Enhancing Dendritic Cell Vaccination by Immune Checkpoint Blockade as Therapy in AML

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List of Abbreviations

AML	Acute myeloid leukemia
APC	Antigen-presenting cell
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CMV	Cytomegalovirus
CR	Complete remission
CTLA-4	Cytotoxic T lymphocyte associated protein-4
DC	Dendritic cell
FDA	Food and drug administration
FGL-1	Fibrinogen-like Protein 1
GAL-3	Galectin-3
HSCT	Hematological stem cell transplantation
IFN	Interferon
IL	Interleukin
LAA	Leukemia-associated antigen
LAG-3	Lymphocyte activation gene 3
LSECtin	Liver sinusoidal endothelial cell lectin
MHC	Major histocompatibility complex
mRNA	Messenger Ribonucleic acid
NK cell	Natural killer cell
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein ligand 1
PD-L2	Programmed cell death protein ligand 2
PRAME	Preferentially expressed antigen in melanoma
RFS	Relapse-free survival
RNA	Ribonucleic acid
TAA	Tumor-associated antigen
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor

T_{reg}	Regulatory T cell
WT1	Wilms tumor 1

List of Publications

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

Publication I:

„Toll-like receptor 7/8-matured RNA-transduced dendritic cells as post-remission therapy in acute myeloid leukemia: results of a phase I trial."

Felix S. Lichtenegger, Frauke M. Schnorfeil, **Maurine Rothe**, Katrin Deiser, Torben Altmann, Veit L. Bücklein, Thomas Köhnke, Christian Augsberger, Nikola P. Konstandin, Karsten Spiekermann, Andreas Moosmann, Stephan Boehm, Melanie Boxberg, Mirjam H.M. Heemskerk, Dennis Goerlich, Georg Wittmann, Beate Wagner, Wolfgang Hiddemann, Dolores J. Schendel, Gunnar Kvalheim, Iris Bigalke, Marion Subklewe

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Publication II:

„**Targeting LAG-3 and PD-1 to enhance T cell activation by antigen-Presenting cells**."

Maurine Rothe*, Felix S. Lichtenegger*, Frauke M. Schnorfeil, Katrin Deiser, Christina Krupka, Christian Augsberger, Miriam Schlüter, Julia Neitz and Marion Subklewe:

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The proportion and distribution of work that has been contributed by the individual authors is listed in the publication (chapter 3, page 23-24, 39 and 63).

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1. Summary

The success of checkpoint inhibition has changed treatment algorithms in several tumor entities within the past years. Treatment success has mainly been observed in cancers with an inflamed microenvironment and an immune infiltrate leading to upregulation of checkpoint molecules on tumor cells as a means of immune escape. Hence, in tumor entities with a low endogenous anti-tumor response, such as acute myeloid leukemia (AML), checkpoint inhibition as monotherapy has so far shown no clinical benefit. Therapeutic vaccination based on autologous dendritic cells (DCs) pulsed with leukemia-associated antigens (LAA) is able to elicit anti-leukemic immunity. The combination with checkpoint inhibitors might enable to enhanced anti-leukemic immune responses in two ways: First, by blocking the interaction between checkpoint molecules on anti-leukemic T cells and upregulated checkpoint molecules on the leukemic target cells; and second, by enhancing the initial interaction between T cells and DCs which constitutively express inhibitory checkpoint molecules on their surface. Thus, a combinatorial therapy of DC vaccination and checkpoint blockade, in particular for cancers with a low endogenous anti-tumor response is a promising treatment strategy.

We have implemented a phase I/II first-in-human clinical study using monocyte-derived toll-like receptor (TLR) 7/8-matured next-generation DCs loaded with wilms tumor 1 (WT1), preferentially expressed antigen in melanoma (PRAME) and cytomegalovirus (CMV)pp65 RNA as post-remission therapy of AML patients with a non-favorable risk profile.

DC vaccination was feasible and safe and induced antigen-specific immune responses. AML-specific T cell responses correlated with improved relapse-free survival (RFS), especially in younger patients (≤ 65 years).

Despite a strong co-stimulatory profile, DCs also expressed co-inhibitory checkpoint ligands. We examined those inhibitory interactions using an *in vitro* T cell-DC coculture. DC-activated T cells upregulated programmed cell death protein 1 (PD-1) and lymphocyte activation gene 3 (LAG-3), while DCs expressed the respective ligands programmed cell death protein ligand 1 (PD-L1) and major histocompatibility complex (MHC) class-II. As hypothesized, we demonstrated that blockade of PD-1 and particularly of LAG-3 by suitable blocking antibodies enhanced DC-induced T cell activation.

We conclude that TLR7/8-matured next-generation DC vaccination induces vaccine antigen-specific immune responses which may lead to delay or prevention of relapse. Our *in vitro* data supports the rationale of combining DC vaccination with PD-1 and/or LAG-3 blockade to further augment anti-leukemic immune responses and improve clinical outcome.

2. Introduction

2.1 Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a hematological malignancy, which is defined by disrupted differentiation and uncontrolled proliferation of myeloid progenitor cells in bone marrow and blood (Dohner, Weisdorf et al. 2015).This results in proneness to infections and anemia and ultimately in multiple organ failure and death. AML is the most frequent leukemia among adults with a median age of 68 years and an incidence rate of 19,520 new cases and 10,670 deaths in the US in 2018 (Lichtenegger, Krupka et al. 2017, Siegel, Miller et al. 2018).

AML is a genetically and clinically heterogeneous disease with a very poor prognosis. Despite the strong need for improvement, the treatment has barely changed over the past decades: The standard treatment after diagnosis is a high dose induction chemotherapy comprising three days of anthracycline and seven days of cytarabine. This so called “3+7” regimen induces complete remission (CR) in about 80% of the patients (Dohner, Estey et al. 2010, Burnett, Wetzler et al. 2011, Ferrara and Schiffer 2013, Lichtenegger, Krupka et al. 2017). However, the risk of relapse is high due to chemorefractory leukemic cells. A post-remission therapy to eliminate residual leukemic cells is therefore mandatory (Reinisch, Chan et al. 2015).

Usually, patients with a favorable genetic risk profile get additional cycles of chemotherapy as consolidation, whereas the method of choice for AML patients with high relapse risk is allogeneic hematopoietic stem cell transfer (HSCT). HSCT was the first curative immunotherapy for patients with hematological malignancies (cure rate over 50%). Its clinical benefit relies in particular on the so-called graft-versus-leukemia effect: allogeneic T and natural killer (NK) cells of the donor recognize and target malignant cells of the recipient. However, the beneficial potential of anti-leukemic responses is opposed by the individual risk of graft-versus-host disease. In addition, the donor availability is challenging (Stelljes, Krug et al. 2014, Kassim and Savani 2017). Especially elderly patients (< 60 years) are often not medically fit for intensive therapies including HSCT (Klepin, Rao et al. 2014). Thus, there is a high medical need for the development and improvement of novel therapies.

2.2 Cancer Immunotherapy in AML

Cancer immunotherapy aims to direct the body´s own immune system against malignant tumor cells. It represents one of the most promising novel strategies to cure cancer and to decrease relapse rates (Rusch, Bayry et al. 2018). Various T cell based immunotherapeutic strategies to eliminate chemorefractory leukemic cells are currently under preclinical- and clinical investigation (Lichtenegger, Krupka et al. 2015, Lichtenegger, Krupka et al. 2017). The most prominent are:

- T cell engaging antibody based approaches to recruit T cells to target antigen expressing tumor cells (Jin, Lee et al. 2009, Laszlo, Gudgeon et al. 2014).
- Adoptive T cell transfer (TCR-, or CAR T cell therapy) to augment autologous T cells in number and tumor antigen specificity (Xue, Gao et al. 2005, Spranger, Jeremias et al. 2012, Brenner 2013, Pizzitola, Anjos-Afonso et al. 2014, Prommersberger, Jetani et al. 2018, Gomes-Silva, Atilla et al. 2019)
- Dendritic cell vaccination to induce strong and durable tumor antigen-specific T cell responses (Van Tendeloo, Van de Velde et al. 2010, Anguille, Willemen et al. 2012, Khoury, Collins et al. 2017, Weinstock, Rosenblatt et al. 2017).
- Immune checkpoint blockade to enhance or reactivate preexisting anti-tumor T cell responses (Alatrash, Daver et al. 2016).

The future will show the advantages and disadvantages of each anti-leukemic treatment strategy in AML.

2.3 Dendritic Cell Vaccination in AML

2.3.1 Vaccination Strategies

The induction of tumor antigen-specific immune responses is the primary goal in cancers with a low mutational burden and no (or only low) preexisting endogenous anti-tumor immune responses, such as AML (Yarchoan, Hopkins et al. 2017). Consequently, leukemia-specific neoantigens arised by mutations and restricted to the tumor are very rare in AML.

Leukemia-associated antigens (LAAs) are endogenous antigens which are overexpressed by leukemic cells compared to healthy tissues. Thus, LAAs can be presented via peptide MHC-complexes (pMHC) to T cells. Despite immunological tolerance towards self-antigens, these pMHC-T cell interactions were shown to induce detectable anti-LAA-specific immune responses (Rosenberg 1999, Anguille, Van Tendeloo et al. 2012). In the last decades, numerous LAAs were identified (Greiner, Li et al. 2005). Nevertheless, the clinical outcome of LAA peptide vaccination in AML still remains unsatisfying. A major difficulty of peptide vaccination is to overcome T cell tolerance against self-restricted LAAs and transformation into efficient specific T cell responses eradicating the tumor (Schmitt, Casalegno-Garduno et al. 2009, Subklewe, Geiger et al. 2014).

As the most potent professional antigen-presenting cells (APCs) DCs are capable to initiate both, tolerance as well as strong long-lasting (innate and adaptive) immune responses (Banchereau and Steinman 1998, Steinman 2001). In the context of an inflammatory response, DCs undergo a maturation process that includes upregulation of cell surface MHC and co-stimulatory molecules and secretion of numerous cytokines including IL-12p70 which are crucial for the induction of primary T cell responses (Sallusto and Lanzavecchia 2002). Thus, mature tumor-associated antigen (TAA)-presenting DCs are highly eligible as cellular adjuvant for targeted therapeutic vaccination (Timmerman and Levy 1999). Numerous *in vivo* experiments have already demonstrated the capacity of injected TAA-loaded DCs to induce TAA-specific T cell responses and tumor regression.

The therapy relies on patient-derived DCs that are *ex vivo* manipulated- and TAA -loaded. Crucial parameters for DC vaccination are *inter alia* the source of DC precursors (PBMCs, primary DCs,..), DC maturation protocol (TLR agonist based etc), target antigen (TAA/LAA), way of antigen loading, route of application (peptide pulsing, *in vitro*-transcribed RNA electroporation etc), and application interval

(Saxena and Bhardwaj 2018). Monocyte-derived DCs have been reported to induce the most potent immune responses. However, there are also alternative attempts using DC-like constructs (Rosenblatt, Stone et al. 2016, Lichtenegger, Krupka et al. 2017, Sprooten, Ceusters et al. 2019).

2.3.2 Ongoing Phase II Clinical Trials on DC-Vaccination as Therapy in AML

Different treatment strategies of high-risk AML patients are currently under investigation. Noteworthy is a personalized DC-AML hybridoma vaccination strategy, which relies on the fusion of autologous AML cells with autologous DCs. This hybridoma is thought to stimulate broad anti-tumor responses and was tested in 17 AML patients in CR. It was well tolerated and showed an augmentation of leukemia-specific T cell responses as well as durable remissions (Rosenblatt, Stone et al. 2016, Nahas, Stroopinsky et al. 2019). However, this study comes with a substantial bias regarding the selection of long-term survivors, which complicates further conclusions (Lichtenegger, Krupka et al. 2017).

Most clinical trials are based on *in vitro* differentiated DCs from monocytes, but also some from CD34+ progenitors. Different ways of antigen loading are tested. A common approach is DC pulsing with a single tumor-associated protein or a peptide fragment. Specific immune responses were observed *in vitro*, whereas clinical benefit failed *in vivo* (Lesterhuis, Schreibelt et al. 2011). Major issues with peptide-pulsing are the restriction of T cell responses to the selected epitope and unattended post-translational modifications of the tumor antigen. DC electroporation with mRNAs circumvents this problem (Lesterhuis, De Vries et al. 2010). This approach has already been successfully tested in phase I/II studies for post-remission therapy of AML: A vaccination trial using hTERT mRNA electroporated DCs demonstrated vaccine antigen-specific T cell responses and RFS after a median observation time of 52 months in 58% of the vaccinated patients (n=19) (Khoury, Collins et al. 2017). In another trial, DCs loaded with WT1 mRNA induced anti-leukemic responses in 43% and molecular remission in 30% of the patients (n=30). A correlation between overall survival (OS) and WT1-specific $CD8^+$ T cell responses was thereby demonstrated (Van Tendeloo, Van de Velde et al. 2010, Anguille, Van de Velde et al. 2017).

In those studies DC maturation was performed using the gold standard cocktail consisting of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and prostaglandins

(PGE2) (Jonuleit, Kuhn et al. 1997). While this protocol was developed to increase the expression of DC maturation markers as well as immunostimulatory and migratory capacities, the resulting cells lack secretion of IL-12p70, which is crucial to induce optimal anti-tumor immune responses. IL-12p70 leads to a Th1 polarization of $CD4^+$ cells, which, in turn, support the activation of both, TAA-specific $CD8^+$ T cells as well as of NK cells (Carreno, Becker-Hapak et al. 2013).
Inflammatory cytokine secretion by DCs is typically triggered by activation of toll-like receptors (TLR). Physiologically, TLRs signal upon recognition of pathogen patterns. Many of those receptors exist with different functions and pathways to be involved in (Schreibelt, Tel et al. 2010). In the case of IL-12p70, TLR7/8 and TLR3 signaling pathways need to get activated (Napolitani, Rinaldi et al. 2005). Furthermore, the standard protocols for DC differentiation from monocytes were based on seven days (7d) of cell culture. An accelerated production of clinical-grade mature DCs lowers the expenses of manufacture. In addition, the faster differentiation might reflect more appropriate the situation *in vivo* (Burdek, Spranger et al. 2010, Subklewe, Geiger et al. 2014).

2.3.3 New Generation DC Vaccine for Immunotherapy of AML

Our group developed a three-day (3d) GMP compliant protocol for the generation of DCs based on a TLR7/8 agonist (Subklewe, Geiger et al. 2014). Monocytes are isolated and subsequently stimulated by addition of GM-CSF and IL-4. The following day, DCs are matured by a 24h *in vitro* culture using a cocktail composed of the synthetical TLR7/8 agonist R848 together with PGE2 as well as the cytokines TNF-α, IL-1β and IFN-γ (Zobywalski, Javorovic et al. 2007, Beck, Dorfel et al. 2011, Subklewe, Geiger et al. 2014). We tested our new cocktail in comparison to the gold standard cocktail for the maturation of monocyte-derived 3d- and 7d-DCs, respectively. Similar to DCs generated based on a standard 7d-protocol, 3d-DCs displayed a mature surface phenotype and demonstrated their capacity to take up and present antigens and were able to stimulate antigen-specific T cells. However, they had several superiorities: Increased yields of viable DCs were obtained. The relative surface-expression of the co-stimulatory molecules CD80 and CD86 was higher than the one of the co-inhibitory molecule PD-L1. Dramatically higher levels of IL-12p70 were secreted upon CD40-CD40-ligand interaction whereas IL-10 secretion, which would support an undesired Th2 polarization of $CD4^+$ T cells was

low. Moreover, 3d-DCs demonstrated an enhanced migratory ability *in vitro* compared to 7d-DCs. This observation was in line with a substantial expression of the chemokine receptor CCR7 (Burdek, Spranger et al. 2010, Lichtenegger, Mueller et al. 2012). Thus, TLR7/8-matured 3d-DCs demonstrated a significantly improved ability for Th1 polarization and antigen-specific activation of autologous T cells compared to DCs generated with the standard cocktail. In addition, NK cells were highly increased. The protocol was also evaluated for the generation of mature 3d-DCs from monocytes of AML patients in remission. (Zobywalski, Javorovic et al. 2007, Spranger, Javorovic et al. 2010, Beck, Dorfel et al. 2011, Lichtenegger, Mueller et al. 2012, Subklewe, Geiger et al. 2014).

Based on these DCs we conducted a phase I/II proof-of-concept clinical study, which has been recently completed (publication I). DCs were pulsed with mRNA encoding the LAAs WT1, PRAME, and CMVpp65 as adjuvant and control antigen for vaccination of AML patients in CR with a non-favourable risk profile and not eligible for allogeneic HSCT (NCT01734304).

2.3.4 The Clinical Study Antigens WT1, PRAME, and CMVpp65

A fundamental part of the DC vaccination study design is the selection of suitable TAAs to elicit beneficial anti-tumor immunity and to prevent adverse events. WT1 and PRAME are both oncogenic LAAs with high expression on AML bulk cells (>85% and 65%) and on leukemic stem cells. Both were shown to be immunogenic and have already proven clinical efficacy and safety (Li, Giannopoulos et al. 2006, Keilholz, Letsch et al. 2009, Rezvani, Yong et al. 2009, Maslak, Dao et al. 2010, Quintarelli, Dotti et al. 2011).

WT1 is a zinc finger transcription factor and overexpressed in a wide range of cancers (including ovarian cancer) while it is rarely found in normal adult tissue.

WT1-specific T cells were detected in both, healthy individuals and in AML patients. AML patients treated with WT1 peptide-based vaccines demonstrated immune responses in clinical trials (Mailander, Scheibenbogen et al. 2004, Rezvani, Yong et al. 2008, Keilholz, Letsch et al. 2009, Subklewe, Geiger et al. 2014).

PRAME is a cancer testis antigen. It contributes to oncogenesis by impeding cell differentiation, growth arrest, and programmed cell death/apoptosis. High mRNA amounts of PRAME were detected in AML patients and correlated to the disease

(Greiner, Ringhoffer et al. 2004, Wadelin, Fulton et al. 2010, Subklewe, Geiger et al. 2014).

Human cytomegalovirus (CMV) is a member of the herpes virus family. Its 65-kDa phosphoprotein (pp65) has been verified as a main immunodominant and immunogenic target antigen for CMV-specific $CD8^+$ T cells. Hence, it was selected as a control and helper antigen for the DC study (Grigoleit, Kapp et al. 2007, Subklewe, Geiger et al. 2014).

2.3.5 Boosting DC-induced T Cell Responses

Besides high surface expression of co-stimulatory molecules, co-inhibitory molecules, such as PD-L1, have also been described to be expressed on DCs (Lichtenegger, Mueller et al. 2012). In this regard, it is noteworthy that MHC class-II, which is highly expressed on 3d-DCs can act as an inhibitory ligand of lymphocyte activation gene 3 (LAG-3), an immune checkpoint receptor particularly upregulated on activated T cells (more detailed in 2.4). Blockade of those interactions by monoclonal antibodies is a promising approach to reverse the inhibitory effects (Pardoll 2012). Therefore, combination of DC vaccination with checkpoint inhibition is a promising strategy to further enhance immune responses, particularly in cancer entities with a low endogenous anti-tumor response (Hobo, Maas et al. 2010).

2.4 Immune Checkpoint Blockade in Cancer Therapy

Immune checkpoint molecules are major targets in the field of cancer research. Inhibitory checkpoint receptors are mainly upregulated on activated T cells, while the interacting ligands were found to be constitutively expressed on the surface of APCs and/or upregulated on inflamed tissues usually triggered by inflammatory cytokines (such as IFN-γ). The physiological role of immune checkpoints is silencing of immune responses to protect from collateral tissue damage and autoimmunity. However, inhibitory immune checkpoint ligands can also be expressed or upregulated on cancer cells in the context of inflammation. This provides an escape mechanism for tumor cells from successful immune recognition and elimination (Pardoll 2012, Chen and Flies 2013).

Numerous of those molecules have been discovered in the last decades. Cytotoxic T lymphocyte associated protein-4 (CTLA-4) and programmed cell death protein-1 (PD-1) are the most prominent ones (Ishida, Agata et al. 1992, Krummel and Allison 1995, Weber 2010). The Blockade of the inhibitory interactions with their ligands (CD80/86 and PD-L1/-L2, respectively) using monoclonal antibodies demonstrated successful enhancement of anti-tumor immune responses in preclinical and clinical studies (Hodi, Mihm et al. 2003, Iwai, Terawaki et al. 2005, Pardoll 2012, Kyi and Postow 2014). This resulted in the FDA approval of antibodies against CTLA-4, PD-1 and PD-L1 for application in advanced solid tumors but also in classical Hodgkin lymphoma (Ansell, Lesokhin et al. 2015, Ottaviano, De Placido et al. 2019). In 2018, James P. Allison and Tasuku Honjo won the Nobel Prize in medicine for the discovery of these checkpoint inhibition approaches (Bazhin, Amedei et al. 2018, Smyth and Teng 2018).

Despite the clinical success of checkpoint inhibitors in terms of raised life expectancy, there still remains a large population of cancer patients who does not benefit from the treatment. Responses are limited to cancer entities with a high tumor mutational burden and side effects are still challenging (Topalian, Hodi et al. 2012, Ansell, Lesokhin et al. 2015, Sharma, Hu-Lieskovan et al. 2017, Seidel, Otsuka et al. 2018, Ottaviano, De Placido et al. 2019). Novel targets and combinatorial strategies for a broader application are sought after.

Studies with checkpoint inhibitors for the treatment of AML are still in early development and mainly restricted to PD-1 so far.

2.4.1 PD-1

Programmed cell death protein 1 (PD-1; CD279) belongs to the B7/CD28 family. Besides activated $CD4^+$ and $CD8^+$ T cells, PD-1 expression has been shown by B cells, monocytes, DCs and NK cells (Liang, Latchman et al. 2003, Okazaki and Honjo 2006). PD-1 interacts with PD-L1 (CD274) as well as with PD-L2 (CD273). PD-L1 is basically little expressed on normal tissues. However, it was shown to be upregulated by various tumor entities in inflammatory conditions (Taube, Anders et al. 2012). High PD-L1 surface expression on tumor cells correlates with decreased immune responses. PD-L2 is expressed on APCs and was also detected on certain solid tumors (Hobo, Hutten et al. 2018).

