Dissertation zur Erlangung des Doktorgrades

der Fakultät für Chemie und Pharmazie

der Ludwig-Maximilians-Universität München

Novel multifunctional antibody constructs combining antigen and adjuvant delivery to dendritic cells as a therapeutic vaccine

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aus

Karlstadt, Deutschland

2020

Erklärung

Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Herrn Prof. Dr. Karl-Peter Hopfner betreut.

Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

München, den 10.08.2020

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Dissertation eingereicht am 14.08.2020

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Mündliche Prüfung am 23.10.2020

This thesis has been prepared from April 2016 to August 2020 in the laboratory of Professor Dr. Karl-Peter Hopfner at the Gene Center of the Ludwig-Maximilians-Universität München.

Parts of this thesis are submitted for publication:

Schmitt S, Tahk S, Lohner A, Hänel G, Maiser A, Hauke M, Lubna P, Rothe M, Josenhans C, Leonhardt H, Griffioen M, Deiser K, Fenn NC, Hopfner KP*, Subklewe M*. Fusion of bacterial flagellin to a dendritic cell-targeting αCD40 antibody construct coupled with viral or leukemia-specific antigens enhances dendritic cell maturation and activates peptide-responsive T cells. Frontiers in Immunology, provisionally accepted on 19.10.2020

Parts of this thesis have been and will be presented at retreats or international conferences:

Schmitt S, Fenn N, Deiser K, Subklewe M, Hopfner KP. Multispecific antibody derivatives to enhance dendritic cell-mediated antitumor response
i-Target Begutachtung, 13.12.2016 in Munich, Germany

Herrmann M, Schmitt S, Deiser K, Fenn N, Subklewe M, Hopfner KP. Novel multispecific antibody derivatives for T cell-mediated cancer immunotherapy
Gene Center Retreat, 26.-28.06.2017 on the island Fraueninsel at Lake Chiemsee, Germany

Schmitt S, Lohner A, Deiser K, Rothe M, Augsberger C, Fenn N, Griffioen M, Hopfner KP, Subklewe M. Multifunctional antibody construct for *in vivo* targeting of dendritic cells as a therapeutic vaccination strategy in AML
17th CIMT Annual Meeting, 21.-23.5.2019 in Mainz, Germany

Schmitt S, Lohner A, Deiser K, Maiser A, Augsberger C, Moosmann A, Leonhardt H, Fenn N, Griffioen M, Hopfner KP, Subklewe M. Multifunctional antibody construct for *in vivo* targeting of dendritic cells as a therapeutic vaccination strategy in AML
7th Immunotherapy of Cancer Conference (ITOC), 2.-3.10.2020 in Munich, Germany

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Summary

Conventional *ex vivo* dendritic cell (DC) vaccine strategies for the treatment of cancer are shown to be safe and to expand peptide-specific T cells in some patients. However, the feasibility and clinical efficacy are hampered by a laborious Good Manufacturing Practice (GMP) production and biological issues such as an impaired DC migration capacity. Targeting tumor-derived peptides to DC-associated endocytic receptors *in vivo* by antibody–antigen conjugates therefore represents a promising alternative. To induce a strong and sustained antigen-specific T cell response rather than tolerance, DC-targeting antibodies are commonly co-administered with toll-like receptor (TLR) agonists as adjuvants. In this context, peptide- or antibody-coupled TLR agonists have shown a higher vaccination efficacy and specificity compared to soluble adjuvants. To combine the advantages of *in vivo* DC vaccines with those of conjugated TLR agonists, we generated novel multifunctional antibody constructs (MACs) that simultaneously deliver antigens and TLR-activating adjuvants to DCs in a single molecule.

The engineered MACs consist of an αCD40 single-chain variable fragment (scFv) or an Fc-silenced αCD40 IgG1 antibody fused to a CMV pp65-derived antigen including the immunodominant NLVPMVATV (CMV_{NLV}) epitope for proof-of-principle studies. We showed that the αCD40 scFv bound agonistically to CD40 and highly upregulated maturation markers on monocyte-derived DCs (moDCs). The αCD40 IgG1, in contrast, exhibited low intrinsic agonistic activity. Upon binding, moDCs internalized αCD40 scFv and IgG1 antibodies into early endosomal compartments. As shown by DC–T cell co-cultures, moDCs incubated with CMV-coupled MACs cross-presented the processed CMV_{NLV} peptide on the surface and induced a peptide-specific T cell activation and proliferation. A higher T cell response was obtained for the scFv format. In a next step, different TLR agonists were conjugated to MACs to compare their ability to stimulate DCs. The TLR4 agonist LPS and the TLR5 ligand flagellin further enhanced the DC maturation elicited by αCD40 scFv, but unexpectedly, TLR ligation did not affect or even diminished the T cell response. The TLR8 agonist ssRNA40 in combination with αCD40 scFv led to a minor upregulation of DC maturation markers due to an insufficient uptake of the RNA, however, T cell proliferation was augmented. Notably, the combination of αCD40 IgG1 and flagellin in a single MAC molecule (αCD40.Flg^{CMV}) matured DCs by specifically interacting with TLR5 and enhanced cross-presentation as well as the CMV_{NLV}-specific T cell responses. Based on these results, αCD40.Flg^{CMV} was classified as the most promising molecule to be further characterized.

Finally, we could successfully confirm the functionality of the novel MAC format in a tumor setting by delivering the acute myeloid leukemia (AML)-specific mutated nucleophosmin (mNPM1)-derived neoantigen CLAVEEVSL ($mNPM1_{CLA}$) to DCs. This is the first study to investigate mNPM1 in a DC vaccination context and to fuse a neoantigen to a DC-targeting antibody. DCs loaded with mNPM1-coupled αCD40 scFv and αCD40 IgG1 were able to induce efficient $mNPM1_{CLA}$-directed T cell responses. Again, fusing flagellin to αCD40 IgG1 revealed a beneficial effect on DC maturation and cross-presentation, which demonstrates a high therapeutic potential for αCD40.Flg^{mNPM1}.

The fusion of flagellin to an antigen-conjugated αCD40 IgG1 was identified as the most promising strategy to co-deliver a peptide and a TLR-stimulating signal to DCs that activates DCs and thereby boosts peptide-specific T cell responses. The herein described data prove the functionality of flagellin fusion molecules *in vitro*. They lay the foundation for the therapeutically relevant αCD40.Flg^{mNPM1} MAC to become a promising option for treating AML in the future, especially upon combination with other immunotherapeutic approaches such as adoptive T cell therapy or immune checkpoint blockade.

Zusammenfassung

Konventionelle *ex vivo* Vakzinierungsstrategien mit dendritischen Zellen (DZs) zur Behandlung von Krebs sind erwiesenermaßen sicher und konnten Peptid-spezifische T-Zellen in einigen Patienten expandieren. Jedoch sind die Durchführbarkeit und die klinische Wirksamkeit durch die aufwändige GMP-Produktion der Vakzine sowie durch biologische Probleme, wie die beeinträchtigte Migrationsfähigkeit der DZs, vermindert. Der *in vivo* Antigen-Transfer zu endozytischen Oberflächenrezeptoren auf DZs in Form von Antikörper-Antigen-Konjugaten stellt eine vielversprechende Alternative dar. Um eine starke und langanhaltende Antigen-spezifische T-Zell-Antwort auszulösen und T-Zell-Toleranz zu verhindern, werden DZ-bindende Antikörper für gewöhnlich mit Toll-like Rezeptor (TLR) Agonisten als Adjuvanzien verabreicht. In diesem Zusammenhang wurde bereits gezeigt, dass Peptid- oder Antikörper-gekoppelte TLR Agonisten Vorteile bezüglich der Vakzinierungseffizienz und -spezifität im Vergleich zu löslichen Adjuvanzien aufweisen. Um die potentiellen Vorteile der *in vivo* DZ-Vakzinierung mit denen der gekoppelten TLR Agonisten zu kombinieren, wurden in dieser Arbeit multifunktionelle Antikörperkonstrukte (multifunctional antibody constructs, MACs) generiert, die den zielgerichteten Transport von Antigenen und TLR-aktivierenden Adjuvanzien zu DZs in einem Molekül kombinieren.

Die Antikörperkonstrukte bestehen aus einem αCD40 single-chain variable fragment (scFv) oder einem αCD40 IgG1 mit mutiertem Fc-Teil, an die ein CMV pp65-spezifisches Antigen fusioniert ist, das das immundominante NLVPMVATV (CMV_{NLV}) Epitop enthält. Wir konnten zeigen, dass der αCD40 scFv agonistisch an CD40 band und in hohem Maße Maturierungsmarker auf Monozyten-abgeleiteten DZs (moDZs) hochregulierte. Im Vergleich dazu wies der αCD40 IgG1 nur eine geringe intrinsische agonistische Aktivität auf. Nach Bindung internalisierten moDZs die αCD40 scFv und IgG1 Antikörper in frühe Endosomen. In DZ/T-Zell-Kokulturen wurde gezeigt, dass moDZs, die mit den αCD40 Konstrukten beladen wurden, das prozessierte CMV_{NLV} Peptid an der Oberfläche kreuzpräsentierten und eine Peptid-spezifische T-Zell-Aktivierung und -Proliferation induzierten, wobei das scFv Format eine höhere Antwort auslöste. Anschließend wurden verschiedene TLR Agonisten an die Antikörper gekoppelt und anhand ihres stimulatorischen Potentials verglichen. Der TLR4 Agonist LPS und der TLR5 Ligand Flagellin verstärkten die durch αCD40 scFv hervorgerufene DZ-Maturierung, aber unerwarteterweise beeinflusste die TLR Ligation die T-Zell-Antwort nicht oder reduzierte sie sogar. Die Kopplung von ssRNA40 an αCD40 scFv führte nur zu einer geringen Hochregulation von Maturierungsmarkern aufgrund von einer unzureichenden Aufnahme der RNA, jedoch erhöhte die ssRNA40 die Aktivierung und Proliferation CMV_{NLV}-spezifischer T-Zellen. Bemerkenswerterweise konnte die Fusion von Flagellin an αCD40 IgG1 (αCD40.Flg^{CMV}) sowohl die DZs durch eine spezifische Interaktion mit TLR5 maturieren, aber auch die Kreuzpräsentation von Peptiden und die CMV_{NLV}-spezifische T-Zell-Antwort verstärken. Folglich wurde dieses Molekül als das Vielversprechendste eingestuft, das weiter charakterisiert werden sollte.

In einem nächsten Schritt konnten wir die Funktionalität des Molekülformats auch im Tumorsetting bestätigen, indem wir die virale Antigendomäne gegen ein Akute Myeloische Leukämie (AML)-spezifisches Neoantigen CLAVEEVSL ($mNPM1_{CLA}$) austauschten, das vom mutierten Nukleophosmin (mNPM1) Protein abstammt. Dies ist die erste Studie, die den Einsatz von mNPM1 im Kontext einer DZ-Vakzinierung beschreibt und ein Neoantigen an einen DZ-bindenden Antikörper fusioniert. DZs, die mit mNPM1-gekoppeltem αCD40 scFv und IgG1 beladen wurden, konnten eine effiziente $mNPM1_{CLA}$-spezifische T-Zell-Antwort auslösen. Die Fusion von Flagellin an αCD40^{mNPM1} zeigte abermals einen vorteilhaften Effekt auf DZ-Maturierung und Peptid-Kreuzpräsentation, welcher für ein hohes therapeutisches Potential von αCD40.Flg^{mNPM1} spricht.

In dieser Arbeit wurde die Fusion von Flagellin an einen Antigen-gekoppelten αCD40 IgG1 Antikörper als die aussichtsvollste Methode identifiziert, um sowohl ein Peptid als auch ein TLR-stimulierendes Signal zu DZs zu transferieren und effiziente Peptid-spezifische T-Zell-Antworten zu induzieren. Diese initialen Experimente weisen die Funktionalität von Flagellin-Fusionsmolekülen *in vitro* nach. Sie legen außerdem den Grundstein für das therapeutisch relevante αCD40.Flg^{mNPM1} Molekül, um in der Zukunft eine Behandlungsstrategie der AML zu werden, vor allem in Kombination mit weiteren Immuntherapien wie dem adoptiven T-Zell-Transfer oder der Immuncheckpoint Blockade.

1. Introduction

1.1. Dendritic cells

1.1.1. DC biology

Dendritic cells (DCs) are antigen-presenting cells (APCs) that are characterized by a unique capacity to orchestrate T cell-mediated immune responses. The first subtype of cells with a dendritic phenotype described were Langerhans Cells (LCs) in the skin in 1868, named after his explorer Paul Langerhans.[1] At that time, the distinct function of those cells was not even known. More than one century later, Ralph Steinman and Zanvil A. Cohn shed light upon the cell function by discovering the antigen presentation by DCs.[2] Steinman's later finding that DCs play a crucial rule in the immune system by bridging innate and adaptive immune responses changed the field of immunology.[3, 4] It finally connected the work of Paul Ehrlich and Ilya Metchnikoff from the early 20th century, which was even honored with the Nobel Prize in Physiology or Medicine in 2011.[5]

DCs originate from $CD34^+$ hematopoietic stem cells (HSCs) in the bone marrow and arise from the lympho-myeloid hematopoiesis. They are a heterogeneous population of different subtypes that differ in their function, development as well as their regulation of T cell function.[6] Under steady state, the generation of most DC subsets is controlled by the cytokine Fms-like tyrosine kinase 3 ligand (Flt3L). In addition, during inflammation and infection, granulocyte-macrophage colony-stimulating factor (GM-CSF) mobilizes and stimulates the production of monocyte-derived DCs.[7] In the classical DC life cycle (Figure 1), DCs circulate as precursors through the blood stream. Immature DCs are specialized in antigen capturing, processing and presentation and reside in the peripheral tissues at sites of potential antigen entry, for example in the skin and internal or mucosal surfaces. Once they are activated by inflammatory and pathogen-derived signals (pathogen-associated molecular patterns (PAMPs)) via patter recognition receptors (PRRs), they migrate into the draining lymph nodes or lymphoid tissues. There they get in contact with naïve T cells that are in search for their cognate antigen, but have never been exposed to antigen before. Productive activation of naïve T cells by DCs results in their clonal expansion and differentiation into $CD4^+$ or $CD8^+$ effector and memory T cells, thereby activating effective T cell-specific immune responses.[6-8]

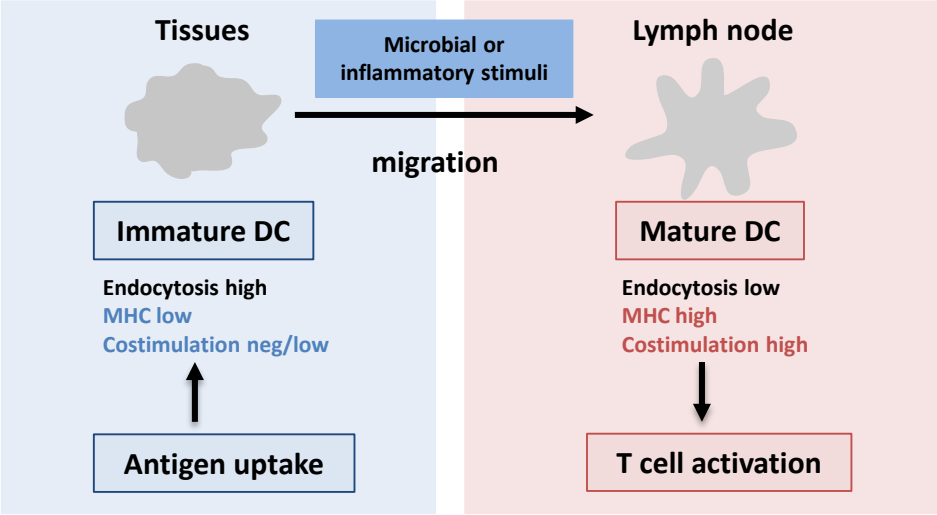

Figure 1. Classical DC life cycle (adapted from Lutz et al.).[9]

1.1.2. DC subtypes

DCs represent just a small population of leukocytes, but they are comprised of various subsets, each of them with an individual combination of surface markers, functions, origin and location (Figure 2). It is commonly accepted that DCs are divided into three major subsets that are derived from a common precursor: plasmocytoid DCs (pDCs), myeloid/conventional DC1 (cDC1) and myeloid/conventional DC2 (cDC2).[10] Each group develops under the control of different levels of the key transcription factors, most prominently of interferon regulatory factors 4 and 8 (IRF4 and IRF8).[11] Besides the aforementioned major subsets, there are other parts of the DC family that are derived from different precursors and share similarities with macrophages or monocytes, respectively.[10, 11] Among those are LCs, a unique population of mononuclear phagocytes restricted to the epidermal skin layer, and monocyte-derived DCs (moDCs) that differentiate from monocytes especially during inflammation.[1, 12] The different DC subtypes will be further characterized in the following sections.

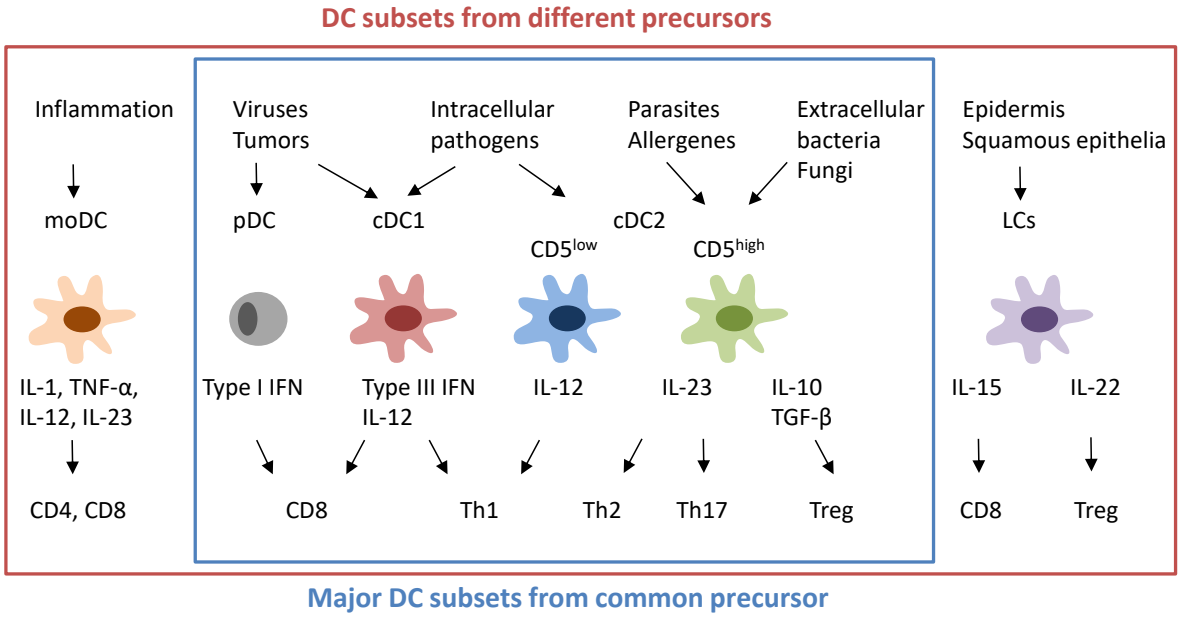

Figure 2. Major subsets of DCs (adapted from Collin et al. and Lutz et al.).[12, 13]

In humans, cDC1 are marked by cluster of differentiation (CD)141 expression and cDC2 by CD1c expression.[14, 15] cDC1 efficiently cross-present antigens to $CD8^+$ T cells and produce high levels of interleukin (IL-)12p70, thus promoting cytotoxic T cells and T helper (Th) 1 cells.[16-19] In contrast, cDC2 support Th1, Th2 and Th17 polarization by being superior in presenting antigens on MHC class II.[17, 20] However, appropriately activated cDC2 cells are also able to secrete high amounts of IL-12 and recent studies suggested that both cross-presentation to $CD8^+$ T cells and Th1 activation are less restricted to the cDC1 lineage in humans compared to what is known in mice.[19, 21]

pDCs participate in the first line defense against viral infections. They act as innate effector cells, which initiate type I interferon (IFN)-induced antiviral responses in adjacent cells and recruit cytotoxic natural killer (NK) cells.[22] Resting and non-stimulated pDCs are weak APCs and do not prime naïve T cells in contrast to cDCs. However, they can acquire the capacity to present antigens after activation. This also confers pDCs the ability to generate efficient effector $CD8^+$ T cell responses against exogenous antigens.[23] In addition, by producing cytokines and chemokines pDCs modulate T cell responses elicited by cDCs.[22]

There has been the discussion in the field whether LCs and moDCs are considered to belong to the DC family or not. They exhibit a distinct gene expression profile that is more similar to that of macrophages or monocytes rather than DCs.[13, 24] However, both also share common features with cDCs. The epidermally resident LCs migrate to skin-draining lymph nodes, have a high functional cross-presentation capacity and MHC class I-related gene expression and a low CD11c expression, what they have in common with cDC1 cells.[25] However, they also share a variety of surface markers, such as FcεR1 and CD39, which cDC2.[26] MoDCs, also termed "inflammatory DCs", are induced *in vivo* under inflammatory and infectious conditions that recruit monocytes into tissues. There, they develop into DCs initiating T cell priming in the draining lymph nodes.[11, 14] MoDCs can be also differentiated from monocytes *in vitro* in the presence of GM-CSF and IL-4.[27, 28] These artificially generated moDCs could serve as a tool for therapeutic strategies and to study DC biology as they resemble a cDC-like cell type *in vitro*. Both *in vivo* naturally occurring and *in vitro* generated moDCs rely on factors, such as GM-CSF, M-CSF and IL-37, however, their precise roles are not fully understood.[13]

1.1.3. Antigen processing and presentation

In general, DCs in the periphery can present antigens on major histocompatibility complex (MHC) class II or I leading to the activation of $CD4^+$ or $CD8^+$ T cells, respectively. For the processing and presentation of endogenous and exogenous antigens, different mechanisms have been described. In DCs, cytosolic self- or virus-derived endogenous antigens are degraded by proteasome degradation into smaller peptides and transferred to the endoplasmatic reticulum (ER) by transporters associated with antigen processing (TAP). Subsequently, peptides are loaded within the ER on MHC class I molecules, which are then rapidly transferred through the Golgi apparatus to the plasma membrane.[29, 30] There, peptide-MHC I complexes interact with $CD8^+$ T cells, thereby allowing the immune system to identify and eliminate virally infected or transformed cancer cells that either display modified self or foreign proteins.[31] MHC II molecules, in contrast, associate with peptides derived from exogenous antigens that come from pathogens or abnormal cells. DCs constitutively scan peripheral areas for those cells or particles that are internalized by clathrin-mediated endocytosis, phagocytosis, micropinocytosis or trogocytosis into the endosomes and lysosomes.[32] In this compartments, exogenous antigens are processed into peptide fragments and loaded on MHC class II receptors. After transport to the surface, peptides bound to MHC II molecules are presented to $CD4^+$ T cells.[30, 33]

In addition to the classical MHC class I and II pathway, DCs also exhibit the special feature to present exogenous proteins on their surface on MHC I molecules, a process called cross-presentation.[34] Two major pathways of antigen cross-presentation can be distinguished (Figure 3). In the vacuolar pathway, antigen processing, degradation by lysosomal proteases and loading on MHC I molecules occurs within the endo/lysosomal compartment.[35] In the endosome-to-cytosol pathway, internalized antigens need to be transported from the endosomal compartment into the cytosol, where they are degraded by the proteasome.[36] Afterwards, derived peptides are shuttled by TAP transporters into the ER or back into the antigen-containing endosomes, where they are loaded into MHC molecules and transported to the plasma membrane.[37, 38] Subsequently, interaction with antigen-specific T cells takes place.

Conclusively, this process of cross-presentation is particularly important not only *in vivo* during infectious and tumor conditions but also for therapeutic approaches, to transform naïve $CD8^+$ T cells into effector cytotoxic T lymphocytes (CTLs) and therefore eliminate harmful target cells.[39, 40] Moreover, the ability to cross-present is not a feature of specialized DC subsets, but is rather tuned by and dependent on several factors, such as DC location and DC maturation status.[41]

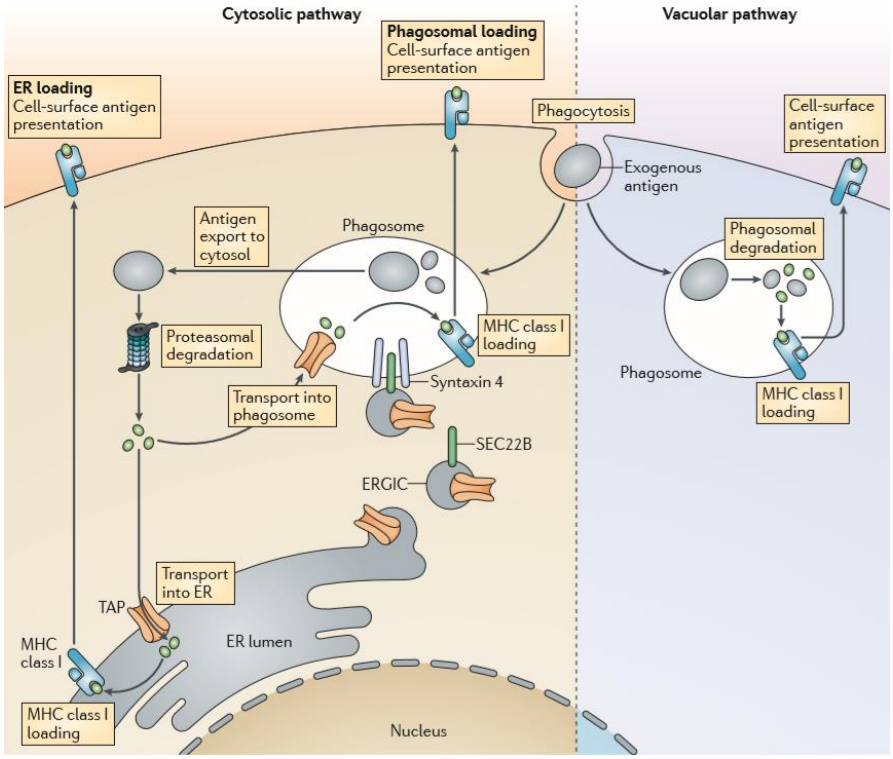

Figure 3. Pathways of peptide cross-presentation via MHC I.[31]

1.1.4. "Three-signal" hypothesis of T cell activation

The presentation of peptides on the DC surface on MHC I or II molecules as peptide–MHC complexes (p–MHC) is followed by interaction with specific T cell receptors (TCRs) on $CD8^+$ or $CD4^+$ T cells, respectively. If $CD8^+$ T cells are activated, they differentiate into CTLs that can give rise to both effector and memory cells.[42] $CD4^+$ T cells, also called Th cells, can either stimulate cytotoxic responses (Th1, Th17), antibody responses (Th2) or inhibit immune responses (regulatory T cells, Tregs).[43] The fate of differentiation will strongly depend on the type of cytokines and chemokines present during DC contact.

It is hypothesized that three signals are necessary to effectively activate T cells (Figure 4). The initial engagement via p-MHC and TCR, that forms the immunological synapse between DCs and T cells, confers "signal 1" of T cell activation, which is also commonly termed "T cell priming".[43] Triggering of the TCR alone is insufficient und usually leads to T cell anergy as indicated by a limited T cell expansion followed by unresponsiveness after reencountering antigen.[44, 45] To activate T cells, a second signal called "co-stimulation" is necessary that is delivered by the interaction of T cell surface receptors with their ligands on the DC plasma membrane. Two classes of co-stimulatory molecules exist on the surface of T cells. Those include the immunoglobulin (Ig) superfamily members, such as CD28, that interact with several members of the B7 family (CD80, CD86) on the DC.[46-48] The other class consists of members of the tumor necrosis factor (TNF) receptor superfamily (CD27, CD40) that bind to membrane-attached proteins of the TNF superfamily.[46, 49] Especially the engagement of CD28 is important, as it alters the threshold level of TLR ligation required for activation, reduces the time needed to stimulate naïve T cells and enhances the magnitude of T cell responses.[6] Signal 1 and 2 may already activate T cells. However, an additional signal has to be provided which will polarise T cell differentiation. Therefore, "signal 3" is delivered by DCs via the production of pro- or anti-inflammatory cytokines to regulate and control the type and quality of triggered T cell responses. One

well-studied third signal agent is IL-12p70 that is involved in the induction of Th1 and CTLs. Both are essential for efficient pathogen, but also tumor rejection.[50, 51] Type I interferons (IFN) contribute similarly to Th1 and CTL expansion and differentiation *in vitro* and *in vivo*.[52, 53] In addition, the secretion of IL-4 drives Th2 differentiation and the combination of IL-6 and TGF-β together with IL-23 promote Th17 responses.[54, 55] Of note is that also NK cells and B cells respond to DC-derived cytokines, especially IL-12p70, indirectly promoting adaptive immunity.[56, 57]

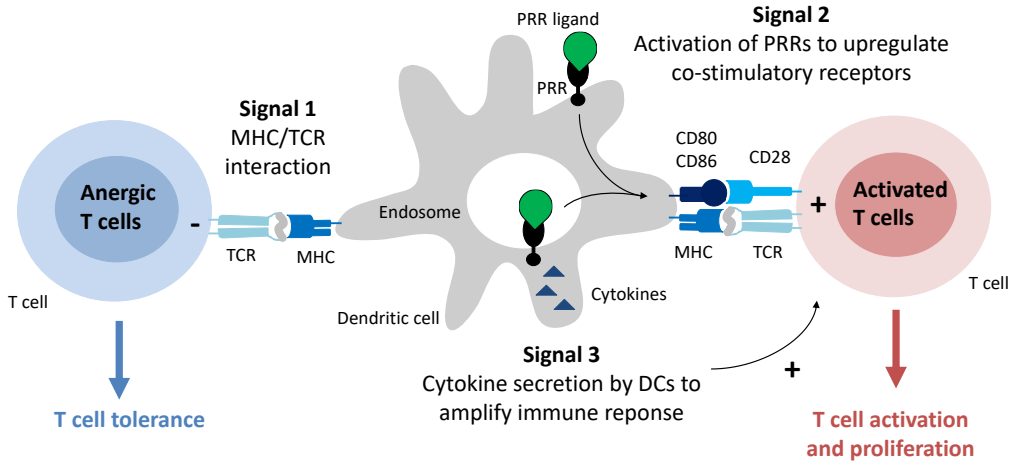

Figure 4. "Three-signal hypothesis" of T cell activation.

Signal 2 and 3 are delivered by the maturation of DCs. The latter is stimulated by inflammatory and pathogen-derived signals, more exactly the PAMPs recognized via PRRs. Upon activation, immature DCs are matured resulting in the expression of co-stimulatory molecules, such as CD80, CD83, CD86 and CD40, secretion of cytokines and migration to lymphoid organs where DCs can interact with antigen-specific T cells.[58] As DCs also process and present autoantigens, the contribution of signal 2 and 3 is particularly important for the overall immune response to prevent autoimmunity and to establish immunological tolerance in the absence of a pathogen-derived signal.[43] In this context, not only the absence of positive co-stimulation triggered by DC maturation, but also the presence of negative co-stimulation and the secretion of inhibitory cytokines might alter the immune response. Exemplarily, CD80/CD86 can engage inhibitory receptors on T cells such as cytotoxic T-lymphocyte antigen 4 (CTLA4) leading to anergic T cells or Tregs.[59]

Collectively, the complex interplay of activating and inhibiting factors determines the fate of a T cell response after encountering an antigen-presenting DC.

1.2. DC-based cancer vaccines

1.2.1. General concept

Reprogramming of the immune system against cancer by immunotherapeutic approaches has shown clinical promise in recent decades. In this context, therapeutic cancer vaccines are developed including virus vector vaccines, molecular vaccines comprised of peptide, DNA or RNA vaccines, and cellular vaccines.[60] Among those, DC-based cancer vaccines are intensely investigated. DCs represent a powerful tool for cancer immunotherapy due to their high capacity to take up and present antigens, for example through cross-presentation. Besides direct antigen presentation, additional DC-intrinsic

properties are relevant for immunotherapy, such as the capacity for migration between lymphoid and non-lymphoid tissues as well as the modulation of cytokine and chemokine gradients to control inflammation and lymphocyte homing. All those factors are presumably important to promote systemic and long-lasting antitumor effects.[61]

In many tumors, immune responses are ineffective as the tumor lacks immunogenicity and often creates an immunosuppressive environment.[62, 63] The tumor microenvironment promotes exhaustion of $CD8^+$ T cells directly or hampers the recruitment of cDC1 cells, thereby inhibiting priming and accumulation of tumor-infiltrating T cells.[64, 65] DC vaccines aim to reverse the ineffective immune responses and the ignorance of the immune system to malignant cells. To achieve this, DCs are stimulated with danger signals and loaded with tumor-specific antigens on MHC molecules. By activating antigen-specific T cells, antigen-bearing cancer cells are supposed to be selectively eliminated.[66] Ideally, therapeutic vaccines should both prime naïve antigen-specific $CD8^+$ T cells to generate CTLs, but also modulate existing memory T cells and induce the transition from chronically activated non-protective $CD8^+$ T cells to healthy $CD8^+$ T cells. These, in turn, generate CTLs that reject cancer but also provide long-lived memory $CD8^+$ T cells to prevent relapse.[67] In addition, the activation of $CD4^+$ T cells by the vaccine has shown therapeutic benefits by producing cytokines to support $CD8^+$ T cell proliferation and differentiation.[68]

Thus, the prerequisites of all DC-based cancer vaccines include a large source of DCs that exhibit a high cross-presenting capacity and are sufficiently matured to induce an efficient and sustained T cell response. Two different vaccination strategies have been developed and already implemented as part of clinical trials. In one approach, DCs are generated and loaded with the desired antigen *ex vivo* followed by re-infusion into the patient. Alternatively, antigens are targeted to DCs *in vivo* by fusing or complexing it with antibodies or other DC-targeting vectors.[69]

1.2.2. Target selection and antigen source

The selection of an appropriate target represents one of the most important steps towards vaccine development. Targets for tumor vaccines can be divided into two classes: tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) (Figure 5).[60]

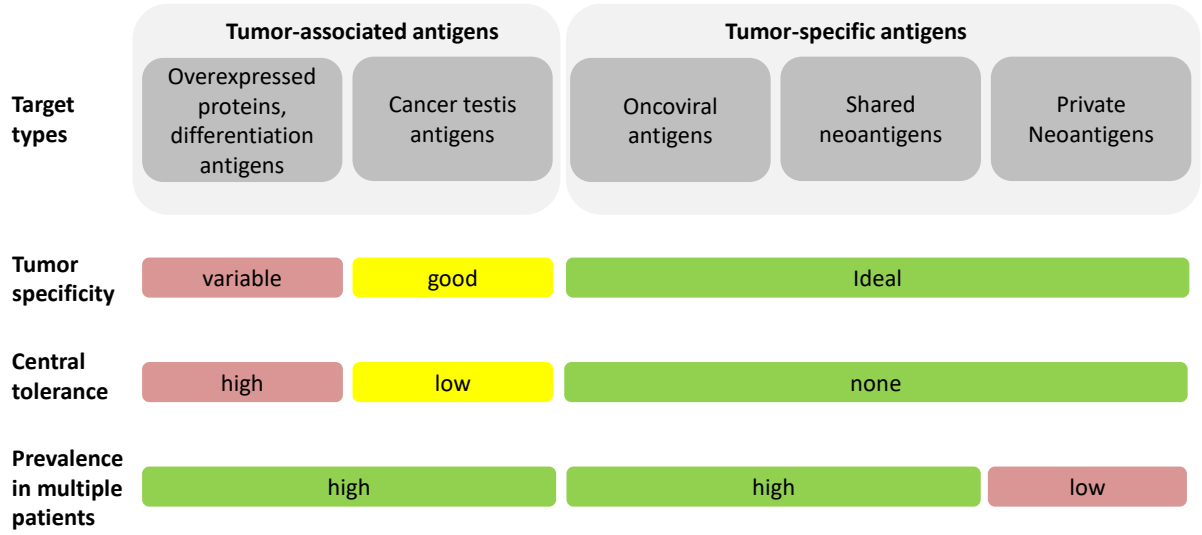

Figure 5. Tumor-associated vs. tumor-specific antigens (adapted from Hollingsworth et al.).[60]

To date, most cancer vaccines target TAAs. These are self-antigens that are either preferentially or abnormally expressed on tumor cells, but may be present at some level also on normal cells. T cells that bind with high affinity to TAAs are typically deleted from the immune repertoire by central and peripheral tolerance mechanisms. Thus, a cancer vaccine targeting these antigens must be potent enough to "break tolerance" and to stimulate the low affinity or rare TAA-reactive T cells that haven't been eliminated.[70] This seems to be difficult to achieve, as several clinical trials targeting TAAs have shown that long-term therapeutic effects are often lacking.[71] As a second challenge, expression of TAAs on normal cells may lead to on-target off-tumor toxicity. This hasn't been described for cancer vaccines so far due to lacking potency, but toxicities are a common observation for other TAA-targeted therapies.[60]

In contrast to TAAs, TSAs are truly tumor-specific and are recognized by the immune system as foreign. As such, high-affinity T cells may be present and strongly activated by these antigens. TSAs can be either antigens expressed by oncoviruses, for example human papilloma virus (HPV) responsible for cervical cancer, or antigens encoded by tumor-specific mutations, the so called neoantigens. Oncoviral antigens are commonly expressed in many patients and so are some neoantigens that are derived from hotspot mutations occurring in multiple cancer patients. However, the majority of neoantigens are unique to individual patient's tumor (private neoantigens) and require the generation of personalized therapy.[60] Early clinical trial results testing personalized neoantigen vaccines, for example in melanoma patients, are very promising and state reasons to further develop these therapies.[72-76] The generation of "off-the-shelf" vaccines employing neoantigens that are shared between patients is still in the early stages. As those neoantigens are quite rare, clinical results are missing even if a high therapeutic potential can be expected.[77, 78]

The DC vaccine can consist of either a single MHC I-restricted epitope as target source or several epitopes.[79] Loading of DCs with epitopes derived from multiple antigenic proteins is suggested to be beneficial over single antigen vaccination. This offers a better chance of recognition and less chance of escape by epitope mutations.[80, 81] In this context, also a combination of MHC class I and II epitopes, for example in so-called long synthetic peptides (LSPs), might be applied. Due to the significant contribution of $CD4^+$ T cells to the overall immune response, vaccination with LSPs has been shown to generate robust and long-term $CD4^+$ and $CD8^+$ T cell responses.[82] Selected antigen candidates are always specific for an individual human leukocyte antigen (HLA) type, which makes it hard to produce "off-the-shelf" vaccines. To overcome this problem, overlapping peptide libraries, tumor cell lysates or nucleic acids encoding tumor antigens can be used to cover all possible tumor epitopes without HLA restriction.[85, 86] Even if vaccination with multiple peptides and different epitopes has shown promising results, a lot of clinical trials investigate single antigen vaccination. These are also able to induce clinical responses and their efficacy can be enhanced by a phenomenon called "epitope spreading". As a vaccine that targets just one antigen can result in tumor cell lysis by specific T cells, new tumor antigens are released, thereby broadening and spreading the response from one to multiple antigens.[83, 84]

1.2.3. *Ex vivo* DC vaccination

The process of *ex vivo* DC vaccination involves the generation of patient-derived autologous DCs, that are matured with danger signals, loaded with selected candidate peptides and re-infused to induce a tumor-specific T cell response (Figure 6). Pioneering work of Inaba, Steinman and colleagues from 1992 ushered the era of *ex vivo* DC vaccines as they demonstrated that mouse DCs can be cultured *ex vivo* from bone marrow precursors.[85]

The generation of sufficient numbers of human DCs for vaccination purposes was one major challenge, as DCs comprise <1% of peripheral blood mononuclear cells (PBMCs).[11] Most commonly moDCs are used that can be generated *ex vivo* in large numbers from purified monocytes that were cultured in presence of GM-CSF and IL-4.[27] These were first used as a source for therapeutic DCs to treat lymphoma patients over two decades ago.[86] Recent work of Sharma and colleagues found out that monocytes cultured with GM-CSF, IL-6 and IFN-γ give rise to a newly described mo-cDC1 population that share even more similarities with cDC1s.[87] Additionally, cDCs and pDCs can be generated from $CD34^+$ hematopoietic stem cells using Flt3L.[81, 88] The phenotype, function and ability to induce T cell responses by *in vitro* generated DCs is highly dependent on the culture methods but also the cytokines and growth factors used for differentiation.[89] Even different techniques for monocyte isolation are shown to influence the antitumor immunogenicity and cytokine production of generated moDCs.[90, 91]

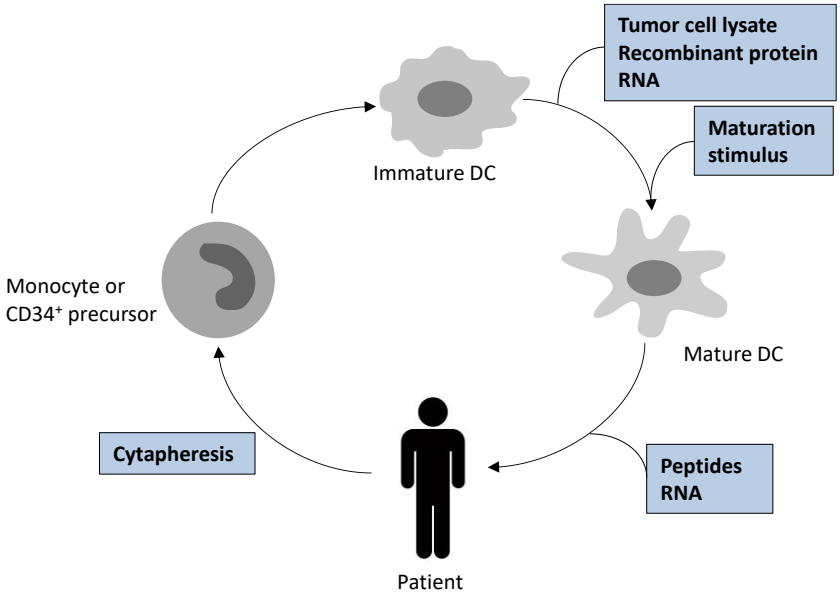

Figure 6. *Ex vivo* DC vaccination (adapted from Tacken et al.).[92]

The generated DC-like cells with an immature phenotype need to be matured prior to administration. When vaccinating in the absence of adjuvants as proper maturation factors, tolerance has been observed.[93, 94] Only DCs matured by danger signals are immunological competent, able to induce an efficient T cell response and exhibit a high migratory capacity towards draining lymph nodes. Various maturation cocktails have been used in the clinic to stimulate moDCs. These generally consist of different ligands for PRRs and cytokines, often in combination with co-stimulatory proteins such as CD40 ligand (CD40L or CD154).[61]

Antigen loading occurs at either the immature or mature DC stage. To load DCs with selected tumor antigens, different techniques are available. Antigens may be supplied by pulsing or electroporation with single peptides or tumor cell lysates, by transfection with an antigen-encoding RNA or DNA or by transduction using viral vectors that express tumor antigens.[95-100]

DCs also need to reach the lymph nodes *in vivo* in order to present antigen to cognate antigen-specific T cells and to induce an immune response. DC-trafficking to lymph nodes is not only dependent on the DC maturation state, but might be also influenced by the injection routes and strategy. Different vaccine administration routes were tested, ranging from intra-dermal (i.d.), intra-nodal (i.n.) to intra-

tumoral injection. However, the data didn't show clear a benefit of any administration route that was consistent among clinical trials. Therefore, the superior site of injection is still unknown.[11]

Numerous clinical trials have established the safety and efficiency of moDC vaccines.[101] However, this approach is quite laborious and expensive, as the DCs have to be generated for each patient individually. Therefore, protocols need to be optimized and accelerated. Most protocols for the generation of clinical-grade moDCs require 7 days of cell culture.[102] New methods have been developed to ensures a less time- and labor-intensive production of DCs within just 2-3 days. The resulting DCs had a mature phenotype and were equally potent in inducing antigen-specific T cell responses compared to DCs generated according to the long protocol.[103, 104] As an example, moDCs generated in this thesis for *in vitro* studies were based on a three-day protocol developed before. This starts with monocyte differentiation into immature DCs for 48 h with GM-CSF and IL-4 followed by DC maturation for 24 h with a TLR7/8 agonist-containing cytokine cocktail for the generation of Th1-polarizing DCs.[105, 106]

1.2.4. *In vivo* DC vaccination

More recently, *in vivo* loading of DCs is being exploited as an alternative approach to *ex vivo* DC vaccination as targeting DCs *in vivo* with antigens and adjuvants may simplify vaccination and produce more physiological DC maturation.[80, 92] The vaccine can be produced on a larger scale since it is applicable to a larger patient cohort. Steinman and his colleague Nussenzweig were the first to demonstrate the principle of targeting antigens to DCs *in vivo* through coupling of antigens to antibodies specific for DC surface receptors.[107-109] After binding its cognate receptor, the antibody is internalized into intracellular compartments, the attached peptide is processed and (cross-)presented on MHC I or II molecules to activate peptide-specific $CD4^+$ or $CD8^+$ T cells (Figure 7).

