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Harnessing T cells for immunotherapy in AML: from molecule to therapy

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List of abbreviations

ALL	acute lymphoblastic leukemia
Allo-SCT	allogenic stem cell transplantation
AML	acute myeloid leukemia
AML-PDX	patient-derived AML xenograft
BDC	blood dendritic cell
BiTE	bispecific T-cell engager
CAR-T cell	Chimeric Antigen Receptor T cell
cDC	conventional dendritic cell
CpG	CpG oligodeoxynucleotide
CTL	cytotoxic $CD8^+$ T lymphocytes
CSC	Cell Surface Capture technology
DC	Dendritic cell
DCleu	leukemia-derived DCs
ELN	European LeukemiaNet
FAB	French-American-British
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GvHD	graft-versus-host-disease
GvL	graft-versus-leukemia
HLA	human leukocyte antigen
HMA	hypomethylating agent
ImmTACs	immune-mobilizing monoclonal TCRs against cancer
LC-MS	liquid chromatography-mass spectrometry
MHC	major-histocompatibility complex
moDC	monocyte-derived dendritic cell
MRD	minimal residual disease
pDC	plasmacytoid dendritic cell
poly(I:C)	polyinosinic:polycytidylic acid
R848	Resiquimod
scFv	single-chain variable fragment
SCT	stem-cell transplantation
TAA	tumor-associated antigen
TCB	T-cell bispecific antibody
TCR	T-cell receptor
TLR	toll-like receptor
TSA	tumor-specific antigen
WHO	World Health Organization
WT1	Wilms' tumor 1

List of publications

This thesis includes two publications that have been accepted for publication in peer-reviewed journals:

"Blood DCs activated with R848 and poly(I:C) induce antigen-specific immune responses against viral and tumor-associated antigens"

Gerulf Hänel, Caroline Angerer, Katja Petry, Felix S. Lichtenegger*, Marion Subklewe*

Cancer Immunol Immunother. 2022 Jul; 71(7):1705-1718. doi: 10.1007/s00262-021-03109-w

*contributed equally

"Integrated multiomic approach for identification of novel immunotherapeutic targets in AML"

Thomas Köhnke, Xilong Liu, Sascha Haubner, Veit Bücklein, **Gerulf Hänel**, Christina Krupka, Victor, Solis-Mezarino, Franz Herzog, Marion Subklewe

Biomark Res. 2022 Jun 10; 10(1):43. doi: 10.1186/s40364-022-00390-4

During my thesis work I contributed significantly to the following publication in a peer-reviewed journal:

Targeting intracellular WT1 in AML with a novel RMF-peptide-MHC specific T-cell bispecific antibody

Christian Augsberger*, **Gerulf Hänel***, Wei Xu, Vesna Pulko, Lydia Jasmin Hanisch, Angelique Augustin, John Challier, Katharina Hunt, Binje Vick, Pier Eduardo Rovatti, Christina Krupka, Maurine Rothe, Anne Schönle, Johannes Sam, Emmanuelle Lezan, Axel Ducret, Daniela Ortiz-Franyuti, Antje-Christine Walz, Jörg Benz, Alexander Bujotzek, Felix S. Lichtenegger, Christian Gassner, Alejandro Carpy, Victor Lyamichev, Jigar Patel, Nikola P konstandin, Antje Tunger, Marc Schmitz, Michael von Bergwelt-Baildon, Karsten Spiekermann, Luca Vago, Irmela Jeremias, Estelle Marrer-Berger, Pablo Umaña, Christian Klein, Marion Subklewe

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*contributed equally

1. Introductory summary

1.1 Acute myeloid leukemia

Acute Myeloid Leukemia (AML) is a clonal malignancy of myeloid origin characterized by uncontrolled proliferation and differentiation of myeloid precursor cells in bone marrow and blood. This leads to the suppression of healthy hematopoiesis and causes neutropenia with the increased risk of fatal infections, thrombocytopenia with the risk of bleeding complications, anemia resulting into fatigue and possibly cardiovascular complications. Untreated AML is usually fatal within three months upon initial diagnosis[1]. The annual incidence of AML in the USA from 2014-2018 was 4.3 cases per 100 000 inhabitants, with a median age at diagnosis of 68 years, whereby 4.4% were younger than 20 years[2]. Initial diagnosis of AML relies on a cytomorphological analysis of blood and bone marrow, followed by cytochemistry and multi parameter flow cytometry for lineage determination[3,4]. Treatment decisions are based on further characterization of genetic aberrations, including chromosomal translocations, inversions or other rearrangements and molecular mutations (e.g. NPM1, CEBPA, FLT3, TP53) and gene rearrangements (e.g. RUNX1-RUNX1T1, BCR-ABL1)[3]. Several classification systems have been established for AML due to the heterogenous character of the disease and the diverse treatment outcome. The historic French-American-British (FAB)-classification system divides AML into subtypes M0-M7 based on morphological and cytochemical properties[5,6]. However, this system is not accounting for cytogenetic diversity and molecular abnormalities that were identified as important clinical markers with ongoing research, leading to the development of the classification system by the World Health Organization (WHO) in 2001 with updates in 2008, 2016 and 2022[4,7–9]. The latest update groups the disease into AML with defining genetic abnormalities and AML defined by differentiation[4]. An additional classification system established by the European LeukemiaNet (ELN) is dividing AML based on genetical characteristics into the three groups: favorable, intermediate and adverse. This has been shown to be prognostically relevant and is the basis for post-remission treatment decisions[3,10,11].

Since the 1970s, standard of care consisted of an intensive induction chemotherapy with cytarabine and anthracyclines (Daunorubicin, Idarubicin or Mitoxantron) commonly known as “7+3” treatment regimen, irrespective of the AML subtype. However, the treatment landscape has become more diverse with advances especially in the field of targeted therapeutic agents[12,13]. Therefore, the 7+3 chemotherapy is now frequently combined with other therapeutic agents, such as the antibody drug conjugate gemtuzumab ozogamicin and the tyrosine kinase inhibitor midostaurin, depending on molecular stratifiers. Complete responses are achieved in 40-60% of patients older than 60 years and up to 85% of patients under 61 years[14].

Patients unfit for intensive chemotherapy received so far hypomethylating agents (HMA), low dose cytarabine or only palliative care, aiming for prolongation of life while maintaining a high quality of life. However, the combination of Azacytidine and Venetoclax has recently been demonstrated to improve median overall survival, to achieve a significant higher complete remission rate compared to the control group receiving only Azacytidine (36.7% vs. 17.9%, $p<0.001$) and to be well tolerated[15]. It has therefore become the standard of care in this subgroup of patients[13,16].

Despite high response rates to induction therapies, disease relapse is frequent due to chemo-refractory leukemic cells. Post-remission therapy therefore consists of additional chemotherapy

with cytarabine in patients with favorable ELN risk profile achieving cure rates of 60-70% in patients under 61 years[14]. In contrast, allogenic stem cell transplantation (allo-SCT) remains the dominant treatment option in patients with intermediate or adverse risk profile providing the best anti-leukemic activity. The curative effect consists especially of the graft-versus-leukemia (GvL) effect mediated by alloreactive lymphocytes eliminating residual tumor cells and possibly by tumor-specific immune responses in additon[17]. Nevertheless, alloreactivity against healthy tissue can result in graft-versus-host-disease (GvHD) and severe complications post transplantation[17]. Accordingly, only a minority of patients are eligible for allogeneic SCT concepts due to age, comorbidities and less common, missing donor.

Overall, cure rates of AML patients strongly depend on the individual risk profile. The five-year relative survival rate was below 10% in the 1970s and has increased to about 30% today[2], whereby a cure rate of 35-40% has been reached in patients younger than 61 years but remains low (5-15%) in patients older than 60 years[14]. Therefore, novel therapeutic approaches are urgently needed.

1.2 Immunotherapeutic targets in AML

Immunotherapies represent promising novel treatment options in many tumor entities including AML. However, developing immunotherapies for AML is challenged by the heterogeneity of the disease, the identification of a suitable tumor antigen and a low mutational burden resulting in low immunogenicity[18]. Numerous tumor targets are currently preclinically and clinically evaluated that can be grouped into different categories (Figure 1): (1) lineage-restricted antigens, (2) tumor-associated antigens (TAAs), and (3) tumor-specific antigens (TSAs)[19,20].

Lineage antigens are non-mutated proteins ubiquitously expressed by leukemic cells, with expression being largely unaffected during disease progression and independent of genetic characteristics[21,22]. Targeting of lineage antigens has been proven especially successful in B-cell malignancies by targeting of CD19 or CD20 with monoclonal antibody constructs (e.g. Tafasitamab, Blinatumomab) and Chimeric Antigen Receptor (CAR)-T cells (e.g. Tisagenlecleucel, Axicabtagen ciloleucel). Accordingly, CD33, CD123, CLL-1, FLT-3 and others are intensively studied in AML aiming for translation of the success seen in B-cell malignancies. However, targeting of lineage antigens is associated with strong on-target off-tumor cytotoxicity leading to the eradication of the whole cell lineage including healthy cells. Whereas this is a controllable problem in B-cell malignancies, targeting of lineage antigens in AML, such as CD33 and CD123, can lead to prolonged cytopenias[23,24]. In addition, lineage antigens may not be expressed on leukemic stem and progenitor cells, thereby impairing clinical efficacy.

TAAs are non-lineage restricted antigens overexpressed by the tumor compared to healthy cells. They have the advantage of a better safety profile when their expression is low or absent on healthy tissue. In addition, TAAs containing oncogenic function are more likely to be expressed also on leukemic stem and progenitor cells improving efficacy. Multiple TAAs are currently evaluated, whereof WT1 has attracted major research interest in AML and is further discussed in section 1.4. Another intensively studied target in AML is PRAME, which is overexpressed in a majority of AML patients. Targeting of this intracellular protein is currently investigated by Dendritic Cell (DC) vaccination and T-cell receptor (TCR)-transgenic T cells[25–27]. However, a major challenge of all target antigens and therapy platforms is downregulation of the target antigen in response to immunotherapy. This might be overcome by dual targeting approaches in

which T-cell bispecific antibodies (TCBs) or CAR-T cells with different target specificity are combined. In addition, conditional targeting of two antigens might increase specificity and lower the risk of on-target off tumor toxicity. Several possible target antigen combinations have already been identified including CD33/TIM3 and CLL-1/TIM3, based on the expression profile on AML compared to healthy cells[21].

Alternatively, targeting neoepitopes from chromosomal rearrangements and gene mutations can increase leukemia-specificity even further. Suitable tumor-restricted epitopes have already been reported from fusion proteins DEK-CAN and PML-RARα, as well as mutations in FLT3 and NPM1[20]. As these oncogenes are expressed intracellularly, the presentation is restricted to defined HLA molecules and is often limited in the amount of molecules presented on the cell surface. Identification of suitable neoepitope targets is therefore challenging[28]. However, several neoantigens derived from the mutations A and D of NPM1 have been confirmed to be presented on HLA-A*02:01. TCR-transgenic T cells targeting CLAVEEVSL presented on NPM1-mutated OCI-AML3 cells were reported to be functional *in vivo*[29].

Despite all progress, HLA-restriction and dependency on recurrent mutations limit the development and broad application of immunotherapeutic approaches targeting neoantigens. Novel immunotherapeutic targets in AML are therefore urgently awaited. Using an optimized multiomic approach, we aimed for an unbiased identification of novel immunotherapeutic targets expressed on AML cells, further described in section 1.5.2.

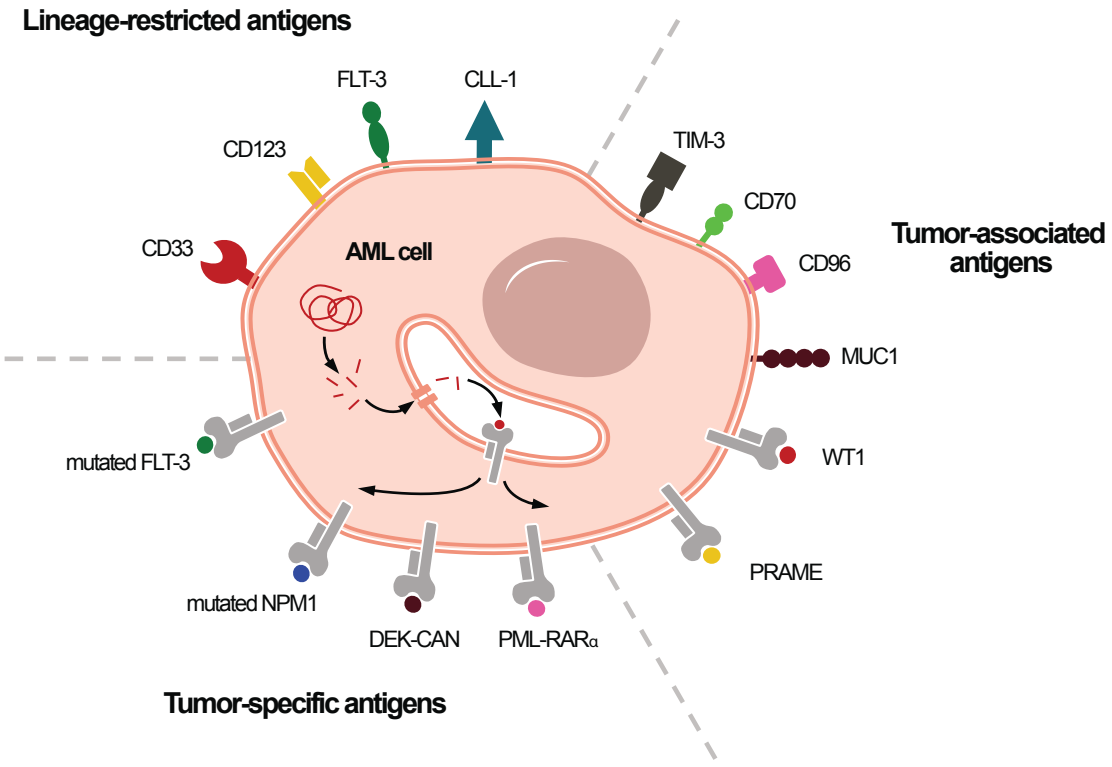

Figure 1: Examples for tumor antigens in AML. Target antigens can be grouped into lineage-restricted antigens, tumor-associated antigens overexpressed by tumor cells compared to healthy tissue and tumor-specific antigens, also known as neoepitopes generated by gene mutations and chromosomal rearrangements. Figure created according to Daver et al[20].

1.3 The toolbox of T-cell target immunotherapy in AML

Numerous immunotherapy concepts, including monoclonal antibodies mediating antibody-dependent cellular cytotoxicity (ADCC) and antibody drug conjugates (ADCs) have been developed for treatment of cancers. In addition, T cells have gained major research interest in the development of cancer therapies, as they are considered to have a major role in anti-tumor immune responses. This has been especially demonstrated by the observation that lymphocytes and IFN-γ dependent-effector functions collaborate in the formation of tumor-specific immune surveillance[30–32]. T-cell targeted immunotherapy platforms include (1) DC vaccination, which aims to induce tumor-specific T-cell responses, (2) immune checkpoint inhibitors, which reinvigorates pre-existing T-cell responses, and also (3) T-cell bispecific antibodies and (4) adoptive T-cell therapies mediating tumor-specific cell lysis by T cells (Figure 2)[20].

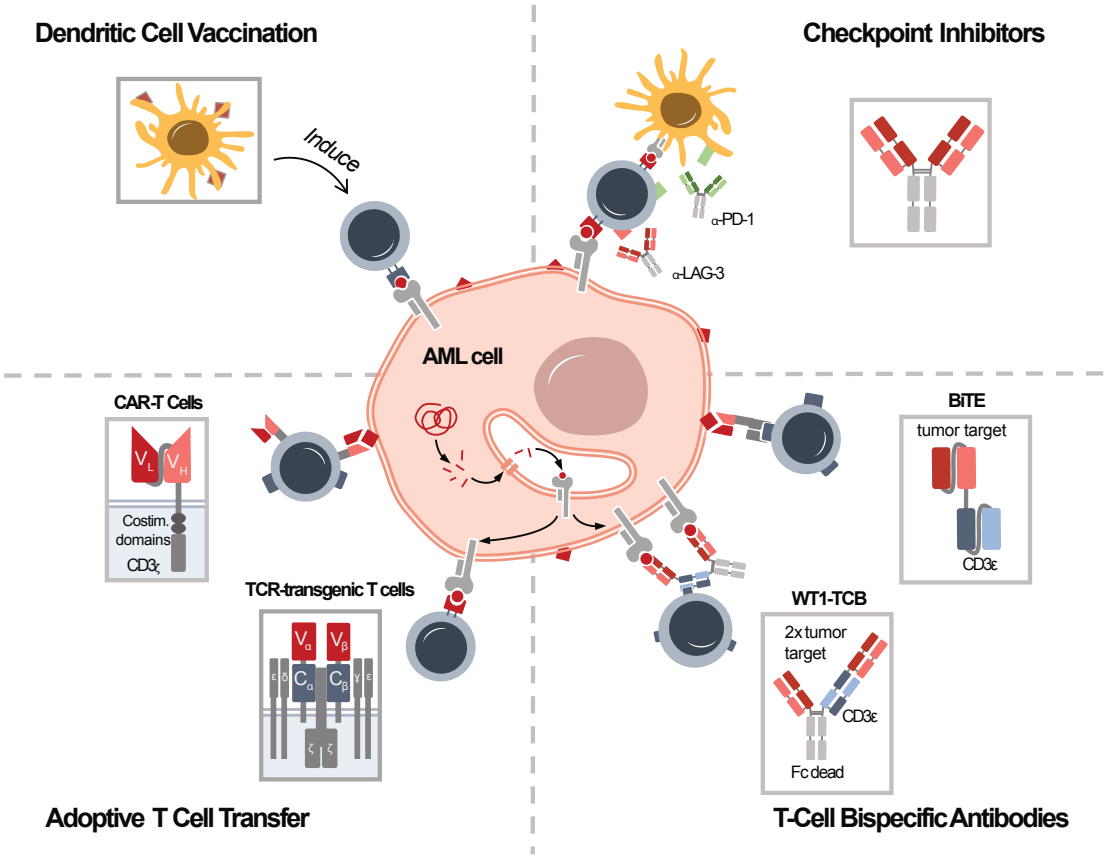

Figure 2: Overview of T-cell targeted immunotherapy in AML. DC vaccination aims for induction of novel tumor-specific immune responses against intracellular target antigens like WT1, PRAME and NPM1[33]. Checkpoint inhibitors targeting PD1/PD-L1, LAG3/HLA-DR or other inhibitory immune signaling pathways can enhance pre-existing immune responses. T-cell bispecific antibodies activate and recruit T cells to tumor cells by simultaneous binding to the T-cell receptor and extracellular (e.g. CD33, CD123) or intracellular (WT1 presented on HLA-A*02) targeting antigens on AML cells, which leads to target cell lysis. Adoptive T cell transfer consists of *in vitro* manipulated and expanded T cells modified with two main techniques. TCR-transgenic T cells express a TCR targeting intracellular antigens presented on HLA molecules. CAR-T cells recognize extracellular target antigens by a fusion of an antibody-derived single-chain variable fragment and an intracellular T-cell signaling domain[20,26]. Figure modified from Subklewe[34].