Some AML mouse models demonstrated the enhancement of anti-leukemic immune responses through blockade of the PD-1/PD-L1 pathway (Saudemont and Quesnel 2004, Zhang, Gajewski et al. 2009). The detection of PD-L1 and PD-L2 expression on human AML cells is heterogeneously reported among different studies (Chen, Liu et al. 2008, Zhang, Zhang et al. 2015, Annibali, Crescenzi et al. 2018). Nevertheless, PD-L1 was inducible upon stimulation with proinflammatory cytokines (Kronig, Kremmler et al. 2014). Similarly, PD-1 expression on T cells from AML patients was not always increased compared to healthy controls depending on the status of the disease, origin and T cell population (Schnorfeil, Lichtenegger et al. 2015, Tan, Chen et al. 2017, Jia, Wang et al. 2018). In a phase I clinical study of patients with advanced hematologic malignancies, the PD-1 modulating antibody Pidilizumab (CT-011) showed only one minimal response among the examined AML patients (Berger, Rotem-Yehudar et al. 2008). However, recent evidence suggests that Pidilizumab binds primary another target Delta-like 1 while the effects on PD-1 are only secondary and restricted to non-glycosylated and hypoglycosylated forms of this checkpoint molecule. Nevertheless, this underlines the acknowledged findings that response to checkpoint inhibition requires endogenous anti-tumor immune responses and correlates with the tumor mutational burden (Rizvi, Hellmann et al. 2015, Schumacher and Schreiber 2015, Yarchoan, Hopkins et al. 2017). As AML belongs to the cancer entities with low mutational rates, the clinical use of checkpoint inhibitors as a monotherapy is less encouraging in comparison to other hemato-oncological malignancies (Boddu, Kantarjian et al. 2018, Seidel, Otsuka et al. 2018, Curran and Glisson 2019) ASH

abstract/Blood (2016) 128(22):764).

The combination of checkpoint inhibitors with T cell inducing strategies are therefore of high interest. DC vaccination is a promising approach to induce T cell responses. T cell responses can be enhanced through blockade of upregulated checkpoint molecules (Ribas, Comin-Anduix et al. 2009, Curran and Glisson 2019). We examined the expression of several inhibitory checkpoint molecules on TLR7/8-matured next-generation DCs. PD-L1 and in particular MHC class-II which also acts as an inhibitory ligand of LAG-3, were highly expressed. DC-activated T cells upregulated corresponding receptors PD-1 and LAG-3. Therefore, we hypothesized that blockade of those interactions with suitable blocking antibodies further increase T cell activation by DCs (ASH abstract/Blood (2016) 128(22):764).

2.4.2 LAG-3

The inhibitory checkpoint molecule lymphocyte activation gene 3 (LAG-3; CD223) is a member of the immunoglobulin superfamily. LAG-3 is related to CD4 and also interacts with MHC class-II molecules on APCs, however with a higher affinity (Triebel, Jitsukawa et al. 1990, Demeure, Wolfers et al. 2001, Li, Wang et al. 2007). LAG-3 is expressed on activated $CD4^+$ and $CD8^+$ T cells and T_{regs} (Huang, Workman et al. 2004), as well as on certain NK cells (Huard, Tournier et al. 1998), B cells (Kisielow, Kisielow et al. 2005) and plasmacytoid DCs (Andreae, Buisson et al. 2003, Workman, Wang et al. 2009).

LAG-3 is localized in endosomal compartments in resting T cells, but it gets quickly upregulated on the T cell surface upon activation (Bae, Lee et al. 2014). Metalloproteases control surface expression of LAG-3 via cleavage from the membrane (Li, Wang et al. 2007). The soluble LAG-3 isoform was shown to activate APCs but also the proliferation of tumor cells particularly with regard to chronic lymphocytic leukemia (Shapiro, Herishanu et al. 2017). LAG-3 signaling silences activation and expansion of $CD4^+$ and $CD8^+$ T cells (Workman and Vignali 2003). Therefore, LAG-3 receptors associate with the CD3-TCR complex during formation of the immunological synapse (Hannier and Triebel 1999). The interaction of LAG-3 with MHC class-II molecules was shown to impede antigen-dependent activation of $CD4^+$ T cells (Hannier, Tournier et al. 1998, Macon-Lemaitre and Triebel 2005, Hobo, Hutten et al. 2018) and a similar role of MHC class-II for $CD8^+$ T cell activation is presumed (Matsuzaki, Gnjatic et al. 2010, Andrews, Marciscano et al.

2017). Nevertheless, the search for other binding partners is pursued. Galectin-3 has been shown to mediate inhibition of $CD8^+$ T cell responses via LAG-3 binding (Kouo, Huang et al. 2015). Furthermore, a role of LSECtin has been demonstrated in melanoma (Hemon, Jean-Louis et al. 2011) and Fibrinogen-like Protein 1 (FGL-1) was recently discovered as a major inhibitory ligand of LAG-3 (Figure 1) (Wang, Sanmamed et al. 2019). LAG-3 were found to be co-expressed with PD-1 by T cells in numerous viral and tumor murine models and human *ex vivo* experiments (Wherry, Ha et al. 2007, Tian, Zhang et al. 2015, Zarour 2016). Consequently, double knockouts or double blockade showed synergy in anti-virus and anti-tumor immune responses (Woo, Turnis et al. 2012, Huang, Eppolito et al. 2015, Foy, Sennino et al. 2016).

Monoclonal blocking antibodies targeting LAG-3 (relatlimab/BMS-986016, LAG525) alone or in combination with anti-PD-1 are currently assessed in clinical trials of patients with hematologic neoplasms/malignancies (NCT02061761, NCT03365791) (Long, Zhang et al. 2018, Andrews, Yano et al. 2019). Furthermore, dual-affinity re-targeting (DART) proteins targeting PD-1 and LAG-3 (MGD013 and FS118) are under current investigation. MGD013 is evaluated in phase I clinical studies for hematologic neoplasms (NCT03219268).

Initial data from a clinical trial using relatlimab in combination with nivolumab demonstrated efficacy in melanoma patients refractory to immunotherapeutic pretreatments (NCT01968109). The combined therapy was safe, with a similar risk profile to nivolumab alone. In total, the treatment resulted in responses of 11.5% of the patients (n=68). In addition, a correlation between LAG-3 expression (≥1%) and therapeutic success has been observed (ESMO abstract/ Ann Oncol (2017) 28(Suppl_5):v605–49. doi:10.1093).

A broad multiparameter flow cytometry analysis showed presence of LAG-3 positive T cells in bone marrow samples from AML patients. Notably, it was shown that the frequency of PD-1/LAG-3 double-positive $CD8^+$ and $CD4^+$ effector T cells was increased in bone marrow of AML patients compared to healthy donor controls (Williams, Basu et al. 2019). However, to this day, no clinical trials are testing the potential of LAG-3 blockade in AML.

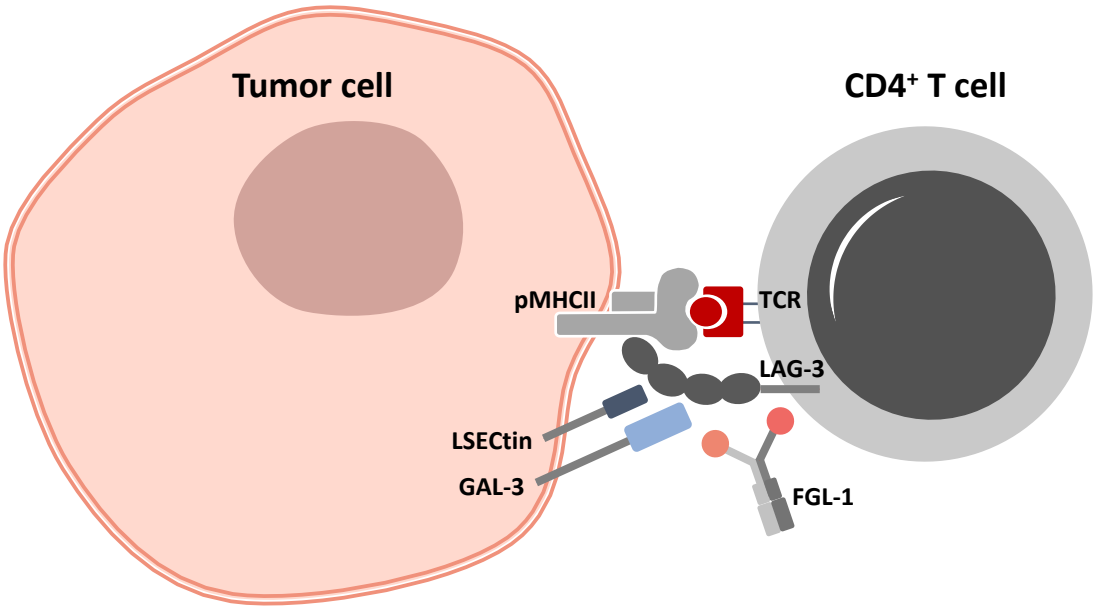

Figure 1: Lymphocyte activation gene 3 (LAG-3) receptor and its ligands. *Major histocompatibility complex class II (MHCII), Fibrinogen-like Protein 1 (FGL-1), Galectin-3 (GAL-3), Liver sinusoidal endothelial cell lectin (LSECtin)*

2.5 Aim of this Thesis

In the first part of this doctoral thesis I conducted the analysis of patient data from our DC vaccination clinical trial with respect to vaccine antigen-specific immune responses, OS and RFS (publication I). In the second part of the thesis, my focus was on studying the enhancement of T cell responses through the addition of checkpoint inhibitors to T cell – DC interaction. In particular, I evaluated the augmentation of antigen-specific T cell responses through DCs with or without checkpoint inhibition, with focus on PD-1 and LAG-3 to assess weather blockade of inhibitory checkpoint interactions enhances DC-induced T cell activation (publication II).

Results support the combination of therapeutic vaccines with checkpoint inhibition to augment antigen-specific T cell responses and reverse adaptive immune escape. The two publications are presented in the following chapter.

3. Publications

3.1 Author Contributions Publication I

„Toll-like receptor 7/8-matured RNA-transduced dendritic cells as post-remission therapy in acute myeloid leukemia: results of a phase I trial."

Felix S. Lichtenegger, Frauke M. Schnorfeil, **Maurine Rothe**, Katrin Deiser, Torben Altmann, Veit L. Bücklein, Thomas Köhnke, Christian Augsberger, Nikola P. Konstandin, Karsten Spiekermann, Andreas Moosmann, Stephan Boehm, Melanie Boxberg, Mirjam H.M. Heemskerk, Dennis Goerlich, Georg Wittmann, Beate Wagner, Wolfgang Hiddemann, Dolores J. Schendel, Gunnar Kvalheim, Iris Bigalke, Marion Subklewe

Journal of Clinical & Translational Immunology. 2020 Feb; doi: 10.1002/cti2.1117

The initial project idea of the clinical trial came from Felix Lichtenegger and Marion Subklewe. Together they planned the concept, all necessary requirements and designed the study protocol. Certain processes were supported by Wolfgang Hiddemann, Dolores Schendel, Gunnar Kvalheim and Iris Bigalke.

The clinical trial was performed by medical doctors at the University Hospital Munich. Mainly by members of the Subklewe group: Felix Lichtenegger, Torben Altmann, Veit Bücklein, Thomas Köhnke, Georg Wittmann, Beate Wagner, and Marion Subklewe. Generation of the vaccine was performed at the Oslo University Hospital by Gunnar Kvalheim and Iris Bigalke.

Immunomonitoring/Data acquisition was largely performed by Frauke Schnorfeil. The establishment and performance of the qPCR for the patients´ PRAME status on mRNA-levels, was done by me (Table 1). This included data analyses using the corresponding software.

WT1-specific T cells (Figure 1f) were generated by Christian Augsberger and me.

The data analysis/interpretation of all other experiments (Figure 1-5) was performed by Frauke Schnorfeil, Felix Lichtenegger, Marion Subklewe and me. Together, we evaluated and discussed the results. In particular, I created the swimmer plot (Figure 4) and the survival curves (Figure 5), and I analyzed and assigned the data of the matched AML-CG cohort (Figure 5 and Table S3) which was acquired by Felix Lichtenegger. Katrin Deiser, Chistian Augsberger, Nikola Konstandin, Karsten Spiekermann, Andreas Moosmann, Stephan Böhm, Melanie

Boxberg, and Mirjam Heemskerk were also involved in certain data acquisitions and/or interpretations.
The final statistical analysis for the manuscript were performed by me. Former statistical analyses were performed by Felix Lichtenegger and Frauke Schnorfeil, Katrin Deiser, and Dennis Görlich.
I designed all final figures and completely created the swimmer plot and the survival curves after consultation with Felix Lichtenegger and Marion Subklewe. Frauke Schnorfeil and Katrin Deiser performed the initial figure design. The manuscript was written by Felix Lichtenegger, Frauke Schnorfeil, Marion Subklewe, and me.

3.2 Author Contributions Publication II

„**Targeting LAG-3 and PD-1 to enhance T cell activation by antigen-Presenting cells**."

Maurine Rothe*, Felix S. Lichtenegger*, Frauke M. Schnorfeil, Katrin Deiser, Christina Krupka, Christian Augsberger, Miriam Schlüter, Julia Neitz and Marion Subklewe:

Frontiers in Immunology. 2018 Feb; doi: 10.3389/ fimmu.2018.00385

*contributed equally

Based on the vaccination trial (publication I), my supervisors, Felix Lichtenegger Marion Subklewe, and I conceptualized this project. Together, we conceived and designed the experiments.
All experiments (Figure 1-7) were performed either completely or mainly by me.
Frauke Schnorfeil, Katrin Deiser and Christina Krupka constructively supported this project in terms of technical questions and data analyses.
Christian Augsberger supported and guided me by the generation of WT1-specific T cells (Figure 7).
Miriam Schlüter and Julia Neitz performed a few of the replicates (Figure 1-3).
I performed data analysed in consultation with Felix Lichtenegger.
Based on the data Felix Lichtenegger, Marion Subklewe, and I planned the figure design, which was independently executed by me.
Felix Lichtenegger, Marion Subklewe, and I wrote the manuscript.

3.3 Publication I

Clinical & Translational Immunology

Clinical & Translational Immunology 2020; e1117. doi: 10.1002/cti2.1117
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ORIGINAL ARTICLE

Toll-like receptor 7/8-matured RNA-transduced dendritic cells as post-remission therapy in acute myeloid leukaemia: results of a phase I trial

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Abstract

Objectives. Innovative post-remission therapies are needed to eliminate residual AML cells. DC vaccination is a promising strategy to induce anti-leukaemic immune responses. **Methods.** We conducted a first-in-human phase I study using TLR7/8-matured DCs transfected with RNA encoding the two AML-associated antigens WT1 and PRAME as well as CMVpp65. AML patients in CR at high risk of relapse were vaccinated 10× over 26 weeks. **Results.** Despite heavy pretreatment, DCs of sufficient number and quality were generated from a single leukapheresis in 11/12 cases, and 10 patients were vaccinated. Administration was safe and resulted in local inflammatory responses with dense T-cell infiltration. In peripheral blood, increased antigen-specific $CD8^+$ T cells were seen for WT1 (2/10), PRAME (4/10) and CMVpp65 (9/10). For CMVpp65, increased $CD4^+$ T cells were detected in 4/7 patients, and an antibody response was induced in 3/7 initially seronegative patients. Median OS was not reached after 1057 days; median RFS was 1084 days. A positive correlation was observed between clinical benefit and younger age as well as mounting of antigen-specific immune responses. **Conclusions.** Administration of TLR7/8-matured DCs to AML patients in CR at high risk of relapse was feasible and safe and resulted in induction of antigen-specific immune responses. Clinical benefit appeared to occur more likely

in patients <65 and in patients mounting an immune response. Our observations need to be validated in a larger patient cohort. We hypothesise that TLR7/8 DC vaccination strategies should be combined with hypomethylating agents or checkpoint inhibition to augment immune responses. **Trial registration.** The study was registered at https://clinicaltrials.gov on 17 October 2012 (NCT01734304) and at https://www.clinicaltrialsregister.eu (EudraCT-Number 2010-022446-24) on 10 October 2013.

Keywords: acute myeloid leukaemia, cancer vaccines, clinical trials, dendritic cell vaccination, immunotherapy

INTRODUCTION

Despite improvements in outcome over the past decades, with 5-year survival rates climbing from 6.2% in 1975–1977 to 28.1% in 2008–2014[1] acute myeloid leukaemia (AML) still has a dismal prognosis.[2] The major reason for the poor survival rate is the high risk of relapse after intensive induction therapy. The most successful strategy to reduce the relapse rate is allogeneic haematopoietic stem cell transplantation (allo-HSCT).[3] This potentially curative cellular immunotherapy is based on the graft-versus-leukaemia effect of allogeneic T cells. However, because of high morbidity and mortality of this therapy, there is a large group of AML patients without this therapeutic option. Alternative strategies for the activation of the immune system aiming at eradication of chemorefractory residual disease are therefore urgently sought after. Vaccines induce and enhance autologous T cells targeting intracellular leukaemia-associated antigens (LAAs) and represent a promising strategy. Immunisation with LAA peptides has been studied in several clinical trials with moderate clinical success so far.[4,5] Optimisation of vaccination might be achieved by the use of DCs. As professional antigen-presenting cells, they represent physiological candidates to induce strong and durable immune responses.[6,7] Several strategies have been applied including hybridomas of autologous DCs fused with leukaemic blasts from primary diagnosis as a vaccine in 17 AML patients in CR. Immunological responses were observed, and 71% of the patients were still in CR at a median follow-up of almost 5 years.[8] Results of two major studies using monocyte-derived DCs loaded with LAAs for post-remission treatment of AML patients have been reported: vaccination with DCs electroporated with mRNA encoding *hTERT* resulted in antigen-specific T-cell responses in 11/19 patients; RFS after a median observation time of 52 months was 58%.[9] Within a phase II trial, an anti-leukaemic response was detected in 13/30 patients vaccinated with DCs loaded with *wilms tumor 1* (*WT1*) mRNA. A molecular remission defined by WT1 qPCR in the peripheral blood was achieved in 9/30 patients, and RFS and OS at 5 years were 30.8% and 50.0%, respectively.[10] In both publications, DC maturation was achieved by a combination of pro-inflammatory cytokines and prostaglandins.[11] While this protocol was designed to promote migratory and immunostimulatory properties of DCs, no IL-12p70 production was induced. However, IL-12 is a crucial cytokine for both Th1 polarisation and NK cell activation. In preclinical work comparing DCs generated from peripheral blood mononuclear cells (PBMCs) of healthy controls using different maturation cocktails, we could show that the addition of a toll-like receptor (TLR) 7/8 ligand to the DC maturation cocktail results in enhanced T-cell stimulation. In direct comparison to DCs matured without a TLR agonist, the resulting DCs are characterised by a higher expression of the costimulatory molecules CD80 and CD86 and very high production of bioactive IL-12p70. Both *in vitro* and *in vivo*, we could show that these DCs stimulate strong immune responses including polarisation of $CD4^+$ T cells to Th1, induction of antigen-specific $CD8^+$ T cells and activation of NK cells.[12,13] This approach can be translated to monocytes derived from AML patients in CR, also resulting in IL12p70-producing DCs with very similar functional characteristics.[14]

Hence, we have developed a good manufacturing practice (GMP)-compliant protocol for the generation of next-generation DCs, combining a short, only 3-day differentiation period with a novel maturation cocktail that includes the TLR 7/8 agonist R848.[15] As accounted for in detail previously[16] mRNAs encoding the LAAs WT1 and preferentially expressed antigen in melanoma (PRAME) as well as the viral control antigen cytomegalovirus (CMV)pp65 were chosen for antigen loading of three separate batches of DCs by electroporation. Here, we describe the results of a phase I first-in-human proof-of-concept trial using next-generation DCs for post-remission therapy of 10 AML patients in first CR with a high risk of relapse (non-favorable risk group or MRD positivity).

RESULTS

Patient characteristics

The characteristics of the 13 patients who were enrolled into the study are shown in Table 1. Twelve patients were positive for WT1 by qPCR at primary diagnosis, four were positive for PRAME by qPCR, and CMV serostatus was positive in four patients before vaccination. Eastern Cooperative Oncology Group (ECOG) performance status was 0 in two patients, 1 in 10 patients and 2 in one patient.

Feasibility of vaccine generation and administration

Twelve patients underwent leukapheresis for production of the DC vaccine; patient #5 developed a leukaemia relapse in the short time span between screening and planned leukapheresis and was excluded from the study before leukapheresis. Key figures of the leukapheresis product are presented in Supplementary table 1. A median of 1.25×10^{10} (range $0.6–2.8 \times 10^{10}$) viable white blood cells was collected per patient. Median monocyte yield was 3.6×10^9 (range $1.0–7.5 \times 10^9$). Median DC yield after electroporation was 3.65×10^8 (range $1.27–5.68 \times 10^8$). After quality control and removal of retain samples, sufficient DCs for the full schedule of 10 vaccinations (1.5×10^8 DCs) were produced for 11 of 12 patients. For patient #2, only six vaccinations were available as the monocyte yield was low because of an unexpected decrease in leucocyte count between screening and leukapheresis (from 5.9 to 3.0 G L^{-1}), and as DC recovery after electroporation was suboptimal. Two patients completed leukapheresis but were not vaccinated because of early relapse during vaccine production (#3) and because of characteristics of the vaccine (#8, see below). Of the 10 patients who actually initiated vaccination, seven underwent the complete regular schedule of 10 vaccinations. Patient #2 received all six vaccinations that were available, which was the minimum required by the study protocol; patient #4 developed a relapse after seven vaccinations and received two further vaccinations in combination with one cycle of 5-azacytidine; and patient #7 also developed a relapse after seven vaccinations and received three further vaccinations in combination with two cycles of 5-azacytidine. Two patients received further DC vaccinations after the end of the study in combination with 5-azacytidine in view of an impending or established relapse: eight vaccinations with five cycles of 5-azacytidine in patient #1 and two vaccinations with one cycle of 5-azacytidine in patient #11. Median time from CR/CR_i to first vaccination was 110 days (range 34–205 days), mainly because of further cycles of consolidation therapy; median time from leukapheresis to first vaccination was 25 days (range 18–38 days).