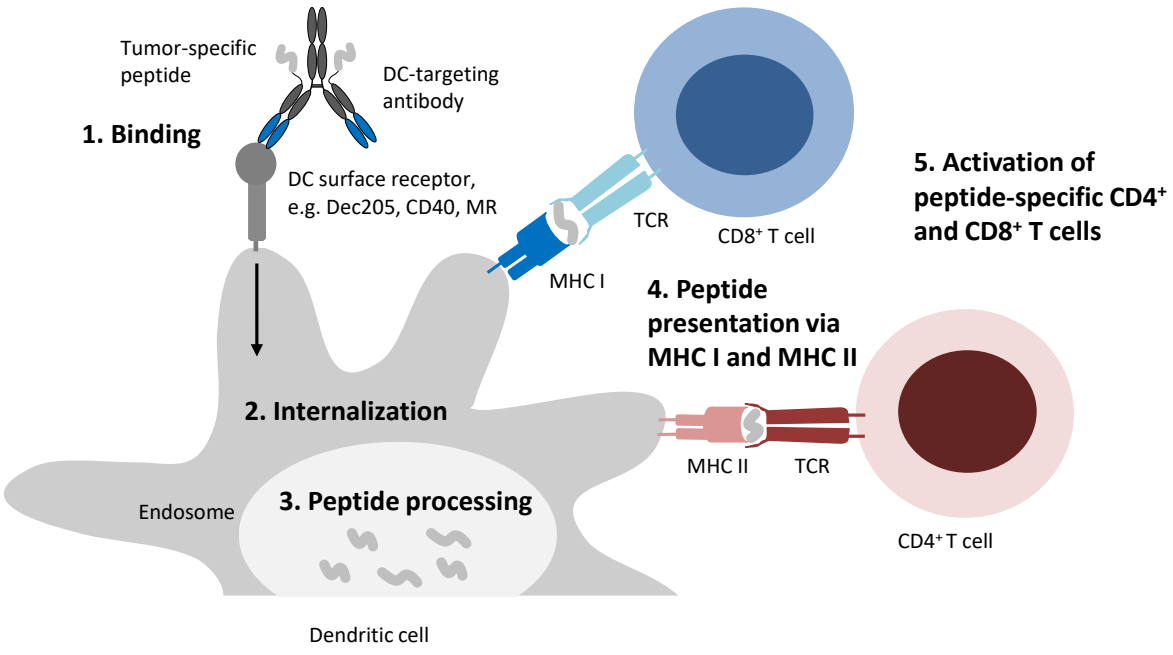

Figure 7. *In vivo* DC vaccination.

Highly investigated endocytic receptors are receptors of the C-type lectin receptor family (CLRs) that serve as PRRs on macrophages and DCs. These bind specifically to glycan structures derived from PAMPs via their carbohydrate recognition domains (CRD) or self-antigen released from dead cells.[110]

The most commonly used CLRs for antibody-targeted approaches are the mannose receptor (MR), 205 kD membrane protein (Dec205), Dectin 1 and 2, dendritic cell natural killer lectin group receptor-1 (DNGR-1 or Clec9A) and DC-specific ICAM-3 grabbing non-integrin (DC-SIGN).[111] Some CLRs serve as phagocytic receptors only, whereas other CLRs activate a signaling cascade via immune-receptor tyrosin-based activation motifs (ITAMs) to initiate immune responses.[112] Further CLRs contain immune-receptor tyrosine based inhibitory motifs (ITIMs) that inhibit cellular activation to prevent uncontrolled immune responses.[113] Hence, various CLRs differ in the ability to induce an immune response in addition to its endocytic function. In addition to targeting CLRs, antibodies against Fc receptors (FcR) and CD40 can also be used. The latter exhibits an exceptional position among DC receptors targeted for vaccination approaches.[111] CD40 not only serves as an endocytic receptor, but also exhibits intrinsic activating function after cross-linking of CD40 on the DC surface either by interacting with CD40L on activated T cells or by agonistic antibody constructs.[114]

All endocytic receptors have differential levels of expression, internalization patterns and downstream trafficking routes. The amount of internalized antigen and receptor expression level was shown not to correlate with the antigen presentation level in previous studies.[115] Exemplarily, Chatterjee et al. described that an anti-MR (αMR) antibody was more efficiently accumulated intracellularly than αCD40 or αDec205. However, CD40 was the best receptor to target to induce antigen cross-presentation, which was not mediated by αCD40-dependent DC activation. They suggested that rather its relatively poor uptake or intra-endosomal degradation in early endosomes is beneficial over a high uptake and trafficking to late endosomes as seen for MR- and Dec205-targeting.[116] The used antibody might also determine which DC subtype, but also which other cells to be targeted. Dec205 is expressed at high levels on moDCs and monocytes, at intermediate levels by B cells and at low levels on NK cells, T cells and pDCs.[117] CD40, in turn, is expressed not only on all DC subsets, B cells, monocytes and macrophages, but also by hematopoietic progenitors, fibroblasts, endothelial and epithelial cells.[118-120] As PRRs are also differentially expressed on DC subtypes and cellular compartments, the adjuvant and DC-targeting antibody should be selected well-orchestrated. This ensures that PRR ligation and antigen loading is found on the same DC and in the correct cellular compartments.[41]

DCs can be targeted by different antibody formats including an IgG1-based conventional antibody, Fab and F(ab')2 fragments or single-chain variable fragments (scFv) (Figure 8). The Fc domain of IgG1 antibodies commonly conjugates them to activating FcγR on immune effector cells. This triggers immune responses, such as antibody-dependent cellular cytotoxicity (ADCC), phagocytosis (ADCP) or complement-dependent cytotoxicity (CDC). Engagement of inhibitory FcγR dampens immune responses.[121] For DC-based vaccines, using scFv offers some advantages over the IgG format. Due to smaller size, scFv penetrate the tissue much better.[122] Since they lack an Fc domain, they do not bind to FcRs and hence do not induce unwanted effector functions and provide specific antigen delivery by reducing non-specific uptake of antigen by other effector cells.[123] As a drawback to antibody constructs with bigger size, scFv have a shorter half-life due to a fast renal clearance.[122] Therefore, silencing Fc-mediated functions by introducing PGLALA (P329G, L234A and L235A) mutations would represent an alternative approach to using Fab- or scFv-based molecules.[124]

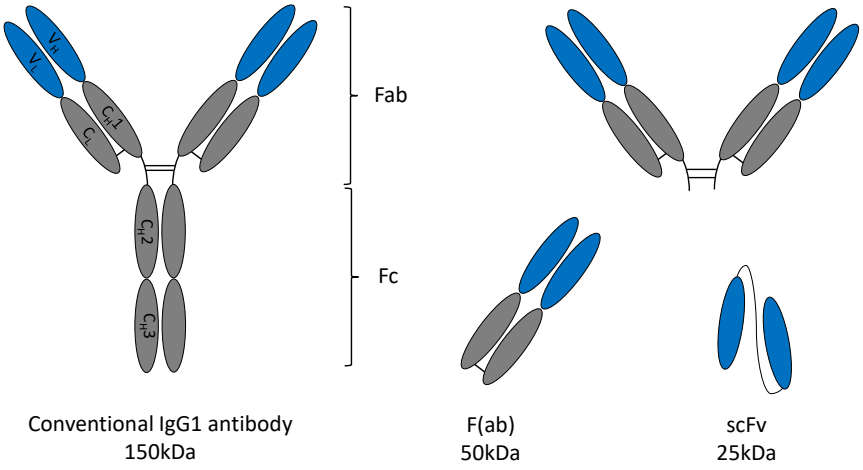

Figure 8. Different antibody formats to be used to target DCs *in vivo* (adapted from De Groof et al.).[125]

In accordance to the "three-signal" hypothesis of T cell activation and similar to *ex vivo* DC vaccination, also *in vivo* vaccines need co-stimulation and DC maturation besides antigen loading. Therefore, most of antibody–antigen conjugates are co-administered with adjuvants, which are commonly ligands for PRRs. This is particularly necessary for targeting for example Dec205, as in the absence of an adjuvant antigen-specific tolerance is induced that could serve as a treatment against autoimmune diseases.[108, 126] As agonistic αCD40 and some αCLR antibodies already elicit co-stimulatory functions, DC-targeting and adjuvant are combined in one molecule.[127, 128] Still, the addition of an additional maturating agent might show synergistic effects. In line with this, the combination of αCD40 with other PRR ligands further boosted the immune response and synergized to stimulate $CD8^+$ T cells in response to peptide and DC vaccines in previous studies.[129-131] Aside from the co-administration of the DC vaccine and the adjuvant, DC-activating factors can also be directly targeted to the antibody and the DC by genetic fusion or covalent coupling. This is expected to reduce off-target effects of soluble adjuvants by activating only DCs presenting the desired antigen.[132, 133] More importantly, it is also shown to enhance therapeutic efficacy.[133, 134]

Conclusively, the DC-targeting antibody in combination with the adjuvant selected for the *in vivo* DC vaccination approach determines the fate of loaded DCs and therefore the specificity and efficiency of the induced immune response. As pre-clinical evaluations showed promising outcomes and encouraged further optimization of *in vivo* DC vaccination strategies, the first clinical trials are ongoing.[135, 136]

1.3. Pattern recognition receptors

1.3.1. Pattern recognition receptor biology

The innate immune system serves as the body's first line of defense and is the major contributor to acute inflammation induced by microbial infection and tissue damage.[137, 138] Innate immunity is also important for the activation of acquired immunity. The innate immune system is essentially made up of barriers to repel pathogens, including skin, mucosa and secretions.[139] Immune effector cells are also involved that encompass phagocytic cells, epithelial and endothelial cells, NK cells, innate lymphoid cells and platelets. Phagocytic cells consist of granulocytes, monocytes, macrophages and DCs. The majority of this cells expresses PRRs on the cell surface that recognize not only PAMPs but also cellular

damage-associated molecular patterns (DAMPs) derived from the host cell. All PRRs share common characteristics, such as their constitutive expression in the host and the detection of pathogen regardless of their life-cycle stage. They are germline encoded, nonclonal, expressed on either all cells or a given type and independent of immunologic memory. Depending on the PRR subtype and the nature of PAMP recognized, distinct signaling pathways and antipathogenic responses are induced, but the basic machineries of PAMP recognition are highly conserved among species.[137]

PRRs include toll-like receptors (TLR), Nod-like receptors (NLR), CLRs, RIG-I-like receptors (RLR), AIM2-like receptors (ALR) and various DNA-recognizing receptors, such as cyclic GMP-AMP synthase (cGAS), DNA-dependent activator of IFN-regulatory factors (DAI), interferon-γ inducible protein 16 (IFI16), DEAD box polypeptide 41 (DDX41) and stimulator of interferon genes (STING).[137, 139-141] While TLRs and CLRs are transmembrane proteins found in the plasma membrane, RLR, ALR, NLR and DNA receptors are located in intracellular compartments, namely the endosome or cytosol.[142] PRRs use specific adapter proteins to recruit various protein kinases, ubiquitin ligases and transcription factors to the signaling complex. Subsequently, activated transcription factors translocate to the nucleus and induce transcriptions of genes encoding inflammatory cytokines, IFN, chemokines and antimicrobial proteins.[142, 143] The inflammatory response is orchestrated by proinflammatory cytokines such as TNF, IL-1 and IL-6. These are pleiotropic proteins that regulate cell death and apoptosis of inflammatory tissues and recruit immune cells to inflamed tissues thereby stimulating adaptive immunity.[142, 144]

In summary, PRRs play a key role in first-line defense by initiation of innate immunity. This makes them a promising target for therapeutic interventions to manipulate the immune system. PRR-targeting strategies can either consist of blocking approaches to reduce excessive signaling present for example in autoimmune disorders.[145] Alternatively, ligation of PRRs can be therapeutically used in the context of antitumor treatments, which were pioneered by Coley's development of sarcoma treatment with a mixture of bacterial toxins.[146] Due to their capacity to induce activation and maturation of phagocytes, PRR agonists are also used as adjuvants for DC vaccination.[147] However, as PRR stimulation has also been described to exhibit protumor effects by inducing chronic inflammation, the amount and duration of activation needs to be carefully titrated.[148]

1.3.2. Toll-like receptors

TLRs, like most of PRRs, are evolutionarily conserved from the worm *Caenorhabditis elegans* to mammals.[139, 149] They received their name from the similarity to the Toll protein that is essential for the development of embryonic dorsoventral polarity and for the antifungal response in Drosophila.[150-152] TLRs are type I transmembrane proteins that are characterized by varying numbers of leucine-rich-repeat (LRR)-containing ectodomains and an intracellular Toll/IL-1R homology (TIR) signaling domain.[153] The LRR domains are composed of various numbers of tandem LRR motifs, each of which is 24-29 amino acids in length and consists of a β strand and an α helix connected by loops. Ligands for TLRs are recognized either by TLR homodimers, heterodimers or by individual TLRs in combination with coreceptors.[154] TLR signaling is triggered by the ectodomain-mediated dimerization of TLRs and involves two distinct pathways (Figure 9): the myeloid differentiation factor 88 (MyD88)-dependent and TIR domain-containing adapter-inducing IFN-β (TRIF)-dependent pathway. Both signaling pathways lead to the activation of NF-κB and the MAPK pathway to induce inflammatory cytokines.[155] While MyD88 is utilized by all TLRs with the exception of TLR3, TLR3 and TLR4 utilize the TRIF-dependent pathway.[156, 157]

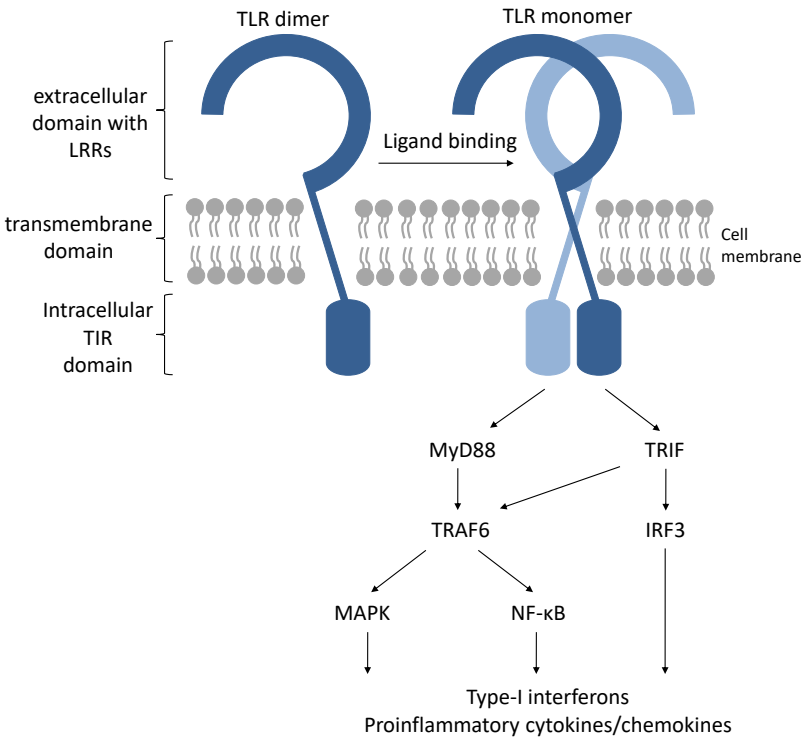

Figure 9. Simplified scheme of TLR engagement and signaling.

To date, thirteen TLR family members are described in mammals, TLR1-TLR10 are found in humans and TLR1-9 and TLR11-13 in mice.[154] These TLRs are expressed on various immune cells including macrophages, DCs, B cells, specific types of T cells and nonimmune cells such as fibroblasts and epithelial cells. Expression of TLRs is not static but rather modulated rapidly in response to pathogens and cytokines. They can be found both extra- and intracellularly and their cellular compartmentalization is modulated by transmembrane and membrane-proximal regions.
While certain TLRs (TLR1, 2, 4, 5 and 6) are expressed predominantly on the cell surface, others (TLRs 3, 7, 8 and 9) are present almost exclusively in intracellular compartments such as endosomes. This requires internalization of their ligands for signaling processes.[137]
All TLRs are activated by different ligands or PAMPs. Human TLRs are divided into three groups: those that recognize lipids or lipopeptides (TLR1, 2, 4 and 6), proteins (TLR5) and nucleic acids (TLR3, 7, 8 and 9).[158] TLR1 forms heterodimers with TLR2 (TLR1/2) and recognizes triacyl lipopeptides.[159] In concert with TLR1 and TLR6 it binds a wide variety of PAMPs including peptidoglycan and lipoproteins of Gram-positive bacteria, but also mycoplasma lipopeptides and fungal zymosan.[160] TLR4 is crucial for the detection of LPS, which is present in the cell wall of Gram-negative bacteria.[161] It can also be activated by structurally unrelated non-bacterial ligands, such as the plant diterpene paclitaxel or viral motifs from the respiratory syncytial virus.[137, 162] The highly related nucleic acid binding TLRs found in cytoplasmic compartments interact with DNA and RNA derived from viruses or bacteria. TLR3 recognizes double-stranded RNA (dsRNA), while TLR7 and 8 are responsive to the single-stranded RNA (ssRNA) found during viral replication.[163, 164] Unmethylated deoxycytidyl-phosphate-deoxyguanosine (CpG) motifs that are commonly present in bacterial and viral genomes are recognized by TLR9.[165] Flagellin, which is a principal component of the flagella of both Gram-positive and -negative bacteria, interacts with TLR5.[166]
TLR10 has also been found to homodimerize or heterodimerize with TLR1 and 2, however its ligand and functions are poorly understood.[167] Just recently, human immunodeficiency virus (HIV) gp41 protein was identified as a TLR10 ligand.[168]

1.3.3. Agonists of pattern recognition receptors as adjuvants

Adjuvants can augment the immune responses to vaccines through a variety of mechanisms, including deposition, increased uptake and stability of the antigen and the activation of the immune system. The latter is performed by agonists of PRRs.[169] Even if nearly all PRRs are potential targets for adjuvants, the main focus is put on TLR agonists given the recent success in ongoing phase I and II clinical trials. In those, TLR agonists are being used as an adjuvant for peptide vaccinations or in combination with radiotherapy.[170]

To date, three TLR ligands are FDA-approved. Bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, was initially described to act via TLR2/4 ligation, but was recently shown to also interact with TLR9.[171] It is mainly used as a vaccine against tuberculosis, but also for the immunotherapy of *in situ* bladder carcinoma.[172] Imiquimod, a small molecule TLR7 agonist, has been successfully used to treat human papilloma virus (HPV)-induced genital warts and certain skin cancers.[173] Its use as vaccine adjuvant, for example in melanoma treatment, is still under investigation.[174, 175] However, a recent clinical trial has demonstrated that pre-treatment with topical imiquimod significantly enhanced the immunogenicity of the intradermal trivalent influenza vaccine.[176] The TLR4 agonist monophosphoryl lipid A (MPL) is a chemically detoxified form of LPS derived from *Salmonella Minnesota*.[177] This is still under investigation as an adjuvant in clinical trials testing cancer vaccines, but it has been already approved as an adjuvant for a preventive vaccine against human papilloma virus (Cervarix™) and Hepatitis B for pre- and haemodialysis patients (Fendrix™).[178, 179]

Several other TLR agonists, that are not approved yet, are currently tested in pre-clinical and clinical settings. Numerous clinical trials explored the potential of using CpG oligodeoxynucleotides (ODNs) as immunoadjuvants for cancer vaccines. Even if vaccination using CpG7909 administered together with synthetic peptide antigens was able to expand antigen-specific $CD8^+$ T cells in several cancer entities, clinical outcomes were not convincing enough.[180, 181] The synthetic and stabilized analog of dsRNA poly(I:C) interacts with TLR3 and has been developed to mimic the response to RNA virus infection. Under the commercial name Hiltonol it has been used in combination with DC-based vaccines in glioblastoma and pancreatic cancer, which showed a good safety profile and disease stabilization in some patients.[182, 183] The TLR5 adjuvant entolimod is a pharmacologically optimized flagellin derivative of *Salmonella dublin* that retains the two constant regions (D0/D1) essential for TLR5 binding. In the first instance, studies have established entolimod as a potential treatment for lethal radiation exposure, for which it had an excellent safety profile even after systemic delivery.[184, 185] It demonstrated promising activity as an adjuvant and induced potent anti-viral immune responses in animal and clinical trials, when it was either fused to antigens or administered separately with vaccines.[186-188] As it has been shown to elicit direct antitumoral effects as well, a combination with DC vaccines in the cancer setting would be a logical consequence.[189-191] Due to the most recent pandemic outbreak of COVID-19, great hopes are placed on the mRNA-based vaccines, which are among others developed by the German biotech companies CureVac and BioNTech. The mRNA not only encodes for the antigen that is expressed by the host translation machinery and subsequently processed and presented. Importantly, it also elicits self-adjuvant effects, as the ssRNA is recognized by TLR7/8. Initially being developed as individualized cancer vaccines mostly encoding for neoantigens, their evaluation in first clinical trials showed encouraging results.[192] As this still represents a new approach, the efficacy of mRNA-based vaccines both in the treatment of diseases but also in the prevention of (viral) diseases has yet to be proven.

Other PRR agonists can also be used as adjuvants. The cytosolic PRR STING has attracted attention as another promising target for anticancer drug development. In a mouse model, the STING activator c-

di-AMP exerted superior adjuvant properties compared to poly(I:C)/CpG formulation when it was combined with a soluble protein vaccine or Dec205-mediated antigen targeting to DCs.[193] DC vaccination adjuvanted with the STING agonist 2´3´-cGAMP elicited protective antitumor and antiviral $CD8^+$ T cell responses both *in vitro* and *in vivo*.[194] Still, clinical translations were not successful so far and need further investigation.[195]

1.4. Acute myeloid leukemia

1.4.1. AML pathogenesis and therapy options

Acute myeloid leukemia (AML) is the most common form of acute leukemia with an incidence that increases with age. The disorder arises in a malignantly transformed multipotential hematopoietic stem cell that acquires successive genomic alterations leading to abnormal differentiation and proliferation properties.[196] The leukemic blasts expand clonally and accumulate in the bone marrow, blood and extramedullary tissues. There, they repress cells of normal hematopoiesis resulting in anemia, granulocytopenia and thrombocytopenia.[197, 198] AML is a remarkably complex malignancy with a high amount of genetic, epigenetic and phenotypic heterogeneity. Typically, multiple malignant clones coexist in patients. As each subclone exhibits a unique pattern of genetic and epigenetic abnormalities, they also differ in the treatment response. The molecular and cytogenetic abnormalities of AML cells involve mutations in critical genes of normal cell development and cellular survival. Thus, targeting this pathways without inducing side effects remains a challenge.[196] This may explain why AML therapy has not significantly improved over the last 30 years and why advancing the treatment has been an extraordinary challenge.[197]

Patients are categorized according to cytomorphologic and cytochemical characteristics to improve risk-stratification and identification of the best therapeutic options.[199] Conventional treatment consists of intensive induction chemotherapy using cytarabine and anthracycline that induces complete cytomorphological remission in up to 80% of patients. Subsequently, patients undergo a consolidation or post-remission therapy that is critical for elimination of minimal residual disease (MRD). It consist of several cycles of intermediate-dose cytarabine chemotherapy or, depending on the genetic risk profile, allogeneic hematopoietic stem cell transplantation (HSCT).[197, 200] Standard therapy cures 35 to 40% in adult patients with age younger than 60.[197] However, most patients experience recurrence of the disease, especially in older patients that are not eligible for intensive chemotherapy and HSCT. In this patients, remaining leukemic cells reconstituting the MRD can be found in the bone marrow, that are of high prognostic value to assess the risk of relapse and long-term survival.[201] These cells presumably originate from chemoresistant leukemic stem cells (LSCs) with the ability to reinitiate and sustain the disease.[202, 203]

Novel targeted therapies and immunotherapies may provide suitable alternate therapeutic approaches especially in patients not eligible for HSCT and to eliminate MRD to prevent relapse.[204] Among targeted therapy substances, seven are already approved by regulatory authorities: the antibody-drug conjugate gemtuzumab ozogamicin (GO) and several small molecule immune modulators. All act as kinase or cell pathway inhibitors, including the FLT3 inhibitor midostaurin, the isocitrate dehydrogenase (IDH) 2 inhibitors enasidenib and the B cell lymohoma 2 (BCL-2) inhibitor venetoclax.[205, 206] Immunotherapeutic approaches, that primarily target the patients' immune system to indirectly kill the cancer cells, are under continuous development, but not approved so far. Monoclonal antibodies, checkpoint inhibitors, NK cell add-back, T cell-based therapies using chimeric antigen receptor (CAR) T cells and AML-specific T cell engaging bispecific antibodies (BiTEs) as well as

vaccines are promising tools aiming to induce long-lasting leukemia remission.[207-210] Among those, AMG-330 or flotetuzumab are examples for clinical-stage molecules that engage $CD3^+$ T cells and tumor cells by targeting surface antigens CD33 and CD123, respectively.[211, 212] Due to the heterogeneity of AML, researchers have to move beyond the "one-size-fits-all" approach and individualize therapeutic strategies depending on the patients' disease phenotype and risk factors.[213] Given the variety of options, a combinatorial therapy of different immunotherapies with a focus on checkpoint inhibitors might be a highly potent strategy for the treatment of AML in the future.[214]

1.4.2. Leukemia-associated antigens, neoantigens and therapeutic use

All immunotherapeutic approaches for AML rely on the recognition of tumor antigens by active immune effector cells in the absence of overwhelming counter-regulatory mechanisms.[215] Suitable antigens are ideally selectively expressed or overexpressed by the malignant cell. Membrane-expressed and cell lineage antigens for AML, such as the already mentioned CD33 or CD123 suitable for all antibody-based therapies, are rarely cancer-specific. Targeting intracellular leukemia-associated antigens (LAAs) provides higher specificity as most of them are typically overexpressed on leukemic cells. In addition, it enlarges the total number of possible antigens. Recently, several LAAs have been identified including Wilm's tumor protein 1 (WT1), preferentially expressed antigen in melanoma (PRAME) and Fms-like tyrosine kinase 3 internal tandem duplication (FLT3ITD).[216]

Highest specificity is provided by targeting neoantigens that arise from tumor-specific mutations or gene fusions identified via whole genome and exome sequencing. They can be either restricted to individual patients or common for a population of patients. Individual neoantigens in the context of AML are produced for example by fusions involving the mixed lineage leukemia (MLL) and histone-lysine N-methyltransferase 2A (KMT2A) gene. They can occur at multiple breakpoints and with multiple partner genes.[217, 218] Mutations in WT1 produce semi-personal rather than shared neoantigens.[219, 220] At the other end of the spectrum are highly recurrent mutations, exemplified by missense mutations in IDH 1 or 2 and exon 12 mutations in nucleophosmin 1 (NPM1).[221-223] The latter one is arguably the most prominent target in this regard, as it is an essential AML driver gene and mutations thereof occur in the same hotspot in 30-35% of all AMLs.[224] The 4 basepair frameshift mutation leads to a C-terminal alternative reading frame of 11 amino acids that can be specifically recognized by CTLs. It has been also proposed that the immunogenicity of mutated NPM1 (mNPM1)-derived neoepitopes might add to the favorable prognosis of AML patients with NPM1 mutations.[225] There are also limitations of targeting neoantigens in hematologic malignancies, such as the low number of protein-coding mutations and the HLA-restriction.[226] However, neoantigen-directed immunotherapy is exceptionally promising, not only because of the high tumor specificity of neoantigens, but also because the aberrant protein often plays an indispensable role in the malignant phenotype.[227] This led to the assumption that targeting a single high-quality neoantigen can be sufficient for disease control or even cure.[228-230]

Therapeutic targeting of LAAs or neoantigens can either involve vaccination strategies using peptides and DCs or T cell-based therapies such as adoptive T cell transfer. LAAs are therapeutically used most commonly for peptide or *ex vivo* DC vaccines. For several LAAs vaccination trials have already been initiated. So far, clinical Phase I/II trials investigating LAA-specific peptide vaccines have demonstrated immunological and molecular responses that translate into clinical efficacy in up to half of the vaccinated AML patients.[231] DC vaccines were also tested in the post-HSCT and advanced disease setting as well as after chemotherapy-induced remission to prevent relapse.[232] Especially in the post-remission setting of AML, treatment with moDCs loaded for example with WT1 or PRAME or fused to AML cells can produce durable remissions and prevent or delay relapse in some high-risk patients.[232-

[235] An *in vivo* DC vaccination strategy with New York esophageal squamous cell carcinoma-1 (NY-ESO-1) as peptide domain has induced a clinical response in patients with myelodisplastic syndrome (MDS) or low blast count AML.[236, 237] Besides targeting LAAs by DC vaccination strategies, also T cell-based therapies are developed. Chapuis et al. recently showed that WT1-directed TCR gene therapy prevents AML relapse after allogeneic HSCT.[238] Further, a new therapy has been investigated *in vitro* and in pre-clinical models utilizing a T cell bispecific antibody construct that recognizes WT1 in the context of HLA-A*02:01.[239] As not all patients experienced overt clinical benefit, there is a big need to optimize this type of immunotherapy, especially by targeting neoantigens. To our knowledge, no AML-specific neoantigen vaccine is investigated so far, neither *in vitro* nor *in vivo*. The only immunotherapeutic approach in AML targeting a neoantigen was recently developed by Van der Lee et al. They generated mNPM1-specific TCR-transduced T cells that showed antitumor efficacy in an AML xenograft mouse model.[224] As targeting neoantigens by either adoptive T cell therapy or vaccines has demonstrated early promise in the clinic in advanced solid tumors, the development of neoantigen-directed (DC) vaccines and T cell therapies in AML is further encouraged.[72-76, 240, 241]

Collectively, AML antigens exhibit a broad therapeutic application. Different approaches targeting LAAs showed clinical responses with room for optimizations, for example by combining it with other immunotherapies such as checkpoint inhibitors or antibodies directed against surface receptors with killing activity to release neoantigens.[232] However, as neoantigen-directed vaccines or T cell-based therapies are expected to be more promising per se, a lot of research effort is focused on those and first clinical results are eagerly anticipated.

2. Objectives

Dendritic cells (DCs) play a key role at the interface between the native and adaptive immune systems, and therefore hold potential for use in the immunotherapy of diseases such as cancer. In particular, the high capacity of DCs for processing and presenting antigens makes them an attractive target for antigen-delivery. Targeting antigens to DC-specific endocytic receptors by antigen–antibody fusions along with immunostimulatory adjuvants have been recently recognized as a promising strategy for an effective vaccine that elicits a strong and sustained T cell response against cancer cells. A variety of DC-targeting antibodies and formats have been tested in combination with different TLR agonists that elicit DC stimulatory potential. In general practice, the adjuvant has been co-administered together with the DC-targeting antibody or the peptide vaccine. However, soluble adjuvants led to an antigen-independent activation of bystander immune cells bearing the risk of adverse events. Also, the co-delivery of an antigen and adjuvant into the same APC in form of a conjugate resulted in superior cross-presentation and peptide-specific T cell activation compared to separate molecule administration *in vivo*.[133, 134, 242-244]

We developed multifunctional antibody constructs (MACs) to target and activate DCs *in vivo* and thus combine DC-targeting of antigens and stimulation by TLR ligation in one molecule (Figure 10). We aimed to identify a potent combination of antibody and adjuvant while reducing adverse events and increasing therapeutic efficacy. MACs consist of an antigen domain including a variable T cell epitope that is fused to an αCD40 antibody construct with agonistic function. The constructs were tested in a scFv or Fc-silenced IgG1 format. To potentiate therapeutic efficacy, different TLR-ligating domains were attached acting as a vaccine adjuvant. Thereby, the TLR4 agonist fluorescein isothiocyanate (FITC)-LPS and TLR8 agonist FITC-ssRNA40 were ligated to the scFv-format antibody by interacting with an αFITC scFv that was located C-terminally of αCD40. The attachment of the D0/D1 domain of the TLR5 agonist flagellin was achieved by genetic fusion to the IgG1- and scFv-format molecules. The different MACs were initially compared *in vitro* in a viral setting by conjugating a cytomegalovirus (CMV)-specific peptide derived from the pp65 protein to the antibody. In these proof-of-principle studies, the most promising candidate was selected based on capability to enhance DC maturation as well as activation and proliferation of antigen-specific T cells.

The concept was replicated in the leukemia setting by fusing an AML-specific mNPM1-derived neoantigen to the DC-targeting antibody. This is of high therapeutic interest, as novel treatment options in AML are urgently needed especially in high risk patients. In these experiments, the functionality of an mNPM1-coupled αCD40 scFv and IgG1 alone was analyzed and in combination with the most potent TLR agonist fusion identified before.

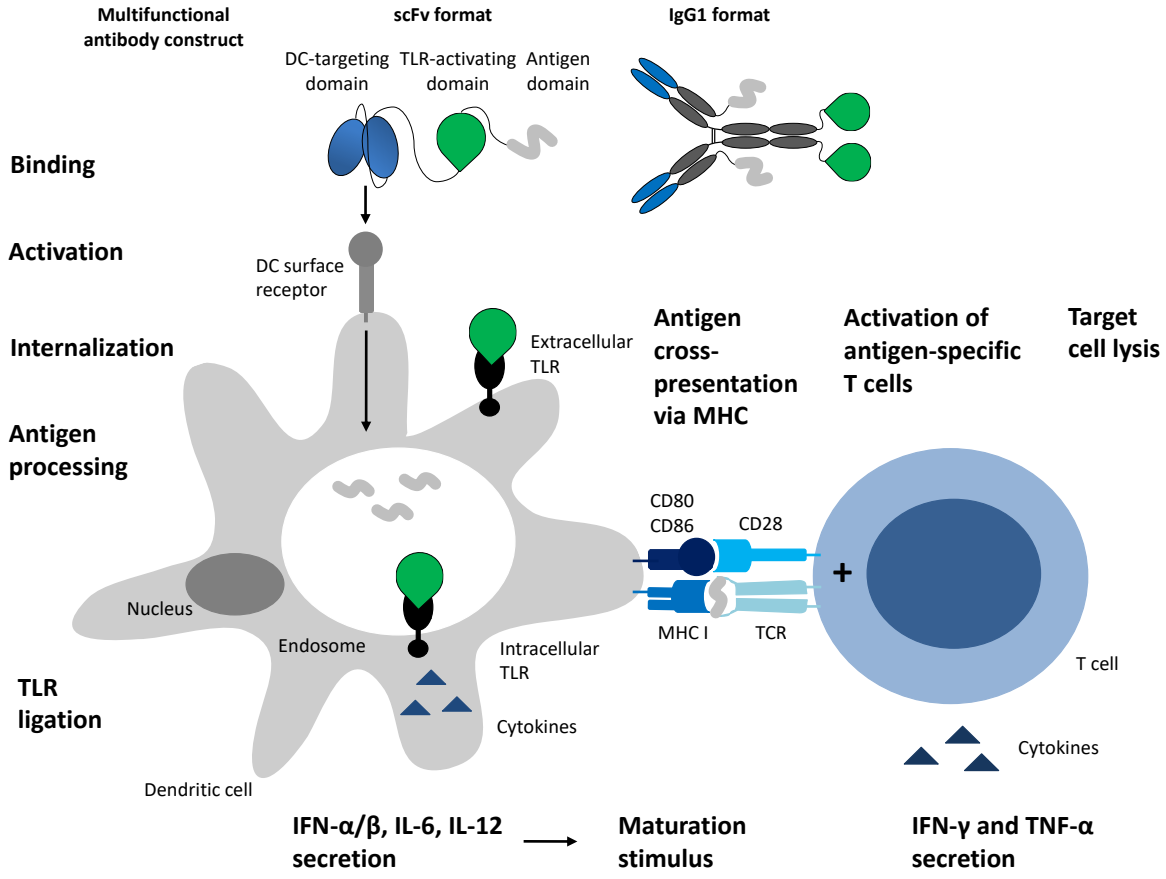

Figure 10. Mode of action of multifunctional antibody constructs.

3. Materials and Methods

3.1. Materials

Unless otherwise stated, all chemicals were purchased from Carl Roth, Merck or Sigma–Aldrich. Restriction enzymes for microbiological applications were obtained from Thermo Fisher Scientific and primers from Metabion. Cell culture media were obtained from Thermo Fisher Scientific and cell culture supplies from Sarstedt and VWR unless indicated otherwise.

3.1.1. *E.coli* strains

The *E.coli* strain XL1 Blue used for cloning was purchased from Stratagen and made chemically competent as previously described.[245] This strain was cultured in lysogeny broth (LB) media (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 1.3 ml/l NaOH) and plated in LB agar (LB-Lennox media with 15 g/l agar).

3.1.2. Healthy donor's material

Peripheral blood samples were collected from healthy donors after written informed consent in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of Ludwig-Maximilians-University. Peripheral blood from healthy donors was the source of PBMCs, monocytes and T cells.

3.1.3. FITC-coupled and uncoupled TLR agonists

The following commercial TLR agonists, optionally FITC-labeled, were used during this study (Table 1). Dried ssRNA40 from Metabion was re-constituted in nuclease-free IDTE pH 7.5 buffer (Integrated DNA Technologies) to a stock concentration of 100 µM. For microscopy studies 6´FAM-ssRNA40-Atto488-3´, a FITC-coupled ssRNA40 with an additional 3´Atto488 label, was used based on an expected quenching of the FITC fluorescence and to ensure sufficient fluorescence stability during microscopy.

Table 1. Commercial TLR agonists.

TLR agonist	Company
6´FAM-ssRNA40 (hereafter named FITC-ssRNA40)	Metabion
6´FAM-ssRNA40-Atto488-3´ (hereafter named FITC-ssRNA40-Atto488)	Metabion
FITC-LPS	Sigma–Aldrich
LPS	Sigma–Aldrich
ssRNA40	Metabion

3.1.4. Plasmids

Table 2. Vector backbones used for protein expression.

Vector	Company
pFUSE-CHIg-hG1	InvivoGen
pFUSE-CLIg-hk	InvivoGen
pSecTag2/Hygro C	Life Technologies

Table 3. Expression vectors.

Name	Encoded sequence	Tag
pFUSE-CHIg - αCD40 V_H	Heavy chain of hCD40-specific IgG1	Fc
pFUSE-CHIg - αCD40 V_H^{Flg}	Heavy chain of hCD40-specific IgG1/flagellin D0/D1 domain	Fc
pFUSE-CHIg - αCD40 V_H^{mFlg}	Heavy chain of hCD40-specific IgG1/flagellin D0/D1 domain R90A E114A	Fc
pFUSE-CHIg - αHer2 V_H	Heavy chain of hHer2-specific IgG1	Fc
pFUSE-CHIg - αHer2 V_H^{Flg}	Heavy chain of hHer2-specific IgG1/flagellin D0/D1 domain	Fc
pFUSE-CHIg - αHer2 V_H^{mFlg}	Heavy chain of hHer2-specific IgG1/flagellin D0/D1 domain R90A E114A	Fc
pFUSE-CLIg - αCD40 V_L^{CMV}	Light chain of hCD40-specific IgG1/amino acid sequence 487–508 of CMV pp65 protein	Fc
pFUSE-CLIg - αCD40 V_L^{mNPM1}	Light chain of hCD40-specific IgG1/amino acid sequence 277–298 of mutated NPM1 protein	Fc
pFUSE-CLIg - αHer2 V_L^{CMV}	Light chain of hHer2-specific IgG1/amino acid sequence 487–508 of CMV pp65 protein	Fc
pFUSE-CLIg - αHer2 V_L^{mNPM1}	Light chain of hCD40-specific IgG1/amino acid sequence 277–298 of mutated NPM1 protein	Fc
pSecTag2 - αCD40.Flg.αFITC^{CMV}	hCD40-specific scFv/flagellin D0/D1 domain/FITC-specific scFv/amino acid sequence 487–508 of CMV pp65 protein	N-His_6
pSecTag2 - αCD40.mFlg.αFITC^{CMV}	hCD40-specific scFv/flagellin D0/D1 domain R90A E114A/FITC-specific scFv/amino acid sequence 487–508 of CMV pp65 protein	N-His_6
pSecTag2 - αCD40.αFITC.TAT^{CMV}	hCD40-specific scFv/FITC-specific scFv/HIV TAT CPP/amino acid sequence 487–508 of CMV pp65 protein	N-His_6
pSecTag2 - αCD40.αFITC^{CMV}	hCD40-specific scFv/FITC-specific scFv/amino acid sequence 487–508 of CMV pp65 protein	N-His_6
pSecTag2 - αCD40.αFITC^{mNPM1}	hCD40-specific scFv/FITC-specific scFv/amino acid sequence 277–298 of mutated NPM1 protein	N-His_6
pSecTag2 - αHer2.Flg.αFITC^{CMV}	hHer2-specific scFv/flagellin D0/D1 domain/FITC-specific scFv/amino acid sequence 487–508 of CMV pp65 protein	N-His_6
pSecTag2 - αHer2.mFlg.αFITC^{CMV}	hHer2-specific scFv/flagellin D0/D1 domain R90A E114A/FITC-specific scFv/amino acid sequence 487–508 of CMV pp65 protein	N-His_6
pSecTag2 - αHer2.αFITC ^{mNPM1}	hHer2-specific scFv/FITC-specific scFv/amino acid sequence 277–298 of mutated NPM1 protein	N-His_6
pSecTag2 - αHer2.αFITC.TAT^{CMV}	hHer2-specific scFv/FITC-specific scFv/HIV TAT CPP/amino acid sequence 487–508 of CMV pp65 protein	N-His_6
pSecTag2 - αHer2.αFITC^{CMV}	hHer2-specific scFv/FITC-specific scFv/amino acid sequence 487–508 of CMV pp65 protein	N-His_6

3.1.5. Amino acid sequences

Table 4. Amino acid sequences of binding modules and ligands.

