6). Interestingly, T-cell proliferation upon Blinatumomab stimulation was significantly higher compared to T-cell proliferation induced by Staphylococcal Enterotoxin B (SEB) stimulation used as positive control. On average 21.90% (±1.98%) of Blinatumomab-stimulated $CD3^+$ T cells proliferated compared to 14.57% (± 3.90%) of SEB stimulated T cells ($P = 0.0441$).

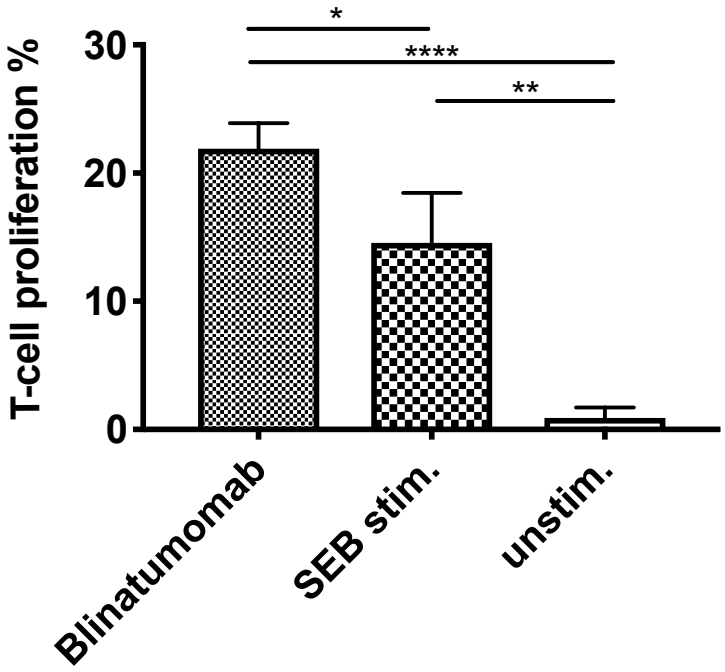


Figure 6: Blinatumomab-mediated proliferation of primary T cells.

PBMCs of healthy donors were incubated with Nalm6 cells (effector/target ratio: 10/1) and were stimulated with Blinatumomab or SEB as positive control. Flow cytometry-based cell proliferation assay (determined with CellTrace Violet™) was performed at 5 days of co-culture. Bars represent mean values ± S.D. of 3 healthy donors. For each donor 3 independent experiments were performed in technical triplicates. * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$.

5.2.2 Blinatumomab-mediated proliferation of T-cell subpopulations

The influence of Blinatumomab stimulation on T-cell proliferation was further investigated by evaluating the proliferation of T-cell subpopulations, $CD4^+$ and $CD8^+$ T cells. Blinatumomab stimulation induced a differing proliferation pattern of $CD4^+$ and $CD8^+$ T cells.

Upon Blinatumomab stimulation, proliferation of $CD8^+$ T cells was significantly higher as compared with $CD4^+$ T-cell proliferation. At day 3, 5.99% (±3.99%) of $CD8^+$ T cells proliferated in comparison to 2.16% (±1.48%) of $CD4^+$ T cells (P = 0.0159). $CD8^+$ T-cell proliferation was 34.98% (±9.29%) compared to $CD4^+$ T-cell proliferation of 11.56% at day 5 (±5.66%; P < 0.0001; Figure 8).

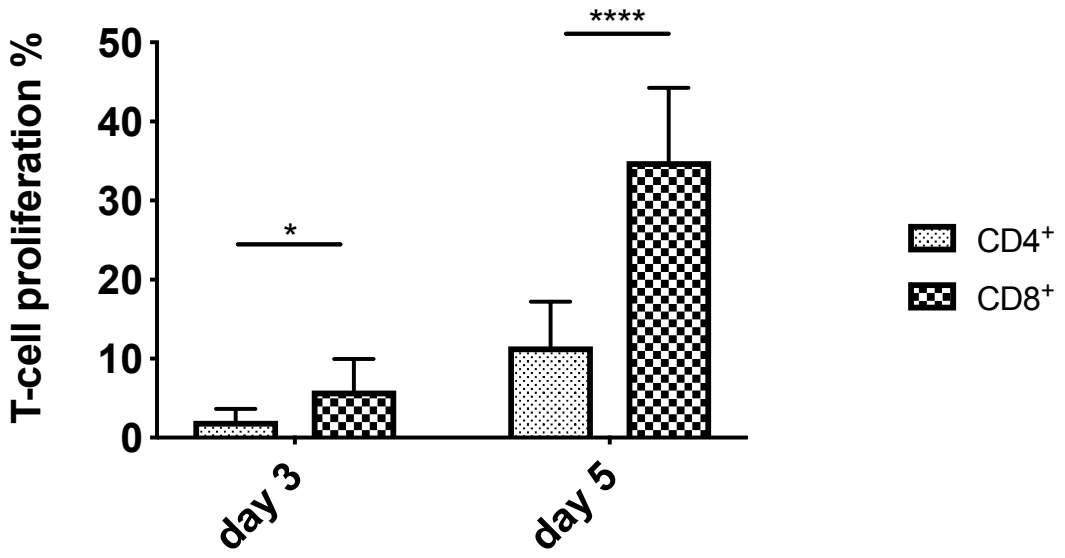


Figure 7: Blinatumomab-induced proliferation of T-cell subpopulations

Blinatumomab-stimulated primary PBMCs were co-cultured with Nalm6 cells in an effector/target ratio of 10/1. Flow cytometry-based CellTrace Violet™ T-cell proliferation assay was performed to determine the proliferation of $CD4^+$ and $CD8^+$ T cells at day 3 and 5 of co-culture. Bars represent mean values ± S.D. of 3 healthy donors. For each donor 3 independent experiments were performed. * $P \leq 0.05$, **** $P \leq 0.0001$.

5.2.3 Influence of immune checkpoints on Blinatumomab-mediated proliferation of primary T cells

To further assess the influence of immune checkpoints on Blinatumomab-mediated T-cell proliferation, proliferation assays were performed on Blinatumomab-stimulated primary human PBMCs co-cultured with Nalm6 or Raji cell lines as target cells stably transfected with the stimulatory immune checkpoint CD80 or inhibitory checkpoint PD‑L1.

Co-culture of Nalm6 cells transfected with CD80 significantly increased Blinatumomab-mediated T-cell expansion within 5 days (Figure 7A and B). CD80 expression on Nalm6 cells led to an averaged T-cell proliferation of 32.76% (±2.08%) compared to 21.90% (±1.98%) in the wildtype controls at day 5 (P = 0.0028). In contrast, the expression of the inhibitory immune checkpoint PD‑L1 on Nalm6 cells did not significantly influence Blinatumomab-induced T-cell proliferation. (Figure 7A and B).

However, Blinatumomab-induced proliferation of T cells co-cultured with Raji cells was significantly higher as compared with T cell expansion in the presence of Nalm6 cells. Co-culture with Raji wildtype cells led to a T-cell proliferation of 38.59% (±4.59%) compared to 21.9% (±1.97%) T-cell proliferation in the presence of Nalm6 wildtype cells (P = 0.0044). Expression of PD‑L1 on Raji cells significantly decreased the proliferation of primary T cells leading to a T-cell expansion of 21.27% (±7.75%) as compared with 38.59% (±4.58%) in the wildtype controls (P = 0.0291; Figure 7C).

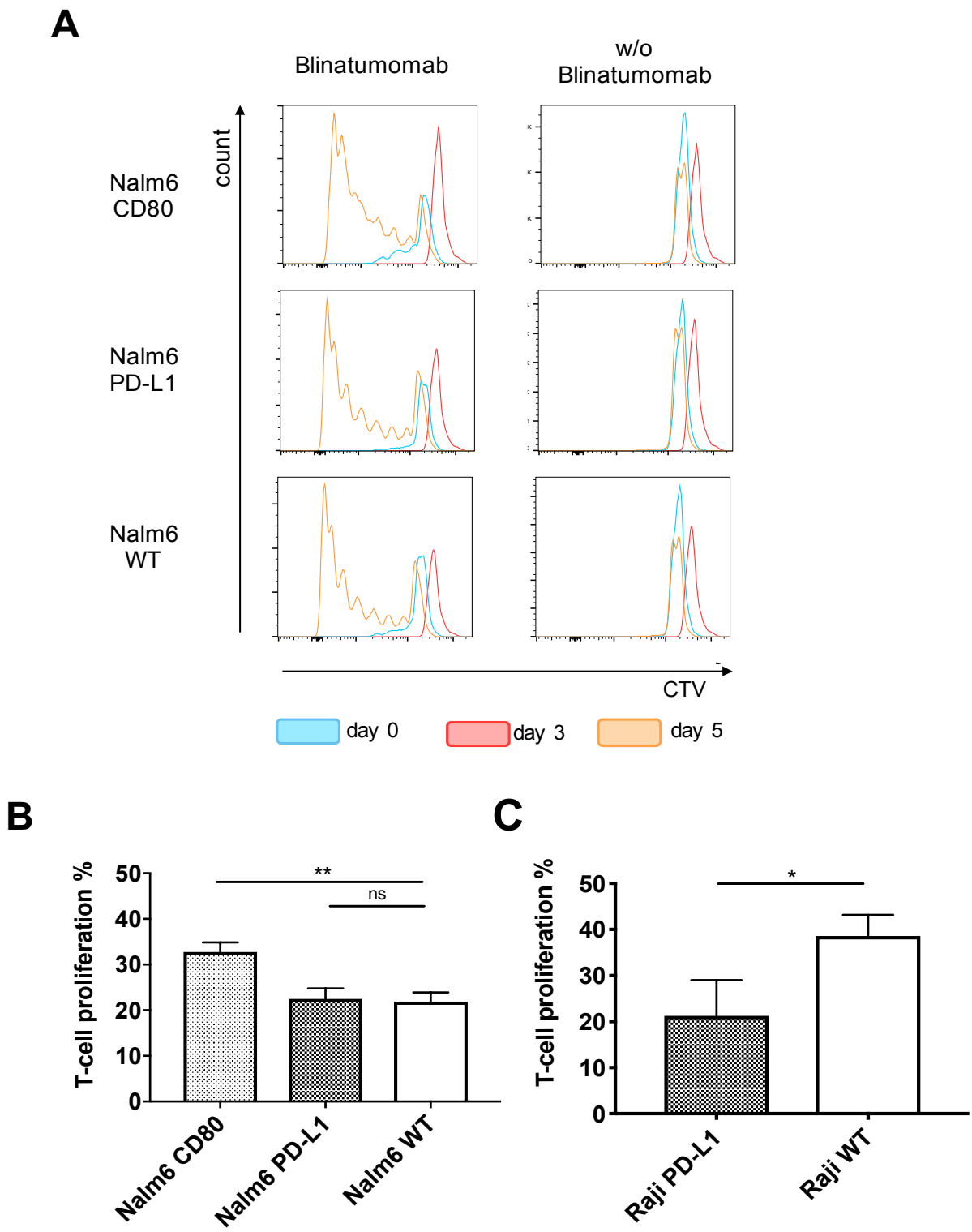


Figure 8: Influence of immune checkpoints on Blinatumomab-mediated T-cell proliferation.

Blinatumomab-stimulated primary PBMCs were incubated with Nalm6 or Raji cell lines stably transfected with CD80 or PD-L1 in an effector/target ratio of 10/1. Flow cytometry-based cell proliferation assays using CellTrace Violet™ were performed at day 0, 3 and 5 of co-culture. **(A)** Representative overlay histogram of T-cell proliferation. The number of peaks correlates with the number of cell divisions. **(B and C)** Proliferation of T cells within a period of 5 days represented as percentage of proliferating $CD3^+$ T cells among all T cells. Bars represent mean values of percentage of proliferated T cells ± S.D. of 3 healthy donors. For each donor 3 independent experiments were performed. * $P \leq 0.05$, ** $P \leq 0.01$.

5.2.4 Influence of immune checkpoints on Blinatumomab-mediated proliferation of T-cell subpopulations

Since Blinatumomab stimulation induced a differing proliferation pattern of T-cell subpopulations (see 5.2.2), the influence of immune checkpoints on Blinatumomab-mediated proliferation of $CD4^+$ and $CD8^+$ T cells was further evaluated.

Expression of CD80 on Nalm6 cell lines significantly enhanced $CD4^+$ T-cell proliferation from 2.16% (Nalm6 WT co-culture) to 13.53% (Nalm6 CD80 co-culture) at day 3 of co-culture and from 11.56% to 29.83% at day 5 respectively ($P = 0.004$ and $P < 0.001$). In contrast, CD80 expression did not significantly influence $CD8^+$ cell proliferation with a $CD8^+$ proliferation rate of 32.26% (±4.41%) versus 34.98% (±1.62%) in the controls at day 5 ($P = 0.228$; Figure 9A).

PD-L1 expression on Nalm6 and Raji cells affected the proliferation of T-cell subpopulations equally. PD-L1 expression on lymphoblastic cell lines (Nalm6 and Raji) did not significantly reduce the proliferation of $CD4^+$ and $CD8^+$ T cells compared to wildtype controls (Figure 9A and B).

Evaluation the ratio of $CD4^+$ to $CD8^+$ cell proliferation revealed that CD80 expression on Nalm6 cells led to an identical proliferation of both T-cell subpopulations with a $CD4^+/CD8^+$ ratio of 1:1.08. Co-culture with Nalm6 stably transfected with PD-L1 and wildtypes resulted in a T-cell proliferation with a $CD4^+/CD8^+$ ratio of 1:3.15 and 1:3.02 respectively (Figure 10A).

The characteristic proliferation pattern of $CD4^+$ and $CD8^+$ T-cell subpopulations was consistent among all three healthy human donors. Representative flow cytometric analyses on T-cell proliferation demonstrate a donor-independent T-cell proliferation pattern which is distinctly influenced by the expression of CD80 and PD-L1 upon Blinatumomab stimulation (Figure 10B).

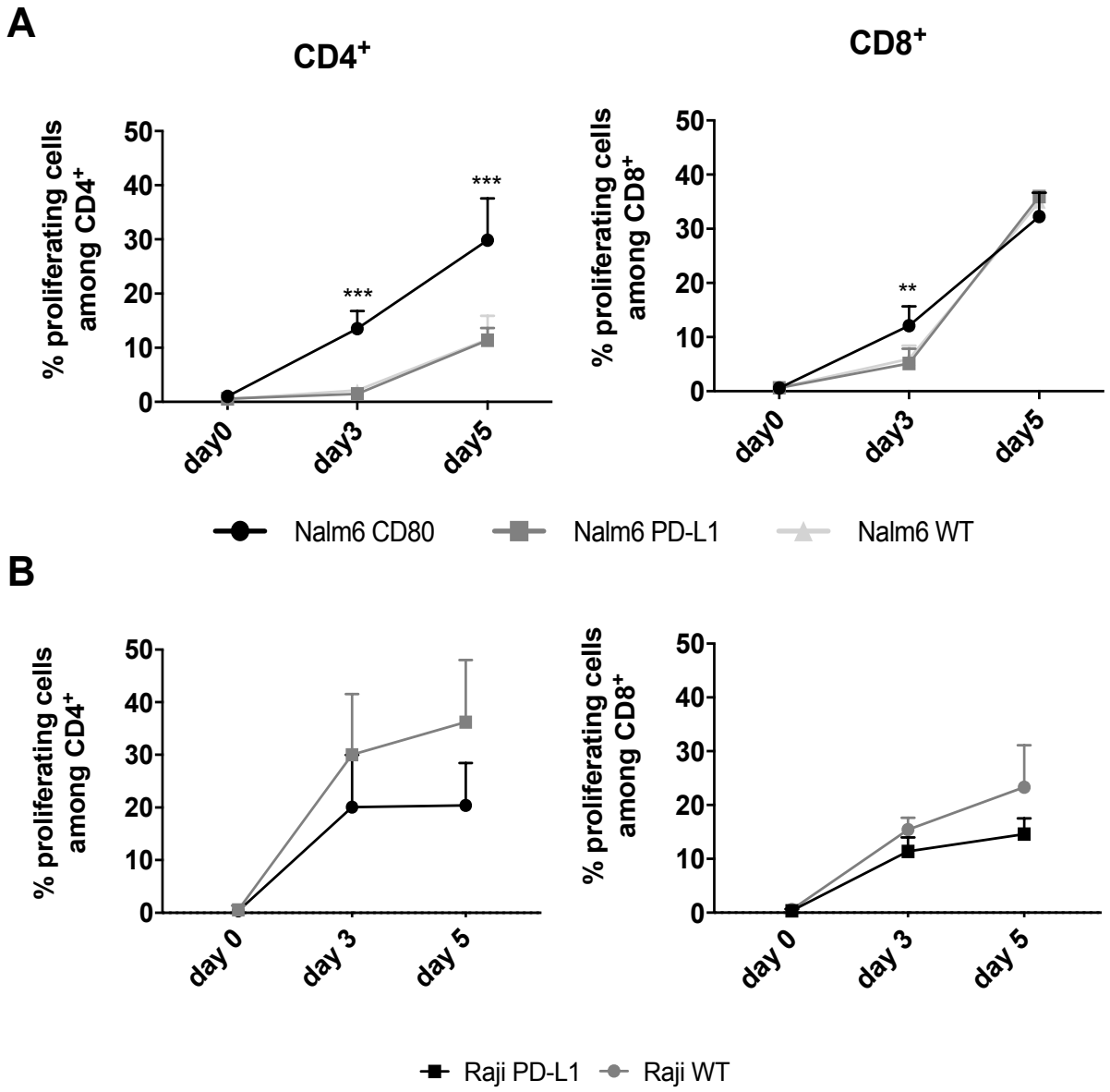


Figure 9: Influence of immune checkpoint expression on lymphoblastic cell lines on Blinatumomab-mediated proliferation of CD4+ and CD8+ T-cells.