Vaccine characterisation

All 12 generated DC preparations were tested for their phenotype, migration capacity, cytokine secretion, and processing and presentation of the three selected antigens after RNA electroporation (Figure 1 and Supplementary figure 1). For all patients, the cells showed a typical DC phenotype ($CD14^{low}$ and $CD83^+$; Figure 1a). Expression of various costimulatory or chemokine receptor molecules was measured, and the specific fluorescence intensity (SFI) was calculated (Figure 1b). Median SFI was 124.6 for HLA-DR, 4.1 for CCR7, 35.6 for CD40, 31.6 for CD80, 35.4 for CD86, and 21.5 for PD-L1. The ratio of CD86 to PD-L1 expression as a potential measure of positive costimulation was 1.25 in median. A median of 74.5% (range 38.3–98.4%) of DCs showed migration towards a CCL19 gradient (Figure 1c). Ten of 12 DC preparations secreted relatively high amounts of IL-12p70 (median of

Table 1. Patient characteristics

	Gender	Age (years)	FAB	Cytogenetics	Molecular genetics	ELN risk group	Status of disease at SV1	WT1 expr prim dx	PRAME expr prim dx	CMV serostatus study start	ECOG	Leukocytes at dx (G L^{-1})	Tx prior DC vx
#1	m	72	M1	Complex karyotype	NPM1 wt CEBPA wt MLL neg	Adverse	CR	pos	neg	pos	1	2.6	s-HAM, TAD-9
#2	m	54	s-AML (MDS)	del(12)(p13p13)(ETV6-)	NPM1 wt FLT3-ITD neg CEBPA wt MLL neg	Intermediate II	CRi	pos	pos	neg	1	3.7	s-HAM, TAD-9
#3	m	62	M4	Normal karyotype	NPM1 mut FLT3-ITD+, FLT3-TKD+ MLL neg	Intermediate I	beginning relapse	pos	pos	neg	1	93.9	s-HAM, TAD-9, AD, Vidaza
#4	f	48	M0	Normal karyotype	NPM1 wt CEBPA wt MLL neg, FLT3-TKD-, FLT3-ITD-, CEBPA wt	Intermediate I	beginning relapse	pos	neg	neg	1	0.9	s-HAM, TAD-9, AD
#5	f	44	M1	Normal karyotype	NPM1 wt CEBPA wt MLL neg	Intermediate I	beginning r elapse	pos	neg	pos	1	1.6	s-HAM, TAD-9
#6	m	65	M2	Normal karyotype	NPM1 wt MLL-PTD+, FLT3-ITD, CEBPA wt	Intermediate II	CR	pos	neg	pos	1	13.9	7 + 3, HAM, 2 × HD-Ara-C
#7	f	74	M1	del(7q)	NPM1 wt FLT3 neg	Intermediate II	CR	neg	neg	neg	1	1.2	s-HAM, TAD-9
#8	f	79	s-AML (MDS)	Normal karyotype	n.a.	Intermediate I	CR	pos	neg	neg	2	n.a.	Vidaza
#9	m	64	s-AML (MDS)	Normal karyotype	NPM1 wt MLL-PTD neg, FLT3 neg, CEBPA wt	Intermediate I	CR	pos	neg	neg	1	n.a.	s-HAM, TAD-9
#10	m	50	M1	Complex karyotype with inv(16)	NPM1wt, MLL-PTD neg, inv16, FLT3-ITD+, FLT3-TKD+, CBFß-MYH11 fusion transcript	Favorable	CR, MRD+	pos	pos	neg	1	75.1	AraC, sHAM, TAD-9
#11	m	69	M1	inv(16)	NPM1 wt, FLT3-ITD neg, FLT3-TKD neg, MLL neg, CBFß-MYH11 fusion transcript, inv16	Favorable	CRi, MRD+	pos	pos	neg	1	3.7	s-HAM, TAD-9
#12	m	55	M2	Normal karyotype	NPM1 wt, FLT3-ITD neg, FLT3-TKD neg, MLL-PTD neg, CEBPA + mt	Intermediate I	CR	pos	neg	pos	0	2.8	s-HAM, TAD-9, AD, AC

(Continued)

Table 1. Continued.

	Gender	Age (years)	FAB	Cytogenetics	Molecular genetics	ELN risk group	Status of disease at SV1	WT1 expr prim dx	PRAME expr prim dx	CMV serostatus study start	ECOG	Leukocytes at dx (G L^{-1})	Tx prior DC vx
#13	m	47	M0	Normal karyotype	NPM1 wt, FLT3-ITD neg, FLT3-TKD neg, MLL-PTD neg, CEBPA wt	Intermediate I	CR	pos	neg	neg	0	1.6	s-HAM, 3 days Fludarabin

AC, cytotoxic regimen consisting of cytarabine and cyclophosphamide; AD, cytotoxic regimen consisting of cytarabine and daunorubicin; CEBPA, CCAAT/enhancer-binding protein alpha; CR, complete response; CRi, complete response with incomplete haematologic recovery; ECOG, Eastern Cooperative Oncology Group; ELN, European Leukemia Net; FAB, French–American–British classification; FLT3, fms-like tyrosine kinase 3; ITD, internal tandem duplication; MLL, mixed-lineage leukaemia; MRD, minimal residual disease; NPM1, nucleophosmin; s-HAM, double induction regimen consisting of sequential high-dose cytarabine and mitoxantrone; SV1, Screening Visit 1; TAD-9, cytotoxic regimen consisting of thioguanine, cytarabine and daunorubicin; TKD, tyrosine kinase domain.

1845 pg/5 $\times$ 10^6 DC/24 h; range 470–4525 pg/5 $\times$ 10^6 DC/24 h) and low amounts of IL-10 (median of 17.3 pg/5 $\times$ 10^6 DC/24 h; range 0–241 pg/5 $\times$ 10^6 DC/24 h), as expected from our previous experiments.[12] DCs of patient #7 showed very low IL-12p70 production (81.5 pg/5 $\times$ 10^6 DC/24 h) and no IL-10 production. DCs of patient #8 showed high IL-12p70 production (1969 pg/5 $\times$ 10^6 DC/24 h), but even higher IL-10 production (3031 pg/5 $\times$ 10^6 DC/24 h; Figure 1d). Because of the unknown effects of vaccinations with IL-10-producing DCs in the AML setting, this patient was excluded from the study and not vaccinated, although all release criteria for the vaccine were fulfilled. Successful translation of the electroporated RNA was proven by intracellular staining of the DCs for the resulting proteins (median SFI 2.36 for WT1, 1.44 for PRAME, 1.53 for CMVpp65); DCs electroporated with one of the other two RNA molecules served as control (Figure 1e and Supplementary figure 2). Presentation of the antigens in the context of HLA molecules was functionally proven by IFN-γ secretion of specific T-cell clones after coculture with the different DC batches. Each T-cell clone was preferentially stimulated by the respective DC batch (Figure 1f).

Vaccine-induced immune responses

For all 10 vaccinated patients, local immune response was measured 48 h after the fifth vaccination by size of local erythema and induration (Figure 2a). Vaccine site reaction was detectable for all patients and all antigens. Variability between patients was high, but no significant differences were found between the three antigens (WT1: median of 1.43 cm^2, range 0.38–4.15 cm^2; PRAME: median of 1.04 cm^2, range 0.28–3.46 cm^2; CMV: median of 1.24 cm^2, range 0.38–3.14 cm^2; Figure 2b). Skin biopsies were taken from nine patients. Dense $CD4^+$ and $CD8^+$ T-cell infiltration was seen by immunohistochemistry (Figure 2c).

Immunomonitoring was performed on PBMCs and plasma samples obtained before vaccination, after five vaccinations and at the end of the study. We found no major changes in the course of the therapy with respect to absolute and relative numbers of leucocytes, granulocytes, monocytes, lymphocytes, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells or $CD3^-$/$CD16\_56^+$ NK cells (data not shown). Antigen-specific T-cell

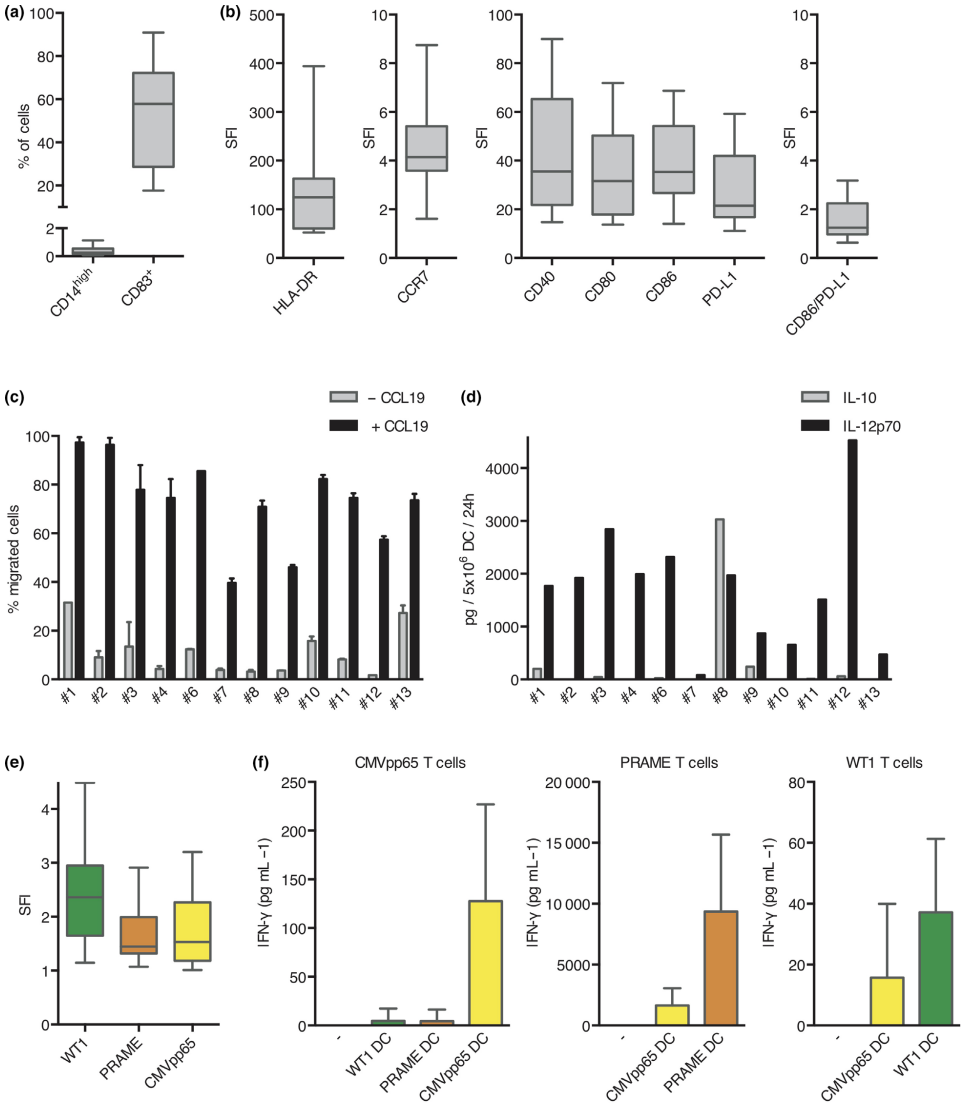

Figure 1. Characterisation of DC phenotype, migration capacity, cytokine secretion and antigen processing and presentation. For all 12 generated DC preparations, surface expression of **(a)** the DC markers CD14 and CD83 and **(b)** various costimulatory or chemokine receptor molecules was determined by flow cytometry. **(c)** Migration towards a CCL19 gradient was measured in a trans-well assay (2 technical replicates per sample). **(d)** Secretion of IL-10 and IL-12p70 after CD40 ligation was analysed. To prove successful antigen translation and presentation after RNA electroporation, DCs were **(e)** intracellularly stained for the resulting proteins and **(f)** used for stimulation of specific T-cell clones as measured by IFN-γ secretion (n = 3–7). For **a**, **b** and **e**, results are presented in box-and-whisker plots, with boxes representing the lower quartile, the median and the upper quartile, while the whiskers show the minimal and the maximal values. For all other graphs, data shown reflect mean and standard deviation.

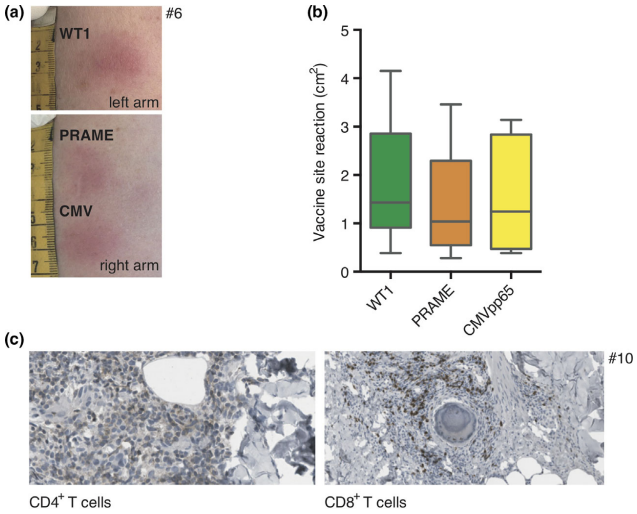

Figure 2. Vaccine site reaction. **(a)** For all 10 vaccinated patients and all antigens, erythema and induration of the vaccine sites were observed. **(b)** There was high variability between patients, but no significant difference between the three antigens in size of local reaction. **(c)** Immunohistochemical analysis of skin biopsies at the vaccine sites revealed dense $CD4^+$ and $CD8^+$ T-cell infiltration (one representative example shown).

responses were measured by ELISpot and by multimer staining, as shown for representative patients in Figure 3 (complete immunomonitoring data of these patients is presented in Supplementary figure 3). An increased ELISpot response after vaccination as defined by a $\geq$ 1.5-fold increase of antigen-specific spot count was detected in 2/10 patients for WT1 (Figure 3a), in 4/10 patients for PRAME (Figure 3b), and in 9/10 patients for CMV (Figure 3c and d; Table 2). These results were largely reflected by multimer staining: an increased response as defined by a $\geq$ 2-fold increase of multimer-positive $CD8^+$ T cells was detected in 1/6 patients for WT1, in 0/3 patients for PRAME, and in 6/8 patients for CMV, with limitations because of the availability of multimers for the various HLA types (Table 2 and Supplementary figure 4). CMV responses were generally very high, with up to 15.9% of all $CD8^+$ T cells stained with a single CMV multimer after vaccination in a primarily seropositive patient (#6; Figure 3g), and up to 9.6% of all $CD8^+$ T cells stained with a single CMV multimer after vaccination in a primarily seronegative patient (#10). Of note, also decreased frequencies after vaccination were observed (Supplementary figure 3). Post-vaccination LAA-specific T-cell responses were significantly lower, but still clearly detectable in some patients (Figure 3e and f). In 4/7 patients where a CMV-specific multimer for HLA type II was available, an increase in antigen-specific $CD4^+$ T cells could be detected as well (Figure 3i; Table 2).

Vaccine-induced B-cell responses were measured by detection of CMV antibodies. Of seven patients who were CMV seronegative before vaccination, antibodies against CMV were detected in three patients after vaccination (#7, #10, #13), and one patient had a borderline reaction after vaccination (#2), while no antibodies against CMV were detectable in three patients (#4, #9, #11). Seroconversion as a result of primary CMV infection was excluded by the methodology.

Clinical responses to vaccination

The vaccination protocol was generally very well tolerated. All patients observed transient vaccine site reactions (erythema, induration, pruritus) of grade 1 intensity. Other frequent adverse events were musculoskeletal pain (6/10), skin reactions outside of vaccine sites (5/10), diarrhoea (4/10) and fatigue (4/10). All potentially treatment-

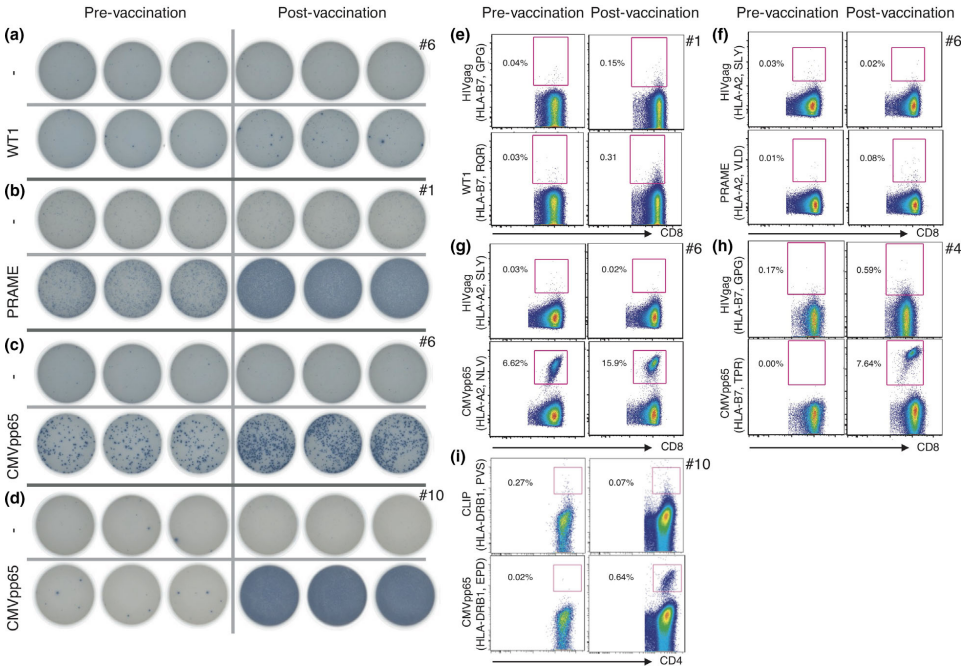

Figure 3. Representative examples of vaccine-induced immune responses. **(a–d)** PBMCs isolated before and after vaccination were tested for antigen-specific T cells by ELISpot. Increased immune responses were detected for the LAAs WT1 **(a)** and PRAME **(b)** as well as for CMVpp65 **(c, d)**. Both expansion of pre-existing immune responses **(c)** and induction of novel immune responses **(d)** were observed. **(e–h)** PBMCs isolated before and after vaccination were tested for antigen-specific $CD8^+$ T cells by multimer staining. Increased immune responses were detected for the LAAs WT1 **(e)** and PRAME **(f)** as well as for CMVpp65 **(g, h)**. Both expansion of pre-existing immune responses **(g)** and induction of novel immune responses **(h)** were observed. **(i)** For CMVpp65, induction of antigen-specific $CD4^+$ cells was also detected.

related adverse events reported by ≥ 2/10 patients are listed in Supplementary table 2. All adverse events were transient, and except for one grade 3 pyrexia, all adverse events were graded 1–2.

Because of limited patient numbers in the phase I setting, clinical efficacy analysis was purely exploratory. Vaccinated patients have been observed for a median of 1057 (range 424–1449) days since primary diagnosis and a median of 811.5 (range 293–1267) days since first vaccination, with the cut-off on 31 March 2018. A swimmer plot of all 10 vaccinated patients is depicted in Figure 4. Three patients (#4, #7 and #11) relapsed already in the course of the scheduled vaccinations, and two patients (#1 and #2) relapsed after the end of the trial. Of these five patients, only one (#4) is still alive after several salvage therapies. The other five vaccinated patients are still alive and in ongoing CR. Aggregated survival data are shown in Figure 5. Median OS has not yet been reached (Figure 5a), and median RFS was 1084 days (Figure 5b), with 50% of patients still relapse-free at the end of observation. In a hypothesis-generating analysis, these survival data compare favorably to a closely matched patient cohort from the AML-Cooperative Group (AML-CG) registry (see Supplementary table 3 for patient characteristics), where median OS was also not yet reached at the end of observation (*P*-value = 0.53; Figure 5a) and median RFS was only 396 days, closely missing out on statistical significance in spite of the small trial group (*P*-value = 0.09; Figure 5b). Exploratory subgroup analysis within the study cohort showed that patients ≤ 65 years had significantly better OS

Table 2. Immune responses to the study antigens

		ELISpot						Multimer					
		WT1		PRAME		CMVpp65			WT1		PRAME		CMVpp65
Pt	Immune responses	Prior vx	Post vx	Prior vx	Post vx	Prior vx	Post vx	MHC	Prior vx	Post vx	Prior vx	Post vx	Prior vx	Post vx
#1	WT1/PRAME/CMV	–	↑	++	↑	++	↑	–	–	↑			++	→
								=					++	→
#2	CMV	–	=	–	=	–	↑	–	–	=	–	=	–	↑
								=					–	=
#4	CMV	–	=	–	=	–	↑	–	–	=			–	↑
								=					–	↑
#6	WT1/PRAME/CMV	–	↑	+	↑	++	↑	–	+	=	–	↑	++	↑
								=						
#7	CMV	–	=	–	=	–	↑	–						
								=					–	↑
#9	PRAME/CMV	–	=	+	↑	–	↑	–						
								=						
#10	PRAME/CMV	–	=	–	↑	–	↑	–					–	↑
								=					–	↑
#11	–	–	=	–	=	–	=	–	–	=	+	→	–	=
								=					–	=
#12	CMV	–	=	–	=	++	↑	–					++	↑
								=					–	↑
#13	CMV	–	=	–	=	–	↑	–	+	=			–	↑
								=						

Prior vaccination (vx): –, no immune response; +, pre-existing immune response; ++, strong pre-existing immune response. Post vx: ↑, increase in multimer-positive T cells or ELISpot response; =, no increase or decrease in multimer-positive T cells or ELISpot response; ↓, decrease in multimer-positive T cells or ELISpot response. For definitions, see Methods.

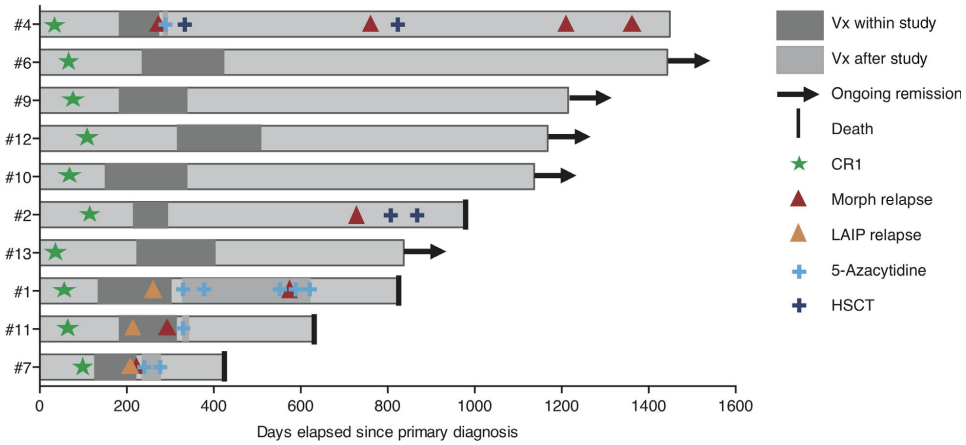

Figure 4. Swimmer plot. Time point of first CR, vaccinations, potential other treatment modalities, and relapses, death or ongoing remission are depicted for all patients treated within the trial.