1.3.1 DC vaccination

DCs are professional antigen-presenting cells that orchestrate adaptive and innate immunity by presenting antigens on major histocompatibility complex class I and II, thereby activating different types of immune effector cells. Antigen cross-presentation on MHC class I leads to the induction of cytotoxic $CD8^+$ T lymphocytes (CTLs), whereas antigens presented in the context of MHC class II lead to the induction of $CD4^+$ T helper (Th) cell responses, which is furthermore shaped by adjunct cytokines released by the DCs. In addition, DCs are activating NK cells by secreting cytokines hence resulting in a multifaceted immune response with involvement of various immune effector cell types[35]. Because of this central role in the immune response, DCs have become into focus for the development of cancer immunotherapies. Furthermore, DCs have the potential to elicit immune responses against tumor-specific neoantigens resulting in improved specificity of immunotherapy approaches[36].

DC vaccinations aim for the induction of novel immune responses and amplification of pre-existing immune responses that might have been dampened by the tumor and the tumor microenvironment. Two major strategies of DC vaccinations are existing: (1) *in situ* targeting of DCs with off-the shelf therapies and (2) canonical vaccination with DCs prepared and modified *ex vivo*[33]. Various *in situ* vaccination strategies have been evaluated, aiming among others for stimulation of DCs by stand-alone administration of Toll-like receptor (TLR) ligands or agonistic antibodies targeting CD40 or Dec205[37,38]. Moreover, vaccinations with tumor-associated or tumor-specific peptides have been evaluated, however with only limited clinical efficacy due to their low immunogenicity and the requirement of adjuvant coadministration[39]. Another approach are antibody-antigen fusion constructs combining DC stimulation and peptide vaccination in a single molecule. A construct of an agonistic αCD40 antibody fused to the TLR5 agonist flagellin and an antigen domain activated DCs and antigen-specific T cells *in vitro*[40]. Similarly, a cDC1-targeting vaccine, consisting of an αCD103 antibody domain fused to a cholera toxin adjuvant and an ovalbumin peptide mediated anti-tumor immunity in a mouse model[41]. In addition, RNA encapsulated into lipoplexes (RNA-LPX) has been shown to efficiently target DCs *in situ* leading to IFN-α secretion by plasmacytoid DCs and induction of strong antigen-specific T cell responses *in vivo*[42].

In contrast, canonical vaccination approaches involve DCs activated and modified *ex vivo*, aiming for the induction of a tumor-antigen specific T-cell response in the lymph node and subsequent anti-tumor activity[43] (Figure 3A). Most clinical trials in AML have evaluated autologous monocyte-derived DCs (moDCs), differing in the duration of their differentiation and cytokines used, as well as the route of antigen loading, DC administration, and tumor target antigens[44]. Administration of DCs has been demonstrated to be overall safe and to induce antigen-specific immune responses in numerous clinical trials[44]. A clinical trial with mRNA-electroporated moDCs, generated in three days by the addition of GM-CSF, IL-4 and activation with the TLR-7/8 ligand R848, induced antigen-specific immune responses in AML patients and was associated with a favorable safety profile[27]. Whereas peptide-pulsing or mRNA electroporation for antigen loading results in pre-defined antigen-specific immune responses, other approaches are better suited to account for the heterogeneity of tumor cells resulting in potentially higher antitumor immunogenicity[45]. A clinical trial investigating hybridomas of autologous moDCs and AML cells observed a persistent rise in leukemia-specific T cells[46]. Another approach is the use of leukemia-derived DCs (DCleu) differentiated from leukemic blast cells[45]. Nevertheless, moDCs have been shown to be superior in activating antigen-specific T cells compared to DCleu. A reason might be a reduced expression of co-stimulatory molecules on DCleu and a higher tolerogenic potential due to expression of

IDO-1 on leukemic blast cells[45,47]. However, moDCs and DCleu require extensive *ex vivo* manufacturing processes potentially negatively affecting effector functions and objective clinical responses have just been observed in a minority of patients[48].

Thus, other DC sources have gained research interest. Primary blood DCs (BDCs) are considered ideal because they differentiate *in vivo* and require only a short manipulation *ex vivo* for activation and antigen loading. They are therefore considered to better retain their functional capacities and to survive longer *in vivo*[49]. BDCs can be subdivided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs)[50]. The latter are involved in anti-viral immune responses through secretion of type I interferons after activation of TLR7 or TLR9[51]. cDCs can be further divided into subpopulations: cDC1s are a rare population among BDCs that express CLEC9A, which is promoting antigen-cross presentation to CTLs[52–54]. In contrast, cDC2s are expressing TLRs involved in sensing, among others, bacteria and fungi and can induce different T-helper cell responses[55]. Due to their versatile capabilites, BDCs are believed to elicit a wider range of immune responses when applied in vaccination approaches compared to moDCs (Figure 3B). First clinical trials have evaluated cDCs and pDCs in solid tumors and observed induction of antigen-specific immune responses. In melanoma patients, Tick-borne encephalitis-activated pDCs secreted high levels of type I IFNs, thereby triggering a tumor-specific immune response[56]. Furthermore, GM-CSF activated cDC2s induced antigen-specific T cell responses in some patients with metastatic melanoma, albeit the immune response might be optimized by an improved activation protocol[57]. However, the combination of all three BDC subsets could potentially further improve induction of anti-tumor immune responses by taking advantage of the individual effector functions conveyed by each BDC subset[49]. Clinical trials combining pDCs and cDC2s are currently evaluated in melanoma and prostate cancer patients, however no results have been published yet[58]. Furthermore, these clinical trials do not account for cDC1s that are considered to be important due to their antigen cross-presentation capacities[59]. We have therefore aimed to develop a tailored protocol for simultaneous activation of all BDCs subsets to trigger strong and robust antigen-specific immune responses further described in section 1.5.1.

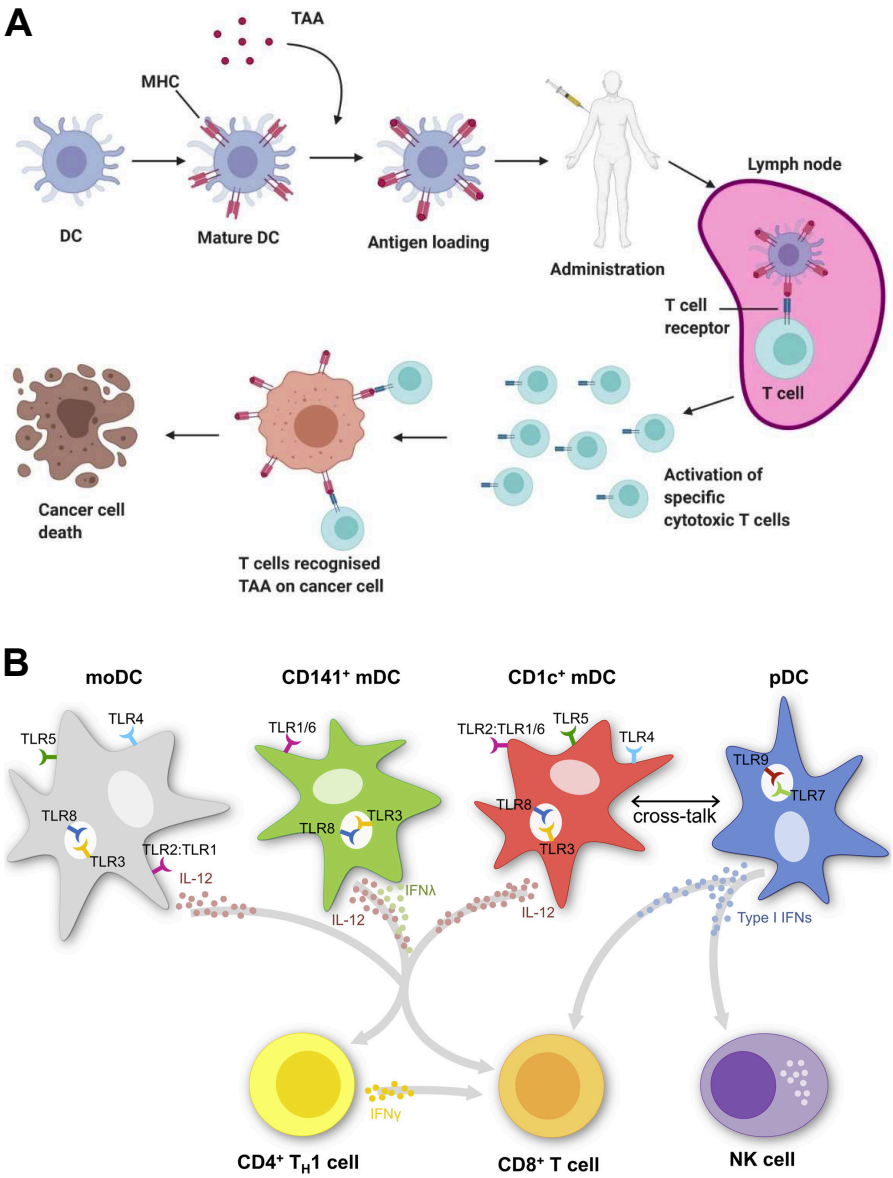

Figure 3: (A) Canonical vaccination with *ex vivo* modified DCs. Re-administration of DCs aims for T-cell priming in the lymph node, leading to the generation of a tumor-antigen specific T-cell response and subsequent anti-tumor activity[43]. **(B)** Comparison of DC subsets evaluated for DC vaccination approaches. *Ex vivo* generated moDCs have been studied in the majority of clinical trials. In contrast, BDCs are fully differentiated and are divided into pDCs, cDC1s and cDC2s, whereby each subset confers different effector functions[58]. Figures adopted from Alard et al. and Bol et al.[43,58].

1.3.2 T-cell bispecific antibodies

T-cell bispecific antibodies (TCBs) exploit the property of T cells to mediate specific cell lysis by simultaneously binding the T-cell receptor (CD3) of the T cell and a tumor antigen. Thereby, T cells are brought close to the tumor cell and activated, leading to subsequent cell lysis by granule-mediated pathways (i.e. perforin/granzyme) and death-receptor pathways, regardless of T-cell specificity (Figure 4A)[60–63]. Moreover, TCBs have been demonstrated to mediate killing of bystander cells lacking target antigen expression, reducing tumor escape by TAA-negative cancer cells[63]. Several different formats of TCBs have been developed (Figure 4B). The most prominent representatives of this antibody class are bispecific T-cell engagers (BiTEs), which comprise two single-chain variable fragments (scFv) connected by a short flexible linker[64]. Blinatumomab, a CD19xCD3 BiTE was the first approved T-cell bispecific antibody in hematologic malignancies, used for treatment of acute lymphoblastic leukemia (ALL)[65]. In order to translate the success of Blinatumomab, several different TCB formats targeting especially lineage antigens are currently evaluated for the treatment of AML[20].

CD33 is among the most prominent antibody targets in AML due to its ubiquitous expression on AML cells and increased expression on leukemic stem cells[66], leading to the development of the antibody drug conjugate gemtuzumab ozogamicin (GO) that became part of the standard therapy in AML[23]. Accordingly, a CD33-targeting BiTE (AMG 330) has been developed showing promising results *ex vivo* and was investigated in a phase I clinical trial (NCT02520427)[66–68]. Moreover, tandem diabodies specific for CD33 are evaluated[22]. Another common lineage marker is CD123, which is expressed in about 60-80% of AML patients with increased prevalence in patients that were resistant to primary induction therapy or that experienced an early relapse[69,70]. Flotetuzumab, a dual affinity retargeting antibody (DART), is investigated at the moment in a phase I/II clinical trial showing first promising results[70,71]. Further prominent target antigens for TCBs currently under investigation in AML are among others CLL-1, FLT-3 and TIM-3[72–74].

A major innovation in improving tumor specificity is the development of TCBs targeting peptides derived from tumor-associated antigens in the context of MHC, comparable to a native TCR-peptide-MHC interaction. These TCR-like antibodies cannot only target intracellular proteins greatly extending the pool of possible tumor targets but can also target mutation-associated neoantigens. Recently, antibodies targeting mutated p53 and *RAS* neoantigens have been described by using classical phage display[75–77]. In contrast, immune-mobilizing monoclonal TCRs against cancer (ImmTACs) represent an alternative platform to target peptide-MHC complexes. ImmTACs consist of an affinity-enhanced T-cell receptor recognizing the tumor target, fused to an anti-CD3 domain[78]. Tebentafusp is the first representative of its class approved recently for uveal melanoma, targeting gp100 in the context of HLA-A*02:01[79]. Another prominent target for TCR-like TCBs is Wilms' Tumor 1 (WT1) further described in section 1.4.

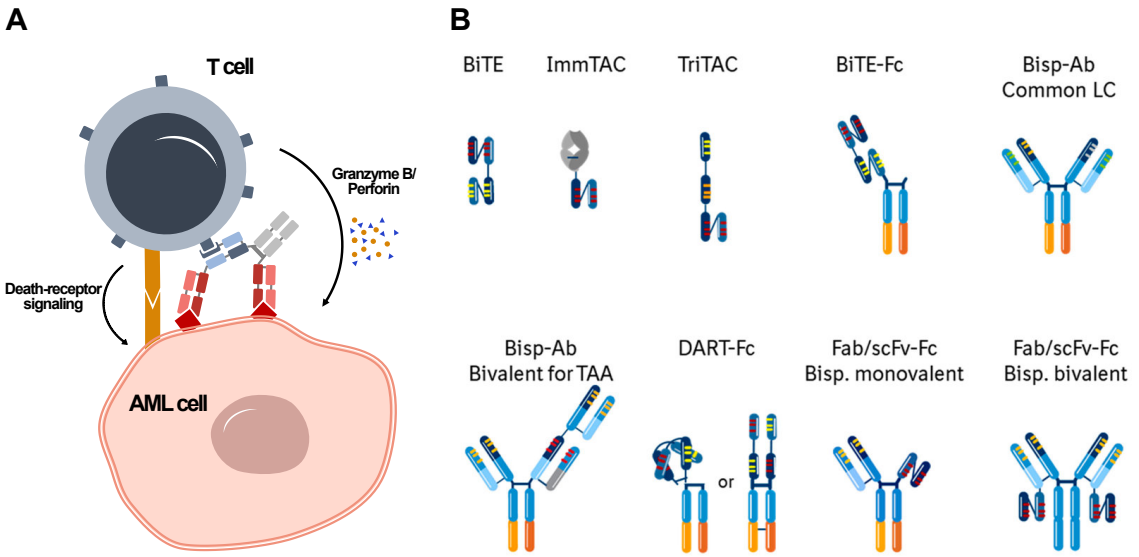

Figure 4: (A) Mode of action of a TCB with bivalent target antigen binding mediating AML cell lysis by Granzyme B and Perforin secreted by T cells and death receptor pathways. Figure created according to Baeuerle et al[62] and Ross et al[63]. **(B)** Overview of common TCB formats. BiTEs consist only of two scFvs and have gained major interest by the approval of Blinatumomab. Addition of an Fc-part results in half-life extended BiTEs with lower serum clearance rates in humans. Tebentafusp is an ImmTAC molecule consisting of an affinity-matured TCR fused to an anti-CD3 domain. Advanced antibody engineering also allows the generation of multivalent antibodies with multiple tumor-target binding sites[80]. Figure adopted from Voynov et al.[80].

1.4 The tumor oncogene Wilms' Tumor 1

Wilms' Tumor 1 (WT1) is a transcriptional and post-transcriptional regulator that has been initially described to be involved in the formation of Wilms' tumor, a pediatric kidney cancer type. WT1 is only expressed in few healthy tissues but in a variety of solid tumors and about 90% of acute leukemias. WT1 expression is correlating with the abundance of AML blasts with high expression at initial diagnosis and in relapse or refractory AML in contrast to complete remission[81]. Therefore, WT1 has been investigated as a marker for minimal residual disease (MRD) monitoring from bone marrow but also peripheral blood[82,83].