Name	sequence
αCD40 scFv disufide stabilized (V_H – $(G_4S)_3$ – V_L)	QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQAPGQCLEWMGWIN PDSGGTNYAQKFQGRVTMTRDTSISTAYMELNRLRSDDTAVYYCARDQPLGYCTN GVCSYFDYWGQGTLVTVSSGGGGSGGGGSGGGGSDIQMTQSPSSVSASVGDRV TITCRASQGIYSWLAWYQQKPGKAPNLLIYTASTLQSGVPSRFSGSGSGTDFTLTISS LQPEDFATYYCQQANIFPLTFGCGTKVEIK
αCD40 V_H	QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMHWVRQAPGQGLEWMGWIN PDSGGTNYAQKFQGRVTMTRDTSISTAYMELNRLRSDDTAVYYCARDQPLGYCTN GVCSYFDYWGQGTLVTVSS
αCD40 V_L	DIQMTQSPSSVSASVGDRVTITCRASQGIYSWLAWYQQKPGKAPNLLIYTASTLQS GVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQANIFPLTFGGGTKVEIK
αHer2 scFv (V_L – $(G_4S)_4$ – V_H) (4D5-8-derived, kindly provided by Prof. Matthias Peipp)	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYS GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRGGGGS GGGGSGGGGSGGGGSEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQ APGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYY CSRWGGDGFYAMDYWGQGTLVTVS
αHer2 V_H	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTN GYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDY WGQGTLVTVS
αHer2 V_L	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYS GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKR
αFITC scFv (V_H – $(G_4S)_3$ – V_L) (kindly provided by Prof. Andreas Plückthun)	QVQLVESGGNLVQPGGSLRLSCAASGFTFGSFAMSWVRQAPGGGLEWVAGLSA RSSLTHYADSVKGRFTISRDNAKNSVYLQMNSLRVEDTAVYYCARRSYDSSGYWGH FYSYMDVWGQGTLVTVSSGGGGSGGGGSGGGGSQSVLTQPSSVSAAPGQKVTIS CSGSTSNIGNNYVSWYQQHPGKAPKLMIYDVSKRPSGVPDRFSGSKSGNSASLDIS GLQSEDEADYYCAAWDDSLSEFLFGTGTKLTVL
Flagellin D0/D1 domain separated by a GSGGG linker (± R90A and E114A) (kindly provided by Prof. Bärbel Stecher)	AQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIK GLTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANSTNSQSDLDSIQAEI TQRLNEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDT GSGGGAEAAATTTENPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNL TSARSRIEDSDYATEVSNMSRAQILQQAGTSVLAQANQVPQNVLSLLR
Extracellular domain of CD40	EPPTACREKQYLINSQCCSLCQPGQKLVSDCTEFTETECLPCGESEFLDTWNRETHC HQHKYCDPNLGLRVQQKGTSETDTICTCEEGWHCTSEACESCVLHRSCSPGFGVK QIATGVSDTICEPCPVGFFSNVSSAFEKCHPWTSCETKDLVVQQAGTNKTDVVCGP QDRLR
Human IgG1 Fc	GADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ
HIV-derived TAT CPP sequence[246]	YGRKKRRQRRRA
sequence 487–508 of CMV pp65 protein (CMV_{NLV} epitope sequence bold)	QAGILAR**NLVPMVATV**QGQN
sequence 277–298 of mutated NPM1 protein ($mNPM1_{CLA}$ epitope sequence bold)	RMTDQEAIQDL**CLAVEEVSL**RK

3.1.6. Commercial antibodies and live/dead stains

Table 5. Commercial antibodies for flow cytometry.

Antigen	Fluorophore	Reactivity	Isotype	Clone	Company
CD2	APC	human	Mouse IgG1, κ	RPA2.10	BioLegend
CD3	APC	human	Mouse IgG2a, κ	HIT3a	BioLegend
CD8	FITC	human	Mouse IgG1, κ	SK1	BioLegend
	PerCP-eFluor710	human	Mouse IgG1, κ	SK1	eBioscience
CMV_{NLV}-specific TCR (dextramer)	PE	human			Immudex
IFN-γ	PE	human	Mouse IgG1, κ	B27	BioLegend
TNF-α	APC	human	Mouse IgG1, κ	MAb11	BioLegend
CD80	BV510	human	Mouse IgG1, κ	2D10	BioLegend
CD83	PerCP-Cy5.5	human	Mouse IgG1, κ	HB15e	BioLegend
CD86	APC	human	Mouse IgG1, κ	BU63	BioLegend
Dec205	PE	human	Mouse IgG1, κ	HD30	BioLegend
HLA-DR	Pacific Blue	human	Mouse IgG2a, κ	L243	BioLegend
Penta·His	AF488 or AF647	human	Mouse IgG1		Qiagen
IgG1 Fc	FITC	human	Rat polyclonal IgG		BioLegend

Table 6. Commercial live/dead stains for flow cytometry.

Live/dead stain	Company
7-AAD	BioLegend
LIVE/DEAD™ Fixable Aqua	Thermo Fisher Scientific
Zombie Green™ Fixable Viability Kit	BioLegend

3.1.7. Buffers and media

Table 7. List of standard buffers used for biochemical and cell culture methods.

Buffer	Components
10×PBS (1 l)	80 g NaCl, 2 g KCl, 14.4 g Na_2-HPO_4 x 2 H_2=, 2 g KH_2PO_4 (pH 7.4)
20×SDS running buffer	0.2% SDS, 179 g/l triethanolamine, 143.3 g/l tricine
4×Laemmli buffer	0.11 M Tris base (pH 6.8), 16% /v/v) glycerol, 4% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue
Biacore regeneration buffer	3 M $MgCl_2$
Biacore sample and running buffer	HBS-EP pH 7.4
Coomassie stain	50% (v/v) ethanol, 7% (v/v) acetic acid, 0.2% (w/v) Coomassie Brilliant Blue R250
Elution buffer for Fc-based purification	0.1 M Citrate, pH 3

Elution buffer for His_6-purification	1×PBS + 200 mM Imidazole, pH 7.4
FACS Buffer	1% (v/v) FBS, 1 mM EDTA in 1×PBS
Neutralization buffer for Fc-based purification	1 M Tris-HCl, pH 9
Wash buffer for Fc-based purification	50 mM Tris-HCl, pH 7.5, ± 500 mM NaCl
Wash buffer for His_6-purification	1×PBS + 10 mM Imidazole

Table 8. List of commercial media and buffers for cell culture.

Media and buffers	Company
DMEM	Thermo Fisher Scientific
DPBS	Thermo Fisher Scientific
Expi293 Expression Medium	Thermo Fisher Scientific
Nuclease-free water pH 7.5	Integrated DNA Technologies (IDT)
RPMI 1640 + GlutaMAX	Gibco, Thermo Fisher Scientific
VLE RPMI	Biochrom
X-VIVO	Lonza

Table 9. List of media and buffers for *E.coli*.

Buffer	Components
LB medium (1 l)	10 g bacto tryptone, 5g yeast extract, 5 g NaCl, 1.3 ml NaOH
LB agar (1 l)	LB medium + 15 g agar
TSS buffer	LB medium with 10% (w/v) PEG 6000, 5% (v/v) DMSO, 5 mM $MgSO_4$ (pH 6.5-6.8), frozen at -20°C

3.1.8. Technical equipment

Table 10. List of technical equipment.

Equipment	Company
Aekta Purifier 100	GE Healthcare
Agarose gel electrophoresis system	Bio-Rad
Biacore X100	GE Healthcare
Cascade II EMCCD camera	Photometrics
Cell culture laminar-flow	BDK Luft- und Reinraumtechnik GmbH
CM5 Chip	GE Healthcare
Countess automated cell counter	Thermo Fisher Scientific
Cytoflex LX	Beckmann Coulter
Deltavision OMX V3 microscope	General Electric
FACSAriaIII	BD Biosciences
Guava easyCyte 6HT	Merck Millipore
Hemacytometer Neubauer improved	Brand GmbH and Co KG
HeraCell CO_2 incubator	Thermo Fisher Scientific
Innova 44 Shaker	New Brunswick Scientific

Inverted laboratory microscope Leica DM IL LED	Leica
Irradiation device XStrahl RS225	XStrahl
Microplate reader Infinite M1000 Pro	Tecan
Mr. Frosty freezing container	Thermo Fisher Scientific
Multitron cell incubator	Infors HT
Nanodrop ND-1000	Peqlab Biotechnologies GmbH
pH-meter 766	Knick
Rotana 460 RT centrifuge	Hettich
Sartorius scale LE 22025	Sartorius AG
Sorvall RC6+ centrifuge	Thermo Fisher Scientific
T personal thermocycler	Biometra
Tabletop centrifuges	Eppendorf
Thermomixer F1.5	Eppendorf
UPlanSApo objective	Olympus

3.1.9. Software

Table 11. List of software.

Software	Company
ApE – A plasmid Editor version 2.0.36	M. Wayne Davis
GraphPad Prism version 6 and 8.2	GraphPad Software Inc.
InCyte Software version 3.1.1.	Merck Millipore
ImageJ	Wayne Rasband (NIH)
softWoRx 6.0 Beta 19	unreleased

3.2. Molecular biology methods

3.2.1. Molecular cloning

All MACs were generated using conventional molecular biology methods. The αCD40 scFv was derived from the αCD40 antibody CP-870,893 (selicrelumab, clone 21.4.1, Hoffman-La Roche) with variable heavy and variable light chains connected by a $(G_4S)_3$ linker.[247, 248] It was disulfide stabilized via cysteins introduced at V_H44 and V_L100.[249] The sequence of the αFITC scFv was published by Plückthun et al.[250] The specificity control contained an αHer2 scFv derived from the 4D5-8 clone (trastuzumab, Hoffman-La Roche) that was published elsewere.[251, 252] The flagellin D0/D1 domain, separated by a GSGGG linker, was cloned by Siret Tahk from genomic DNA of *Salmonella typhimurium* strain SL1344 that was kindly provided by Prof. Dr. Bärbel Stecher. Coding sequences for all scFv molecules were cloned into the mammalian expression vector pSecTag2/HygroC (Thermo Fisher Scientific) containing the Ig kappa (Igκ) leader sequence and an N-terminal His_6-tag. The first scFv was flanked by SfiI restriction sites for possible module exchanges between CD40 and Her2. Coding sequences for IgG1 format molecules were cloned into mammalian pFUSE expression vectors, pFUSE2-CLIg-hκ for light chain sequences and pFUSE-CHIg-hG1 for heavy chain sequences. The Fc-region of the IgG1 backbone was silenced using PGLALA mutations.[124] The antigen domain contained either a sequence of the CMV-specific pp65 protein ($CMV_{487–508}$, abbreviated with CMV) including the immunodominant $CMV_{495–503}$ epitope (NLVPMVATV, abbreviated with CMV_{NLV}), or the mutated NPM1-derived $NPM1_{277–298}$ protein (mNPM1) that comprises the $mNPM1_{288–296}$ epitope (CLAVEEVSL, abbreviated with $mNPM1_{CLA}$). In the scFv

format, the αFITC was cloned C-terminally of αCD40 and separated by a $(G_4S)_4$ linker and the peptide domain at the C-terminus was separated by a G_4S linker. In the IgG1 format, the antigen domain was cloned to the C-termini of the light chains and flagellin to the heavy chains, both separated by $(G_4S)_4$ linkers.

General molecular cloning techniques such as site-specific cleavage of DNA with restriction enzymes, dephosphorylation and ligation as well as size-dependent separation of DNA fragments by agarose gel electrophoresis were conducted according to standard protocols.[253] Commercially available enzymes and ready-made kits were used following manufacturer's instructions. Plasmid DNA was isolated from *E. coli* XL1 blue using NucleoSpin Plasmid EasyPure kit (Macherey-Nagel). For the purification of DNA from agarose gels the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel) was used. Sequencing of all generated DNA vectors was performed at Eurofins Genomics to ensure correct cloning.

3.2.2. PCR and site-directed mutagenesis

Polymerase chain reaction (PCR) was utilized to amplify the coding sequences of different modules from plasmid DNA. If necessary, primer sequences included sequences encoding affinity tags, leader sequences or linkers. PCR reactions were performed using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) or KOD Hot Start Master Mix (Merck Millipore), which was used in combination with primers exhibiting high melting temperatures. The following table shows common PCR reactions with Phusion Flash in comparison to KOD Hot Start Master Mix that both contained 20-50 ng DNA template and 0.5 µM of each primer.

Table 12. Conventional PCR programs for Phusion Flash and KOD Hot Start Master mix.

Step	**Phusion Flash**		**KOD Hot Start**	
	Temperature	**Time**	**Temperature**	**Time**
Initial denaturation	98°C	30 s	95°C	2 min
Denaturation Annealing Extension (20-30 cycles)	98°C T_a of primers 72°C	30 s 30 s 15 s/kb	95°C T_a of primers 70°C	20 s 10 s 10-25 s/kp
Final extension	72°C	5-10 min	70°C	5-10 min
Hold	16°C			

T_a: annealing temperature

Site-directed mutagenesis was used to generate point mutations, in particular PGLALA for Fc-silencing and disulfide stabilization of the αCD40 scFv, but also to delete parts of DNA sequences by introducing stop codons. The 5´and 3´end of primer pairs surrounding the mutation should allow homology pairing of at least 20 bp. For the mutagenesis PCR reaction, 20-50 ng DNA template were incubated with 0.05 µM of each primer and Phusion Flash Master Mix following the protocol in table 12. To remove template DNA, the reaction was subsequently digested with FastDigest DpnI for 30 min at 37°C. Afterwards, the DNA was transformed into chemically competent XL1 blue cells and the DNA isolated from single clones was sequenced according to 3.2.1.

3.2.3. Transformation of *E.coli*

Chemically competent XL1 blue bacterial cells were generated as described previously.[245] Briefly, 200 ml of LB medium containing the appropriate antibiotics were inoculated with 2 ml of an overnight

culture and grown until an OD_{600} of 0.3-0.5 was reached. Then, the cells were centrifuged at 3000 g for 5 min at 4°C, resuspended in ice-cold TSS buffer, aliquoted, shock-frozen in liquid nitrogen and stored at -80°C until further usage.

For transformation, 10-100 ng of plasmid DNA was added to 75 µl of competent bacterial cells followed by incubation on ice for 15-30 min. Bacteria were heat-shocked for 42 s at 42°C and subsequently incubation on ice for 2 min. Subsequently, they recovered in 300 µl LB medium at 37°C for 1 h while shaking at 600 rpm. Afterwards, the cells were centrifuged briefly, most of the supernatant was removed and the cells were resuspended in the remaining LB medium before plating them on LB agar plates supplemented with the appropriate antibiotics.

Single colonies were picked and inoculated in 5 ml of LB medium containing the appropriate antibiotics. The cells were shaken over night at 37°C and plasmid DNA was isolated the next day.

Table 13. Antibiotics and dilutions used for different vectors.

Vector	Antibiotics	Dilution
pSecTag2	Ampicillin (100 mg/ml)	1:1000
pFUSE-CHIg	Zeocin (100 mg/ml)	1:4000
pFUSE-CLIg	Blasticidin	Ready to use pouches at 100 µg/ml

3.3. Cell culture methods

3.3.1. Cell lines and maintenance

The L-428 cell line derived from a Hodgkin lymphoma was a kind gift from Prof. Marion Subklewe and purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). It was cultured in RPMI 1640 + GlutaMAX (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific). Expi293F cells were purchased from Thermo Fisher Scientific and cultured in Expi293 Expression Medium (Thermo Fisher Scientific).

All cell lines were grown until the recommended cell density and passaged twice a week. They were routinely tested for mycoplasma contaminations.

3.3.2. Recombinant protein expression in Expi293F cells

All molecules were expressed in Expi293F cells that were co-transfected with the corresponding vectors using ExpiFectamine 293 transfection kit (Thermo Fisher Scientific) following the manufacturer's instructions. For the generation of IgG1-format antibodies, heavy and light chains were transfected in a 1:1 ratio. Transient expression of all MACs was performed for 5 to 6 days after transfection. Due to the Igκ leader sequence in the vectors, the molecules could subsequently be purified from the supernatants. For low-expressing plasmids and to generate higher amounts of protein, stable cell pools of Expi293F cells expressing scFv contructs were generated. Therefore, 1-2 days after transfection 300 µl of Expi293F cells were transferred to 3 ml DMEM + GlutaMAX medium supplemented with 10% FBS and 50 µg/ml hygromycin B gold (InvivoGen) in a 6-well plate (standard, Sarstedt). By changing the medium with antibiotics and removing dead cells, cells that stably integrated the plasmid were selected, adhered and grew out in clones. After approximately 3 weeks, the stable cell pool could be transferred into shaking culture with Expi293 Expression Medium and

cultivated in the presence of 50 µg/ml hygromycin. Subsequently, stable cells were either frozen or expanded for protein expression.

3.3.3. Isolation of peripheral blood mononuclear cells from heparinized blood

PBMCs from healthy donors were separated from heparinized peripheral blood using the Biocoll separating solution (Biochrom) and Leucosep tubes (Greiner Bio-One) according to the manufacturer's instructions. In brief, 15 ml of Biocoll solution was preloaded in a 50 ml Leucosep tube by centrifugation for 30 s at 1000 g. Heparinized whole-blood samples were diluted with equal volumes of Dulbecco's phosphate buffered saline (DPBS, Thermo Fisher Scientific) and 30ml of the diluted blood was added to the Leucosep tube. Tubes were centrifuged for 10 min at 1000 g without breaking. Buffy coat containing PBMCs was collected, washed and re-suspended in RPMI 1640 + 10% FBS for further use.

3.3.4. Generation of monocyte-derived dendritic cells

MoDCs were generated within 3 days as described before.[106] In brief, monocytes were enriched from PBMCs by plastic adherence in flat bottom 6- or 12-well plates with a surface treated for maximum adhesion (Nunc, Thermo Fisher Scientific) at a concentration of 0.5-1×10^7 cells/ml in Very Low Endotoxin (VLE) RPMI (Biochrom) supplemented with 1.5% human serum (HS, serum pool of AB positive adult males; Institute for Transfusion Medicine, Suhl) – hereafter named DC medium. If required, non-adherent cells (NACs) were kept at 37°C until further use three days later. For some experiments requiring pure monocytes or DCs (for example for microscopy), monocytes were isolated from PBMCs using the Classical Monocyte Isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Afterwards, monocytes were seeded in Nunc 24-well plates and cultured for 48 h at 37°C in DC medium supplemented with 800 IU/ml GM-CSF (Peprotech) and 580 IU/ml IL-4 (Peprotech) (2d-moDCs). For some experiments, 2d-moDCs were then loaded with MACs ± TLR agonists for 24 h followed by different cytokine treatments, depending on which DC maturation state should be achieved. If antibody constructs were combined with TLR agonists and if immature moDCs (iDCs) were needed, 800 IU/ml GM-CSF, 580 IU/ml IL-4 was added for another 24 h together with 250 ng/ml PGE_2 (Sigma–Aldrich). To generate fully mature moDCs (mDCs), maturation was achieved within 24 h by the addition of 800 IU/ml GM-CSF, 580 IU/ml IL-4, 250 ng/ml PGE2, 2000 IU/ml IL-1β (R&D Systems), 1100 IU/ml TNF-α (Peprotech), 5000 IU/ml IFN-γ (Peprotech), 1 µg/ml R848 (InvivoGen).[105, 106] For some experiments, iDCs and mDCs were loaded with the processed HLA-A*02:01-restricted peptides (CMV_{NLV}, $mNPM1_{CLA}$, JPT Peptide Technologies) for 1.5 h at 37°C in serum-free medium.

As the VLE RPMI was discontinued at the end of 2019, regular RPMI 1640 + GlutaMAX was used for the last experiments while no differences in DC quality were detected.

3.3.5. Generation and expansion of CMV_{NLV}- and $mNPM1_{CLA}$-specific T cells

CMV $pp65_{487–495}$-peptide specific $CD8^+$ T cells (CMV_{NLV}-specific T cells) were generated by Alina Lohner from the group of Prof. Marion Subklewe. For this, PBMCs from an HLA-A*02:01^+ donor with previous CMV infection (CMV^+) were isolated. MoDCs were matured as described above, pulsed with 1 µM of CMV_{NLV} for 90 min and irradiated with 30 Gy (XStrahl RS225, XStrahl). Autologous $CD8^+$ T cells were isolated from NACs using the $CD8^+$ T cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. T cells and pulsed DCs were co-cultivated at a T cell:DC ratio of 4:1 in RPMI 1640 with 5% HS and 30 ng/ml IL-21 (Peprotech) for 72 h. On days 3 and 6, co-cultures were expanded 1:1 by adding

medium supplemented with 10 ng/ml IL-15 and IL-7 (Peprotech). On day 9, CMV_{NLV}-specific CMV_{NLV}-specific dextramer$^+$ and $CD8^+$ T cells were sorted on a FACSAriaIII (BD Biosciences).

$mNPM1_{288–296}$-specific $CD8^+$ T cells ($mNPM1_{CLA}$-specific T cells) were kindly provided by Dr. Marieke Griffioen and generated as described previously by the transduction of $CD8^+$ T cells with an $mNPM1_{CLA}$-specific TCR.[224]

For the expansion of specific T cells, PBMC feeders of two HLA-A*02:01$^+$ and two HLA-A*02:01$^-$ donors were mixed in equal amounts and pulsed with 1 µM CMV_{NLV} or $mNPM1_{CLA}$ peptide in X-VIVO-15 medium (Lonza) for 2 h at 37°C. After irradiation with 30 Gy, feeders at a concentration of 2×10^6 cells/ml were cultivated with specific T cells at 0.4×10^6 cells/ml in X-VIVO 15 + 5% HS supplemented with 10 ng/ml IL-7 and IL-15 and 0.5 µg/ml PHA-L (Sigma–Aldrich) in a 6-well plate for suspension cells (Sarstedt). After 3 days, cultures were fed by replacing half volume of medium with fresh X-VIVO 15 + 5% HS supplemented with 50 U/ml IL-2 (Peprotech), 20 ng/ml IL-7 and 20 ng/ml IL-15. Using this medium T cells were splitted and expanded every 3-6 days. Experiments were performed 9-21 days after expansion. Feeding was necessary every 14-21 days.

3.4. Protein biochemistry methods

3.4.1. Purification of Fc-fusion and IgG1 antibodies from cell culture supernatant

To purify IgG1-format molecules, cell culture supernatants containing the desired antibodies were incubated over night at 4°C with an appropriate amount of nProtein A sepharose 4FF beads (GE Healthcare) on a rotating wheel. Beads were collected by centrifugation and loaded into a Bio-Spin chromatography column (Thermo Fisher Scientific). Washing steps were performed with 4 column volumes of wash buffer for Fc-based purification (see table 7, 50 mM Tris-HCl pH 7.5). Subsequently, antibodies were eluted from beads using 6 column volumes of elution buffer (0.1 M citrate pH 3.0) followed by neutralization of elution fractions with neutralization buffer (1 M Tris-HCl pH 9.0). All wash and elution fractions were collected separately and evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue stain (see chapter 3.4.3), fractions containing the antibodies of interest were pooled and concentrated using Amicon spin concentrators (Merck Millipore). These concentrators were also used to exchange the buffers against phosphate buffered saline (PBS) by adding a surplus of PBS during initial centrifugation steps.

If impurities on the first gel analysis were seen and to remove dimer fractions, a size exclusion chromatography (SEC) using a Superdex 200 Increase 10/300 GL column (GE Healthcare) was performed as a second polishing step. SEC fractions containing the antibodies were pooled, visualized by SDS-PAGE and concentrated. Protein concentration was measured with a spectrophotometer (Nanodrop ND-100, Peqlab Biotechnologies GmbH) followed by sample aliquotation, shock freezing in liquid nitrogen and storage at -80°C.

3.4.2. Purification of poly-Histidine tagged proteins from cell culture supernatant

For the purification of His-tagged proteins, cell culture supernatants of transient or stable transfections were incubated with Ni-NTA agarose beads (Qiagen) for 2 h at 4°C. After collection, the beads were transferred into a Bio-Spin chromatography column and washed with 4 column volumes of wash buffer (see table 7, 1×PBS + 10 mM Imidazole) to remove non-specific binding. Subsequently, proteins were eluted with 6 column volumes of elution buffer (1×PBS + 200 mM Imidazole). All further

steps, including SDS-PAGE, concentration and SEC, were performed as described in the previous section.

3.4.3. Denaturing polyacrylamide gel electrophoresis

The purity of protein samples after purification steps or interaction studies were evaluated by SDS-PAGE using precast 4-20% Bis-Tris gels of the RunBlue SDS-PAGE Gel System (Expedeon). Protein samples were mixed with Laemmli buffer and denatured for 5 min at 95°C. After loading the samples onto the gel, it was run at 140 V for 10 min and for another 40-50 min at 180 V in 1×SDS Running Buffer. Afterwards, proteins were stained for 30 min using Coomassie Brilliant Blue staining solution followed by destaining in water. PageRuler Unstained Protein Ladder (Thermo Fisher Scientific) served as size standard.

3.4.4. Thermal unfolding analysis by nanoDSF

The thermal stability of proteins was determined using nano differential scanning fluorimetry (nanoDSF) that measures temperature-dependent changes in the intrinsic fluorescence of tryptophane and tyrosine residues (Tycho NT.6, NanoTemper Technologies). For this, 1 µM of protein in PBS was absorbed by a capillary that was subsequently placed into the reader. Afterwards, the intrinsic protein fluorescence was measured at 330 nM and 350 nM while incubating at increasing temperatures. Changes in fluorescence signal indicated transitions in the folding state of the proteins and the temperatures at which a transition occurred are named as inflection temperatures (T_i) or also melting temperatures (T_m).[254]

3.5. Binding and interaction studies

3.5.1. Binding studies by flow cytometry

Binding analysis of all MACs to CD40 on the DC surface was assessed by incubating CD40-expressing cells, such as L-428 cells or iDCs and mDCs, with the respective protein at saturating concentration. His-tagged proteins were subsequently detected by secondary staining with a Penta·His Alexa Fluor (AF)488 or AF647 conjugate antibody (Qiagen), IgG1 antibodies by a FITC-coupled αhuman IgG Fc (BioLegend). Briefly, 0.5×10^5 cells were stained with 50 µl of 200 nM MACs, diluted in FACS buffer, if not otherwise stated. Cells were incubated for 30 min at 4°C, washed and afterwards stained for another 30 min at 4°C in 50 µl of 1:200 diluted Penta·His or 1:100 diluted αhuman IgG Fc antibody in FACS buffer. Subsequently, cells were washed, resuspended in FACS buffer and measured on a Guava easyCyte 6HT instrument (Merck Millipore). As analysis, the mean fluorescence intensity (MFI) ratio was calculated dividing MFI of the antibody by the MFI of the isotype control.

3.5.2. K_D determination by flow cytometry

To determine equilibrium binding constants (K_D, as an affinity and avidity measurement) by flow cytometry, L-428 cells were incubated with MACs in a concentration range of 0.005 to 200 nM for 30 min at 4°C. Cells were washed and subsequently stained with the corresponding secondary antibody as indicated before. For evaluation, the maximum MFI was set to 100% and all data points were normalized accordingly. The data was fitted with a non-linear regression curve using a one-site specific binding model.

3.5.3. K_D determination by surface plasmon resonance

For K_D assessment by surface plasmon resonance (SPR) using a Biacore X100 machine (GE Healthcare), a CM5 chip (GE Healthcare) was coated with an αhuman Fc capture (GE Healthcare) that reacts with the chip's amino groups at a level of around 8000 response units (RU). The ligand αCD40 IgG1 was immobilized on the chip at a level of 100 RU, which was achieved after a contact time of 180 s and with an antibody concentration of 1.25 nM. The extracellular domain of CD40 was passed over the antibody-coated chip at concentrations ranging from 15.62 to 1000 nM with an association time of 180 s and a dissociation time of 600 s. The K_D was determined by the ratio of the association rate constant (k_{on}) and the dissociation rate constant (k_{off}).

3.5.4. Internalization studies by structured illumination microscopy

To assess internalization of MACs on DCs, moDCs differentiated from magnetically isolated monocytes were used. αCD40.αFITC^{CMV}, αCD40^{CMV} and the αHer2 non-specific binding controls were labeled with an AF594 Antibody Labeling Kit (Thermo Fisher Scientific) and excess of dye was removed according to the manufacturer's instructions. Similar degrees of labeling were obtained for all antibodies. 200 nM of MACs and the non-specific binding controls that had been pre-coupled for 30 min with FITC-ssRNA40 for some experiments were incubated for 90 min at 4°C or at 37°C with iDC and mDCs. For samples incubated at 4°C as well and also the αHer2 controls at either temperature, membrane staining with an AF488-labeled αHLA-DR antibody was performed for 30 min at 4°C. Next, moDCs were transferred onto coverslips using a Shandon Cytospin 3 cytocentrifuge (Thermo Fisher Scientific) for 10 min at 800 rpm. Cells with the exception of those subjected to membrane staining were prepared for intracellular staining by fixation with 4% paraformaldehyde (Sigma–Aldrich) in DPBS followed by permeabilization with 0.1% Triton X-100 (Sigma–Aldrich) in DPBS; both treatments were performed for 10 min at room temperature. Lysosomes were stained for 30 min at 4°C using αLAMP1 (Novus Biologicals) or αEEA1 antibody (Thermo Fisher Scientific), which were detected in a subsequent step using a secondary donkey αrabbit AF488 antibody (Thermo Fisher Scientific) for 30 min at 4°C. Post-fixation took place for 10 min at room temperature with 4% paraformaldehyde followed by nuclear staining with 1 µg/ml DAPI (Thermo Fisher Scientific). Finally, the coverslips were mounted onto glass slides using Vectashield (Vectorlabs) and sealed with transparent nail polish.

3D structured illumination microscopy (SIM) acquisition was done in the laboratory of Prof. Heinrich Leonhardt on a Deltavision OMX V3 microscope (General Electric) equipped with a 100 × 1.4 oil immersion objective UPlanSApo (Olympus), 405 nm, 488 nm and 593 nm diode lasers and Cascade II EMCCD cameras (Photometrics). Raw data were first reconstructed and corrected for colour shifts with the help of the provided software softWoRx 6.0 Beta 19 (unreleased). In a second step, a custom-made macro in Fiji finalized the channel alignment and established composite TIFF stacks.[255] For better presentation of the images all channels were individually adjusted in brightness (exceptions are indicated).

3.5.5. Interaction studies by size exclusion chromatography

SEC techniques were used in order to study the interaction between the αFITC scFv and FITC-labeled nucleic acids. 30 µg of αCD40.αFITC^{CMV} and of a FITC-labeled 42 mer dsDNA (ca. 25 kDa) were loaded independently on a Superdex 200 Increase 5/150 GL column. Afterwards, 30 µg of the two components were mixed, meaning an approximately 2-fold excess of DNA, incubated for 30 min at room temperature and run over the column. Elution at a lower volume compared to the single components indicated a complex formation.

3.5.6. Interaction studies by fluorescence quenching

To study interaction of FITC-ssRNA40 with of αCD40.αFITC^{CMV} by fluorescence quenching, 10 nM of FITC-ssRNA40, diluted in nuclease free water pH 7.5, were incubated with different antibody concentrations, ranging from 0.5 to 160 nM in DPBS, for 30 min at room temperature. The samples were placed in a black non-binding 96-well plate (Greiner Bio One) and subsequently fluorescence intensity with the excitation wavelength of 480 nM and emission wavelength of 520 nM and anisotropy as the inversely related readout was measured via the Infinite M1000 Pro reader (Tecan) at optimal gain. Quenching of FITC fluorescence in combination with escalating anisotropy at increasing antibody concentrations provided evidence for occurring interaction between FITC-ssRNA40 and the αFITC scFv. The same experiment was also performed using FITC-LPS.

3.5.7. Signaling studies using hTLR5-HEK293 cells

Signaling studies were performed in the laboratory of Prof. Christine Josenhans using a human TLR5-expression HEK293-T cell line (hTLR5-HEK293). As a readout, IL-8 secretion was determined using a commercially available enzyme-linked immunosorbent assay (ELISA).[256]

3.6. Functional assays

3.6.1. Maturation and peptide-loading of immature moDCs

As described in 3.3.4. the 2d-moDCs were incubated for 24 h with 200 nM antibody constructs including CMV- or the mNPM1-specific domains to load the cells with the respective peptide. In some experiments, TLR agonists were added either by genetic fusion or by coupling them via an αFITC scFv. If mDCs should be generated, a maturation cocktail including IL-4, GM-CSF, PGE2, IL-1β, IFN-γ, TNF-α and R848 was added for 24 h. However, if DCs should be left in the immature stage or if the DC maturing effect of different stimulations should be investigated, only a reduced cytokine cocktail (IL-4, GM-CSF and PGE2) was appended. For some experiments, iDCs and mDCs were loaded with the already processed peptides (CMV_{NLV}, $mNPM1_{CLA}$) for 1.5 h.

In some assays, in which the flagellin-induced DC maturation should be assessed, iDCs were pre-incubated with a 4-fold excess (200 nM) of an IgA2 αTLR5-blocking antibody or the respective isotype control (InvivoGen) for 30 min at 37°C before the addition of 50 nM flagellin fusion molecules to determine whether this effect was TLR5-driven.

To investigate the maturation stage of DCs, that was either induced by the αCD40 binder, the TLR ligating domains or the R848-containing cytokine cocktail, expression of maturation markers and cytokine secretion was quantified by flow cytometry and compared to iDCs. For this, immunofluorescent staining of the cell surface antigens CD80, CD83, CD86 and HLA-DR was performed using a panel of fluorescently-conjugated monoclonal antibodies as listed in table 5. Corresponding isotype controls were used to determine the MFI ratio. In parallel, IL-6 secretion into the supernatant was quantified via cytometric bead array (BD Biosciences) according to the manufacturer's instructions. Surface stained DCs were acquired on a Cytoflex LX flow cytometer (Beckmann Coulter), cytokine-bound beads on the Guava easyCyte 6HT.

3.6.2. Allogeneic DC–T cell co-cultures

Immature and mature DCs of an HLA-A*02:01 positive donor were loaded with 200 nM of indicated MACs ± TLR agonists or peptides as described in the previous section. Before setting up a co-culture, MACs, maturation reagents and peptides were removed by harvesting and washing of the DCs. The DCs were cultivated with allogeneic CMV_{NLV}- and $mNPM1_{CLA}$-specific T cells, that were generated and expanded according to section 3.3.5., in a 1:5 ratio for 4-6 h in DC medium containing monesin at 25 µM and brefeldin A at 10 µg/ml (both Sigma–Aldrich) at 37°C. As a readout for T cell activation, the percentage of IFN-γ and TNF-α double positive cells within $CD8^+$ cells was determined by intracellular cytokine staining (ICCS). To this end, cells were stained for CD8, fixed and permeabilized using the BD Cytofix/Cytoperm Kit according to the manufacturer's instructions (BD Biosciences) followed by intracellular IFN-γ and TNF-α staining and flow cytometric readout on the Guava easyCyte 6HT.

3.6.3. Autologous DC–T cell co-cultures

DCs were prepared as described in the previous section. Antibody- or peptide-loaded iDCs or mDCs were incubated with autologous NACs of a CMV^+ and HLA-A*02:01^+ donor in a 1:10 ratio in DC medium for 6 days. To analyze T cell proliferation, the percentage of CMV_{NLV}-specific T cells within $CD8^+$ T cells was determined by staining with a CMV_{NLV}-specific dextramer (Immudex). Cells were acquired on the Guava easyCyte 6HT.

3.7. Data plotting and statistical analysis

Data was analyzed and plotted with GraphPad Prism (GraphPad Software Inc.). Differences in DC maturation, T cell activation and proliferation were assessed by the Wilcoxon signed-rank test comparing dependent samples of unknown distribution with each other. Statistical significance was considered for *p*-value < 0.05 (*), <0.01 (**), <0.001 (***) and <0.0001 (****), ns = not significant.

4. Results

4.1. Generation and stability of MACs

4.1.1. Molecule design and cloning

The systemic co-delivery of TLR agonists together with a DC-based vaccine is a promising strategy to enhance vaccine efficacy and to break tolerance to antigens.[257] However, systemic administration of different immune-stimulatory agents increase the risk of broadly distributed adverse events and, in case of small molecules, are often limited by a fast renal clearance. We developed multifunctional antibody constructs (MACs) that deliver antigens together with a TLR agonist to DCs. All molecules target CD40 on the surface of DCs and consist either of an αCD40 Fc-silenced IgG1 antibody or a single-chain variable fragment (scFv) thereof. The utilized binding sequences originate from the agonistic therapeutic antibody CP-870,893 (clone 21.4.1.) that has been disulfide stabilized in the scFv format.[247, 248] For proof-of-principle experiments, the antigen domain contained a sequence of the CMV-specific pp65 protein ($CMV_{487–508}$, CMV) which includes the $CMV_{495–503}$ epitope (NLVPMVATV, CMV_{NLV}). In the second part of the thesis, IgG1 format molecules were investigated using the $mNPM1_{277–298}$ peptide (mNPM1) as an antigen domain that comprises the $mNPM1_{288–296}$ epitope (CLAVEEVSL, $mNPM1_{CLA}$). To couple TLR agonists two different strategies were applied. In one approach, FITC-labeled TLR 4 and 8 agonists (FITC-LPS and FITC-ssRNA40) were non-covalently linked to the antibody via an αFITC scFv that is flanked by the αCD40 scFv on the N-terminus and the antigen domain on the C-terminus ($αCD40.αFITC^{CMV}$). Alternatively, a truncated version of the TLR5 agonist flagellin (Flg) was genetically fused to the antibody ($αCD40.Flg.αFITC^{CMV}$ for scFv format and $αCD40.Flg^{CMV}$ for IgG1 format). The flagellin region, including the D0/D1 domain connected by a GSGGG linker, was cloned from genomic DNA of *Salmonella typhimurium* by Siret Tahk and was designed analogous to the therapeutic drug entolimod.[184, 185] To control specificity of all candidate molecules, the CD40-targeting domain was exchanged by an αHer2 binder that was derived from the therapeutic antibody trastuzumab. Figure 11 shows the schematic composition of all the tested molecules. ScFv-based molecules were cloned into a pSecTag2 vector and for the IgG1 format pFUSE vectors for heavy chain and light chain were used. Both contained the Igκ leader sequence to promote secretion of the protein into the cell culture media. All domains targeting different entities were separated by one or more polyglycine–serine (G_4S) units as indicated.

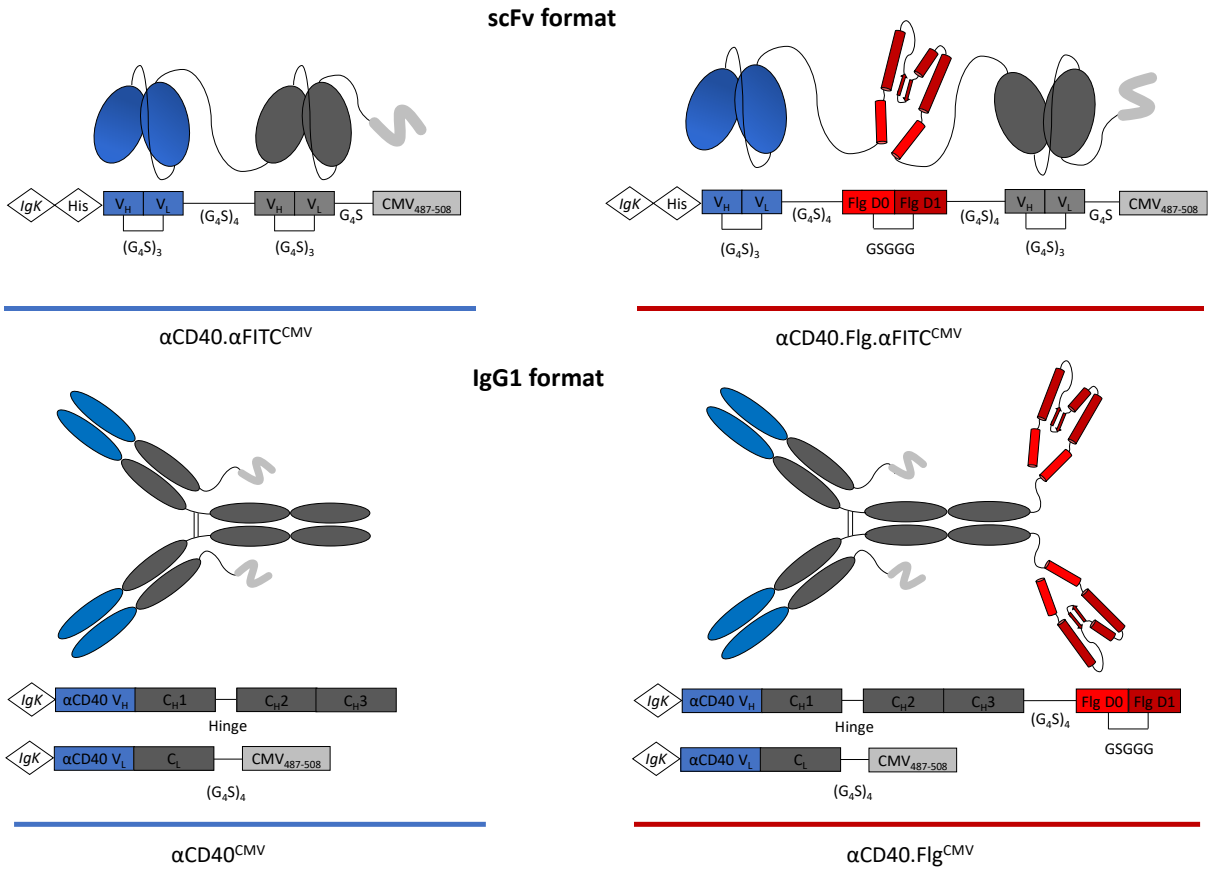

Figure 11. Schematic view of MACs.
The modular composition of the MACs includes an αCD40 scFv or an Fc-silenced IgG1 DC-targeting domain, a CMV-specific antigen domain and an immune-stimulatory domain. The latter consisted of either an αFITC scFv to bind FITC-labeled TLR agonists or of genetically fused flagellin. As non-specific binding controls, all MACs were also generated with an αHer2 binding module. The molecules were also cloned with an mNPM1-derived peptide as an antigen domain.

4.1.2. Expression and purification

The multifunctional antibodies were purified from transiently transfected Expi293F cells in a two-step procedure. ScFv molecules were captured from the cell culture supernatant using Ni-NTA agarose beads, IgG1 antibodies using protein A sepharose beads. SEC with a Superdex 200 Increase 10/300 GL column was performed as a second purification step. αCD40.αFITC^{CMV}, αCD40.Flg.αFITC^{CMV} and αCD40^{CMV} did not show any impurities after the bead-based affinity purification (data not shown), but for αCD40.Flg^{CMV} two peaks were visible on the chromatogram of the preparative SEC (Figure 12A). SDS-PAGE analysis revealed that peak 1 contained a protein of bigger molecular weight, namely αCD40.Flg^{CMV} with flagellin fusion, and peak 2 contained only αCD40^{CMV} (Figure 12B). The fractions of the first peak were pooled to obtain pure αCD40.Flg^{CMV} protein.