Blinatumomab-stimulated PBMCs of healthy donors were incubated with lymphoblastic Nalm6 cell lines (Nalm6 or Raji) stably transfected with CD80 or PD‑L1 (effector/target ratio: 10/1). Flow cytometry-based CellTrace Violet™ cell proliferation assays were performed at day 0, 3 and 5 of co-culture with Nalm6 (A) and Raji (B) cells. Bars represent mean values ± S.D. of 3 healthy donors. For each donor 3 independent experiments were performed. ** $P \leq 0.01$, *** $P \leq 0.001$.

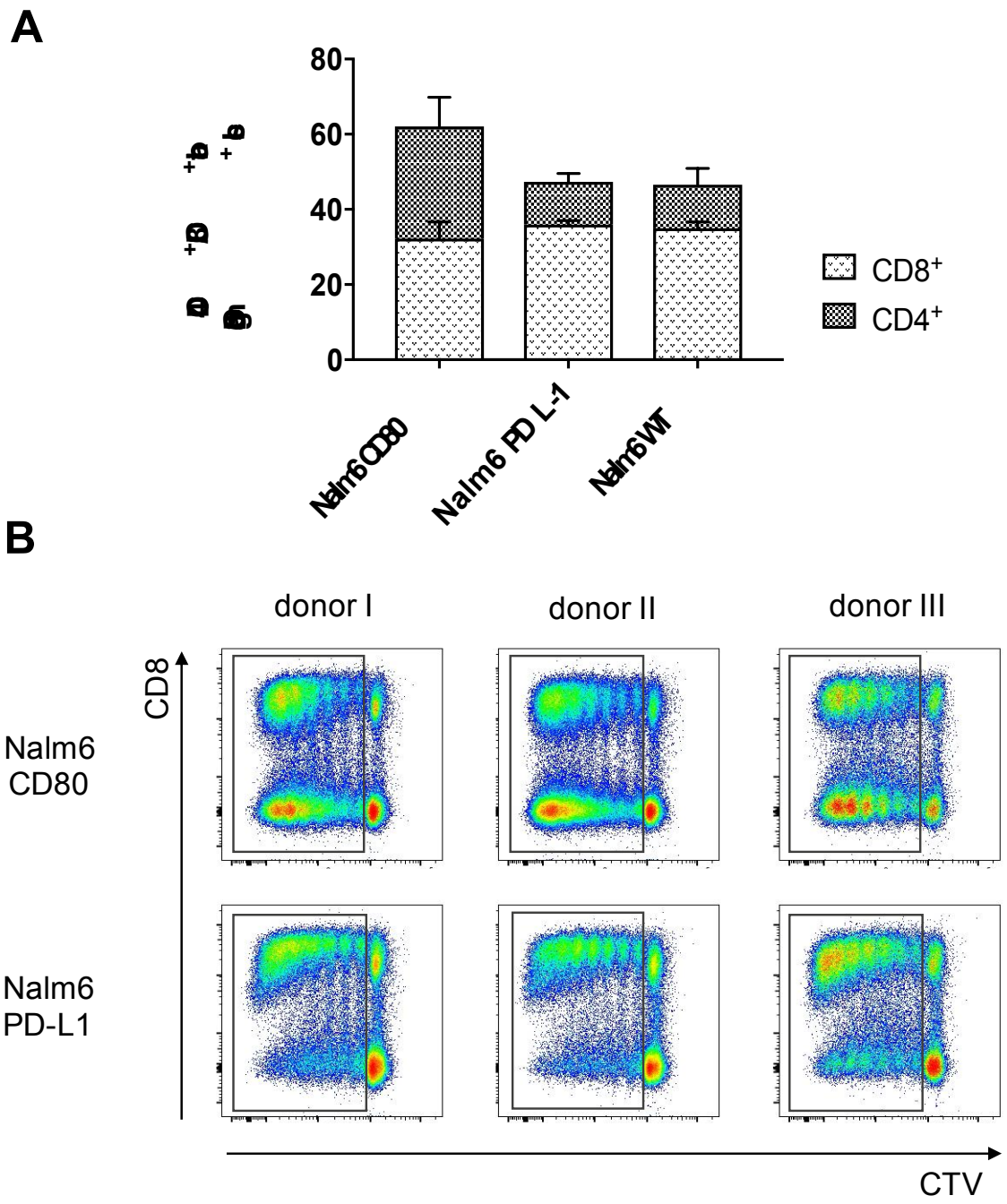


Figure 10: Influence of immune checkpoints on Blinatumomab-mediated proliferation of T-cell subpopulations

Blinatumomab-stimulated PBMCs of healthy donors were incubated with lymphoblastic Nalm6 cell lines stably transfected with CD80 and PD-L1 in an effector/target ratio of 10/1. Flow cytometry-based cell proliferation assay (determined with CellTrace Violet™) was performed after 0, 3 and 5 days of co-culture. **(A)** Ratio of $CD4^+/CD8^+$ proliferating T cells co-cultured at day 5. **(B)** Representative flow cytometric analysis representing T-cell proliferation of 3 different donors (donor I, II, III) after 5 days. Bars represent mean values ± S.D. of 3 healthy donors. For each donor 3 independent experiments were performed.

5.3 Influence of immune checkpoints on Blinatumomab-mediated cytokine production of primary T cells

5.3.1 Blinatumomab-mediated effects on cytokine production of primary T cells

Since cytokine secretion of T cells plays a pivotal role in the regulation of endogenous anti-tumor immunity by modulating the activity of other types of immune cells, intracellular staining of co-cultures for Th_1 (IFNγ, TNFα) and Th_2 (IL-4, IL-10) cytokines after Blinatumomab stimulation was performed. Upon stimulation with Blinatumomab, T cells significantly increased the production of IFNγ and TNFα while Th_2 cytokine levels were unaffected. Blinatumomab stimulation increased IFNγ production of T cells 10.29-fold (from 0.10% (±0.04%) to 1.05% (±0.38%); P=0,013) and TNFα production 114.34-fold (from 0.04% (±0.04%) to 4.31% (±0.47%); P < 0.0001; Figure 11).

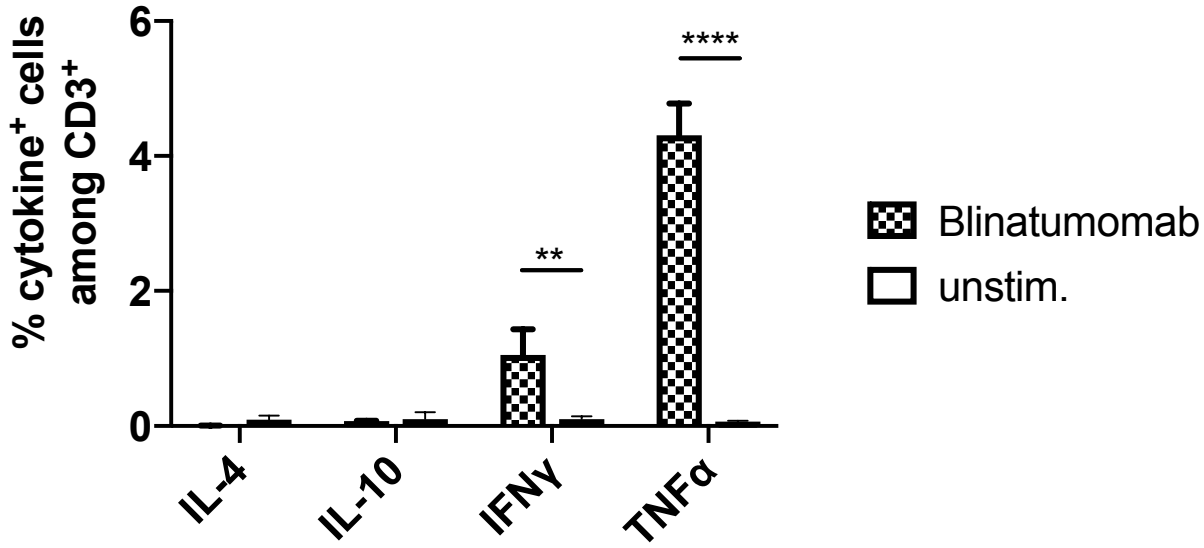


Figure 11: Blinatumomab-mediated effect on cytokine production of human T cells.

PBMCs of healthy donors co-cultured with Nalm6 cells in an effector/target ratio of 10/1 were stimulated with Blinatumomab. Intracellular staining for IL-4, IL-10, IFNγ and TNFα was performed and flow-cytometric analysis were assessed after 24 hours of stimulation. Bars represent mean values ± S.D. of 3 independent experiments of 3 healthy donors. ** P ≤ 0.01, **** P ≤ 0.0001.

5.3.2 Influence of immune checkpoints on Blinatumomab-mediated cytokine production of primary T cells

The influence of immune checkpoints on Blinatumomab-induced cytokine production was assessed by flow cytometry-based analyses after intracellular cytokine staining of Blinatumomab-stimulated primary human PBMCs co-cultured with Nalm6 cell lines stably transfected with CD80 or PD-L1.

Mediated by Blinatumomab, co-culture of Nalm6 cells stably transfected with CD80 significantly enhanced the production of the pro-inflammatory Th_1 cytokines TNFα and IFNγ. CD80 expression on Nalm6 cells led to a 5.54-fold IFNγ production by T cells (5.82% (±1.96%)) compared to T cells co-cultured with Nalm6 wildtype (1.05% (±0.38%); P = 0.0144). TNFα production of T cells co-cultured with Nalm6 stably transfected with CD80 was increased 3.23-fold (13.93% (±0.98%)) compared to the respective wildtype controls (4.31% (±0.47%); P = 0.0001; Figure 12A and B).

Furthermore, the expression of PD-L1 on Nalm6 cells significantly reduced TNFα production of T cells mediated by Blinatumomab. PD-L1 expression resulted in a TNFα production of 3.00% (±0.63%) compared to 4.31% (±0.47%) of T cells in the controls (P = 0.454). PD-L1 expression on Nalm6 cells did not significantly affect production of IFNγ (Figure 12A and B).

Expression of CD80 or PD-L1 on Nalm6 cells did not significantly affect Blinatumomab-mediated production of anti-inflammatory Th_2 cytokines (IL-4 and IL-10). After co-culture with CD80 and PD-L1 expressing Nalm6 cells, 0.009% (± 0.004%) and 0.004% (±0.002%) of T cells produced IL-4 respectively, compared to 0.002% (±0.002%) in the wildtype control (P = 0.0634 and P = 0.0481). Blinatumomab-mediated IL-10 production upon CD80 and PD-L1 expression on Nalm6 cells was 0.145% (±0.057%) and 0.043% (±0.007%) compared to 0.051% (±0.026%) in the controls (P = 0.0469 and P = 0,0087; Figure 12A).

There was no significant difference in the Th_1 cytokine production of T cell subpopulations. CD80 expression on Nalm6 cells lead to an IFNγ production by $CD4^+$ T cell of 5.49% (±2.04%) versus 10.45% (±4.79%; P = 0.073) and TNFα secretion of 27.43 (±9.41%) versus 23.63% (±8.85%; P = 0.581). PD-L1 expression on Nalm6 cells induced a $CD4^+$ IFNγ production of 0.91% (±1.05%) versus $CD8^+$ IFNγ production of 2.65% (±1.23%; P = 0.388). Co-culture with Nalm6 WT resulted in an IFNγ secretion by $CD4^+$ T cell of 0.56% (±0.08%) versus 3.59% (±2.06%;

P = 0.144) and an TNFα secretion of 10.82 (±9.94%) versus 14.81% (±9.12%; P = 0.562; **Figure 12B**).

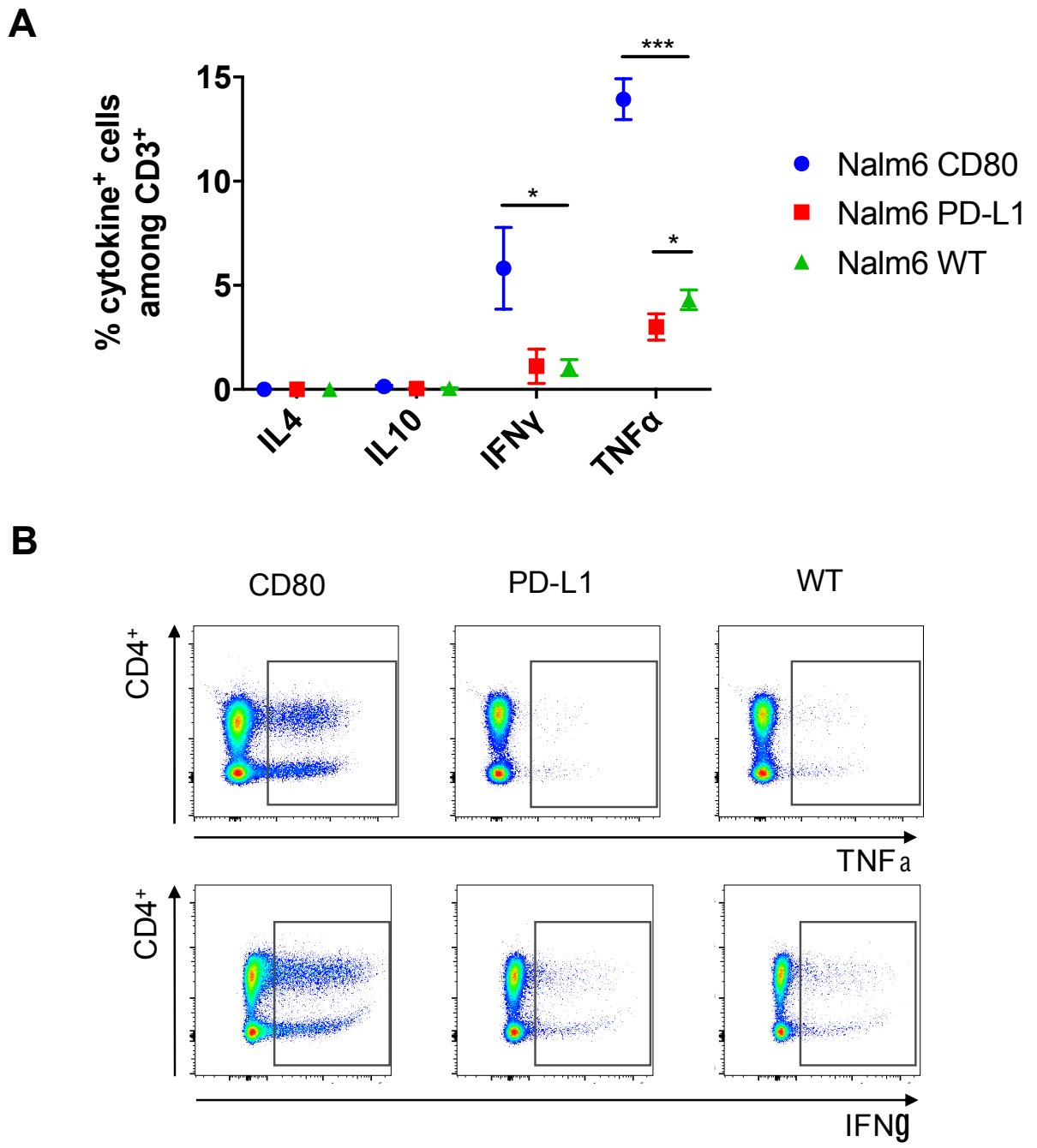


Figure 12: Blinatumomab-mediated cytokine production of primary T cells co-cultured with target cells expressing immune checkpoints.