The WT1 gene encodes ten exons and is translated into numerous isoforms caused by alternative splicing, transcription start sites and the use of an alternative start codon upstream of the actual ATG start codon (Figure 5A). WT1 encodes for an activation and a repression domain, as well as four Kruppel-like zing finger domains located at the C-terminus, involved in DNA and RNA binding[84]. Four major isoforms (A–D) have been identified that differ in a 17 amino acid insertion in exon 5 and a three amino acid insertion termed KTS in exon 9[85,86]. The +KTS isoforms have been found to increase cell growth, whereas the +17aa isoforms are considered to have antiapoptotic function[87,88]. The exact functional relevance of the isoforms remains however unclear.

WT1 has been initially described as a tumor suppressor due to its role in Wilms' Tumor, where a germline mutation and inactivation of WT1 promotes the disease[84]. In contrast, WT1 has been shown to provide oncogenic function as observed by abnormal expression in tumor tissues. This is furthermore underlined by the observation that silencing of WT1 in response to siRNAs leads to cell cycle arrest, increased apoptosis and reduced cell growth[87,89].

The role of WT1 in tumorigenesis in combination with its disease restricted expression profile, including overexpression in leukemic stem cells, has led to the investigation of WT1 as a target in different immunotherapeutic approaches[90]. Numerous clinical trials have evaluated WT1 as tumor target in peptide- and DC-vaccinations observing objective clinical and immunological responses against various epitopes in a majority of patients with hematologic malignancies underlining furthermore the immunogenicity of WT1[91]. Accordingly, WT1 has been ranked highest in a prioritization of cancer vaccine antigens based among others on therapeutic function, immunogenicity, oncogenicity, specificity and expression levels[92]. WT1 is also studied as a target for T-cell based immunotherapies, most commonly targeting the WT1 derived peptide RMFPNAPYL (RMF peptide) presented on HLA-A*02. Adoptive transfer of *ex vivo* generated $CD8^+$ T cell clones after SCT showed antileukemic activity in some leukemia patients, especially when T cell clones were generated in the presence of IL-21[93]. Since avidity of T cell clones for WT1 was varying, this approach was later refined by the use of TCR-transgenic T cells expressing a well characterized high affinity TCR. A prophylactic treatment of AML patients post SCT with these TCR-transgenic T cells showed after a median follow-up of 44 months a remarkable relapse-free survival rate of 100%, while a control group exhibited a significant higher relapse-rate[94].

At the same time, efforts have been made to generate an antibody targeting WT1, which is challenged by the fact that only WT1-derived peptides presented on MHC molecules are available for antibody targeting, due to the intracellular localization of WT1. Nevertheless, an antibody termed ESK1 targeting the RMF peptide was generated by phage display and showed high avidity and *in vivo* activity against tumor cells[95]. This antibody has been subsequently converted into a BiTE-like antibody showing efficacy against leukemias and solid tumors. Analysis of the crystal

structure of ESK1 binding HLA-A*02 and alanine scanning revealed however that this antibody is almost exclusively recognizing peptide residue 1 of the RMF peptide and binds twisted to the peptide-MHC complex compared to a TCR, resulting in low specificity[96,97]. These problems were solved by the WT1-TCB, where I was involved in the preclinical characterization together with my colleague Christian Augsberger[81]. The WT1-TCB is a 2+1 TCB engineered using Crossmab technology with bivalent targeting of the $WT1_{RMF}$/HLA-A*02 complex and monovalent affinity for the T cell (Figure 5B). WT1-TCB mediates efficient killing of primary AML cells by allogenic healthy donor T cells and autologous T cells, as well as inhibition of tumor growth in AML patient-derived xenograft mouse models[81]. Analysis of the crystal structure showed in contrast to ESK1, a TCR-like binding to the peptide-MHC complex with high contribution of the peptide to antibody binding. Accordingly, peptide-specificity screenings and experiments for on-target off-tumor cytotoxicity of $CD34^+$ stem cells confirmed the antibody specificity[81]. Furthermore, RMF peptide presentation was confirmed for the first time on a primary AML sample by mass spectrometry after peptide enrichment using the WT1-TCB[81]. These promising results therefore led to the initiation of the first clinical trial investigating a TCR-like antibody (NCT04580121).

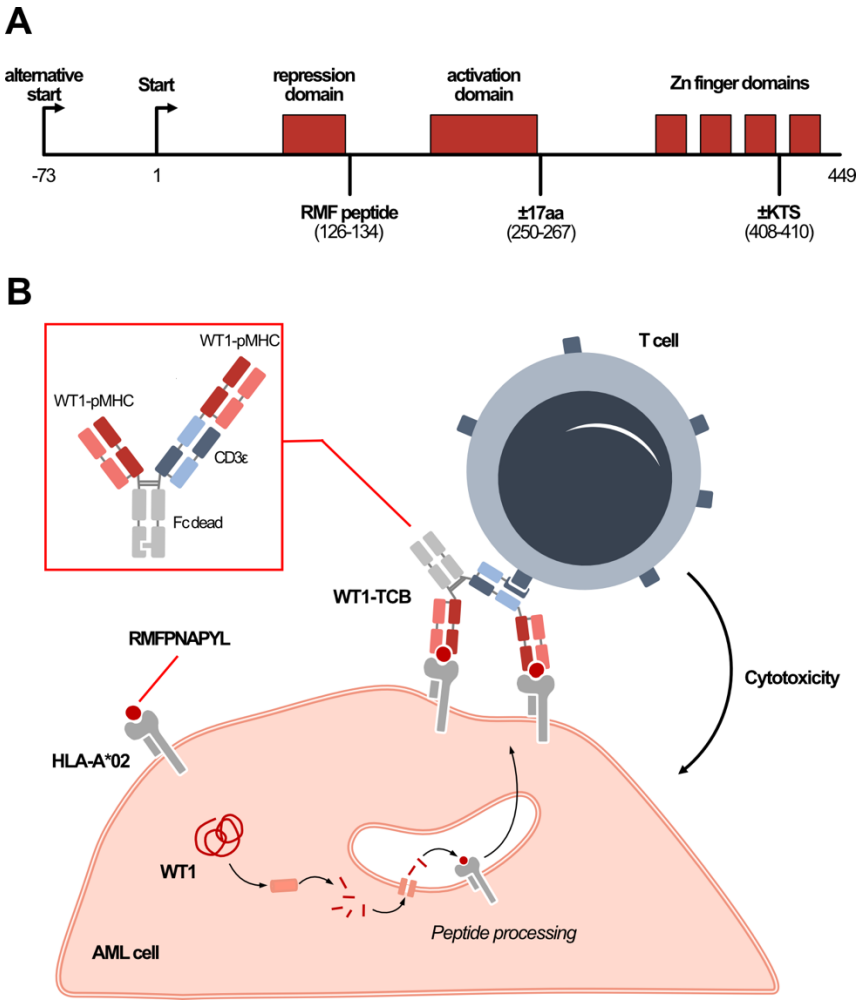

Figure 5: (A) Schematic representation of the WT1 protein indicating the most important functional domains and the location of the RMF peptide at amino acid position 126-134. Different isoforms arise by the use of an alternative translation start site and the insertion of the KTS motif. Figure created according to Yang et al[86]. **(B)** Mode of action of the WT1-TCB targeting the RMF peptide presented on HLA-A*02. WT1-TCB bivalently recognizes the tumor antigen and has monovalent affinity for CD3ε. Adopted from Augsberger, Hänel et al[81].

1.5 Summary of publications

1.5.1 Publication I: Blood DCs activated with R848 and poly(I:C) induce antigen-specific immune responses against viral and tumor-associated antigens

In the first publication we evaluated Blood DCs (BDCs) as an alternative to moDCs for vaccination approaches. To this end, we first screened different combinations of synthetic TLR agonists and activation protocols for optimal activation of all BDC subsets simultaneously. We found that a combination of TLR ligands is required to induce maximal cytokine secretion. While CpG+poly(I:C) induced highest IFN-α secretion, R848+poly(I:C) was optimal for high IL-12p70 secretion. Since we aimed especially for induction of Th1 immune responses mediated by IL-12p70, we focused in our subsequent analysis on R848+poly(I:C); an activation cocktail that also led to intermediate IFN-α secretion. We furthermore analyzed, how the duration of BDC activation affects cytokine secretion of BDCs and the T-cell stimulatory capacity. We observed that BDCs secrete IFN-α already 3 h after activation with R848+poly(I:C), whereas IL-12p70 secretion was found to occur at later time points.

In addition, we evaluated activation of BDC subsets separately with tailored TLR agonists in comparison to simultaneous activation of all BDCs. We found that BDCs secreted higher amounts of IL-12p70 and induced stronger T-cell responses than cDCs, pDCs or a combination of cDCs and pDCs suggesting a cross-talk of BDC subsets during activation. In a final step, we observed that activation of BDCs with R848+poly(I:C) significantly improves BDC migration and activation of NK cells. Moreover, activation of BDCs improved the expansion of T cells specific for viral antigens, as well as the tumor oncogene WT1.

I contributed to this paper by conducting and analyzing all experiments and generating the entire data published in the manuscript. Furthermore, I wrote the first draft of the manuscript and conveyed the review process until final publication.

1.5.2 Publication II: Integrated multiomic approach for identification of novel immunotherapeutic targets in AML

Since AML is a highly heterogenous disease identifying suitable target antigens for immunotherapy is challenging. By using a novel multiomic approach based on mass spectrometry we aimed for an unbiased approach to identify novel target antigens in AML. The Cell Surface Capture (CSC) technology relies on biotinylation of lysine residues (Lys-CSC) or glycosylation sites (Glyco-CSC) of proteins on the cell surface and subsequent cell lysis. The labeled proteins are then enriched by Streptavidin and analyzed by liquid chromatography-mass spectrometry (LC-MS)[98,99]. However, the application of this technology with primary AML samples is hampered by high number of viable cells needed for the analysis. Therefore, this method has until now only been successfully performed using cell lines or xeno-amplified ALL patient samples.

In an attempt to use the CSC technology with primary AML cells, we optimized the protocol using OCI-AML3 cells for protein recovery by improving cell homogenization and protein digestion. Furthermore, we observed in a direct comparison that most proteins were detected by Glyco-CSC, while only few additional proteins were identified by using Lys-CSC or a combination of

both methods (Cys-Glyco-CSC; labeling of cysteine residues and glycosylation sites). In a next step, we applied the improved Glyco-CSC protocol on patient-derived AML xenograft samples (AML-PDX samples), as well as primary AML samples pre-cultivated *ex vivo* for three days on irradiated MS-5 feeder cells. We detected 621 surface proteins with some proteins being exclusively expressed on either OCI-AML3 cells or primary AML cells. Moreover, the number of detected proteins was similar between primary AML samples and AML-PDX samples although analyzing higher cell numbers for the latter. Next, the detected proteins were filtered for putative immunotherapeutic targets by excluding proteins highly expressed on hematopoietic stem and progenitor cells, monocytes and non-hematopoietic tissues, as wells as proteins that were only identified in less than half of the samples. The resulting list of 76 proteins was then further analyzed based on a large patient cohort for their rate of nonsynonymous mutations to identify target proteins likely functionally relevant for leukemogenesis. Expression of five novel putative targets was subsequently validated using unrelated primary AML samples by flow cytometry, observing three of five targets to be consistently expressed. Thereof, CD148 and ITGA4 were found to be additionally expressed on granulocytes and monocytes or hematopoietic stem- and progenitor cells, respectively. In contrast, Integrin beta-7 was not or only lowly expressed on healthy hematopoietic cells but uniformly expressed on AML cells, therefore representing a promising new target antigen.

I prepared and expanded primary AML samples on MS-5 feeder cells which was necessary to obtain the sufficient cell numbers for subsequent analysis. I performed and analyzed the flow cytometry data and participated in concept and writing of the full-text publication.

2. Publications

2.1 Publication I

Cancer Immunology, Immunotherapy
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ORIGINAL ARTICLE

Blood DCs activated with R848 and poly(I:C) induce antigen-specific immune responses against viral and tumor-associated antigens

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Abstract
Monocyte-derived Dendritic cells (DCs) have successfully been employed to induce immune responses against tumor-associated antigens in patients with various cancer entities. However, objective clinical responses have only been achieved in a minority of patients. Additionally, generation of GMP-compliant DCs requires time- and labor-intensive cell differentiation. In contrast, Blood DCs (BDCs) require only minimal ex vivo handling, as differentiation occurs in vivo resulting in potentially better functional capacities and survival. We aimed to identify a protocol for optimal in vitro activation of BDCs including the three subsets pDCs, cDC1s, and cDC2s. We evaluated several TLR ligand combinations and demonstrated that polyinosinic:polycytidylic acid [poly(I:C)] and R848, ligands for TLR3 and TLR7/8, respectively, constituted the optimal combination for inducing a positive co-stimulatory profile in all BDC subsets. In addition, TLR3 and TLR7/8 activation led to high secretion of IFN-α and IL-12p70. Simultaneous as opposed to separate tailored activation of pDCs and cDCs increased immunostimulatory capacities, suggesting that BDC subsets engage in synergistic cross-talk during activation. Stimulation of BDCs with this protocol resulted in enhanced migration, high NK-cell activation, and potent antigen-specific T-cell induction.
We conclude that simultaneous activation of all BDC subsets with a combination of R848 + poly(I:C) generates highly immunostimulatory DCs. These results support further investigation and clinical testing, as standalone or in conjunction with other immunotherapeutic strategies including adoptive T-cell transfer and checkpoint inhibition.

Keywords Immunotherapy · Blood dendritic cells · Toll-like receptors · Plasmacytoid dendritic cells · Conventional dendritic cells

Abbreviations

AML	Acute myeloid leukemia
BDC	Blood dendritic cell
cDC	Conventional dendritic cell
CEF	Cytomegalovirus (CMV), Epstein–Barr virus (EBV), influenza
CEFT	Cytomegalovirus (CMV), Epstein–Barr virus (EBV), influenza, tetanus
CpG	CpG oligodeoxynucleotide
GM-CSF	Granulocyte–macrophage colony-stimulating factor
HS	Human serum
MFI	Median fluorescence intensity
moDC	Monocyte-derived dendritic cell
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
poly(I:C)	Polyinosinic:polycytidylic acid
R848	Resiquimod
TLR	Toll-like receptor
WT1	Wilms tumor 1

Felix S. Lichtenegger and Marion Subklewe contributed equally to this work.

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Introduction

Generating, enhancing, and maintaining tumor-specific immune responses is a major challenge in the search for cures for cancer. Dendritic cells (DCs) orchestrate innate and adaptive immunity, thereby inducing tailored, strong, and durable immune responses. They have therefore been extensively evaluated as an immunotherapeutic tool, including various in vivo targeting approaches [1, 2]. However, most pre-clinical and clinical studies have focused on ex vivo DC activation and antigen-loading approaches [3, 4].

Numerous clinical trials have evaluated monocyte-derived DCs (moDCs) against different cancer entities, including multiple myeloma and Acute myeloid leukemia (AML). Their overall safety and induction of tumor-specific immune responses have been demonstrated [4–8]. However, objective clinical responses have only been obtained for a minority of patients [9, 10]. In addition, the generation of moDCs is labor- and time-consuming as the differentiation of monocytes into DCs takes several days. Furthermore, transcriptional comparisons of moDCs and naturally occurring DC subsets indicate great functional differences [11].

Therefore, other sources of DCs have recently been evaluated. Primary blood DCs (BDCs) are hypothesized to be ideal candidates for inducing anticancer immune responses since they differentiate in vivo and require only brief ex vivo handling, resulting potentially in better preservation of functional capacities and longer in vivo survival [12]. At least three BDC populations can be distinguished: $CD11c^+$ $CD141^+$ $CLEC9A^+$ conventional DCs (cDC1s), $CD11c^+$ $CD1c^+$ cDC2s, and $CD11c^-$ $CD303^+$ $CD123^+$ plasmacytoid DCs (pDCs) [13].

pDCs are specialized to recognize viral infections via toll-like receptor (TLR) 7 or TLR9, which results in strong production of type I interferons (IFNs). These cytokines modulate adaptive and innate immunity by inducing, for example, NK-cell activation, B-cell differentiation, and Th1 polarization [14]. In contrast, cDC2s express TLRs that recognize lipopolysaccharides (TLR4), flagellin (TLR5), and the lipoproteins of bacteria and fungi (TLR2 and TLR6). They also express TLR8 and TLR9 that are activated by single-stranded RNA and DNA, respectively. Thus, different $CD4^+$ T helper subsets and $CD8^+$ T cells are induced depending on the circumstances [15]. cDC1s represent a rare BDC subset that expresses, among others, TLR3 that is activated by viral double-stranded RNAs leading to secretion of interleukin (IL) 12 [16]. They also express TLR8, which is closely related to TLR7, and the endocytic receptor CLEC9A that promotes antigen cross-presentation of intracellular pathogens or necrotic cells on MHC class I, thereby activating cytotoxic $CD8^+$ T cells [17]. cDC1s have therefore attracted much interest in the context of cancer immunotherapy [18].

Owing to their multifaceted properties, BDCs are considered to induce a greater diversity of immune responses compared to moDCs when used in vaccination approaches. The first clinical trials evaluating pDCs and cDCs in solid tumors have already demonstrated safety and, to some extent, induction of tumor-specific immune responses. Tick-borne encephalitis vaccine-activated pDCs demonstrated a mature phenotype and produced large amounts of type I IFNs in melanoma patients, inducing tumor-specific immune responses [19]; this is in contrast to antigen-loaded, but non-activated cDC2s administered to prostate cancer patients [20]. In a clinical trial of GM-CSF-activated cDC2s, three of 14 metastatic melanoma patients exhibited functional tumor-specific T cells. However, the authors postulated that immune responses might be optimized by a better-tailored activation stimulus [21]. In line with this hypothesis, we evaluated different protocols for in vitro activation of BDCs based on TLR ligands.