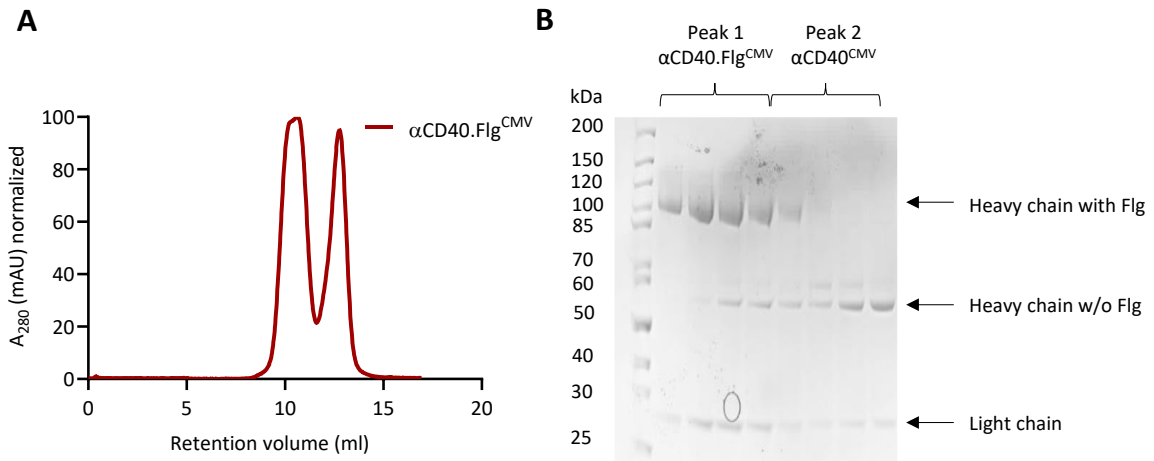

Figure 12. Preparative gel filtration profile and SDS-PAGE analysis of αCD40.Flg^{CMV} after protein A-based affinity chromatography.
(A) Chromatogram of preparative SEC with a Superdex 200 Increase 10/300 GL column and **(B)** SDS-PAGE analysis of αCD40.Flg^{CMV} purified via Protein A beads from the supernatant of transiently transfected Expi293F cells. Two peaks of different molecular weight were eluting from the column, one peak including heavy chain with flagellin fusion (αCD40.Flg^{CMV}) and the other one without flagellin attached (αCD40^{CMV}).

Molecule purity subsequent to the two-step purification was further evaluated by SDS-PAGE analysis and analytical gel filtration (Figure 13). As depicted on SDS-PAGE, all molecules without flagellin were clean and corresponded to the computed masses. Both molecules with flagellin fusion (αCD40.Flg.αFITC^{CMV} and αCD40.Flg^{CMV}) showed a higher apparent molecular weight than the theoretical value due to glycosylation of flagellin (for example 90 kDa determined by SDS-PAGE vs 80 kDa computed mass for heavy chain fusion) (Figure 13A-B). αCD40.Flg.αFITC^{CMV} was visible as a double band on SDS-PAGE, which could not be separated by SEC due to just a minimal difference in size. For αCD40.Flg^{CMV} in the IgG1 format, SDS-PAGE analysis revealed that still a low amount of heavy chain lacking the flagellin domain was present after preparative SEC (Figure 13A). Nevertheless, analytical gel filtration as a second quality control of the proteins demonstrated clean chromatograms for all molecules including flagellin fusions (Figure 13C-D). αCD40^{CMV} and αCD40.αFITC^{CMV} could be produced in reasonable amounts. Notably, the insertion of the flagellin domain reduced the yield in the scFv format by approx. 2-fold and in the IgG1 format by 6-fold (Table 14).

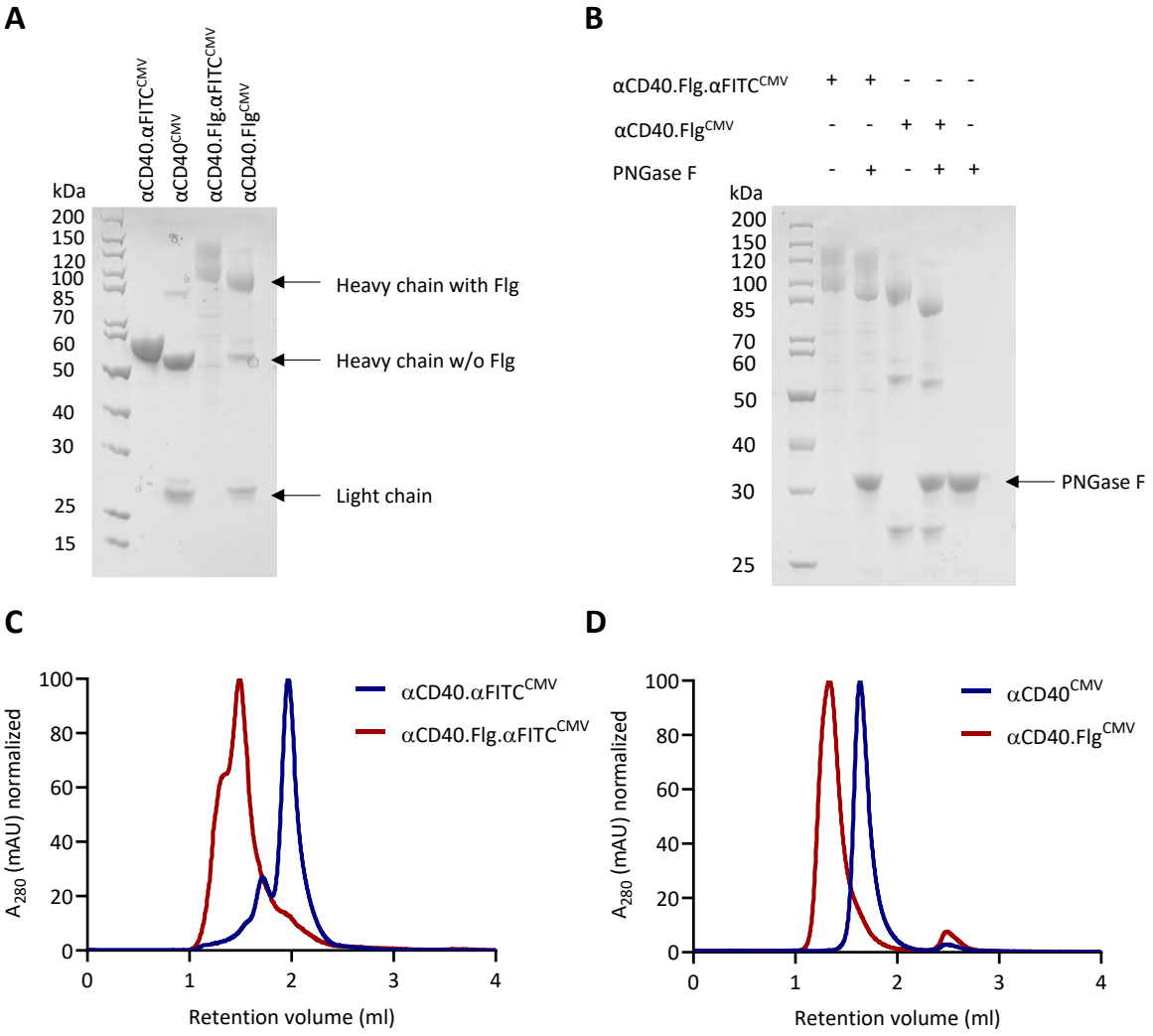

Figure 13. SDS-PAGE analysis and analytical gel filtration profile of purified and concentrated MACs subsequent to the two-step purification procedure.
(A) SDS-PAGE evaluation of purified proteins (3 µg). **(B)** PNGase F digestion of αCD40.Flg.αFITC^{CMV} and αCD40.Flg^{CMV} (3 µg) for 1 h at 37°C and subsequent SDS-PAGE evaluation resulted in a reduction of molecular weights due to removal of N-linked glycosylations. **(C, D)** Analytical SEC chromatograms of αCD40 scFv **(C)** and IgG1 **(D)** antibodies (30 µg) with and without flagellin fusion using a Superdex 200 Increase 5/150 GL column.

Table 14. Expression yields.

Molecule	**Yield** (Mean ± SD of *n* = 3 purifications)
αCD40.αFITC^{CMV}	13.3 ± 0.8 mg/ml
αCD40.Flg.αFITC^{CMV}	6.0 ± 3.0 mg/ml
αCD40^{CMV}	19.5 ± 8.5 mg/ml
αCD40.Flg^{CMV}	3.3 ± 1.2 mg/ml

4.1.3. Thermal stability and unfolding

The thermal stability and unfolding of the molecules were investigated using nanoDSF that employs intrinsic tryptophan or tyrosin fluorescence. Figure 14 shows the unfolding profile and table 15 the melting temperatures of all molecules. αCD40 IgG1 antibodies exhibited higher melting transitions compared to scFv format molecules. The insertion of the flagellin domain did not influence thermal stability of both scFv- and IgG1-based molecules.

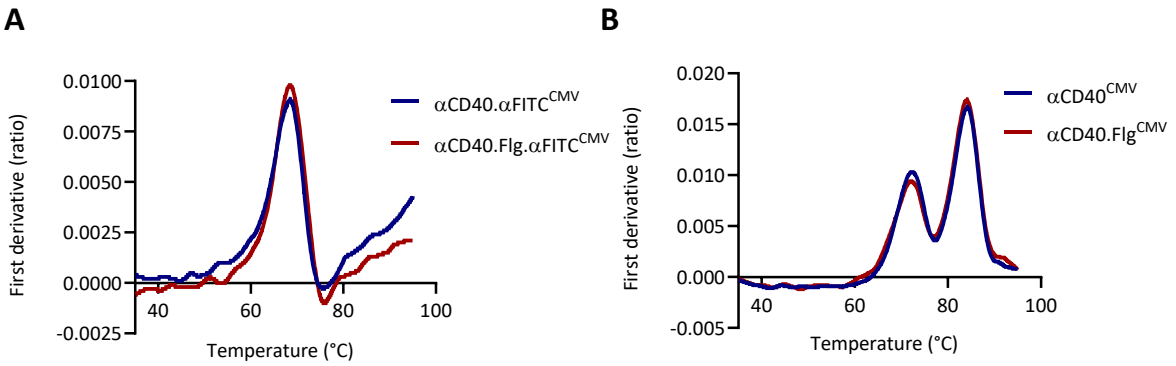

Figure 14. Thermal unfolding of MACs.
Thermal stability of αCD40.αFITC^{CMV} and αCD40.Flg.αFITC^{CMV} **(A)** as well as of αCD40^{CMV} and αCD40.Flg^{CMV} **(B)** as determined by nanoDSF.

Table 15. Melting temperatures (T_m)

Molecule	T_m (1)	T_m (2)
αCD40.αFITC^{CMV}	68.8°C	76.0°C
αCD40.Flg.αFITC^{CMV}	68.5°C	75.6°C
αCD40^{CMV}	72.5°C	84.1°C
αCD40.Flg^{CMV}	71.7°C	84.1°C

To determine time- and temperature-dependent stability, proteins were stored for up to 14 days at 4°C and for up to 7 days 37°C, large aggregates were separated by centrifugation and the supernatant was evaluated by SDS-PAGE. None of the molecules showed any substantial degradation compared to freshly thawed proteins and all antibodies remained intact at least for 7 days at 37°C and for 14 days at 4°C (Figure 15). This confirms that the proteins were sufficiently stable at physiological conditions.

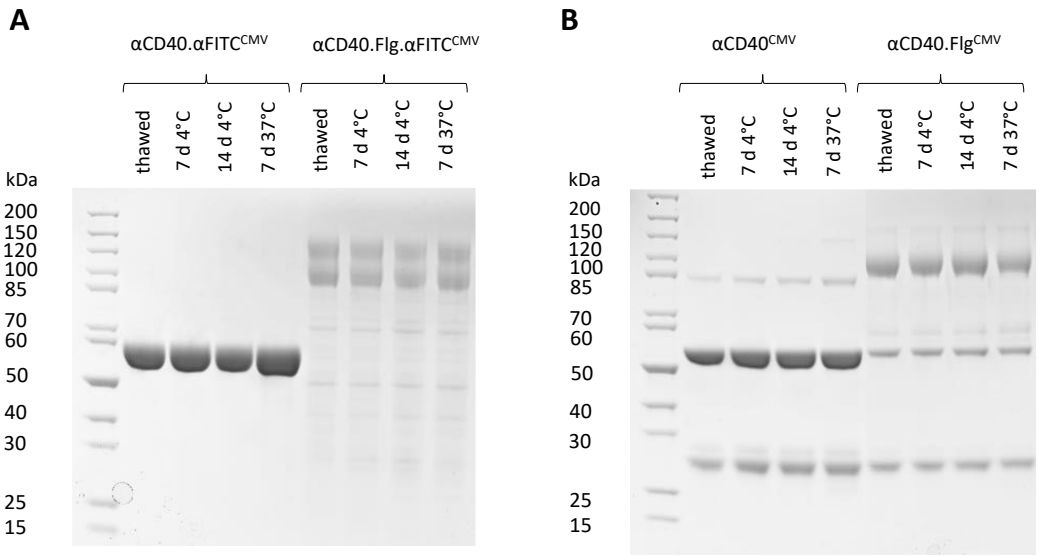

Figure 15. Time- and temperature-dependent stability of MACs.
SDS-PAGE analysis of 3 µg of αCD40.αFITC^{CMV} and αCD40.Flg.αFITC^{CMV} **(A)** as well as of αCD40^{CMV} and αCD40.Flg^{CMV} **(B)** freshly thawed and after incubation at 4°C and 37°C for indicated time points in PBS.

4.1.4. Functionality of the αFITC domain

To prove functionality of the αFITC scFv domain, the interaction of αCD40.αFITC^{CMV} with a FITC-labeled DNA and ssRNA was verified via analytical SEC and measurement of fluorescence quenching and anisotropy. Figure 16A shows that incubation of αCD40.αFITC^{CMV} with a FITC-labeled 42 mer DNA oligonucleotide results in the formation of a complex that elutes at an earlier volume indicating a higher molecular weight from the Superdex 200 Increase 5/150 GL column compared to the single molecules. As a 2-fold excess of DNA was used, remaining unbound DNA was eluted subsequently to the complex.

For the fluorescence quenching experiment, increasing concentrations of αFITC scFv were incubated with 10 nM of FITC-ssRNA40 leading to a decrease in FITC fluorescence (Figure 16B). In parallel, anisotropy as the inversely related readout increased indicating an interaction of the FITC-ssRNA with the αFITC module. A complete quenching was observed at a 4-fold excess of αFITC scFv, which could be due to the presence of some non-functional protein. When the assay was performed by replacing FITC-ssRNA40 with FITC-LPS, the same results were obtained (data not shown).

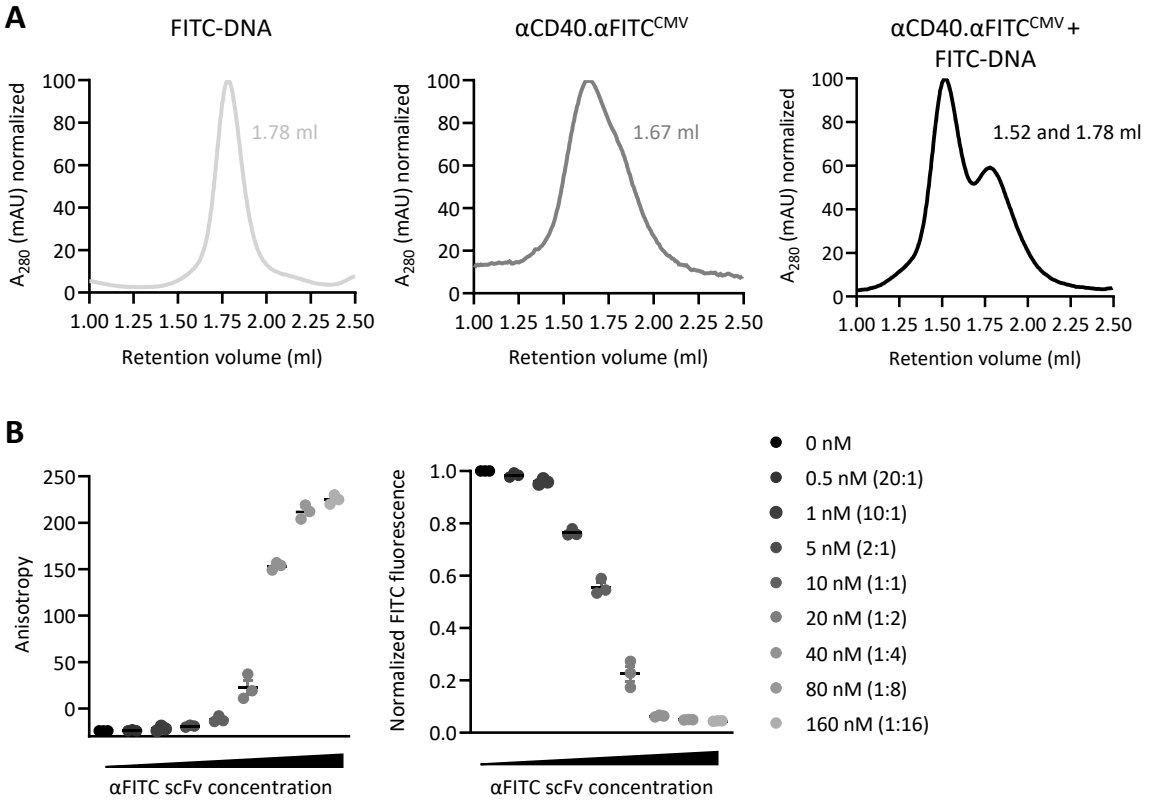

Figure 16. Interaction studies of αCD40.αFITC^{CMV} and FITC-coupled nucleic acids.
(A) 30 µg of αCD40.αFITC^{CMV}, a FITC-labeled 42 mer DNA or the complex of both were run over a Superdex 200 Increase 5/150 GL column. The complex formation of αFITC scFv together with the FITC-DNA was proven by eluting at a lower volume compared to the single molecules. **(B)** 10 nM of FITC-ssRNA40 were incubated with different concentrations of αCD40.αFITC^{CMV} (n = 3). Subsequently, fluorescence and anisotropy were measured. A concentration-dependent decrease of FITC fluorescence (quenching) as well as increase of anisotropy with escalating concentrations of αFITC scFv was detected.

4.2. Binding and internalization

4.2.1. Binding to primary DCs and L-428 cells

MACs were designed to bind to the DC surface marker CD40 and thereby bringing both a TLR agonist and a protein-specific antigen domain to the cell. To evaluate the binding properties of the αCD40-targeting module, the interaction of the molecules in saturating concentrations with the CD40-expressing L-428 cell line, but also with primary iDCs and mDCs matured using a TLR8 agonist-containing cytokine cocktail was investigated by flow cytometry-based binding studies (Figure 17). For this and further functional assays, αHer2 scFv or IgG1 antibodies served as non-specific binding controls since the L-428 cell line and DCs are Her2 negative. Figure 17A-B shows representative histograms of the molecules on the different cell types, Figure 17C-D displays mean MFI ratios on iDCs from different donors. All molecules bound specifically to CD40 on L-428 cells and DCs with higher binding to mDCs compared to iDCs as a result of CD40 upregulation during the maturation process. The insertion of the flagellin domain did not alter binding of the IgG1 format molecule on DCs and L-428 cells. However, αCD40.Flg.αFITC^{CMV} showed reduced MFI ratios on both cell types compared to αCD40.αFITC^{CMV} maybe due to steric hindrance of flagellin and the CD40-binding site.

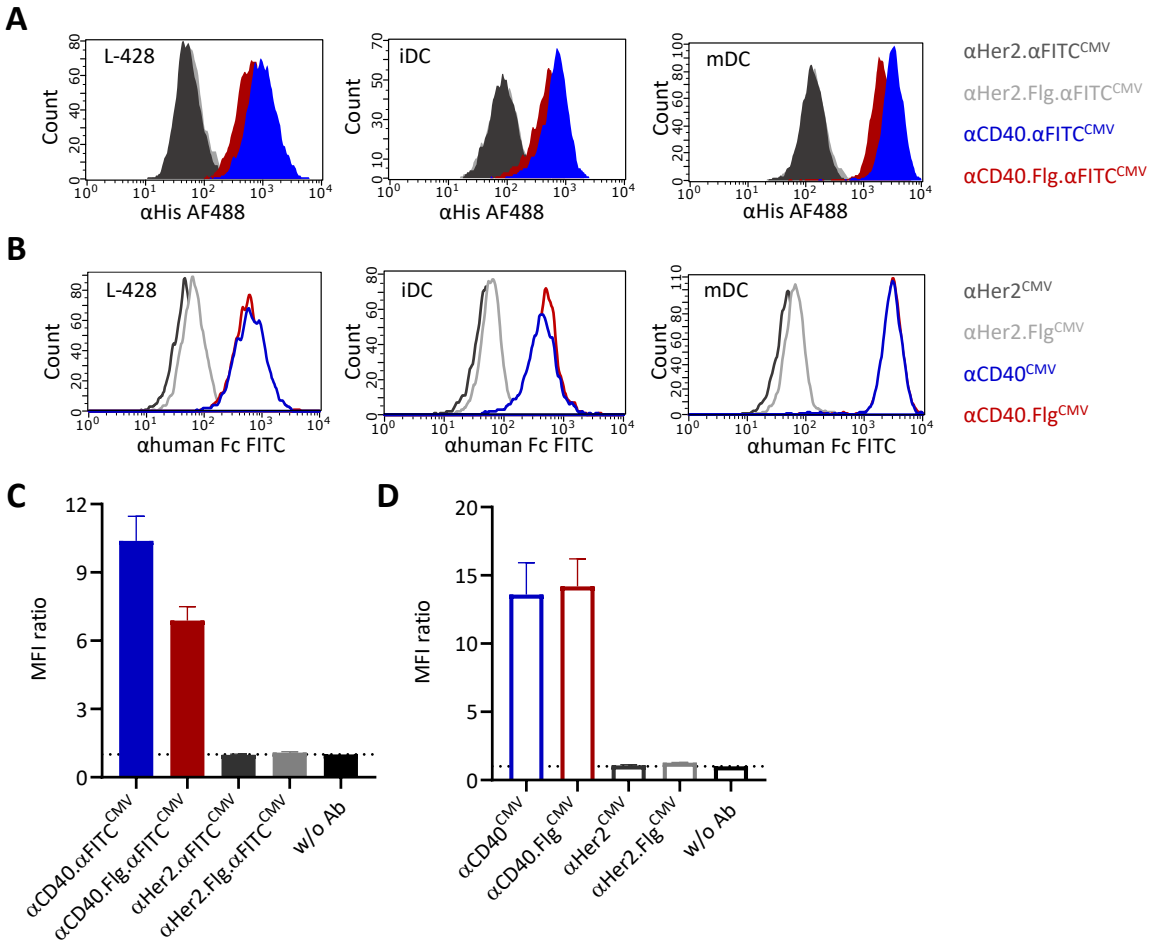

Figure 17. Binding analysis of MACs to L-428 cells and primary iDCs and mDCs.
(A, B) Representative histograms of scFv- **(A)** and IgG1-based antibodies **(B)** to iDCs, mDCs and L-428 cells. Binding was detected by flow cytometry using either a FITC-conjugated αhuman Fc secondary antibody or an AF488-labeled αHis secondary antibody. They grey line shows non-specific stainings of the αHer2 non-specific binding controls. **(C, D)** Binding of scFv- **(C)** and IgG1-format **(D)** MACs to iDCs from different donors. Mean fluorescence intensity (MFI) ratio was calculated by dividing the specific antibody staining by the non-specific staining of the secondary antibody. Graphs display mean of $n = 4$ different donors with SEM as error bars.

4.2.2. K_D determination via flow cytometry and surface plasmon resonance

After describing the ability of MACs to bind to L-428 cells, a quantitative characterization of the binding strength of αCD40.αFITC^{CMV}, αCD40.Flg.αFITC^{CMV}, αCD40^{CMV} and αCD40.Flg^{CMV} was performed using two techniques. For the flow cytometry-based method, L-428 cells were incubated with increasing antibody concentrations up to saturation and binding was quantified. Subsequently, the dissociation constants (K_D) were determined as an affinity or avidity measurement (Figure 18A and Table 16). As expected, a higher dissociation constant was detected for IgG1 format molecules compared to the scFv format probably based on bivalent binding. In correlation with the previously shown binding data, the fusion of flagellin domains reduced the affinity of the αCD40 scFv, but slightly lowered the dissociation constant for the IgG1 format molecule indicating better binding.

As a more precise method to determine affinity including association and dissociation kinetics, an SPR analysis was performed for αCD40^{CMV} and αCD40.Flg^{CMV} (Figure 18B and Table 16). Besides detecting a fast on-rate and off-rate, mean K_D values of 27.4 nM were obtained for αCD40^{CMV} and 21.8 nM for

αCD40.Flg^{CMV}. These values were slightly higher not only compared to the flow cytometry-based affinity determination, but also in comparison to the K_D of this αCD40 clone published in a patent.[248] This might be explained by methodological differences of the SPR analyses. Additionally, the binding kinetics of the clone were investigated in an IgG2 backbone in the patent.

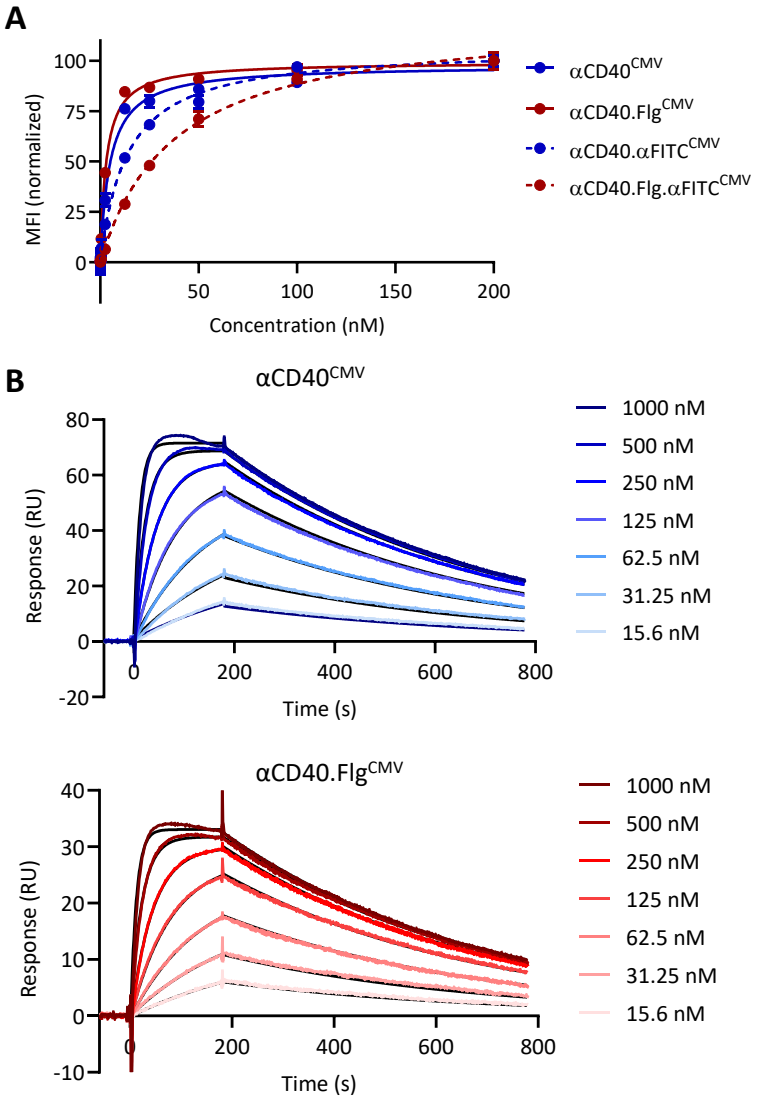

Figure 18. K_D measurements of MACs via flow cytometry and Biacore.
(A) Concentration-dependent binding of αCD40.αFITC^{CMV}, αCD40.Flg.αFITC^{CMV}, αCD40^{CMV} and αCD40.Flg^{CMV} on L-428 cells was analyzed by flow cytometry. Graphs show mean values of *n* = 3 independent experiments with SEM as error bars. **(B)** Representative SPR profiles for αCD40^{CMV} and αCD40.Flg^{CMV}. Colored curves represent raw data and black curves fitted data. An αhuman Fc antibody was coated onto a CM5 chip to immobilize αCD40 IgG1 antibodies. As an analyte the extracellular domain of CD40 was injected and the concentration-dependent on- and off-rates were determined.

Table 16. K_D values measured by flow cytometry and SPR (Mean ± SD of *n* = 3 experiments)

Molecule	K_D (flow cytometry)	K_D (SPR)
αCD40.αFITC^{CMV}	13.3 ± 1.3 nM	n.d.
αCD40.Flg.αFITC^{CMV}	37.2 ± 2.1 nM	n.d.
αCD40^{CMV}	4.9 ± 0.3 nM	27.4 ± 3.7 nM
αCD40.Flg^{CMV}	3.0 ± 0.2 nM	21.8 ± 1.1 nM

n.d. = not determined

4.2.3. CD40-dependent internalization

To ensure processing of the attached antigenic peptide in the endosome, MACs need to internalize after binding their cognate receptor. The uptake of CD40 upon bivalent binding of antibodies has already been investigated.[116, 258] To study the intracellular translocation of this αCD40 clone and to detect possible differences between the IgG1- and scFv-format molecules, we incubated moDCs at different temperatures with directly AF594-labeled αCD40.αFITC^{CMV} and αCD40^{CMV}, respectively, and co-stained with the expected cellular compartment. As target cells both iDCs and mDCs were compared, as it was generally assumed that all forms of endocytosis are downregulated during the maturation process.[259] Incubation of DCs with AF594-labeled antibodies at 4°C resulted in distinct cell membrane labeling, as indicated by a co-localization with HLA-DR on the cell surface (Figure 19). At 37°C, internalization of αCD40 IgG1 and scFv molecules was detected within 1.5 hours. It turned out that αCD40 specifically translocated to EEA1^{+} early endosomal compartments, but not to late endosomes. With this, results by Chatterjee et al. could be confirmed.[116] Furthermore, no obvious difference was observed between iDCs and mDCs, which is in line with published results by Platt et al.[259] Both maturation stages equally internalized the antibodies, even if a higher CD40 surface staining is observed for mature DCs as expected. Therefore, in our hands, DC maturation did not obviously affect the level of receptor-mediated endocytosis. For all conditions only a minimal amount of non-specific internalization occurred for the αHer2 non-specific binding controls (data not shown).

We tested in a next step whether the FITC-labeled ssRNA40, that is derived from HIV and ligates intracellular TLR8, is taken up in complex with αCD40.αFITC^{CMV} into early endosomes.[164] Since the FITC fluorescence was shown to be quenched after interacting with the αFITC scFv, another Atto488 fluorescence tag was added to the 3´end of the RNA. Figure 20 shows the internalization of FITC-ssRNA40-Atto488 together with AF594-αCD40.αFITC^{CMV} that were pre-incubated for 30 min to facilitate complex formation. Indeed, ssRNA40 was taken up and co-localized with the αCD40 antibody. If pre-incubated with the αHer2.αFITC^{CMV} non-specific binding control, less spots of fluorescent ssRNA40 could be found intracellularly.

This leads to the conclusion that CD40-targeting MACs not only bound specifically to CD40 and were internalized upon binding into early endosomes, but they could also function as a carrier for a FITC-labeled TLR agonist via the αFITC scFv.

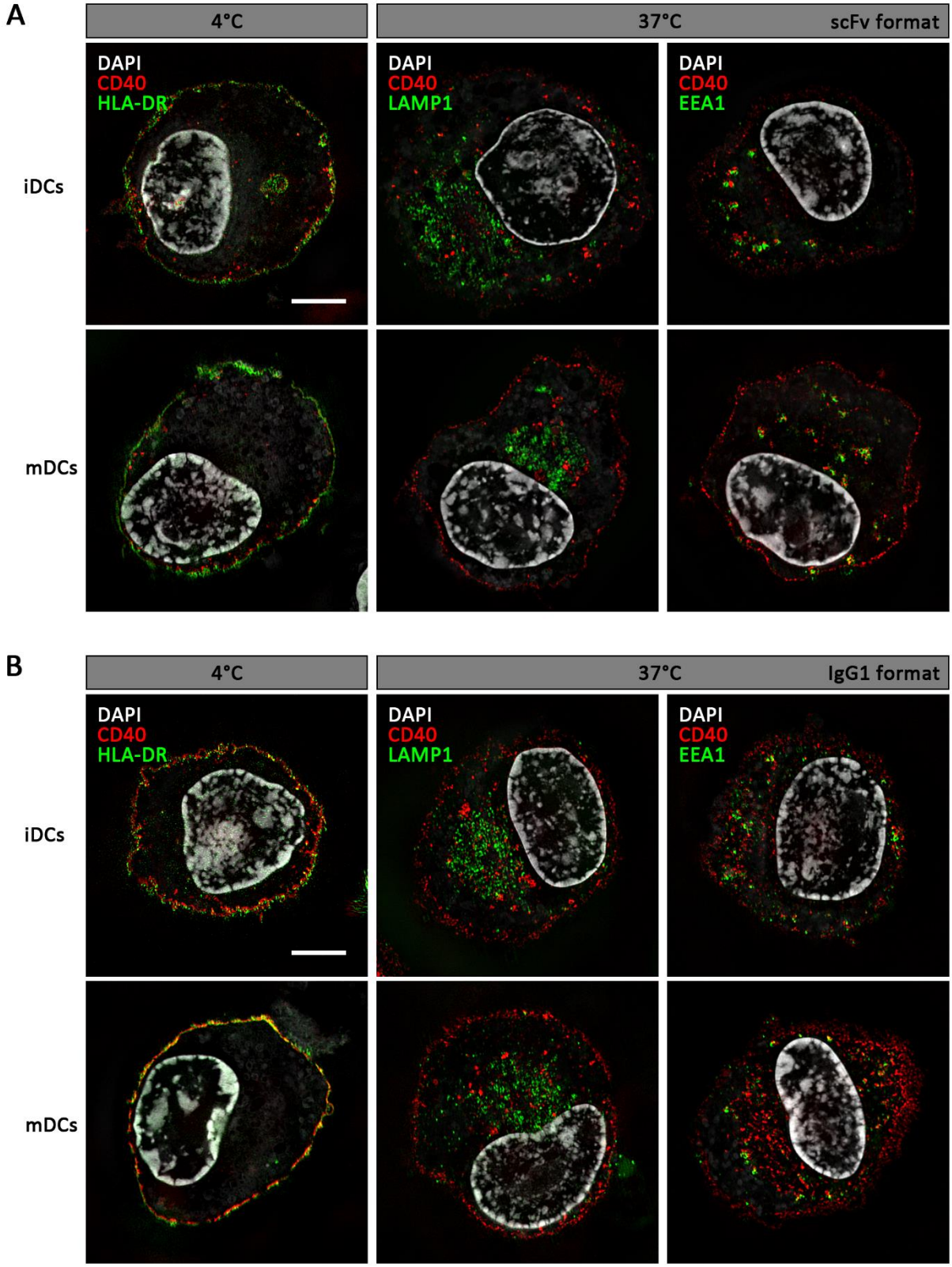

Figure 19. CD40-dependent internalization of αCD40.αFITC^{CMV} and αCD40^{CMV}.
Internalization of AF594-labeled αCD40.αFITC^{CMV} scFv **(A)** and αCD40^{CMV} IgG1 **(B)** after incubation for 1.5 h with iDCs and mDCs at 4°C and 37°C. At 4°C co-staining of the membrane with αHLA-DR-AF488 was performed. At 37°C the early endosomes were visualized by αEEA1 and late endosomes by αLAMP1 that were both detected by an AF488-labeled donkey αrabbit secondary antibody. All conditions were counterstained with DAPI. The scale bar represents 5 µM. The acquisition was performed in the laboratory of Prof. Heinrich Leonhardt.

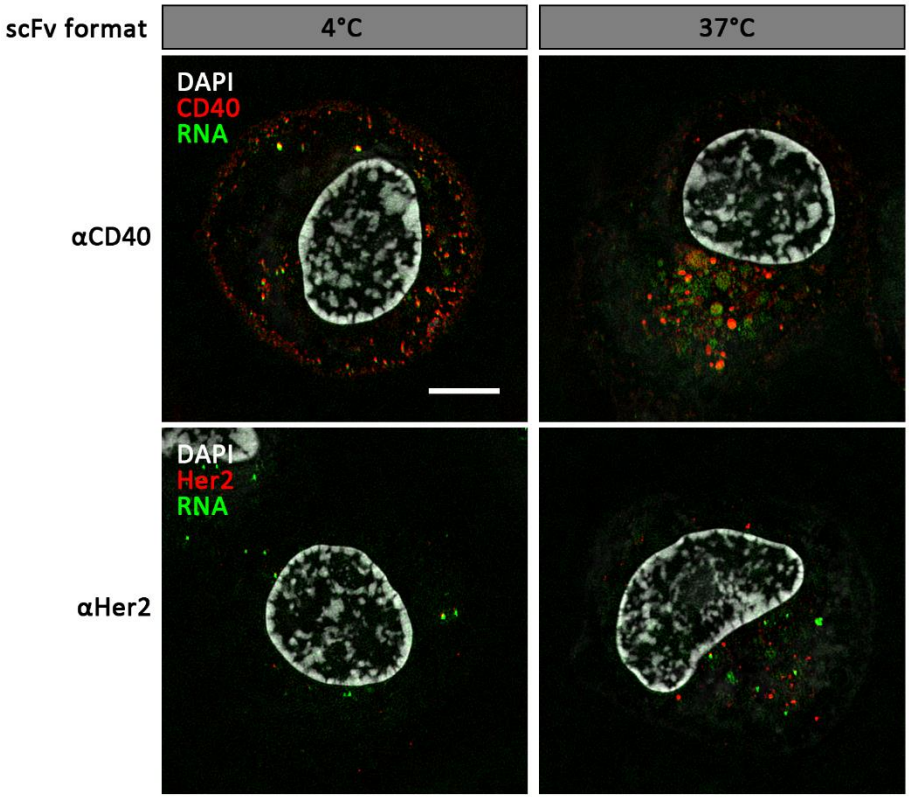

Figure 20. Internalization of FITC-ssRNA40-Atto488 together with AF594-labeled αCD40.αFITC^{CMV} and αHer2.αFITC^{CMV}
AF594-αCD40.αFITC^{CMV} or the αHer2 non-specific binding control were pre-incubated for 30 min with FITC-ssRNA40-Atto488 at 37°C, followed by incubation for 1.5 h at 4°C or 37°C on iDCs. All conditions were counterstained with DAPI und the green fluorescence intensity was adjusted on an equal level to compare different conditions. The scale bar represents 5 µM. The acquisition was performed in the laboratory of Prof. Heinrich Leonhardt.

4.3. Functional characterization of MACs

4.3.1. Induction of DC maturation

The αCD40 antibody CP-870,893 used in thesis has been shown to bind agonistically to CD40 in its parental IgG2 backbone.[247, 248] Therefore, we expected that maturation of DCs is already induced by the αCD40 binding module itself and is further enhanced by the ligation of TLRs.

The agonistic activity of the antibody was confirmed and potential differences between scFv- and IgG1-format molecules investigated. For this, iDCs were incubated with αCD40.αFITC^{CMV} and αCD40^{CMV} or the respective non-specific binding controls and screened for the upregulation of maturation markers as well as for IL-6 secretion. TLR8 agonist-matured mDCs were included as positive control. The scFv-based αCD40.αFITC^{CMV} antibody led to a significant increase in CD80, CD83 and CD86 expression and the secretion of IL-6 compared to the αHer2 control (Figure 21A). αCD40^{CMV} induced a much lower, but still significant upregulation of maturation markers on the DC surface (Figure 21B). Thus, without further stimulation by TLR agonists, αCD40.αFITC^{CMV} in the scFv format shows a clear benefit over αCD40^{CMV} in the IgG1 format.

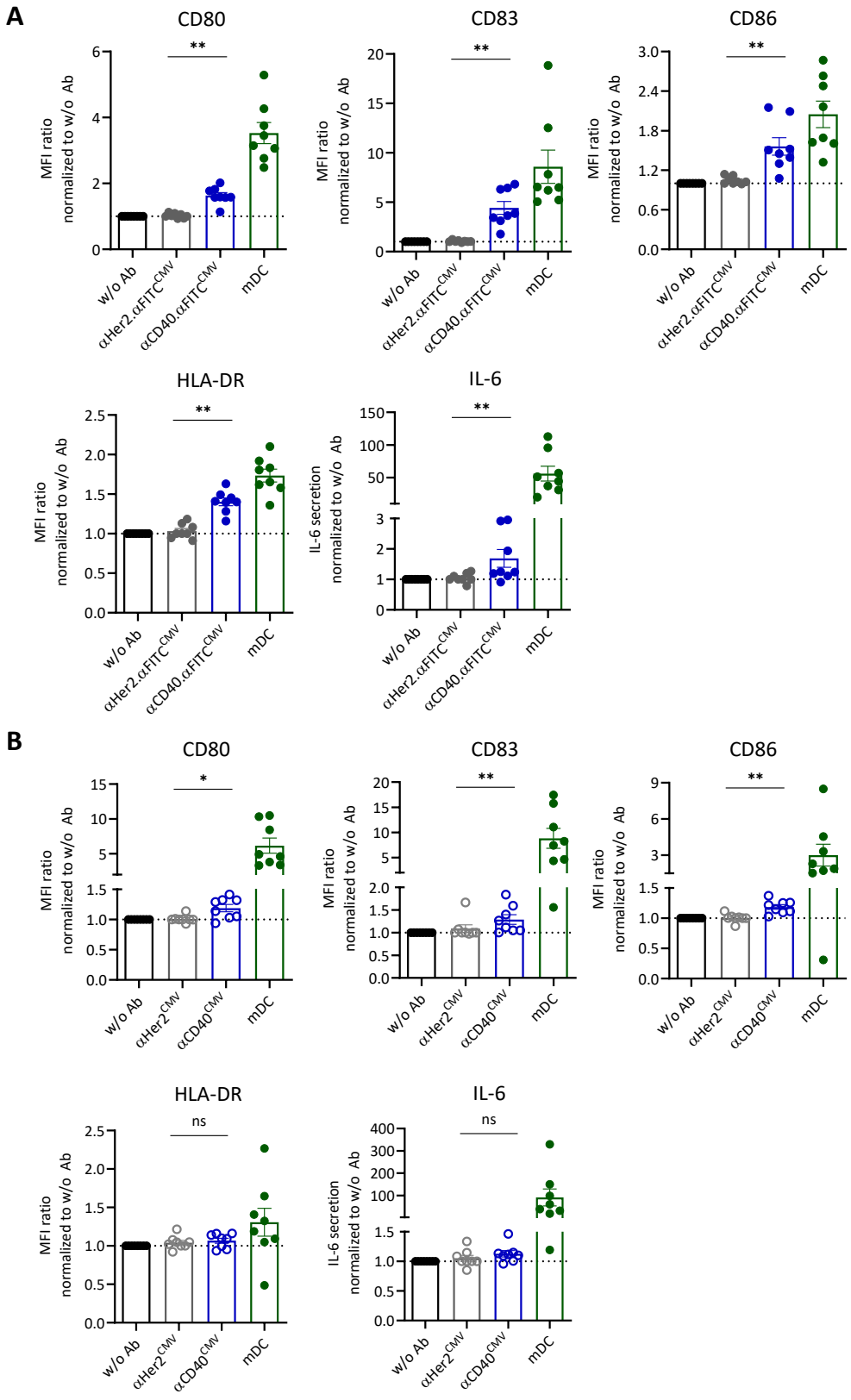

Figure 21. Effect of αCD40.αFITC^{CMV} scFv and αCD40^{CMV} IgG1 on DC maturation markers and cytokine secretion.
iDCs were incubated with αCD40.αFITC^{CMV} **(A)** or αCD40^{CMV} IgG1 **(B)** for 24 h. ● refers to scFv format, ○ to IgG1 format. DCs were analyzed based on surface expression of CD80, CD83, CD86 and HLA-DR as well as IL-6 cytokine secretion into the supernatant by flow cytometry. Surface markers and cytokine secretion are normalized to the control without antibody addition (w/o Ab). The graphs show means of *n* = 8 different donors with SEM as error bars. For statistical analysis, a Wilcoxon-signed rank test was applied.