(median not yet reached vs. 628 days; *P*-value = 0.0008; Figure 5c) and RFS (median not yet reached vs. 294 days; *P*-value = 0.0122; Figure 5d) than patients > 65 years. Immune responders as defined by expansion of antigen-specific T cells against WT1 or PRAME showed a trend towards better OS (median not yet reached vs. 976 days; Figure 5e) and RFS (median not yet reached vs. 509 days; Figure 5f) than immune non-responders, but statistical significance was not reached because of the low patient number. Specifically, the three patients ≤ 65 years who showed an LAA-specific immune response (#6, #9 and #10) are all in ongoing CR.

Combination of 5-azacytidine with DC vaccination as individual treatment attempt

Towards the end of the study treatment, patient #1 developed an increase in MRD load, for both WT1 copy number and frequency of leukaemia-associated immunophenotype (LAIP), predicting an impending relapse (Supplementary figure 4a). After positive discussion with the ethics committee of the LMU Munich and written informed consent by the patient, we started an individual treatment attempt combining 5-azacytidine in the approved dose and schedule (75 mg m^{-2} s.c. on days 1–7 of a 28-day cycle) with next-generation DC vaccination on day 8 and day 15 (Supplementary figure 4a). Vaccine site reactions were found to be considerably enhanced (Supplementary figure 4b), and the frequency of LAA-specific T cells was increased (Supplementary figure 4c). Two cycles of this combination therapy lead to MRD conversion (Supplementary figure 4a), which lasted for some time before the patient relapsed almost a year later. Similar treatment attempts were later repeated for patients #4, #7 and #11, however not in MRD situation, but in overt relapse. Similar results in terms of local reaction and reduction of disease burden were not observed in these cases.

DISCUSSION

As detailed above, two clinical trials using monocyte-derived DCs loaded with LAA-specific mRNA have already been published.[9,10] In both studies, DCs were activated by the classical combination of pro-inflammatory cytokines and prostaglandins,[11] and mRNA encoding a single LAA (*hTERT* and *WT1*, respectively) was used for electroporation. Our trial decisively differed in two important respects. First, the TLR7/8 ligand R848 was included into the maturation protocol, resulting in DCs with improved immunostimulatory properties including secretion of IL-12p70, as demonstrated in detail previously.[12] This study represents the first-in-human trial applying these

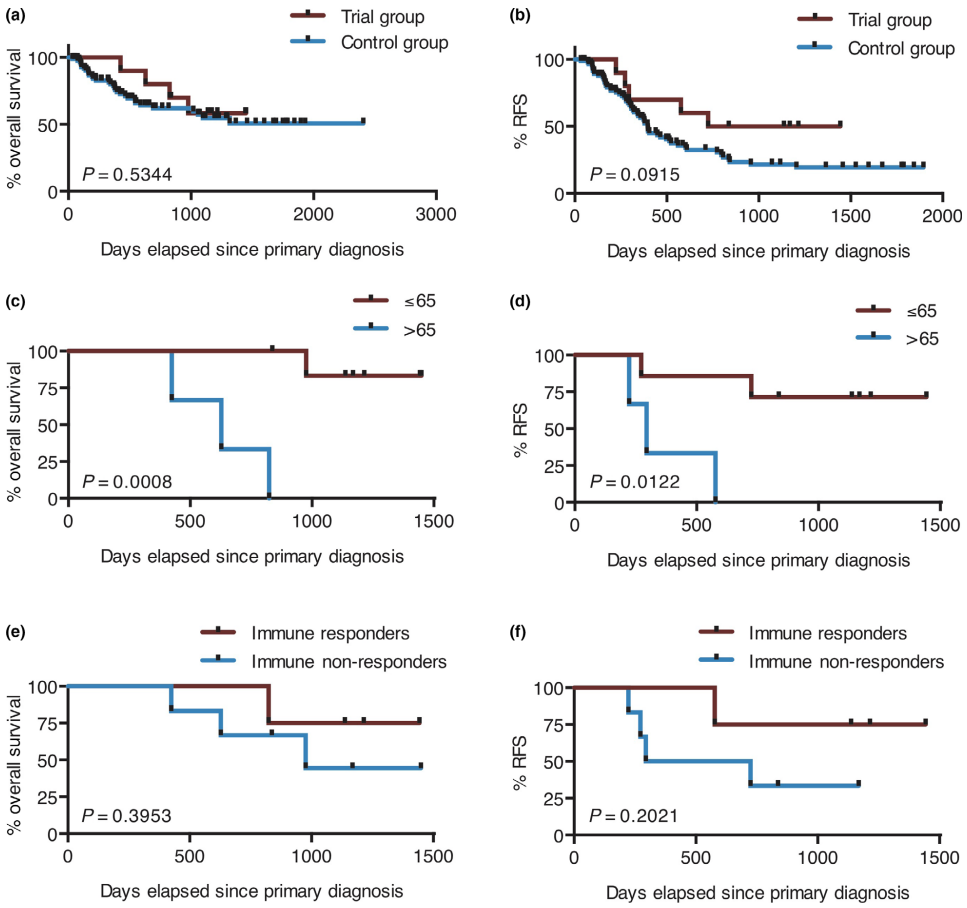

Figure 5. Survival analysis. OS **(a, c, e)** and RFS **(b, d, f)** of the vaccinated patients were depicted by Kaplan–Meier plots and compared by the log-rank test. **(a, b)** Patients treated within the trial were compared to a closely matched cohort of 88 patients from the AML-CG registry. **(c, d)** Within the study cohort, patients ≤ 65 years and > 65 years at time of diagnosis were compared. **(e, f)** Immune responders as defined by an increase in LAA-specific T cells after vaccination were compared to immune non-responders.

next-generation DCs to patients. Second, three antigens were chosen for loading of separate DC batches.[16] Next to WT1, which is very frequently overexpressed in AML and the most prominent antigen in vaccination trials for AML, both for DC vaccination[10,17,18] and for peptide vaccination,[5] we decided to add a second LAA in order to broaden anti-leukaemic responses and to decrease the possibility of immune escape. We chose PRAME as the most prominent cancer–testis antigen in AML.[19,20] CMVpp65 as a very abundant and immunogenic viral antigen was added for loading of a third batch of DCs, allowing us to differentiate between the induction of primary and secondary immune responses by comparison of CMV-seronegative and CMV-seropositive patients.

The primary objective of this trial using next-generation DCs for post-remission therapy of AML patients was to explore the feasibility of DC generation as well as the safety of the

vaccinations. Patients in first CR after intensive chemotherapy, but with a high risk of relapse, could be included. Three of the 13 patients who were enrolled did not proceed to vaccination because of disease-related factors (very early relapse prior to the first vaccination; $n = 2$) or because of factors related to vaccine production (high IL-10 secretion by DCs; $n = 1$). The high production of IL-10 by the DC vaccine produced for patient #8 was unique and had never been seen before in preclinical experiments. This accentuates both the very high relapse risk of the enrolled patients and the high success rate (> 90%) in production of DCs secreting high amounts of IL-12p70 and low amounts of IL-10. For the other 10 patients, the generated DCs sufficed for vaccination of all three antigens at the minimum of six specified time points. Median time between leukapheresis and start of the vaccination was 25 days (Supplementary table 1). Eight of these 10 patients completed the full study protocol, while two were taken off study because of early relapse, again highlighting the unfavorable prognosis of the included patients. We conclude that generation and administration of next-generation DCs are feasible in AML patients after intensive chemotherapy, albeit early relapse can prevent successful administration in very high-risk patients. Judging from the 105 vaccinations that were administered in total, tolerability of the protocol was excellent. Only transient adverse events were observed, and except for one grade 3 pyrexia, all adverse events were graded 1–2 (Supplementary table 2). Despite using DCs with stronger immunostimulatory capacity compared to prior vaccination studies, our data showed an excellent safety profile.

As a secondary objective of the trial, we studied immunological responses to the DC vaccinations. Antigen loading was done by electroporation of mRNA in order to allow for HLA-independent, multiple-epitope antigen presentation. T-cell responses before and after the vaccinations were detected by multimer staining and by ELISpot. The analysis of CMVpp65-specific T-cell responses allowed us to distinguish between T- and B-cell responses in latent CMV carriers in comparison with CMV negative patients. Within our cohort, 3/10 patients were seropositive for antibodies against human CMV. Before the vaccinations, we detected antigen-specific T cells by tetramer and ELISpot in all three seropositive patients (Table 2). Interestingly, we observed an induction of a T-cell response to CMVpp65 in all but one patient after vaccination, and an expansion of CMVpp65-specific T cells in seropositive patients. For one patient (#1), we observed divergent results between ELISpot and multimer assays, with strong upregulation of the ELISpot response and downregulation of the multimer-positive population. We hypothesise that this might be interpreted as a selective expansion of antigen-specific T cells not detected by the available multimers or possible determinant spreading to T cells recognising an alternative epitope. Using major histocompatibility complex (MHC) class II multimers, we found an increase in antigen-specific $CD4^+$ T cells in four of seven applicable patients (Table 2). In two of these patients (#7, #10), this correlated with development of antibodies against CMVpp65. A physiological seroconversion as a result of primary CMV infection was ruled out by missing detection of the CMV-associated protein p150. From the data on CMVpp65 immunomonitoring, we conclude that next-generation DCs are capable of inducing primary and secondary immune responses. These are not restricted to $CD8^+$ T-cell responses, but also comprise $CD4^+$ T-cell and antibody responses.

Similarly, we were able to show the induction of LAA-specific T-cell responses. However, in contrast to the immune responses against CMVpp65, the responses directed against WT1 and PRAME were lower in frequency and not detected in all patients. This might partially be attributed to restricted availability of HLA-specific multimers and a random mix of peptides with different lengths for the ELISpot assays. Therefore, it is likely that not all LAA-specific T cells were detected in spite of the two complementary methods. However, differences between a viral antigen and autoantigens certainly play a role, with high-affinity T cells against the latter being negatively selected in the thymus during T-cell development. Our immunomonitoring data provide evidence that the immunostimulatory capacity of next-generation TLR7/8-matured DCs is very high. Further work is needed to identify the optimal setting for DC application, for example induction of neoantigen-specific T cells or boosting of genetically engineered T cells for adoptive transfer.

In spite of the single-arm phase I design and the limited patient number in this first-in-human trial, we believe it is highly relevant to report the safety and tolerability of a TLR7/8-matured DC vaccine. The successful application of more

than 100 vaccines demonstrates the suitability of the vaccine, which is also applicable in other cancer entities. Besides, we demonstrated the induction of immunological responses. Promising clinical outcome is suggested by the comparison to a closely matched patient cohort. A beneficial effect of vaccination was observed with respect to RFS with a median survival of 1084 compared to 396 days. This effect was more pronounced for patients of younger age and with vaccine-induced immune responses. Patients $\leq$ 65 years showed significantly better OS and RFS than patients > 65 years. Two of the patients in the older cohort relapsed quickly without detection of a LAA-specific immune response, and the third patient relapsed shortly after termination of the vaccination protocol. In the younger cohort, however, only two of seven patients relapsed, and six of seven were still alive at data cut-off. This is in line with a recent publication, in which an overall survival benefit was dominantly observed in the patient cohort below 65 years of age.[10] This might be related to the larger pool of naive T cells in younger AML patients, which are required for the induction of novel anti-leukaemic immune responses.[21] Moreover, immune responses against WT1 and PRAME correlated to prolonged OS and RFS (Figure 5e and f). Specifically, all three patients of the younger age group that showed a leukaemia-specific immune response remained in ongoing CR until data cut-off. Our data support the hypothesis that TLR7/8-matured DCs induce protective LAA-specific immune response in patients $\leq$ 65 years. However, frequency and strength of LAA-specific immune responses need to be enhanced in order to improve clinical benefit.

Of note, because of the very small patient number, the comparison of survival data with the matched patient cohort is purely exploratory and hypothesis-generating. There was therefore no formal statistical analysis plan for this comparison, and multiple testing was not compensated for.

Several factors might have contributed to the fact that the immunological and clinical effects in this study were lower than might have been expected. The use of autoantigens for vaccination has been discussed above. Second, a comparison of the DC characterisation within this trial with the results of our preclinical experiments[12,13,15] showed considerably lower CD86/PD-L1 ratio and IL-12p70 secretion. This might be due to the upscaling of the DC generation process including elutriation of a leukapheresis product after overnight storage instead of plastic adherence of freshly isolated PBMCs.

However, we believe that combinatorial approaches are the most promising strategy to further enhance immune responses and hence clinical benefit. Epigenetic modifiers such as DNA methyltransferase inhibitors and histone deacetylase inhibitors are suitable combination partners because of an enhancement in antigen processing and presentation of malignant cells.[22–25] In the setting of myelodysplastic syndrome, the combination of vaccination against NY-ESO-1 and decitabine resulted in an increased antigen-specific immune response.[26] In our hands, the combination of next-generation DC vaccination with 5-azacytidine resulted in a striking increase in local and systemic immune responses. This translated into a temporary MRD conversion in a single patient. We suggest pursuing this approach in further clinical trials. Immune checkpoint blockade is another strategy for combinational approaches. Early clinical trials are already combining vaccines with programmed cell death protein 1 (PD-1) blockers for treatment of various malignancies including AML.[27] The combination of both epigenetic modification by azacytidine and PD-1 blockade by nivolumab was recently shown to be a safe and effective therapy for relapsed AML.[28] However, other checkpoint molecules might be even more relevant as suggested by our preclinical data showing that blockade of lymphocyte activation gene 3 (LAG-3) strongly enhances DC-induced immune responses against viral and leukaemia-associated antigens.[29]

CONCLUSIONS

Vaccination of high-risk AML patients with TLR7/8-matured RNA-loaded DCs was feasible, safe and resulted in induction of leukaemia-specific immune responses. Explorative comparison to a matched cohort suggests a benefit on the clinical outcome; positive effects of vaccination on survival were particularly seen for immune responders and patients $\leq$ 65 years. Perspectively, immune responses can be further augmented by combining TLR7/8-matured DCs with immunomodulatory drugs like hypomethylating agents or checkpoint inhibitors.

METHODS

Study design

We here report results of a phase I trial, with clinical efficacy analysis being purely explorative. AML (excluding acute promyelocytic leukaemia) patients at the age of 18–75 with a non-favorable risk profile (intermediate I, intermediate II or adverse according to European LeukemiaNet (ELN) classification of 2010;[30] or with a favorable risk according to ELN and MRD positivity) in CR/CR_i after at least one cycle of intensive induction therapy including an anthracycline and cytarabine were eligible for enrolment. Patients with prior allo-HSCT, severe organ dysfunction or active clinically relevant autoimmune disease were excluded. None of the patients were eligible for an allo-HSCT, either because of comorbidities, lack of donor or missing consent. The primary objective of the study was to determine safety and feasibility of immunotherapy with autologous DCs, resulting in the endpoints of frequency of adverse events and percentage of patients in whom treatment with the scheduled number of immunotherapies (10 DC vaccinations) was feasible. As a secondary objective, we explored the induction of immunological responses to the DC vaccination. Clinical responses were estimated by comparing RFS and OS between immune responders and non-responders as well as between all vaccinated study patients and matched control patients of the AML-CG registry. The vaccine was administered intradermally up to 10 times within 26 weeks at 5×10^6 DCs for each antigen (three batches at three separate sites) and time point, starting at weekly intervals and continuing at four-week intervals (see Supplementary figure 5). No other anti-leukaemia therapy was permitted in parallel as long as the patient was in remission, but 5-azacytidine was added to the ongoing vaccination strategy in some patients when the criteria for a leukaemia relapse were met. The study was mono-centric, open-label, prospective and non-randomised. All patients with successful vaccine generation who still met the eligibility criteria after this process were vaccinated at the Department of Medicine III, University Hospital, LMU Munich.

Vaccine generation

Peripheral blood mononuclear cells were collected by leukapheresis and transported to the GMP facility of the Department of Cellular Therapy at The Norwegian Radium Hospital in Oslo. Monocytes were enriched from leukapheresis using elutriation (ELUTRA, Caridian) and cultured in RPMI 1640 medium with very low endotoxin (Biochrom, Berlin, Germany) plus 1.5% human AB serum (Institute of Transfusion Medicine, Suhl, Germany), supplemented with 560 IU mL^{-1} GM-CSF (Leukine®, Bayer, Leverkusen, Germany) and 20 ng mL^{-1} interleukin-4 (R&D Systems, Wiesbaden, Germany) for 40–72 h. Thereafter, 10 ng mL^{-1} TNF-α, 10 ng mL^{-1} IL1-β (both R&D Systems, Wiesbaden, Germany), 5000 IU mL^{-1} interferon-γ (Imukin®, Boehringer Ingelheim, Ingelheim, Germany), 250 ng mL^{-1} PGE2 (Prostine® E2; Pfizer, Kent, UK) and 1 μg mL^{-1} R848 (3M Pharmaceuticals, St. Paul, MN, USA) were added to the culture medium for another 20–26 h.[16] Mature DCs were thoroughly washed and electroporated in three different batches, each transduced with *in vitro* transcribed (ivt) codon-optimised RNA (produced at Oslo University Hospital in clinical grade) encoding for either human *WT1* (isoform A, NP_000369.3), *PRAME* (NP_006106.1) or *CMVpp65* (P06725.2). After 2–6 h, DCs were harvested and cryopreserved. Before the first batch of DCs was administered to the individual patient, release criteria including total cell number, viability, and CD80 positivity, as well as lack of excessive contaminating cells, microbiological contamination and mycoplasma, were controlled (see Supplementary table 5 for details). Before administration, cells were resuspended with 200 μL DPBS each.

Vaccine characterisation

Expression of DC surface antigens was measured by flow cytometry using a panel of fluorescence-conjugated monoclonal antibodies (Supplementary table 6). Dead cells were excluded by Live/Dead Aqua (Invitrogen, Carlsbad, CA, USA) staining and only singlets gated. Corresponding mouse IgG isotype controls were used. After washing, cells were analysed using a FACS LSR II (BD Biosciences). Post-acquisition analysis was performed using FlowJo software (version 9.7.6; Tree Star, Ashland, OR, USA). The percentage of positive cells was determined by setting the gate at or below 1% in the respective isotype control. SFI was calculated as the ratio of the median fluorescence intensity of the test sample to its corresponding isotype control. Migration and cytokine secretion capacity of DCs were analysed as described previously.[14] To assess protein expression of transfected RNA in DCs, the freshly thawed cells were fixed using Foxp3 Staining Buffer Set (eBioscience). After FcR blocking, intracellular antigen staining was performed with anti-HCMV, anti-WT1 or anti-PRAME, and AF647-conjugated anti-mouse F(ab)2 as secondary antibody (Supplementary table 6). DC antigen presentation capacity was tested in an human leucocyte antigen (HLA)-matched 24h coculture of *CMVpp65*, *WT1* or *PRAME* RNA-transfected DCs with CMV-specific T cells (kindly provided by A. Moosmann), WT1-specific T cells (generated in our laboratory as previously described[31]) or PRAME-specific T cells (generated as previously described[32]), respectively, at a 1:10 ratio. IFN-γ secretion into the supernatant was analysed by cytometric bead array (CBA) Human IFN-γ Flex Set (BD Biosciences).

Measurement of immune responses

Local reactions at the vaccine sites were assessed by measuring the diameter of the erythema 48h after the fifth vaccination. Skin biopsies were taken and analysed by immunohistochemistry for $CD4^+$ and $CD8^+$ T-cell infiltration. Patients' lymphocyte subpopulations in peripheral blood were analysed according to standard procedures. Human IFN-γ single-colour ELISpot assays (CTL, Bonn, Germany) were performed following the manufacturer's recommendations with 2μg mL^{-1} CMVpp65, WT1 or PRAME peptide pools (JPT, Berlin, Germany) in triplicates. Resulting spots were counted using the ImmunoSpot S6 Analyzer's (CTL) Smart Count

Mode. Multimer staining was performed depending on the patient's HLA (Supplementary table 4) and availability of corresponding multimers. PE-labelled multimers (Supplementary table 6) were used for identification of vaccine-induced $CD4^+$ and $CD8^+$ T cells specific for CMVpp65, WT1 and PRAME. Multimers for HIV-Gag and CLIP were used as controls. For detection of CMV-specific $CD4^+$ T cells by MHC class II multimers, PBMCs were expanded for 7 days in the presence of 2.5 μM CMVpp65 peptide EPDVYYTSAFVFPTK (JPT) with 5 ng mL^{-1} IL-7 und IL-15 (PeproTech) added during the last three days. T-cell surfaces were additionally stained for CD3, CD4 and CD8. Patient sera were analysed for antibodies against the single antigens of human CMV before and after vaccination using the recomLine CMV IgG, IgM Immunoassay (MIKROGEN, Neuried, Germany) and the Enzygnost® (Siemens Healthcare GmbH, Erlangen, Germany). Primary CMV infection during the trial was excluded by assessment of the study-specific p65 protein without concomitant detection of the p150 protein.

Clinical assessments

Patients were monitored for adverse events starting from the first screening visit until 4 weeks after the last vaccination. All toxicities were graded according to the National Cancer Institute Common Toxicity Criteria version 5.0. Leukaemia was assessed by routine bone marrow diagnostics including determination of MRD by available molecular markers and by LAIP. RFS and OS were followed until the cut-off date of 31 March 2018 and depicted by swimmer plot for individual patients and by Kaplan–Meier plots.

Statistical analysis

For the analysis of ELISpot responses, the frequency of antigen-specific T cells was calculated by subtracting the mean number of spots in the control wells from the mean number of spots observed in response to antigen. Prior to the vaccination, ≥ 5 antigen-specific T cells were considered a positive response (+ in Table 2) and ≥ 100 antigen-specific T cells were considered a highly positive response (++). Upregulation of an immune response to the vaccinations (↑) was defined to be a ≥ 1.5-fold increase of antigen-specific spot count and ≥ 5 antigen-specific T cells after vaccinations. For determination of antigen-specific T cells by multimer staining, the percentage of $CD8^+$ or $CD4^+$ T cells stained positive with a control multimer was subtracted from the percentage of cells stained positive with the specific multimer. Prior to the vaccination, ≥ 0.1% antigen-specific T cells were considered a positive response (+ in Table 2) and ≥ 1% antigen-specific T cells were considered a highly positive response (++). Upregulation of an immune response to the vaccinations (↑) was defined to be a ≥ 2-fold increase of multimer-positive $CD8^+$ or $CD4^+$ T cells and ≥ 0.1% antigen-specific T cells after vaccinations. Downregulation of an immune response to the vaccinations (↓) was defined to be a ≥ 2-fold decrease of multimer-positive $CD8^+$ or $CD4^+$ T cells. An immune response to a specific antigen was defined by upregulation of the ELISpot and/or multimer response to the respective antigen (Table 2). In order to compare survival data of this single-arm trial to that of AML patients with very similar characteristics, a carefully matched cohort of 88 patients from the AML-CG registry was selected according to the following criteria: CR/CRi/CRp after intensive induction therapy; no allo-HSCT in CR1; duration of remission at least as long as in the trial population; non-favorable risk type; ECOG 0 or 1; and age at diagnosis 18–75. A comparison of patient characteristics between the DC study cohort and the AML-CG registry cohort is depicted in Supplementary table 3. Differences in survival between different groups were tested by log-rank test.