Here, we report the first protocol for achieving simultaneous in vitro activation of all BDC subsets based on activation of TLR3 and TLR7/8 with polyinosinic:polycytidylic acid [poly(I:C)] and R848 (Resiquimod), respectively. Our data suggest this to be the optimal combination for inducing a positive co-stimulatory profile in all BDC subsets, high secretion of IFN-α, and maximal secretion of IL-12p70. Activating all BDC subpopulations together increased immunostimulatory capacities compared to separately activating pDCs and cDCs with tailored protocols, indicating a synergistic cross-talk between BDC subsets during activation. Moreover, we demonstrate that activation of BDCs with this protocol results in enhanced migration, high NK-cell activation, and potent antigen-specific T-cell induction.

Methods

Healthy donors

Heparinized peripheral blood was collected from healthy donors after informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig-Maximilians-Universität (Munich, Germany).

Isolation and culturing of BDCs

BDCs were isolated from peripheral blood mononuclear cells (PBMCs) using the human Blood Dendritic Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in DC medium [X-Vivo 15 medium (Lonza, Verviers, Belgium), 2% human serum (HS;

Sigma–Aldrich, Steinheim, Germany), 800 U/mL GM-CSF, 10 ng/mL IL-3 (both Peprotech, Rocky Hill, NJ, USA)].

Unless otherwise indicated, BDCs were activated for 20 h with 0.5 µM CpG (ODN 21,798, Miltenyi Biotec), 5 µg/mL R848, 25 µg/mL poly(I:C)-LMV (both Invivogen, Toulouse, France), 5 ng/mL IFN-γ (Peprotech), 2 µg/mL CD40L (BioCat, Heidelberg, Germany), 5 µg/mL protamine-RNA or combinations thereof.

Protamine-RNA complexes were formed as previously described [22] using mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific, Vilnius, Lithuania) for mRNA generation from the supplied control template.

Flow cytometry and cytokine quantification

Expression of immune checkpoint molecules on BDCs was assessed using Aqua-LIVE/DEAD (Life Technologies, Eugene, OR, USA) and the antibodies and isotype controls listed in Table 1. Median fluorescence intensity (MFI) ratios were calculated based on respective isotype controls.

Cytokine concentrations were quantified using the human MACSPlex Cytokine 12 Kit (Miltenyi Biotec).

Flow cytometry measurements were performed on a CytoFLEX S (Beckman Coulter, Krefeld, Germany). Data were analyzed using FlowJo v10.7 (BD, Ashland, OR, USA) and Prism v9.0.1 (GraphPad Software, San Diego, CA, USA). All stated values are reported as mean ± SEM.

Table 1 Antibodies used for characterization of BDC immune checkpoints

Type	Antigen	Dye	Clone	Manufacturer
Lineage markers	CD3	VioGreen	REA613	Miltenyi Biotec
	CD11c	PE/Vio770	REA618	Miltenyi Biotec
	CD14	VioGreen	REA599	Miltenyi Biotec
	CD19	VioGreen	REA675	Miltenyi Biotec
	CD123	APC/Vio770	REA918	Miltenyi Biotec
	CD141	PerCP/Vio700	REA674	Miltenyi Biotec
Immune checkpoints	CD40	APC	REA733	Miltenyi Biotec
	CD70	FITC	REA292	Miltenyi Biotec
	CD80	PE	REA661	Miltenyi Biotec
	CD86	VioBlue	REA968	Miltenyi Biotec
	CD197	APC	REA546	Miltenyi Biotec
	CD252	PE	11C3.1	Biolegend
	CD270	PE	RE247	Miltenyi Biotec
	CD273	FITC	RE985	Miltenyi Biotec
	CD274	BV421	MIH3	Biolegend
	CD275	PE	REA991	Miltenyi Biotec
	CD276	FITC	REA1094	Miltenyi Biotec
	CD279	BV421	EHA12.2H7	Biolegend
	B7-H4	FITC	MIH43	AbD Serotec
	B7-H5	APC	730,804	R&D
	Gal-9	FITC	REA435	Miltenyi Biotec
	GITRL	APC	REA841	Miltenyi Biotec
	HLA-DR	VioBlue	REA968	Miltenyi Biotec
Isotype controls	mIgG1	BV421	MOPC-21	Biolegend
	mIgG1	FITC	MOPC-21	Biolegend
	mIgG1	PE	MOPC-21	Biolegend
	mIgG2b	APC	MPC-11	Biolegend
	REA control (S)	APC	REA293	Miltenyi Biotec
	REA control (S)	FITC	REA293	Miltenyi Biotec
	REA control (S)	PE	REA293	Miltenyi Biotec
	REA control (S)	VioBlue	REA293	Miltenyi Biotec

Isolation and culturing of pDCs and cDCs

pDCs were isolated from PBMCs by using the human CD304 MicroBead Kit, followed by isolation of cDCs from the flow-through by using the human Myeloid Dendritic Cell Isolation Kit (both Miltenyi Biotec). Alternatively, cDCs were isolated from whole PBMCs. pDCs were cultured for 16 h at 37 °C in DC medium, followed by stimulation with CpG for 3 h. cDCs were resuspended in DC medium and stimulated for 20 h with R848 and poly(I:C) immediately after isolation.

T-cell stimulation assay

Activated BDCs, cDCs, pDCs, or a 1:1 mixture of cDCs and pDCs were pulsed with a cytomegalovirus (CMV), Epstein–Barr virus (EBV), influenza, and tetanus (CEFT) peptide pool (0.25 µg/mL/peptide, JPT, Berlin, Germany) for 2 h. T cells were isolated from PBMCs using human CD3 MicroBeads (Miltenyi Biotec) and stained using CellTrace Far Red (Life Technologies). DCs and autologous T cells were co-cultured at a ratio of 1:10 in R10 medium [RPMI-1640 (PAN-Biotech, Aidenbach, Germany), 10% fetal bovine serum, penicillin–streptomycin–glutamine (both Life Technologies)] for 5 days and subsequently stained for CD3 (PerCP/Vio700, REA613), CD4 (APC/Vio770, REA623), and CD8 (PE/Vio770, REA734) (all Miltenyi Biotec).

Migration assay

The lower part of a 96-transwell plate (5 µm pore size, Corning, Kennebunk, ME, USA) was filled with 200 µL X-Vivo15 medium supplemented with 2% HS and either 200 ng/mL CCL19 (R&D Systems), 200 ng/mL CCL21 (Biolegend) or no chemokine. BDCs were seeded in the upper chamber in technical duplicates. Cells were harvested after 3 h incubation at 37 °C from both chambers, stained for CD11c and CD123, and analyzed by flow cytometry together with Precision Count Beads (Biolegend).

NK-cell activation assay

NK cells were isolated from fresh PBMCs using the human NK Cell Isolation Kit (Miltenyi Biotec). BDCs were co-cultured with autologous NK cells for 24 h at a ratio of 1:10 in R10 medium. NK-cell activation was assessed by staining for CD69 (APC, FN50) or mIgG1 isotype (APC, MOPC-21) on cells positive for CD16 (FITC, 3G8) and CD56 (PE, MEM-188; all Biolegend).

Expansion of antigen-specific T cells

$CD8^+$ T cells were isolated using CD8 T Cell Isolation Kit (Miltenyi Biotec) and cultured in T-cell medium (X-Vivo 15 medium, 5% HS, penicillin–streptomycin–glutamine) supplemented with 5 ng/mL IL-7 (Peprotech) for 20 h. Autologous BDCs, activated with R848 + poly(I:C), were pulsed for 2 h with 1 µg/mL/peptide of PepTivator CEF MHC Class I Plus, WT1, or SARS-CoV-2 Prot_M, Prot_N and Prot_S (all Miltenyi Biotec). T cells and pulsed BDCs were co-cultured at a T cell/BDC ratio of 4:1 in T-cell medium containing 30 ng/mL IL-21 (Peprotech). On days 3, 5, and 7, co-cultures were expanded 1:1 with medium containing 10 ng/mL IL-7 and IL-15 (Peprotech). Antigen-specific T-cell expansion was monitored after 10 days by restimulation for 4 h with equal numbers of autologous PBMCs and 1 µg/mL peptides in medium containing 25 µM monensin and 10 µg/mL brefeldin A (both Sigma–Aldrich). Cells were stained for CD3 (FITC, UCHT1, Biolegend) and CD8 (VioBlue, REA734, Miltenyi Biotec). The percentage of cytokine-secreting $CD8^+$ T cells was analyzed by intracellular staining for IFN-γ (PE, B27) and TNF-α (APC, MAb11; both Biolegend).

Results

Isolated BDCs express positive immune checkpoint molecules

BDCs were isolated from PBMCs with a median yield of 0.69% (Fig. 1a). The expression of surface markers by BDCs was analyzed by flow cytometry based on the gating strategy shown in Supplementary Fig. 1. The median relative frequencies of pDCs, cDC1s, and cDC2s among BDCs were 47.6%, 2.6%, and 49.4%, respectively (Fig. 1b).

Analysis by flow cytometry and calculation of MFI ratios revealed that all BDC subsets expressed HLA-DR and several positive co-stimulatory immune checkpoint molecules. All subsets expressed high levels of CD80 (pDC: 38.8 ± 6.3; cDC1: 27.5 ± 5.4; cDC2: 37.7 ± 6.4), whereas CD86 was only weakly expressed on cDC1s and cDC2s, and absent on pDCs. ICOSL was highly expressed by pDCs (24.4 ± 4.8) and cDC1s (53.1 ± 17.2), whereas CD40 was present predominantly on cDC1s (16.3 ± 2.7). Furthermore, BDCs expressed no or only minimal levels of the immune checkpoint molecules CD70, CD137L, GITRL, and OX40L. We detected no expression of co-inhibitory markers, including

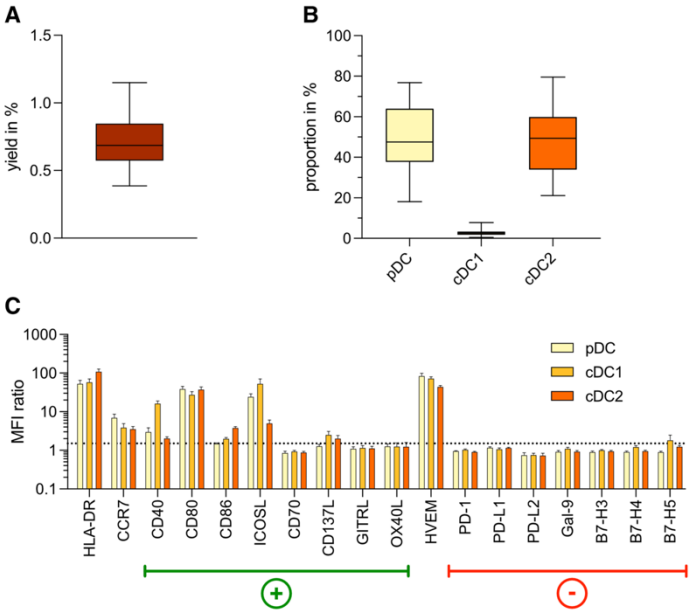


Fig. 1 Characterization of BDCs isolated from peripheral blood. **a** Yield of BDCs isolated from healthy donor PBMCs ($n=30$). **b** Proportion of DC subsets among isolated BDCs ($n=18$). **c** MFI ratios of co-stimulatory and co-inhibitory immune checkpoints expressed by pDCs (light yellow), cDC1s (light orange), and cDC2s (dark orange) isolated from peripheral blood ($n=6$). Bars represent mean ± SEM. Box-and-whisker plots show 5th and 95th percentile

PD-L1, PD-L2, Gal-9, B7-H3, B7-H4, and B7-H5. All BDC subsets expressed high levels of HVEM (pDC: 83.8 ± 14.3; cDC1: 73.0 ± 6.8; cDC2: 44.1 ± 3.3). The migratory receptor CCR7 was weakly expressed on all subsets, with its highest expression on pDCs (7.0 ± 1.6) (Fig. 1c).

A combination of TLR ligands is required to induce a positive co-stimulatory profile on all BDCs

To identify a protocol for activating all BDC subsets simultaneously, we analyzed the expression of immune checkpoint molecules by BDCs in response to different TLR ligands and combinations thereof (Fig. 2a–d). Supplementary Table 1 reports expression of the immune checkpoint molecules CD80, CD40, PD-L1, and the migratory receptor CCR7 for all BDC subsets in response to different combinations of TLR ligands. Activation with TLR3 ligand poly(I:C) caused high expression of CD80 and CD40 and the highest expression of CCR7 on cDC1s and cDC2s. However, expression of CD80 and CD40 was further increased on cDC subsets if poly(I:C) was combined with other TLR ligands. The combination of poly(I:C) with the TLR9 ligand CpG consistently caused the highest expression of these markers on pDCs (CD80: 191.1 ± 34.8; CD40: 129.2 ± 44.7; CCR7: 70.9 ± 13.2). The combination of poly(I:C) with the TLR7/8 ligand R848 resulted in consistently high expression of CD80, CD40, and CCR7 on all BDC subsets (Fig. 2a–c).

The co-inhibitory immune checkpoint PD-L1 was not expressed on pDCs and only weakly expressed by cDC1s and cDC2s cultured without TLR ligands. Activation with CpG + R848 + poly(I:C) led to the highest expression of PD-L1 on all BDC subsets (pDCs: 13.1 ± 1.8; cDC1s: 22.9 ± 3.3; cDC2s: 26.0 ± 3.6), whereas R848 + poly(I:C) caused only intermediate levels of expression (pDCs: 11.8 ± 2.4; cDC1s: 14.7 ± 1.6; cDC2s: 13.8 ± 1.7) (Fig. 2d).

TLR stimulation of BDCs results in distinct cytokine secretion

Next, we evaluated the secretion of cytokines by BDCs in response to TLR activation. We observed the highest secretion of IL-12p70 by simultaneous triggering of TLR3 and TLR7/8 with R848 + poly(I:C). Interestingly, adding CpG led to significantly reduced IL-12 levels ($4.9\times10^3 \pm 8.2\times10^2$ vs. $2.8\times10^3 \pm 6.2\times10^2$ pg/mL; $p=0.014$). Using poly(I:C) ± CpG caused intermediate IL-12 levels (Fig. 2e). Stimulation with only R848 or CpG or

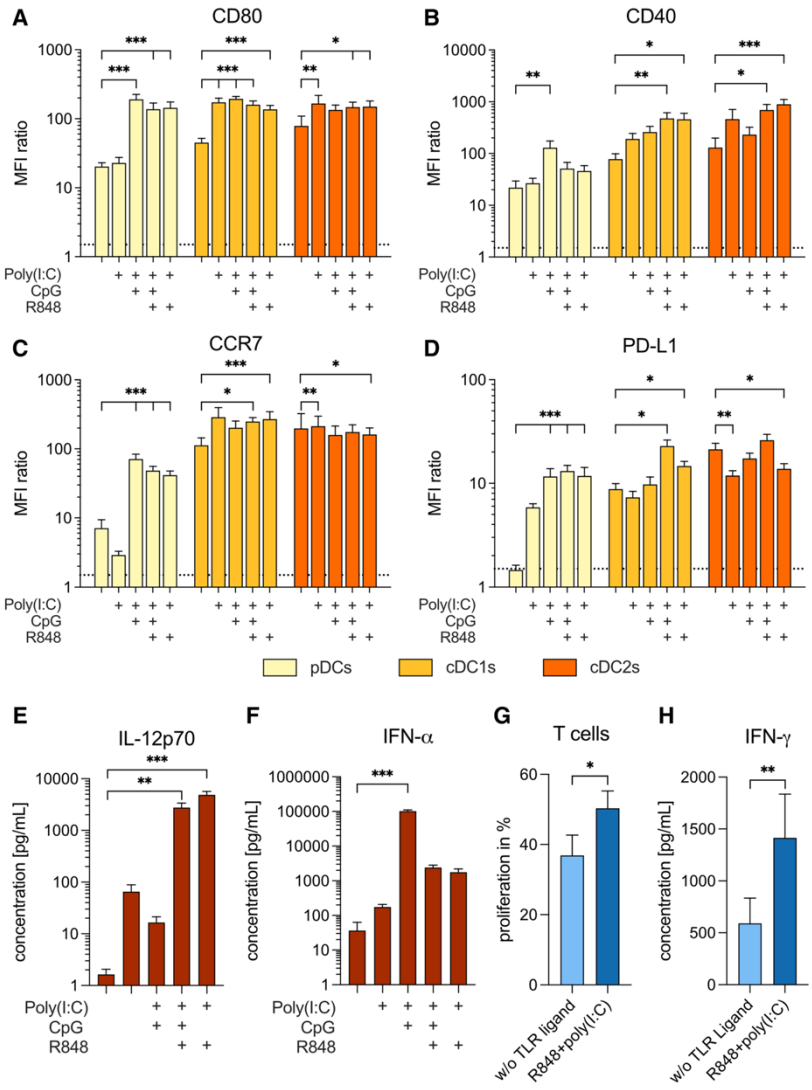


Fig. 2 Comparison of TLR ligand cocktails for simultaneous activation of all BDC subsets. Expression of CD80 (**a**), CD40 (**b**), CCR7 (**c**), and PD-L1 (**d**) on BDC subsets, and secretion of IL-12p70 (**e**) and IFN-α (**f**) by BDCs in response to activation with different TLR ligand cocktails ($n = 6$). (**g**) T-cell proliferation and (**h**) secretion of IFN-γ by T cells induced by BDCs activated without TLR ligands (light blue) or R848 + poly(I:C) (dark blue) ($n = 14$–16). Bars represent mean ± SEM. Statistical differences compared to the control without TLR ligand were analyzed by paired one-way ANOVA with Bonferroni's multiple comparison test (**a–f**) or Wilcoxon matched-pairs signed-rank test (**g, h**). Only significant differences are shown: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

the combination of the two did not lead to IL-12 production in a parallel study, nor did protamine-RNA (Supplementary Fig. 2a). Addition of IFN-γ, but not CD40L, to the combination of R848 and poly(I:C) further enhanced secretion of IL-12 (Supplementary Fig. 3a).