Next, the stimulatory potential of fused TLR agonists was analyzed. To this end, iDCs were treated with αCD40.αFITC^{CMV} with (FITC)-LPS and (FITC)-ssRNA40 or with flagellin fusion molecules (αCD40.Flg^{CMV} and αCD40.Flg.αFITC^{CMV}). Maturation marker expression and cytokine secretion induced by all mentioned molecules were compared with αCD40.αFITC^{CMV} and αCD40^{CMV} without TLR ligation and the respective αHer2 controls to identify potential beneficial effects of TLR fusions.

For FITC-LPS, all maturation markers were highly upregulated and an elevated IL-6 secretion was measured independent of the combination with αCD40.αFITC^{CMV} or with αHer2.αFITC^{CMV} (Figure 22A). This indicates that TLR4 ligation by LPS saturates DC activation, which is not further boosted by a second stimulus given through the agonistic αCD40 scFv. No difference was seen between FITC-LPS and LPS in the low amounts of donors tested (data not shown). This could be expected as LPS binds to extracellular TLR4 independent of the MAC fusion.

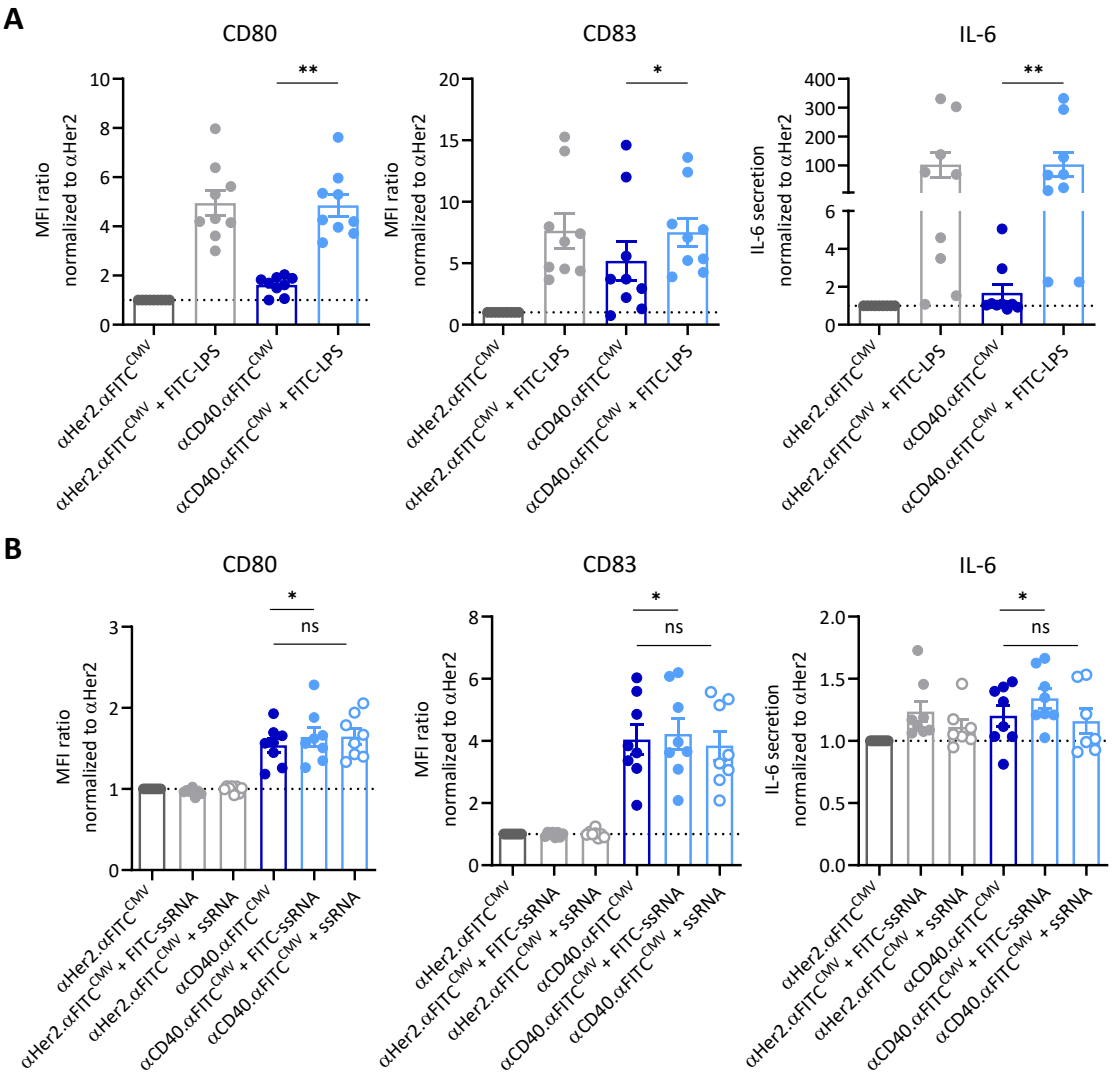

Figure 22. Effect of TLR ligation by FITC-LPS and (FITC-)ssRNA40 in combination with αCD40.αFITC^{CMV} on DC maturation markers and cytokine secretion.
iDCs were incubated with αCD40.αFITC^{CMV} or the αHer2 control for 24 h that had been pre-incubated with equimolar amounts of FITC-LPS **(A)** and (FITC-)ssRNA40 **(B)** for 30 min at 37°C for complex formation. ● refers to FITC-ssRNA40, ○ to unlabeled ssRNA40. DCs were analyzed based on surface expression of CD80 and CD83 as well as IL-6 cytokine secretion into the supernatant by flow cytometry. MFI ratios of surface markers and cytokine secretion are normalized to the αHer2 control. The graphs show means of *n* = 7-9 different donors with SEM as error bars. For statistical analysis, a Wilcoxon-signed rank test was applied.

The coupling of HIV-derived FITC-ssRNA40 to αCD40.αFITC^{CMV} induced a low, but significant increase in CD80 and CD83 expression as well as IL-6 secretion (Figure 22B). The addition of ssRNA40, that is not fused to αCD40.αFITC^{CMV} and might be taken up independently from the antibody, did not bring a clear beneficial effect over αCD40.αFITC^{CMV}. The overall stimulating effects of ssRNA40, however, were quite low compared to the TLR4 stimulation by LPS.

To prove that the FITC-ssRNA40 is also effective, its functionality was tested by complexing the RNA with poly-L-arginine (PLA) to facilitate the endosomal transport.[260] In fact, incubation of FITC-ssRNA40 with PLA in complex with αCD40.αFITC^{CMV} prior to adding it to the DCs increased CD80 expression in relation to αCD40 scFv only (Figure 23A). This uptake, however, was mostly independent of αCD40.αFITC^{CMV}, since the PLA-complexed ssRNA40 in combination with the αHer2 non-specific binding control led to a similar DC activation. These results indicate that low effects of the ssRNA40 in combination with αCD40.αFITC^{CMV} on DC maturation as seen in Figure 22B could be based on an insufficient uptake of the ssRNA40 by the DCs.

A further attempt was made to facilitate the uptake of the ssRNA by cloning a HIV TAT protein-derived cell-penetrating peptide (CPP) sequence C-terminally of the αFITC scFv, thereby introducing a hydrophobic patch.[246] Unfortunately, this attempt didn't show any benefit (Figure 23B). In addition, these molecules were not able to induce any T cell response (data not shown). That's why, we continued with the ssRNA40 to see whether the low increase in DC maturation was sufficient to enhance T cell responses in next experiments.

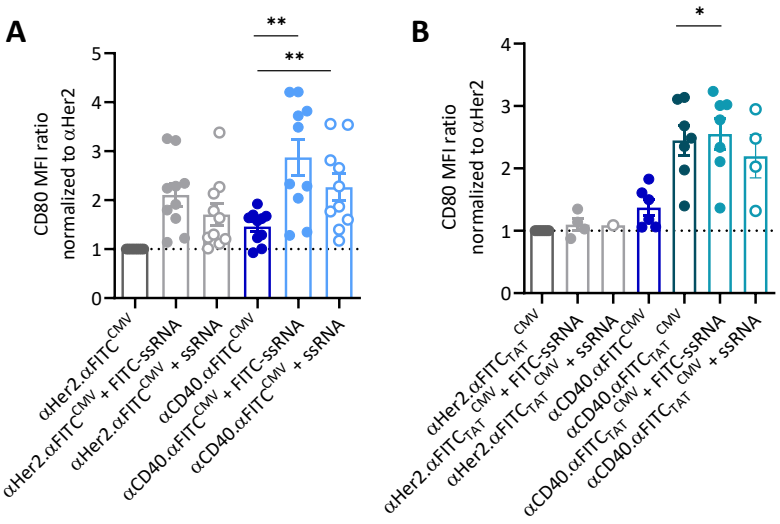

Figure 23. Attempts to facilitate ssRNA40 uptake into the DCs.
(A) (FITC-)ssRNA40 was complexed with PLA in combination with αCD40.αFITC^{CMV} or αHer2.αFITC^{CMV} prior to adding it to the cells. **(B)** A CPP sequence derived from the HIV TAT protein was introduced C-terminally of the αFITC scFv. ● refers to FITC-ssRNA, ○ to unlabeled ssRNA. CD80 expression was determined by flow cytometry. MFI ratios are normalized to the αHer2 control. The bars show means of *n* = 1-12 different donors with SEM as error bars. For statistical analysis, a Wilcoxon-signed rank test was applied.

Fusing the D0/D1 domain of flagellin to αCD40.αFITC^{CMV} induced a significant upregulation of DC maturation markers as well as enhanced IL-6 secretion compared to the αCD40 control (Figure 24A). To investigate TLR5-specific effects, the two mutations R90A and E114A were introduced (mFlg) that are shown to highly reduce binding to TLR5.[261, 262] As expected, mutated flagellin elicited a lower DC maturation state compared to molecules with wild-type flagellin. The R90A single mutant was not able to reduce DC maturation (data not shown). In addition, αHer2.Flg.αFITC^{CMV} increased CD80, CD83

expression and IL-6 secretion compared to αHer2.αFITC^{CMV}. As the non-specific binding control itself was not able to bind DCs, the effects of αHer2.Flg.αFITC^{CMV} on DC maturation indicated the stimulatory potential of flagellin that was activating DCs to a similar extent as the αCD40.αFITC^{CMV}. In line with the scFv results, flagellin fusion to αCD40^{CMV} upregulated surface maturation markers and enhanced IL-6 secretion, which was lowered by mutating flagellin (Figure 24B). To investigate whether the genetic fusion of αCD40^{CMV} and flagellin maintains functionalities of both fusion partners, we compared the activity of αCD40.Flg^{CMV} to co-administered Fc.Flg and αCD40^{CMV} (Figure 24C). Even if a difference between the two variants was seen for the αHer2 control molecule, treatment with αCD40.Flg^{CMV} and also the combination of Fc.Flg and αCD40^{CMV} led to similar DC maturation states, as reflected by CD80 and CD83 expression. This indicates that fusion of flagellin to αCD40^{CMV} does not greatly affect its interplay with TLR5 and that neither antibody nor flagellin integrity is impaired.

The flagellin fusion molecules were also tested in the scFv format without the αFITC scFv as this would be redundant in this molecule. In this format, the flagellin domain and the CMV-specific peptide were connected by either a G_4S or a $(G_4S)_4$ linker. The αCD40 module was also exchanged by a different DC-targeting binder, namely αDec205, that is not activating the DCs itself. In all of the tested fusion molecules, flagellin-specific TLR5 ligation and DC maturation was achieved as predicted and the flagellin mutant abolished the activating effects (data not shown). However, none of the molecules was able to elicit a T cell response maybe due to suboptimal uptake and therefore processing of the peptide (data not shown). Hence, only the most promising flagellin fusion candidates αCD40.Flg.αFITC^{CMV} and αCD40.Flg^{CMV} were investigated further.

In summary, FITC-LPS highly activated DCs. This was mainly independent of the DC-targeting antibody, since LPS binds TLR4 on the DC surface without the need of being targeted to the DC by interacting with αCD40.αFITC^{CMV} *in vitro*. TLR8 ligation by FITC-ssRNA40 in combination with αCD40.αFITC^{CMV} induced a slightly higher DC maturation compared to uncoupled ssRNA40 due to targeted delivery. However, the beneficial effect of TLR8 ligation compared to αCD40.αFITC^{CMV} without TLR stimulation was low. In further experiments using complexed ssRNA, it turned out that the uptake of FITC-ssRNA40 in combination with the αFITC scFv was not optimal, even if microscopic investigation of ssRNA40 internalization looked promising. The fusion of the TLR5-binding domain of flagellin to the non-targeting αHer2 scFv or IgG1 molecule already activated DCs significantly, which was even further boosted by combining it with the DC-targeting αCD40 binder. The flagellin mutant with impaired TLR5 binding diminished these effects, which indicates a TLR5-specific DC activation mechanism.

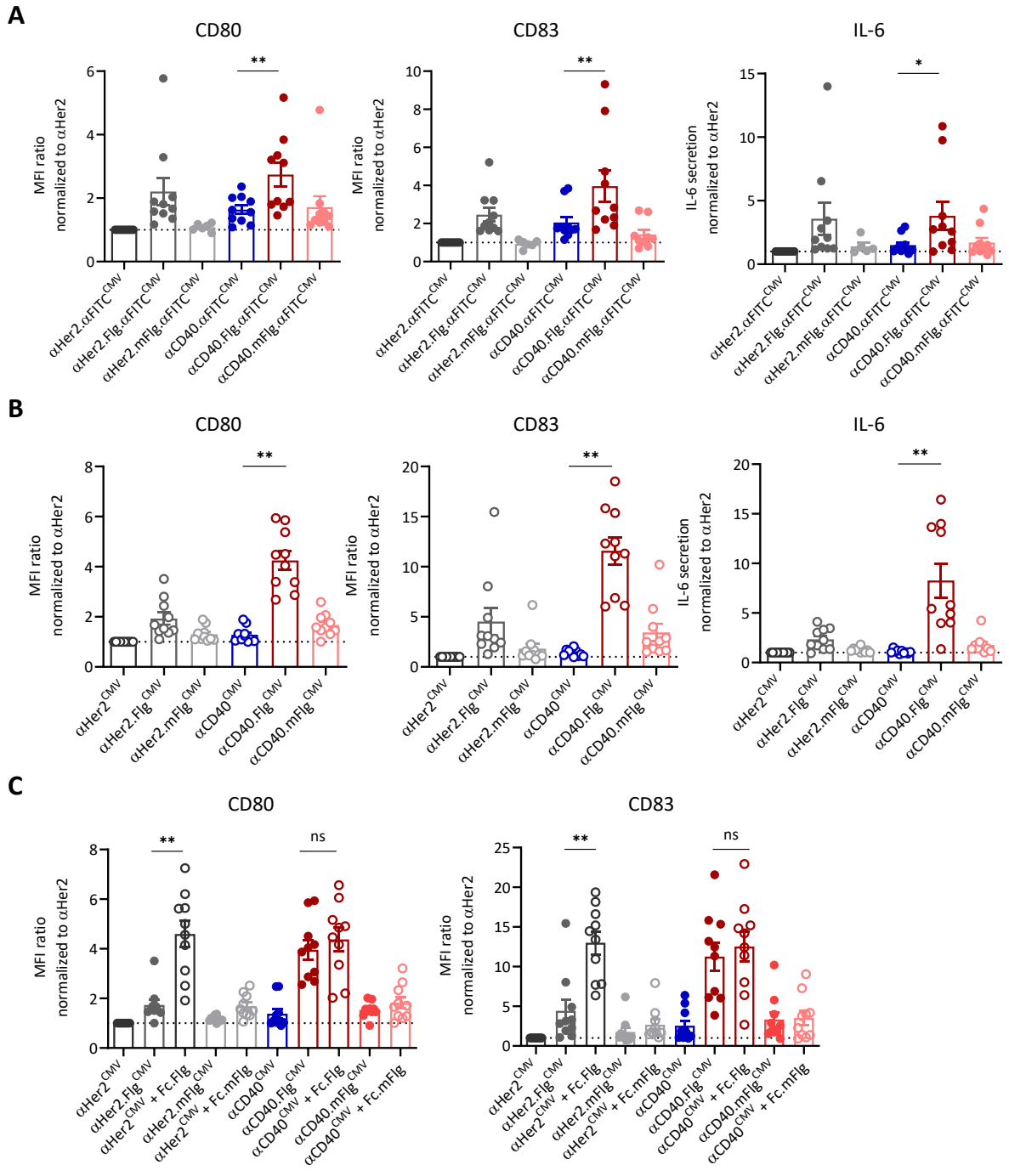

Figure 24. Effect of TLR5 ligation on DC maturation.
iDCs were incubated with αCD40.Flg.αFITC^{CMV} **(A)** or αCD40.Flg^{CMV} **(B)** or their controls without flagellin or mFlg as well as the αHer2 non-specific binding controls. ● refers to scFv format, ○ to IgG1 format. **(C)** Difference between the genetic fusion of flagellin and the co-administration of the DC-targeting antibody and Fc.Flg. iDCs were incubated with the flagellin fusion molecule (αCD40.Flg^{CMV}) or the combination of αCD40^{CMV} and Fc.Flg or the respective controls. ● refers to flagellin fusion molecules, ○ to the combination of αCD40/Her2 and Fc.Flg. Surface maturation marker expression and secretion of IL-6 was determined by flow cytometry. MFI ratios were normalized to the non-specific binding control. The graphs show means of *n* = 10 different donors with SEM as error bars. For statistical analysis, a Wilcoxon-signed rank test was applied.

4.3.2. Interaction with TLR5

As the genetic fusion of flagellin to a DC-targeting antibody construct might interfere with TLR5 interaction, binding to its receptor and the induction of downstream signaling was analyzed. First, flagellin-induced DC maturation was investigated with and without the presence of an IgA2 αTLR5-blocking and neutralizing antibody. To this end, iDCs were preincubated with either the blocking antibody or the isotype control followed by the addition of flagellin fusion molecules or the respective mutants and controls. Figure 25 shows that the flagellin-induced upregulation of DC maturation markers was greatly diminished by the presence of the αTLR5 antibody, but not by the isotype control. Thus, the TLR5 interaction was necessary for the stimulation of DC maturation by αCD40.Flg.αFITC^{CMV}, αCD40.Flg^{CMV} and Fc.Flg.

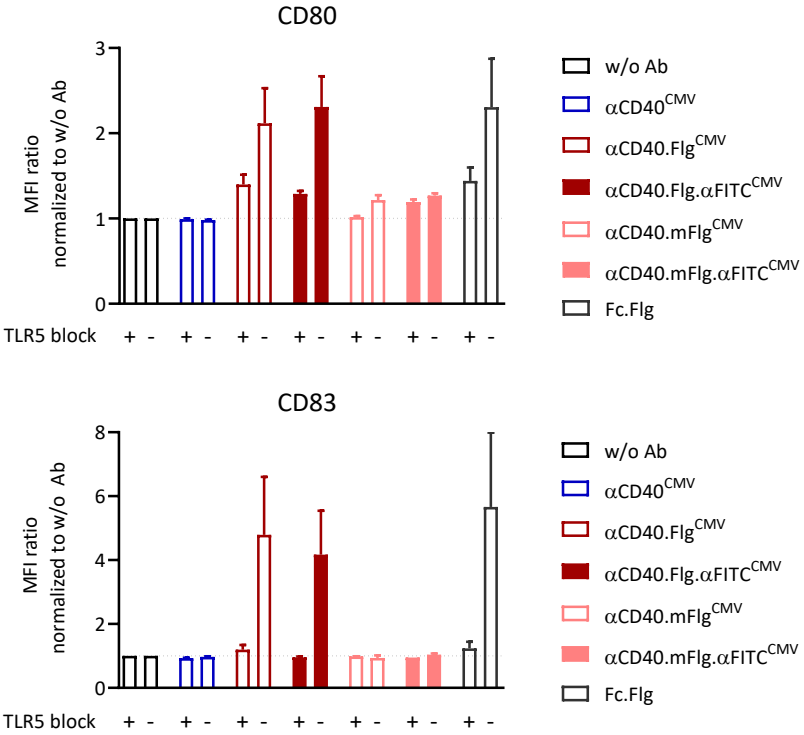

Figure 25. Flagellin-induced DC maturation in presence of an αTLR5-blocking antibody.
iDCs were pre-treated with either an IgA2 αTLR5-blocking antibody or the IgA2 isotype control with subsequent administration of flagellin fusion molecules or controls as indicated. Filled bars refer to scFv format, open bars to IgG1 format. CD80 and CD83 expression was measured on the DC surface by flow cytometry. MFI ratios of surface markers are normalized to the non-specific binding control. The bars show means of *n* = 3 different donors with SEM as error bars.

Second, the activation of TLR5 downstream signaling processes by flagellin fusion molecules was studied. A hTLR5-HEK293 reporter cell line, transiently transfected with human full-length TLR5, was incubated with abovementioned molecules and signal transduction was analyzed based on IL-8 secretion. As expected, αCD40.Flg^{CMV} and Fc.Flg induced TLR5 signaling in a similar extent, whereas αCD40.mFlg.αFITC^{CMV} and αCD40.mFlg^{CMV} did not (Figure 26). Interestingly, αCD40.Flg.αFITC^{CMV} showed a lower IL-8 secretion compared to the IgG1 fusion.

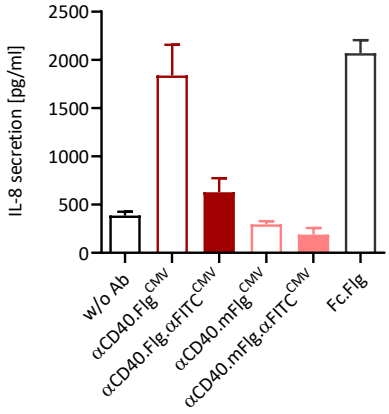

Figure 26. Induction of TLR5 signaling by flagellin fusion molecules.
HEK293-T cells transiently transfected with hTLR5 (hTLR5-HEK293) were incubated with αCD40.Flg^{CMV}, αCD40.mFlg^{CMV} or Fc.Flg or the scFv format flagellin fusions. Filled bars refer to scFv format, open bars to IgG1 format. IL-8 secretion into the supernatants was determined by ELISA. Graph displays mean values of biological duplicates each measured in triplicates. This experiment was performed in the laboratory of Prof. Christine Josenhans.

Taken together, the data show that αCD40.αFITC.Flg^{CMV} and αCD40.Flg^{CMV} molecules enhanced DC maturation by specifically interacting with TLR5 and were able to induce downstream signaling processes.

4.3.3. MAC-mediated CMV$_{NLV}$-specific T cell activation and proliferation

The main goal of this thesis was to generate a DC-based vaccine that specifically brings a peptide as payload to the DC, simultaneously activates an adjacent TLR and thereby induces an efficient peptide-specific T cell response. Here, the ability of αCD40.αFITC^{CMV} and αCD40^{CMV} to induce T cell activation and proliferation was characterized in allogeneic as well as autologous DC–T cell co-culture experiments (Figure 27). The modulation of the T cell response by the addition of different TLR-ligating domains was further investigated.

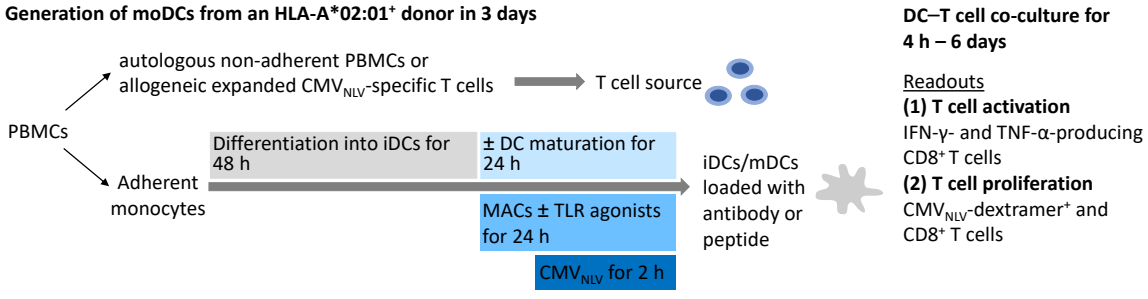

Figure 27. Experimental setup of DCs co-cultivated with autologous non-adherent PBMCs (NACs) or allogeneic expanded CMV$_{NLV}$-specific T cells with readouts for T cell activation and proliferation.

First, we addressed the question of whether the CMV-specific peptide fused to αCD40 is correctly processed into the epitope sequence NLVPMVATV (CMV$_{NLV}$) and subsequently cross-presented on the DC surface via MHC I. iDCs and mDCs from HLA-A*02:01$^+$ donors that were pre-loaded with αCD40.αFITC^{CMV}, the αHer2 control (Figure 28A) or with the respective IgG1 format molecules (Figure

28B) were cultivated with allogeneic expanded CMV_{NLV}-specific $CD8^+$ T cells. To rule out the possibility of T cell activation triggered by αCD40-mediated DC maturation and to confirm antigen specificity, an αCD40 antibody coupled with a non-stimulating peptide was included as a control (αCD40.αFITC^{mNPM1} and αCD40^{mNPM1}, vide infra). T cell functionality was validated by pulsing the DCs with the already processed CMV_{NLV} peptide. Subsequently, T cell activation was measured by intracellular IFN-γ and TNF-α staining of $CD8^+$ T cells via flow cytometry. Independent of the molecule formats and the DC maturation state, αCD40.αFITC^{CMV}- and αCD40^{CMV}-loaded iDCs and mDCs elicited significantly higher T cell activation compared to those treated with the αCD40 and αHer2 controls. As expected, the highest secretion of proinflammatory cytokines was achieved for the CMV_{NLV}-peptide pulsed DCs as a positive control that were loaded with the peptide from the outside without requiring internalization and processing. This proved the specificity of the T cell population. Notably, the αHer2 non-specific binding controls induced a low amount of non-specific T cell activation especially if loaded on mDCs.

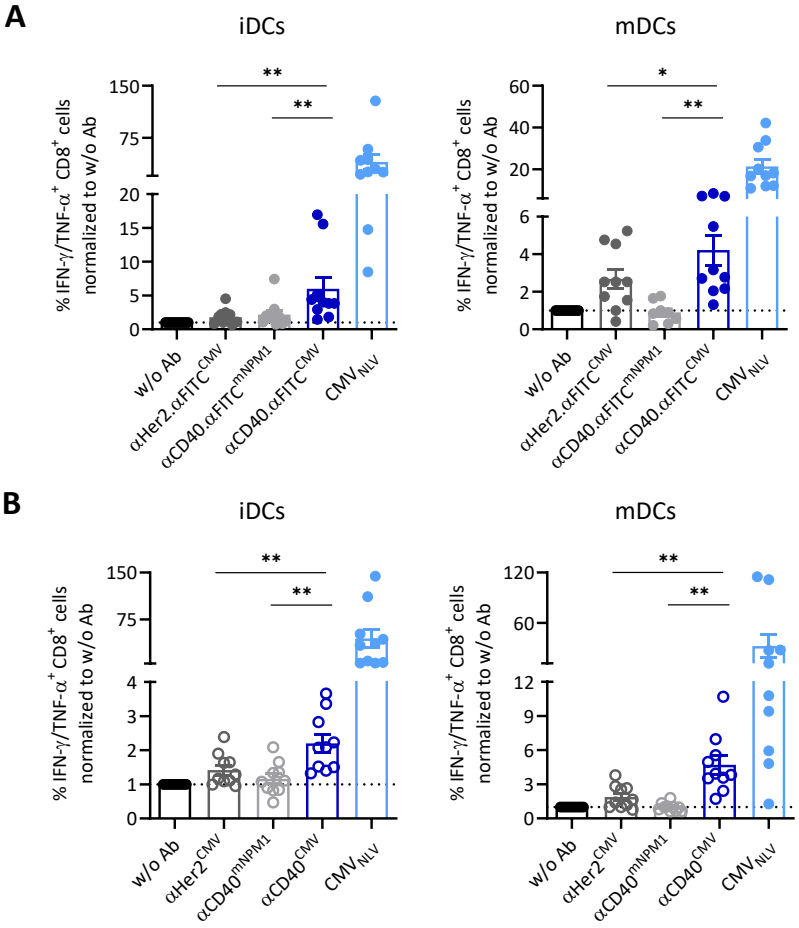

Figure 28. Co-culture of allogeneic CMV_{NLV}-specific T cells and HLA-A*02:01$^+$ iDCs or mDCs pre-incubated with MACs or peptide.

DCs were loaded with αCD40.αFITC^{CMV} scFv **(A)** and αCD40^{CMV} IgG1 **(B)**, αCD40 conjugated to a control peptide (αCD40.αFITC^{mNPM1} or αCD40^{mNPM1}), the αHer2 controls or the processed CMV_{NLV} peptide prior to starting the cultivation. As a readout for T cell activation, IFN-γ- and TNF-α-producing $CD8^+$ T cells were quantified by flow cytometry. ● refers to scFv format, ○ to IgG1 format. T cell activation is normalized to w/o Ab. Bars show means of *n* = 8-12 donors with SEM as error bars. For statistical analysis, a Wilcoxon-signed rank test was applied.

We next analyzed, whether T cells were stimulated sufficiently to proliferate after encountering CMV_{NLV}-presenting DCs (Figure 29). The non-adherent PBMC fraction (NACs) of an HLA-A*02:01$^+$ donor with previous CMV infection was co-cultured with autologous DCs. The DCs were pre-loaded with the molecules as described for the allogeneic setting. CMV_{NLV}-specific dextramer staining was performed to determine the number of expanded CMV_{NLV}-specific T cells. In line with the results obtained for allogeneic co-cultures, αCD40.αFITC^{CMV}- and αCD40^{CMV}-loaded iDCs and mDCs led to a significantly higher CMV_{NLV}-specific T cell proliferation compared to the control molecules in the autologous setting. Again, mDCs loaded with the αHer2 non-specific binding controls triggered some amount of T cell proliferation. T cells proliferated the most after interaction with CMV_{NLV}-loaded DCs. Thus, the αCD40 IgG1 and the scFv format molecules were able to induce a T cell response, but αCD40.αFITC^{CMV} was more potent in delivering the peptide to DCs.

Taken together, targeting CMV peptides to DCs via αCD40 leads to correct antigen processing and cross-presentation and induces activation and proliferation of antigen-specific T cells. Consistent with previous results, this validates the targeting approach and confirms a functional molecule design.

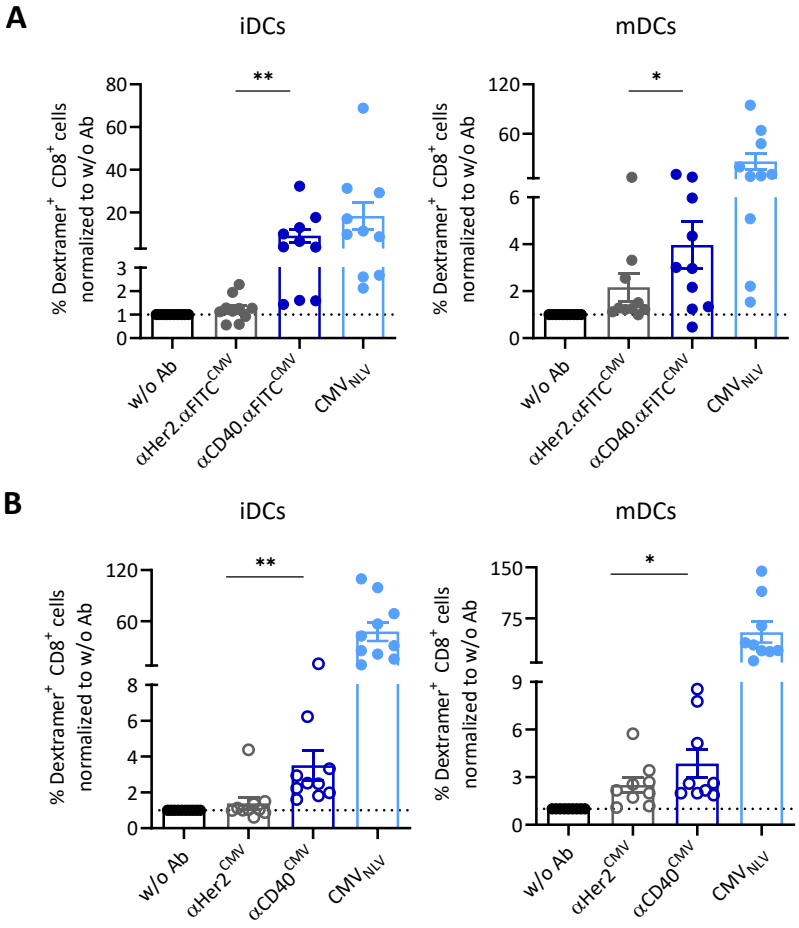

Figure 29. Co-culture of autologous NACs of an HLA-A*02:01$^+$ and CMV$^+$ donor and iDCs or mDCs pre-incubated with MACs or peptide.

DCs were loaded with αCD40.αFITC^{CMV} scFv **(A)** and αCD40^{CMV} IgG1 **(B)**, the αHer2 controls or the processed CMV_{NLV} peptide prior to starting the cultivation. T cell proliferation was determined by CMV_{NLV}-specific dextramer staining of CD8$^+$ T cells and measured by flow cytometry. ● refers to scFv format, ○ to IgG1 format. T cell proliferation is normalized to w/o Ab. Bars show mean values of *n* = 10 donors with SEM as error bars. For statistical analysis, a Wilcoxon-signed rank test was applied.

In the following, different TLR agonists as adjuvants were fused to αCD40.αFITC^{CMV} and αCD40^{CMV} to identify which antibody–adjuvant combination is most beneficial for cross-presentation and T cell activation *in vitro*. Autologous and allogeneic co-culture experiments were performed as previously described. In addition to the molecules without TLR ligation, iDCs were pre-incubated with antibody–adjuvant fusion molecules to identify the impact of TLR stimulation on T cell responses and to investigate potential differences between TLR agonists.

In contrast to results expected from the DC maturation data, the combination of αCD40.αFITC^{CMV} with FITC-LPS to target TLR4 on the DC surface led to a slight reduction of the T cell response compared to αCD40.αFITC^{CMV} as indicated by a diminished cross-presentation and T cell proliferation (Figure 30A).

Intracellular TLR8 was ligated by ssRNA40. In this setting, the fusion of the RNA to the antibody is exceptionally important for *in vitro* experiments, as this directs the ssRNA40 specifically to early endosomal compartments as shown by microscopy. The combination of αCD40.αFITC^{CMV} with FITC-ssRNA40 induced a significantly higher T cell proliferation compared to αCD40.αFITC^{CMV}, which could not be achieved by uncoupled ssRNA40 (Figure 30B). By using allogeneic CMV$_{NLV}$-specific cells as T cell source, a slight, but not significant increase of T cell activation could be detected as well. This was surprising as previous data showed that FITC-ssRNA40 was only able to induce a minimal amount of DC maturation due to an inefficient uptake into the endosomes. However, the activity of ssRNA40 seems to be enough to induce a T cell response. As the RNA uptake could be boosted by the complexation with PLA, which leads to intracellular translocation of the ssRNA40 independent of the antibody, the combination of PLA, ssRNA40 and antibody was also tested in the T cell readouts (Figure 30C). Surprisingly, if higher amounts of ssRNA40 were present in the endosome to interact with TLR8, the beneficial effect of the ssRNA40 was lost since αCD40.αFITC^{CMV} plus PLA-complexed FITC-ssRNA40 did not induce a higher T cell response compared to αCD40.αFITC^{CMV}.

Extracellular TLR5 was ligated by fusing flagellin either C-terminally of an αCD40 scFv (αCD40.Flg.αFITC^{CMV}) or to the αCD40 heavy chain (αCD40.Flg^{CMV}). Even if αCD40.Flg.αFITC^{CMV} and αCD40.Flg^{CMV} were able to induce similar DC maturation states, functional T cell assays revealed differences between both formats. When conjugated to the αCD40 scFv antibody, the flagellin domain impaired the T cell response (Figure 31A). However, αCD40.Flg^{CMV} in the IgG1 format significantly enhanced cross-presentation and T cell proliferation compared to αCD40^{CMV} that was dampened by the flagellin mutant (Figure 31B). In line with the maturation data, no difference in T cell response was observed between αCD40.Flg^{CMV} and co-administered αCD40^{CMV} and Fc.Flg highlighting that coupling of the activating flagellin domain to the DC-targeting αCD40 antibody does not alter its functionality (Figure 31C).

Taken together, αCD40.Flg^{CMV} was rated as the most promising molecule that combined the benefit in peptide cross-presentation and T cell activation together with a potent DC maturing activity in one molecule.

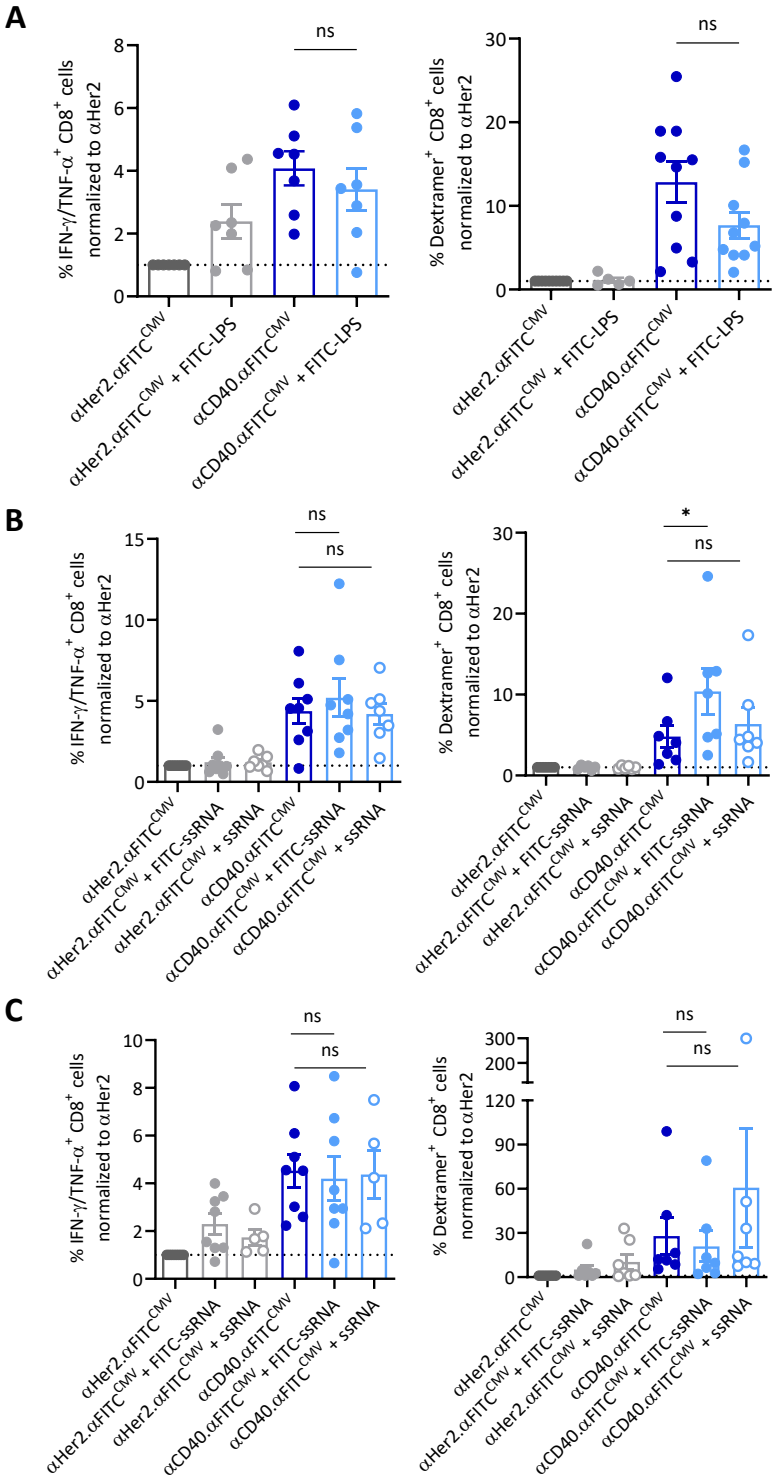

Figure 30. Co-cultures of allogeneic or autologous T cells and iDCs loaded with αCD40.αFITC^{CMV} and simultaneous co-administration of TLR4 and 8 ligands.

Autologous and allogeneic co-cultures were performed as previously described. iDCs were incubated with αCD40.αFITC^{CMV} or the non-specific binding control and stimulated by FITC-LPS **(A)**, (FITC-)ssRNA40 **(B)** or by PLA-complexed (FITC-)ssRNA40 **(C)**. T cell proliferation was analyzed by CMV_{NLV}-specific dextramer staining of CD8$^+$ T cells and activated T cells were quantified via intracellular IFN-γ and TNF-α staining of CD8$^+$ T cells, both measured by flow cytometry. The T cell response is normalized to the αHer2 control. ● refers to FITC-ssRNA, ○ to unlabeled ssRNA. Graphs indicate means of *n* = 5-10 donors with SEM as error bars. For statistical analysis, a Wilcoxon-signed rank test was applied.

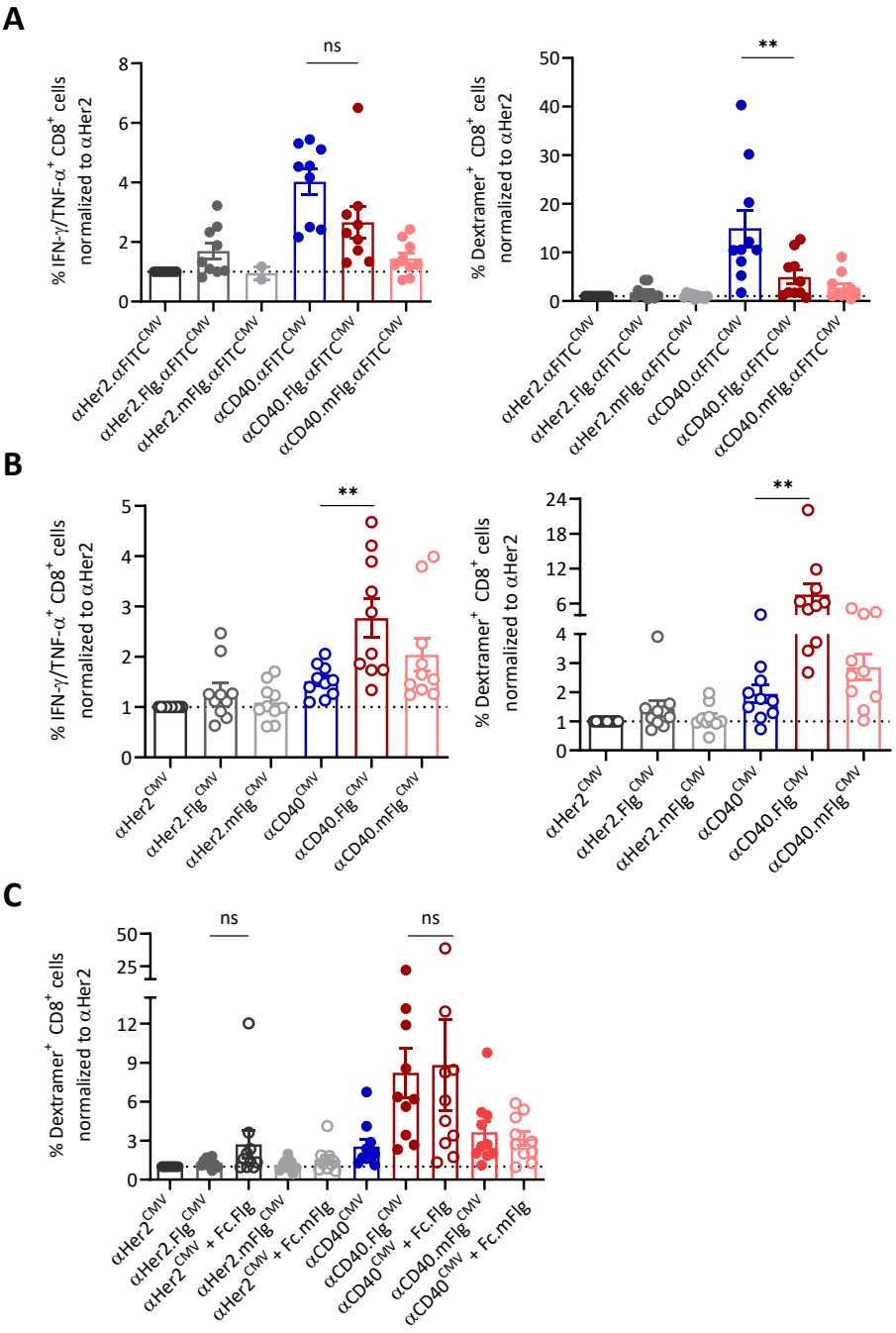

Figure 31. Co-cultures of allogeneic or autologous T cells and iDCs loaded with αCD40.αFITC^{CMV} or αCD40^{CMV} with and without fusion of the flagellin D0/D1 domain.