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CONFLICT OF INTEREST

DJS is employed by Medigene Immunotherapies GmbH, and holds patents and receives royalties for DC vaccines. All other authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

FSL, WH, DJS, GK, IB and MS designed the clinical trial. FSL, TA, VLB, TK, GW, BW, GK, IB and MS performed the clinical trial. FMS, FSL, MR, KD, CA, NPK, KS, AM, SB, MBo, MBr, MHMH and MS acquired and analysed the data. FSL, FMS, MR, KD and DG performed the statistical analysis. FSL, FMS, MR, KD, and MS designed the figures. FSL, FMS, MR and MS wrote the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the ethics committee of the LMU Munich and by the Paul-Ehrlich-Institute in Langen, Germany.

CONSENT FOR PUBLICATION

All patients provided written informed consent.

AVAILABILITY OF DATA AND MATERIAL

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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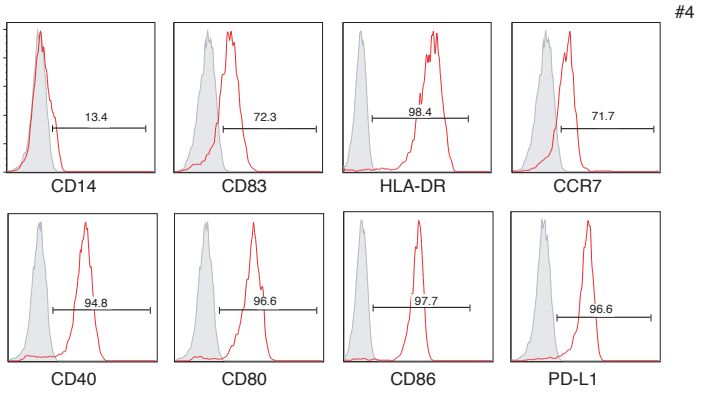
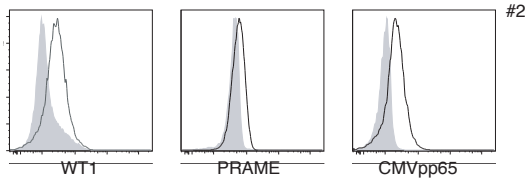
Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

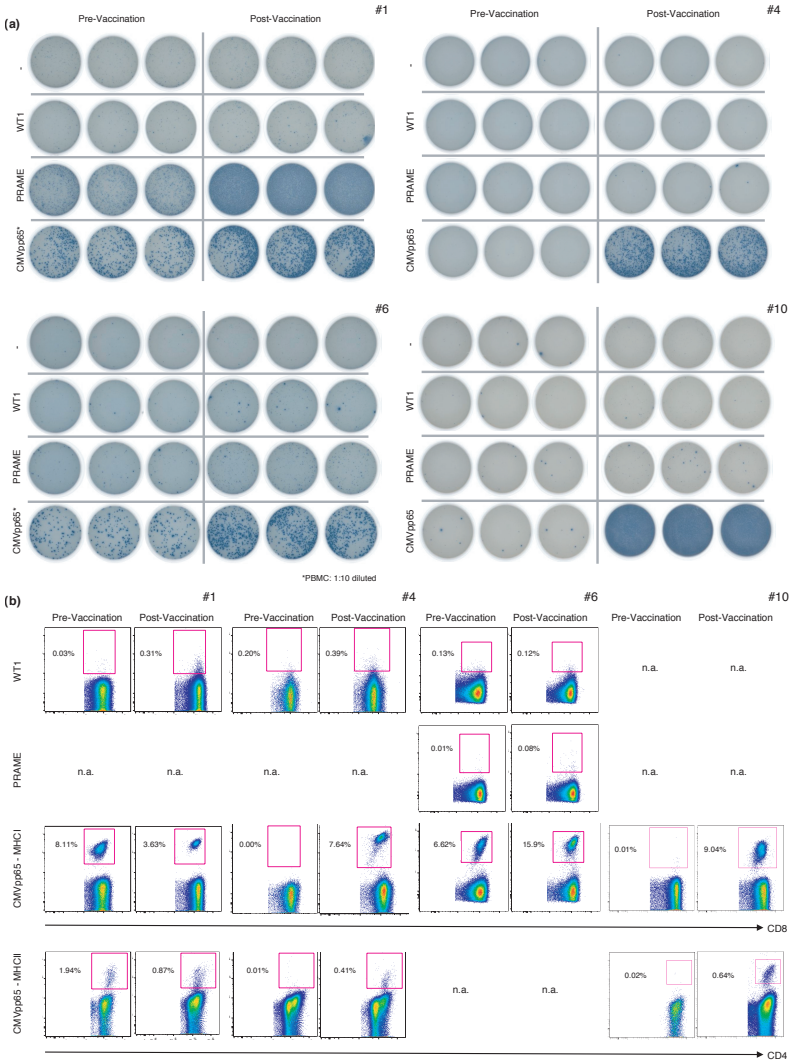
Supplementary Materials:



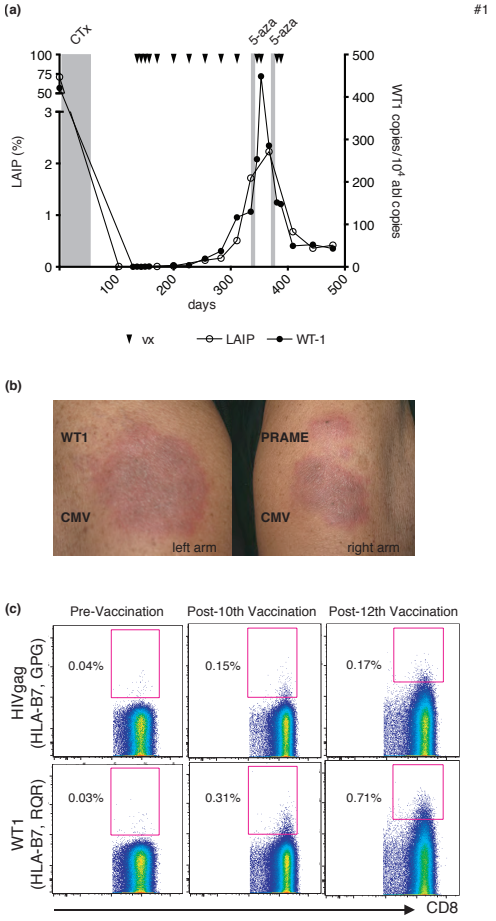
Supplementary figure 1. DC phenotype: Expression of surface molecules detected on DCs by flow cytometry in a representative patient sample.



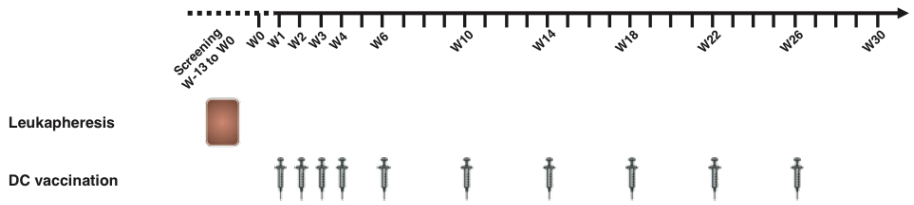
Supplementary figure 2. DC antigen expression: Freshly thawed DCs were intracellularly stained for the proteins translated from the electroporated RNA. DCs electroporated with one of the other two RNA species (PRAME as control for WT-1, CMV as control for PRAME, and WT1 as control for CMVpp65) served as control. A representative patient sample is shown.



Supplementary figure 3. Immunmonitoring data for patients #1, #4, #6, and #10. PBMCs isolated before and after vaccination were tested for antigen-specific T cells **(a)** by Elispot and **(b)** by multimer staining.



Supplementary figure 4. Individual treatment attempt with combination of 5-azacytidine and DC vaccination in patient #1. (a) Course of LAIP and WT1 MRD, showing MRD relapse after 10 vaccinations and MRD conversion after the combination therapy. **(b)** Enhanced local reaction to the vaccination after preceding 5-azacytidine therapy. **(c)** Increase in WT1-specific T cells after the combination therapy as measured by multimer.



Supplementary figure 5. Vaccination schedule

	Leukocyte count (GxL^-1)	Monocytes (%)	WBC (x10^10)	Monocyte yield (x10^9)	DC recovery after electroporation (x10^8)	Potential vaccinations	Leukapheresis to Vx1 (d)	CR to Vx1 (d)
#1	7.6	11	1.4	3.4	3.76	14	24	82
#2	5.9	7	1	2.2	1.27	6	25	104
#3	2.3	7	0.8	2.7	3.96	14	n.a.	n.a.
#4	3.7	11	1	3.4	5.45	22	32	149
#5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
#6	10.5	4	1.7	5.1	5.68	25	24	176
#7	5.9	13	1.5	3.6	1.85	10	18	34
#8	9.4	6	2.7	7.5	2.86	10	n.a.	n.a.
#9	4.1	10	1.1	3.6	3.39	14	25	97
#10	2.6	11	1.4	3.8	3.54	16	38	96
#11	5.19	7	0.9	3.9	4.84	24	25	116
#12	4.45	7	2.8	6.6	5.22	24	26	205
#13	3.26	9	0.6	1.0	2.38	11	19	185

Supplementary table 1. Leukapheresis data. Leukocyte count and Monocytes refer to the patient's peripheral blood values at the screening visit before leukapheresis. WBC = White blood cells and Monocyte yield refer to the leukapheresis product. Potential vaccinations = Number of sets of three batches each for vaccinations with DCs loaded with all three antigens. Vx1, First vaccination.

Adverse Event	Grade	n (% of n=10)
Vaccine site reaction (erythema, induration, pruritus)	1	10 (100)
Musculoskeletal pain	1–2	6 (60)
Skin reactions (erythema, pruritus) outside of vaccine sites	1	5 (50)
Diarrhea	1–2	4 (40)
Fatigue	1	4 (40)
Headache	1–2	3 (30)
Vertigo	1–2	3 (30)
Arthralgia	1	3 (30)
Cough	1	3 (30)
Nausea and vomiting	1	3 (30)
Respiratory infection	1	3 (30)
Night sweats	1	2 (20)
Pyrexia	1, 3	2 (20)

Supplementary table 2. Adverse events reported during the study. Listed are all adverse events that were observed in ≥ 2 patients and were at least possibly related to the investigational medicinal product.

	DC study cohort	AML-CG cohort
No. of patients	13	88
Age, years Median (range)	62 (44-79)	62 (25-75)
Patients age, N (%)		
≤ 65 years	9 (69)	52 (59)
> 65 years	4 (31)	36 (41)
Sex Female/male, N (%)	4/9 (31/69)	40/48 (45/55)
ECOG, N (%)		
0	2 (15)	24 (27)
1	10 (77)	64 (73)
2	1 (8)	0 (0)
ELN, N (%)		
Favorable	2 (15)	0 (0)
IntermediateI	7 (54)	42 (48)
intermediateII	3 (23)	30 (34)
Adverse	1 (8)	16 (18)
Molecular aberrations, N (%):		
NPM1 mutation		
Pos.	1 (8)	28 (35)
Neg.	11 (92)	53 (65)
Missing/unknown	1	7
FLT3-ITD		
Pos.	3 (33)	23 (28)
Neg.	6 (67)	58 (72)
Missing/unknown	4	7
FAB, N (%)		
M0	2 (15)	7 (9)
M1	5 (38)	18 (22)
M2	2 (15)	23 (28)
M3	0 (0)	0 (0)
M4	1 (8)	13 (16)
M5	0 (0)	10 (12)
M6	0 (0)	0 (0)
M7	0 (0)	1 (1)
s-AML (MDS)	3 (23)	9 (11)
Missing/unknown	0	7
Leukocytes at dx (GxL^-1) Median (range)	3 (1-94)	8 (1-292)
LDH, UxL^-1 Median (range)	232 (181-2401)	388 (63-8078)

Supplementary table 3. Patient characteristics of the trial cohort in comparison with the matched cohort from the AML-CG registry.

	A	B	C	DRB1	DQB1	DPB1
#1	24:02, 30:01	**07:02**, 13:02	06:02, 07:02	**07:01**, 15:01	02:02, 06:02	04:01, 04:02
#2	**02:01**, 03:01	44:05, 56:01	01:02, 02:02	**07:01**, 16:01	05:02, 03:03	04:01, 10:01
#3	02:01, 24:02	14:01, 40:01	03:04, 08:02	07:01	02:02, 03:03	04:01
#4	03:01, 23:01	**07:02**, 44:03	04:01, 07:02	**07:01**, 15:01	02:02, 06:02	04:01
#5	02:01, 03:01	07:02	07:02	15:01	06:02	02:01, 04:01
#6	**02:01**	15:01, 18:01	03:04, 07:01	04:01, 14:54	03:02, 05:03	04:01
#7	03:01, 25:01	13:02, 18:01	06:02, 12:03	**07:01**, 15:01	02:02, 06:02	03:01, 04:01
#8	01:01, 03:01	08:01, 40:01	07:01, 15:02	08:01, 15:01	04:02, 06:02	04:01, 04:02
#9	03:01, 26:08	15:01, 40:01	03:04, 04:01	01:01, 11:03	03:01, 05:01	03:01, 04:02
#10	**01:01**	49:01, 57:01	06:02, 07:22	**07:01,** 13:02	03:03, 06:04	02:01, 04:01
#11	**02:01**, 32:01	44:02, 51:01	05:01, 15:02	**07:01**, 11:01	03:01, 03:03	03:01, 04:01
#12	**01:01**, 31:01	27:05, 39:01	01:02, 12:03	07:01**, 13:01**	02:02, 06:03	04:01
#13	11:01, **24:02**	**07:02**, 27:05	02:02, 07:02	13:01, 15:01	06:02, 06:03	03:01, 04:02

Supplementary table 4. HLA-typing of the patients. Multimer staining was performed against the HLAs highlighted in bold with corresponding multimers.

Parameter	Method	Specification
Total cell count in 500 µL	Cell Dyn Ruby (Abott)	> 2x10^6
Viability	Tryphan blue (Ph. Eur.)	> 60%
CD80 positive	Flow Cytometry (BD)	> 60%
Microbiological contamination	Bactec System (BD)	Negative
Contaminating cells (NK, T and B cells)	Flow Cytometry (BD)	< 20%
Mycoplasma testing	16SrDNA PCR and sequencing	Negative

Supplementary table 5. Release Criteria for the DC vaccine.

	Reagent	Manufacturer	City, Country
Antibodies	anti-CD14 (FITC, 61D3)	eBioscience	San Diego, CA, USA
	anti-CD40 (PE, clone 5C3)	eBioscience	San Diego, CA, USA
	anti-CD80 (PE, L307.4)	BD Biosciences	Heidelberg, Germany
	anti-CD83 (APC, HB15)	BD Biosciences	Heidelberg, Germany
	anti-CD86 (PB, IT2.2)	BioLegend	San Diego, CA, USA
	anti-CD274 (FITC, MIH1)	BD Biosciences	Heidelberg, Germany
	anti-CCR7 (APC, FR11-11E8)	Miltenyi Biotec	Bergisch Gladbach, Germany
	anti-HLA-DR (PE, LN3)	BioLegend	San Diego, CA, USA
	anti-CD3 (APC, UCHT1)	BioLegend	San Diego, CA, USA
	anti-CD4 (FITC, VIT4)	Miltenyi Biotec	Bergisch Gladbach, Germany
	anti-CD8 (PerCP-eFluor710, SK1)	eBioscience	San Diego, CA, USA
	Live/Dead Aqua	Invitrogen	Carlsbad, CA, USA
	FcR Blocking Reagent	Miltenyi Biotec	Bergisch Gladbach, Germany
	anti-HCMV ppUL83	Biomerieux	Marcy-l'Étoile, France
	anti-WT1 (6F-H2)	Agilent	Santa Clara, CA, USA
	anti-PRAME (ab89097)	Abcam	Cambridge, UK
	AF647-conjugated anti-mouse F(ab)2	Dianova	Hamburg, Germany
	CMVpp65 (A*01:01-YSEHPTFTSQY)	ProImmune	Oxford, UK
	CMVpp65 (A*02:01-NLVPMVATV)	Immudex	Copenhagen, Denmark
	CMVpp65 (A*24:02-QYDPVAALF)	Immudex	Copenhagen, Denmark
	CMVpp65 (B*07:02- TPRVTGGGAM)	Immudex	Copenhagen, Denmark
	CMVpp65 (DRB1*07:01-EPDVYYTSAFVFPTK)	NIH Tetramer Facility	Atlanta, GA, USA
	WT1 (A*02:01-RMFPNAPYL)	Immudex	Copenhagen, Denmark
	WT1 (A*02:01-VLDFAPPGA)	Immudex	Copenhagen, Denmark
	WT1 (A*24:02-CYTWNQMNL)	Immudex	Copenhagen, Denmark
	WT1 (B*07:02- RQRPHPGAL)	Immudex	Copenhagen, Denmark
	PRAME (A*02:01-VLDGLDVLL)	Immudex	Copenhagen, Denmark
	PRAME (A*02:01-ALYVDSLFFL)	Immudex	Copenhagen, Denmark
	HIV-Gag (A*02:01-SLYNTVATL)	Immudex	Copenhagen, Denmark
	HIV-Gag (A*24:02-RYLKDQQLL)	Immudex	Copenhagen, Denmark
	HIV-Gag (B*07:02-GPGHKARVL)	Immudex	Copenhagen, Denmark
	CLIP (DRB1*07:01-PVSKMRMATPLLMQA)	NIH Tetramer Facility	Atlanta, GA, USA
Primer	Roche FastStart Essential DNA Probes Master (# 06402682001)	Roche Diagnostics	Basel, Switzerland
	Real time ready singe Assays - Roche PRAME Assay ID: 117436, config. # 100104279	Roche Diagnostics	Basel, Switzerland
	Real time ready singe Assays - Roche Abl1 Assay ID: 144473, config. # 100104288	Roche Diagnostics	Basel, Switzerland
	WT1 forward primer 5´-cgctattcgcaatcagggtta-3´	MetaBion International AG	Martinsried, Germany
	WT1 reverse primer 5´-gggcgtgtgaccgtagct-3´	MetaBion International AG	Martinsried, Germany
	WT1 probe 5´-FAM-agcacggtcaccttcgacgg-BHQ-1-3´	MetaBion International AG	Martinsried, Germany
	cABL Taq forward primer 5´-cct ttt cgt tgc act gta tga ttt-3´	MetaBion International AG	Martinsried, Germany
	cABL Taq reverse primer 5´-cgcc taa gac ccg gag ctt tt-3´	MetaBion International AG	Martinsried, Germany
	ABL1 probe 5´-FAM-tgg cca gtg gag ata aca ctc taa gca taa cta aag g-BHQ-1-3´	MetaBion International AG	Martinsried, Germany

Supplementary table 6. List of antibodies and primers used in the study.

3.4 Publication II

frontiers in Immunology

ORIGINAL RESEARCH
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Targeting LAG-3 and PD-1 to Enhance T Cell Activation by Antigen-Presenting Cells

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Immune checkpoint inhibition has been shown to successfully reactivate endogenous T cell responses directed against tumor-associated antigens, resulting in significantly prolonged overall survival in patients with various tumor entities. For malignancies with low endogenous immune responses, this approach has not shown a clear clinical benefit so far. Therapeutic vaccination, particularly dendritic cell (DC) vaccination, is a strategy to induce T cell responses. Interaction of DCs and T cells is dependent on receptor–ligand interactions of various immune checkpoints. In this study, we analyzed the influence of blocking antibodies targeting programmed cell death protein 1 (PD-1), HVEM, CD244, TIM-3, and lymphocyte activation gene 3 (LAG-3) on the proliferation and cytokine secretion of T cells after stimulation with autologous TLR-matured DCs. In this context, we found that LAG-3 blockade resulted in superior T cell activation compared to inhibition of other pathways, including PD-1/PD-L1. This result was consistent across different methods to measure T cell stimulation (proliferation, IFN-γ secretion), various stimulatory antigens (viral and bacterial peptide pool, specific viral antigen, specific tumor antigen), and seen for both $CD4^+$ and $CD8^+$ T cells. Only under conditions with a weak antigenic stimulus, particularly when combining antigen presentation by peripheral blood mononuclear cells with low concentrations of peptides, we observed the highest T cell stimulation with dual blockade of LAG-3 and PD-1 blockade. We conclude that priming of novel immune responses can be strongly enhanced by blockade of LAG-3 or dual blockade of LAG-3 and PD-1, depending on the strength of the antigenic stimulus.

Keywords: cancer immunotherapy, dendritic cell, immune checkpoint molecules, LAG-3, PD-1, T cell response

Abbreviations: AML, acute myeloid leukemia; APC, antigen-presenting cell; CBA, cytometric bead array; CEFT, CMV, EBV, influenza, tetanus; CFSE, carboxyfluorescein N-succinimidyl ester; DC, dendritic cell; FLR, Epstein–Barr nuclear Ag 3 A peptide FLRGRAYGL; HD, healthy donor; LAG-3, lymphocyte activation gene 3; MACS, magnetic activated cell sorting; MFI, median fluorescence intensity; MHC, major histocompatibility complex; NAC, non-adherent cell; PB, peripheral blood; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death protein 1; PRAME, preferentially expressed antigen in melanoma; ORR, objective response rate; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{EMRA}, effector memory RA T cell; T_{naive}, naive T cell; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; TLR, toll-like receptor; TLR-3-DCs, dendritic cells generated within 3 days based on a TLR7/8 ligand; VLD, WT1 peptide VLDFAPPGA; WT1, Wilms Tumor 1.

INTRODUCTION

Immunotherapy has changed our approach to anti-cancer treatment in recent years. Checkpoint inhibitors have particularly been in the focus of clinical development and have shown remarkable success as monotherapy or as combination partners for various tumor entities. This has resulted in approval for different solid tumor entities, but also for Hodgkin lymphoma (1–4). Checkpoint blockade is thought to reactivate endogenous T cell responses directed against tumor neoantigens presented in the context of major histocompatibility complex (MHC) molecules. In tumors with low endogenous T cell responses, however, the primary goal of immunotherapy needs to be the initiation of T cell responses directed against tumor-associated antigens. Various vaccination concepts are being pursued, and only recently, personalized neoantigen-based vaccines were shown to efficiently trigger T cell responses and lead to improved clinical outcome in patients with malignant melanoma (5, 6).