By contrast, we observed the highest IFN-α concentrations using poly(I:C) + CpG (Fig. 2f). Adding R848 to this combination caused a significant reduction of IFN-α levels ($1.0 \times 10^5 \pm 8.3 \times 10^3$ pg/mL vs. $2.4 \times 10^3 \pm 4.2 \times 10^2$; $p < 0.001$), similar to activating with R848 + poly(I:C) ($1.8 \times 10^3 \pm 4.4 \times 10^2$ pg/mL). Poly(I:C) induced only negligible IFN-α secretion. Activation with CpG, R848, or protamine-RNA caused notable IFN-α secretion (Supplementary Fig. 2b). Addition of IFN-γ or CD40L to the combination of R848 and poly(I:C) did not change the IFN-α response (Supplementary Fig. 3b).

Since R848 + poly(I:C) caused an overall positive co-stimulatory expression on BDCs, high IL-12 secretion, and intermediate levels of IFN-α secretion, we focused on this combination for subsequent experiments.

Activation of BDCs with R848 + poly(I:C) increases T-cell responses

Next, we measured the T-cell responses induced by TLR-activated BDCs. To do so, we co-cultured BDCs with autologous T cells and measured T-cell proliferation and IFN-γ secretion after five and four days, respectively. Nonactivated BDCs induced a T-cell proliferation of 36.9 ± 5.8%. This was significantly increased to 50.3 ± 5.0% ($p = 0.018$) if BDCs were activated with R848 + poly(I:C) (Fig. 2g). Similarly, T cells secreted significantly higher amounts of IFN-γ in response to R848 + poly(I:C)-activated BDCs compared to nonactivated BDCs ($1.4 \times 10^3 \pm 4.2 \times 10^2$ vs. $5.9 \times 10^2 \pm 2.4 \times 10^2$ pg/mL; $p = 0.008$) (Fig. 2h).

IL-12 and IFN-α are secreted at different time points

To determine when cytokines are secreted by BDCs, we analyzed cytokine secretion 1, 3, 6, and 20 h after TLR activation. Owing to time constraints, we introduced an additional resting step of 16 h between BDC isolation and activation. As a control, we also evaluated a 20-h activation without the resting step.

We observed no secretion of IL-12 within the first 6 h of activation and only a small response after 20 h (32.2 ± 10.4 pg/mL). In contrast, when we added the R848 + poly(I:C) directly after cell isolation, we observed strong IL-12 secretion for the same donors ($2.1 \times 10^3 \pm 1.1 \times 10^3$ pg/mL) (Fig. 3a). For IFN-α, as early as 3 h after adding R848 + poly(I:C), we measured a mean concentration of $3.8 \times 10^3 \pm 1.3 \times 10^2$ pg/mL that remained comparable after 6 and 20 h. Activation for 20 h directly after isolation resulted in lower IFN-α secretion compared to no resting after 20 h ($4.0 \times 10^3 \pm 1.2 \times 10^2$ vs. $6.0 \times 10^2 \pm 5.9 \times 10^1$ pg/mL) (Fig. 3b).

A shorter BDC activation time does not increase T-cell responses

To determine if a shorter duration of BDC activation improves T-cell responses, we prepared co-cultures of T cells and autologous BDCs activated with R848 + poly(I:C) for 1, 3, 6, and 20 h after 16 h "resting" or for 20 h directly after cell isolation. We analyzed T-cell proliferation and IFN-γ secretion after five and four days, respectively. BDCs activated for 20 h induced a T-cell proliferation of 59.0 ± 6.0%. This was significantly higher compared to shorter activation periods of between 1 and 6 h (range: 32.3–36.9%; $p < 0.001$). "Resting" versus "no resting" prior to 20-h activation did not result in a significant difference ($p = 0.781$) (Fig. 3c). In contrast, maximum IFN-γ secretion by T cells was detected with BDCs activated for 20 h directly after cell isolation ($1.6 \times 10^3 \pm 4.3 \times 10^2$ pg/mL), compared to BDCs rested for 16 h (for 20 h: $5.2 \times 10^2 \pm 5.6 \times 10^1$ pg/mL; $p = 0.007$). No significant differences in IFN-γ secretion between BDCs activated for 1, 3, 6, or 20 h after the resting step was observed (Fig. 3d).

Individual activation of cDCs and pDCs with a tailored protocol is not superior to a combined activation of all BDCs

An important translational question is whether separate activation of cDCs and pDCs with a tailored activation protocol is superior to the combined activation of all BDC subsets. To address this question, we isolated pDCs, cDCs, and entire BDCs from the same donors and activated cDCs and BDCs with R848 + poly(I:C) for 20 h immediately after isolation. By contrast, pDCs were rested for 16 h before exposing them to CpG + poly(I:C) for 3 h, as described for optimal IFN-α secretion above. Secretion of IL-12 was higher by BDCs than by cDCs ($3.5 \times 10^3 \pm 1.5 \times 10^3$ and $1.9 \times 10^3 \pm 7.4 \times 10^2$ pg/mL, respectively). Activation with CpG + poly(I:C) resulted in strong secretion of IFN-α by pDCs ($4.9 \times 10^3 \pm 9.2 \times 10^2$ pg/mL), while observed cytokine concentrations for R848 + poly(I:C)-activated BDCs were lower ($2.9 \times 10^3 \pm 8.1 \times 10^2$ pg/mL). We did not observe any cytokine secretion in controls without TLR ligands (Fig. 4a, b).

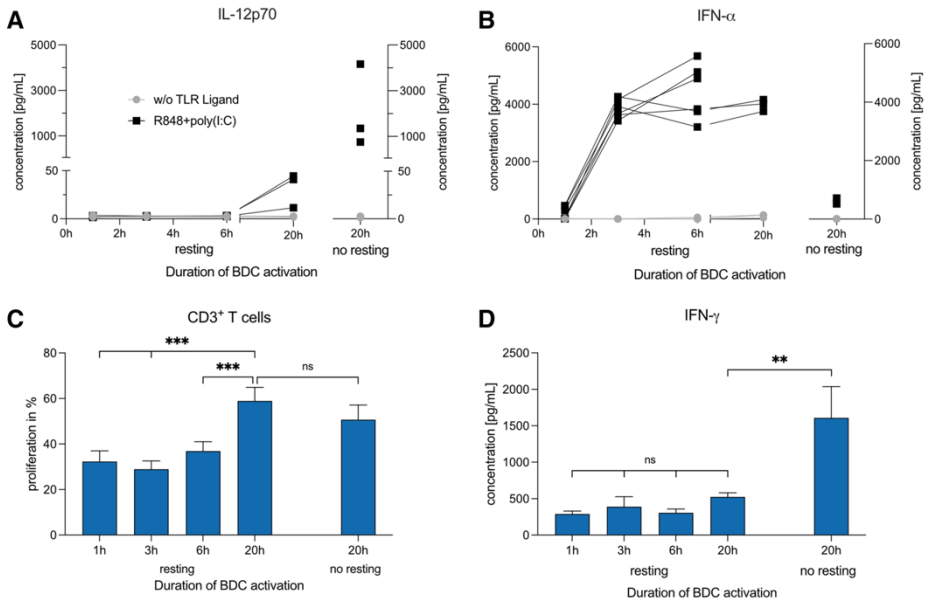


Fig. 3 Evaluation of shorter durations of BDC activation for differences in cytokine secretion and induction of T-cell responses. Cumulative secretion of IL-12p70 (**a**) and IFN-α (**b**) by BDCs after 1, 3, 6, and 20 h of activation with R848 and poly(I:C). BDCs were "rested" for 16 h before the TLR ligand was added. In addition, BDCs were activated directly after isolation for 20 h ($n=3$–6). **c** Induction of T-cell proliferation by BDCs activated for various time periods ($n=9$). **d** Secretion of IFN-γ in co-cultures of T cells and BDCs that were activated for various periods of time ($n=6$). Bars represent mean ± SEM. Statistical differences compared to 20-h activation with TLR ligands after resting for 16 h were analyzed by paired one-way ANOVA with Bonferroni's multiple comparison test. Only significant differences are shown: $^{**}p<0.01$, $^{***}p<0.001$

In co-culture experiments of autologous T cells and BDCs, pDCs, cDCs, or a 1:1 mixture of pDCs and cDCs, T-cell proliferation was significantly higher when using BDCs collectively activated, compared to a mixture of separately activated pDCs and cDCs (20.8 ± 5.0% vs. 7.9 ± 1.7%, $p=0.044$) (Fig. 4c). Similarly, IFN-γ secretion by T cells was highest in response to BDCs ($8.0\times10^2\pm3.2\times10^2$ pg/mL), whereas the mixture of pDCs and cDCs exhibited a 1.8-fold reduction ($4.4\times10^2\pm1.5\times10^2$ pg/mL) (Fig. 4d).

IFN-α supplementation of cDCs activated with R848 + poly(I:C) significantly increased IL-12 secretion ($2.4\times10^3\pm6.1\times10^2$ vs. $1.9\times10^3\pm6.2\times10^3$ pg/mL; $p=0.040$) (Fig. 4e).

TLR-activated BDCs expand antigen-specific CD8$^+$ T cells

In order to test the capacity of BDCs to induce antigen-specific T-cell responses, autologous CD8$^+$ T cells were co-cultured with BDCs pulsed with viral peptides derived from CMV, EBV, and influenza A (CEF peptides) or SARS-CoV-2, or peptides derived from the tumor oncogene WT1 (Fig. 5a, b).

R848 + poly(I:C)-activated BDCs demonstrated enhanced expansion of CEF-specific CD8$^+$ T cells, compared to nonactivated BDCs (mean fold change of

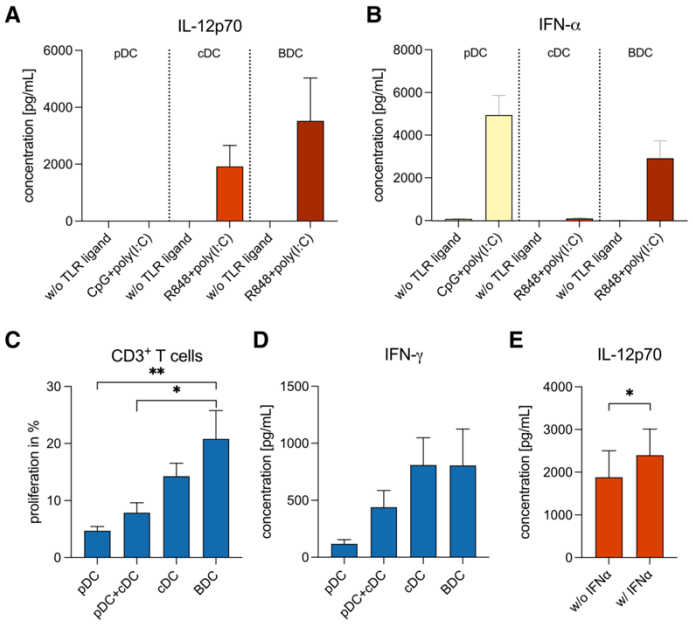


Fig. 4 Comparison between tailored activation protocols for cDCs and pDCs and simultaneous activation of BDCs. **a, b** Secretion of IL-12p70 and IFN-α by pDCs, cDCs, and BDCs upon activation with tailored protocols for TLR stimulation ($n=6$). **c, d** T-cell proliferation and IFN-γ secretion induced in co-culture experiments with autologous pDCs, cDCs, BDCs, or a 1:1 mixture of pDCs and cDCs ($n=6$). Bars represent mean ± SEM. Statistical test: paired one-way ANOVA with Bonferroni's multiple comparison test; $*p<0.05$, $**p<0.01$. **e** Effect of IFN-α addition on IL-12p70 secretion by cDCs activated with R848 + poly(I:C) ($n=10$). Bars represent mean ± SEM. Statistical test: Wilcoxon matched-pairs signed-rank test, $*p<0.05$

antigen-specific T cells compared to respective controls with unpulsed BDCs: 21.3 ± 6.5 vs. 13.3 ± 1.9; ± SEM). Similarly, expansion of T cells specific for SARS-CoV-2 from donors that had been previously infected by the virus was higher by R848 + poly(I:C)-activated BDCs compared to nonactivated BDCs (9.0 ± 2.1 vs. 8.2 ± 1.2). For WT1-specific T cells, R848 + poly(I:C)-activated BDCs similarly caused an improved expansion of antigen-specific T cells compared to non-activated BDCs (2.9 ± 1.1 vs. 1.6 ± 0.5).

BDCs activated with R848 + poly(I:C) results in increased NK-cell activation

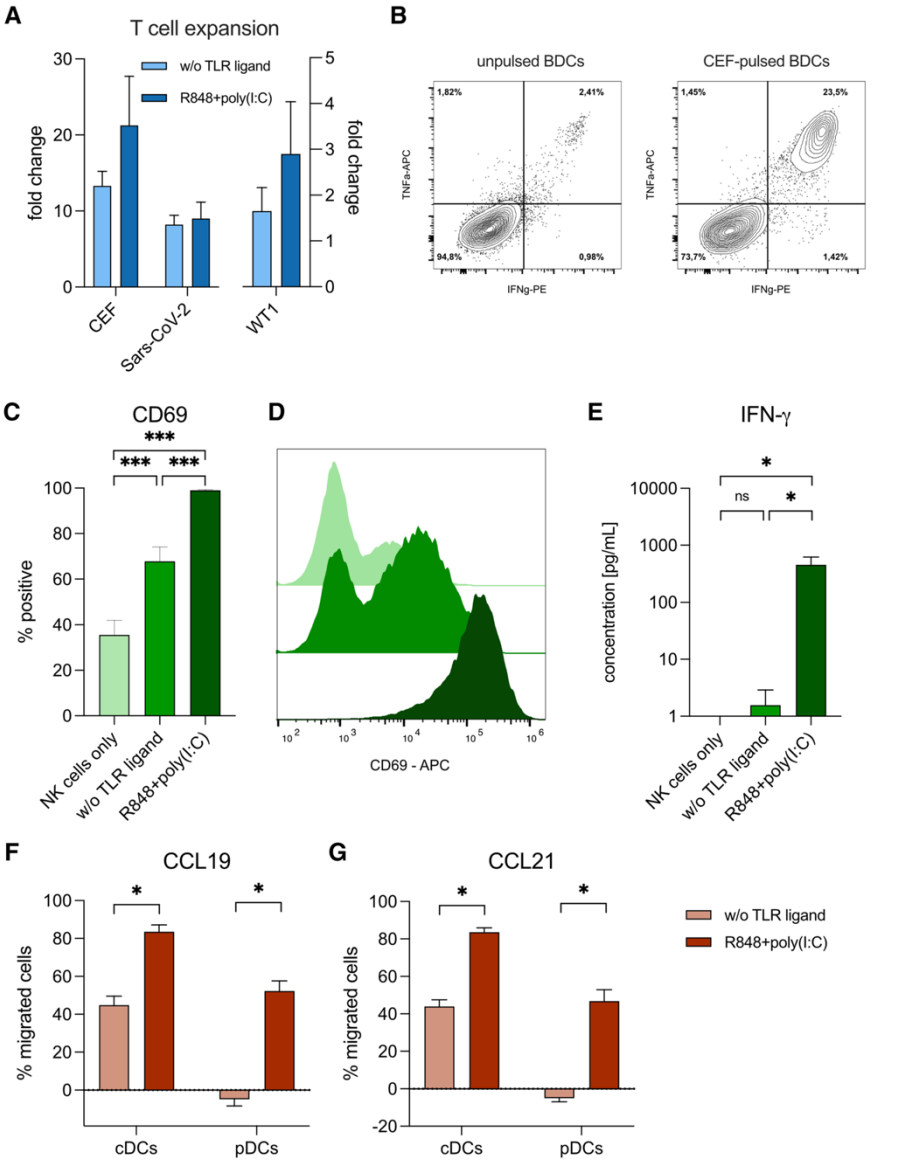
We tested the activation of NK cells co-cultured with autologous BDCs. Co-cultivation with nonactivated BDCs resulted in an increased frequency of CD69-expressing NK cells compared to controls with only NK cells (70.7 ± 6.1 vs. 41.6 ± 7.9%). Addition of R848 + poly(I:C)-activated BDCs led to CD69 expression by almost all NK cells (99.1 ± 0.2%; $p<0.001$) at very high levels (Fig. 5c, d). Similarly, TLR-activated BDCs resulted in a significant increase of IFN-γ secretion by NK cells compared to nonactivated BDCs ($4.5\times10^2 \pm 1.7\times10^2$ vs. 1.6 ± 1.3 pg/mL; $p=0.019$) (Fig. 5e).

R848 + poly(I:C) increases specific migration of BDCs

Finally, we analyzed whether R848 + poly(I:C) increased the specific migration of DCs toward the chemokines CCL-19 and CCL-21. pDCs migrated toward CCL-19 only upon activation with R848 + poly(I:C) (52.3 ± 5.4%). For cDCs, TLR-activation increased migration from 44.9 ± 4.8% to 83.5 ± 3.6% ($p=0.031$) upon TLR activation. Similar results were obtained for CCL-21 (Fig. 5f, g).