PBMCs form healthy donors were incubated with Nalm6 cells stably transfected with CD80 and PD-L1 in an effector/target ratio of 10/1. Intracellular staining for IL-4, IL-10, IFNγ and TNFα was performed and flow-cytometric analysis was assessed after 24 hours of Blinatumomab stimulation **(A)**. IFNγ and TNFα production of T cells is shown in representative flow cytometry blots **(B)**. Interleaved symbols represent mean values ± S.D. of 3 independent experiments of 3 healthy donors. * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

5.4 Expression of PD-1 on primary T cells upon Blinatumomab-mediated T-cell activation

PD-L1 expression on target cells significantly attenuated Blinatumomab-mediated T-cell effector function in terms of target cells lysis, T-cell proliferation and production of pro-inflammatory cytokines. It is consequently crucial to investigate the expression of PD-1, the ligand for PD-L1, on Blinatumomab-stimulated T cells in co-culture with lymphoblastic target cells.

For this purpose, human PBMCs of three healthy donors were co-cultured with Raji and Nalm6 cell lines in presence or absence of Blinatumomab. PD-1 expression on T cells was analyzed *via* flow cytometry after 24 hours of stimulation. Blinatumomab stimulation induced an upregulation of PD-1 on T cells in the presence of lymphoblastic cells. PD-1 expression of Blinatumomab-stimulated T cells co-cultured with Raji cells was 14.73% (±6.71%) compared to 4.40% (±2.2%) in the unstimulated controls (P = 0.0588; **Figure 13**).

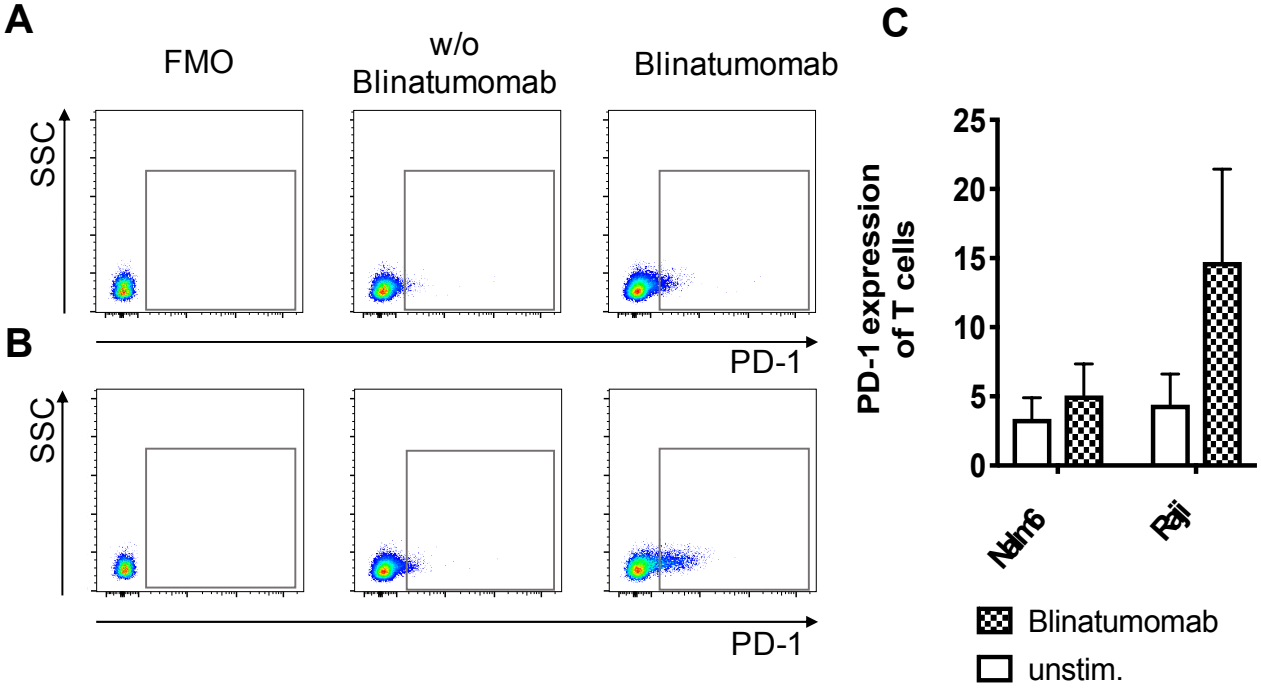


Figure 13: Blinatumomab-mediated effect on T-cell PD-1 expression.

Co-culture of primary human PBMCs with target cells were performed in an effector/target ratio of 10/1 and stimulated with Blinatumomab for 24 hours. PD-1 expression of T cells in co-culture with Nalm6 **(A)** and Raji cells **(B)** and unstimulated controls are shown in representative density plots of flow cytometric analyses. PD-1 expression of T cells upon Blinatumomab stimulation is shown as percentage of PD-1 expressing T cells among all T cells **(C)**. Bars represent mean values ± S.D. of 2 independent experiments of 3 healthy donors.

5.5 Method comparison

5.5.1 Comparison of cytotoxicity assays: Luciferase versus CellTrace Violet™ cytotoxicity assay

Blinatumomab-mediated cytotoxicity of T cells was analyzed by performing luciferase- and flow cytometry-based CellTrace Violet™ (CTV) cytotoxicity assays to confirm validity of the results. The assays differ in their staining as well as detection method as described in the respective method section (3.4.1 and 3.4.2).

Since biological standard deviation describes intra-individual variances among the donors, assessment on the technical replicates further represents an appropriate approach for the evaluation of the comparability between both assays. Independently on the cell line or expressing checkpoints, both assays technically detected comparable data (Figure 14A).

To statistically evaluate the comparability between Luciferase and CTV cytotoxicity assays, assessment for goodness-of-fit was performed. Linear regression analysis was calculated on the equation of the cytotoxicity measurements of both assays on Nalm6 cell lines (Nalm6 CD80, Nalm6 PD-L1 and wildtypes). Goodness of fit was assessed by the coefficient of correlation R^2 value of 0,8358 (Figure 14B).

For the evaluation of measurement precision, cytotoxicity analyses were performed three times under the same conditions for both assays. The mean variance coefficients (VC) for measurement precision was higher for the Luciferase cytotoxicity assay (7.78%) compared to the VC for the CTV cytotoxicity assay (3.66%). The VC of all evaluated measurements of both assays was within the standard acceptance of a variety coefficient of 25% (Table 8).

The method precision of both cytotoxicity assays was performed by evaluating the results of three experiments performed in replicates. The mean variance coefficients for measurement precision of the Luciferase cytotoxicity assay was 11.06% compared to the VC of the CTV cytotoxicity assay of 12.05%. The VC of both measurements was within the standard acceptance of a variety coefficient of 25% (Table 9).

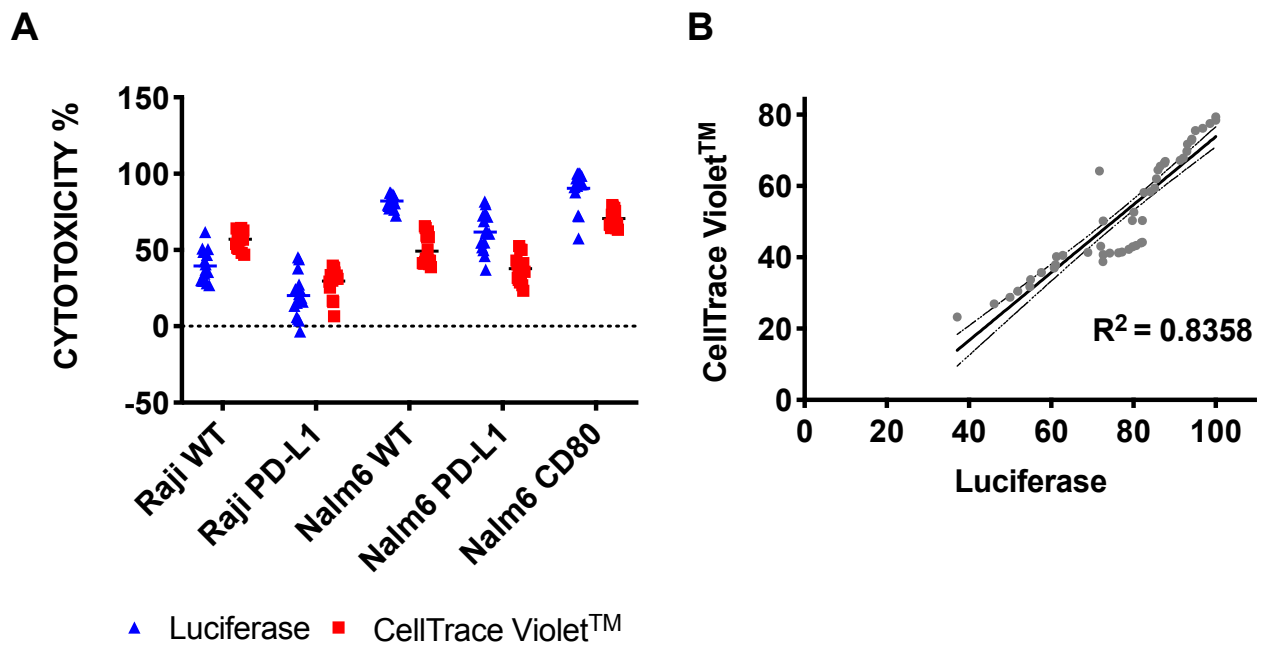


Figure 14: Comparison of Luciferase- and flow cytometry-based CellTrace Violet™ assay for detection of Blinatumomab-mediated cytotoxicity.

PBMCs were co-cultured with Nalm6 cells stably transfected with PD-L1 and CD80 and with Raji cells transfected with PD-L1 for 24 hours. Stimulation was performed with 500 pg/ml Blinatumomab. **(A)** Results of Luciferase and CTV cytotoxicity assays. Each dot represents one measurement performed on 3 different healthy donors in each 2 independent experiments. **(B)** Linear regression analysis of the results of CellTrace Violet™ and Luciferase cytotoxicity assays of PBMCs co-cultured with Nalm6 cells stably transfected with PD-L1 and CD80. Dashed lines represent the 90% confidence bands of the best-fit line.

Table 6: Measurement precision

Evaluation of measurement precision was performed by analyzing the same sample three times under the same conditions.

	Luciferase cytotoxicity assay					CellTrace Violet cytotoxicity assay			
	Donor	Cytotox. % of each measurement	Mean	S.D.	VC	Cytotox. % of each measurement	Mean	S.D.	VC
Nalm6 WT	I	85,97	85,79	0,26	**0,31**	41,26	42,01	1,76	**4,19**
		83,33				40,76			
		85,60				44,02			
	II	82,16	79,42	2,91	**3,66**	58,43	58,67	0,61	**1,04**
		79,72				59,37			
		76,37				58,21			
	III	77,21	79,53	2,71	**3,41**	65,57	64,01	1,82	**2,84**
		82,52				64,46			
		78,87				62,01			
Nalm6 PD-L1	I	60,96	51,03	12,40	**24,30**	30,48	28,74	1,77	**6,17**
		54,81				28,82			
		46,13				26,93			
	II	68,90	58,01	7,04	**12,14**	40,23	37,18	3,27	**8,79**
		72,58				37,59			
		60,69				33,73			
	III	61,03	78,70	3,96	**5,04**	76,20	51,03	1,40	**2,74**
		71,74				73,16			
		51,83				71,71			
Nalm6 CD80	I	92,11	85,94	11,56	**13,45**	78,45	73,69	2,29	**3,11**
		72,60				79,39			
		93,10				77,51			
	II	91,30	97,10	5,02	**5,17**	72,79	78,45	0,94	**1,20**
		100,00				75,55			
		100,00				71,51			
	III	100,00	97,29	2,51	**2,58**	76,20	73,29	2,07	**2,82**
		95,05				73,16			
		96,84				71,71			

Table 7: Method precision

Evaluation of method precision was performed by analyzing the cytotoxicity assays in replicates.

	Luciferase cytotoxicity assay					CellTrace Violet cytotoxicity assay			
	Donor	Cytotox. % of replicates	Mean	S.D.	VC	Cytotox. % of replicates	Mean	S.D.	VC
Nalm6 WT	I	68.02	80,07	10,44	**13,04**	42.01	39,06	3,12	**7,98**
		85.79				39.36			
		73.03				35.80			
	II	88.78	84,27	4,69	**5,57**	58.67	50,80	8,53	**16,79**
		79.42				41.74			
		79.53				51.98			
	III	77.21	80,07	10,44	**13,04**	64.01	53,96	9,58	**17,76**
		84.60				44.93			
		77.69				52.93			
Nalm6 PD-L1	I	51.03	59,95	15,93	**26,58**	28,87	31,08	3,94	**12,68**
		50.47				28,74			
		78.70				35,63			
	II	58.01	63,80	5,06	**7,93**	38,99	37,11	1,92	**5,18**
		67.39				37,18			
		61.53				35.15			
	III	61.03	69,50	8,65	**12,45**	56.82	49,11	8,83	**17,98**
		65.99				51.03			
		68.27				39.47			
Nalm6 CD80	I	92.60	89,60	8,35	**9,32**	64,86	69,28	6,24	**9,01**
		86.59				73,69			
	II	97.10	91,19	8,35	**9,16**	67.56	73,01	7,70	**10,55**
		85.28				78.45			
	III	97.29	95,62	2,37	**2,48**	66.34	46,54	4,91	**10,55**
		93.93				73,29			

5.5.2 Comparison of proliferation assays: CFSE versus CellTrace Violet™ T-cell proliferation assay

CFSE and CellTrace Violet™ (CTV) proliferation assays represent two flow cytometry-based approaches to analyze T-cell proliferation differing in the fluorescent dye labelling as described in the method section (3.5.1 and 3.5.2). To assess the comparability of both assays, CFSE or CTV labeled T cells were stimulated with SEB and were analyzed under identical conditions.

Both assays demonstrated a significantly enhanced T-cell proliferation over a period of 5 days. Detected by CFSE proliferation assay, SEB stimulation induced to a T-cell proliferation at day 3 and day 5 of 39,33% (±2,07%) and of 41,16% (±5,14%) compared to 0,78% (±0,64%) and 1,39% (±0,45%) in the unstimulated controls at the respective days ($P < 0,0001$ and $P = 0,0001$). Analyzed by CTV proliferation assay, SEB stimulation led to a T-cell proliferation of 30,37% (±7,68%) at day 3 and 38,97% (±0,87%) at day 5 compared to 0,31% (±0,41%) and 1,13% (±1,9%) respectively ($P = 0,0024$ and $P < 0,0001$; Figure 15A).

Cell proliferation detected by flow cytometry was assessed by measuring a decrease of fluorescence as the number of fluorescent molecules is constantly divided from one proliferating cell generation to another. Therefore, the quality of proliferation assessment depends on the stability of the fluorescent dye over the period of measurement. CellTrace Violet™ dye demonstrates in the representative histogram of unstimulated and SEB stimulated T-cell proliferation a more stable fluorescence quality compared to CFSE (Figure 15B).