Dendritic cells (DCs) are particularly eligible to induce strong and durable immune responses. Over the years, multiple different maturation protocols have been used to generate DCs from monocytes *ex vivo* (7), and the resulting DCs differ considerably in their immunostimulatory capacities. We have developed a GMP-compliant 3-day protocol for the generation of DCs with improved immunogenicity based on a toll-like receptor (TLR) 7/8 ligand (TLR-3-DCs) (8). These DCs express higher numbers of co-stimulatory molecules and secrete higher levels of IL-12p70 compared to DCs generated with the standard protocol (9). Currently, we are conducting a phase I/II study on vaccination with DCs loaded with Wilms Tumor 1 (WT1) and preferentially expressed antigen in melanoma as leukemia-associated antigens for postremission therapy of acute myeloid leukemia (AML) patients (10).

In order to further enhance immunological and clinical responses, multiple combinatorial approaches with DC vaccination can be considered. These include, but are not restricted to chemotherapy and radiotherapy, cytokines and TLR agonists, hypomethylating agents, but also more targeted strategies, such as elimination of immunosuppressive cell types (e.g., myeloid-derived suppressor cells, regulatory T cells), molecularly targeted therapies and adoptive cell therapy (11, 12).

Another promising approach is the combination of DC vaccination with immune checkpoint inhibitors (13). Activated or chronically stimulated T cells upregulate various co-inhibitory molecules, such as programmed cell death protein 1 (PD-1), CD244 (2B4), CD160, T-cell immunoglobulin and mucin-domain containing-3 (TIM-3, CD366), and lymphocyte activation gene 3 (LAG-3, CD223) (14, 15). Their ligands are expressed both on antigen-presenting cells (APCs) and tumor cells. The inhibition of these checkpoints by blocking antibodies can, thus, enhance a vaccination-induced anti-cancer immune response in two ways. On the one hand, checkpoint inhibitors influence the interaction between T cells and cancer cells, resulting in enhanced anti-cancer T cell responses. On the other hand, checkpoint blockade may enhance the antigen-specific activation of T cells by DCs or other APCs. Studies performed in this field so far mainly focus on the inhibition of the PD-1/PD-L1 pathway (16–21).

Other co-inhibitory molecules, however, are also expressed on APCs, even on DCs after maturation with a TLR ligand (9). We, therefore, analyzed the effects of blocking various immune checkpoints on the stimulation of T cells by autologous TLR-3-DCs, mainly using virus antigens as a model system. Besides PD-1, we tested HVEM, CD244, TIM-3, and particularly LAG-3.

LAG-3 is a member of the Ig superfamily that was identified in 1990 (22). It is structurally similar to CD4 and binds MHC class II with a higher affinity than CD4 (23, 24). LAG-3 is expressed on activated $CD4^+$ and $CD8^+$ T cells as well as on a subset of natural killer cells (22). By using a knock-out mouse model, LAG-3 was found to impede T cell expansion and to control the number of memory T cells (25). Besides effector cells, LAG-3 can also be found on the surface of T regulatory cells and seems to be instrumental for their suppressive activity (26) as well as for T cell homeostasis (27). Finally, LAG-3 is also expressed on plasmacytoid DCs (28). Thus, modulation of the LAG-3 pathway has the potential to impact autoimmunity and infections as well as cancer (29, 30). In three distinct transplantable tumor models, LAG-3 and PD-1 have been shown to be co-expressed on tumor-infiltrating lymphocytes, and blockade of both pathways had synergistic effects on the anti-tumor $CD8^+$ T cell response (31). Similarly in ovarian cancer patients, co-expression of LAG-3 and PD-1 was found on antigen-specific $CD8^+$ T cells, and co-blockade of both lead to improved proliferation and cytokine production (32). Accordingly, different LAG-3 antibodies as monotherapy or in combination with anti-PD-1 have entered clinical trials for various cancer entities focusing on solid tumors.

In our model, we found that priming of T cells by DCs is significantly enhanced by blockade of LAG-3. We, therefore, propose the combination of DC vaccination and LAG-3 blockade as a promising approach for the initiation of novel immune responses, particularly in tumors with low endogenous immune responses including AML.

MATERIALS AND METHODS

Media and Reagents

Very low endotoxin RPMI 1640 medium (FG 1415; Biochrom) supplemented with 1.5% human serum (serum pool of AB positive adult males; Institute for Transfusion Medicine)—hereafter named DC medium—was used for the generation of DCs and all coculture experiments. The following reagents were used to generate DCs: GM-CSF (300-03), rhIL-4 (200-04), IFN-γ (300-02; all PeproTech), rhIL-1β (201-LB), TNF-α (210-TA/CF; both R&D Systems), PGE2 (P5640; Sigma-Aldrich), and R848 (tlrl-r848; InvivoGen).

Cell Isolation and Generation of DCs

After written informed consent, peripheral blood (PB) samples were collected from healthy donors (HDs) under a clinical protocol entitled "*in vitro* studies to establish new immunotherapies for AML and other hematological neoplasias." Both the consent form and the protocol were approved by the institutional review board (Ethikkommission bei der LMU München). Both cell isolation and generation of DCs were performed as described previously

for TLR-3-DCs (9) with the exception of polyI:C, which was not included in the maturation cocktail.

Coculture of DCs and T Cells

Dendritic cells were pulsed with a mixed CMV, EBV, influenza, and tetanus (CEFT) peptide pool (2 µg/ml; PM-CEFT; JPT) for 2 h at 37°C, 5% CO_2, incubated for 10 min on ice and subsequently washed. $CD3^+$ T cells were isolated from autologous non-adherent cells (NACs) by magnetic activated cell sorting (MACS, 130-050-101; Miltenyi Biotec) according to the manufacturer's protocol. CEFT-pulsed DCs and $CD3^+$ T cells were cocultured at a ratio of 1:10 in 96-well round bottom plates for 4 days at 37°C, 5% CO_2. For blocking experiments, the following monoclonal blocking antibodies were added at 10 µg/ml: α-CD244 (PP35; 16-2449-81; eBioscience), α-HVEM (122; 318802), α-TIM-3 (F38-2E2; 345003), α-PD-1 (EH12.27H7; 329911; all BioLegend), α-LAG-3 (17B4; AG-20B-0012PF; AdipoGen or ab40466; Abcam). The blocking antibody concentration of 10 µg/ml that we used was based on prior experiments demonstrating antibody blockade of immune checkpoints (21). Reducing the antibody concentration to 5 µg/ml did not alter our results (data not shown).

Coculture of DCs and NACs

Dendritic cells were pulsed with the Epstein–Barr nuclear Ag 3 A peptide FLRGRAYGL (FLR) (2 µg/ml; JPT) for 2 h at 37°C, 5% CO_2 and subsequently washed. FLR-pulsed DCs and autologous NACs were cocultured at a ratio of 1:80 in 96-well round bottom plates for 6 days at 37°C, 5% CO_2. For blocking experiments, α-PD-1 and α-LAG-3 were added as above.

Culture of PBMCs

Peripheral blood mononuclear cells (PBMCs) were loaded with FLR and cultured in 96-well round bottom plates (5×10^5/well) in the presence or absence of α-PD-1 and α-LAG-3 for 6–8 days at 37°C, 5% CO_2.

Surface Phenotyping of DCs and T Cells

Immunofluorescent staining of DC surface antigens was performed using a panel of fluorescence-conjugated monoclonal antibodies: CD80 (PE, L307.4; 560925), CD83 (APC, HB15e; 551073) CD86 (FITC, 2331 (FUN-1); 557343), CD273 (APC, MIH18; 557926), CD274 (FITC, MIH1; 558065; all BD Biosciences), Galectin-9 (PE, 9M1-3; 348906), CD48 (FITC, BJ40; 336706), HLA-DR (Pacific Blue, LN3; 327016; all BioLegend), HVEM (APC, 94801; FAB356A; R&D Systems). Corresponding isotype controls were used.

Immunofluorescent staining of T-cell surface antigens was performed using the following fluorescence-conjugated monoclonal antibodies: CD244 (PE, C1.7; 329507 or APC, C1.7; 329511), PD-1 (Brilliant Violet 421, EH12.7H7; 329919), CD3 (FITC, UCHT1; 300406), CD45RA (Brilliant Violet 421, HI100; 304129; all BioLegend), CD160 (APC, 688327; FAB6700A), TIM-3 (PE, 344823; FAB2365P; both R&D Systems), CD8 (PerCP-eFluor 710, SK1; 8046-0087; eBioscience), CD4 (APC-H7, RPA-T4; 560158; BD Biosciences), LAG-3 (ATTO 647N, 17B4; AG-20B-0012TS AdipoGen), CCR7 (CD197, APC, FR 11-11E8; 130-098-125; Miltenyi Biotec). Corresponding isotype controls were used. Intracellular FoxP3 staining was performed according to the manufacturer's instructions (APC, 3G3; Miltenyi Biotec).

Cells were analyzed using a FACS LSR II (BD Biosciences). Post-acquisition analysis was performed using FlowJo software (version 9.7.6; Tree Star). The median fluorescence intensity (MFI) ratio was calculated by dividing the MFI of the measured population by the MFI of cells stained with the isotype-matched antibody. For the upregulation of checkpoint molecules, the percentage of positive cells (% positive) was obtained by setting the gate at or below 1% in the respective isotype control.

Cytokine Secretion Measurement by Bead-Based Immunoassay

Secretion of IFN-γ and TNF-α was quantified by cytometric bead array (CBA) Flex Set (560111; BD Biosciences) according to the manufacturer's instructions.

CFSE Proliferation Assay

Isolated $CD3^+$ T cells were labeled with carboxyfluorescein N-succinimidyl ester (CFSE, C34554; Life Technologies) and cultured in the presence of autologous DCs. Unstimulated T cells served as negative control. Harvested cells were then stained with antibodies for CD3 (APC, UCHT1; 300412; BioLegend), CD4 (APC-H7), and CD8 (PerCP-eFluor 710). The percentage of divided cells (% divided) was analyzed using FlowJo software.

Fluorescence-Based Cell Sorting

Magnetic activated cell sorting-enriched $CD3^+$ T cells were sorted according to CCR7 and CD45RA expression levels into naive T cells (T_{naive}), central memory T cells (T_{CM}), effector memory T cells (T_{EM}), and effector memory RA T cells (T_{EMRA}) using an Aria III (BD Biosciences).

Expansion of WT1 Peptide-Specific T Cells

Wilms Tumor 1 antigen VLD (VLD = WT1 peptide VLDFAPPGA)-specific T cells were generated as previously described (33). Briefly, DCs were matured as described above. Autologous $CD8^+$ T cells were isolated from NACs using the $CD8^+$ T Cell Isolation Kit (130-096-495; Miltenyi Biotec) and incubated overnight in VLE-RPMI medium supplemented with 5% human serum and 5 ng/ml of IL-7 (200-07; Peprotech). DCs were pulsed with 2.5 µL/ml of the HLA-A*02:01-restricted VLD peptide (VLDFAPPGA; JPT) for 90 min and irradiated with 30 Gy. $CD8^+$ T cells and DCs were cocultivated in a 4:1 T cell:DC ratio and incubated with 30 ng/ml of IL-21 (200-21; Peprotech) in the presence or absence of 10 µg/ml LAG-3 or PD-1 blocking antibodies for 72 h. On day 3, cocultures were expanded 1:1 by adding medium supplemented with 10 ng/ml IL-15 and IL-7 (200-07, 200-15; both Peprotech) and 10 µg/ml blocking antibodies. On days 6–7, cells were analyzed by flow cytometry using VLD multimer (WB3469; Immudex) and fluorescence-conjugated monoclonal antibodies (see above).

Statistical Analysis

Data were analyzed using Prism 6 (GraphPad Software). All results are presented in box-and-whisker plots, with boxes

representing the lower quartile, the median and the upper quartile, while the whiskers show the minimal and the maximal value. The significance of differences for pairwise comparison was determined using the two-tailed Wilcoxon signed rank test. $p < 0.05$ was considered statistically significant (* in all figures), while $p < 0.01$ is termed highly significant (** in all figures).

RESULTS

TLR-3-DCs Expressed PD-L1 and HLA-DR

TLR-3-DCs were generated from PB of HDs. The characteristic phenotype of these DCs, with high expression of CD83, CD86, and CD80 and downregulation of CD14 is shown in **Figure 1A**. Expression of various inhibitory checkpoint molecules on DCs was analyzed by flow cytometry on 3–10 of these samples. HLA-DR was added to the panel as ligand for lymphocyte activation gene 3 (LAG-3) on T cells. MFI ratio of the expression data is presented in **Figure 1B**, statistical significance was tested against a theoretical median of 1.5. The expression of PD-L1 (median 6.2; $n = 7$; $p = 0.004$) and HLA-DR (median 184.5; $n = 7$; $p = 0.016$) on TLR-3-DCs was found to be (highly) significant. By contrast, HVEM (median 2.0; $n = 10$), CD48 (median 2.5; $n = 7$), Gal-9 (median 0.8; $n = 7$), and PD-L2 (median 0.9; $n = 3$) were not significantly expressed (**Figure 1B**).

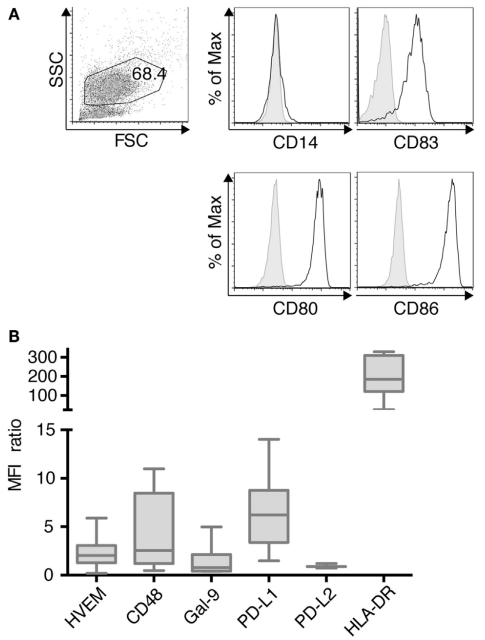

FIGURE 1 | Immunophenotypic characterization of dendritic cells generated within 3 days based on a TLR7/8 ligand (TLR-3-DCs). TLR-3-DCs were generated from peripheral blood of healthy donor (HDs), and surface marker expression was measured by flow cytometry. **(A)** The characteristic phenotype of a dendritic cell population (FSChi/SSChi/CD14-/CD83+/CD80+/CD86+) is shown for one representative donor. **(B)** Expression of various inhibitory checkpoint molecules was analyzed on TLR-3-DCs of 3–10 donors, and MFI ratio of the expression is presented as box-and-whisker plots.

CD244, TIM-3, PD-1, and LAG-3 Were Upregulated on T Cells after Stimulation with TLR-3-DCs

Expression of the respective co-inhibitory ligands was determined on T cells with and without stimulation by DCs. TLR-3-DCs were generated from PB of HDs and pulsed with CEFT peptide pool. $CD3^+$ T cells were isolated from PB of the same HDs and cocultured with autologous DCs or with CEFT peptide pool alone for 96 h. Expression of various inhibitory checkpoint molecules was analyzed on T cells by flow cytometry for 7–14 HDs. The percentage of positive cells is presented for $CD4^+$ (**Figure 2A**; Figure S1 in Supplementary Material) and $CD8^+$ (**Figure 2B**; Figure S1 in Supplementary Material) T cells. Statistical significance was tested between stimulation with pulsed DCs and CEFT stimulation alone as a control. $CD4^+$ T cells showed a (highly) significant

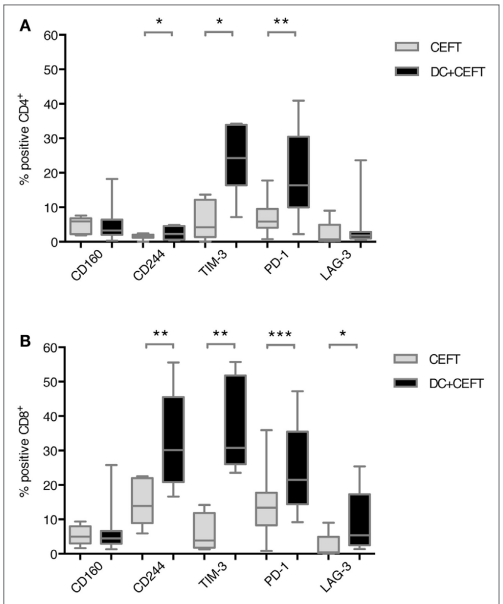

FIGURE 2 | Upregulation of immune checkpoint ligands on T cells after dendritic cell stimulation. T cells of 7–14 healthy donor were cocultured with autologous TLR-3-DCs pulsed with CMV, EBV, influenza, tetanus (CEFT) peptide pool or with CEFT peptide pool alone. Expression of various inhibitory checkpoint molecules was analyzed by flow cytometry. The percentage of positive cells is presented as box-and-whisker plots for $CD4^+$ **(A)** and for $CD8^+$ **(B)** T cells. *$p < 0.05$; **$p < 0.01$.

upregulation of CD244 (median of 2.3 vs. 1.5%; $n = 7$; $p = 0.047$), TIM-3 (median of 24.3 vs. 4.2%; $n = 7$; $p = 0.016$) and PD-1 (median of 16.4 vs. 5.9%; $n = 13$; $p = 0.003$) after stimulation with TLR-DCs, while expression of CD160 (median of 3.3 vs. 5.9%; $n = 7$) and LAG-3 (median of 1.8 vs. 0.7%; $n = 9$) were not changed (**Figure 2A**). On $CD8^+$ T cells, we found (highly) significant upregulation of CD244 (median of 30.2 vs. 13.9%; $n = 8$; $p = 0.008$), TIM-3 (median of 30.8 vs. 3.9%; $n = 8$; $p = 0.008$), PD-1 (median of 21.5 vs. 13.4%; $n = 14$; $p < 0.001$) and LAG-3 (median of 5.4 vs. 0.4%; $n = 9$; $p = 0.027$), but not of CD160 (median of 4.5% vs. 5.0%; $n = 8$) (**Figure 2B**).

Blockade of PD-1 and LAG-3, but Not HVEM, CD244 or TIM-3, Enhanced Proliferation of T Cells after Stimulation with TLR-3-DCs

In order to determine the functional relevance of co-inhibitory molecule interaction between TLR-3-DCs and T cells, we first tested the influence of checkpoint blockade on proliferation of T cells after DC stimulation. $CD3^+$ T cells isolated from PB of HDs were labeled with CFSE and cocultured with autologous CEFT-pulsed TLR-3-DCs for 5 days in the presence or absence of respective blocking antibodies. The percentage of divided cells was determined by flow cytometry. The ratio between the percentages of divided cells with and without blocking antibody was calculated. Data for 4–13 samples is presented in **Figure 3A** for $CD4^+$ T cells and in **Figure 3B** for $CD8^+$ T cells, original data is shown in Table S1 in Supplementary Material. Statistical significance was calculated against a fold change of 1.0, equal to no effect of the blocking antibody on proliferation. For $CD4^+$ T cells, no effect of checkpoint blockade on proliferation was found for HVEM (fold change 0.91; $n = 6$), CD244 (fold change 1.05; $n = 4$) and TIM-3 (fold change 1.02; $n = 4$). Blockade of PD-1 resulted in slightly enhanced proliferation (fold change 1.15; $n = 13$; $p = 0.002$), and blockade of LAG-3 lead to markedly enhanced proliferation (fold change 1.44; $n = 9$; $p = 0.002$), both statistically highly significant (**Figure 3A**). Similarly, for $CD8^+$ T cells, blockade of PD-1 resulted in slightly enhanced proliferation (fold change 1.08; $n = 13$; $p = 0.003$), and blockade of LAG-3 lead to markedly enhanced proliferation (fold change 1.24; $n = 9$; $p = 0.002$), both statistically highly significant, while no effect of checkpoint blockade on proliferation was found for HVEM (fold change 0.88; $n = 6$), CD244 (fold change 0.96; $n = 4$) and TIM-3 (fold change 0.91; $n = 4$) (**Figure 3B**).

Blockade of PD-1 and LAG-3, but Not HVEM, CD244 or TIM-3, Enhanced IFN-γ and TNF-α Secretion by T Cells after Stimulation with TLR-3-DCs

Next, we determined whether checkpoint blockade also influenced IFN-γ and TNF-α secretion by T cells after DC stimulation. $CD3^+$ T cells isolated from PB of HDs were cocultured with autologous CEFT-pulsed TLR-3-DCs for 96 h in the presence or absence of respective blocking antibodies. The concentration of IFN-γ and TNF-α in the culture supernatant was determined by CBA. IFN-γ and TNF-α fold change was calculated by dividing the concentration of the coculture with blocking antibody by the concentration of the control coculture without antibody. Data for 5–14 samples is presented for IFN-γ in **Figure 3C**, statistical significance was calculated against a fold change of 1.0, original data are shown in Table S1 in Supplementary Material. No effect of checkpoint blockade on IFN-γ secretion was found for HVEM (fold change 0.63; $n = 7$), CD244 (fold change 0.86; $n = 5$), and TIM-3 (fold change 1.01; $n = 5$). Blockade of PD-1 resulted in enhanced IFN-γ secretion (fold change 1.50; $n = 14$; $p = 0.002$) and blockade of LAG-3 lead to markedly enhanced IFN-γ secretion (fold change 5.00; $n = 9$; $p = 0.004$), both statistically highly significant (**Figure 3C**). Similarly, no effect of checkpoint blockade on TNF-α secretion was found for HVEM (fold change 0.89; $n = 7$), CD244 (fold change 1.01; $n = 5$) and TIM-3 (fold change 0.92; $n = 5$), while blockade of PD-1 (fold change 1.69; $n = 14$; $p = 0.002$), and blockade of LAG-3 (fold change 5.29; $n = 9$; $p = 0.008$) resulted in enhanced TNF-α secretion, both statistically highly significant (Figure S2 in Supplementary Material).