Autologous and allogeneic co-cultures were performed as previously described. **(A, B)** iDCs were incubated with αCD40.Flg.αFITC^{CMV} **(A)** or αCD40.Flg^{CMV} **(B)** for TLR5 activation or their controls without flagellin or mFlg as well as the αHer2 non-specific binding controls. ● refers to scFv format, ○ to IgG1 format. **(C)** Difference between genetic fusion of flagellin and co-administration of DC-targeting antibody and Fc.Flg. iDCs were incubated with the flagellin fusion molecule (αCD40.Flg^{CMV}) or the combination of αCD40^{CMV} with Fc.Flg or the respective controls. ● refers to flagellin fusion molecules, ○ to the combination of αCD40/Her2^{CMV} with Fc.Flg. T cell proliferation was analyzed by CMV_{NLV}-specific dextramer staining of CD8$^+$ T cells and activated T cells were quantified via intracellular IFN-γ and TNF-α staining of CD8$^+$ T cells, both measured by flow cytometry. The T cell response is normalized to the αHer2 control. Graphs show means of *n* = 2-10 donors with SEM as error bars. For statistical analysis, a Wilcoxon-signed rank test was applied.

4.3.4. MAC-mediated mNPM1$_{CLA}$-specific T cell activation

The before-mentioned experiments served as an important groundwork, on which the development of a novel therapeutic molecule to treat AML could be based. In the following part of the thesis, the vaccine concept was replicated in the leukemia setting by replacing the CMV-specific peptide with an mNPM1-derived neoantigen. The functionality of αCD40.αFITC^{mNPM1} scFv and αCD40^{mNPM1} IgG1 was proven by verifying that the fused mNPM1$_{277–298}$ sequence was correctly processed and cross-presented as CLAVEEVSL (mNPM1$_{CLA}$) on HLA-A*02:01.

For co-culture experiments, CD8$^+$ T cells were used that were transduced with a TCR recognizing this antigen in the context of HLA-A*02:01. These showed reactivity against HLA-A*02:01$^+$ and mNPM1$^+$ AML cell lines and primary patient cells.[224] The mNPM1$_{CLA}$-specific T cell population was co-cultured with DCs that had been pre-incubated with αCD40.αFITC^{mNPM1}, the αHer2 control or with an αCD40 conjugated to a control peptide (αCD40.αFITC^{CMV}). T cell functionality was validated using mNPM1$_{CLA}$-pulsed DCs. In fact, the mNPM1$_{CLA}$-specific T cells recognized the presented epitope on αCD40.αFITC^{mNPM1}-incubated DCs as detected by IFN-γ and TNF-α secretion (Figure 32A). Pulsing of DCs with the processed mNPM1$_{CLA}$ peptide as positive control led to the highest response and confirmed the specificity of the T cells. When the same experiment was performed using IgG1 format molecules, DCs incubated with αCD40^{mNPM1} also successfully cross-presented the mNPM1$_{CLA}$ peptide on MHC I and led to a higher T cell activation compared to the negative controls (Figure 32B). In line with the findings for the CMV setting, the αCD40^{mNPM1} was inferior to αCD40.αFITC^{mNPM1} in the scFv format regarding T cell activation.

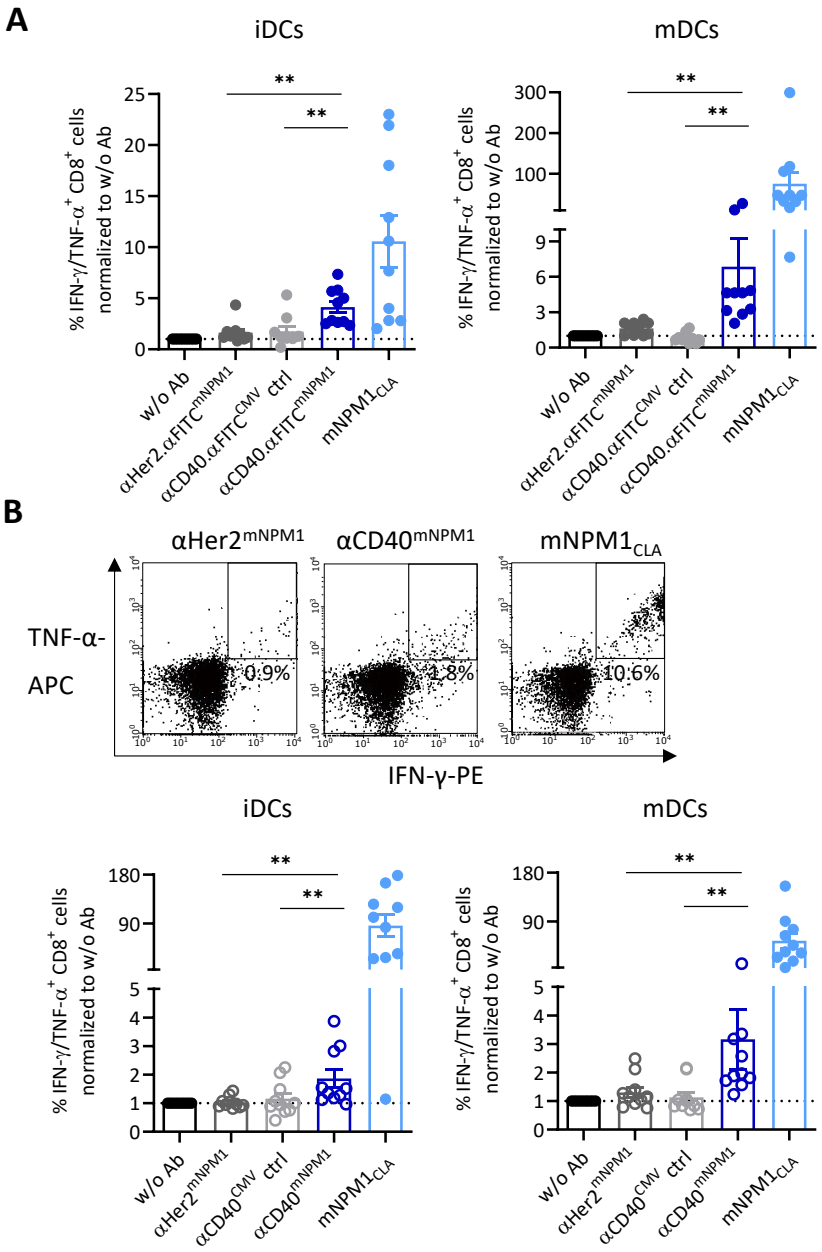

Figure 32. Co-culture of allogeneic mNPM1$_{CLA}$-specific T cells and HLA-A*02:01$^+$ iDCs and mDCs pre-incubated with MACs or peptide.
DCs were loaded with αCD40.αFITC^{mNPM1} scFv **(A)** and αCD40^{mNPM1} IgG1 **(B)**, αCD40 conjugated to a control peptide (αCD40.αFITC^{CMV} or αCD40^{CMV}), the αHer2 controls or the processed mNPM1$_{CLA}$ peptide prior to starting the cultivation. As a readout for T cell activation, IFN-γ- and TNF-α-producing CD8$^+$ T cells were quantified by flow cytometry. In B, representative flow cytometry dot plots of co-cultures of T cells and iDCs are shown. ● refers to scFv format, ○ to IgG1 format. T cell activation is normalized to w/o Ab. Bars represent means of *n* = 10 donors with SEM as error bars. For statistical analysis, a Wilcoxon-signed rank test was applied.

As αCD40.Flg^{CMV} was the most promising molecule in previous experiments with the flagellin domain enhancing both DC maturation and cross-presentation to T cells, its stimulatory potential was also investigated in the leukemia setting. For this purpose, iDCs are loaded with αCD40^{mNPM1}, αCD40.Flg^{mNPM1} or αCD40.mFlg^{mNPM1} as well as the respective αHer2 controls and cultivated with mNPM1$_{CLA}$-specific T cells (Figure 33). Again, fusing flagellin to αCD40^{mNPM1} significantly enhanced the

T cell response compared to the control without flagellin. The beneficial effect of the TLR5 ligation could be reversed by the insertion of R90A/E114A mutations into the flagellin domain.

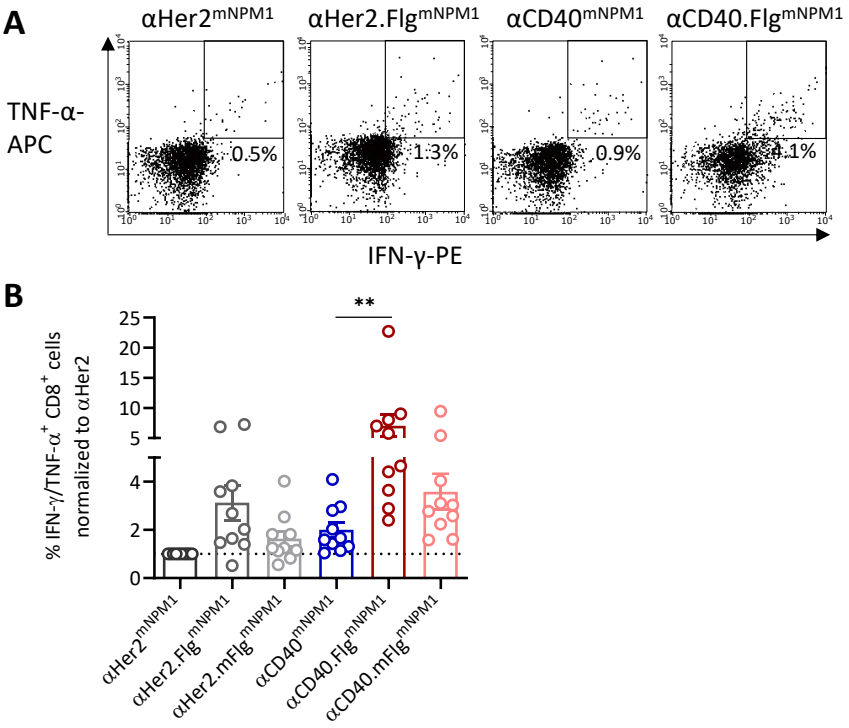

Figure 33. Co-culture of allogeneic mNPM1$_{CLA}$-specific T cells and iDCs loaded with αCD40^{mNPM1} with and without flagellin fusion to ligate TLR5.
Allogeneic co-cultures were performed as previously described. iDCs were incubated with αCD40^{mNPM1}, αCD40.Flg^{mNPM1} or αCD40.mFlg^{mNPM1} as well as the respective αHer2 controls. Activated T cells were quantified by counting IFN-γ- and TNF-α-producing CD8$^+$ T cells by flow cytometry. **(A)** Representative flow cytometry dot plots for one donor are shown. **(B)** Summarized T cell activation data normalized to the non-specific binding control. Bars indicate means of *n* = 10 donors with SEM as error bars. For statistical analysis, a Wilcoxon-signed rank test was applied.

Taken together, targeting a mNPM1-derived peptide to DCs by conjugation to an αCD40 scFv and IgG1 antibody led to an efficient mNPM1$_{CLA}$-specific T cell response. Fusion of the TLR5 agonist flagellin to αCD40^{mNPM1} was able to stimulate DCs thereby enhancing cross-presentation of conjugated neoantigens to CD8$^+$ T cells. These results confirm the functionality of the molecule *in vitro* and motivate for further evaluation *in vivo* to prove it a promising treatment option for AML.

5. Discussion

5.1. Rationale for MACs

DC vaccination strategies are widely considered as a powerful tool in immunotherapy of cancer and other diseases. Numerous clinical studies utilizing the initially developed *ex vivo* DC vaccination have been employed in different cancer entities and demonstrated safety and immunogenicity of the vaccines.[61] At the same time, these clinical trials have often demonstrated a poor efficacy.[263] One of the major factors restricting *in vivo* potency is the inefficient migration of administered DCs to the lymph node, wherein DCs activate antigen-specific T cells. The *ex vivo* DC generation process is also challenged by the expensive and labor-intensive GMP production that is difficult to standardize as the vaccines need to be generated for each patient individually. Therefore, *in vivo* targeting of DCs was more recently being exploited. This allows for large-scale vaccine production since it can be applied to a larger patient cohort. More importantly, it also exhibits biological advantages as it exploits the intrinsic migratory capacity of DCs and directly activates natural DC subsets at multiple sites *in vivo*, thereby producing a more physiological DC maturation.[80, 92, 264] Even if clinical data are still rare, *in vivo* vaccination is considered as a promising strategy for eliciting strong and lasting T cell response against intracellular pathogens and cancer.[263, 265]

Common to all vaccination approaches, DCs targeted by the antigen need to gain optimal T cell stimulating capacities. It is well known that immature DCs tend to induce tolerogenic responses at steady state while in the presence of adjuvants, maturation markers are upregulated and tolerance induction is abrogated.[126] Thus, to augment therapeutic efficacy, peptide-based cancer vaccines and DC-targeting antibodies are usually co-administered with adjuvants such as TLR agonists in a uncoupled manner.[266] Soluble adjuvants, however, allow for the activation of immune cells that do not present the delivered antigen, which implicates the risk of counterproductive side effects such as cytokine release or autoimmunity.[132, 133] To improve the safety profiles and to reduce the necessary dose, vaccines have been generated that combine the antigen and adjuvant in one delivery system or even in one molecule. Approaches such as exosome-based tumor antigens–adjuvant co-delivery systems or polymeric nanoparticles encapsulating both peptides and TLR agonists have been developed.[133, 267, 268] Alternatively, the adjuvant can be also directly fused to the peptide vaccine or DC-targeting antibody.[134, 242-244, 269] Targeted delivery of TLR ligands was not only associated with reduced serum cytokine release and related toxicity, but also reduced their dose requirement by 100-fold.[133] Furthermore, co-delivery of antigen and adjuvant into the same APC resulted in superior cross-presentation and peptide-specific T cell activation compared to separate molecule administration *in vivo*.[133, 134, 242-244] This implicates that direct coupling of the adjuvant increases vaccine efficacy while providing highest specificity of targeting and DC activation. In parallel, it allows the reduction of the adjuvant dose making side effects less likely.[134]

The benefits observed for both *in vivo* DC vaccination and for antigen-adjuvant conjugates served as rationales for this project. We generated multifunctional antibody constructs that combine *in vivo* targeting of antigens to DCs and stimulation by TLR agonists in one molecule. CD40 was selected as DC surface receptor to be targeted by either IgG1- or scFv-based constructs that exhibit themselves adjuvant activity. To further amplify the system, the stimulation by agonistic αCD40 constructs was combined with additional activating signals delivered by different TLR agonists (LPS, flagellin or ssRNA40). By using a CMV-specific antigen domain for proof-of-principle studies, the best molecule combination that elicited highest DC maturation with correlating T cell response was identified, namely αCD40.Flg^{CMV} in the IgG1 format. This molecule was further tested in a tumor setting by delivering the mNPM1-derived neoantigen to DCs aiming to develop a potent AML-directed DC vaccine. The novelty of the therapeutically relevant and most promising candidate molecule

αCD40.Flg^{mNPM1} is not only the combination of DC-targeting of antigens and the adjuvant in one molecule, but also the use of mNPM1 as an AML-specific neoantigen in a vaccination concept. This is the first study to investigate neoantigen delivery to a DC-targeting antibody, which was motivated by the encouraging results from clinical trials that investigated personalized neoantigen vaccines in other cancer entities.[72-75] Despite their even greater impact, data on therapies employing shared neoantigen vaccines are sparely available.[77, 78] Since mNPM1 is a shared neoantigen of 30% of AML patients, the groundwork for the development of a "off-the-shelf" vaccine to treat AML should be laid with the mNPM1-directed vaccine.

5.2. Comparative analysis of MACs activating different TLRs

MACs were generated by fusing various TLR agonists to a peptide-coupled DC-targeting antibody construct of either an αCD40 Fc-silenced IgG1 or scFv format. The TLR4 agonist LPS and TLR8 agonist ssRNA40 were linked via an αFITC scFv to αCD40.αFITC^{CMV}, whereas the TLR5 agonist flagellin was genetically fused to αCD40 IgG1 (αCD40.Flg^{CMV}) and scFv (αCD40.Flg.αFITC^{CMV}). A comparative analysis of the molecules was performed to rate them based on protein yield, the ability to induce DC maturation, T cell activation and proliferation as well as the innovation and impact (Figure 34).

	TLR4	TLR5		TLR8	
Molecule	αCD40.αFITC^{CMV} + FITC-LPS	αCD40.Flg.αFITC^{CMV} scFv	αCD40.Flg^{CMV} IgG1	αCD40.αFITC^{CMV} + FITC-ssRNA40	αCD40.αFITC^{CMV} + FITC-ssRNA40 + PLA
Protein yield					
DC maturation					
Cross-presentation and T cell activation					
T cell proliferation					
Innovation/Impact					

Best Worst

Figure 34. Comparative analysis of MACs activating different TLRs.

Concerning protein yield, the αCD40 antibody constructs without flagellin fusion were advantageous over αCD40.Flg.αFITC^{CMV} and αCD40.Flg^{CMV} and could be expressed in reasonable amounts. However, genetic fusion of the flagellin domain reduced expression yields by approximately 2-fold in the scFv format and by 6-fold in the IgG1 format.

Next, the induction of DC maturation by MACs with or without TLR agonists was investigated. The αCD40 variable regions used in these studies have been derived from CP-870,893 (clone 21.4.1) that was shown to bind agonistically to CD40 in its original IgG2 format.[247, 248] αCD40.αFITC^{CMV} in the scFv format showed a high agonistic potential. Surprisingly, the αCD40^{CMV} in the IgG1 format displayed a minor activating potential as shown by a low, but still significant upregulation of maturation markers. The agonistic activity of αCD40 antibodies, but also of other agonistic receptor-engaging antibodies,

is in general dependent on their potential to crosslink CD40. It has long been believed to rely predominantly on crosslinking by the inhibitory FcγRIIb to deliver their activity.[270, 271] Clone CP-870,893 of the IgG2 isotype just reveals low affinity binding to FcγRs and still exhibits immune activating potential. White et al. postulated that the agonistic activity was solely mediated by unique hinge properties of the IgG2 subclass.[272] This could be disproven two years later, as CP-870,893 in the IgG1 format with a different hinge region showed even higher immune activation, while the activity of IgG1 was completely abolished by the Fc-silencing N297A mutation.[273] Both publications indicated that the potency of agonistic CD40 antibodies can be influenced by the level of Fc crosslinking and by different hinge regions. However, the CD40 epitope recognized and the strength of the signal achieved seem to be the more important determinants.[274] In line with this, it was shown in this thesis that the αCD40.αFITC^{CMV} was able to highly activate DCs even without an Fc portion present. The exact mechanisms that explain, why the scFv format was even more efficient in immune cell activation compared to the Fc-silenced IgG1 format, remains elusive, but both antibody formats seem to bind in a different manner to CD40. Importantly, all TLR agonists were able to upregulate DC maturation markers and enhanced IL-6 secretion. Flagellin and LPS were similarly able to stimulate DCs. The lowest DC maturating activity was seen for the ssRNA40 probably due to an inefficient uptake of the FITC-ssRNA in combination with αCD40.αFITC^{CMV}. When the RNA was complexed with PLA to facilitate the endosomal transport, ssRNA40 highly increased in DC maturation.

We further assessed the ability of antibody-peptide conjugates of different formats to induce T cell activation and proliferation without an adjuvant. Our *in vitro* studies have shown that αCD40.αFITC^{CMV}- and αCD40.αFITC^{mNPM1}-loaded DCs more efficiently cross-presented the peptides and triggered T cell responses compared to αCD40^{CMV} and αCD40^{mNPM1}. This indicated that the scFv format was more potent if used without additional stimulation, probably because of a higher intrinsic agonistic activity. When the T cell responses with TLR ligation were explored in a following step, it turned out that an increased DC maturation does not automatically correlate with enhanced cross-presentation and T cell activation. PLA-complexed FITC-ssRNA40, that highly activated DCs, combined with αCD40.αFITC^{CMV} was not able to boost T cell responses. FITC-LPS resulted in a slightly lower T cell activation and proliferation compared to αCD40.αFITC^{CMV}, although TLR4 ligation considerably upregulated DC maturation markers. The fusion of flagellin to the scFv format MAC (αCD40.Flg.αFITC^{CMV}) even led to a significantly decreased T cell proliferation compared to αCD40.αFITC^{CMV}. In contrast, the combination of FITC-ssRNA40 and αCD40.αFITC^{CMV} did not greatly enhance DC maturation and T cell activation, but significantly increased T cell proliferation compared to αCD40.αFITC^{CMV} alone as well as with addition of uncoupled ssRNA40. Importantly, the αCD40.Flg^{CMV} MAC in the IgG1 format both upregulated DC maturation markers and enhanced the T cell response compared to αCD40^{CMV}.

In general, DC maturation is characterized by an enhancement in antigen processing and (cross-)presentation for example by the increased synthesis and surface levels of MHC class I and II molecules.[275, 276] DC maturation further stimulates T cells, which is mediated by the upregulation of co-stimulatory surface molecules and the secretion of proinflammatory cytokines.[277] However, the functional capability of DCs to induce T cell activation does not always directly correlate with common maturation markers, for example because Tregs use some of the same signaling pathways including CD80/CD86.[278-280] In addition, it was generally presumed that DC maturation downregulates the potential of DCs to take up antigens and all forms of endocytosis. Platt et al. reported that DCs indeed shut down micropinocytosis responsible for the non-selective uptake of extracellular material, but they continue to accumulate antigens especially by receptor-mediated endocytosis and phagocytosis.[259] This has also been seen in our microscopy analysis showing that iDCs and mDCs highly internalized antibodies and that the DC maturation state did not obviously affect the level of receptor-

mediated endocytosis. Still, the observations that TLR activation induces DC maturation while occasionally impairing T cell activation and proliferation might be a combinatorial effect of reduced antigen accumulation and cross-presentation as well as the induction of Tregs that compromise $CD8^+$ T cell responses. The latter should not have a high influence in allogeneic co-cultures of DCs and expanded peptide-specific T cells that mostly consist of a $CD8^+$ population. But Treg triggering might be a problem in the autologous setting using NACs, even if this could not be observed in some initial experiments (data not shown). In addition, overactivation of DCs that leads to activation-induced cell death (AICD), as for example described after LPS challenge, might limit the ability to take up antigens and stimulate T cells.[281, 282] Especially the combination of the highly activating αCD40 scFv format with other TLR ligands might have been an overkill. Hence, it would have been interesting to couple FITC-LPS to an αCD40 IgG1 antibodies with αFITC scFvs fused to the light chains and to investigate LPS induced T cell response in combination with a low maturating αCD40 format. However, since impaired T cell activation as response to highly matured DCs is barely described in the literature, these results would maybe not be translatable into *in vivo* systems and would clinically not have any significance.

Not only differences in DC maturation might have affected the internalization process of MACs and therefore the availability of processed peptides. The finding that αCD40.Flg.αFITC^{CMV}-loaded iDCs less efficiently interacted with T cells might be the result of the diminished binding to DCs and therefore impaired internalization compared to αCD40.αFITC^{CMV}, which would need to be proven by microscopy. Also, the interaction of flagellin and LPS with surface TLRs might disturb antibody internalization and lead to reduced T cell activation. This is, however, contradicted by the beneficial effects observed for flagellin fusion to the αCD40^{CMV} IgG1 and by the fact that ssRNA40 in combination with PLA targeting intracellular TLR8 showed the same inhibitory trend.

To sum up the *in vitro* findings, all MACs elicited DC maturation but differed in their properties to induce T cell activation and proliferation. The flagellin fusion molecule αCD40.Flg^{CMV} was the only MAC that showed consistent results in DC maturation and T cell response. It is also biochemically and clinically the most relevant molecule due to the genetic flagellin fusion and the stable interaction between TLR agonist and antibody. Together with the novelty of the format, αCD40.Flg^{CMV} is considered as the most promising candidate to be further investigated.

5.3. Advantages and limitations

As mentioned before, the constructs developed in this thesis offer important benefits compared to conventional *ex vivo* DC strategies and compared to therapeutic regiments where the adjuvant and the antibody–antigen conjugate are co-administered instead of being fused.

All MACs target CD40 on the DC surface. The delivery of antigens to DCs by CD40-targeting antibodies was shown to be more efficient in eliciting MHC I cross-presentation and to induce the highest levels of $CD8^+$ T cell responses in comparison to other receptors.[116, 283, 284] Chatterjee et al. demonstrated that this effect was predominantly promoted by the intracellular trafficking pathway.[116] αCD40 antibodies not only elicit DC-targeting function, but also induce CD40 signaling in the case of an agonistic binder, such as the one used in this work. As this delivers an intrinsic co-stimulatory signal to DCs, the use of αCD40 antibodies might also be advantageous over antibodies directed against other endocytic receptors. However, especially the context of immune cell activation, agonistic αCD40 binders including the one used in this studies have a somewhat bad reputation. Being initially developed to suppress tumor growth as a monotherapy for different cancer entities with promising antitumor effects, agonistic αCD40 antibodies have been also used for vaccination approaches.[285-287]

In combination with fused or soluble antigens, they exhibited strong and promising adjuvant activity even at low doses.[114] However, in clinics, severe treatment-related adverse events such as cytokine release syndrome and hepatotoxicity have been reported for the parental antibody version CP-870,893 limiting the dosage and the treatment.[247, 285] This antibody also induced chronic B-cell activation associated with diminished circulating T cell numbers in some patients, that was also observed in mouse studies with different αCD40 clones.[288-290] This might have resulted from AICD, since CD40 is also expressed on activated T cells.[291] In addition, hyper immune stimulation by CD40 agonists also poses the risk of T cell anergy, which might be particularly detrimental if those agonistic αCD40 antibodies are used in the context of DC vaccines.[289] As a consequence, the dose needs to be tightly titrated or the affinity to CD40 adjusted to reduce systemic toxicity induced by overactivation and to maintain the vaccine efficacy. In our experiments, $\alpha CD40^{CMV}$ in the Fc-silenced IgG1 format displayed only low agonistic activity, whereas $\alpha CD40.\alpha FITC^{CMV}$ with a αCD40 scFv upregulated DC maturation markers more strongly. Especially if combined with another TLR ligating stimulus, the $\alpha CD40^{CMV}$ is presumably beneficial over the scFv format in regard to adverse events. It might combine the advantage of low intrinsic agonistic activity with the benefit of targeting antigens to CD40 concerning cross-presentation and T cell activation. However, if used without additional stimulation by TLR agonists, the scFv format was more potent.

The two formats reveal additional differences. Due to their smaller size, scFvs enter into the tissues, where targeted iDCs are resident, more easily compared to antibodies in the IgG1 format. Molecules of smaller size are faster cleared from the plasma and reveal shorter half-lives.[122] Importantly, as scFvs lack Fc domains and the αCD40 IgG1 displays a effector-silent Fc region, both formats do not bind to Fcγ receptors and provide therefore CD40-specific antigen delivery by reducing non-specific uptake of antigens.[123] The PGLALA-mutated αCD40 IgG1 is still able to bind FcRn resulting in the same favourable pharmacokinetic profile as wild-type IgG1 molecules.[124]

Regarding cell selectivity, αCD40 antibodies would target not only DCs but also other CD40-expressing cells including predominantly B cells, monocytes, macrophages, hematopoietic progenitors, fibroblasts, endothelial and epithelial cells.[118-120] To achieve DC-specific targeting and to overcome problems related to potential antigen sinks, other groups have shown that the administration route plays an important role. In particular, by intra-dermal (i.d.) administration of CD40-targeting agents and by relative over-expression of CD40 on DCs, any binding that might occur to other cells doesn't interfere with efficient vaccine delivery to DCs.[292, 293] The DC selectivity is also necessary to reduce side effects, as agonistic αCD40 antibodies for example were shown to induce polyclonal stimulation of B cells leading to splenomegaly *in vivo*.[294, 295] On the other hand, targeting B cells might contribute to the vaccine efficiency of αCD40-antigen conjugates.[114] In addition, dual targeting of DC surface receptors and TLRs, that are both specifically expressed on DCs, may provide further selectivity. Since TLR5 is not expressed on B cells but on DCs, the $\alpha CD40.Flg^{CMV/mNPM1}$ construct might preferentially target DCs.

All MACS were site-specifically coupled to the adjuvant, either by genetic fusion or by an αFITC scFv. It was shown that the flagellin fusion to $\alpha CD40^{CMV}$ didn't interfere with the αCD40 binding site and didn't affect protein integrity. This was an important finding, as other coupling methods might have disadvantages. In a similar approach, Kreutz et al. developed an antibody–antigen–adjuvant conjugate consisting of an αDec205-specific antibody conjugated via sulfo-SMCC linkers to the model antigen ovalbumin (OVA) and CpG oligodeoxynucleotides (ODN). Although they could show the functionality of their molecule, they encountered the problem that CpG fusion altered the antibody binding and uptake. The fusion allowed the delivery of both antigen and adjuvant to cells partially independently of the DC-targeting antibody.[134] Also, the covalent linkage via amine-to-sulfhydryl crosslinkers might

be too strong to release the TLR agonist in the endosome for optimal interaction with its receptor, whereas the FITC-ligated TLR agonist should be released by acidic pH. However, this interaction might not be strong enough to be used in a clinically relevant molecule. Coupling the TLR agonist via FITC-αFITC interaction was mainly an elegant way to test the potency of different FITC-TLR agonists *in vitro*. The genetic fusion variant of flagellin, on the other hand, might be used in a clinical setting. As flagellin anyway interacts with a TLR located on the cell surface, an intracellular cleavage is not necessary. Moreover, the capacity of genetically fused flagellin to interact with TLR5 could already be validated in functional assays.

The combination of αCD40$^{CMV/mNPM1}$ and flagellin revealed the most promising results concerning DC maturation and T cell response when used in an IgG1 format. TLR5 agonists are favourably positioned among other TLR ligands, even if used systemically, because of a restricted expression pattern in tissues (primarily in the gut, liver and bladder) and an uniquely safe profile of induced cytokines.[190] Flagellin is not only recognized at the cell surface by TLR5, but also by the cytoplasmic neuronal apoptosis inhibitory protein (NAIP) and NLR-containing a CARD domain (NLRC)-4, which activates the inflammasome pathway and thereby enhances their effects on innate and antigen-specific cellular immunity.[296, 297] A derivative of flagellin called entolimod, which is similar to the truncated flagellin version used in our molecule, has been initially established as a potential treatment for lethal radiation exposure.[184, 185] Flagellin was also shown to induce potent anti-viral immune responses in animals and in clinical trials, when it was used as a fusion protein with antigens or as a separate adjuvant combined with vaccines.[186-188, 298, 299] In mouse studies, TLR5 ligation by flagellin was able to convert tolerogenic DCs into activating APCs that preferentially induce Th1 responses.[300] As flagellin is also reported to elicit direct antitumoral effects, a combination with DC vaccines in the cancer setting was a logical consequence. [189-191, 301, 302] Data from this work proves that flagellin performs well if combined with a DC-targeting antibody-peptide conjugate. Unfortunately, the flagellin fusion drastically decreased the expression yield of the αCD40 antibody by around 6-fold. For its application in the clinics, the cell system or transfection efficiency would need to be optimized for higher expression levels.

Since the other tested TLR agonists, LPS and ssRNA40, were indirectly linked to the antibody via an αFITC scFv, the fusion did not affect expression yields. LPS has been developed and tested mostly as monotherapeutic for the treatment of cancer.[303-305] In these trials, toxicities were reported and LPS has long been considered too toxic for human use. After the formulation of LPS have been optimized, the present GMP grade endotoxin has a stronger safety profile and causes a transient, but well tolerated systemic inflammatory response in a dose-dependent manner.[306, 307] Further attempts were made to replace LPS by detoxified versions. Among those, MPL is particularly attractive as an adjuvant for anti-cancer vaccination due to its capacity to induce robust Th1-polarized and cell-mediated immunity while reducing toxicity.[308] MPL has already been evaluated as a cancer vaccine adjuvant in published clinical trials and is the only defined TLR ligand approved as part of a vaccine.[178, 179]

The phosphothioate-protected RNA oligonucleotide ssRNA40 from the U5 region of HIV-1 was first synthesized by Heil et al. in 2004. It was shown to stimulate DCs and macrophages to secrete proinflammatory cytokines by interacting with human TLR8.[164] The combination of nucleic acid-based TLR agonists, such as ssRNAs, with *in vivo* DC vaccination approaches appears feasible since their cognate TLRs are located in the endosomal compartments. These TLR agonists cannot easily cross membranes due to their size and negative net charge.[309] If the ssRNA is fused to a DC-targeting antibody, it should be specifically internalized together with the antibody into the endosome. Presumably, only the DCs that have taken up the antigen become activated leading to a highly cell-specific immune response. For other vaccination approaches, cationic carrier systems have been employed to enable cellular uptake and increase half-life in the circulation.[310] Complexing the ssRNA40

with PLA acts in a similar way, but unspecific uptake in other cells is commonly observed using these techniques that leads to unwanted off-target effects.[309] Combining DC-targeting antibodies with nucleic acid-based TLR agonists might be also beneficial for reducing side effects. However, our data have shown that the uptake of the ssRNA40 into the DC is not efficient, if the RNA is coupled via an αFITC scFv. Thus, a high dose of ssRNA40 would be necessary to reach a level of intracellular ssRNA40 that is sufficient to induce immune responses. This would make it a highly expensive and not clinically feasible approach. Alternatively, direct conjugation of ssRNAs or small molecule TLR8 ligands have been already applied for peptide-based vaccines and might also be a good strategy for *in vivo* DC vaccination.[243, 311, 312]

5.4. State of the art and optimization potential

DC vaccines are in general safe and able to promote antitumor immunity. However, many previous attempts to develop effective therapeutic cancer vaccines yielded disappointing results as the vaccines produced durable objective clinical responses in only a minority of patients with measurable disease.[313] This was not only observed for *ex vivo* DC vaccination approaches that have been vigorously investigated in clinical trials for decades, but also for *in vivo* DC-targeting.[69] Most of the clinically used antibody-based vaccines include carrier systems such as liposomes, but only two of them are pure antibody–peptide conjugates. CDX-1307 and CDX-1401 from Celldex Therapeutics target either MR or Dec205 and are tested in phase I or II clinical trials to treat advanced epithelial malignancies.[135, 136] Both are administered together with the TLR agonists poly(I:C) and R848 as adjuvants. No dose-limiting toxicities were observed, but only a low number of patients responded, mostly only with a disease stabilization. Therefore, new vaccine designs are needed to improve the induction of an immune response and its durability.

The key lessons learned from failed vaccine attempts include: the need for multiple, immunogenic antigens, the importance of potent vaccine technologies and co-stimulation, and the need to abolish tumor-mediated immunosuppression.[60] New strategies, such as ligandome analyses or in silico tools using high-throughput sequencing data, are enabling the selection and construction of more immunogenic TAAs and the identification of tumor-specific neoantigens.[60, 314] Targeting multiple peptides or long proteins to DCs including both MHC I and II epitopes is presumably beneficial over single peptide vaccines. Although $CD4^+$ T cells do not exhibit direct antitumor activity, their presence amplifies antitumor immune responses by boosting $CD8^+$ T cells. In addition, $CD4^+$ T cells are major players in the transition of the tumor microenvironment towards an antitumor milieu.[315] The reversion of immunosuppression can also be achieved by combination of DC vaccines with immune checkpoint inhibitors (ICIs) and other immunomodulators, which has already shown promising pre-clinical results and is currently tested in clinical trials.[316, 317] Further, the success of a DC vaccine depends on the amount of delivered co-stimulatory signals. A combination of different TLR ligands with potential synergistic activity might provide advantages.[318] TLR3 and TLR9 agonists exhibited good synergies in human DCs as shown by previous studies.[319, 320] The clinically approved vaccine adjuvants AS01, AS02 and AS04 indicate that a combination of one TLR ligand with different adjuvants, such as saponins or alum, can be potent and safe to use in the clinic.[41] Besides TLR ligation, activating the STING pathway is a promising strategy to induce co-stimulation of T cells by DCs. In a recent study, the STING activator c-di-AMP elicited superior adjuvant properties than the formulation poly(I:C)/CpG after vaccination with soluble protein antigen or Dec205-mediated antigen targeting to DCs.[193] Fu et al. have shown in pre-clinical models that cancer vaccines formulated with cytosolic cyclic dinucleotides (CDNs) can cure established tumors resistant to PD-1 blockade, highlighting again the adjuvant potential of STING

agonists.[321] For adjuvants directly acting on DCs and for the agents modulating the tumor microenvironment, more work needs to be done to determine which combinations are most effective and to find out the optimal dose scheduling for each component.[60]

A further optimization of *in vivo* DC vaccines might be achieved by targeting several receptors in a vaccination approach. There is still no consensus on which DC receptor would be a choice of priority in terms of targeting antigen. In addition, the selected DC receptor determines not only the targeted DC subtype but also the intracellular trafficking pathway. This might be important if multipeptide vaccines including also MHC II epitopes are investigated, as MHC II predominantly resides in late endosomes.[32] Thus, targeting multiple receptors instead of a single receptor could give a synergistic boost to generate activated T cells.[111]

These are general improvements to optimize vaccination approaches, which can be also transferred to the MACs developed in this thesis. In addition to the aforementioned points, the MAC activating TLR4 could be optimized by exchanging LPS by its detoxified version MPL, if its fusion is possible. Ligation of TLR8 could be achieved by covalent coupling of a small molecule TLR8 agonist, such as thiol-functionalized 3M-012. This is a closely related analogue of R848 and could be conjugated via thiol-maleimide chemistry to amine groups of antibodies.[322] A more promising method to couple agonists of intracellular TLRs, however, is a pH-sensitive linker that is cleaved in the endosome to release the payload.[323] This technique, which has been initially developed for their application in antibody–drug conjugates, ensures optimal ligand interaction with the intracellular TLR that is not sterically hindered by the attached antibody. To activate STING as an alternative PRR pathway, the generation of an αCD40 antibody coupled to a chemically modified cGAMP version is already planned in collaboration with the chemistry department.

The most promising and neoantigen-directed molecule αCD40.Flg^{mNPM1} has still some optimization potential. A longer linker between the heavy chain and flagellin might facilitate proper folding and secretion thereby possibly increasing the expression yield. The N-terminal fusion of flagellin to an Fc domain has been shown to elicit slightly superior activity compared to the C-terminal fusion (data not shown). Therefore, fusing flagellin to the N-terminus of the heavy chain might also be a version to be tested in the future, even if this comprises the risk of interfering with the CD40-binding site. Alternatively, in the C-terminal fusion molecule the D0 and D1 domain could be swapped to mimic the naturally occurring terminal position of D0.

The therapeutic outcomes cannot only be ameliorated by optimizing the antibody-based vaccine itself, but also by adapting therapeutic regimens. In particular, different immunotherapeutic approaches have the potential to synergize.[324] Therefore, the best strategy would be to not to investigate DC vaccines as stand-alone drugs, but to combine it with other immunotherapies such as the already mentioned ICI. Especially if ICI was used with neoepitope-based vaccines, the repertoire of neoantigen-specific T cells was enhanced, which resulted in sustained progression-free survival in clinical trials.[72, 73] This shows that the combination of DC vaccination with ICIs might overcome the anergic state of vaccine-induced T cells.[325] In addition, several clinical trials explored the potential of post-transfer vaccination to enhance clinical efficacy of adoptively transferred T cells expressing a TCR specific for an intracellular tumor antigen.[326, 327] In line with this, combining αCD40.Flg^{mNPM1} with TCR gene therapy, in particular with mNPM1$_{CLA}$-specific T cells generated by Van der Lee et al., could be a promising approach to expand transferred T cell *in vivo* in order to generate a long-lasting response.[224]

5.5. Concluding remarks and future perspectives

In the present study, MACs were generated to target and activate DCs *in vivo*. These combined an αCD40-DC-targeting domain with the CMV- or mNPM1-specific peptide and the adjuvant in one molecule. The coupling of three different TLR agonists as adjuvants was analyzed and compared by means of DC maturation and T cell activating potential, but also in respect to molecule design and innovation. Fusing flagellin to the Fc-silenced αCD40 IgG1 antibody (αCD40.Flg^{mNPM1}) turned out to be the most promising molecule, as it potently increased DC maturation by specifically activating TLR5 and enhanced the mNPM1-directed T cell response. Thus, we propose αCD40.Flg^{mNPM1} as a highly promising therapeutic approach for AML treatment. We hypothesize that fusing the flagellin domain to the antibody leads to decreased side effects and increased clinical efficacy compared to the administration of two separate molecules.

To gain more insights into its mode of action and functionality, αCD40.Flg^{mNPM1} needs to be further investigated. The following experiments need to verify whether the molecule also induce substantial expansion of the small pool of mNPM1$_{CLA}$-specific T cells present in AML patients. This would be a prerequisite for the induction of an AML-directed antitumor response *in vivo*. Finally, the functionality of αCD40.Flg^{mNPM1} needs to be analyzed in a pre-clinical AML mouse model in order to prove whether the *in vitro* results can also be confirmed *in vivo* and whether the molecule is able to elicit an AML-directed tumor regression. *In vivo* studies should also evaluate the potential benefit of αCD40.Flg^{mNPM1} in reducing side effects and increasing efficacy compared to the co-administration of the DC-targeting antibody and the adjuvant. It would be interesting to explore the efficacy of therapies combining αCD40.Flg^{mNPM1} with ICI or adoptively transferred mNPM1$_{CLA}$-specific T cells that recognize and kill primary AML cells both *in vitro* and *in vivo*.

Collectively, the *in vitro* evaluation of αCD40.Flg^{mNPM1} performed in this thesis demonstrated the functionality of this novel antibody format and its ability to induce efficient mNPM1-specific T cell responses. Thus, the first investigation of neoantigen-delivery to a DC-targeting antibody and of mNPM1 as a shared neoantigen in a DC vaccination approach was successful. The positive results motivate for further studies and provide the rational for αCD40.Flg^{mNPM1} to become a future treatment option for AML patients.

6. References

1. Schuler, G.; Steinman, R. M., Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J Exp Med* **1985,** *161* (3), 526-46.