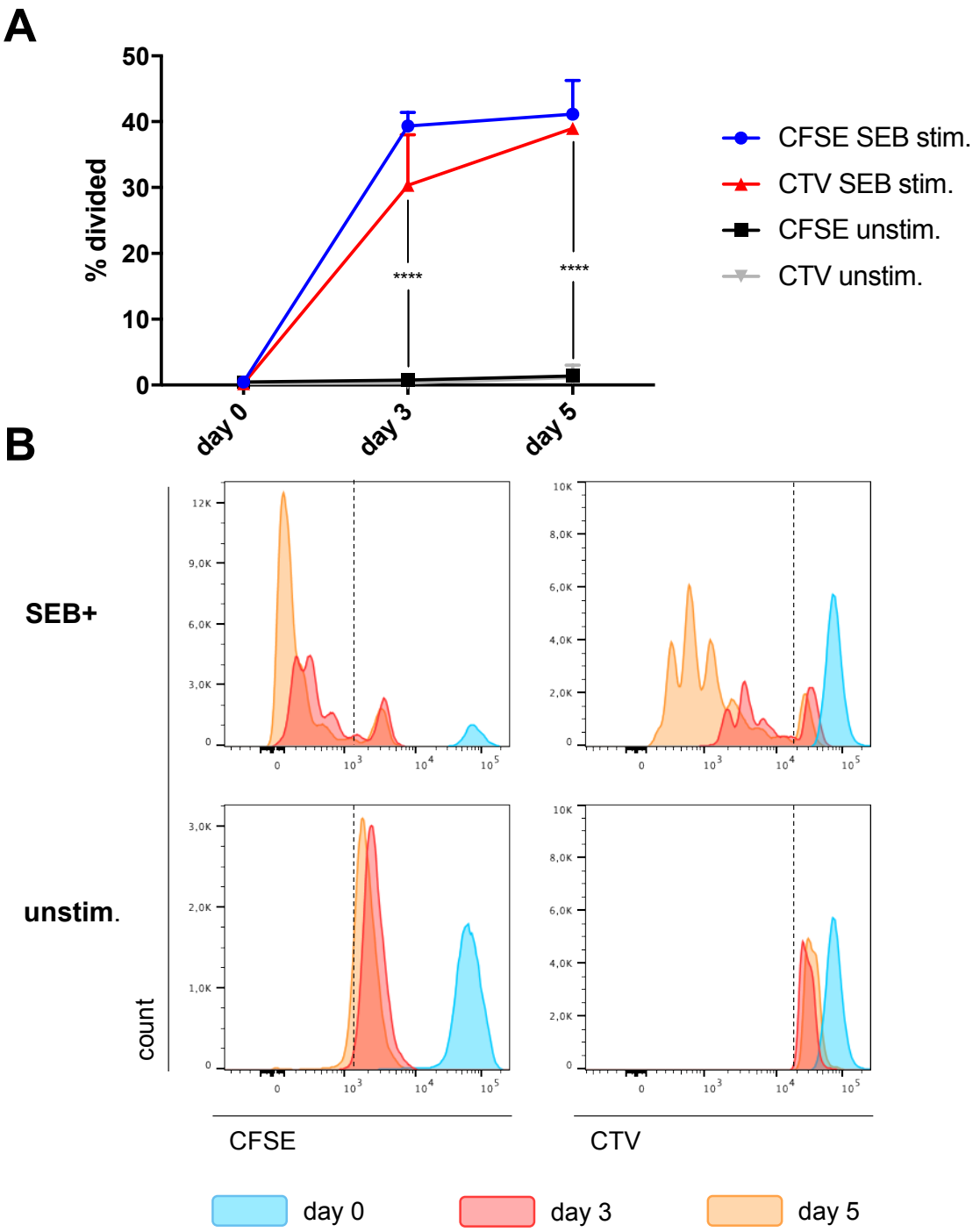


Figure 15: Comparison of flow cytometry-based analysis of T-cell proliferation detected by CFSE and CellTrace Violet.

PBMCs from healthy donors were stimulated with SEB and incubated for 5 days. **(A)** Flow cytometric analysis were performed after 0, 3 and 5 days. **(B)** Overlay histogram showing T-cell proliferation over period of 5 days. T-cell proliferation is described by percent divided cells defining the fraction of the origin population that divided at least once. Points represent mean values ± S.D. of 3 healthy donors. For each donor 3 independent experiments were performed in technical triplicates. ** $P \leq 0.01$ **** $P \leq 0.0001$.

6 Discussion

Relapse and non-responsiveness to standard chemotherapy remains a major cause of treatment failure in pediatric B-lineage acute lymphoblastic leukemia and is associated with dismal prognosis (Schrappe, Reiter et al. 2000). The bi-specific, CD3/CD19 targeting antibody construct Blinatumomab represents a novel immunotherapeutic approach to enhance endogenous T-cell driven anti-tumor activity against lymphoblastic cells (Gruen, Bommert et al. 2004). Clinical trials have highlighted the potential of Blinatumomab to improve the outcome of patients with refractory or relapsed ALL who would otherwise face particularly poor prognosis (Topp, Gokbuget et al. 2015, Barlev, Lin et al. 2017). Nevertheless, there is still a significant subset of patients who does not respond to Blinatumomab treatment and ultimately relapses.

Since the upregulation of inhibitory immune checkpoints on cancer cells represents a highly effective mechanism to undermine endogenous cancer immunosurveillance (Pardoll 2012), there is an urgent need to further investigate the immune-suppressive influence of inhibitory immune checkpoints on Blinatumomab-mediated anti-leukemic activity. Profound understanding on the influence of inhibitory immune checkpoints on Blinatumomab-mediated effects is pivotal in order to develop novel immunotherapeutic approaches to finally improve the outcome of children with relapsed and refractory B-lineage ALL.

In this study, firstly Blinatumomab-mediated effects on T-cell effector functions in the presence of lymphoblastic cells were evaluated. Furthermore, the influence of immune checkpoints on Blinatumomab-mediated effects on primary human T cells were investigated. The strong immune stimulatory effect of Blinatumomab on T-cell effector functions has been confirmed and further, it has been demonstrated that the expression of the stimulatory immune checkpoint CD80 and the inhibitory checkpoint PD‑L1 contrarily influenced Blinatumomab-mediated T-cell functions including target cells lysis, T-cell proliferation and cytokine production.

6.1 Specification of leukemia model and Blinatumomab concentration

To most closely mimic the Blinatumomab-exerted influence on T-cell effector functions in the setting of childhood B-lineage ALL, co-cultures of primary human PBMCs with lymphoblastic cell lines Nalm6 or Raji were performed.

Nalm6 and Raji cell lines were used as model for malignant lymphoblastic cells. Nalm6 cells represent B-cell precursor ALL cells which originate from the peripheral blood of a male patient with relapsed B-lineage ALL. Raji cell line was established from a patient with Burkitt lymphoma and was used as a model of mature B-cell ALL. Nalm6 and Raji cell lines are frequently used by numerous study groups for experiments on ALL (Dreier, Lorenczewski et al. 2002, Hoffmann, Hofmeister et al. 2005, Brandl, Haas et al. 2007, Feucht, Kayser et al. 2016). Since Raji and Nalm6 highly express CD19, these cell lines were used as targets in a co-culture model to investigate Blinatumomab-mediated effects *in vitro*.

In this study, PBMCs rather than purified T cells were used to most closely mimic the actual situation in the peripheral blood of leukemia patients during Blinatumomab treatment. Secondary effects by other co-stimulated cells, for instance natural killer cells, B cells, monocytes and macrophages, are therefore captured as well. Consequently, target cells for Blinatumomab were a mix of normal and lymphoblastic B cells. Dreier and colleagues showed that healthy and malignant B cells are equally susceptible to Blinatumomab-exerted target cells lysis (Dreier, Lorenczewski et al. 2002).

Compared to previously published studies, Blinatumomab stimulation was performed in a relatively low antibody concentration. A significant Blinatumomab-mediated cytotoxic activity against lymphoblastic cell lines at concentrations of 10 to 100 pg/ml was demonstrated by Loffler and colleagues (Loffler, Kufer et al. 2000). Moreover, an ongoing phase I clinical study demonstrated that a constant serum Blinatumomab level of 500 to 600 ng/ml induced partial and complete tumor response in refractory non-Hodgkin lymphoma patients (Bargou 2006). In accordance with the current clinical application of Blinatumomab, Blinatumomab stimulation was performed in a concentration of 500pg/ml within this study.

6.2 Blinatumomab-mediated effects on T-cell effector functions

6.2.1 Blinatumomab stimulation increases the cytotoxic activity of human T cells against lymphoblastic cells

To assess Blinatumomab-mediated cytolytic capacity, cytotoxicity assays were performed on co-cultures of primary human PBMCs and lymphoblastic cell lines in the presence of Blinatumomab. Blinatumomab stimulation induced a significantly enhanced target cells lysis. Furthermore, Blinatumomab-induced target cell killing was effector to target cell dependent.

The findings brought out by this study are consistent with different studies demonstrating Blinatumomab as a potent stimulus for T cell-exerted target cells lysis. Hoffman and colleagues showed that $CD8^+$ cytotoxic T cell clones induced complete target cells lysis in an effector to target ratio of 1/5 (Hoffmann, Hofmeister et al. 2005). Another group demonstrated complete target cells lysis by purified T cells at lower effector to target ratios ranging from 1/20 to 1/5 (Dreier, Lorenczewski et al. 2002). Since Blinatumomab-mediated cytolytic effects are mainly exerted by $CD8^+$ cytotoxic T cells (Dreier, Lorenczewski et al. 2002), the use of $CD8^+$ cell clones or purified T cells might explain the complete target cell lysis at these low effector to target ratios. Furthermore, in these studies Blinatumomab stimulation was used in a considerably higher concentration compared to the one used in the present study. Loffler and colleagues stimulated their co-cultures in the same antibody concentration as used in the present study (Loffler, Kufer et al. 2000). Remarkably, the results on effector to target cell-dependent cytotoxicity revealed by Loffler et al. are consistent with the findings of this study.

Despite varying cell lines, Blinatumomab concentrations and cytotoxicity assays, the findings of this study confirm previously published results.

Taking together, this study confirms that Blinatumomab represents a potent stimulus of T cell exerted target cells lysis against leukemic cells.

6.2.2 Blinatumomab stimulation increases cell proliferation of human T cells

Blinatumomab-mediated effects on T-cell proliferation were assessed by performing flow cytometry-based cell proliferation assays on primary human T cells in co-culture with lymphoblastic cells stimulated with Blinatumomab. Blinatumomab stimulation significantly increased T-cell proliferation in the presence of lymphoblastic cells.

Previously published studies on Blinatumomab-mediated effects on T-cell proliferation substantially corroborate the results brought out by this study. Haagen and colleagues demonstrated the ability of the CD19/CD3 bi-specific antibody construct to initiate T-cell proliferation. T-cell expansion was observed for healthy donors as well as non-Hodgkin's lymphoma patients upon stimulation with Blinatumomab within 3 days (Haagen, de Lau et al. 1994). These findings were further confirmed by Loffler and colleagues. This study group observed Blinatumomab enhanced T-cell proliferation in the presence of autologous B cells, while upon depletion of B cells almost no T-cell expansion was exerted (Loffler, Kufer et al. 2000). In the presence of lymphoblastic cell lines the same Blinatumomab construct, as used in the present study, induced a strong expansion of primary human T cells within 5 days (Brandl, Haas et al. 2007). The antibody concentrations used in these previously published studies was considerably higher than the one used in the present study. The present study thus reveals that Blinatumomab is even able to enhance T-cell proliferation at lower antibody concentrations being equivalent to serum concentrations after clinical application.

Although several trials outlined Blinatumomab as a potent stimulus for T-cell proliferation in general, evaluation of cell proliferation of T-cell subsets is currently missing. In this study Blinatumomab stimulation led to a significantly increased proliferation of $CD8^+$ T cells compared to $CD4^+$ T cells. This finding is in accordance with the fact that Blinatumomab-mediated target cell lysis is predominantly exerted by $CD8^+$ effector cells (Hoffmann, Hofmeister et al. 2005). A recent study by Smith et al. postulated that the threshold for initiation the expansion of T-cell subpopulations is contrarily affected by mitogenic cytokines (IL-2) and TCR signaling. $CD8^+$ cell proliferation is positively influenced by co-stimulatory environmental influences while $CD4^+$ cell proliferation is independent of antigen affinity, abundance and mitogenic cytokines (Au-Yeung, Smith et al. 2017). Since Blinatumomab-stimulation induces a strong secretion of IFNγ and TNFα (see 6.2.3.) this study suggests that pro-inflammatory cytokines similarly influence the proliferation of T-cell subpopulations.

Notably, Blinatumomab-mediated enhanced T-cell proliferation appears to be beneficial for the clinical application of the bi-specific antibody. Specific target cell lysis is crucial to eliminate leukemic blast. Blinatumomab-stimulated T-cell expansion increases the effector to target ratio and consequently counteracts T-cell apoptosis which is crucial to pursue target cell killing.

The findings of this study are consistent with previously published studies confirming Blinatumomab as a potent stimulus for T-cell proliferation in the presence of lymphoblastic cells and show a distinct influence of Blinatumomab on the expansion of T-cell subpopulations.

6.2.3 Blinatumomab stimulation increases production of pro-inflammatory cytokines of primary T cells

The evaluation of Blinatumomab-mediated cytokine production was performed on primary human PBMCs in presence of lymphoblastic cells stimulated with Blinatumomab. Blinatumomab acted as a strong T-cell stimulus to produce pro-inflammatory Th_1 cytokines (IFNγ and TNFα) while the production of anti-inflammatory Th_2 cytokines (IL-4 and IL-10) was not affected upon Blinatumomab stimulation. The dominance of pro-inflammatory cytokines provides vital evidence of the strong immune stimulatory effect of Blinatumomab.

Under inflammatory conditions, pro-inflammatory cytokines are crucial to modulate and activate T-cell effector functions. IFNγ and TNFα secretion is predominantly exerted by $CD4^+$ helper T cells (Th_2 cells), $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD56^+$ natural killer cells (Bruggemann, Schrauder et al.). For this study, $CD56^+$ NK cells were excluded in flow cytometric analyses. Consequently, increased IFNγ and TNFα production was predominantly driven by CTLs. This observation is further consistent with the findings of Dreier and colleagues who demonstrated that Blinatumomab-mediated target cell killing is mainly driven by $CD8^+$ cytotoxic T cells (Dreier, Lorenczewski et al. 2002).

Secretion of IFNγ and TNFα by T cells results in an increased activation of effector T cells which consequently induces granzyme- and perforin-driven apoptosis of tumor cells. Moreover, IFNγ is potentially able to directly enhance apoptotic signaling in tumor cells by induction of a subset of genes, called IFN stimulated genes, which modulate pro-apoptotic pathways in cancer cells. (Chawla-Sarkar, Lindner et al. 2003). Since Blinatumomab-mediated target cell killing is mainly exerted by secretion of granzymes and perforin (Gruen, Bommert et al. 2004), the enhanced

pro-apoptotic signaling in cancer cells induced by IFNγ may additionally contribute to Blinatumomab-mediated cell lysis.

Contrasted by the strong stimulatory effect of Blinatumomab on pro-inflammatory cytokine production, Blinatumomab did not considerably affect the production of Th_2 cytokines (IL-4 and IL-10). IL-10 functions as an inhibitor of pro-inflammatory cytokine production and Th_1 cell activity. Thus, low IL-10 levels concur well the dominance of Blinatumomab-mediated immune stimulation. Anti-inflammatory cytokines are predominantly secreted by $CD4^+$ Th_2 cells. Consequently, the lack of IL-4 and IL-10 production contrasted by elevated levels of IFNγ and TNFα indicate that Blinatumomab may predominantly affects $CD4^+$ Th_1 cells and CTLs.

The results on Blinatumomab-mediated cytokine production are partly consistent with the study of Brandl et al. who observed Blinatumomab-induced cytokine release of both Th_1 (IFNγ, TNFα) and Th_2 (IL-4, IL-10) cytokines (Brandl, Haas et al. 2007). Notably, Brandl and colleagues used twice the Blinatumomab concentration than used in the present study. Since it was shown that such high Blinatumomab concentration has stronger effects on target cells lysis (Dreier, Lorenczewski et al. 2002), it might be also coherent with additionally enhanced levels of Th_2 cytokines.

Since enhanced target cell lysis and T-cell proliferation are necessary for an efficient anti-leukemic immune response, one should consider a dual role of cytokine secretion in the setting of leukemia patients treated with Blinatumomab. It was previously reported that leukemic blast T cells upregulate the inhibitory immune checkpoint PD-L1 as a consequence of constant IFNγ and TNFα stimulation (Feucht, Kayser et al. 2016). Furthermore, Kronig and colleagues observed an enhanced PD-L1 upregulation on leukemic blasts in AML patients during chemotherapy due to constantly high IFNγ levels (Kronig, Kremmler et al. 2014). Thus, Blinatumomab-mediated production of pro-inflammatory cytokines potentially promotes the upregulation of inhibitory molecules on leukemic blasts, which consequently may give rise to treatment failure.

Furthermore, co-medication of Blinatumomab with glucocorticoids might inhibit the upregulation of inhibitory immune checkpoints via suppression of cytokine release. Brandl and colleagues reported that dexamethasone considerable reduced Blinatumomab-induced Th_1 and Th_2 cytokine production (Brandl, Haas et al. 2007). Most interestingly, dexamethasone did not inhibit the cytolytic capacity of Blinatumomab-stimulated T cells against human lymphoblastic

cells. Consequently, combinatory treatment with glucocorticoids potentially ameliorates the responsiveness to Blinatumomab treatment.

While cytokines play a pivotal role in modulating T-cell effector functions and recruitment of other kinds of immune cells, aberrantly enhanced levels of cytokines, also known as cytokine release syndrome (CRS), represent a most serious adverse event due to Blinatumomab application. CRS is a potentially life threatening systemic inflammatory immune response characterized by the elevation of IL-6, IL-10 and IFNγ (Klinger, Brandl et al. 2012).