Discussion

BDCs have become a promising alternative to moDCs in cancer immunotherapies owing to their multifaceted properties. However, results from first clinical trials indicate the need for improved activation protocols. Here, we systematically established a protocol for the simultaneous activation of all BDC subsets *in vitro* using a combination of two TLR ligands. Although BDCs represent only about 1% of


A
T cell expansion
w/o TLR ligand
R848+poly(I:C)
fold change
fold change
CEF
Sars-CoV-2
WT1
B
unpulsed BDCs
CEF-pulsed BDCs
TNFa-APC
IFNg-PE
1,82%
2,41%
94,8%
0,98%
1,45%
23,5%
73,7%
1,42%
C
CD69
% positive
NK cells only
w/o TLR ligand
R848+poly(I:C)
D
CD69 - APC
E
IFN-γ
concentration [pg/mL]
ns
F
CCL19
% migrated cells
cDCs
pDCs
G
CCL21
w/o TLR ligand
R848+poly(I:C)

◂**Fig. 5** Migration, NK-cell activation, and T-cell expansion by BDCs. **a** Expansion of T cells specific for CEF, WT1, and SARS-CoV-2 by BDCs activated with TLR ligands ($n=3$–4). **b** Representative flow cytometry analysis of antigen-specific T-cell expansion by BDCs activated with R848 + poly(I:C). BDCs were either not pulsed (left) or pulsed with peptides (right) prior to co-culturing with autologous T cells. **c** Activation of NK cells in co-cultures with autologous BDCs activated with TLR ligands ($n=9$). **d** Representative example for NK-cell activation by BDCs. Color coding: NK cells only (light green), BDCs activated without TLR ligands (green) and with R848 + poly(I:C) (dark green). **e** Secretion of IFN-γ in co-cultures of NK cells and autologous BDCs ($n=7$). Migration of BDC subsets towards CCL-19 (**f**) and CCL-21 (**g**) upon activation with TLR ligands in a transwell assay ($n=6$). Bars represent mean ± SEM. Statistical tests: paired one-way ANOVA with Bonferroni's multiple comparison test (**c**, **e**), Wilcoxon matched-pairs signed-rank test (**f**, **g**), $^{*}p<0.05$, $^{***}p<0.001$

all PBMCs, automated systems allow their rapid isolation from buffy coats or leukapheresis products to high purity in a closed system [23].

We observed low to intermediate expression of positive co-stimulatory immune checkpoint molecules on BDCs after isolation from peripheral blood. Stimulation with TLR ligands induced high expression of the co-stimulatory molecules CD80 and CD40 and the migratory receptor CCR7, whereby a combination of a TLR3 with a TLR7/8 ligand proofed to be optimal for all BDC subsets. We also observed upregulation of PD-L1 in all BDC subsets, providing the rationale to combine BDC vaccination with PD-1/PD-L1 blockade. This has already been successfully evaluated in pre-clinical studies, however, results from clinical trials are scarce [24–26]. Nevertheless, the feasibility of a similar approach has been demonstrated in metastatic melanoma patients by blockade of CTLA-4 in combination with moDCs [27].

TLR ligands also caused strong secretion of IL-12 and IFN-α by BDCs. Whereas IFN-α secretion was at its highest level upon activation with CpG + poly(I:C), IL-12 secretion was maximal with R848 + poly(I:C). In contrast, single TLR stimulation-induced generally lower BDC responses. This is in line with previous results, describing TLR synergy between TLR3 or TLR4 and TLR8 in human moDCs and cDC2s resulting in potent IL-12p70 induction [28]. Addition of IFN-γ to the combination of R848 and poly(I:C) led to further enhanced secretion of IL-12p70, similar to previous results in moDCs [28].

We decided to focus on R848 + poly(I:C) to optimize Th1 differentiation, which is induced by IL-12. Similar to our results, R848 + poly(I:C) has been reported to upregulate co-stimulatory immune checkpoints in BDC subsets and to induce cytokine and chemokine secretion (i.e., type I IFNs, IL-12) in a humanized mouse model [29]. Addition of CpG to R848 + poly(I:C) provided no benefit with respect to immune checkpoint expression and cytokine secretion by BDCs. This reflects the previous finding that single-stranded DNA oligonucleotides such as CpG inhibit the activation of moDCs and nonhematopoietic cells by poly(I:C) [30].

By analyzing the kinetics of cytokine secretion, we found that IFN-α production by BDCs starts only a few hours after stimulation with R848 + poly(I:C), whereas the onset of IL-12 secretion appears to be later. This is consistent with results from a humanized mouse model [29]. Unexpectedly, the introduction of a resting step before TLR activation of BDCs strongly increased IFN-α secretion, whereas IL-12 was reduced. Spontaneous apoptosis of cDCs during ex vivo handling might explain this observation, whereas cultivation with IL-3 during the resting step might have promoted survival of pDCs. Conversely, preincubation with IL-3 for longer periods was reported to decrease the IFN-α secretion capacity of pDCs [31], however, as appropriate controls were missing in that study, spontaneous apoptosis might have been at play. Furthermore, we observed that reducing the time span of BDC activation and introducing the resting step resulted in reduced T-cell responses compared to BDC activation for 20 h immediately after their isolation.

Evaluation of tailored activation protocols for BDC subsets allowed us to elucidate the individual contributions of pDCs and cDCs to induce immune responses. cDCs induced stronger T-cell responses than pDCs, correlating with IL-12 secretion. The role assigned to pDCs in shaping the immune response in oncological malignancies remains controversial, with contradicting correlations between pDC infiltration and disease prognosis [32, 33]. Recently, the results of a clinical trial of protamine-RNA-activated pDCs, cDCs, or a combination of pDCs and cDCs in prostate cancer were reported. In all three treatment arms, antigen-specific T-cell responses were induced and correlated with radiographic progression-free survival, but no significant differences were observed [34]. Interestingly, when the authors used protamine-RNA for DC activation, cDCs did not secrete IL-12, but pDCs did, albeit at a low level. This might have been caused by a rare contaminating cDC population sharing common pDC markers [35], as the purity of the pDC vaccine was only modest. However, in our study, BDCs elicited higher immunostimulatory capacities than a combination of pDCs and cDCs activated with tailored protocols, underlining the rationale for activating all BDC subsets simultaneously.

DC vaccines aim to elicit and maintain T-cell responses against tumor-specific antigens. WT1 is a universal tumor target antigen due to its expression pattern in multiple different cancer entities, thus having great potential for developing immunotherapies [36]. However, inducing sufficient immune responses is a major challenge owing to the low immunogenicity of nonmutated tumor antigens and clonal deletion of self-reactive T cells [37]. R848 + poly(I:C)-activated BDCs were able to expand WT1-specific T cells, providing a rationale for their further development as an immunotherapeutic tool. In addition to their

use as monotherapy, DC vaccines might enhance existing immune responses in the context of Chimeric antigen receptor (CAR) T-cell therapies. Accordingly, moDCs have been shown to boost WT1-specific CAR T cells in a humanized mouse model, resulting in enhanced inhibition of tumor growth [38].

DC vaccination trials to date have mainly focused on induction of T-cell responses, however, NK-cell activation by DCs constitutes an interesting upside for immunotherapies. Not only do NK cells recruit and activate further DCs, promoting T-cell responses, they can also eliminate tumor cells directly. Thus, tumor-cell material is released that can be processed by further DCs and be presented to T cells [39]. As activation of NK cells is mediated by IL-12 and type I interferons [40], BDCs activated with R848 + poly(I:C) demonstrated potent NK-cell activation with respect to CD69 expression and IFN-γ secretion. Importantly, NK-cell activation has been shown to correlate with clinical outcomes in several clinical trials, including those of a DC vaccine targeting WT1 in AML [39, 41, 42].

Migration of DCs to draining lymph nodes relies on the migratory receptor CCR7 and is central to induce adaptive immune responses. CCL-19 and CCL-21 promote the migration of DCs to the lymph nodes themselves and to the T-cell zone within the lymph nodes, enabling T-cell activation by DCs [43]. Only a few clinical trials have monitored the migration of their DC vaccine, revealing that only a small percentage of the injected DCs successfully migrated to the lymph nodes, underlining the need for enhanced DC migration capacities [44–46]. Activation with R848 + poly(I:C) upregulated expression of CCR7 on all BDCs. As a consequence, we observed improved migration of BDCs toward CCL-19 and CCL-21. Importantly, pDCs migrated only upon TLR activation.

Our investigations show R848 + poly(I:C) to be an optimized cocktail for ex vivo activation of all BDC subsets. Our findings support the further investigation and usage in early clinical trials – as standalone or in conjunction with other immunotherapeutic strategies including adoptive T-cell transfer and checkpoint inhibition.

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Author contributions FSL and MS designed the study and supervised the project. GH and FSL wrote the manuscript and together with CA and KP were involved in research design and data interpretation. GH performed experiments and analyzed the data.

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Data availability All data generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflicts of interests G.H. declares no conflicts of interest. C.A. and K.P. are employed by Miltenyi Biotec. F.S.L. is employed by Hoffmann-La Roche. M.S. has received industry research support from Amgen, Gilead, Miltenyi Biotec, Morphosys, Roche, and Seattle Genetics, and has served as a consultant/advisor to Amgen, BMS, Celgene, Gilead, Pfizer, Novartis, and Roche. She sits on the advisory boards of Amgen, Celgene, Gilead, Janssen, Novartis, Pfizer, and Seattle Genetics, and serves on the speakers' bureau at Amgen, Celgene, Gilead, Janssen, and Pfizer.

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was obtained by the Institutional Review Board of the LMU Munich.

Consent to participate Informed consent was obtained from all individual participants included in the study.

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2.2 Publication II

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Integrated multiomic approach for identification of novel immunotherapeutic targets in AML

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Abstract

Background: Immunotherapy of acute myeloid leukemia has experienced considerable advances, however novel target antigens continue to be sought after. To this end, unbiased approaches for surface protein detection are limited and integration with other data types, such as gene expression and somatic mutational burden, are poorly utilized. The Cell Surface Capture technology provides an unbiased, discovery-driven approach to map the surface proteins on cells of interest. Yet, direct utilization of primary patient samples has been limited by the considerable number of viable cells needed.

Methods: Here, we optimized the Cell Surface Capture protocol to enable direct interrogation of primary patient samples and applied our optimized protocol to a set of samples from patients with acute myeloid leukemia (AML) to generate the AML surfaceome. We then further curated this AML surfaceome to exclude antigens expressed on healthy tissues and integrated mutational burden data from hematologic cancers to further enrich for targets which are likely to be essential to leukemia biology. Finally, we validated our findings in a separate cohort of AML patient samples.

Results: Our protocol modifications allowed us to double the yield in identified proteins and increased the specificity from 54 to 80.4% compared to previous approaches. Using primary AML patient samples, we were able to identify a total of 621 surface proteins comprising the AML surfaceome. We integrated this data with gene expression and mutational burden data to curate a set of robust putative target antigens. Seventy-six proteins were selected as potential candidates for further investigation of which we validated the most promising novel candidate markers, and identified CD148, ITGA4 and Integrin beta-7 as promising targets in AML. Integrin beta-7 showed the most promising combination of expression in patient AML samples, and low or absent expression on healthy hematopoietic tissue.

Conclusion: Taken together, we demonstrate the feasibility of a highly optimized surfaceome detection method to interrogate the entire AML surfaceome directly from primary patient samples and integrate this data with gene expression and mutational burden data to achieve a robust, multiomic target identification platform. This approach has the potential to accelerate the unbiased target identification for immunotherapy of AML.

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Background

Immunotherapeutic strategies including chimeric antigen receptor (CAR) T cells or antibody based therapies have transformed treatment outcomes in cancer. For acute myeloid leukemia (AML) however, while there have been considerable advances in the field of immunotherapy, lack of efficacy or on-target off-leukemia toxicity remain a challenge. Several studies have recently demonstrated patterns of immune-activation and -evasion in AML [1, 2], highlighting the complex interaction of genomic lesions and immune activation. However, despite these advances, identification of suitable targets have remained challenging. Thus, novel target antigens with more favorable expression characteristics are sought after. Genome-wide assessment of mutational burden and gene expression directly from primary patient samples have delivered significant insight into AML biology and prognosis. However, only recent technical advances in proteomics have allowed for unbiased and sensitive analysis of the cancer proteome. For the development of novel immunotherapeutic strategies, the choice of which surface antigen to target is arguably the most important design step. Efficient and unbiased interrogation of the entirety of surface proteins has been technically challenging. While previous reports have characterized the AML surfaceome in cell lines [3, 4], cell-surface capture proteomics directly on primary patient samples has only been successfully performed in xeno-amplified acute lymphoblastic leukemia [5]. However, both clonal composition as well as surface antigen expression have been reported to change upon xenotransplantation in AML [6, 7]. Other approaches aiming to increase throughput of surface protein detection have either adapted traditional flow cytometry [8] or mass cytometry based approaches [9]. However, these methodologies are dependent on the existence of antibodies against a-priori defined target antigens, outlining inherent limitations for de novo target discovery. We therefore aimed to close this gap by improving the current methodologies to enable unbiased, whole-surfaceome detection directly from xeno-amplification free AML patient samples.

Additionally, while several studies have incorporated gene expression data to identify putative immunotherapeutic targets [3], gene sequencing of somatic mutations contributed significantly to our understanding of underlying biologic mechanisms of leukemogenesis [10]. These sequencing efforts have been performed in large patient cohorts, primarily with the goal to identify drivers of disease. However, this data type has not been systematically incorporated into the discovery of immunotherapeutic targets. We believe integration into target-discovery platforms should improve their robustness, since effective immunotherapeutic targets ought not to only be expressed uniformly, these targets should also have *functional* relevance to the tumor, thus *rarely* be deleted or affected by change-of-function variants. We postulate that mutation data might be incorporated into a target discovery platform and implement a strategy to achieve this goal.

In this study, we optimized a targeted proteomics assay for the efficient interrogation of the entire surface proteome (surfaceome) of primary acute myeloid leukemia samples without the necessity of xenograft amplification. To avoid xenotransplantation, we employed our short-term *in vitro* culturing system, which we previously demonstrated does not show large shifts in clonal composition or antigen expression [11]. Next, we integrated our proteomics data into a comprehensive set of gene expression as well as mutational burden data to enrich for expressed and functionally relevant targets. We then validated a set of high-scoring putative targets identified by our approach in independent primary AML samples.

This integrated approach allows for unbiased, genome-wide screening from all three modalities – mutation, transcription and surface protein expression – for target discovery in immunotherapy of AML.

Methods

Cell culture

HL-60 and OCI-AML3 AML cell lines were grown in RPMI 1640 medium plus 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin/streptomycin and 5 μM β-mercaptoethanol at 37 °C and 5% CO_2.

Primary patient cell culture

Peripheral blood (PB) or bone marrow (BM) samples were collected at the Laboratory of Leukemia Diagnostics at the University Hospital Munich with Institutional Review Board approval. Ficoll density gradient centrifugation was performed to isolate mononuclear cells and patient specimens were subsequently stored in liquid nitrogen until further use. *In vitro* culture of patient samples was performed as previously reported [12]. Specifically, irradiated MS-5 stromal cells were seeded in 6-well plates at 2.5×10^5 cells/well 1 day prior to addition of patient specimens in α-MEM with 12.5% FBS, 12.5% horse serum, 1% glutamine, and 1% penicillin/streptomycin. $1–1.5 \times 10^7$ primary patient cells per well were added

into the plate and the media was supplemented with IL-3, TPO and G-CSF (all 20 ng/ml). After 3 days, the non-adherent cells were subjected to the Glyco-CSC protocol as described below. Detailed patient information is summarized in Table S1.

Patient-derived xenograft (PDX) cells

Patient-derived xenograft (PDX) cells were collected from mice as previously described by Vick et al. [13]. Specifically, primary AML patient mononuclear cells were transplanted i.v. into sublethally irradiated NSG mice. After 6–20 weeks, prior to mice showing clinical signs of illness, mice were euthanized and human cells were isolated from spleen and bone marrow.

Cell Surface Capture (CSC)

The original Glyco-CSC, Cys-Glyco-CSC as well as Lys-CSC protocols were performed as described by Wollscheid et al. as well as Bausch-Fluck et al. [14, 15]. Specifically, 1×10^8 cells were selectively labeled after sodium-meta-periodate oxidation with biocytin hydrazide (for Glyco-CSC and Cys-Glyco-CSC protocols, Biomol) or directly without oxidation using NHS-SS-biotin (Lys-CSC protocol, ThermoFisher). Then, cells were lysed in hypotonic lysis buffer with a Dounce homogenizer on ice. Cell debris was removed by centrifugation and the supernatant (containing the membrane fraction) collected. Next, the cell membrane fraction was subjected to ultracentrifugation either in a buffer consisting of a 1:1 ratio with lysis buffer and membrane preparation buffer (for Glyco-CSC and Cys-Glyco-CSC), or in a sucrose gradient (Lys-CSC). The resulting membrane pellets were washed and dissolved in ammonium bicarbonate solution by indirect sonication.

For the Glyco-CSC protocol, the membrane proteins were reduced using tris(2-carboxyethyl)phosphine and then alkylated by iodoacetamide. Membrane proteins were digested overnight with trypsin (Promega). The biotinylated membrane proteins were then bound to a Streptavidin Plus UltraLink Resin, washed and either released by PNGase F (Glyco-CSC and Cys-Glyco-CSC) or by reducing agents (cysteine contained peptides in Cys-Glyco-CSC and Lys-CSC). Peptides were desalted and washed in C18 columns (ThermoFisher) and concentrated in a SpeedVac concentrator (Uniequip). Lastly, dried peptides were resuspended in 20 µl LC–MS grade water (supplemented with 0.1% formic acid and 5% acetonitrile) and stored at -20 °C.