Combination with PD-1 Blockade Resulted in an Increase of IFN-γ Secretion, but Not in an Enhanced Proliferation of T Cells after Stimulation with TLR-3-DCs Compared to LAG-3 Blockade Alone

We tested the hypothesis that blockade of PD-1 and LAG-3 has additive or synergistic effects on proliferation or IFN-γ secretion by T cells after stimulation with TLR-3-DCs. For proliferation assays, $CD3^+$ T cells isolated from PB of 7 HDs were labeled with CFSE and cocultured with autologous TLR-3-DCs for 5 days in the presence or absence of blocking antibodies for PD-1 and LAG-3, both alone and in combination. As above, the percentage of divided cells was determined by flow cytometry for the different conditions, and the ratio between the percentages of divided cells with and without blocking antibody was calculated. Data are presented in **Figure 3D** for $CD4^+$ T cells and in **Figure 3E** for $CD8^+$ T cells, statistical significance was calculated for the combination of blocking antibodies vs. single antibody blockade, original data are shown in Table S1 in Supplementary Material. For the combination of PD-1 and LAG-3 blockade (median fold change of 1.37 for $CD4^+$ and 1.26 for $CD8^+$), we found significantly higher T cell proliferation compared to PD-1 blockade alone (median fold change of 1.02 for $CD4^+$; $p = 0.016$; 1.02 for $CD8^+$; $p = 0.016$), but no difference to LAG-3 blockade alone (median fold change of 1.31 for $CD4^+$; $p = 0.094$; 1.20 for $CD8^+$; $p = 0.250$).

Similarly, for IFN-γ secretion assays, $CD3^+$ T cells isolated from PB of 8 HDs were cocultured with autologous CEFT-pulsed TLR-3-DCs for 96 h in the presence or absence of blocking antibodies for PD-1 and LAG-3, both alone and in combination. The concentration of IFN-γ in the culture supernatant was determined by CBA. IFN-γ fold change was calculated as a ratio between the IFN-γ concentration of the coculture with and without blocking antibody. Statistical significance was calculated for the combination of blocking antibodies vs. single antibody blockade. For the combination of PD-1 and LAG-3 blockade (median fold change

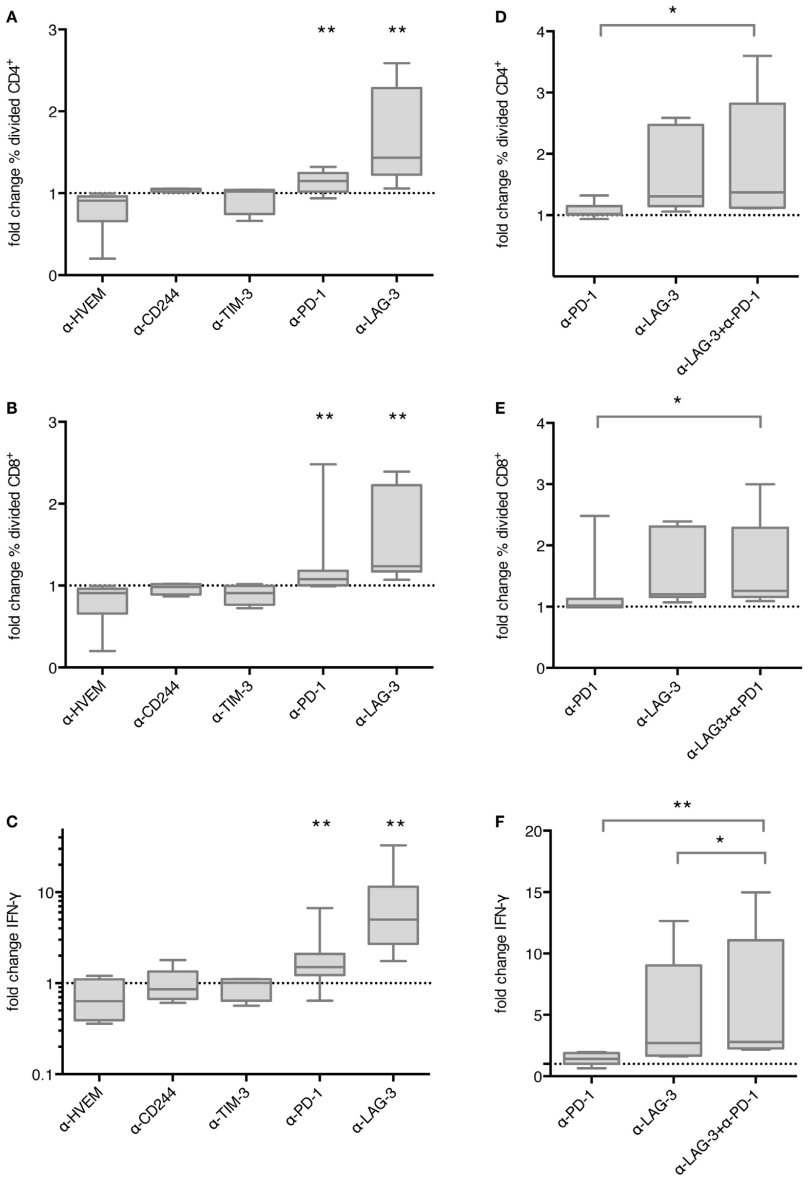

FIGURE 3 | Effect of immune checkpoint blockade on proliferation and IFN-γ secretion of T cells after stimulation with TLR-3-DCs. $CD3^+$ T cells of 4–14 healthy donor (HDs) were cocultured with autologous CMV, EBV, influenza, tetanus (CEFT)-pulsed TLR-3-DCs in the presence or absence of immune checkpoint blocking antibodies, either for individual antibodies **(A–C)** or in different combinations of α-PD-1 and α-LAG-3 antibodies **(D–F)**. Proliferation of $CD4^+$ **(A,D)** and $CD8^+$ T cells **(B,E)** was analyzed by carboxyfluorescein N-succinimidyl ester (CFSE) assay, and the ratio between the percentages of divided cells with and without blocking antibody was calculated. IFN-γ secretion of $CD3^+$ T cells **(C,F)** was determined by cytometric bead array (CBA) assay, and the ratio between concentration with and without blocking antibody was calculated. All data are presented as box-and-whisker plots, and statistical significance was calculated against a fold change of 1.0. $^*p < 0.05$; $^{**}p < 0.01$.

of 2.80), the increase in IFN-γ secretion compared to PD-1 blockade alone was statistically highly significant (median fold change of 1.41; $p = 0.008$). In comparison to LAG-3 blockade alone, we found a slight, but statistically significant enhancement (median fold change of 2.70; $p = 0.016$) (**Figure 3F**). Taken together, LAG-3 blockade alone resulted in strong enhancement of T cell proliferation and IFN-γ secretion. The effect on IFN-γ secretion was slightly increased by the combination with PD-1 blockade, while no additional effect was seen for T cell proliferation.

LAG-3 Blockade Mainly Enhanced IFN-γ Secretion by Naive and T_{CM}, While PD-1 Blockade Also Resulted in an Increase of IFN-γ Secretion by Effector Memory Cells

Next, we analyzed the differential effect of PD-1 and LAG-3 blockade on T cell subpopulations. MACS-enriched $CD3^+$ T cells were sorted according to CCR7 and CD45RA expression levels into T_{naive}, T_{CM}, T_{EM}, and T_{EMRA} T cells (**Figure 4A**). The various T cell populations were cocultured with autologous CEFT-pulsed TLR-3-DCs for 96 h in the absence of presence of blocking antibodies for PD-1 and LAG-3. Again, the concentration of IFN-γ in the culture supernatant was determined by CBA. IFN-γ fold change was calculated by dividing the concentration of the coculture with blocking antibody by the concentration of the control coculture without antibody. Data for six samples is presented for PD-1 (**Figure 4B**) and for LAG-3 (**Figure 4C**) blockade. Statistical significance was calculated against a fold change of 1.0. We found that PD-1 blockade lead to significantly increased IFN-γ secretion of T_{naive} (median fold change of 1.41; $p = 0.031$), T_{CM} (median fold change of 1.43; $p = 0.031$), and T_{EM} (median fold change of 1.47; $p = 0.031$), while the increased secretion of T_{EMRA} was not statistically significant (median fold change of 1.96; $p = 0.156$) (**Figure 4B**). By contrast, LAG-3 blockade had significant effects on IFN-γ secretion of T_{naive} (median fold change of 2.04; $p = 0.031$) and T_{CM} (median fold change of 1.71; $p = 0.031$), but not on T_{EM} (median fold change of 1.34; $p = 0.094$) and T_{EMRA} (median fold change of 1.33; $p = 0.094$) (**Figure 4C**). With respect to the $CD25^+$/$FoxP3^+$ regulatory T cell subpopulation of $CD4^+$ T cells, we saw a tendency toward a higher percentage after LAG-3 blockade (Figure S3 in Supplementary Material).

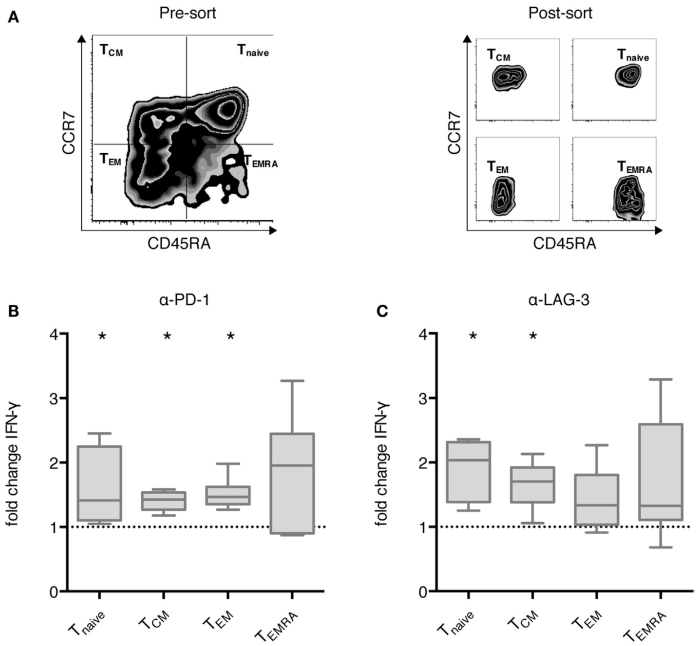

FIGURE 4 | Effect of programmed cell death protein 1 (PD-1) and lymphocyte activation gene 3 (LAG-3) blockade on IFN-γ secretion of different T cell subpopulations after stimulation with TLR-3-DCs. MACS-enriched $CD3^+$ T cells of 8 healthy donor (HDs) were sorted according to CCR7 and CD45 RA expression **(A)**. The various T cell subpopulations were cocultured with autologous CMV, EBV, influenza, tetanus (CEFT)-pulsed TLR-3-DCs in the presence or absence of α-PD-1 **(B)** and α-LAG-3 **(C)** antibody. IFN-γ secretion was determined by cytometric bead array (CBA) assay, and the ratio between concentration with and without blocking antibody was calculated. All data are presented as box-and-whisker plots, and statistical significance was calculated against a fold change of 1.0. *$p < 0.05$.

Blockade of LAG-3, but Not PD-1, Enhanced Proliferation of EBV Antigen-Specific T Cells after Stimulation with TLR-3-DCs

Next, we tested whether blockade of PD-1 and LAG-3 also enhances the proliferation of antigen-specific T cells after stimulation with TLR-3-DCs. NACs (mainly consisting of T cells) of 9 HDs were cocultured with autologous FLR-pulsed TLR-3-DCs for 144 h in the presence or absence of blocking antibodies for PD-1 and LAG-3, both alone and in combination. The percentage of FLR tetramer positive (Tet^+) cells within the $CD8^+$ T cell population was determined by flow cytometry. Tet^+ fold change was calculated by dividing the percentage in the condition with blocking antibody by the percentage in the condition without any antibody. Statistical significance was calculated against a fold change of 1.0. Blockade of LAG-3 resulted in a significantly increased percentage of Tet^+ $CD8^+$ T cells (median fold change 1.69; $p = 0.039$), while blockade of PD-1 (median fold change 0.79) and the combination of LAG-3 and PD-1 blockade (median fold change 0.61) did not enhance the percentage of antigen-specific T cells (**Figure 5A**). This was not due to a lack of PD-1 expression on T cells, as further analysis of the Tet^+ $CD8^+$ T cells after stimulation with FLR-pulsed DCs revealed that PD-1 was expressed on 92.6% of the T cells, while LAG-3 was found on only 49.1% of T cells (**Figure 5B**).

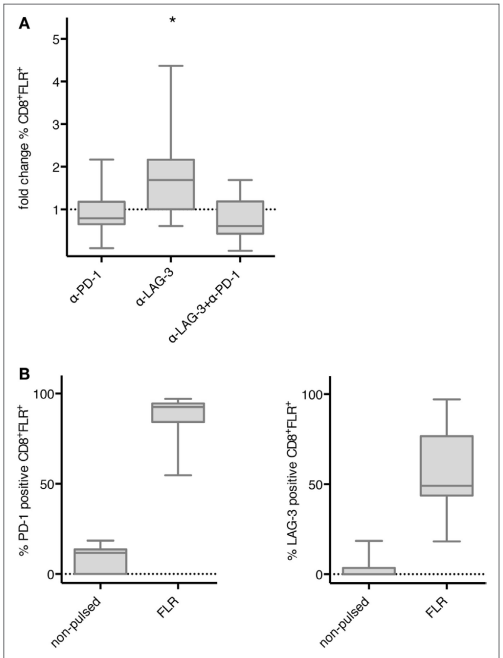

FIGURE 5 | Effect of programmed cell death protein 1 (PD-1) and lymphocyte activation gene 3 (LAG-3) blockade on proliferation of EBV antigen-specific T cells after stimulation with TLR-3-DCs. non-adherent cell (NACs) of 9 healthy donor (HDs) were cocultured with autologous Epstein–Barr nuclear Ag 3 A peptide FLRGRAYGL (FLR)-pulsed TLR-3-DCs in the presence or absence of α-PD-1 and α-LAG-3 antibody. **(A)** The percentage of FLR tetramer positive cells within the $CD8^+$ T cell population was determined by flow cytometry. Data for fold change to the condition without blocking antibody are presented as box-and-whisker plots, and statistical significance was calculated against a fold change of 1.0. $^*p < 0.05$. **(B)** PD-1 and LAG-3 expression was determined for FLR tetramer positive $CD8^+$ T cells after stimulation with non-pulsed or FLR-pulsed TLR-3-DCs.

Blockade of LAG-3, but Not PD-1, Enhanced Proliferation and IFN-γ Secretion of T Cells after Stimulation with FLR-Pulsed APCs within PBMCs

We then asked if the effect of LAG-3 blockade on proliferation and IFN-γ secretion also holds true, if T cells are not stimulated by TLR-3-DCs, but by the various APCs naturally occurring within PBMCs. PBMCs of 8 HDs were pulsed with FLR peptide and cultured for 6 days in the presence or absence of blocking antibodies for PD-1 and LAG-3, both alone and in combination. Thereafter, the percentage of FLR tetramer positive cells (Tet^+) within the $CD8^+$ T cell population was determined by flow cytometry. Tet^+ fold change was calculated by dividing the percentage in the condition with blocking antibody by the percentage in the condition without any antibody. Statistical significance was calculated against a fold change of 1.0. Blockade of LAG-3 resulted in a significantly increased percentage of Tet^+ $CD8^+$ T cells (median fold change 1.80; $p = 0.023$), while blockade of PD-1 (median fold change 1.05) did not enhance the percentage of antigen-specific T cells. The combination of LAG-3 and PD-1 blockade also significantly enhanced the percentage of Tet^+ $CD8^+$ T cells (median fold change 1.74; $p = 0.016$), but this was not different from LAG-3 alone ($p = 0.461$) (**Figure 6A**).

The concentration of IFN-γ was determined in the culture supernatant after 6–8 days of coculture by CBA. IFN-γ fold change was calculated by dividing the concentration of the coculture with blocking antibody by the concentration of the control coculture without antibody. Data for the same eight samples is presented in **Figure 6B**, statistical significance was calculated against a fold change of 1.0. Blockade of PD-1 (median fold change 0.96) did not enhance IFN-γ secretion, while increase of IFN-γ secretion after blockade of LAG-3 was highly significant (median fold change 4.07; $p = 0.008$). The combination of LAG-3 and PD-1 blockade also enhanced IFN-γ secretion highly significantly (median fold change 6.88; $p = 0.008$), but the difference to LAG-3 blockade alone was not significant ($p = 0.188$) (**Figure 6B**).

Further analysis of the Tet^+ $CD8^+$ T cells after stimulation with FLR-pulsed PBMCs revealed that PD-1 was expressed on almost all of the T cells (93.3%), while LAG-3 was found on only 10.5% of T cells (**Figure 6C**). Therefore, the non-existent effect of PD-1 blockade in this setting was not due to an absence of PD-1 on the T cell surface.

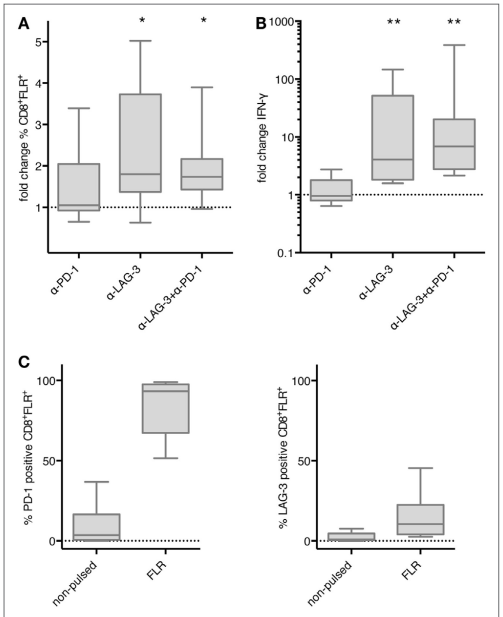

FIGURE 6 | Effect of programmed cell death protein 1 (PD-1) and lymphocyte activation gene 3 (LAG-3) blockade on proliferation and IFN-γ secretion of EBV antigen-specific T cells after stimulation with antigen-presenting cells (APC) within peripheral blood mononuclear cells (PBMCs). Epstein–Barr nuclear Ag 3 A peptide FLRGRAYGL (FLR) peptide-pulsed PBMCs of 8 healthy donor (HDs) were cultered in the presence or absence of α-PD-1 and α-LAG-3 antibody. The percentage of FLR tetramer positive cells within the $CD8^+$ T cell population was determined by flow cytometry **(A)**, and IFN-γ secretion was determined by cytometric bead array (CBA) assay **(B)**. Data for fold change to the condition without blocking antibody are presented as box-and-whisker plots, and statistical significance was calculated against a fold change of 1.0. $^*p < 0.05$; $^{**}p < 0.01$. **(C)** PD-1 and LAG-3 expression was determined for FLR tetramer positive $CD8^+$ T cells after stimulation with non-pulsed or FLR-pulsed PBMCs.

Blockade of LAG-3, More than PD-1, Enhanced Expansion of WT1 Tumor Antigen-Specific T Cells after Stimulation with TLR-3-DCs

Finally, we tested the hypothesis that the effect of LAG-3 blockade on proliferation of antigen-specific T cells can also be transferred to tumor antigen specificity. $CD8^+$ T cells of 3 HDs were cocultured with autologous TLR-3-DCs pulsed with a WT1 antigen (VLD peptide) for 6–7 days in the presence or absence of blocking antibodies for PD-1 and LAG-3. The percentage of VLD tetramer positive (Tet^+) cells within the $CD8^+$ T cell population was determined by flow cytometry. Results for all three donors are presented in **Figure 7**. Blockade of LAG-3 resulted in an increased percentage of Tet^+ $CD8^+$ T cells in two of three cases, while blockade of PD-1 resulted in an increase in Tet^+ $CD8^+$ T cells in only one case, and to a lesser extent.

DISCUSSION

Over the last decades, DCs generated *in vitro* for the vaccination of tumor patients have been optimized with respect to cytokine production as well as co-stimulatory molecule expression. However, even TLR-3-DCs, which demonstrate an improved phenotype and functional profile, express co-inhibitory molecules (9). Combining DC vaccination with checkpoint inhibition is, therefore, conceivable and might enhance T cell responses.

In this study, we systematically analyzed the effect of different checkpoint inhibitors on T cell stimulation by TLR-3-DCs. We found that within our experimental settings, blockade of LAG-3 was consistently superior to PD-1 blockade, independently of the method to measure T cell stimulation (proliferation, IFN-γ secretion), the stimulating antigen (viral and bacterial peptide pool, specific viral antigen, specific tumor antigen), and the type of T cell ($CD4^+$ and $CD8^+$ T cells) used. This was not expected, as LAG-3 expression on T cells is relatively low compared to PD-1 expression and only slightly upregulated after stimulation. However, it has to be considered that checkpoint molecules are often upregulated on antigen-specific T cells only (Figure S4A in Supplementary Material) and, thus, the assessment of bulk T cell populations might only insufficiently reflect checkpoint molecule expression. Besides, HLA-DR as the main ligand for LAG-3 is much higher expressed on APCs including TLR-3-DCs than any co-inhibitory molecules (**Figure 1**), conceivably resulting in numerous receptor–ligand interactions with T cells that help to explain the strong effects seen in our blocking experiments. LSECtin, a cell surface lectin of the DC-SIGN family, has been identified as an alternative ligand for LAG-3, and LAG-3 blockade has been shown to result in abrogation of immunoinhibitory effects of LSECtin in a melanoma mouse model (34). As LSECtin is only marginally expressed on TLR-3-DCs (Figure S5 in Supplementary Material), the effects of LAG-3 blockade demonstrated here are more likely due to interaction with HLA-DR. Similarly, the low PD-L2 expression on DCs suggests that PD-L1 is more relevant for the interaction with PD-1 in our setting. However, we cannot rule out that other receptor–ligand interactions between DCs and T cells that have not yet been explored are responsible for the effects on T cell responses that we describe. In order to further elucidate the mechanism of action, a potential approach could be the application of MHC class II blocking antibodies. In a model using COS-7 cells transfected with human LAG-3 and MHC class II-expressing human B lymphoblastic cell lines, it could be shown that both blocking antibodies against LAG-3 and HLA-DR were able to disrupt the rosettes formed by these cells (23). However, the exact binding site on MHC class II for LAG-3 is still unknown making the choice of an antibody that specifically blocks the interaction of MHC II with LAG-3 technically challenging.