Interestingly, the same cytokine pattern is pathologically elevated in hemophagocytic lymphohistiocytosis (HLH), also referred to as macrophage activation syndrome (Kantarjian, Stein et al.) (Gokbuget, Kneba et al. , Xu, Tang et al. 2012). HLH/MAS is characterized by abnormally increased macrophage activity leading to an inappropriate immune activation and cytokine release. Teachey et al. hypothesized that high levels of pro-inflammatory cytokines (CRS) induced by Blinatumomab might initiate aberrantly increased macrophage activation and subsequently promote HLH/MAS (Teachey, Rheingold et al. 2013). The findings on Blinatumomab-mediated cytokine production revealed within this study support this hypothesis. IL-10 is an important inhibitor of macrophage activity and is therefore highly released by T cells to dampen abnormally activated macrophages in HLH/MAS. An increased IL-10 production might therefore suggest abnormal macrophage activity. In the present study, Blinatumomab-induced an enhanced production of IFNγ, while leaving IL-10 production unaffected. The distinct constellation of high IFNγ and low IL-10 levels might therefore indicate a strong immune stimulatory effect and an appropriate macrophage function. This effect was observed for PBMCs from healthy donors without any macrophage dysfunction. Consequently, the results brought out by this study can support the hypothesis that high levels of pro-inflammatory cytokines induced by Blinatumomab potentially initiate abnormally enhanced macrophage activation and therefore might promote HLH/MAS.

6.2.4 Blinatumomab induces upregulation of immune checkpoints on T cells in the presence of lymphoblastic cells

This study confirmed that Blinatumomab significantly enhances several T-cell effector functions. Blinatumomab stimulation constantly activates T cells in the presence of lymphoblastic cells. However, as a consequence of permanently mediated T-cell stimulation, Blinatumomab might

also promote T-cell exhaustion. T-cell exhaustion is a state of T-cell dysfunction which is characterized by poor effector functions and the upregulation of several exhaustion markers including PD-1 (Wherry and Kurachi 2015).

To investigate whether Blinatumomab is able to initiate T-cell exhaustion, flow cytometric analyses on PD-1 expression of Blinatumomab-stimulated PBMCs in the presence with lymphoblastic cells were performed.

Blinatumomab-mediated recognition of lymphoblastic target cells led to considerably increased expression of PD‑1 on T cells. Interestingly, Blinatumomab-induced PD‑1 expression was higher on T cells in the presence of Raji cells compared to T cells co-cultured with Nalm6 cells. Raji cells express various additional stimulatory immune checkpoints and therefore trigger a stronger T-cell response (see 3.1). Consequently, Raji cells are potentially more susceptible to Blinatumomab stimulation compared to Nalm6 cells lacking further expression of stimulatory molecules. The influence of these different expression pattern of stimulatory immune checkpoints might explain a higher PD-1 expression on T cells interacting with Raji cells.

6.3 Influence of immune checkpoints on Blinatumomab-mediated effects on T-cell effector functions

The upregulation of inhibitory immune checkpoints on cancer cells represents a highly effective mechanism to undermine endogenous cancer immunosurveillance (Pardoll 2012). Since this study confirmed Blinatumomab as a potent stimulus to enhance anti-leukemic activity of primary T cells, it is of particular importance to provide deeper insights into the influence of immune checkpoints on Blinatumomab-mediated anti-leukemic effects.

The evaluation of the influence of lymphoblastic CD80 and PD‑L1 expression on Blinatumomab-mediated T-cell effector functions (target cell lysis, cell proliferation and cytokine production) was assessed on primary human T cells stimulated with Blinatumomab in the presence of lymphoblastic cells stably transfected with either the stimulatory checkpoint CD80 or the inhibitory molecule PD‑L1.

6.3.1 Expression of CD80 and PD-L1 influences Blinatumomab-mediated cytotoxic activity of human T cells

Evaluation of the influence of these immune checkpoints revealed that CD80 expression on Nalm6 cells enhanced Blinatumomab-exerted target cells lysis. PD-L1 expression on lymphoblastic cell lines significantly reduced Blinatumomab-mediated T cell cytotoxicity in Raji cells stably transfected with the inhibitory immune checkpoint.

Since Raji cells constantly express various additional co-stimulatory molecules, including HVEM, CD80, CD86, CD27, CD40, CD70, they trigger a stronger stimulatory effect on T-cell function compared to Nalm6 cells (see 3.1). Remarkably, the expression of one single inhibitory immune checkpoint, PD-L1, predominated the effect of numerous stimulatory immune checkpoints and sufficiently initiated a considerable reduction of Blinatumomab-mediated T-cell cytotoxicity. This finding demonstrates the high immune suppressive influence of PD-L1 as a frequently misemployed immune evasive mechanism of cancer cells. Furthermore, these results provide evidence that the overexpression of one single stimulatory or inhibitory immune checkpoint can substantially influence the Blinatumomab-mediated effects on T-cell effector functions.

The results brought out by this study are in good agreement with Feucht et al.'s findings on the influence of immune checkpoints on Blinatumomab-mediated effects (Feucht, Kayser et al. 2016). This study was performed under similar experimental conditions using the same transfected cell lines. Feucht and colleagues outlined CD80 and PD-L1 expression as effective co-variables with potent stimulatory and inhibitory effects on Blinatumomab-induced cytolytic capacity which is well consistent with the results of the present study.

This study provided evidence of CD80 expression on leukemic cells to enhance Blinatumomab-induced cytotoxic capacity of T cells against lymphoblastic cells. Furthermore, the results brought out by this study outline PD-L1 upregulation as a highly potent cancer immune evasive mechanism able to considerably attenuate Blinatumomab-mediated cytotoxic activity against leukemia cells.

6.3.2 Expression of CD80 and PD-L1 contrarily influence Blinatumomab-mediated T-cell proliferation

Results brought out by this study showed that CD80 expression on Nalm6 cells enhanced Blinatumomab-induced T-cell proliferation. PD-L1 expression on Raji cells led to a significant suppression of Blinatumomab-mediated T-cell proliferation in Raji cells stably transfected with the inhibitory immune checkpoint.

As described above, Raji cells expressing numerous additional stimulatory immune checkpoints are more immunogenic compared to Nalm6 cells with predominantly absent co-stimulatory molecules. The presence of stimulatory checkpoints substantiates the higher susceptibility of Raji cells to Blinatumomab-induced T-cell proliferation in comparison to Nalm6 cells. This finding is consistent with previously published work of Feucht and colleagues who observed that lymphoblastic target cells expressing stimulatory molecules are more susceptible to T-cell attack compared to less immunogenic cell lines (Feucht, Kayser et al. 2016).

The ability of PD-L1 expression to diminish the stimulatory effect of numerous co-stimulatory checkpoints impressively demonstrates PD-L1 up-regulation as an effective immune evasive mechanism to attenuate Blinatumomab-mediated T-cell expansion. In the setting of peripheral blood of leukemia patients, Blinatumomab-induced T-cell proliferation increases the ratio of T cells to leukemic blasts favoring T cell-mediated anti-leukemic activity. Suppression of T-cell proliferation via target cell dependent PD-L1/PD-1 interaction is thus potentially able to diminish Blinatumomab-mediated anti-leukemic effects and therefore potentially able to induce treatment failure. The inhibitory effect of PD-L1 expression on lymphoblastic cells was observed on Blinatumomab-induced target cells lysis as well. Hence the findings demonstrate the immune suppressive effect of PD-L1/PD-1 interaction on Blinatumomab-mediated T-cell effector functions.

In contrast, CD80 expression on lymphoblastic cells drastically enhanced Blinatumomab-mediated T-cell proliferation. It was previously reported that CD80 expression can initiate and maintain $CD4^+$ T-cell proliferation upon phytohaemagglutinin (PHA) stimulation (Vasilevko, Ghochikyan et al. 2002). Additionally, the stimulatory effect of CD80 expression on Blinatumomab-mediated T-cell activity was confirmed in cytotoxicity assays performed within this study. The strong conformity of cytotoxicity and proliferation results within this study

provides evidence that target cell related co-stimulatory signals positively determine Blinatumomab-mediated effects on T-cell effector functions.

Interestingly, the expression of CD80 on lymphoblastic cells distinctly influenced the proliferation of T-cell subpopulations. CD80 expression considerably enhanced $CD4^+$ cell proliferation, while leaving $CD8^+$ cell expansion unaffected.

It was recently reported by Chan and colleagues that the inhibitory molecule CTLA-4 is considerably higher expressed on activated $CD4^+$ T cells compared to $CD8^+$ T cells (Chan, Gibson et al. 2014). The influence of CTLA-4 expression on T-cell proliferation was demonstrated as CTLA-4 blockade led to enhanced $CD4^+$ cell expansion. Furthermore, a recent study showed that Blinatumomab-induced recognition of lymphoblastic cells initiates CTLA-4 expression on T cells (Feucht, Kayser et al. 2016). CTLA-4 expression is initiated after T-cell activation leading to decreased expression of CD28 and attenuated T-cell effector function (Chen and Flies 2013). Moreover, CTLA-4 can remove CD80 from the surface of antigen-presenting cells through trans-endocytosis (Qureshi, Zheng et al. 2011). The co-stimulatory CD28 and the co-inhibitory CTLA-4 share the same ligand CD80. Consequently, there is an intrinsic competition between CD28 and CTLA-4 molecules for the binding of CD80. Since CD80 expression on target cells resulted in an enhanced $CD4^+$ proliferation in this study, the co-stimulatory effect upon Blinatumomab stimulation might outperform the inhibitory influence of CTLA-4 expression.

Taken together, the results brought out by this study highlight lymphoblastic PD-L1 and CD80 expression as potent co-variables able to contrarily regulate Blinatumomab-mediated T-cell proliferation.

Furthermore, this study showed a diverse effect of Blinatumomab-mediated proliferation of T-cell subpopulations favoring $CD8^+$ cell proliferation. However, CD80 expression on target cells diminished this effect resulting in an enhanced proliferation of $CD4^+$ T cells. Analyses on Blinatumomab-mediated cytotoxicity showed that CD80 expression on target cells significantly enhanced the elimination of lymphoblastic cells. Since CD80 expression also induces an increased $CD4^+$ cell proliferation leaving $CD8^+$ cell proliferation unaffected, $CD4^+$ T cells might play a pivotal role in elimination of lymphoblastic tumor cells. Recent studies mainly focused on the evaluation cytotoxic capacity of $CD8^+$ T cells paying less attention to $CD4^+$ T cells. However, the results brought out by is study propose a major role of $CD4^+$ cells in cancer cell elimination, especially in the setting of Blinatumomab treatment.

6.3.3 Expression of CD80 and PD-L1 contrarily influence Blinatumomab-mediated production of pro-inflammatory Th_1 cytokines

To determine the influence of immune checkpoints on Blinatumomab-mediated cytokine production, intracellular cytokine production was assessed on Blinatumomab-stimulated T cells in the presence of lymphoblastic cells stably transfected with either CD80 or PD-L1. CD80 expression on target cells further increased Blinatumomab-mediated induction of IFNγ and TNFα production in T cells. In contrast, T cells diminished Blinatumomab-mediated TNFα induction in the presence of lymphoblastic cells stably transfected with PD-L1 while IFNγ production remained unaffected. Independent on immune checkpoint expression of target cells, Blinatumomab did not detectably induce anti-inflammatory Th_2 cytokine production.

Co-stimulatory signaling via the CD80/CD28 axis enhances T-cell effector functions (Chen and Flies 2013). This study confirmed CD80 expression as a potent co-stimulus for target cell lysis and T-cell proliferation upon Blinatumomab stimulation. CD80 expression leading to a significantly increased Blinatumomab-mediated production of pro-inflammatory cytokines concurs therefore well with the results brought out by this study so far. These findings further emphasize CD80 expression on leukemic blasts as a potentially favorable prognostic factor for response to Blinatumomab treatment. Even though CD80 expression on leukemic cells is able to augment Blinatumomab-exerted anti-tumor activity, it's immune stimulatory capacity might also trigger aberrantly elevated cytokine levels during Blinatumomab treatment leading to CRS or HLH/MAS (Teachey, Rheingold et al. 2013).

Furthermore, PD-L1 is frequently upregulated on leukemic blasts as a consequence to permanent IFNγ stimulation (Feucht, Kayser et al. 2016). Consequently, high level of IFNγ might initiate inhibitory immune checkpoint upregulation and promote T-cell exhaustion in the course of Blinatumomab treatment.

Persistent exposure to antigens and inflammatory signals frequently leads to a state of dysfunctional T-cell function, also referred to as T cell exhaustion (Wherry and Kurachi 2015). T-cell exhaustion is characterized by diminished effector function and expression of various inhibitory signals, including PD-1, BTLA, CTLA-4, LAG-3, and Tim-3 (Blackburn, Shin et al. 2009, Fourcade, Sun et al. 2012, Woo, Turnis et al. 2012). PD-L1/PD-1 interaction represents a pivotal role as regulator of T-cell exhaustion responsible for an attenuated production of pro-inflammatory cytokines (Li, Jie et al. 2015). In chronic viral infection T-cell exhaustion occurs in a

hierarchical manner. At early stages cytolytic capacity, T-cell proliferation and IL-2 production appears to be lost followed by a deficiency of TNFα production. Finally, at severe stages of T-cell exhaustion, the ability of IFNγ production is extinguished (Shin and Wherry 2007). Interestingly, in this study PD-L1 expression lead to a diminished production of TNFα while leaving IFNγ production unaffected. This can be attributed to a state of T-cell exhaustion with most effector functions being compromised while the ability of IFNγ production is still preserved. Therefore, these results not only outline PD-L1 expression as a possible trigger for T-cell dysfunction during Blinatumomab treatment, but also emphasize the hypothesis of a hierarchically conducted T-cell exhaustion in the setting of pediatric leukemia.

These observations are furthermore consistent with the study of Kong and colleagues who investigated the functional status of exhausted T cells deriving from relapsed AML patients (Kong, Zhang et al. 2015). T cells were classified as exhausted by high expression of PD-1 and Tim3. Following CD3/CD28 stimulation, exhausted T cells produced extremely low levels of IL-2, TNFα and IFNγ.

In conclusion, present results provide evidence of the stimulatory and inhibitory effect of lymphoblastic CD80 and PD-L1 expression on Blinatumomab-induced production of pro-inflammatory cytokines.

6.4 Comparison of assays for the evaluation of cytotoxicity and T-cell proliferation

6.4.1 Comparison of cytotoxicity assays: Luciferase- versus CellTrace Violet™ assay

In this study, T cell mediated target cell lysis upon Blinatumomab stimulation was assessed by two cytotoxicity assays which differed fundamentally in their technical mode of operation. This study demonstrated comparable results on the evaluation of T-cell cytotoxicity in direct comparison of luciferase-based and CTV cytotoxicity assays.

The luciferase cytotoxicity assay basically measures luminescence emitted from luciferase transduced viable target cells. After administration of substrate light is emitted by luciferase mediated enzymatic reactions of viable cells. Since luciferase activity is ATP-dependent, cytotoxicity evaluation relies on decreased luciferase activity by depletion of intracellular ATP of a dying cell (Karimi, Lee et al. 2014). The luminescence emission is directly proportional to the viability of the target cells (Crouch, Kozlowski et al. 1993). Hence, luciferase cytotoxicity assay

provides fundamental information on the integrity of the biological functionality of the target cells. Since ATP-dependent luminescence emission relies on the metabolic activity of target cells, it is not possible to distinguish between cytostatic and cytotoxic effects of drugs (Adan, Kiraz et al. 2016).

In contrast to the luciferase-based cytotoxicity assay, T-cell cytotoxicity was assessed performing flow cytometric analyses. Flow cytometry-based cytotoxicity assays basically detect previously labeled, viable target cells. Uptake of the DNA intercalating fluorescent agent propidium iodide (Bruggemann, Schrauder et al.) due to increased cell membrane permeability of dying target cells leads to a bright signal which allows a very specific differentiation between dead and viable cells. To further distinguish living target from effector cells, target cells have been labeled with a bright fluorescent dye (CellTrace Violet) prior to the measurement. Flow cytometry-based cytotoxicity assays measure target cells lysis regardless to the metabolic activity of the target cells and allow cytotoxicity evaluation on the single cell level. Furthermore flow cytometry offers the possibility to determine the phenotype of involved cells (Zaritskaya, Shurin et al. 2010). In contrast to luciferase cytotoxicity assay, this assay does not depend on the biological functionality of the target cells.

Despite varying mode of actions, this study revealed that both, the luciferase as well as the flow cytometry-based cytotoxicity assays, are appropriate and sensitive approaches for the evaluation of T cell mediated target cells lysis.

6.4.2 Comparison of proliferation assays: CFSE versus CellTrace Violet™ cell assay

T-cell proliferation was assessed by two flow cytometry-based cell proliferation assays which basically differed in the fluorochromes used for labeling cells of interest. This study provided comparable results on T-cell proliferation in direct comparison of CFSE and CTV cell proliferation assays. Furthermore, CTV was demonstrated to have more stable fluorescence properties compared to CFSE.