Modifications to the CSC protocol

To improve the CSC protocol and enable direct utilization of primary patients specimens, we modified the original protocols to incorporate the following changes: the concentration of biocytin hydrazide was reduced to 5.4 mM (original 6.5 mM); samples were homogenized using a Bioruptor device (Diagenode) instead of a dounce homogenizer; trypsin digestion was performed twice; and finally the NH_4HCO_3 solution was replaced with a digestion buffer (containing 1 mM iodoacetamide, 100 mM NH_4HCO_3, and 1 mM BHES for Glyco-CSC and Cys-Glyco-CSC, or 1.25 mM iodoacetamide, 100 mM NH4HCO3, 2 mg/ml RapiGest surfactant, and 1.25 mM BHES for Lys-CSC).

Liquid chromatography–mass spectrometry

Peptide samples were separated by a 70 min gradient on a reversed-phase nano HPLC and analyzed by an Orbitrap Elite instrument. MS1 spectra were acquired at 120,000 resolution in data-dependent acquisition mode (top 10) and MS2 spectra at low resolution in the ion trap as well as the following settings: activation—10 ms; maximum injection time—100 ms; AGC target value—104 ions; and minimum ion count—500. Each sample was analyzed in duplicate and technical replicates were averaged.

Raw data files were loaded into MaxQuant for protein identification against the reviewed human proteome (UniProt August-2015). Maximum precursor mass error was set to 4.5 ppm and the fragment mass error was 0.5 Da. The following search parameters were used as variable modification: protein N-terminal acetylation, methionine oxidation and N-deamidation. Carbamidomethylation on cysteines was set as a fixed modification. The final peptide and protein lists were filtered using a false discovery rate (FDR) of 0.01.

Next, the results were further refined using annotations from the Cell Surface Protein Atlas and UniProt. Specifically, we validated deamidation events as N-glycosylation if they fulfilled the following criteria: 1) the deamidation had a MaxQuant localization probability > 0.75 and the deamidation occurred in a NXS/T glycosylation motif; 2) the identified protein contained at least one transmembrane (TM) domain and/or signal peptide; and 3) the NXS/T glycosylation motif did not occur within the TM domain. Intensities between different samples were compared at the deamidation site level as well as protein level and intensities of N-glycosylations of the same site but in different tryptic peptides were summed. N-glycosylations at the same protein were averaged. Finally, intensities were log2-transformed and normalized to the median between samples. Protein level clustering was performed by hierarchical clustering either using Cosine correlation distance or Euclidean distance. Edges with a cosine correlation lower than 0.85 in the network plot were filtered out. Each sample was tested at least

twice and intensities of the identified peptides were averaged.

Filtering of putative targets

Next, we filtered our list of putative surfaceome targets further to eliminate genes that fulfilled the were either highly expressed on healthy hematopoietic cells and/or highly expressed on other normal human tissues. Specifically, we utilized two gene expression databases: one derived from healthy blood cells in different stages of hematopoiesis (BloodSpot—HemaExplorer) and one derived from normal human tissues (Genotype-Tissue Expression Project (GTEx)). The mean and standard deviation for all of our surfaceome genes was enumerated and those genes with expression on three hematopoietic subpopulations (hematopoietic stem cells, early hematopoietic progenitors and CD14 + monocytes) beyond the upper standard deviation of the mean were eliminated (Supplementary Figure S1A). Similarly, the maximal tissue expression on non-hematologic tissue was enumerated for our surfaceome genes and those genes, where the maximal expression found in any non-hematologic tissue was beyond the upper standard deviation of the mean were eliminated (Supplementary Figure S1B).

Mutational burden analysis

Somatic mutations were downloaded from the Catalogue Of Somatic Mutations In Cancer (COSMIC, V91). Tumor types were grouped in non-hematologic malignancies (Non-Heme) and hematologic malignancies (Heme). Finally, the ratio of non-synonymous vs. synonymous mutations (dN/dS-ratio) was enumerated for all genes detected in our AML surfaceome.

FACS analysis

Flow cytometric validation of our putative surfaceome target antigens was performed using an antibody panel shown in Supplementary Table S4. Independent AML patient samples were analyzed in the Laboratory of Leukemia Diagnostics at the University Hospital Munich. After thawing, patient samples were washed with PBS and 1×10^6 cells were resuspended in 100 µl of FACS buffer. After staining with the appropriate amount of antibody or isotype control, cells were washed and data was acquired using a Navios flow cytometer (Beckman Coulter). Subpopulations were gated as outlined in Supplementary Figure S2. The target antigen expression levels were determined by median fluorescence intensity (MFI) ratio by dividing the MFI value of the antigen-specific antibody by the MFI value of the respective isotype control.

Statistics

Venn Diagrams were generated using nVenn [16] and statistical analyses for significant differences between samples/groups were performed using unpaired two-tailed t-tests using GraphPad Prism 6, unless otherwise stated.

Results

Cell Surface Capture Technology

In this study, we aimed to interrogate the AML surfaceome from primary AML samples. The technology, initially described by Wollscheid et al. [14], relies on the labelling of accessible glycosylation sites or Lysine residues (Fig. 1). In theory, this approach thus allows to characterize the entirety of expressed surface proteins without the need of antibodies or any knowledge of the expected targets. However, the most relevant limitation for the applicability in primary patient samples is that the established protocol requires substantial cell numbers in excess of 10^8—10^9 cells. We thus set out to optimize the protocol for the interrogation of AML cells.

Protocol Optimization increases protein recovery

We set out to optimize the protocol by step-wise introduction of protocol modifications as outlined in Fig. 2A. While a slight reduction of the biocytin hydrazide concentration did not increase yield alone, together with improved mechanical homogenization the number of detected peptides increased by approx. 30% (Fig. 2B). However, the most pronounced improvement was detectable by including a second digestion step, and finally a modified digestion buffer markedly increased yield for both cluster of differentiation (CD) proteins (Fig. 2C) as well as Non-CD proteins (Fig. 2D). This highlights the importance of optimized digestion conditions for a maximum of mass spectrometric identifications. Importantly, these modifications to the original Glyco-CSC protocol also improved the specificity of the assay: We reasoned that peptides were likely specifically captured in the assay if they displayed the characteristic mass shift (0.984 Da) associated with successful N-glycosylation and the corresponding protein contained a transmembrane domain or signal peptide. Using this metric of specificity, 54% of all identified peptides in the unmodified Glyco-CSC protocol fulfilled these criteria. Notably, our modified protocol was able to increase this metric to 80.4%.

Next, we aimed to interrogate whether Glyco-CSC is sufficient to detect the majority of cell surface proteins or whether the CSC-variants Lys-CSC or Cys-Glyco-CSC drastically increase the number of identified proteins in AML. Using our modified protocol, we noted that the vast majority of proteins were identified using

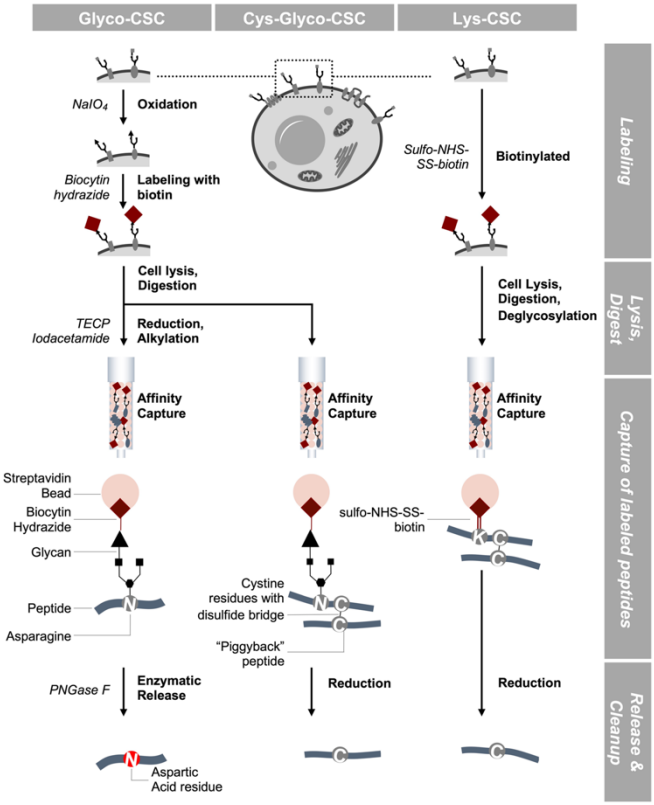


Fig. 1 Outline of Glyco-Cell Surface Capture (CSC) and its variants Cys-Glyco-CSC and Lys-CSC. The workflows adapted from the protocols by Wollscheid et al. as well as Bausch-Fluck et al. [14, 15] and are comprised of labeling, digestion, affinity capture, peptide release and peptide identification by tandem mass spectrometry

the Glyco-CSC technology, whereas the number of additional proteins identified by either Lys-CSC or Cys-Glyco-CSC were only modest (Fig. 2E and F). These two observations allowed us to conclude that our protocol modifications drastically increased the number of identified proteins and that the vast majority of proteins was obtained by the Glyco-CSC method.

Our Modified CSC-workflow allows for the efficient capture of the AML surfaceome from primary patient samples

Next, we applied our modified Glyco-CSC methodology to primary patient samples. In previous studies in acute lymphoblastic leukemia, investigators (using the traditional Glyco-CSC protocol) used a xenoamplification strategy to obtain sufficient cell material for CSC [5]. We thus initially applied a similar strategy and obtained cells from two patient-derived AML xenograft samples and subjected them to our modified Glyco-CSC protocol. In parallel, our laboratory has established a robust culture system that allows for culturing and functional interrogation of primary AML samples *in vitro* [12]. We previously validated that this culture system retains antigen expression levels, preserves the relative frequency of genetic (sub)clones and retains the frequency of

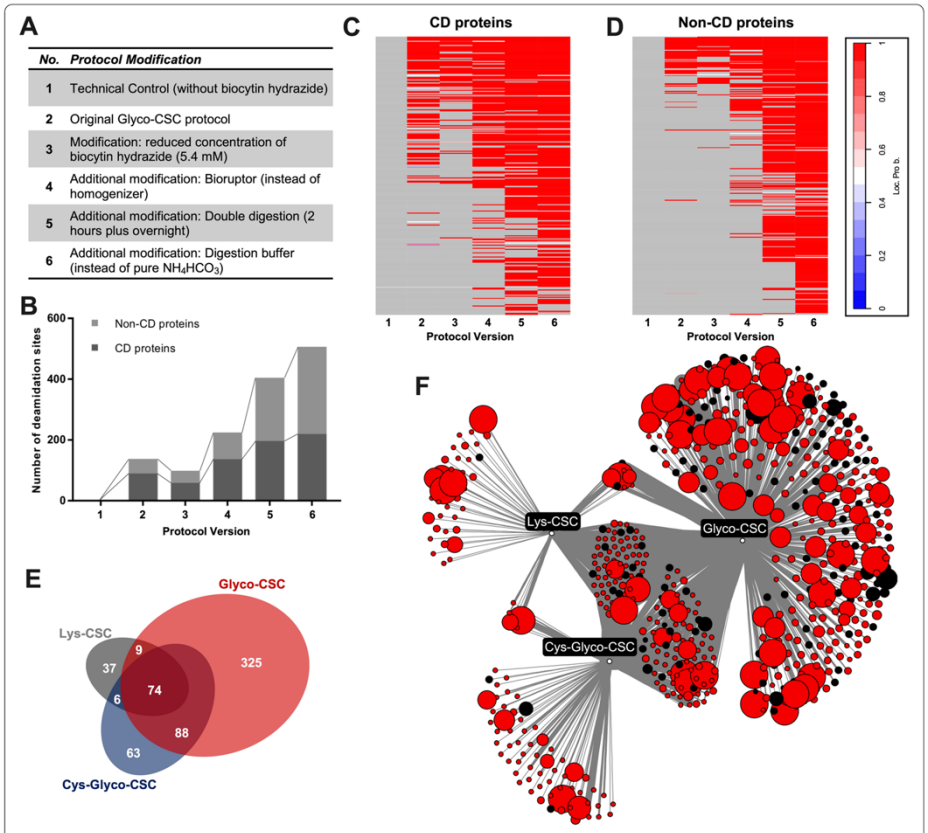


Fig. 2 Protocol Optimization Steps using OCI-AML3 cells. **A** Protocol optimization steps for Glyco-CSC examined. **B** Bar graph of number of deamidation sites from the OCI-AML3 cell line according to our improved protocol versions. No. 6 yielded > 500 deamidation sites, representing 252 proteins. **C** & **D** Probability of N-glycosylation events being localized at the expected position of identified peptides in each group (Loc. Prob., threshold ≥ 0.75) for CD proteins (C) and Non-CD proteins (D). **E** Comparison of detected proteins between Glyco-CSC, Lys-CSC and Cys-Glyco-CSC. Numbers refer to detected proteins. **F** Detailed view of detected proteins in comparison experiment summarized in (E). Nodes representing CD proteins (black) and non-CD proteins (red). Thickness of connecting lines is proportional to the number of peptides identified for the associated protein and size of the node indicates the number of transmembrane domains (TMs). Proteins with ≥ 10 TMs were limited to 10

leukemia-initiating cells [11]. Given our extensive experience with this technique and our successful modifications of the Glyco-CSC protocol, we next subjected cells cultured *in vitro* for 3 days to the Glyco-CSC protocol.

Results for these experiments are summarized in Figs. 3A & B. In total, we identified 621 surface proteins on our primary patient samples. Importantly however, not all proteins were present on all samples (Fig. 3B). While the cell line experiments for OCI-AML3 cells (included in the heatmaps for reference) showed some overlap with the primary patient samples, we also identified surface proteins in several of the patient samples that were not present in the cell line data. Importantly, while the cell number obtained from the patient-derived xenografts was higher than what was available from short-term cultured primary patient

(See figure on next page.)

Fig. 3 Modified CSC-workflow allows for the interrogation of the AML surfaceome in primary patient samples. **A** 621 surface proteins identified with our modified CSC technology 7 clinical samples and 1 AML cell line (OCI-AML3), separated into CD proteins (black, 163) and non-CD proteins (red, 458). **B** Expression levels of CD proteins identified by CSC. Heatmap intensity indicates the log10 average of iBAQ. **C** Overview of filtering strategy to eliminate targets with abundant expression on normal healthy tissue using publicly available gene expression databases. Furthermore, only proteins that were detected in at least half of the primary patient samples were considered. As a result, 76 proteins remain as potential candidates for manual evaluation. **D** Protein expression of 5 new putative targets (top, in green) and 6 markers currently being investigated (bottom, in grey) as immunotherapeutic targets in AML samples

material directly, our data shows detection of similar total numbers of proteins (Fig. 3B), highlighting that the combination of protocol improvements and our *in vitro* culturing conditions allows for the robust interrogation of the AML surfaceome directly from primary patient material. Of note, we observed a > 90% overlap in detected proteins in technical replicates, underlining the robustness of our technique (Supplementary Figure S3).