In a recently published study that analyzed the effects of checkpoint blockade on T cell stimulation by allogeneic DCs, the addition of an antibody directed against LAG-3 to the coculture

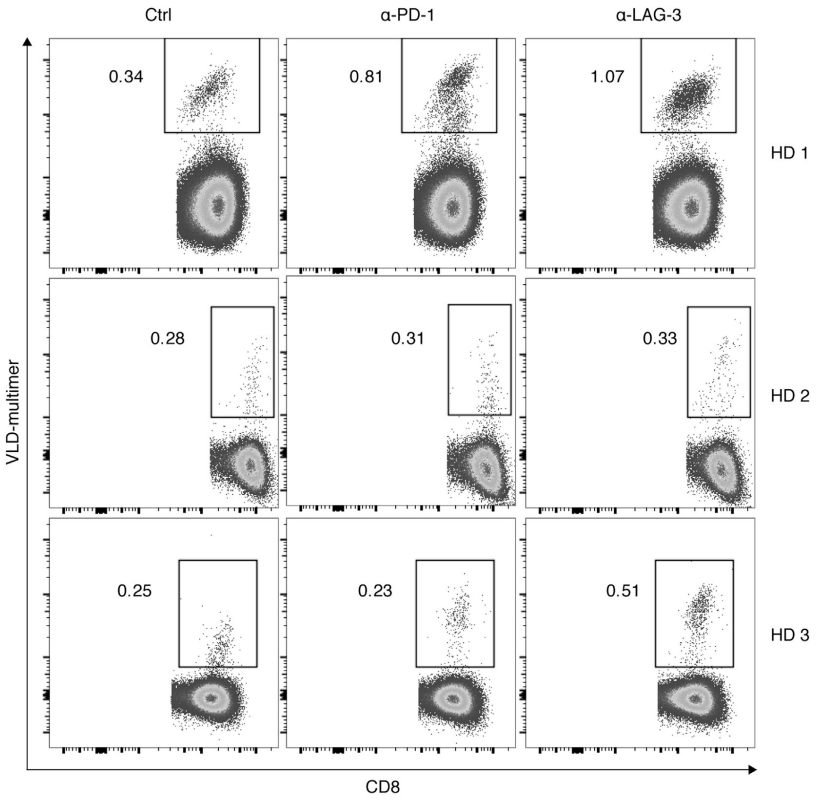

FIGURE 7 | Effect of programmed cell death protein 1 (PD-1) and lymphocyte activation gene 3 (LAG-3) blockade on proliferation of Wilms Tumor 1 tumor-antigen-specific T cells after stimulation with TLR-3-DCs. $CD8^+$ T cells of three healthy donor (HDs) were cocultured with autologous VLD-pulsed. TLR-3-DCs in the presence or absence of α-PD-1 and α-LAG-3 antibody. The percentage of VLD tetramer positive cells within the $CD8^+$ T cell population was determined by flow cytometry. Data for all three donors are shown.

did not result in significant changes in T cell proliferation or cytokine secretion (21). As the setting of these experiments differed from ours in the origin of the blood donors (allogeneic vs. autologous), maturation protocol of the DCs and target antigens, there are multiple reasons for the diverging results. However, it is also important to notice that the LAG-3 antibody used is of a different clone and its blockade of the ligand–receptor interactions might be less effective than in our experiments. While we did not directly proof that the antibodies we used were blocking the interaction with their ligands, we only chose antibodies that had been described in the literature to have this capacity. Besides, we showed that addition of the blocking antibodies reduced the capacity of the respective staining antibody to bind to the receptor (Figure S4B in Supplementary Material).

While the effects of PD-1 blockade on T cell stimulation by TLR-3-DCs were less pronounced than those of LAG-3 blockade in our experiments, they were still significant. Surprisingly, however, the combination of both blocking antibodies did not result in a relevant increase in T cell stimulation compared to the LAG-3 antibody alone. In the analysis of viral antigen-specific T cell stimulation, it was even deleterious (**Figure 5**). Several murine tumor models, including a B16 melanoma and an MC38 colon adenocarcinoma model (31) demonstrated synergistic anti-tumor immunity by dual blockade of PD-1 and LAG-3. One possible explanation for our observation is an overstimulation of T cells by the combination of the immunostimulatory TLR-3-DCs with two effective checkpoint inhibitors. This is in line with data published for chronic lymphocytic leukemia, where PD-1 blockade abolished the positive effect induced by anti-LAG-3 antibodies in combination with CD3/CD28 beads as a very strong stimulus (35).

This hypothesis was substantiated in our experiments using PBMCs, comprising APCs that are relatively less

immunostimulatory compared to TLR-3-DCs. Here, the combination of both blocking antibodies resulted in T cell stimulation that was at least similar to the LAG-3 antibody alone (**Figure 6**). As the strength of the antigen stimulus is also dependent on peptide concentration, we conducted peptide titration assays in the setting of viral antigen-specific T cell stimulation both by TLR-3-DCs and by PBMCs (Figure S6 in Supplementary Material). At the lowest peptide concentration, the combinatorial blockade was equally effective to LAG-3 blockade alone for DCs, while the effect of LAG-3 blockade on PBMCs was strongly increased by the addition of PD-1 blockade. Thus, we provide evidence that LAG-3 blockade alone is effective in boosting of T cell stimulation by a strong antigenic stimulus, while the combination of LAG-3 and PD-1 blockade is more effective in the setting of weak T cell stimulation. This observation is in line with *ex vivo* T cell stimulation experiments with tumor-infiltrating lymphocytes of epithelial ovarian cancer patients, where dual blockade of LAG-3 and PD-1 during priming of tumor antigen-specific T cells with tumor-derived APCs as weak stimulators increased T cell effector function to the levels observed with PB-derived APCs as stronger stimulators (32).

Our data set is focused on the priming phase of the immune response rather than the effector phase. The expression levels of checkpoint molecules on APCs clearly differ from those on tumor cells. Therefore, it is not surprising that the dominant effect of LAG-3 blockade and the relatively low effect of PD-1 blockade that we see deviates from the results in animal studies (36) and the outstanding clinical effects observed with PD-1 blockade as monotherapy or in combination with antineoplastic agents in clinical trials for various tumor entities. The effects observed in those studies rely on the effector phase of the immune response and are dependent on pre-existing effector T cells. Different immune checkpoints seem to be of importance in priming and effector phase, as directly shown for the epithelial ovarian cancer model, where LAG-3 blockade did not influence the effector function of already primed tumor-infiltrating T cells (32). Similarly, a 4-1BB agonist was more effective than an anti-LAG-3 blocking antibody as a combination partner for PD-1 blockade in a melanoma mouse model in the absence of any cancer vaccine (37). Recently, first data was published from an ongoing clinical trial (NCT01968109), in which anti-LAG-3 in combination with anti-PD-1 showed activity in melanoma patients who were relapsed or refractory to anti-PD-1/-PD-L1 therapy. The objective response rate (ORR) was 11.5% in 61 efficacy-evaluable patients, and a correlation of higher ORR with a LAG-3 expression above 1% on tumor-associated immune cells was shown (38).

Checkpoint blockade has revolutionized cancer therapy in several entities, including melanoma, lung cancer, and urothelial carcinoma. To our current understanding, these results primarily rely on reversing adaptive immune escape mechanisms of the tumor cells in the context of an immune response. Our data, however, support the relevance of checkpoint inhibition within the induction of primary or secondary anti-tumor immune responses. Thus, checkpoint inhibitors might also be therapeutically beneficial in tumor entities with a non-immunogenic microenvironment. Further studies will be needed to address the question of checkpoint inhibition within the priming versus effector phase of T cell responses. The sequencing and exact timing of LAG-3 and PD-1 blockade might be of particular relevance for the induction for optimal anti-tumor T cell responses.

AUTHOR CONTRIBUTIONS

FL, MR and MS conceived and designed the experiments. MR, FS, KD, CK, CA, MS, and JN performed the experiments. FL, MR, and MS analyzed the data and designed the figures. FL and MS wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00385/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

Targeting LAG-3 and PD-1 to Enhance T Cell Activation by Antigen-Presenting Cells

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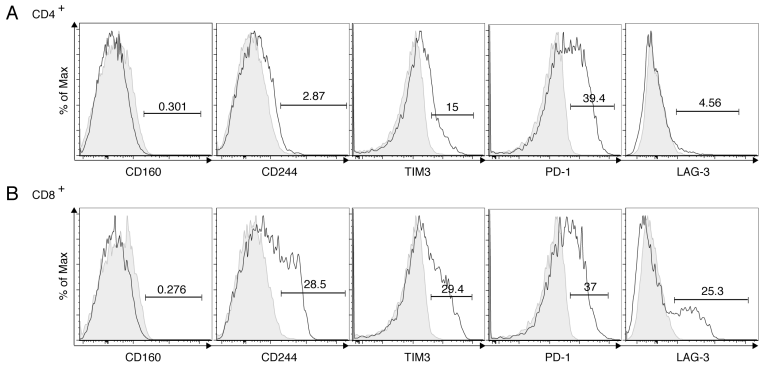


Figure S1. Upregulation of immune checkpoint ligands on T cells after DC stimulation. T cells were cocultured with autologous TLR-3-DCs pulsed with CEFT peptide pool. Expression of various inhibitory checkpoint molecules after 4 days of coculture was analyzed by flow cytometry. Original histogram data are shown for one representative donor.

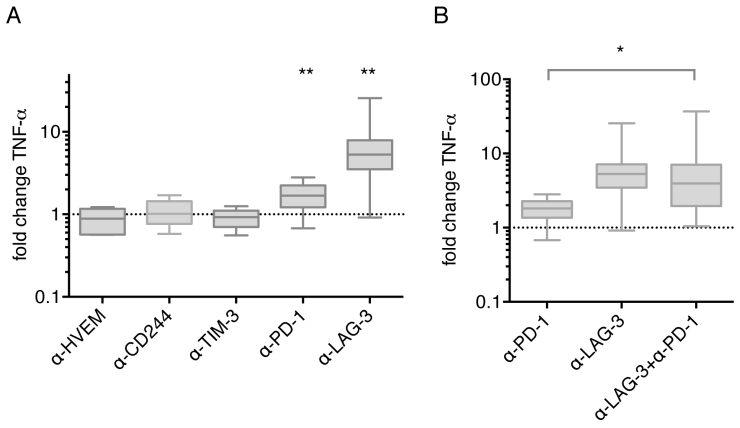


Figure S2. Effect of immune checkpoint blockade on TNF-α secretion of T cells after stimulation with TLR-3-DCs. $CD3^+$ T cells of 4–14 HDs were cocultured with autologous CEFT-pulsed TLR-3-DCs in the presence or absence of immune checkpoint blocking antibodies, either for individual antibodies (A) or in different combinations of α-PD-1 and α-LAG-3 antibodies (B). TNF-α secretion of $CD3^+$ T cells was determined by CBA assay, and the ratio between concentration with and without blocking antibody was calculated. All data are presented as box-and-whisker plots, and statistical significance was calculated against a fold change of 1.0. *, $p<0.05$; **, $p<0.01$.

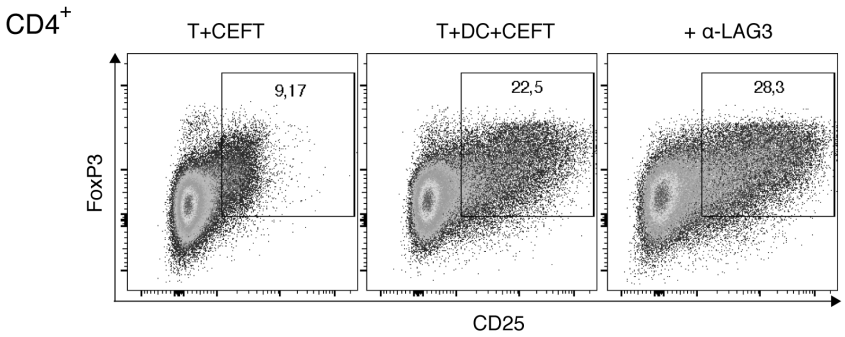
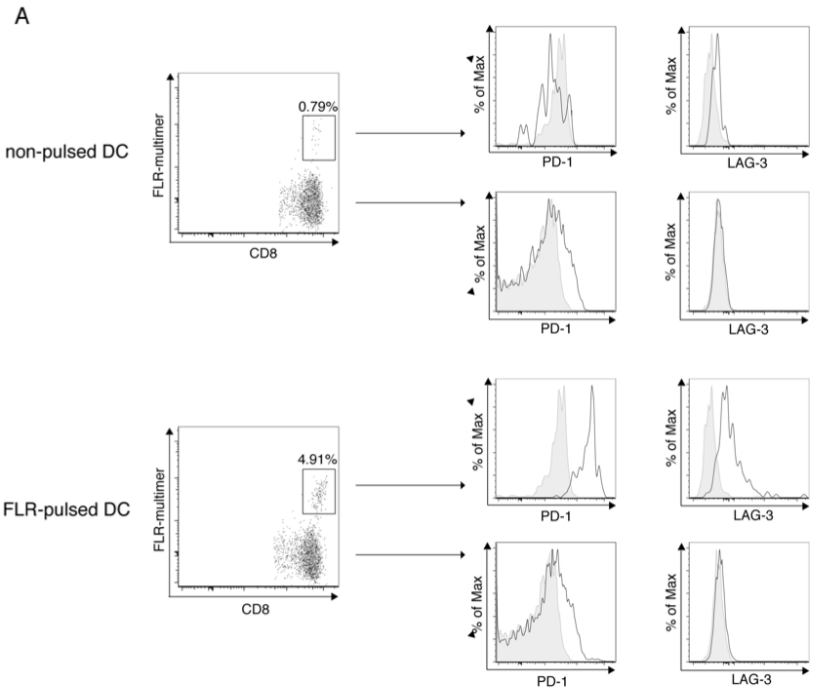


Figure S3. Effect of LAG-3 blockade on percentage of regulatory T cells after stimulation with TLR-3-DCs. $CD3^+$ T cells were cocultured with autologous CEFT-pulsed TLR-3-DCs in the presence or absence of α-LAG-3 antibody. Within the $CD4^+$ T cell population, co-expression of CD25 and FoxP3 was determined by flow cytometry. Data are shown for one representative donor, demonstrating a relative increase in percentage of regulatory T cells after LAG-3 blockade.


A
non-pulsed DC
FLR-pulsed DC
FLR-multimer
CD8
0.79%
4.91%
% of Max
PD-1
LAG-3

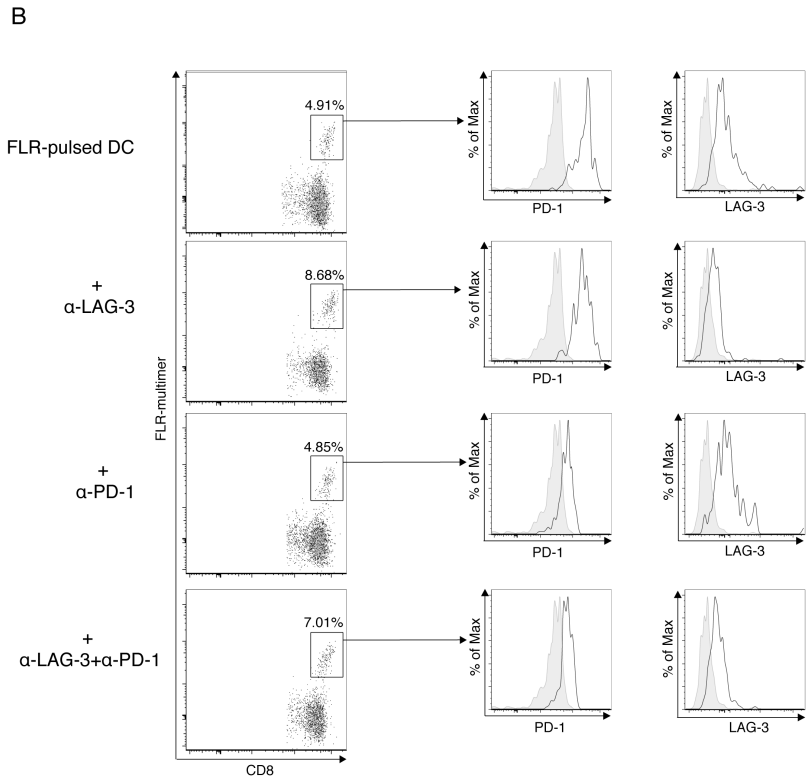


Figure S4. Competition between blocking antibodies and staining antibodies for LAG-3 and PD-1 on EBV antigen-specific T cells after stimulation with TLR-3-DCs. DCs were pulsed with FLR peptide and cocultured with autologous NAC in the presence or absence of α-PD-1 and α-LAG-3 blocking antibodies. Expression of PD-1 and LAG-3 positive cells within the FLR tetramer-positive cells of the $CD8^+$ T cell population was determined by flow cytometric measurements with respective staining antibodies. Data are shown for one representative donor.

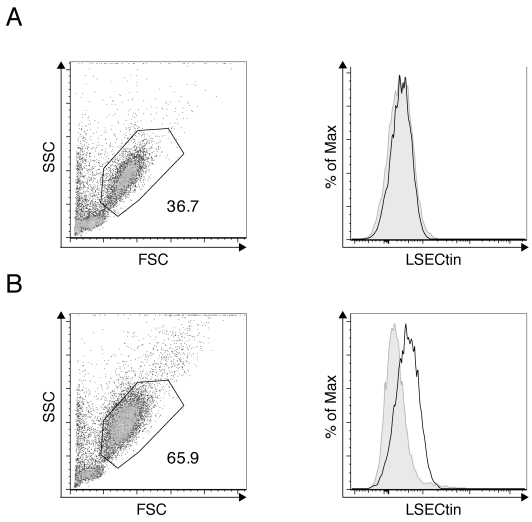


Figure S5. LSECtin expression of immature DCs and TLR-3-DCs. DCs were generated from PB of HDs, and surface marker expression was measured by flow cytometry. LSECtin expression of immature DCs (A), harvested on d2 of DC generation, before addition of maturation cocktail, and LSECtin expression of mature TLR-3-DCs (B), harvested on d3, after 24 hours with maturation cocktail, is shown for one representative donor.

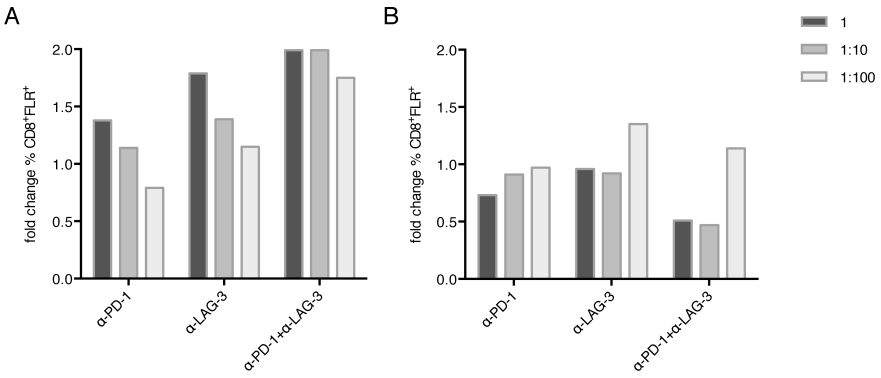


Figure S6. Effect of PD-1 and LAG-3 blockade on proliferation of EBV antigen-specific T cells after stimulation with PBMCs or TLR-3-DCs using different peptide concentrations. PBMCs (A) and TLR-3-DCs (B) were pulsed with FLR peptide in different dilutions and cocultured with NACs in the presence or absence of α-PD-1 and α-LAG-3 antibodies. The percentage of FLR tetramer-positive cells within the $CD8^+$ T cell population was determined by flow cytometry. Data for fold change to the condition without blocking antibody are shown for one representative donor.

A+D	$CD4^+$+DC^{+CEFT}	Ctrl	αHVEM	αCD244	αTIM3	αPD-1	αLAG-3	αPD-1+ αLAG-3
HD1	% divided	32.20	32.10	32.40	23.30	35.10		
HD2	% divided	10.80	10.60	10.40	11.00	13.30		
HD3	% divided	11.40	8.94	9.85	10.50	12.30		
HD4	% divided	13.60	15.10	13.90	12.10	15.30		
HD5	% divided	33.10					38.90	
HD6	% divided	16.90				17.20	37.20	
HD7	% divided	10.00				12.40	21.80	
HD8	% divided	21.70	3.30			21.40	50.10	49.80
HD9	% divided	42.70	13.50			44.70	54.20	51.60
HD10	% divided	15.70				39.00	37.50	47.10
HD11	% divided	30.10				34.10	35.70	38.00
HD12	% divided	50.00				51.00	59.80	70.80
HD13	% divided	37.00				36.50	42.80	43.10
B+E	$CD8^+$+DC^{+CEFT}	Ctrl	αHVEM	αCD244	αTIM3	αPD-1	αLAG-3	αPD-1+ αLAG-3
HD15	% divided	29.60	29.50	31.10	19.60	32.10		
HD16	% divided	11.50	10.90	11.80	12.00	14.10		
HD17	% divided	9.33	8.26	9.81	9.30	11.80		
HD18	% divided	12.70	11.80	13.40	13.20	15.10		
HD19	% divided	35.10					43.70	
HD20	% divided	10.30				12.00	22.80	
HD21	% divided	10.90				13.80	24.20	
HD22	% divided	14.60	2.99			16.30	36.10	53.30
HD23	% divided	39.00	31.60			39.40	61.00	60.90
HD24	% divided	8.82				11.60	22.80	24.90
HD25	% divided	19.50				19.80	24.60	25.10
HD26	% divided	41.60				39.00	44.10	46.70
HD27	% divided	37.90				38.50	43.60	41.90
C+F	$CD3^+$ +DC+CEFT IFNγ	Ctrl	αHVEM	αCD244	αTIM3	αPD-1	αLAG-3	αPD-1+ αLAG-3
HD29	pg/mL	398.63	387.30	715.76	402.48	964.74		
HD30	pg/mL	1381.55	541.91	1229.15	1523.45	9228.35		
HD31	pg/mL	194.64	123.56	142.63	138.33	256.26		
HD32	pg/mL	313.29	172.33	268.29	177.56	483.00		
HD33	pg/mL	851.05	304.34	516.54	946.29	787.98		
HD34	pg/mL	3485.22				4411.02		
HD35	pg/mL	594.75					2846.31	
HD36	pg/mL	378.06				620.73	3666.30	
HD37	pg/mL	8291.48				28491.01	271494.80	
HD38	pg/mL	2009.66				2733.42	5047.71	5513.86
HD39	pg/mL	15948.00				10193.00	27906.00	39155.00
HD40	pg/mL	937.11				1370.81	9714.00	12249.00
HD41	pg/mL	62772.98				69980.57	793222.10	940013.10
HD42	pg/mL	704.90	777.35			1340.94	3522.41	3609.71
HD43	pg/mL	1922.88	2321.78			3813.53	5553.99	5483.80
HD44	pg/mL	989.40				958.00	1593.60	2145.30
HD45	pg/mL	1121.40				2089.00	1837.00	2488.20

Table S1. Effect of immune checkpoint blockade on proliferation and IFN-γ secretion of T cells after stimulation with TLR-3-DCs. Original data on which Figure 3 is based.

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