CFSE cell proliferation assay is widely used for investigating cell proliferation. Numerous publications are published using CFSE cell proliferation assay to assess lymphocyte proliferation (Fulcher and Wong 1999, Hilchey and Bernstein 2007, Venken, Thewissen et al. 2007). Yet, in this study working with GFP transduced targets cells as indispensable part of co-culture experiments represented a challenging task because GFP and CFSE share the same spectral properties and are therefore both detected by the same fluorescein filter (FITC) (Lyons 2000,

Walker, Lukyanov et al. 2015). Furthermore, CFSE is a highly bright fluorescent dye with strong spectral overlap in various other filters.

Therefore, the fluorescent dye CellTrace Violet™ (CTV) was used to assess T-cell proliferation. While CTV is chemically related to CFSE, it differs in excitation and emission spectra to CFSE and consequently is compatible with GFP. Parish and colleagues describe CTV in direct comparison with CFSE as a novel alternative dye for evaluating cell proliferation (Quah and Parish 2012). CTV can resolve cell divisions to a comparable level as CFSE with the advantage of being compatible with fluorochromes detected in the FITC channel, such as GFP.

Results on the comparison of CFSE and CTV cell proliferation assays suggest CTV as a novel and attractive fluorescent dye which can be clearly used for the evaluation of T-cell proliferation. CTV therefore represents a promising alternative to standard CFSE cell proliferation assay.

6.5 Outlook

First, regarding the small sample size of three individuals, caution must be applied when generalizing the result brought out by this study. Further research including results of *ex vivo* or *in vivo* experiments will allow a transfer of the present findings into clinical application of pediatric ALL patients receiving Blinatumomab.

Furthermore, the present work focused on the two most prominent inhibitory and stimulatory immune checkpoints. Since the role of immune checkpoints in cancer is not yet fully understood, possible interactions between various other immune checkpoints and the ones investigated in the present study still need to be investigated.

Finally, the present study evaluates phenotypic functionality of T cells. Further evaluation on the influence of immune checkpoints and Blinatumomab on gene expression and intracellular signaling pathways will provide fundamental insights into molecular mechanism of T-cell response to the respective conditions.

6.6 Conclusion

In summary, this study *in vitro* examined the influence of stimulatory and inhibitory immune checkpoint expression of lymphoblastic cells on Blinatumomab-mediated T-cell functions in terms of specific target cell lysis, T-cell proliferation and cytokine production.

As conclusion, the study demonstrated that the upregulation of immune checkpoints by lymphoblastic cells substantially influences Blinatumomab-induced anti-leukemic T-cell effector functions.

The results suggest, that inhibitory signaling *via* PD-1/PD-L1 interactions or the loss of co-stimulation through CD80 represent highly potent cancer immune evasive mechanisms with the ability to induce *in vivo* resistance to Blinatumomab treatment. Combinatory therapy approaches with PD-1/PD-L1 blocking antibodies might therefore represent a novel and highly promising therapeutic strategy which may open the possibility to further improve the outcome of children with relapsed/refractory B-lineage ALL.

7 List of figures

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9 List of abbreviations

AEs	adverse events
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
BiTE	bi-specific T cell engager
BTLA	B and T lymphocyte attenuator
CAR	chimeric antigen receptor
CD	cluster of differ
CFSE	carboxyfluorescein succinimidyl ester
CLL	chronic lymphoblastic leukemia
CNS	central nervous system
COG	Children's Oncology Group
CR	complete remission
CRS	cytokine release syndrome
CTLA-4	cytotoxic T-lymphocyte-associated Protein 4
CTV	CellTrace Violet
DAMP	damage-associated molecular pattern molecule
DC	dendritic cells
EFS	event-free-survival
EGIL	European Group for the Immunological Characterization of Leukemia
EMA	European Medicines Agency
FAB	French-American-British
FDA	US Food and Drug Administration
FSC	forward scatter
GFP	green fluorescent protein
HLA	human leukocyte antigen
HSA	human serum albumin
HSCT	allogenic hematopoietic stem cell transplant
HVEM	herpesvirus entry mediator
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin

JAK	Janus kinases
LAG3	lymphocyte-activation protein 3
mAB	monoclonal antibody
MDSC	myeloid-derived suppressor cell
MHC	major histocompability complex
MLL	mixed lineage leukemia
MRD	manual residual disease
MT103	Blinatumomab
Mϕ	macrophages
NHL	Non-Hodgkin Lymphoma
NK	natural killer
OS	overall survival
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD-1	programmed death 1
PD-L1	programmed death 1 ligand one
Ph	Philadelphia translocation
r/r	refractory or relapsed
scFv	single-chain variable fragment
SSC	side scatter
STAT5	signal transducer and activator of transcription 5
TAA	tumor-associated antigen
TCR	T-cell receptor
TGF-β	transforming growth factor-β
Th	T-helper cell
TIM3	T-cell immunoglobulin and mucin domain 3
TNF	tumor necrosis factor
T_{Reg} cell	regulatory T-cell
VEGF	vascular endothelial growth factor
WBC	white blood cell count
WHO	World Health Organization
WT	wildtype

10 References

Adan, A., Y. Kiraz and Y. Baran (2016). "Cell Proliferation and Cytotoxicity Assays." Curr Pharm Biotechnol **17**(14): 1213-1221.

Aldoss, I., R. C. Bargou, D. Nagorsen, G. R. Friberg, P. A. Baeuerle and S. J. Forman (2017). "Redirecting T cells to eradicate B-cell acute lymphoblastic leukemia: bispecific T-cell engagers and chimeric antigen receptors." Leukemia **31**(4): 777-787.

Amor-Guéret, M. (2006). "Bloom syndrome, genomic instability and cancer: the SOS-like hypothesis." Cancer Lett **236**(1): 1-12.

Ansell, S. M., A. M. Lesokhin, I. Borrello, A. Halwani, E. C. Scott, M. Gutierrez, S. J. Schuster, M. M. Millenson, D. Cattry, G. J. Freeman, S. J. Rodig, B. Chapuy, A. H. Ligon, L. Zhu, J. F. Grosso, S. Y. Kim, J. M. Timmerman, M. A. Shipp and P. Armand (2015). "PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma." N Engl J Med **372**(4): 311-319.

Au-Yeung, B. B., G. A. Smith, J. L. Mueller, C. S. Heyn, R. G. Jaszczak, A. Weiss and J. Zikherman (2017). "IL-2 Modulates the TCR Signaling Threshold for CD8 but Not CD4 T Cell Proliferation on a Single-Cell Level." J Immunol **198**(6): 2445-2456.

Bargou, R., Kufer P, Kirchinger P, Noppeney R, Schuler M, Viardot A, Weigang-Koehler K, Zettl F, Gerecke C, Libicher M, Einsele H, Riethmuller G, Lutterbuese R, Klinger M, Baeuerle PA, Schlereth B, Reinhardt C (2006). "MT103 (anti-CD19 × anti-CD3-BiTE) induces B cell depletion, clearance of bone marrow infiltration and clinical responses in heavily pre-treated NHL patients: first data from dose-escalation study MT103-104. In: 11th congress of European hematology association. Ref type: Abstract."

Barlev, A., V. W. Lin, A. Katz, K. Hu, Z. Cong and B. Barber (2017). "Estimating Long-Term Survival of Adults with Philadelphia Chromosome-Negative Relapsed/Refractory B-Precursor Acute Lymphoblastic Leukemia Treated with Blinatumomab Using Historical Data." Adv Ther **34**(1): 148-155.

Bene, M. C., G. Castoldi, W. Knapp, W. D. Ludwig, E. Matutes, A. Orfao and M. B. van't Veer (1995). "Proposals for the immunological classification of acute leukemias. European Group for the Immunological Characterization of Leukemias (EGIL)." Leukemia **9**(10): 1783-1786.

Blackburn, S. D., H. Shin, W. N. Haining, T. Zou, C. J. Workman, A. Polley, M. R. Betts, G. J. Freeman, D. A. Vignali and E. J. Wherry (2009). "Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection." Nat Immunol **10**(1): 29-37.

Borowitz, M. J., B. L. Wood, M. Devidas, M. L. Loh, E. A. Raetz, W. L. Salzer, J. B. Nachman, A. J. Carroll, N. A. Heerema, J. M. Gastier-Foster, C. L. Willman, Y. Dai, N. J. Winick, S. P. Hunger, W. L. Carroll and E. Larsen (2015). "Prognostic significance of minimal residual disease in high risk B-ALL: a report from Children's Oncology Group study AALL0232." Blood **126**(8): 964-971.

Brahmer, J., K. L. Reckamp, P. Baas, L. Crino, W. E. Eberhardt, E. Poddubskaya, S. Antonia, A. Pluzanski, E. E. Vokes, E. Holgado, D. Waterhouse, N. Ready, J. Gainor, O. Aren Frontera, L. Havel, M. Steins, M. C. Garassino, J. G. Aerts, M. Domine, L. Paz-Ares, M. Reck, C. Baudelet, C.

T. Harbison, B. Lestini and D. R. Spigel (2015). "Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer." N Engl J Med **373**(2): 123-135.

Braig, F., A. Brandt, M. Goebeler, H. P. Tony, A. K. Kurze, P. Nollau, T. Bumm, S. Bottcher, R. C. Bargou and M. Binder (2017). "Resistance to anti-CD19/CD3 BiTE in acute lymphoblastic leukemia may be mediated by disrupted CD19 membrane trafficking." Blood **129**(1): 100-104.

Brandl, C., C. Haas, S. d'Argouges, T. Fisch, P. Kufer, K. Brischwein, N. Prang, R. Bargou, J. Suzich, P. A. Baeuerle and R. Hofmeister (2007). "The effect of dexamethasone on polyclonal T cell activation and redirected target cell lysis as induced by a CD19/CD3-bispecific single-chain antibody construct." Cancer Immunol Immunother **56**(10): 1551-1563.

Bruggemann, M., A. Schrauder, T. Raff, H. Pfeifer, M. Dworzak, O. G. Ottmann, V. Asnafi, A. Baruchel, R. Bassan, Y. Benoit, A. Biondi, H. Cave, H. Dombret, A. K. Fielding, R. Foa, N. Gokbuget, A. H. Goldstone, N. Goulden, G. Henze, D. Hoelzer, G. E. Janka-Schaub, E. A. Macintyre, R. Pieters, A. Rambaldi, J. M. Ribera, K. Schmiegelow, O. Spinelli, J. Stary, A. von Stackelberg, M. Kneba, M. Schrappe and J. J. van Dongen (2010). "Standardized MRD quantification in European ALL trials: proceedings of the Second International Symposium on MRD assessment in Kiel, Germany, 18-20 September 2008." Leukemia **24**(3): 521-535.

Chan, D. V., H. M. Gibson, B. M. Aufiero, A. J. Wilson, M. S. Hafner, Q. S. Mi and H. K. Wong (2014). "Differential CTLA-4 expression in human CD4+ versus CD8+ T cells is associated with increased NFAT1 and inhibition of CD4+ proliferation." Genes Immun **15**(1): 25-32.

Chawla-Sarkar, M., D. J. Lindner, Y. F. Liu, B. R. Williams, G. C. Sen, R. H. Silverman and E. C. Borden (2003). "Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis." Apoptosis **8**(3): 237-249.

Chen, L. and D. B. Flies (2013). "Molecular mechanisms of T cell co-stimulation and co-inhibition." Nat Rev Immunol **13**(4): 227-242.

Chen, X., S. Liu, L. Wang, W. Zhang, Y. Ji and X. Ma (2008). "Clinical significance of B7-H1 (PD-L1) expression in human acute leukemia." Cancer Biol Ther **7**(5): 622-627.

Crouch, S. P., R. Kozlowski, K. J. Slater and J. Fletcher (1993). "The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity." J Immunol Methods **160**(1): 81-88.

Dreier, T., G. Lorenczewski, C. Brandl, P. Hoffmann, U. Syring, F. Hanakam, P. Kufer, G. Riethmuller, R. Bargou and P. A. Baeuerle (2002). "Extremely potent, rapid and costimulation-independent cytotoxic T-cell response against lymphoma cells catalyzed by a single-chain bispecific antibody." Int J Cancer **100**(6): 690-697.

Esparza, S. D. and K. M. Sakamoto (2005). "Topics in pediatric leukemia--acute lymphoblastic leukemia." MedGenMed **7**(1): 23.

Feucht, J., S. Kayser, D. Gorodezki, M. Hamieh, M. Doring, F. Blaeschke, P. Schlegel, H. Bosmuller, L. Quintanilla-Fend, M. Ebinger, P. Lang, R. Handgretinger and T. Feuchtinger (2016). "T-cell responses against CD19+ pediatric acute lymphoblastic leukemia mediated by bispecific T-cell engager (BiTE) are regulated contrarily by PD-L1 and CD80/CD86 on leukemic blasts." Oncotarget **7**(47): 76902-76919.

Fourcade, J., Z. Sun, O. Pagliano, P. Guillaume, I. F. Luescher, C. Sander, J. M. Kirkwood, D. Olive, V. Kuchroo and H. M. Zarour (2012). "CD8(+) T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1." Cancer Res **72**(4): 887-896.

Francisco, L. M., V. H. Salinas, K. E. Brown, V. K. Vanguri, G. J. Freeman, V. K. Kuchroo and A. H. Sharpe (2009). "PD-L1 regulates the development, maintenance, and function of induced regulatory T cells." J Exp Med **206**(13): 3015-3029.

Fulcher, D. and S. Wong (1999). "Carboxyfluorescein succinimidyl ester-based proliferative assays for assessment of T cell function in the diagnostic laboratory." Immunol Cell Biol **77**(6): 559-564.

Gardner, R., D. Wu, S. Cherian, M. Fang, L. A. Hanafi, O. Finney, H. Smithers, M. C. Jensen, S. R. Riddell, D. G. Maloney and C. J. Turtle (2016). "Acquisition of a CD19-negative myeloid phenotype allows immune escape of MLL-rearranged B-ALL from CD19 CAR-T-cell therapy." Blood **127**(20): 2406-2410.

Gokbuget, N., M. Kneba, T. Raff, H. Trautmann, C. R. Bartram, R. Arnold, R. Fietkau, M. Freund, A. Ganser, W. D. Ludwig, G. Maschmeyer, H. Rieder, S. Schwartz, H. Serve, E. Thiel, M. Bruggemann and D. Hoelzer (2012). "Adult patients with acute lymphoblastic leukemia and molecular failure display a poor prognosis and are candidates for stem cell transplantation and targeted therapies." Blood **120**(9): 1868-1876.

Gokbuget, N., G. Zugmaier, M. Klinger, P. Kufer, M. Stelljes, A. Viardot, H. A. Horst, S. Neumann, M. Bruggemann, O. G. Ottmann, T. Burmeister, D. Wessiepe, M. S. Topp and R. Bargou (2017). "Long-term relapse-free survival in a phase 2 study of blinatumomab for the treatment of patients with minimal residual disease in B-lineage acute lymphoblastic leukemia." Haematologica **102**(4): e132-e135.

Group, C. A. C. (1996). "Duration and intensity of maintenance chemotherapy in acute lymphoblastic leukaemia: overview of 42 trials involving 12 000 randomised children." Lancet **347**(9018): 1783-1788.

Gruen, M., K. Bommert and R. C. Bargou (2004). "T-cell-mediated lysis of B cells induced by a CD19xCD3 bispecific single-chain antibody is perforin dependent and death receptor independent." Cancer Immunol Immunother **53**(7): 625-632.

Grupp, S. A., M. Kalos, D. Barrett, R. Aplenc, D. L. Porter, S. R. Rheingold, D. T. Teachey, A. Chew, B. Hauck, J. F. Wright, M. C. Milone, B. L. Levine and C. H. June (2013). "Chimeric antigen receptor-modified T cells for acute lymphoid leukemia." N Engl J Med **368**(16): 1509-1518.

Haagen, I. A., W. B. de Lau, B. J. Bast, A. J. Geerars, M. R. Clark and B. C. de Gast (1994). "Unprimed CD4+ and CD8+ T cells can be rapidly activated by a CD3 x CD19 bispecific antibody to proliferate and become cytotoxic." Cancer Immunol Immunother **39**(6): 391-396.

Haas, C., E. Krinner, K. Brischwein, P. Hoffmann, R. Lutterbuse, B. Schlereth, P. Kufer and P. A. Baeuerle (2009). "Mode of cytotoxic action of T cell-engaging BiTE antibody MT110." Immunobiology **214**(6): 441-453.