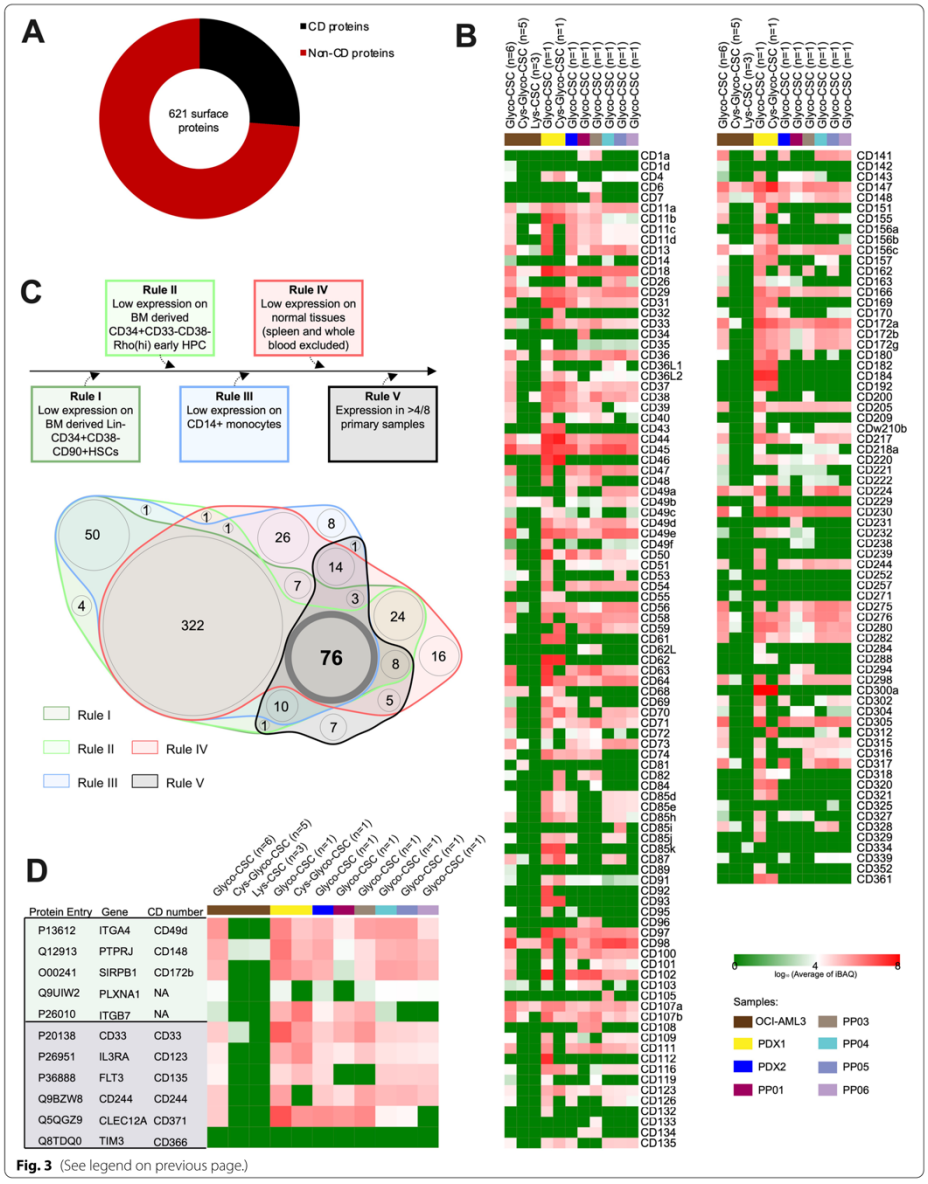
Filtering of putative targets

Next we set out to identify a shortlist of potential targets for immunotherapy in AML. Unlike the B cell target antigen CD19, where significant on-target, off-tumor activity is well tolerated, targeting of antigens that are widely expressed on myeloid lineage cells is more problematic. The main concern in this context remains toxicity against healthy cells but also widely expressed antigens acting as an 'antigen-sink', which depletes the amount of bioavailable therapeutic agent [17]. We thus set out to eliminate antigens that are highly expressed on cells within the hematopoietic stem cell compartment, on mature monocytes and on non-hematopoietic tissues. Our approach involved the mining of publicly available gene expression data of these tissue types. We defined a set of 5 rules, which focused on different cellular compartments, where expression of our putative targets might limit therapeutic efficacy. These were immature hematopoietic stem cells as well as early hematopoietic progenitor cells, CD14 + monocytes, and non-hematopoietic normal tissues. Specifically, we determined expression levels of our surfaceome candidate genes in these hematologic subpopulations and eliminated those genes with expression levels beyond the upper standard deviation of the mean from our cohort (Supplementary Figure S1A). Finally, we required that the putative target needed to be detected in our Glyco-CSC surfaceome dataset in more than half of the primary patient samples investigated (Fig. 3C, top panel). Interestingly, the largest number of excluded targets was eliminated during this last step (322 targets), further illustrating that AML remains an immunophenotypically heterogeneous disease. Applying this set of filters to our dataset, we obtained a shortlist of 76 putative targets (Fig. 3C, bottom panel), which can now be subjected to manual curation and further validation (Supplementary Table 2). Of note, in our dataset, many immunotherapeutic targets currently investigated for the therapy of AML were also detected, including CD33, CD123 (IL3RA), and CLL-1 (CLEC12A), amongst others. Detection of our putative list of targets was robust across the set of samples investigated, with few outliers (Fig. 3D). The data for the AML Surfaceome and the respective gene expression values from healthy tissues are summarized in Supplementary Table 3.

Mutational burden analysis

To further scrutinize our list of detected surface proteins, we aimed to interrogate whether these genes are likely functionally relevant and thus expected to be of low risk to escape under therapeutic pressure. By enumerating the rate of nonsynonymous mutations observed in large patient cohorts, we aim to identify whether *expressed* genes of interest are *hypo*mutated and thus might serve an *essential* role in leukemic cell survival. We interrogated the database curated by the Catalogue Of Somatic Mutations In Cancer (COSMIC, V91) and specifically enumerated the non-synonymous vs. synonymous mutations (dN/dS-ratio) found in the set of genes identified in the AML surfaceome. We excluded the known driver genes in FLT3 and CALR for this analysis. Most genes showed a low non-synonymous mutation rate (Fig. 4A), only few genes were found to have high rates of non-synonymous mutations. Compared to non-hematologic malignancies, synonymous mutation rates in our surfaceome genes were lower, possibly reflecting the more essential role of these genes in hematologic cancers (Fig. 4B).

Most notably, genes currently under investigation as immunotherapeutic targets in AML had low dN/dS-ratios, indicating their relative stability. Importantly, our putative novel targets identified in this study reliably showed a very low dN/dS-ratio, in line with genes that are of functional relevance for hematologic malignancies.



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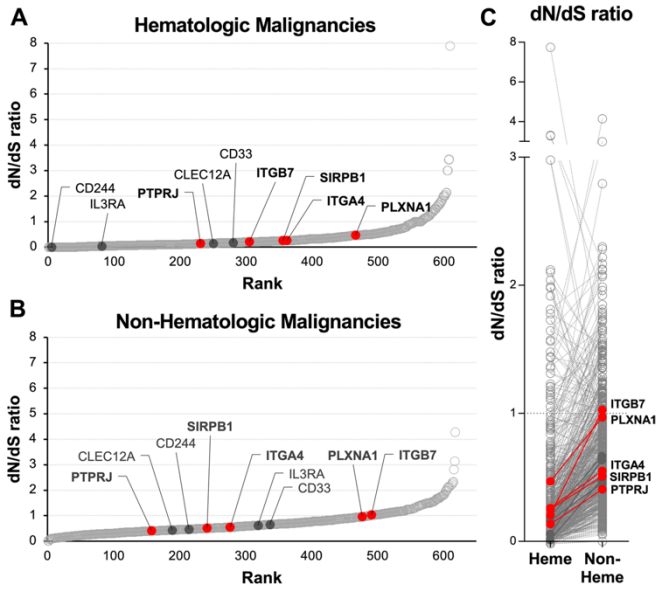

Fig. 4 Candidate antigens display low frequency of non-synonymous mutations, suggesting their functional relevance in hematologic malignancies. **A** Ratio of Non-synonymous to synonymous mutations in hematologic malignancies found in genome-wide screens in the COSMIC database in genes represented in our surfaceome dataset. Genes are sorted by rank of dN/dS ratio. Selected immunotherapy targets are highlighted. **B** Ratio of Non-synonymous to synonymous mutations in Non-hematologic malignancies in the COSMIC database. Genes are the same as in A. **C** Pairwise comparison of relative Non-synonymous mutation rates amongst surfaceome genes between Hematologic and Non-Hematologic malignancies

Validation in independent patient samples and healthy donors

Encouraged by these results we next validated the protein expression of a small set of novel, putative targets on a set of independent AML samples obtained during routine clinical diagnosis at our clinic. Of note, while the cell number required for our CSC-workflow required a short *in vitro* expansion, these independent patient samples used for target validation by flow cytometry were measured directly without any *in vitro* culture. We found that amongst the 5 targets investigated here, CD148, ITGA4 and to a lesser degree Integrin beta-7 (encoded by the ITGB7 gene) were consistently expressed on the cell surface in primary AML samples (Fig. 5A). Next, we measured the abundance of surface expression of these three targets on healthy hematopoietic stem and progenitor cells as well as mature granulocytes, monocytes and lymphocytes (Supplementary Figure S2). Interestingly, CD148 was found to show significant expression in both the mature lymphocyte, but more pronounced in the monocyte and granulocyte compartment, and ITGA4 showed moderate expression in the healthy HSPC compartment (Fig. 5A). These results are noteworthy, since our own selection process involved the removal of proteins found to be highly expressed based on gene expression levels in some of these subsets. This highlights the necessity to perform protein-level validation in healthy cell populations of interest even if gene expression data is available. Out of our list of putative targets, Integrin beta-7 showed the most promising combination of uniform – albeit moderate – expression in patient AML samples, and low or absent expression on healthy hematopoietic tissue (Fig. 5A and B).

Discussion

Immunotherapeutic approaches targeting cell surface proteins have shown remarkable success in a variety of cancers. AML, has thus far remained a challenging entity for successful targeting. This is thought to be due to the choice

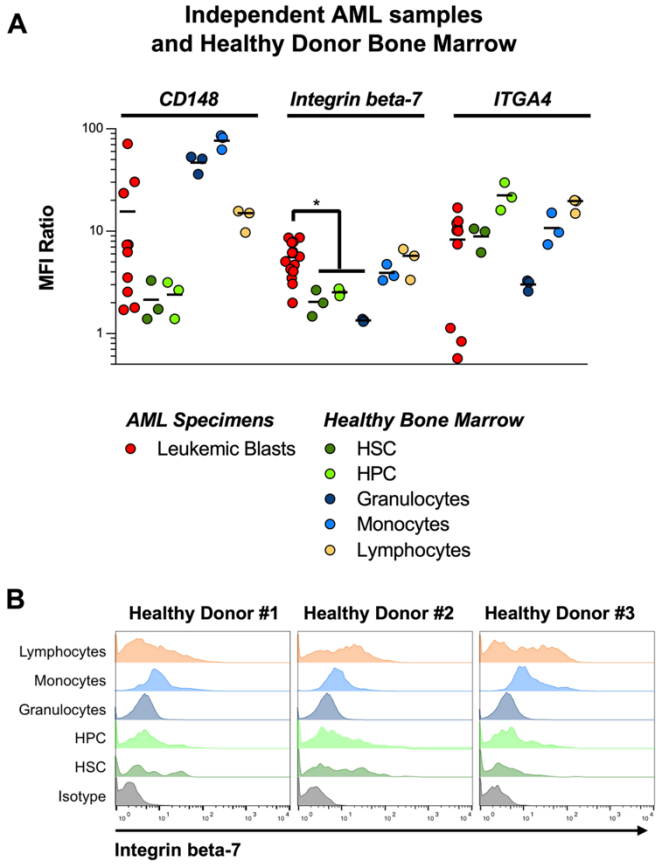

Fig. 5 Validation of putative targets in independent samples by flow cytometry. **A** Expression of potential targets on independent patient samples and healthy donor bone marrow specimens by FACS, showing significant expression on healthy mature monocytes and granulocytes for CD148 or HSPCs for ITGA4. *$p < 0.05$ **B** Distribution of Integrin beta-7 on healthy donors showing favorable (low) expression on healthy hematopoietic tissues

of antigen that is being targeted, with most approaches focusing on myeloid antigens. While there has been some success in early trials targeting CD33 and CD123, on-target off-tumor toxicity has been of concern [17]. Thus, identification of novel target antigens is of great interest to the field. In this study, we optimized the cell-surface capture technology to detect the surfaceome of primary human AML. Importantly, compared to prior reports on either cell lines or xenoamplified ALL samples, our optimized system is capable of reliably detecting a high number of proteins directly from AML patient specimens, including especially less well characterized non-CD proteins. This allowed us to explore novel target antigens without the use of a-priori defined antibody panels. Using this approach, we identified 621 surface antigens expressed on our primary AML samples. We next devised a strategy to curate promising candidates for further evaluation. To serve as an ideal target antigen, putative targets have to show uniform expression, have low or absent expression on non-tumor tissue and ideally be functionally relevant to

the tumor, i.e. loss of expression should confer a disadvantage to the tumor cells. We thus employed several steps to fulfill these criteria: 1) we demonstrate expression of our novel putative target antigens on a cohort of independent patient samples, 2) we demonstrate low expression levels on healthy tissue, including hematopoietic stem and progenitor cells by mRNA and protein, and 3) devise a strategy to anticipate functional relevance in tumor biology by determining the dN/dS-ratio of our putative targets.

Here, we identify three putative targets—CD148, ITGA4 and Integrin beta-7—previously not described for AML. As a membrane protein tyrosine phosphatase, CD148 is expressed on all hematopoietic lineages and plays multiple roles relevant to cell adhesion, migration, proliferation and differentiation [18–20]. Besides those functions on immune cells, CD148 has also been implicated to regulate the activation of platelets through the Src family kinases [21, 22]. It has also been considered as a potentially useful discriminating marker for the diagnosis of mantle cell lymphoma [23] to characterize mature B cell lymphoid neoplasms infiltrating blood and bone marrow [24]. Finally, CD148 has also been implicated as a prognostic marker of gastric cancer [25]. Similar to this plethora of roles, we also detected expression of CD148 on various immune cell populations including monocytes, granulocytes and lymphocytes. Therefore, we hypothesize that CD148 might be best suited as a therapeutic target in combinatorial strategies [26].

ITGA4 (CD49d) acts as a part of the very late antigen-4 (VLA-4) and has been studied in the context of chronic lymphocytic leukemia (CLL), where it has been found to represent an adverse prognostic marker [27–29]. Similarly, ITGA4 has been shown to correlate with prognosis in patients with early Oral Squamous Cell Carcinoma [30]. In our analysis, expression of ITGA4 was less homogenous, similarly to the reported binary expression pattern in CLL. However, ITGA4 expression was also detected on several healthy hematopoietic cells, which might impact the suitability of ITGA4 as a single target antigen. Studies on the prognostic role of ITGA4 expression in AML are very limited [31], thus we expect future studies to illuminate its role further.

Integrin beta-7 has been suggested to play a role in high-risk multiple myeloma and overexpression of Integrin beta-7 has been associated with DNA-hypomethylation [32]. In addition to its cell adhesion, migration and homing roles, Integrin beta-7 has also been suggested to participate in regulating the immune microenvironment as well as suppressing cell proliferation through the glycolysis/glucose metabolism pathway [33, 34]. However, the exact role of Integrin beta-7 specifically in AML is unknown. Interestingly, proteins encoded by ITGB7 and ITGA4 form a heterodimer known as LPAM-1, thus likely explaining the coincidence on our list of putative targets. At the same time, Integrin beta-7 is an exclusive dimerization partner ITAE [35]. Therefore, targeting of heterodimers formed by Integrin beta-7 could improve specificity of immunotherapeutic approaches.

We aimed to determine the feasibility of our approach in a diverse set of primary patient samples, representing a variety of genetic backgrounds and wanted to explore whether we might be able to identify common target antigens using this approach. Further studies will have to determine if genetically defined subgroups of AML cases will reveal differentially expressed target antigens. Our method represents a proof-of-concept approach to evaluate this in future studies.

Taken together, this study established an improved proteomics assay for the efficient interrogation of the entire surface proteome (surfaceome) of primary acute myeloid leukemia without the necessity of xenograft amplification. We demonstrate how these datasets can be integrated with other modalities to comprehensively explore putative novel antigens. In our dataset, Integrin beta-7 showed the most promising constellation of homogenous expression in our validation cohort and low expression on healthy tissues. Thus, Integrin beta-7 is a potential antigen of interest for AML that has not been proposed yet, warranting further study.

Conclusion

We developed an integrated approach which allows for unbiased, genome-wide screening of all three modalities – mutation, transcription and surface expression – for target discovery in immunotherapy of AML directly from patient specimens. Using this approach, we identify Integrin beta-7 as a potential target antigen for the immunotherapy of AML and validate this candidate antigen in primary AML patient specimens.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40364-022-00390-4.

Additional file 1: Table S1. Clinical characteristics of patients included in study.

Additional file 2: Table S2. Shortlist of 76 putative targets.

Additional file 3: Table S3. AML Surfaceome and gene expression values from healthy tissues.

Additional file 4: Table S4. Antibody panel for flow cytometric validation.

Additional file 5: Figure S1. Expression patterns and cut-off determination for AML Surfaceome. A: Expression of Surfaceome genes in hematopoietic stem cells in the bone marrow (HSCs – top panel), early hematopoietic progenitors in the bone marrow (HPC, middle panel) and CD14+ monocytes (bottom panel). Data derived from BloodSpot

database (HemaExplorer). Cut-off used for filtering of Surfaceome candidates shown (calculated as the mean + SD). B: Expression of Surfaceome genes in non-hematopoietic tissue, displaying average non-heme tissue expression (top panel) or maximal non-heme tissue expression (from Genotype-Tissue Expression Project - GTEx). Cut-off used for filtering of Surfaceome candidates shown (calculated as the mean + SD).

Additional file 6: Figure S2. Gating of subpopulations for FACS validation of putative Surfaceome targets. A: Expression of putative Surfaceome markers was assessed on healthy bone marrow specimens gating for both mature (lymphocyte, monocyte, granulocyte) as well as progenitor (hematopoietic stem cell "HSC", hematopoietic progenitor cell "HPC") populations. B: Gating of "Blast" population in leukemia specimens. "Blasts" were gated based on dim/intermediate CD45 expression.

Additional file 7: Figure S3. Number of proteins identified in technical replicates. Venn Diagram representing number of unique proteins identified by our modified CSC workflow from a xeno-amplified AML specimen (PDX1) processed separately. >90% of proteins were identified in both runs, suggesting highly robust identification of the surface proteome.

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Authors' contributions
T.K. and M.S. conceived the study. T.K. and X.L. designed and performed experiments. C.K., S.H. and G.H. performed experiments. V.S-M. and F.H. performed LC–MS measurements. T.K., V.B., G.H., W.H. and M.S. analyzed data. T.K. and M.S. wrote the manuscript with support from all authors. All authors read and approved the final manuscript.

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Availability of data and materials
All primary data generated in this study is supplied as supplementary information.

Declarations

Ethics approval and consent to participate
Patient specimens used in this study were obtained after informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Ludwig-Maximilians-Universität (Munich, Germany).

Consent for publication
Not applicable.

Competing interests
M.S. has received research support from Amgen, Gilead, Miltenyi Biotec, Morphosys, Roche, and Seattle Genetics, sits on the advisory boards of Amgen, Celgene, Gilead, Janssen, Novartis, Pfizer, and Seattle Genetics, serves on the speakers' bureau at Amgen, Celgene, Gilead, Janssen, and Pfizer, and has served as a consultant/advisor to Amgen, BMS, Celgene, Gilead, Pfizer, Novartis, and Roche.

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