2. Steinman, R. M.; Cohn, Z. A., Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* **1973,** *137* (5), 1142-62.

3. Banchereau, J.; Steinman, R. M., Dendritic cells and the control of immunity. *Nature* **1998,** *392* (6673), 245-52.

4. Mellman, I.; Steinman, R. M., Dendritic cells: specialized and regulated antigen processing machines. *Cell* **2001,** *106* (3), 255-8.

5. Paul, W. E., Bridging innate and adaptive immunity. *Cell* **2011,** *147* (6), 1212-5.

6. Van Brussel, I.; Berneman, Z. N.; Cools, N., Optimizing dendritic cell-based immunotherapy: tackling the complexity of different arms of the immune system. *Mediators Inflamm* **2012,** *2012*, 690643.

7. Steinman, R. M.; Banchereau, J., Taking dendritic cells into medicine. *Nature* **2007,** *449* (7161), 419-426.

8. von Andrian, U. H.; Mempel, T. R., Homing and cellular traffic in lymph nodes. *Nature Reviews Immunology* **2003,** *3* (11), 867-878.

9. Lutz, M. DC tolerance. http://www.virologie.uni-wuerzburg.de/en/immunology/immunological-lab-groups/research-group-prof-dr-manfred-lutz/projects/dc-tolerance-engl/ (accessed 14.07.2020).

10. Guilliams, M.; Ginhoux, F.; Jakubzick, C.; Naik, S. H.; Onai, N.; Schraml, B. U.; Segura, E.; Tussiwand, R.; Yona, S., Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol* **2014,** *14* (8), 571-8.

11. Huber, A.; Dammeijer, F.; Aerts, J. G. J. V.; Vroman, H., Current State of Dendritic Cell-Based Immunotherapy: Opportunities for in vitro Antigen Loading of Different DC Subsets? *Front Immunol* **2018,** *9*, 2804-2804.

12. Collin, M.; Bigley, V., Human dendritic cell subsets: an update. *Immunology* **2018,** *154* (1), 3-20.

13. Lutz, M. B.; Strobl, H.; Schuler, G.; Romani, N., GM-CSF Monocyte-Derived Cells and Langerhans Cells As Part of the Dendritic Cell Family. *Front Immunol* **2017,** *8*, 1388-1388.

14. MacDonald, K. P.; Munster, D. J.; Clark, G. J.; Dzionek, A.; Schmitz, J.; Hart, D. N., Characterization of human blood dendritic cell subsets. *Blood* **2002,** *100* (13), 4512-20.

15. Dzionek, A.; Fuchs, A.; Schmidt, P.; Cremer, S.; Zysk, M.; Miltenyi, S.; Buck, D. W.; Schmitz, J., BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J Immunol* **2000,** *165* (11), 6037-46.

16. den Haan, J. M. M.; Lehar, S. M.; Bevan, M. J., Cd8+ but Not Cd8− Dendritic Cells Cross-Prime Cytotoxic T Cells in Vivo. *Journal of Experimental Medicine* **2000,** *192* (12), 1685-1696.

17. Williams, J. W.; Tjota, M. Y.; Clay, B. S.; Vander Lugt, B.; Bandukwala, H. S.; Hrusch, C. L.; Decker, D. C.; Blaine, K. M.; Fixsen, B. R.; Singh, H.; Sciammas, R.; Sperling, A. I., Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nat Commun* **2013,** *4*, 2990.

18. Shortman, K.; Heath, W. R., The CD8+ dendritic cell subset. *Immunol Rev* **2010,** *234* (1), 18-31.

19. Nizzoli, G.; Krietsch, J.; Weick, A.; Steinfelder, S.; Facciotti, F.; Gruarin, P.; Bianco, A.; Steckel, B.; Moro, M.; Crosti, M.; Romagnani, C.; Stolzel, K.; Torretta, S.; Pignataro, L.; Scheibenbogen, C.; Neddermann, P.; De Francesco, R.; Abrignani, S.; Geginat, J., Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. *Blood* **2013,** *122* (6), 932-42.

20. Schlitzer, A.; McGovern, N.; Teo, P.; Zelante, T.; Atarashi, K.; Low, D.; Ho, A. W.; See, P.; Shin, A.; Wasan, P. S.; Hoeffel, G.; Malleret, B.; Heiseke, A.; Chew, S.; Jardine, L.; Purvis, H. A.; Hilkens, C. M.; Tam, J.; Poidinger, M.; Stanley, E. R.; Krug, A. B.; Renia, L.; Sivasankar, B.; Ng, L. G.; Collin, M.; Ricciardi-Castagnoli, P.; Honda, K.; Haniffa, M.; Ginhoux, F., IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* **2013,** *38* (5), 970-83.

21. Watchmaker, P. B.; Lahl, K.; Lee, M.; Baumjohann, D.; Morton, J.; Kim, S. J.; Zeng, R.; Dent, A.; Ansel, K. M.; Diamond, B.; Hadeiba, H.; Butcher, E. C., Comparative transcriptional and functional profiling defines conserved programs of intestinal DC differentiation in humans and mice. *Nat Immunol* **2014,** *15* (1), 98-108.

22. Swiecki, M.; Gilfillan, S.; Vermi, W.; Wang, Y.; Colonna, M., Plasmacytoid dendritic cell ablation impacts early interferon responses and antiviral NK and CD8(+) T cell accrual. *Immunity* **2010,** *33* (6), 955-66.

23. Mouriès, J.; Moron, G.; Schlecht, G.; Escriou, N.; Dadaglio, G.; Leclerc, C., Plasmacytoid dendritic cells efficiently cross-prime naive T cells in vivo after TLR activation. *Blood* **2008,** *112* (9), 3713-3722.

24. Miller, J. C.; Brown, B. D.; Shay, T.; Gautier, E. L.; Jojic, V.; Cohain, A.; Pandey, G.; Leboeuf, M.; Elpek, K. G.; Helft, J.; Hashimoto, D.; Chow, A.; Price, J.; Greter, M.; Bogunovic, M.; Bellemare-Pelletier, A.; Frenette, P. S.; Randolph, G. J.; Turley, S. J.; Merad, M., Deciphering the transcriptional network of the dendritic cell lineage. *Nat Immunol* **2012,** *13* (9), 888-99.

25. Carpentier, S.; Vu Manh, T. P.; Chelbi, R.; Henri, S.; Malissen, B.; Haniffa, M.; Ginhoux, F.; Dalod, M., Comparative genomics analysis of mononuclear phagocyte subsets confirms homology between lymphoid tissue-resident and dermal XCR1(+) DCs in mouse and human and distinguishes them from Langerhans cells. *J Immunol Methods* **2016,** *432*, 35-49.

26. De Monte, A.; Olivieri, C. V.; Vitale, S.; Bailleux, S.; Castillo, L.; Giordanengo, V.; Maryanski, J. L.; Segura, E.; Doglio, A., CD1c-Related DCs that Express CD207/Langerin, but Are Distinguishable from Langerhans Cells, Are Consistently Present in Human Tonsils. *Front Immunol* **2016,** *7*, 197.

27. Sallusto, F.; Lanzavecchia, A., Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* **1994,** *179* (4), 1109-18.

28. Heystek, H. C.; Mudde, G. C.; Ohler, R.; Kalthoff, F. S., Granulocyte-macrophage colony-stimulating factor (GM-CSF) has opposing effects on the capacity of monocytes versus monocyte-derived dendritic cells to stimulate the antigen-specific proliferation of a human T cell clone. *Clin Exp Immunol* **2000,** *120* (3), 440-447.

29. Cresswell, P.; Bangia, N.; Dick, T.; Diedrich, G., The nature of the MHC class I peptide loading complex. *Immunol Rev* **1999,** *172*, 21-8.

30. Guermonprez, P.; Valladeau, J.; Zitvogel, L.; Thery, C.; Amigorena, S., Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* **2002,** *20*, 621-67.

31. Joffre, O. P.; Segura, E.; Savina, A.; Amigorena, S., Cross-presentation by dendritic cells. *Nature Reviews Immunology* **2012,** *12* (8), 557-569.

32. ten Broeke, T.; Wubbolts, R.; Stoorvogel, W., MHC class II antigen presentation by dendritic cells regulated through endosomal sorting. *Cold Spring Harb Perspect Biol* **2013,** *5* (12), a016873-a016873.

33. Delamarre , L. l.; Holcombe , H.; Mellman , I., Presentation of Exogenous Antigens on Major Histocompatibility Complex (MHC) Class I and MHC Class II Molecules Is Differentially Regulated during Dendritic Cell Maturation. *Journal of Experimental Medicine* **2003,** *198* (1), 111-122.

34. Carbone, F. R.; Bevan, M. J., Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. *J Exp Med* **1990,** *171* (2), 377-87.

35. Shen, L.; Sigal, L. J.; Boes, M.; Rock, K. L., Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity* **2004,** *21* (2), 155-65.

36. Palmowski, M. J.; Gileadi, U.; Salio, M.; Gallimore, A.; Millrain, M.; James, E.; Addey, C.; Scott, D.; Dyson, J.; Simpson, E.; Cerundolo, V., Role of immunoproteasomes in cross-presentation. *J Immunol* **2006,** *177* (2), 983-90.

37. Kovacsovics-Bankowski, M.; Rock, K. L., A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* **1995,** *267* (5195), 243-6.

38. Guermonprez, P.; Saveanu, L.; Kleijmeer, M.; Davoust, J.; Van Endert, P.; Amigorena, S., ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. *Nature* **2003,** *425* (6956), 397-402.

39. Sigal, L. J.; Crotty, S.; Andino, R.; Rock, K. L., Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* **1999,** *398* (6722), 77-80.

40. Huang, A. Y.; Golumbek, P.; Ahmadzadeh, M.; Jaffee, E.; Pardoll, D.; Levitsky, H., Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* **1994,** *264* (5161), 961-5.

41. Ho, N. I.; Huis in 't Veld, L. G. M.; Raaijmakers, T. K.; Adema, G. J., Adjuvants Enhancing Cross-Presentation by Dendritic Cells: The Key to More Effective Vaccines? *Front Immunol* **2018,** *9* (2874).

42. Kaech, S. M.; Cui, W., Transcriptional control of effector and memory CD8+ T cell differentiation. *Nat Rev Immunol* **2012,** *12* (11), 749-61.

43. Liechtenstein, T.; Dufait, I.; Lanna, A.; Breckpot, K.; Escors, D., MODULATING CO-STIMULATION DURING ANTIGEN PRESENTATION TO ENHANCE CANCER IMMUNOTHERAPY. *Immunol Endocr Metab Agents Med Chem* **2012,** *12* (3), 224-235.

44. Bachmaier, K.; Krawczyk, C.; Kozieradzki, I.; Kong, Y. Y.; Sasaki, T.; Oliveira-dos-Santos, A.; Mariathasan, S.; Bouchard, D.; Wakeham, A.; Itie, A.; Le, J.; Ohashi, P. S.; Sarosi, I.; Nishina, H.; Lipkowitz, S.; Penninger, J. M., Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* **2000,** *403* (6766), 211-6.

45. Chiang, Y. J.; Kole, H. K.; Brown, K.; Naramura, M.; Fukuhara, S.; Hu, R. J.; Jang, I. K.; Gutkind, J. S.; Shevach, E.; Gu, H., Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* **2000,** *403* (6766), 216-20.

46. Rowley, T. F.; Al-Shamkhani, A., Stimulation by Soluble CD70 Promotes Strong Primary and Secondary CD8⁺ Cytotoxic T Cell Responses In Vivo. *The Journal of Immunology* **2004,** *172* (10), 6039-6046.

47. Frauwirth, K. A.; Thompson, C. B., Activation and inhibition of lymphocytes by costimulation. *The Journal of Clinical Investigation* **2002,** *109* (3), 295-299.

48. Sharpe, A. H.; Freeman, G. J., The B7–CD28 superfamily. *Nature Reviews Immunology* **2002,** *2* (2), 116-126.

49. Croft, M., Costimulation of T cells by OX40, 4-1BB, and CD27. *Cytokine Growth Factor Rev* **2003,** *14* (3-4), 265-73.

50. Cho, H. J.; Takabayashi, K.; Cheng, P.-M.; Nguyen, M.-D.; Corr, M.; Tuck, S.; Raz, E., Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism. *Nature Biotechnology* **2000,** *18* (5), 509-514.

51. Trinchieri, G.; Pflanz, S.; Kastelein, R. A., The IL-12 Family of Heterodimeric Cytokines: New Players in the Regulation of T Cell Responses. *Immunity* **2003,** *19* (5), 641-644.

52. Curtsinger, J. M.; Schmidt, C. S.; Mondino, A.; Lins, D. C.; Kedl, R. M.; Jenkins, M. K.; Mescher, M. F., Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J Immunol* **1999,** *162* (6), 3256-62.

53. Curtsinger, J. M.; Valenzuela, J. O.; Agarwal, P.; Lins, D.; Mescher, M. F., Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. *J Immunol* **2005,** *174* (8), 4465-9.

54. Swain, S. L.; Weinberg, A. D.; English, M.; Huston, G., IL-4 directs the development of Th2-like helper effectors. *J Immunol* **1990,** *145* (11), 3796-806.

55. Kaiko, G. E.; Horvat, J. C.; Beagley, K. W.; Hansbro, P. M., Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology* **2008,** *123* (3), 326-338.

56. Kalinski, P.; Giermasz, A.; Nakamura, Y.; Basse, P.; Storkus, W. J.; Kirkwood, J. M.; Mailliard, R. B., Helper role of NK cells during the induction of anticancer responses by dendritic cells. *Molecular Immunology* **2005,** *42* (4), 535-539.

57. Schmitt, N.; Morita, R.; Bourdery, L.; Bentebibel, S. E.; Zurawski, S. M.; Banchereau, J.; Ueno, H., Human Dendritic Cells Induce the Differentiation of Interleukin-21-Producing T Follicular Helper-like Cells through Interleukin-12. *Immunity* **2009,** *31* (1), 158-169.

58. Steinman, R. M., The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* **1991,** *9*, 271-96.

59. Fooksman, D. R.; Vardhana, S.; Vasiliver-Shamis, G.; Liese, J.; Blair, D. A.; Waite, J.; Sacristan, C.; Victora, G. D.; Zanin-Zhorov, A.; Dustin, M. L., Functional anatomy of T cell activation and synapse formation. *Annu Rev Immunol* **2010,** *28*, 79-105.

60. Hollingsworth, R. E.; Jansen, K., Turning the corner on therapeutic cancer vaccines. *npj Vaccines* **2019,** *4* (1), 7.

61. Perez, C. R.; De Palma, M., Engineering dendritic cell vaccines to improve cancer immunotherapy. *Nature Communications* **2019,** *10* (1), 5408.

62. Ghirelli, C.; Hagemann, T., Targeting immunosuppression for cancer therapy. *J Clin Invest* **2013,** *123* (6), 2355-7.

63. Blankenstein, T.; Coulie, P. G.; Gilboa, E.; Jaffee, E. M., The determinants of tumour immunogenicity. *Nat Rev Cancer* **2012,** *12* (4), 307-13.

64. Spranger, S.; Bao, R.; Gajewski, T. F., Melanoma-intrinsic beta-catenin signalling prevents anti-tumour immunity. *Nature* **2015,** *523* (7559), 231-5.

65. Davoodzadeh Gholami, M.; Kardar, G. A.; Saeedi, Y.; Heydari, S.; Garssen, J.; Falak, R., Exhaustion of T lymphocytes in the tumor microenvironment: Significance and effective mechanisms. *Cell Immunol* **2017,** *322*, 1-14.

66. van Willigen, W. W.; Bloemendal, M.; Gerritsen, W. R.; Schreibelt, G.; de Vries, I. J. M.; Bol, K. F., Dendritic Cell Cancer Therapy: Vaccinating the Right Patient at the Right Time. *Front Immunol* **2018,** *9* (2265).

67. Palucka, K.; Banchereau, J., Dendritic-cell-based therapeutic cancer vaccines. *Immunity* **2013,** *39* (1), 38-48.

68. Aarntzen, E. H.; De Vries, I. J.; Lesterhuis, W. J.; Schuurhuis, D.; Jacobs, J. F.; Bol, K.; Schreibelt, G.; Mus, R.; De Wilt, J. H.; Haanen, J. B.; Schadendorf, D.; Croockewit, A.; Blokx, W. A.; Van Rossum, M. M.; Kwok, W. W.; Adema, G. J.; Punt, C. J.; Figdor, C. G., Targeting CD4(+) T-helper cells improves the induction of antitumor responses in dendritic cell-based vaccination. *Cancer Res* **2013,** *73* (1), 19-29.

69. Baldin, A. V.; Savvateeva, L. V.; Bazhin, A. V.; Zamyatnin, A. A., Jr., Dendritic Cells in Anticancer Vaccination: Rationale for Ex Vivo Loading or In Vivo Targeting. *Cancers (Basel)* **2020,** *12* (3).

70. Pedersen, S. R.; Sorensen, M. R.; Buus, S.; Christensen, J. P.; Thomsen, A. R., Comparison of vaccine-induced effector CD8 T cell responses directed against self- and non-self-tumor antigens: implications for cancer immunotherapy. *J Immunol* **2013,** *191* (7), 3955-67.

71. Melero, I.; Gaudernack, G.; Gerritsen, W.; Huber, C.; Parmiani, G.; Scholl, S.; Thatcher, N.; Wagstaff, J.; Zielinski, C.; Faulkner, I.; Mellstedt, H., Therapeutic vaccines for cancer: an overview of clinical trials. *Nat Rev Clin Oncol* **2014,** *11* (9), 509-24.

72. Sahin, U.; Derhovanessian, E.; Miller, M.; Kloke, B. P.; Simon, P.; Lower, M.; Bukur, V.; Tadmor, A. D.; Luxemburger, U.; Schrors, B.; Omokoko, T.; Vormehr, M.; Albrecht, C.; Paruzynski, A.; Kuhn, A. N.; Buck, J.; Heesch, S.; Schreeb, K. H.; Muller, F.; Ortseifer, I.; Vogler, I.; Godehardt, E.; Attig, S.; Rae, R.; Breitkreuz, A.; Tolliver, C.; Suchan, M.; Martic, G.; Hohberger, A.; Sorn, P.; Diekmann, J.; Ciesla, J.; Waksmann, O.; Bruck, A. K.; Witt, M.; Zillgen, M.; Rothermel, A.; Kasemann, B.; Langer, D.; Bolte, S.; Diken, M.; Kreiter, S.; Nemecek, R.; Gebhardt, C.; Grabbe, S.; Holler, C.; Utikal, J.; Huber, C.; Loquai, C.; Tureci, O., Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* **2017,** *547* (7662), 222-226.

73. Ott, P. A.; Hu, Z.; Keskin, D. B.; Shukla, S. A.; Sun, J.; Bozym, D. J.; Zhang, W.; Luoma, A.; Giobbie-Hurder, A.; Peter, L.; Chen, C.; Olive, O.; Carter, T. A.; Li, S.; Lieb, D. J.; Eisenhaure, T.; Gjini, E.; Stevens, J.; Lane, W. J.; Javeri, I.; Nellaiappan, K.; Salazar, A. M.; Daley, H.; Seaman, M.; Buchbinder, E. I.; Yoon, C. H.; Harden, M.; Lennon, N.; Gabriel, S.; Rodig, S. J.; Barouch, D. H.; Aster, J. C.; Getz, G.; Wucherpfennig, K.; Neuberg, D.; Ritz, J.; Lander, E. S.; Fritsch, E. F.; Hacohen, N.; Wu, C. J., An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* **2017,** *547* (7662), 217-221.

74. Keskin, D. B.; Anandappa, A. J.; Sun, J.; Tirosh, I.; Mathewson, N. D.; Li, S.; Oliveira, G.; Giobbie-Hurder, A.; Felt, K.; Gjini, E.; Shukla, S. A.; Hu, Z.; Li, L.; Le, P. M.; Allesøe, R. L.; Richman, A. R.; Kowalczyk, M. S.; Abdelrahman, S.; Geduldig, J. E.; Charbonneau, S.; Pelton, K.; Iorgulescu, J. B.; Elagina, L.; Zhang, W.; Olive, O.; McCluskey, C.; Olsen, L. R.; Stevens, J.; Lane, W. J.; Salazar, A. M.; Daley, H.; Wen, P. Y.; Chiocca, E. A.; Harden, M.; Lennon, N. J.; Gabriel, S.; Getz, G.; Lander, E. S.; Regev, A.; Ritz, J.; Neuberg, D.; Rodig, S. J.; Ligon, K. L.; Suvà, M. L.; Wucherpfennig, K. W.; Hacohen, N.; Fritsch, E. F.; Livak, K. J.; Ott, P. A.; Wu, C. J.; Reardon, D. A., Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. *Nature* **2019,** *565* (7738), 234-239.

75. Hilf, N.; Kuttruff-Coqui, S.; Frenzel, K.; Bukur, V.; Stevanović, S.; Gouttefangeas, C.; Platten, M.; Tabatabai, G.; Dutoit, V.; van der Burg, S. H.; Thor Straten, P.; Martínez-Ricarte, F.; Ponsati, B.; Okada, H.; Lassen, U.; Admon, A.; Ottensmeier, C. H.; Ulges, A.; Kreiter, S.; von Deimling, A.; Skardelly, M.; Migliorini, D.; Kroep, J. R.; Idorn, M.; Rodon, J.; Piró, J.; Poulsen, H. S.; Shraibman, B.; McCann, K.; Mendrzyk, R.; Löwer, M.; Stieglbauer, M.; Britten, C. M.; Capper, D.; Welters, M. J. P.; Sahuquillo, J.; Kiesel, K.; Derhovanessian, E.; Rusch, E.; Bunse, L.; Song, C.; Heesch, S.; Wagner, C.; Kemmer-Brück, A.; Ludwig, J.; Castle, J. C.; Schoor, O.; Tadmor, A. D.; Green, E.; Fritsche, J.; Meyer, M.; Pawlowski, N.; Dorner, S.; Hoffgaard, F.; Rössler, B.; Maurer, D.; Weinschenk, T.; Reinhardt, C.; Huber, C.; Rammensee, H. G.; Singh-Jasuja, H.; Sahin, U.; Dietrich, P. Y.; Wick, W., Actively personalized vaccination trial for newly diagnosed glioblastoma. *Nature* **2019,** *565* (7738), 240-245.

76. Carreno, B. M.; Magrini, V.; Becker-Hapak, M.; Kaabinejadian, S.; Hundal, J.; Petti, A. A.; Ly, A.; Lie, W. R.; Hildebrand, W. H.; Mardis, E. R.; Linette, G. P., Cancer immunotherapy. A dendritic cell vaccine increases the breadth and diversity of melanoma neoantigen-specific T cells. *Science* **2015,** *348* (6236), 803-8.

77. Leoni, G.; D'Alise, A. M.; Cotugno, G.; Langone, F.; Garzia, I.; De Lucia, M.; Fichera, I.; Vitale, R.; Bignone, V.; Tucci, F. G.; Mori, F.; Leuzzi, A.; Di Matteo, E.; Troise, F.; Abbate, A.; Merone, R.; Ruzza, V.; Diodoro, M. G.; Yadav, M.; Gordon-Alonso, M.; Vanhaver, C.; Panigada, M.; Soprana, E.; Siccardi, A.; Folgori, A.; Colloca, S.; van der Bruggen, P.; Nicosia, A.; Lahm, A.; Catanese, M. T.; Scarselli, E., A genetic vaccine encoding shared cancer neoantigens to treat tumors with Microsatellite Instability. *Cancer Research* **2020**, canres.1072.2020.

78. Peterson, M.; Murphy, S. N.; Lainson, J.; Zhang, J.; Shen, L.; Diehnelt, C. W.; Johnston, S. A., Comparison of personal and shared frameshift neoantigen vaccines in a mouse mammary cancer model. *BMC Immunol* **2020,** *21* (1), 25-25.

79. Santos, P. M.; Butterfield, L. H., Dendritic Cell-Based Cancer Vaccines. *Journal of immunology (Baltimore, Md. : 1950)* **2018,** *200* (2), 443-449.

80. Turnis, M. E.; Rooney, C. M., Enhancement of dendritic cells as vaccines for cancer. *Immunotherapy* **2010,** *2* (6), 847-862.

81. Banchereau, J.; Palucka, A. K.; Dhodapkar, M.; Burkeholder, S.; Taquet, N.; Rolland, A.; Taquet, S.; Coquery, S.; Wittkowski, K. M.; Bhardwaj, N.; Pineiro, L.; Steinman, R.; Fay, J., Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. *Cancer Res* **2001,** *61* (17), 6451-8.

82. Onodi, F.; Maherzi-Mechalikh, C.; Mougel, A.; Ben Hamouda, N.; Taboas, C.; Gueugnon, F.; Tran, T.; Nozach, H.; Marcon, E.; Gey, A.; Terme, M.; Bouzidi, A.; Maillere, B.; Kerzerho, J.; Tartour, E.; Tanchot, C., High Therapeutic Efficacy of a New Survivin LSP-Cancer Vaccine Containing CD4+ and CD8+ T-Cell Epitopes. *Frontiers in Oncology* **2018,** *8* (517).

83. Disis, M. L., Immunologic biomarkers as correlates of clinical response to cancer immunotherapy. *Cancer Immunol Immunother* **2011,** *60* (3), 433-42.

84. Lehmann, P. V.; Forsthuber, T.; Miller, A.; Sercarz, E. E., Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* **1992,** *358* (6382), 155-7.

85. Inaba, K.; Inaba, M.; Romani, N.; Aya, H.; Deguchi, M.; Ikehara, S.; Muramatsu, S.; Steinman, R. M., Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* **1992,** *176* (6), 1693-702.

86. Hsu, F. J.; Benike, C.; Fagnoni, F.; Liles, T. M.; Czerwinski, D.; Taidi, B.; Engleman, E. G.; Levy, R., Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* **1996,** *2* (1), 52-8.

87. Sharma, M. D.; Rodriguez, P. C.; Koehn, B. H.; Baban, B.; Cui, Y.; Guo, G.; Shimoda, M.; Pacholczyk, R.; Shi, H.; Lee, E. J.; Xu, H.; Johnson, T. S.; He, Y.; Mergoub, T.; Venable, C.; Bronte, V.; Wolchok, J. D.; Blazar, B. R.; Munn, D. H., Activation of p53 in Immature Myeloid Precursor Cells Controls Differentiation into Ly6c(+)CD103(+) Monocytic Antigen-Presenting Cells in Tumors. *Immunity* **2018,** *48* (1), 91-106.e6.

88. Proietto, A. I.; Mittag, D.; Roberts, A. W.; Sprigg, N.; Wu, L., The equivalents of human blood and spleen dendritic cell subtypes can be generated in vitro from human CD34(+) stem cells in the presence of fms-like tyrosine kinase 3 ligand and thrombopoietin. *Cell Mol Immunol* **2012,** *9* (6), 446-54.

89. Vopenkova, K.; Mollova, K.; Buresova, I.; Michalek, J., Complex evaluation of human monocyte-derived dendritic cells for cancer immunotherapy. *J Cell Mol Med* **2012,** *16* (11), 2827-37.

90. Elkord, E.; Williams, P. E.; Kynaston, H.; Rowbottom, A. W., Human monocyte isolation methods influence cytokine production from in vitro generated dendritic cells. *Immunology* **2005,** *114* (2), 204-12.

91. Marques, G. S.; Silva, Z.; Videira, P. A., Antitumor Efficacy of Human Monocyte-Derived Dendritic Cells: Comparing Effects of two Monocyte Isolation Methods. *Biol Proced Online* **2018,** *20*, 4.

92. Tacken, P. J.; de Vries, I. J.; Torensma, R.; Figdor, C. G., Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. *Nat Rev Immunol* **2007,** *7* (10), 790-802.

93. Sweny, P.; Lui, S. F.; Scoble, J. E.; Varghese, Z.; Fernando, O. N.; Moorhead, J. F., Conversion of stable renal allografts at one year from cyclosporin A to azathioprine: a randomized controlled study. *Transpl Int* **1990,** *3* (1), 19-22.

94. Hsu, L.; Zhu, X. N.; Zhao, Y. S., Immunoglobulin E and circulating immune complexes in endolymphatic hydrops. *Ann Otol Rhinol Laryngol* **1990,** *99* (7 Pt 1), 535-8.

95. Dannull, J.; Su, Z.; Rizzieri, D.; Yang, B. K.; Coleman, D.; Yancey, D.; Zhang, A.; Dahm, P.; Chao, N.; Gilboa, E.; Vieweg, J., Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest* **2005,** *115* (12), 3623-33.

96. Palucka, K.; Ueno, H.; Fay, J.; Banchereau, J., Harnessing dendritic cells to generate cancer vaccines. *Ann N Y Acad Sci* **2009,** *1174*, 88-98.

97. Gilboa, E.; Vieweg, J., Cancer immunotherapy with mRNA-transfected dendritic cells. *Immunol Rev* **2004,** *199*, 251-63.

98. Pecher, G.; Haring, A.; Kaiser, L.; Thiel, E., Mucin gene (MUC1) transfected dendritic cells as vaccine: results of a phase I/II clinical trial. *Cancer Immunol Immunother* **2002,** *51* (11-12), 669-73.

99. Butterfield, L. H.; Comin-Anduix, B.; Vujanovic, L.; Lee, Y.; Dissette, V. B.; Yang, J. Q.; Vu, H. T.; Seja, E.; Oseguera, D. K.; Potter, D. M.; Glaspy, J. A.; Economou, J. S.; Ribas, A., Adenovirus MART-1-engineered autologous dendritic cell vaccine for metastatic melanoma. *J Immunother* **2008,** *31* (3), 294-309.

100. Di Nicola, M.; Carlo-Stella, C.; Mortarini, R.; Baldassari, P.; Guidetti, A.; Gallino, G. F.; Del Vecchio, M.; Ravagnani, F.; Magni, M.; Chaplin, P.; Cascinelli, N.; Parmiani, G.; Gianni, A. M.; Anichini, A., Boosting T cell-mediated immunity to tyrosinase by vaccinia virus-transduced, CD34(+)-derived dendritic cell vaccination: a phase I trial in metastatic melanoma. *Clin Cancer Res* **2004,** *10* (16), 5381-90.

101. Ahmed, M. S.; Bae, Y. S., Dendritic cell-based therapeutic cancer vaccines: past, present and future. *Clin Exp Vaccine Res* **2014,** *3* (2), 113-6.

102. Jonuleit, H.; Kühn, U.; Müller, G.; Steinbrink, K.; Paragnik, L.; Schmitt, E.; Knop, J.; Enk, A. H., Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *European Journal of Immunology* **1997,** *27* (12), 3135-3142.

103. Dauer, M.; Obermaier, B.; Herten, J.; Haerle, C.; Pohl, K.; Rothenfusser, S.; Schnurr, M.; Endres, S.; Eigler, A., Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. *J Immunol* **2003,** *170* (8), 4069-76.

104. Kvistborg, P.; Boegh, M.; Pedersen, A. W.; Claesson, M. H.; Zocca, M. B., Fast generation of dendritic cells. *Cell Immunol* **2009,** *260* (1), 56-62.

105. Zobywalski, A.; Javorovic, M.; Frankenberger, B.; Pohla, H.; Kremmer, E.; Bigalke, I.; Schendel, D. J., Generation of clinical grade dendritic cells with capacity to produce biologically active IL-12p70. *J Transl Med* **2007,** *5*, 18.

106. Lichtenegger, F. S.; Mueller, K.; Otte, B.; Beck, B.; Hiddemann, W.; Schendel, D. J.; Subklewe, M., CD86 and IL-12p70 are key players for T helper 1 polarization and natural killer cell activation by Toll-like receptor-induced dendritic cells. *PLoS One* **2012,** *7* (9), e44266-e44266.

107. Bonifaz, L.; Bonnyay, D.; Mahnke, K.; Rivera, M.; Nussenzweig, M. C.; Steinman, R. M., Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J Exp Med* **2002,** *196* (12), 1627-38.

108. Hawiger, D.; Inaba, K.; Dorsett, Y.; Guo, M.; Mahnke, K.; Rivera, M.; Ravetch, J. V.; Steinman, R. M.; Nussenzweig, M. C., Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med* **2001,** *194* (6), 769-79.

109. Soares, H.; Waechter, H.; Glaichenhaus, N.; Mougneau, E.; Yagita, H.; Mizenina, O.; Dudziak, D.; Nussenzweig, M. C.; Steinman, R. M., A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo. *J Exp Med* **2007,** *204* (5), 1095-106.

110. Sancho, D.; Reis e Sousa, C., Sensing of cell death by myeloid C-type lectin receptors. *Curr Opin Immunol* **2013,** *25* (1), 46-52.

111. Hossain, M. K.; Wall, K. A., Use of Dendritic Cell Receptors as Targets for Enhancing Anti-Cancer Immune Responses. *Cancers* **2019,** *11* (3), 418.

112. Robinson, M. J.; Sancho, D.; Slack, E. C.; LeibundGut-Landmann, S.; Reis e Sousa, C., Myeloid C-type lectins in innate immunity. *Nat Immunol* **2006,** *7* (12), 1258-65.

113. Dambuza, I. M.; Brown, G. D., C-type lectins in immunity: recent developments. *Curr Opin Immunol* **2015,** *32*, 21-7.

114. Barr, T. A.; McCormick, A. L.; Carlring, J.; Heath, A. W., A potent adjuvant effect of CD40 antibody attached to antigen. *Immunology* **2003,** *109* (1), 87-92.

115. Reuter, A.; Panozza, S. E.; Macri, C.; Dumont, C.; Li, J.; Liu, H.; Segura, E.; Vega-Ramos, J.; Gupta, N.; Caminschi, I.; Villadangos, J. A.; Johnston, A. P.; Mintern, J. D., Criteria for dendritic cell receptor selection for efficient antibody-targeted vaccination. *J Immunol* **2015,** *194* (6), 2696-705.

116. Chatterjee, B.; Smed-Sorensen, A.; Cohn, L.; Chalouni, C.; Vandlen, R.; Lee, B. C.; Widger, J.; Keler, T.; Delamarre, L.; Mellman, I., Internalization and endosomal degradation of receptor-bound antigens regulate the efficiency of cross presentation by human dendritic cells. *Blood* **2012,** *120* (10), 2011-20.

117. Kato, M.; McDonald, K. J.; Khan, S.; Ross, I. L.; Vuckovic, S.; Chen, K.; Munster, D.; MacDonald, K. P.; Hart, D. N., Expression of human DEC-205 (CD205) multilectin receptor on leukocytes. *Int Immunol* **2006,** *18* (6), 857-69.

118. Van Kooten, C.; Banchereau, J., CD40-CD40 ligand: a multifunctional receptor-ligand pair. *Adv Immunol* **1996,** *61*, 1-77.

119. van Kooten, C.; Banchereau, J., Functions of CD40 on B cells, dendritic cells and other cells. *Curr Opin Immunol* **1997,** *9* (3), 330-7.

120. Galy, A. H.; Spits, H., CD40 is functionally expressed on human thymic epithelial cells. *J Immunol* **1992,** *149* (3), 775-82.

121. Stewart, R.; Hammond, S. A.; Oberst, M.; Wilkinson, R. W., The role of Fc gamma receptors in the activity of immunomodulatory antibodies for cancer. *Journal for ImmunoTherapy of Cancer* **2014,** *2* (1), 29.

122. Beckman, R. A.; Weiner, L. M.; Davis, H. M., Antibody constructs in cancer therapy: protein engineering strategies to improve exposure in solid tumors. *Cancer* **2007,** *109* (2), 170-9.

123. Birkholz, K.; Schwenkert, M.; Kellner, C.; Gross, S.; Fey, G.; Schuler-Thurner, B.; Schuler, G.; Schaft, N.; Dorrie, J., Targeting of DEC-205 on human dendritic cells results in efficient MHC class II-restricted antigen presentation. *Blood* **2010,** *116* (13), 2277-85.

124. Schlothauer, T.; Herter, S.; Koller, C. F.; Grau-Richards, S.; Steinhart, V.; Spick, C.; Kubbies, M.; Klein, C.; Umana, P.; Mossner, E., Novel human IgG1 and IgG4 Fc-engineered antibodies with completely abolished immune effector functions. *Protein Eng Des Sel* **2016,** *29* (10), 457-466.

125. De Groof, T.; Bobkov, V.; Heukers, R.; Smit, M., Nanobodies: New avenues for imaging, stabilizing and modulating GPCRs. *Molecular and Cellular Endocrinology* **2019,** *484*, 15-24.

126. Steinman, R. M., Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol* **2012,** *30*, 1-22.

127. Sanchez, P. J.; McWilliams, J. A.; Haluszczak, C.; Yagita, H.; Kedl, R. M., Combined TLR/CD40 Stimulation Mediates Potent Cellular Immunity by Regulating Dendritic Cell Expression of CD70 In Vivo. *The Journal of Immunology* **2007,** *178* (3), 1564-1572.

128. Fujii, S.; Liu, K.; Smith, C.; Bonito, A. J.; Steinman, R. M., The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* **2004,** *199* (12), 1607-18.

129. Ahonen, C. L.; Doxsee, C. L.; McGurran, S. M.; Riter, T. R.; Wade, W. F.; Barth, R. J.; Vasilakos, J. P.; Noelle, R. J.; Kedl, R. M., Combined TLR and CD40 triggering induces potent CD8+ T cell expansion with variable dependence on type I IFN. *The Journal of experimental medicine* **2004,** *199* (6), 775-784.

130. Kerkmann, M.; Rothenfusser, S.; Hornung, V.; Towarowski, A.; Wagner, M.; Sarris, A.; Giese, T.; Endres, S.; Hartmann, G., Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. *J Immunol* **2003,** *170* (9), 4465-74.

131. Hoki, T.; Yamauchi, T.; Odunsi, K.; Ito, F., Synergistic anti-tumor efficacy of combined TLR3 and CD40 neoantigen vaccine requires Batf3-dependent dendritic cells. *The Journal of Immunology* **2018,** *200* (1 Supplement), 181.7-181.7.

132. Kreutz, M.; Tacken, P. J.; Figdor, C. G., Targeting dendritic cells--why bother? *Blood* **2013,** *121* (15), 2836-44.

133. Tacken, P. J.; Zeelenberg, I. S.; Cruz, L. J.; van Hout-Kuijer, M. A.; van de Glind, G.; Fokkink, R. G.; Lambeck, A. J.; Figdor, C. G., Targeted delivery of TLR ligands to human and mouse dendritic cells strongly enhances adjuvanticity. *Blood* **2011,** *118* (26), 6836-44.

134. Kreutz, M.; Giquel, B.; Hu, Q.; Abuknesha, R.; Uematsu, S.; Akira, S.; Nestle, F. O.; Diebold, S. S., Antibody-Antigen-Adjuvant Conjugates Enable Co-Delivery of Antigen and Adjuvant to Dendritic Cells in Cis but Only Have Partial Targeting Specificity. *PLoS One* **2012,** *7* (7), e40208.

135. Morse, M. A.; Chapman, R.; Powderly, J.; Blackwell, K.; Keler, T.; Green, J.; Riggs, R.; He, L.-Z.; Ramakrishna, V.; Vitale, L.; Zhao, B.; Butler, S. A.; Hobeika, A.; Osada, T.; Davis, T.; Clay, T.; Lyerly, H. K., Phase I study utilizing a novel antigen-presenting cell-targeted vaccine with Toll-like receptor stimulation to induce immunity to self-antigens in cancer patients. *Clinical cancer research : an official journal of the American Association for Cancer Research* **2011,** *17* (14), 4844-4853.

136. Bradley, D.; Morse, M.; Keler, T.; Green, J.; Davis, T.; Inman, B., A randomized phase II study of a novel antigen-presenting cell-targeted hCG-β vaccine (the CDX-1307 regimen) in muscle-invasive bladder cancer. *Journal of Clinical Oncology* **2010,** *28*, TPS228-TPS228.

137. Akira, S.; Uematsu, S.; Takeuchi, O., Pathogen recognition and innate immunity. *Cell* **2006,** *124* (4), 783-801.

138. Beutler, B.; Jiang, Z.; Georgel, P.; Crozat, K.; Croker, B.; Rutschmann, S.; Du, X.; Hoebe, K., Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. *Annu Rev Immunol* **2006,** *24*, 353-89.

139. Janeway, C. A., Jr.; Medzhitov, R., Innate immune recognition. *Annu Rev Immunol* **2002,** *20*, 197-216.

140. Pandey, S.; Kawai, T.; Akira, S., Microbial sensing by Toll-like receptors and intracellular nucleic acid sensors. *Cold Spring Harb Perspect Biol* **2014,** *7* (1), a016246-a016246.

141. Schroder, K.; Tschopp, J., The inflammasomes. *Cell* **2010,** *140* (6), 821-32.

142. Takeuchi, O.; Akira, S., Pattern recognition receptors and inflammation. *Cell* **2010,** *140* (6), 805-20.

143. Wang, X.; Majumdar, T.; Kessler, P.; Ozhegov, E.; Zhang, Y.; Chattopadhyay, S.; Barik, S.; Sen, G. C., STING Requires the Adaptor TRIF to Trigger Innate Immune Responses to Microbial Infection. *Cell Host & Microbe* **2016,** *20* (3), 329-341.

144. Kawai, T.; Akira, S., The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol* **2009,** *21* (4), 317-37.

145. Gao, W.; Xiong, Y.; Li, Q.; Yang, H., Inhibition of Toll-Like Receptor Signaling as a Promising Therapy for Inflammatory Diseases: A Journey from Molecular to Nano Therapeutics. *Front Physiol* **2017,** *8*, 508-508.

146. Coley, W. B., The Treatment of Inoperable Sarcoma by Bacterial Toxins (the Mixed Toxins of the Streptococcus erysipelas and the Bacillus prodigiosus). *Proc R Soc Med* **1910,** *3* (Surg Sect), 1-48.

147. Rezaei, N., Therapeutic targeting of pattern-recognition receptors. *Int Immunopharmacol* **2006,** *6* (6), 863-869.

148. Urban-Wojciuk, Z.; Khan, M. M.; Oyler, B. L.; Fåhraeus, R.; Marek-Trzonkowska, N.; Nita-Lazar, A.; Hupp, T. R.; Goodlett, D. R., The Role of TLRs in Anti-cancer Immunity and Tumor Rejection. *Front Immunol* **2019,** *10* (2388).

149. Akira, S.; Takeda, K., Toll-like receptor signalling. *Nature Reviews Immunology* **2004,** *4* (7), 499-511.

150. Hashimoto, C.; Hudson, K. L.; Anderson, K. V., The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **1988,** *52* (2), 269-79.

151. Lemaitre, B.; Nicolas, E.; Michaut, L.; Reichhart, J.-M.; Hoffmann, J. A., The Dorsoventral Regulatory Gene Cassette spätzle/Toll/cactus Controls the Potent Antifungal Response in Drosophila Adults. *Cell* **1996,** *86* (6), 973-983.

152. Hoffmann, J. A., The immune response of Drosophila. *Nature* **2003,** *426* (6962), 33-38.

153. Bowie, A.; O'Neill, L. A. J., The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *Journal of Leukocyte Biology* **2000,** *67* (4), 508-514.

154. Kawai, T.; Akira, S., Regulation of innate immune signalling pathways by the tripartite motif (TRIM) family proteins. *EMBO Mol Med* **2011,** *3* (9), 513-27.

155. Kawasaki, T.; Kawai, T., Toll-like receptor signaling pathways. *Front Immunol* **2014,** *5*, 461-461.

156. Broad, A.; Kirby, J. A.; Jones, D. E., Toll-like receptor interactions: tolerance of MyD88-dependent cytokines but enhancement of MyD88-independent interferon-beta production. *Immunology* **2007,** *120* (1), 103-11.