Hehn. (2014, 05.10.2015). "Häufigkeit von Leukämien in Deutschland." Retrieved 2017, 08.08, from https://www.kompetenznetz-leukaemie.de/content/aerzte/epidemiologie/.

Hilchey, S. P. and S. H. Bernstein (2007). "Use of CFSE to monitor ex vivo regulatory T-cell suppression of CD4+ and CD8+ T-cell proliferation within unseparated mononuclear cells from malignant and non-malignant human lymph node biopsies." Immunol Invest **36**(5-6): 629-648.

Hino, R., K. Kabashima, Y. Kato, H. Yagi, M. Nakamura, T. Honjo, T. Okazaki and Y. Tokura (2010). "Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma." Cancer **116**(7): 1757-1766.

Hoelzer, D., R. Bassan, H. Dombret, A. Fielding, J. M. Ribera and C. Buske (2016). "Acute lymphoblastic leukaemia in adult patients: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up." Ann Oncol **27**(suppl 5): v69-v82.

Hoelzer, D., J. Walewski, H. Dohner, A. Viardot, W. Hiddemann, K. Spiekermann, H. Serve, U. Duhrsen, A. Huttmann, E. Thiel, J. Dengler, M. Kneba, M. Schaich, I. G. Schmidt-Wolf, J. Beck, B. Hertenstein, A. Reichle, K. Domanska-Czyz, R. Fietkau, H. A. Horst, H. Rieder, S. Schwartz, T. Burmeister, N. Gokbuget and L. German Multicenter Study Group for Adult Acute Lymphoblastic (2014). "Improved outcome of adult Burkitt lymphoma/leukemia with rituximab and chemotherapy: report of a large prospective multicenter trial." Blood **124**(26): 3870-3879.

Hoffmann, P., R. Hofmeister, K. Brischwein, C. Brandl, S. Crommer, R. Bargou, C. Itin, N. Prang and P. A. Baeuerle (2005). "Serial killing of tumor cells by cytotoxic T cells redirected with a CD19-/CD3-bispecific single-chain antibody construct." Int J Cancer **115**(1): 98-104.

Howlader N, N. A., Krapcho M, Garshell J, Miller D, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z,Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA. (April 2014, 17.12.2014). "SEER Cancer Statistics Review, 1975-2011." Retrieved 08.08., 2017, from http://seer.cancer.gov/csr/1975_2011/.

Hunger, S. P., X. Lu, M. Devidas, B. M. Camitta, P. S. Gaynon, N. J. Winick, G. H. Reaman and W. L. Carroll (2012). "Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group." J Clin Oncol **30**(14): 1663-1669.

Hunger, S. P. and C. G. Mullighan (2015). "Acute Lymphoblastic Leukemia in Children." N Engl J Med **373**(16): 1541-1552.

Jacoby, E., S. M. Nguyen, T. J. Fountaine, K. Welp, B. Gryder, H. Qin, Y. Yang, C. D. Chien, A. E. Seif, H. Lei, Y. K. Song, J. Khan, D. W. Lee, C. L. Mackall, R. A. Gardner, M. C. Jensen, J. F. Shern and T. J. Fry (2016). "CD19 CAR immune pressure induces B-precursor acute lymphoblastic leukaemia lineage switch exposing inherent leukaemic plasticity." Nat Commun **7**: 12320.

Jelinek, T., J. Mihalyova, M. Kascak, J. Duras and R. Hajek (2017). "PD-1/PD-L1 inhibitors in haematological malignancies: update 2017." Immunology **152**(3): 357-371.

Jen, E. Y., Q. Xu, A. Schetter, D. Przepiorka, Y. L. Shen, D. Roscoe, R. Sridhara, A. Deisseroth, R. Philip, A. T. Farrell and R. Pazdur (2018). "FDA Approval: Blinatumomab for Patients with B-cell

Precursor Acute Lymphoblastic Leukemia in Morphologic Remission with Minimal Residual Disease." Clin Cancer Res.

Kantarjian, H., A. Stein, N. Gokbuget, A. K. Fielding, A. C. Schuh, J. M. Ribera, A. Wei, H. Dombret, R. Foa, R. Bassan, O. Arslan, M. A. Sanz, J. Bergeron, F. Demirkan, E. Lech-Maranda, A. Rambaldi, X. Thomas, H. A. Horst, M. Bruggemann, W. Klapper, B. L. Wood, A. Fleishman, D. Nagorsen, C. Holland, Z. Zimmerman and M. S. Topp (2017). "Blinatumomab versus Chemotherapy for Advanced Acute Lymphoblastic Leukemia." N Engl J Med **376**(9): 836-847.

Karimi, M. A., E. Lee, M. H. Bachmann, A. M. Salicioni, E. M. Behrens, T. Kambayashi and C. L. Baldwin (2014). "Measuring cytotoxicity by bioluminescence imaging outperforms the standard chromium-51 release assay." PLoS One **9**(2): e89357.

Keir, M. E., M. J. Butte, G. J. Freeman and A. H. Sharpe (2008). "PD-1 and its ligands in tolerance and immunity." Annu Rev Immunol **26**: 677-704.

Klinger, M., C. Brandl, G. Zugmaier, Y. Hijazi, R. C. Bargou, M. S. Topp, N. Gokbuget, S. Neumann, M. Goebeler, A. Viardot, M. Stelljes, M. Bruggemann, D. Hoelzer, E. Degenhard, D. Nagorsen, P. A. Baeuerle, A. Wolf and P. Kufer (2012). "Immunopharmacologic response of patients with B-lineage acute lymphoblastic leukemia to continuous infusion of T cell-engaging CD19/CD3-bispecific BiTE antibody blinatumomab." Blood **119**(26): 6226-6233.

Kohnke, T., C. Krupka, J. Tischer, T. Knosel and M. Subklewe (2015). "Increase of PD-L1 expressing B-precursor ALL cells in a patient resistant to the CD19/CD3-bispecific T cell engager antibody blinatumomab." J Hematol Oncol **8**: 111.

Kong, Y., J. Zhang, D. F. Claxton, W. C. Ehmann, W. B. Rybka, L. Zhu, H. Zeng, T. D. Schell and H. Zheng (2015). "PD-1(hi)TIM-3(+) T cells associate with and predict leukemia relapse in AML patients post allogeneic stem cell transplantation." Blood Cancer J **5**: e330.

Kronig, H., L. Kremmler, B. Haller, C. Englert, C. Peschel, R. Andreesen and C. U. Blank (2014). "Interferon-induced programmed death-ligand 1 (PD-L1/B7-H1) expression increases on human acute myeloid leukemia blast cells during treatment." Eur J Haematol **92**(3): 195-203.

Krupka, C., P. Kufer, R. Kischel, G. Zugmaier, F. S. Lichtenegger, T. Kohnke, B. Vick, I. Jeremias, K. H. Metzeler, T. Altmann, S. Schneider, M. Fiegl, K. Spiekermann, P. A. Bauerle, W. Hiddemann, G. Riethmuller and M. Subklewe (2016). "Blockade of the PD-1/PD-L1 axis augments lysis of AML cells by the CD33/CD3 BiTE antibody construct AMG 330: reversing a T-cell-induced immune escape mechanism." Leukemia **30**(2): 484-491.

Lee, D. W., J. N. Kochenderfer, M. Stetler-Stevenson, Y. K. Cui, C. Delbrook, S. A. Feldman, T. J. Fry, R. Orentas, M. Sabatino, N. N. Shah, S. M. Steinberg, D. Stroncek, N. Tschernia, C. Yuan, H. Zhang, L. Zhang, S. A. Rosenberg, A. S. Wayne and C. L. Mackall (2015). "T cells expressing CD19 chimeric antigen receptors for acute lymphoblastic leukaemia in children and young adults: a phase 1 dose-escalation trial." Lancet **385**(9967): 517-528.

Lee, P., R. Bhansali, S. Izraeli, N. Hijiya and J. D. Crispino (2016). "The biology, pathogenesis and clinical aspects of acute lymphoblastic leukemia in children with Down syndrome." Leukemia **30**(9): 1816-1823.

Li, J., H. B. Jie, Y. Lei, N. Gildener-Leapman, S. Trivedi, T. Green, L. P. Kane and R. L. Ferris (2015). "PD-1/SHP-2 inhibits Tc1/Th1 phenotypic responses and the activation of T cells in the tumor microenvironment." Cancer Res **75**(3): 508-518.

Locatelli, F., M. Schrappe, M. E. Bernardo and S. Rutella (2012). "How I treat relapsed childhood acute lymphoblastic leukemia." Blood **120**(14): 2807-2816.

Loffler, A., P. Kufer, R. Lutterbuse, F. Zettl, P. T. Daniel, J. M. Schwenkenbecher, G. Riethmuller, B. Dorken and R. C. Bargou (2000). "A recombinant bispecific single-chain antibody, CD19 x CD3, induces rapid and high lymphoma-directed cytotoxicity by unstimulated T lymphocytes." Blood **95**(6): 2098-2103.

Lyons, A. B. (2000). "Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution." J Immunol Methods **243**(1-2): 147-154.

Ma, X., M. Edmonson, D. Yergeau, D. M. Muzny, O. A. Hampton, M. Rusch, G. Song, J. Easton, R. C. Harvey, D. A. Wheeler, J. Ma, H. Doddapaneni, B. Vadodaria, G. Wu, P. Nagahawatte, W. L. Carroll, I. M. Chen, J. M. Gastier-Foster, M. V. Relling, M. A. Smith, M. Devidas, J. M. Guidry Auvil, J. R. Downing, M. L. Loh, C. L. Willman, D. S. Gerhard, C. G. Mullighan, S. P. Hunger and J. Zhang (2015). "Rise and fall of subclones from diagnosis to relapse in pediatric B-acute lymphoblastic leukaemia." Nat Commun **6**: 6604.

Martinelli, G., N. Boissel, P. Chevallier, O. Ottmann, N. Gokbuget, M. S. Topp, A. K. Fielding, A. Rambaldi, E. K. Ritchie, C. Papayannidis, L. R. Sterling, J. Benjamin and A. Stein (2017). "Complete Hematologic and Molecular Response in Adult Patients With Relapsed/Refractory Philadelphia Chromosome-Positive B-Precursor Acute Lymphoblastic Leukemia Following Treatment With Blinatumomab: Results From a Phase II, Single-Arm, Multicenter Study." J Clin Oncol **35**(16): 1795-1802.

Maude, S. L., N. Frey, P. A. Shaw, R. Aplenc, D. M. Barrett, N. J. Bunin, A. Chew, V. E. Gonzalez, Z. Zheng, S. F. Lacey, Y. D. Mahnke, J. J. Melenhorst, S. R. Rheingold, A. Shen, D. T. Teachey, B. L. Levine, C. H. June, D. L. Porter and S. A. Grupp (2014). "Chimeric antigen receptor T cells for sustained remissions in leukemia." N Engl J Med **371**(16): 1507-1517.

Menegaux, F., M. Ripert, D. Hémon and J. Clavel (2007). "Maternal alcohol and coffee drinking, parental smoking and childhood leukaemia: a French population-based case-control study." Paediatr Perinat Epidemiol **21**(4): 293-299.

Moorman, A. V., H. M. Ensor, S. M. Richards, L. Chilton, C. Schwab, S. E. Kinsey, A. Vora, C. D. Mitchell and C. J. Harrison (2010). "Prognostic effect of chromosomal abnormalities in childhood B-cell precursor acute lymphoblastic leukaemia: results from the UK Medical Research Council ALL97/99 randomised trial." Lancet Oncol **11**(5): 429-438.

Moricke, A., M. Zimmermann, A. Reiter, H. Gadner, E. Odenwald, J. Harbott, W. D. Ludwig, H. Riehm and M. Schrappe (2005). "Prognostic impact of age in children and adolescents with acute lymphoblastic leukemia: data from the trials ALL-BFM 86, 90, and 95." Klin Padiatr **217**(6): 310-320.

Mosmann, T. R. and S. Sad (1996). "The expanding universe of T-cell subsets: Th1, Th2 and more." Immunol Today **17**(3): 138-146.

Mullighan, C. G., L. A. Phillips, X. Su, J. Ma, C. B. Miller, S. A. Shurtleff and J. R. Downing (2008). "Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia." Science **322**(5906): 1377-1380.

Nagel, I., M. Bartels, J. Duell, H. H. Oberg, S. Ussat, H. Bruckmueller, O. Ottmann, H. Pfeifer, H. Trautmann, N. Gokbuget, A. Caliebe, D. Kabelitz, M. Kneba, H. A. Horst, D. Hoelzer, M. S. Topp, I. Cascorbi, R. Siebert and M. Bruggemann (2017). "Hematopoietic stem cell involvement in BCR-ABL1-positive ALL as potential mechanism of resistance to blinatumomab therapy." Blood.

Nagele, V., A. Kratzer, G. Zugmaier, C. Holland, Y. Hijazi, M. S. Topp, N. Gokbuget, P. A. Baeuerle, P. Kufer, A. Wolf and M. Klinger (2017). "Changes in clinical laboratory parameters and pharmacodynamic markers in response to blinatumomab treatment of patients with relapsed/refractory ALL." Exp Hematol Oncol **6**: 14.

Nicola Goekbuget, H. D., Massimiliano Bonifacio, Albrecht Reichle, Carlos Graux, Violaine Havelange, Eike C. Buss, Christoph Faul, Monika Bruggemann, Arnold Ganser, Julia Stieglmaier, Hendrik Wessels, Vincent Haddad, Gerhard Zugmaier, Dirk Nagorsen and Ralf C. Bargou (2014). "BLAST: A Confirmatory, Single-Arm, Phase 2 Study of Blinatumomab, a Bispecific T-Cell Engager (BiTE®) Antibody Construct, in Patients with Minimal Residual Disease B-Precursor Acute Lymphoblastic Leukemia (ALL)." Blood **124**: 21 379.

Norde, W. J., W. Hobo, R. van der Voort and H. Dolstra (2012). "Coinhibitory molecules in hematologic malignancies: targets for therapeutic intervention." Blood **120**(4): 728-736.

Offner, S., R. Hofmeister, A. Romaniuk, P. Kufer and P. A. Baeuerle (2006). "Induction of regular cytolytic T cell synapses by bispecific single-chain antibody constructs on MHC class I-negative tumor cells." Mol Immunol **43**(6): 763-771.

Pardoll, D. M. (2012). "The blockade of immune checkpoints in cancer immunotherapy." Nat Rev Cancer **12**(4): 252-264.

Patel, A., H. L. Kaufman and M. L. Disis (2017). "Next generation approaches for tumor vaccination." Chin Clin Oncol **6**(2): 19.

Porter, C. C., T. E. Druley, A. Erez, R. P. Kuiper, K. Onel, J. D. Schiffman, K. Wolfe Schneider, S. R. Scollon, H. S. Scott, L. C. Strong, M. F. Walsh and K. E. Nichols (2017). "Recommendations for Surveillance for Children with Leukemia-Predisposing Conditions." Clin Cancer Res **23**(11): e14-e22.

Postow, M. A., J. Chesney, A. C. Pavlick, C. Robert, K. Grossmann, D. McDermott, G. P. Linette, N. Meyer, J. K. Giguere, S. S. Agarwala, M. Shaheen, M. S. Ernstoff, D. Minor, A. K. Salama, M. Taylor, P. A. Ott, L. M. Rollin, C. Horak, P. Gagnier, J. D. Wolchok and F. S. Hodi (2015). "Nivolumab and ipilimumab versus ipilimumab in untreated melanoma." N Engl J Med **372**(21): 2006-2017.

Prucker, C., A. Attarbaschi, C. Peters, M. N. Dworzak, U. Pötschger, C. Urban, F. M. Fink, B. Meister, K. Schmitt, O. A. Haas, H. Gadner, G. Mann and A. B.-F.-M. B. S. Group (2009). "Induction death and treatment-related mortality in first remission of children with acute lymphoblastic leukemia: a population-based analysis of the Austrian Berlin-Frankfurt-Münster study group." Leukemia **23**(7): 1264-1269.

Pui, C. H. and S. Jeha (2007). "New therapeutic strategies for the treatment of acute lymphoblastic leukaemia." Nat Rev Drug Discov **6**(2): 149-165.

Quah, B. J. and C. R. Parish (2012). "New and improved methods for measuring lymphocyte proliferation in vitro and in vivo using CFSE-like fluorescent dyes." J Immunol Methods **379**(1-2): 1-14.

Queudeville, M., R. Handgretinger and M. Ebinger (2017). "Immunotargeting relapsed or refractory precursor B-cell acute lymphoblastic leukemia - role of blinatumomab." Onco Targets Ther **10**: 3567-3578.

Qureshi, O. S., Y. Zheng, K. Nakamura, K. Attridge, C. Manzotti, E. M. Schmidt, J. Baker, L. E. Jeffery, S. Kaur, Z. Briggs, T. Z. Hou, C. E. Futter, G. Anderson, L. S. Walker and D. M. Sansom (2011). "Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4." Science **332**(6029): 600-603.

Rabenstein, H., A. C. Behrendt, J. W. Ellwart, R. Naumann, M. Horsch, J. Beckers and R. Obst (2014). "Differential kinetics of antigen dependency of CD4+ and CD8+ T cells." J Immunol **192**(8): 3507-3517.

Raponi, S., M. S. De Propris, S. Intoppa, M. L. Milani, A. Vitale, L. Elia, O. Perbellini, G. Pizzolo, R. Foa and A. Guarini (2011). "Flow cytometric study of potential target antigens (CD19, CD20, CD22, CD33) for antibody-based immunotherapy in acute lymphoblastic leukemia: analysis of 552 cases." Leuk Lymphoma **52**(6): 1098-1107.

Rayes, A., R. L. McMasters and M. M. O'Brien (2016). "Lineage Switch in MLL-Rearranged Infant Leukemia Following CD19-Directed Therapy." Pediatr Blood Cancer **63**(6): 1113-1115.

Reismuller, B., A. Attarbaschi, C. Peters, M. N. Dworzak, U. Potschger, C. Urban, F. M. Fink, B. Meister, K. Schmitt, K. Dieckmann, G. Henze, O. A. Haas, H. Gadner, G. Mann and G. Austrian Berlin-Frankfurt-Munster Study (2009). "Long-term outcome of initially homogenously treated and relapsed childhood acute lymphoblastic leukaemia in Austria--a population-based report of the Austrian Berlin-Frankfurt-Munster (BFM) Study Group." Br J Haematol **144**(4): 559-570.

Roberts, D. M., R. B. Jones, R. M. Smith, F. Alberici, D. S. Kumaratne, S. Burns and D. R. Jayne (2015). "Immunoglobulin G replacement for the treatment of infective complications of rituximab-associated hypogammaglobulinemia in autoimmune disease: a case series." J Autoimmun **57**: 24-29.

Sadelain, M. (2015). "CAR therapy: the CD19 paradigm." J Clin Invest **125**(9): 3392-3400.

Sakuishi, K., L. Apetoh, J. M. Sullivan, B. R. Blazar, V. K. Kuchroo and A. C. Anderson (2010). "Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity." J Exp Med **207**(10): 2187-2194.

Salzer, W. L., M. Devidas, W. L. Carroll, N. Winick, J. Pullen, S. P. Hunger and B. A. Camitta (2010). "Long-term results of the pediatric oncology group studies for childhood acute lymphoblastic leukemia 1984-2001: a report from the children's oncology group." Leukemia **24**(2): 355-370.

Schoenaker, M. H., F. Suarez, T. Szczepanski, N. Mahlaoui and J. L. Loeffen (2016). "Treatment of acute leukemia in children with ataxia telangiectasia (A-T)." Eur J Med Genet **59**(12): 641-646.

Schrappe, M., S. P. Hunger, C. H. Pui, V. Saha, P. S. Gaynon, A. Baruchel, V. Conter, J. Otten, A. Ohara, A. B. Versluys, G. Escherich, M. Heyman, L. B. Silverman, K. Horibe, G. Mann, B. M. Camitta, J. Harbott, H. Riehm, S. Richards, M. Devidas and M. Zimmermann (2012). "Outcomes after induction failure in childhood acute lymphoblastic leukemia." N Engl J Med **366**(15): 1371-1381.

Schrappe, M., A. Reiter, W. D. Ludwig, J. Harbott, M. Zimmermann, W. Hiddemann, C. Niemeyer, G. Henze, A. Feldges, F. Zintl, B. Kornhuber, J. Ritter, K. Welte, H. Gadner and H. Riehm (2000). "Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM Study Group." Blood **95**(11): 3310-3322.

Schrappe, M., A. Reiter, M. Zimmermann, J. Harbott, W. D. Ludwig, G. Henze, H. Gadner, E. Odenwald and H. Riehm (2000). "Long-term results of four consecutive trials in childhood ALL performed by the ALL-BFM study group from 1981 to 1995. Berlin-Frankfurt-Münster." Leukemia **14**(12): 2205-2222.

Schreiber, R. D., L. J. Old and M. J. Smyth (2011). "Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion." Science **331**(6024): 1565-1570.

Schumacher, T. N. and R. D. Schreiber (2015). "Neoantigens in cancer immunotherapy." Science **348**(6230): 69-74.

Seidel, J. A., A. Otsuka and K. Kabashima (2018). "Anti-PD-1 and Anti-CTLA-4 Therapies in Cancer: Mechanisms of Action, Efficacy, and Limitations." Front Oncol **8**: 86.

Shin, H. and E. J. Wherry (2007). "CD8 T cell dysfunction during chronic viral infection." Curr Opin Immunol **19**(4): 408-415.

Sirvent, N., S. Suciu, X. Rialland, F. Millot, Y. Benoit, D. Plantaz, A. Ferster, A. Robert, P. Lutz, B. Nelken, E. Plouvier, L. Norton, Y. Bertrand and J. Otten (2011). "Prognostic significance of the initial cerebro-spinal fluid (CSF) involvement of children with acute lymphoblastic leukaemia (ALL) treated without cranial irradiation: results of European Organization for Research and Treatment of Cancer (EORTC) Children Leukemia Group study 58881." Eur J Cancer **47**(2): 239-247.

Sotillo, E., D. M. Barrett, K. L. Black, A. Bagashev, D. Oldridge, G. Wu, R. Sussman, C. Lanauze, M. Ruella, M. R. Gazzara, N. M. Martinez, C. T. Harrington, E. Y. Chung, J. Perazzelli, T. J. Hofmann, S. L. Maude, P. Raman, A. Barrera, S. Gill, S. F. Lacey, J. J. Melenhorst, D. Allman, E. Jacoby, T. Fry, C. Mackall, Y. Barash, K. W. Lynch, J. M. Maris, S. A. Grupp and A. Thomas-Tikhonenko (2015). "Convergence of Acquired Mutations and Alternative Splicing of CD19 Enables Resistance to CART-19 Immunotherapy." Cancer Discov **5**(12): 1282-1295.

Stambrook, P. J., J. Maher and F. Farzaneh (2017). "Cancer Immunotherapy: Whence and Whither." Mol Cancer Res **15**(6): 635-650.

Stary, J., M. Zimmermann, M. Campbell, L. Castillo, E. Dibar, S. Donska, A. Gonzalez, S. Izraeli, D. Janic, J. Jazbec, J. Konja, E. Kaiserova, J. Kowalczyk, G. Kovacs, C. K. Li, E. Magyarosy, A. Popa, B. Stark, Y. Jabali, J. Trka, O. Hrusak, H. Riehm, G. Masera and M. Schrappe (2014). "Intensive chemotherapy for childhood acute lymphoblastic leukemia: results of the randomized intercontinental trial ALL IC-BFM 2002." J Clin Oncol **32**(3): 174-184.

Swann, J. B. and M. J. Smyth (2007). "Immune surveillance of tumors." J Clin Invest **117**(5): 1137-1146.

Teachey, D. T., S. R. Rheingold, S. L. Maude, G. Zugmaier, D. M. Barrett, A. E. Seif, K. E. Nichols, E. K. Suppa, M. Kalos, R. A. Berg, J. C. Fitzgerald, R. Aplenc, L. Gore and S. A. Grupp (2013). "Cytokine release syndrome after blinatumomab treatment related to abnormal macrophage activation and ameliorated with cytokine-directed therapy." Blood **121**(26): 5154-5157.

Topp, M. S., N. Gokbuget, A. S. Stein, G. Zugmaier, S. O'Brien, R. C. Bargou, H. Dombret, A. K. Fielding, L. Heffner, R. A. Larson, S. Neumann, R. Foa, M. Litzow, J. M. Ribera, A. Rambaldi, G. Schiller, M. Bruggemann, H. A. Horst, C. Holland, C. Jia, T. Maniar, B. Huber, D. Nagorsen, S. J. Forman and H. M. Kantarjian (2015). "Safety and activity of blinatumomab for adult patients with relapsed or refractory B-precursor acute lymphoblastic leukaemia: a multicentre, single-arm, phase 2 study." Lancet Oncol **16**(1): 57-66.

Topp, M. S., N. Gokbuget, G. Zugmaier, E. Degenhard, M. E. Goebeler, M. Klinger, S. A. Neumann, H. A. Horst, T. Raff, A. Viardot, M. Stelljes, M. Schaich, R. Kohne-Volland, M. Bruggemann, O. G. Ottmann, T. Burmeister, P. A. Baeuerle, D. Nagorsen, M. Schmidt, H. Einsele, G. Riethmuller, M. Kneba, D. Hoelzer, P. Kufer and R. C. Bargou (2012). "Long-term follow-up of hematologic relapse-free survival in a phase 2 study of blinatumomab in patients with MRD in B-lineage ALL." Blood **120**(26): 5185-5187.

Topp, M. S., N. Gokbuget, G. Zugmaier, P. Klappers, M. Stelljes, S. Neumann, A. Viardot, R. Marks, H. Diedrich, C. Faul, A. Reichle, H. A. Horst, M. Bruggemann, D. Wessiepe, C. Holland, S. Alekar, N. Mergen, H. Einsele, D. Hoelzer and R. C. Bargou (2014). "Phase II trial of the anti-CD19 bispecific T cell-engager blinatumomab shows hematologic and molecular remissions in patients with relapsed or refractory B-precursor acute lymphoblastic leukemia." J Clin Oncol **32**(36): 4134-4140.

Topp, M. S., P. Kufer, N. Gokbuget, M. Goebeler, M. Klinger, S. Neumann, H. A. Horst, T. Raff, A. Viardot, M. Schmid, M. Stelljes, M. Schaich, E. Degenhard, R. Kohne-Volland, M. Bruggemann, O. Ottmann, H. Pfeifer, T. Burmeister, D. Nagorsen, M. Schmidt, R. Lutterbuese, C. Reinhardt, P. A. Baeuerle, M. Kneba, H. Einsele, G. Riethmuller, D. Hoelzer, G. Zugmaier and R. C. Bargou (2011). "Targeted therapy with the T-cell-engaging antibody blinatumomab of chemotherapy-refractory minimal residual disease in B-lineage acute lymphoblastic leukemia patients results in high response rate and prolonged leukemia-free survival." J Clin Oncol **29**(18): 2493-2498.

Vaitkeviciene, G., E. Forestier, M. Hellebostad, M. Heyman, O. G. Jonsson, P. M. Lahteenmaki, S. Rosthoej, S. Soderhall and K. Schmiegelow (2011). "High white blood cell count at diagnosis of childhood acute lymphoblastic leukaemia: biological background and prognostic impact. Results from the NOPHO ALL-92 and ALL-2000 studies." Eur J Haematol **86**(1): 38-46.

van Dongen, J. J., T. Seriu, E. R. Panzer-Grümayer, A. Biondi, M. J. Pongers-Willemse, L. Corral, F. Stolz, M. Schrappe, G. Masera, W. A. Kamps, H. Gadner, E. R. van Wering, W. D. Ludwig, G. Basso, M. A. de Bruijn, G. Cazzaniga, K. Hettinger, A. van der Does-van den Berg, W. C. Hop, H. Riehm and C. R. Bartram (1998). "Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood." Lancet **352**(9142): 1731-1738.

van Dongen, J. J., V. H. van der Velden, M. Bruggemann and A. Orfao (2015). "Minimal residual disease diagnostics in acute lymphoblastic leukemia: need for sensitive, fast, and standardized technologies." Blood **125**(26): 3996-4009.

Vasilevko, V., A. Ghochikyan, M. J. Holterman and M. G. Agadjanyan (2002). "CD80 (B7-1) and CD86 (B7-2) are functionally equivalent in the initiation and maintenance of CD4+ T-cell proliferation after activation with suboptimal doses of PHA." DNA Cell Biol **21**(3): 137-149.

Venken, K., M. Thewissen, N. Hellings, V. Somers, K. Hensen, J. L. Rummens and P. Stinissen (2007). "A CFSE based assay for measuring CD4+CD25+ regulatory T cell mediated suppression of auto-antigen specific and polyclonal T cell responses." J Immunol Methods **322**(1-2): 1-11.

Volejnikova, J., E. Mejstrikova, T. Valova, L. Reznickova, L. Hodonska, V. Mihal, J. Sterba, Y. Jabali, D. Prochazkova, B. Blazek, J. Hak, Z. Cerna, O. Hrusak, J. Stary, J. Trka and E. Fronkova (2011). "Minimal residual disease in peripheral blood at day 15 identifies a subgroup of childhood B-cell precursor acute lymphoblastic leukemia with superior prognosis." Haematologica **96**(12): 1815-1821.

von Stackelberg, A., F. Locatelli, G. Zugmaier, R. Handgretinger, T. M. Trippett, C. Rizzari, P. Bader, M. M. O'Brien, B. Brethon, D. Bhojwani, P. G. Schlegel, A. Borkhardt, S. R. Rheingold, T. M. Cooper, C. M. Zwaan, P. Barnette, C. Messina, G. Michel, S. G. DuBois, K. Hu, M. Zhu, J. A. Whitlock and L. Gore (2016). "Phase I/Phase II Study of Blinatumomab in Pediatric Patients With Relapsed/Refractory Acute Lymphoblastic Leukemia." J Clin Oncol **34**(36): 4381-4389.

Vrooman, L. M. and L. B. Silverman (2016). "Treatment of Childhood Acute Lymphoblastic Leukemia: Prognostic Factors and Clinical Advances." Curr Hematol Malig Rep **11**(5): 385-394.

Walker, C. L., K. A. Lukyanov, I. V. Yampolsky, A. S. Mishin, A. S. Bommarius, A. M. Duraj-Thatte, B. Azizi, L. M. Tolbert and K. M. Solntsev (2015). "Fluorescence imaging using synthetic GFP chromophores." Curr Opin Chem Biol **27**: 64-74.

Wherry, E. J. and M. Kurachi (2015). "Molecular and cellular insights into T cell exhaustion." Nat Rev Immunol **15**(8): 486-499.

Woo, S. R., M. E. Turnis, M. V. Goldberg, J. Bankoti, M. Selby, C. J. Nirschl, M. L. Bettini, D. M. Gravano, P. Vogel, C. L. Liu, S. Tangsombatvisit, J. F. Grosso, G. Netto, M. P. Smeltzer, A. Chaux, P. J. Utz, C. J. Workman, D. M. Pardoll, A. J. Korman, C. G. Drake and D. A. Vignali (2012). "Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape." Cancer Res **72**(4): 917-927.

Xu, X. J., Y. M. Tang, H. Song, S. L. Yang, W. Q. Xu, N. Zhao, S. W. Shi, H. P. Shen, J. Q. Mao, L. Y. Zhang and B. H. Pan (2012). "Diagnostic accuracy of a specific cytokine pattern in hemophagocytic lymphohistiocytosis in children." J Pediatr **160**(6): 984-990 e981.

Zaritskaya, L., M. R. Shurin, T. J. Sayers and A. M. Malyguine (2010). "New flow cytometric assays for monitoring cell-mediated cytotoxicity." Expert Rev Vaccines **9**(6): 601-616.

Zhou, J., M. A. Goldwasser, A. Li, S. E. Dahlberg, D. Neuberg, H. Wang, V. Dalton, K. D. McBride, S. E. Sallan, L. B. Silverman, J. G. Gribben and D.-F. C. I. A. Consortium (2007). "Quantitative analysis of minimal residual disease predicts relapse in children with B-lineage acute lymphoblastic leukemia in DFCI ALL Consortium Protocol 95-01." Blood **110**(5): 1607-1611.

Zhou, Y., Y. He, W. Xing, P. Zhang, H. Shi, S. Chen, J. Shi, J. Bai, S. D. Rhodes, F. Zhang, J. Yuan, X. Yang, X. Zhu, Y. Li, H. Hanenberg, M. Xu, K. A. Robertson, W. Yuan, G. Nalepa, T. Cheng, D. W. Clapp and F. C. Yang (2017). "An abnormal bone marrow microenvironment contributes to hematopoietic dysfunction in Fanconi anemia." Haematologica **102**(6): 1017-1027.

Zugmaier, G., M. S. Topp, S. Alekar, A. Viardot, H. A. Horst, S. Neumann, M. Stelljes, R. C. Bargou, M. Goebeler, D. Wessiepe, E. Degenhard, N. Gokbuget and M. Klinger (2014). "Long-term follow-up of serum immunoglobulin levels in blinatumomab-treated patients with minimal residual disease-positive B-precursor acute lymphoblastic leukemia." Blood Cancer J **4**: 244.

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