157. Saito, T.; Hirai, R.; Loo, Y. M.; Owen, D.; Johnson, C. L.; Sinha, S. C.; Akira, S.; Fujita, T.; Gale, M., Jr., Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc Natl Acad Sci U S A* **2007,** *104* (2), 582-7.

158. Kawai, T.; Akira, S., TLR signaling. *Semin Immunol* **2007,** *19* (1), 24-32.

159. Takeuchi, O.; Sato, S.; Horiuchi, T.; Hoshino, K.; Takeda, K.; Dong, Z.; Modlin, R. L.; Akira, S., Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol* **2002,** *169* (1), 10-4.

160. Takeuchi, O.; Hoshino, K.; Kawai, T.; Sanjo, H.; Takada, H.; Ogawa, T.; Takeda, K.; Akira, S., Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **1999,** *11* (4), 443-51.

161. Chow, J. C.; Young, D. W.; Golenbock, D. T.; Christ, W. J.; Gusovsky, F., Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* **1999,** *274* (16), 10689-92.

162. Kurt-Jones, E. A.; Popova, L.; Kwinn, L.; Haynes, L. M.; Jones, L. P.; Tripp, R. A.; Walsh, E. E.; Freeman, M. W.; Golenbock, D. T.; Anderson, L. J.; Finberg, R. W., Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* **2000,** *1* (5), 398-401.

163. Alexopoulou, L.; Holt, A. C.; Medzhitov, R.; Flavell, R. A., Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **2001,** *413* (6857), 732-8.

164. Heil, F.; Hemmi, H.; Hochrein, H.; Ampenberger, F.; Kirschning, C.; Akira, S.; Lipford, G.; Wagner, H.; Bauer, S., Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **2004,** *303* (5663), 1526-9.

165. Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K.; Akira, S., A Toll-like receptor recognizes bacterial DNA. *Nature* **2000,** *408* (6813), 740-5.

166. Hayashi, F.; Smith, K. D.; Ozinsky, A.; Hawn, T. R.; Yi, E. C.; Goodlett, D. R.; Eng, J. K.; Akira, S.; Underhill, D. M.; Aderem, A., The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **2001,** *410* (6832), 1099-103.

167. Hasan, U.; Chaffois, C.; Gaillard, C.; Saulnier, V.; Merck, E.; Tancredi, S.; Guiet, C.; Briere, F.; Vlach, J.; Lebecque, S.; Trinchieri, G.; Bates, E. E., Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol* **2005,** *174* (5), 2942-50.

168. Henrick, B. M.; Yao, X.-D.; Zahoor, M. A.; Abimiku, A. l.; Osawe, S.; Rosenthal, K. L., TLR10 Senses HIV-1 Proteins and Significantly Enhances HIV-1 Infection. *Front Immunol* **2019,** *10* (482).

169. Vasou, A.; Sultanoglu, N.; Goodbourn, S.; Randall, R. E.; Kostrikis, L. G., Targeting Pattern Recognition Receptors (PRR) for Vaccine Adjuvantation: From Synthetic PRR Agonists to the Potential of Defective Interfering Particles of Viruses. *Viruses* **2017,** *9* (7), 186.

170. Kang, J.; Demaria, S.; Formenti, S., Current clinical trials testing the combination of immunotherapy with radiotherapy. *J Immunother Cancer* **2016,** *4*, 51.

171. von Meyenn, F.; Schaefer, M.; Weighardt, H.; Bauer, S.; Kirschning, C. J.; Wagner, H.; Sparwasser, T., Toll-like receptor 9 contributes to recognition of Mycobacterium bovis Bacillus Calmette-Guerin by Flt3-ligand generated dendritic cells. *Immunobiology* **2006,** *211* (6-8), 557-65.

172. Morales, A.; Eidinger, D.; Bruce, A. W., Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. *J Urol* **1976,** *116* (2), 180-3.

173. Lebwohl, M.; Dinehart, S.; Whiting, D.; Lee, P. K.; Tawfik, N.; Jorizzo, J.; Lee, J. H.; Fox, T. L., Imiquimod 5% cream for the treatment of actinic keratosis: results from two phase III, randomized, double-blind, parallel group, vehicle-controlled trials. *J Am Acad Dermatol* **2004,** *50* (5), 714-21.

174. Fehres, C. M.; Bruijns, S. C. M.; van Beelen, A. J.; Kalay, H.; Ambrosini, M.; Hooijberg, E.; Unger, W. W. J.; de Gruijl, T. D.; van Kooyk, Y., Topical rather than intradermal application of the TLR7 ligand imiquimod leads to human dermal dendritic cell maturation and CD8+ T-cell cross-priming. *European Journal of Immunology* **2014,** *44* (8), 2415-2424.

175. Adams, S.; O'Neill, D. W.; Nonaka, D.; Hardin, E.; Chiriboga, L.; Siu, K.; Cruz, C. M.; Angiulli, A.; Angiulli, F.; Ritter, E.; Holman, R. M.; Shapiro, R. L.; Berman, R. S.; Berner, N.; Shao, Y.; Manches, O.; Pan, L.; Venhaus, R. R.; Hoffman, E. W.; Jungbluth, A.; Gnjatic, S.; Old, L.; Pavlick, A. C.; Bhardwaj, N., Immunization of malignant melanoma patients with full-length NY-ESO-1 protein using TLR7 agonist imiquimod as vaccine adjuvant. *J Immunol* **2008,** *181* (1), 776-84.

176. Hung, I. F.; Zhang, A. J.; To, K. K.; Chan, J. F.; Li, C.; Zhu, H. S.; Li, P.; Li, C.; Chan, T. C.; Cheng, V. C.; Chan, K. H.; Yuen, K. Y., Immunogenicity of intradermal trivalent influenza vaccine with topical imiquimod: a double blind randomized controlled trial. *Clin Infect Dis* **2014,** *59* (9), 1246-55.

177. Casella, C. R.; Mitchell, T. C., Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. *Cell Mol Life Sci* **2008,** *65* (20), 3231-40.

178. Cluff, C. W., Monophosphoryl Lipid A (MPL) as an Adjuvant for Anti-Cancer Vaccines: Clinical Results. In *Lipid A in Cancer Therapy*, Jeannin, J.-F., Ed. Springer New York: New York, NY, 2010; pp 111-123.

179. Garçon, N.; Di Pasquale, A., From discovery to licensure, the Adjuvant System story. *Hum Vaccin Immunother* **2017,** *13* (1), 19-33.

180. Speiser, D. E.; Lienard, D.; Rufer, N.; Rubio-Godoy, V.; Rimoldi, D.; Lejeune, F.; Krieg, A. M.; Cerottini, J. C.; Romero, P., Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J Clin Invest* **2005,** *115* (3), 739-46.

181. Karbach, J.; Gnjatic, S.; Bender, A.; Neumann, A.; Weidmann, E.; Yuan, J.; Ferrara, C. A.; Hoffmann, E.; Old, L. J.; Altorki, N. K.; Jager, E., Tumor-reactive CD8+ T-cell responses after vaccination with NY-ESO-1 peptide, CpG 7909 and Montanide ISA-51: association with survival. *Int J Cancer* **2010,** *126* (4), 909-18.

182. Mehrotra, S.; Britten, C. D.; Chin, S.; Garrett-Mayer, E.; Cloud, C. A.; Li, M.; Scurti, G.; Salem, M. L.; Nelson, M. H.; Thomas, M. B.; Paulos, C. M.; Salazar, A. M.; Nishimura, M. I.; Rubinstein, M. P.; Li, Z.; Cole, D. J., Vaccination with poly(IC:LC) and peptide-pulsed autologous dendritic cells in patients with pancreatic cancer. *J Hematol Oncol* **2017,** *10* (1), 82-82.

183. Okada, H.; Kalinski, P.; Ueda, R.; Hoji, A.; Kohanbash, G.; Donegan, T. E.; Mintz, A. H.; Engh, J. A.; Bartlett, D. L.; Brown, C. K.; Zeh, H.; Holtzman, M. P.; Reinhart, T. A.; Whiteside, T. L.; Butterfield, L. H.; Hamilton, R. L.; Potter, D. M.; Pollack, I. F.; Salazar, A. M.; Lieberman, F. S., Induction of CD8+ T-cell responses against novel glioma-associated antigen peptides and clinical activity by vaccinations with {alpha}-type 1 polarized dendritic cells and polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose in patients with recurrent malignant glioma. *J Clin Oncol* **2011,** *29* (3), 330-6.

184. Burdelya, L. G.; Krivokrysenko, V. I.; Tallant, T. C.; Strom, E.; Gleiberman, A. S.; Gupta, D.; Kurnasov, O. V.; Fort, F. L.; Osterman, A. L.; Didonato, J. A.; Feinstein, E.; Gudkov, A. V., An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. *Science* **2008,** *320* (5873), 226-30.

185. Vijay-Kumar, M.; Aitken, J. D.; Sanders, C. J.; Frias, A.; Sloane, V. M.; Xu, J.; Neish, A. S.; Rojas, M.; Gewirtz, A. T., Flagellin treatment protects against chemicals, bacteria, viruses, and radiation. *J Immunol* **2008,** *180* (12), 8280-5.

186. Taylor, D. N.; Treanor, J. J.; Strout, C.; Johnson, C.; Fitzgerald, T.; Kavita, U.; Ozer, K.; Tussey, L.; Shaw, A., Induction of a potent immune response in the elderly using the TLR-5 agonist, flagellin, with a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125, STF2.HA1 SI). *Vaccine* **2011,** *29* (31), 4897-902.

187. Holbrook, B. C.; D'Agostino, R. B., Jr.; Parks, G. D.; Alexander-Miller, M. A., Adjuvanting an inactivated influenza vaccine with flagellin improves the function and quantity of the long-term antibody response in a nonhuman primate neonate model. *Vaccine* **2016,** *34* (39), 4712-4717.

188. Labastida-Conde, R. G.; Ramirez-Pliego, O.; Peleteiro-Olmedo, M.; Lopez-Guerrero, D. V.; Badillo-Godinez, O. D.; Gutierrez-Xicotencatl, M. L.; Rosas-Salgado, G.; Gonzalez-Fernandez, A.; Esquivel-Guadarrama, F. R.; Santana, M. A., Flagellin is a Th1 polarizing factor for human CD4(+) T cells

and induces protection in a murine neonatal vaccination model of rotavirus infection. *Vaccine* **2018,** *36* (29), 4188-4197.

189. Burdelya, L. G.; Brackett, C. M.; Kojouharov, B.; Gitlin, I. I.; Leonova, K. I.; Gleiberman, A. S.; Aygun-Sunar, S.; Veith, J.; Johnson, C.; Haderski, G. J.; Stanhope-Baker, P.; Allamaneni, S.; Skitzki, J.; Zeng, M.; Martsen, E.; Medvedev, A.; Scheblyakov, D.; Artemicheva, N. M.; Logunov, D. Y.; Gintsburg, A. L.; Naroditsky, B. S.; Makarov, S. S.; Gudkov, A. V., Central role of liver in anticancer and radioprotective activities of Toll-like receptor 5 agonist. *Proceedings of the National Academy of Sciences* **2013,** *110* (20), E1857-E1866.

190. Brackett, C. M.; Kojouharov, B.; Veith, J.; Greene, K. F.; Burdelya, L. G.; Gollnick, S. O.; Abrams, S. I.; Gudkov, A. V., Toll-like receptor-5 agonist, entolimod, suppresses metastasis and induces immunity by stimulating an NK-dendritic-CD8+ T-cell axis. *Proceedings of the National Academy of Sciences of the United States of America* **2016,** *113* (7), E874-E883.

191. Haderski, G. J.; Kandar, B. M.; Brackett, C. M.; Toshkov, I. M.; Johnson, C. P.; Paszkiewicz, G. M.; Natarajan, V.; Gleiberman, A. S.; Gudkov, A. V.; Burdelya, L. G., TLR5 agonist entolimod reduces the adverse toxicity of TNF while preserving its antitumor effects. *PLoS One* **2020,** *15* (2), e0227940.

192. Sahin, U.; Derhovanessian, E.; Miller, M.; Kloke, B.-P.; Simon, P.; Löwer, M.; Bukur, V.; Tadmor, A. D.; Luxemburger, U.; Schrörs, B.; Omokoko, T.; Vormehr, M.; Albrecht, C.; Paruzynski, A.; Kuhn, A. N.; Buck, J.; Heesch, S.; Schreeb, K. H.; Müller, F.; Ortseifer, I.; Vogler, I.; Godehardt, E.; Attig, S.; Rae, R.; Breitkreuz, A.; Tolliver, C.; Suchan, M.; Martic, G.; Hohberger, A.; Sorn, P.; Diekmann, J.; Ciesla, J.; Waksmann, O.; Brück, A.-K.; Witt, M.; Zillgen, M.; Rothermel, A.; Kasemann, B.; Langer, D.; Bolte, S.; Diken, M.; Kreiter, S.; Nemecek, R.; Gebhardt, C.; Grabbe, S.; Höller, C.; Utikal, J.; Huber, C.; Loquai, C.; Türeci, Ö., Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* **2017,** *547* (7662), 222-226.

193. Volckmar, J.; Knop, L.; Stegemann-Koniszewski, S.; Schulze, K.; Ebensen, T.; Guzmán, C. A.; Bruder, D., The STING activator c-di-AMP exerts superior adjuvant properties than the formulation poly(I:C)/CpG after subcutaneous vaccination with soluble protein antigen or DEC-205-mediated antigen targeting to dendritic cells. *Vaccine* **2019,** *37* (35), 4963-4974.

194. Gutjahr, A.; Papagno, L.; Nicoli, F.; Kanuma, T.; Kuse, N.; Cabral-Piccin, M. P.; Rochereau, N.; Gostick, E.; Lioux, T.; Perouzel, E.; Price, D. A.; Takiguchi, M.; Verrier, B.; Yamamoto, T.; Paul, S.; Appay, V., The STING ligand cGAMP potentiates the efficacy of vaccine-induced CD8+ T cells. *JCI Insight* **2019,** *4* (7).

195. Su, T.; Zhang, Y.; Valerie, K.; Wang, X.-Y.; Lin, S.; Zhu, G., STING activation in cancer immunotherapy. *Theranostics* **2019,** *9* (25), 7759-7771.

196. Löwenberg, B.; Rowe, J. M., Introduction to the review series on advances in acute myeloid leukemia (AML). *Blood* **2016,** *127* (1), 1-1.

197. Dohner, H.; Weisdorf, D. J.; Bloomfield, C. D., Acute Myeloid Leukemia. *N Engl J Med* **2015,** *373* (12), 1136-52.

198. De Kouchkovsky, I.; Abdul-Hay, M., 'Acute myeloid leukemia: a comprehensive review and 2016 update'. *Blood Cancer J* **2016,** *6* (7), e441.

199. Dohner, H.; Estey, E. H.; Amadori, S.; Appelbaum, F. R.; Buchner, T.; Burnett, A. K.; Dombret, H.; Fenaux, P.; Grimwade, D.; Larson, R. A.; Lo-Coco, F.; Naoe, T.; Niederwieser, D.; Ossenkoppele, G. J.; Sanz, M. A.; Sierra, J.; Tallman, M. S.; Lowenberg, B.; Bloomfield, C. D., Diagnosis and

management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **2010,** *115* (3), 453-74.

200. Roboz, G. J., Current treatment of acute myeloid leukemia. *Curr Opin Oncol* **2012,** *24* (6), 711-9.

201. Feller, N.; van der Pol, M. A.; van Stijn, A.; Weijers, G. W.; Westra, A. H.; Evertse, B. W.; Ossenkoppele, G. J.; Schuurhuis, G. J., MRD parameters using immunophenotypic detection methods are highly reliable in predicting survival in acute myeloid leukaemia. *Leukemia* **2004,** *18* (8), 1380-90.

202. Pollyea, D. A.; Gutman, J. A.; Gore, L.; Smith, C. A.; Jordan, C. T., Targeting acute myeloid leukemia stem cells: a review and principles for the development of clinical trials. *Haematologica* **2014,** *99* (8), 1277-84.

203. Bonnet, D.; Dick, J. E., Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **1997,** *3* (7), 730-7.

204. Lichtenegger, F. S.; Schnorfeil, F. M.; Hiddemann, W.; Subklewe, M., Current strategies in immunotherapy for acute myeloid leukemia. *Immunotherapy* **2013,** *5* (1), 63-78.

205. Guerra, V. A.; DiNardo, C.; Konopleva, M., Venetoclax-based therapies for acute myeloid leukemia. *Best Pract Res Clin Haematol* **2019,** *32* (2), 145-153.

206. Walasek, A., The new perspectives of targeted therapy in acute myeloid leukemia. *Adv Clin Exp Med* **2019,** *28* (2), 271-276.

207. Mahalleh, M.; Shabani, M.; Rayzan, E.; Rezaei, N., Reinforcing the primary immunotherapy modulators against acute leukemia; monoclonal antibodies in AML. *Immunotherapy* **2019,** *11* (18), 1583-1600.

208. Davis, K. L.; Agarwal, A. M.; Verma, A. R., Checkpoint inhibition in pediatric hematologic malignancies. *Pediatr Hematol Oncol* **2017,** *34* (6-7), 379-394.

209. Hansrivijit, P.; Gale, R. P.; Barrett, J.; Ciurea, S. O., Cellular therapy for acute myeloid Leukemia - Current status and future prospects. *Blood Rev* **2019,** *37*, 100578.

210. Epperly, R.; Gottschalk, S.; Velasquez, M. P., Harnessing T Cells to Target Pediatric Acute Myeloid Leukemia: CARs, BiTEs, and Beyond. *Children (Basel)* **2020,** *7* (2), 14.

211. Ravandi, F.; Stein, A. S.; Kantarjian, H. M.; Walter, R. B.; Paschka, P.; Jongen-Lavrencic, M.; Ossenkoppele, G. J.; Yang, Z.; Mehta, B.; Subklewe, M., A Phase 1 First-in-Human Study of AMG 330, an Anti-CD33 Bispecific T-Cell Engager (BiTE®) Antibody Construct, in Relapsed/Refractory Acute Myeloid Leukemia (R/R AML). *Blood* **2018,** *132* (Supplement 1), 25-25.

212. Uy, G. L.; Rettig, M. P.; Vey, N.; Godwin, J.; Foster, M. C.; Rizzieri, D. A.; Arellano, M. L.; Topp, M. S.; Huls, G.; Jongen-Lavrencic, M.; Martinelli, G.; Paolini, S.; Ciceri, F.; Carrabba, M. G.; Sweet, K. L.; Ravandi, F.; Church, S. E.; Vadakekolathu, J.; Rutella, S.; Sun, J.; Yang, K.; Baughman, J.; Curtis, T.; Timmeny, E.; Cali, K.; Tran, K.; Muth, J.; La Motte-Mohs, R.; Poirot, C.; Pallis, A.; Cesano, A.; Bonvini, E.; Wigginton, J.; Lowenberg, B.; Davidson-Moncada, J. K.; DiPersio, J. F., Phase 1 Cohort Expansion of Flotetuzumab, a CD123×CD3 Bispecific Dart® Protein in Patients with Relapsed/Refractory Acute Myeloid Leukemia (AML). *Blood* **2018,** *132* (Supplement 1), 764-764.

213. Jonas, B. A.; Medeiros, B. C., Individualizing Therapeutic Strategies in Acute Myeloid Leukemia: Moving Beyond the 'One-Size-Fits-All' Approach. *Oncology (Williston Park)* **2016,** *30* (4), 330-333.

214. Stahl, M.; Goldberg, A. D., Immune Checkpoint Inhibitors in Acute Myeloid Leukemia: Novel Combinations and Therapeutic Targets. *Current Oncology Reports* **2019,** *21* (4), 37.

215. Beyar-Katz, O.; Gill, S., Novel Approaches to Acute Myeloid Leukemia Immunotherapy. *Clinical Cancer Research* **2018,** *24* (22), 5502-5515.

216. Subklewe, M.; Geiger, C.; Lichtenegger, F. S.; Javorovic, M.; Kvalheim, G.; Schendel, D. J.; Bigalke, I., New generation dendritic cell vaccine for immunotherapy of acute myeloid leukemia. *Cancer Immunol Immunother* **2014,** *63* (10), 1093-103.

217. van Dongen, J. J.; Macintyre, E. A.; Gabert, J. A.; Delabesse, E.; Rossi, V.; Saglio, G.; Gottardi, E.; Rambaldi, A.; Dotti, G.; Griesinger, F.; Parreira, A.; Gameiro, P.; Diaz, M. G.; Malec, M.; Langerak, A. W.; San Miguel, J. F.; Biondi, A., Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. *Leukemia* **1999,** *13* (12), 1901-28.

218. Meyer, C.; Burmeister, T.; Groger, D.; Tsaur, G.; Fechina, L.; Renneville, A.; Sutton, R.; Venn, N. C.; Emerenciano, M.; Pombo-de-Oliveira, M. S.; Barbieri Blunck, C.; Almeida Lopes, B.; Zuna, J.; Trka, J.; Ballerini, P.; Lapillonne, H.; De Braekeleer, M.; Cazzaniga, G.; Corral Abascal, L.; van der Velden, V. H. J.; Delabesse, E.; Park, T. S.; Oh, S. H.; Silva, M. L. M.; Lund-Aho, T.; Juvonen, V.; Moore, A. S.; Heidenreich, O.; Vormoor, J.; Zerkalenkova, E.; Olshanskaya, Y.; Bueno, C.; Menendez, P.; Teigler-Schlegel, A.; Zur Stadt, U.; Lentes, J.; Gohring, G.; Kustanovich, A.; Aleinikova, O.; Schafer, B. W.; Kubetzko, S.; Madsen, H. O.; Gruhn, B.; Duarte, X.; Gameiro, P.; Lippert, E.; Bidet, A.; Cayuela, J. M.; Clappier, E.; Alonso, C. N.; Zwaan, C. M.; van den Heuvel-Eibrink, M. M.; Izraeli, S.; Trakhtenbrot, L.; Archer, P.; Hancock, J.; Moricke, A.; Alten, J.; Schrappe, M.; Stanulla, M.; Strehl, S.; Attarbaschi, A.; Dworzak, M.; Haas, O. A.; Panzer-Grumayer, R.; Sedek, L.; Szczepanski, T.; Caye, A.; Suarez, L.; Cave, H.; Marschalek, R., The MLL recombinome of acute leukemias in 2017. *Leukemia* **2018,** *32* (2), 273-284.

219. King-Underwood, L.; Renshaw, J.; Pritchard-Jones, K., Mutations in the Wilms' tumor gene WT1 in leukemias. *Blood* **1996,** *87* (6), 2171-9.

220. Pronier, E.; Bowman, R. L.; Ahn, J.; Glass, J.; Kandoth, C.; Merlinsky, T. R.; Whitfield, J. T.; Durham, B. H.; Gruet, A.; Hanasoge Somasundara, A. V.; Rampal, R.; Melnick, A.; Koche, R. P.; Taylor, B. S.; Levine, R. L., Genetic and epigenetic evolution as a contributor to WT1-mutant leukemogenesis. *Blood* **2018,** *132* (12), 1265-1278.

221. Paschka, P.; Schlenk, R. F.; Gaidzik, V. I.; Habdank, M.; Kronke, J.; Bullinger, L.; Spath, D.; Kayser, S.; Zucknick, M.; Gotze, K.; Horst, H. A.; Germing, U.; Dohner, H.; Dohner, K., IDH1 and IDH2 mutations are frequent genetic alterations in acute myeloid leukemia and confer adverse prognosis in cytogenetically normal acute myeloid leukemia with NPM1 mutation without FLT3 internal tandem duplication. *J Clin Oncol* **2010,** *28* (22), 3636-43.

222. Falini, B.; Mecucci, C.; Tiacci, E.; Alcalay, M.; Rosati, R.; Pasqualucci, L.; La Starza, R.; Diverio, D.; Colombo, E.; Santucci, A.; Bigerna, B.; Pacini, R.; Pucciarini, A.; Liso, A.; Vignetti, M.; Fazi, P.; Meani, N.; Pettirossi, V.; Saglio, G.; Mandelli, F.; Lo-Coco, F.; Pelicci, P. G.; Martelli, M. F., Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med* **2005,** *352* (3), 254-66.

223. Falini, B.; Martelli, M. P.; Bolli, N.; Sportoletti, P.; Liso, A.; Tiacci, E.; Haferlach, T., Acute myeloid leukemia with mutated nucleophosmin (NPM1): is it a distinct entity? *Blood* **2011,** *117* (4), 1109-20.

224. van der Lee, D. I.; Reijmers, R. M.; Honders, M. W.; Hagedoorn, R. S.; de Jong, R. C. M.; Kester, M. G. D.; van der Steen, D. M.; de Ru, A. H.; Kweekel, C.; Bijen, H. M.; Jedema, I.; Veelken, H.; van Veelen, P. A.; Heemskerk, M. H. M.; Falkenburg, J. H. F.; Griffioen, M., Mutated nucleophosmin 1 as immunotherapy target in acute myeloid leukemia. *The Journal of Clinical Investigation* **2019,** *129* (2), 774-785.

225. Greiner, J.; Ono, Y.; Hofmann, S.; Schmitt, A.; Mehring, E.; Gotz, M.; Guillaume, P.; Dohner, K.; Mytilineos, J.; Dohner, H.; Schmitt, M., Mutated regions of nucleophosmin 1 elicit both CD4(+) and CD8(+) T-cell responses in patients with acute myeloid leukemia. *Blood* **2012,** *120* (6), 1282-9.

226. Biernacki, M. A.; Bleakley, M., Neoantigens in Hematologic Malignancies. *Front Immunol* **2020,** *11* (121).

227. Yamamoto, T. N.; Kishton, R. J.; Restifo, N. P., Developing neoantigen-targeted T cell-based treatments for solid tumors. *Nat Med* **2019,** *25* (10), 1488-1499.

228. Tran, E.; Robbins, P. F.; Lu, Y. C.; Prickett, T. D.; Gartner, J. J.; Jia, L.; Pasetto, A.; Zheng, Z.; Ray, S.; Groh, E. M.; Kriley, I. R.; Rosenberg, S. A., T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer. *N Engl J Med* **2016,** *375* (23), 2255-2262.

229. Tran, E.; Turcotte, S.; Gros, A.; Robbins, P. F.; Lu, Y. C.; Dudley, M. E.; Wunderlich, J. R.; Somerville, R. P.; Hogan, K.; Hinrichs, C. S.; Parkhurst, M. R.; Yang, J. C.; Rosenberg, S. A., Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. *Science* **2014,** *344* (6184), 641-5.

230. Zacharakis, N.; Chinnasamy, H.; Black, M.; Xu, H.; Lu, Y. C.; Zheng, Z.; Pasetto, A.; Langhan, M.; Shelton, T.; Prickett, T.; Gartner, J.; Jia, L.; Trebska-McGowan, K.; Somerville, R. P.; Robbins, P. F.; Rosenberg, S. A.; Goff, S. L.; Feldman, S. A., Immune recognition of somatic mutations leading to complete durable regression in metastatic breast cancer. *Nat Med* **2018,** *24* (6), 724-730.

231. Schmitt, M.; Casalegno-Garduno, R.; Xu, X.; Schmitt, A., Peptide vaccines for patients with acute myeloid leukemia. *Expert Rev Vaccines* **2009,** *8* (10), 1415-25.

232. Van Acker, H. H.; Versteven, M.; Lichtenegger, F. S.; Roex, G.; Campillo-Davo, D.; Lion, E.; Subklewe, M.; Van Tendeloo, V. F.; Berneman, Z. N.; Anguille, S., Dendritic Cell-Based Immunotherapy of Acute Myeloid Leukemia. *J Clin Med* **2019,** *8* (5), 579.

233. Lichtenegger, F. S.; Deiser, K.; Rothe, M.; Schnorfeil, F. M.; Krupka, C.; Augsberger, C.; Köhnke, T.; Bücklein, V. L.; Altmann, T.; Moosmann, A.; Brüggemann, M.; Heemskerk, M. H. M.; Wittmann, G.; Wagner, B.; Hiddemann, W.; Bigalke, I.; Kvalheim, G.; Subklewe, M., Induction of Antigen-Specific T-Cell Responses through Dendritic Cell Vaccination in AML: Results of a Phase I/II Trial and Ex Vivo Enhancement By Checkpoint Blockade. *Blood* **2016,** *128* (22), 764-764.

234. Rosenblatt, J.; Stone, R. M.; Uhl, L.; Neuberg, D.; Joyce, R.; Levine, J. D.; Arnason, J.; McMasters, M.; Luptakova, K.; Jain, S.; Zwicker, J. I.; Hamdan, A.; Boussiotis, V.; Steensma, D. P.; DeAngelo, D. J.; Galinsky, I.; Dutt, P. S.; Logan, E.; Bryant, M. P.; Stroopinsky, D.; Werner, L.; Palmer, K.; Coll, M.; Washington, A.; Cole, L.; Kufe, D.; Avigan, D., Individualized vaccination of AML patients in remission is associated with induction of antileukemia immunity and prolonged remissions. *Science translational medicine* **2016,** *8* (368), 368ra171-368ra171.

235. van de Loosdrecht, A. A.; van Wetering, S.; Santegoets, S. J. A. M.; Singh, S. K.; Eeltink, C. M.; den Hartog, Y.; Koppes, M.; Kaspers, J.; Ossenkoppele, G. J.; Kruisbeek, A. M.; de Gruijl, T. D., A novel allogeneic off-the-shelf dendritic cell vaccine for post-remission treatment of elderly patients with acute myeloid leukemia. *Cancer immunology, immunotherapy : CII* **2018,** *67* (10), 1505-1518.

236. Dhodapkar, M. V.; Sznol, M.; Zhao, B.; Wang, D.; Carvajal, R. D.; Keohan, M. L.; Chuang, E.; Sanborn, R. E.; Lutzky, J.; Powderly, J.; Kluger, H.; Tejwani, S.; Green, J.; Ramakrishna, V.; Crocker, A.; Vitale, L.; Yellin, M.; Davis, T.; Keler, T., Induction of Antigen-Specific Immunity with a Vaccine Targeting NY-ESO-1 to the Dendritic Cell Receptor DEC-205. *Science Translational Medicine* **2014,** *6* (232), 232ra51-232ra51.

237. Griffiths, E. A.; Srivastava, P.; Matsuzaki, J.; Brumberger, Z.; Wang, E. S.; Kocent, J.; Miller, A.; Roloff, G. W.; Wong, H. Y.; Paluch, B. E.; Lutgen-Dunckley, L. G.; Martens, B. L.; Odunsi, K.; Karpf, A. R.; Hourigan, C. S.; Nemeth, M. J., NY-ESO-1 Vaccination in Combination with Decitabine Induces Antigen-Specific T-lymphocyte Responses in Patients with Myelodysplastic Syndrome. *Clin Cancer Res* **2018,** *24* (5), 1019-1029.

238. Chapuis, A. G.; Egan, D. N.; Bar, M.; Schmitt, T. M.; McAfee, M. S.; Paulson, K. G.; Voillet, V.; Gottardo, R.; Ragnarsson, G. B.; Bleakley, M.; Yeung, C. C.; Muhlhauser, P.; Nguyen, H. N.; Kropp, L. A.; Castelli, L.; Wagener, F.; Hunter, D.; Lindberg, M.; Cohen, K.; Seese, A.; McElrath, M. J.; Duerkopp, N.; Gooley, T. A.; Greenberg, P. D., T cell receptor gene therapy targeting WT1 prevents acute myeloid leukemia relapse post-transplant. *Nature Medicine* **2019,** *25* (7), 1064-1072.

239. Klein, C.; Augsberger, C.; Xu, W.; Heitmüller, C.; Hanisch, L.; Sam, J.; Pulko, V.; Schönle, A.; Challier, J.; Carpy, A.; Konstandin, N.; Rothe, M.; Hänel, G.; Bücklein, V.; Lichtenegger, F. S.; Tunger, A.; Schmitz, M.; von Bergwelt, M.; Spiekermann, K.; Umana, P.; Subklewe, M., Targeting Intracellular WT1 in AML Utilizing a T Cell Bispecific Antibody Construct: Augmenting Efficacy through Combination with Lenalidomide. *Blood* **2019,** *134* (Supplement_1), 4450-4450.

240. Armistead, P. M., Cellular therapy against public neoantigens. *The Journal of Clinical Investigation* **2019,** *129* (2), 506-508.

241. Ott, P. A.; Dotti, G.; Yee, C.; Goff, S. L., An Update on Adoptive T-Cell Therapy and Neoantigen Vaccines. *Am Soc Clin Oncol Educ Book* **2019,** *39*, e70-e78.

242. Kastenmüller, K.; Wille-Reece, U.; Lindsay, R. W.; Trager, L. R.; Darrah, P. A.; Flynn, B. J.; Becker, M. R.; Udey, M. C.; Clausen, B. E.; Igyarto, B. Z.; Kaplan, D. H.; Kastenmüller, W.; Germain, R. N.; Seder, R. A., Protective T cell immunity in mice following protein-TLR7/8 agonist-conjugate immunization requires aggregation, type I IFN, and multiple DC subsets. *J Clin Invest* **2011,** *121* (5), 1782-96.

243. Oh, J. Z.; Kedl, R. M., The capacity to induce cross-presentation dictates the success of a TLR7 agonist-conjugate vaccine for eliciting cellular immunity. *Journal of immunology (Baltimore, Md. : 1950)* **2010,** *185* (8), 4602-4608.

244. Maurer, T.; Heit, A.; Hochrein, H.; Ampenberger, F.; O'Keeffe, M.; Bauer, S.; Lipford, G. B.; Vabulas, R. M.; Wagner, H., CpG-DNA aided cross-presentation of soluble antigens by dendritic cells. *European Journal of Immunology* **2002,** *32* (8), 2356-2364.

245. Hanahan, D., Studies on transformation of Escherichia coli with plasmids. *J Mol Biol* **1983,** *166* (4), 557-80.

246. Ziegler, A.; Seelig, J., High Affinity of the Cell-Penetrating Peptide HIV-1 Tat-PTD for DNA. *Biochemistry* **2007,** *46* (27), 8138-8145.

247. Vonderheide, R. H.; Flaherty, K. T.; Khalil, M.; Stumacher, M. S.; Bajor, D. L.; Hutnick, N. A.; Sullivan, P.; Mahany, J. J.; Gallagher, M.; Kramer, A.; Green, S. J.; O'Dwyer, P. J.; Running, K. L.; Huhn, R. D.; Antonia, S. J., Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. *J Clin Oncol* **2007,** *25* (7), 876-83.

248. Bedian, V.; Gladue Ronald, P.; Corvalan, J.; Jia, X.-C.; Feng, X. Antibodies That Bind Cd40 And Methods Of Treating Cancer And Enhancing Immune Responses. US 7618633 B2, 2007/09/25, 2009.

249. Weatherill, E. E.; Cain, K. L.; Heywood, S. P.; Compson, J. E.; Heads, J. T.; Adams, R.; Humphreys, D. P., Towards a universal disulphide stabilised single chain Fv format: importance of interchain disulphide bond location and vL–vH orientation. *Protein Engineering, Design and Selection* **2012,** *25* (7), 321-329.

250. Pedrazzi, G.; Schwesinger, F.; Honegger, A.; Krebber, C.; Plückthun, A., Affinity and folding properties both influence the selection of antibodies with the selectively infective phage (SIP) methodology. *FEBS Letters* **1997,** *415* (3), 289-293.

251. Roskopf, C. C.; Schiller, C. B.; Braciak, T. A.; Kobold, S.; Schubert, I. A.; Fey, G. H.; Hopfner, K.-P.; Oduncu, F. S., T cell-recruiting triplebody 19-3-19 mediates serial lysis of malignant B-lymphoid cells by a single T cell. *Oncotarget* **2014,** *5* (15), 6466-6483.

252. Carter, P.; Presta, L.; Gorman, C. M.; Ridgway, J. B.; Henner, D.; Wong, W. L.; Rowland, A. M.; Kotts, C.; Carver, M. E.; Shepard, H. M., Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci U S A* **1992,** *89* (10), 4285-9.

253. J.F, S.; Russell, D., *Molecular Cloning: A Laboratory Manual (3-Volume Set)*. 2001; Vol. 1.

254. Haffke, M.; Rummel, G.; Boivineau, J.; Münch, A.; Jaakola, V.-P., *nanoDSF: Label-free Thermal Unfolding Assay of G Protein-Coupled Receptors for Compound Screening and Buffer Composition Optimization*. 2016.

255. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J.-Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A., Fiji: an open-source platform for biological-image analysis. *Nat Methods* **2012,** *9* (7), 676-682.

256. Faber, E.; Tedin, K.; Speidel, Y.; Brinkmann, M. M.; Josenhans, C., Functional expression of TLR5 of different vertebrate species and diversification in intestinal pathogen recognition. *Scientific Reports* **2018,** *8* (1), 11287.

257. Lu, H., TLR Agonists for Cancer Immunotherapy: Tipping the Balance between the Immune Stimulatory and Inhibitory Effects. *Front Immunol* **2014,** *5*, 83.

258. Fan, X.; Upadhyaya, B.; Wu, L.; Koh, C.; Santín-Durán, M.; Pittaluga, S.; Uzel, G.; Kleiner, D.; Williams, E.; Ma, C. A.; Bodansky, A.; Oliveira, J. B.; Edmonds, P.; Hornung, R.; Wong, D. W.; Fayer, R.; Fleisher, T.; Heller, T.; Prussin, C.; Jain, A., CD40 agonist antibody mediated improvement of chronic Cryptosporidium infection in patients with X-linked hyper IgM syndrome. *Clin Immunol* **2012,** *143* (2), 152-161.

259. Platt, C. D.; Ma, J. K.; Chalouni, C.; Ebersold, M.; Bou-Reslan, H.; Carano, R. A. D.; Mellman, I.; Delamarre, L., Mature dendritic cells use endocytic receptors to capture and present antigens. *Proceedings of the National Academy of Sciences of the United States of America* **2010,** *107* (9), 4287-4292.

260. Yamaki, T.; Kamiya, Y.; Ohtake, K.; Uchida, M.; Seki, T.; Ueda, H.; Kobayashi, J.; Morimoto, Y.; Natsume, H., A Mechanism Enhancing Macromolecule Transport Through Paracellular Spaces Induced by Poly-L-Arginine: Poly-L-Arginine Induces the Internalization of Tight Junction Proteins via Clathrin-Mediated Endocytosis. *Pharmaceutical Research* **2014,** *31* (9), 2287-2296.

261. Smith, K. D.; Andersen-Nissen, E.; Hayashi, F.; Strobe, K.; Bergman, M. A.; Barrett, S. L. R.; Cookson, B. T.; Aderem, A., Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nature Immunology* **2003,** *4* (12), 1247-1253.

262. Song, W.; Jeon, Y.; Namgung, B.; Hong, M.; Yoon, S., A conserved TLR5 binding and activation hot spot on flagellin. *Scientific Reports* **2017,** *7*, 40878.

263. Kastenmüller, W.; Kastenmüller, K.; Kurts, C.; Seder, R. A., Dendritic cell-targeted vaccines — hope or hype? *Nature Reviews Immunology* **2014,** *14* (10), 705-711.

264. Sabado, R. L.; Balan, S.; Bhardwaj, N., Dendritic cell-based immunotherapy. *Cell Research* **2017,** *27* (1), 74-95.

265. Macri, C.; Dumont, C.; Johnston, A. P.; Mintern, J. D., Targeting dendritic cells: a promising strategy to improve vaccine effectiveness. *Clinical & Translational Immunology* **2016,** *5* (3), e66.

266. Khong, H.; Overwijk, W. W., Adjuvants for peptide-based cancer vaccines. *Journal for immunotherapy of cancer* **2016,** *4*, 56-56.

267. Morishita, M.; Takahashi, Y.; Matsumoto, A.; Nishikawa, M.; Takakura, Y., Exosome-based tumor antigens–adjuvant co-delivery utilizing genetically engineered tumor cell-derived exosomes with immunostimulatory CpG DNA. *Biomaterials* **2016,** *111*, 55-65.

268. Rahimian, S.; Fransen, M. F.; Kleinovink, J. W.; Christensen, J. R.; Amidi, M.; Hennink, W. E.; Ossendorp, F., Polymeric nanoparticles for co-delivery of synthetic long peptide antigen and poly IC as therapeutic cancer vaccine formulation. *J Control Release* **2015,** *203*, 16-22.

269. Ceglia, V.; Zurawski, S.; Montes, M.; Bouteau, A.; Wang, Z.; Ellis, J.; Kroll, M.; Igyarto, B. Z.; Levy, Y.; Zurawski, G., Dendritic cell targeting anti-CD40 antibody-CD40L-HIV-1 vaccines are adjuvant intrinsic. *The Journal of Immunology* **2020,** *204* (1 Supplement), 167.2-167.2.

270. White, A. L.; Chan, H. T. C.; Roghanian, A.; French, R. R.; Mockridge, C. I.; Tutt, A. L.; Dixon, S. V.; Ajona, D.; Verbeek, J. S.; Al-Shamkhani, A.; Cragg, M. S.; Beers, S. A.; Glennie, M. J., Interaction with FcγRIIB Is Critical for the Agonistic Activity of Anti-CD40 Monoclonal Antibody. *The Journal of Immunology* **2011,** *187* (4), 1754-1763.

271. White, A. L.; Dou, L.; Chan, H. T. C.; Field, V. L.; Mockridge, C. I.; Moss, K.; Williams, E. L.; Booth, S. G.; French, R. R.; Potter, E. A.; Butts, C.; Al-Shamkhani, A.; Cragg, M. S.; Verbeek, J. S.; Johnson, P. W. M.; Glennie, M. J.; Beers, S. A., Fcγ Receptor Dependency of Agonistic CD40 Antibody in Lymphoma Therapy Can Be Overcome through Antibody Multimerization. *The Journal of Immunology* **2014,** *193* (4), 1828-1835.

272. White, Ann L.; Chan, H. T. C.; French, Ruth R.; Willoughby, J.; Mockridge, C. I.; Roghanian, A.; Penfold, Christine A.; Booth, Steven G.; Dodhy, A.; Polak, Marta E.; Potter, Elizabeth A.; Ardern-Jones, Michael R.; Verbeek, J. S.; Johnson, Peter W. M.; Al-Shamkhani, A.; Cragg, Mark S.; Beers, Stephen A.; Glennie, Martin J., Conformation of the Human Immunoglobulin G2 Hinge Imparts Superagonistic Properties to Immunostimulatory Anticancer Antibodies. *Cancer Cell* **2015,** *27* (1), 138-148.

273. Dahan, R.; Barnhart, B. C.; Li, F.; Yamniuk, A. P.; Korman, A. J.; Ravetch, J. V., Therapeutic Activity of Agonistic, Human Anti-CD40 Monoclonal Antibodies Requires Selective FcγR Engagement. *Cancer cell* **2016,** *29* (6), 820-831.

274. Richman, L. P.; Vonderheide, R. H., Role of Crosslinking for Agonistic CD40 Monoclonal Antibodies as Immune Therapy of Cancer. *Cancer Immunology Research* **2014,** *2* (1), 19-26.

275. Cella, M.; Engering, A.; Pinet, V.; Pieters, J.; Lanzavecchia, A., Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* **1997,** *388* (6644), 782-7.

276. Cella, M.; Salio, M.; Sakakibara, Y.; Langen, H.; Julkunen, I.; Lanzavecchia, A., Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *The Journal of experimental medicine* **1999,** *189* (5), 821-829.

277. Reis e Sousa, C., Dendritic cells in a mature age. *Nat Rev Immunol* **2006,** *6* (6), 476-83.

278. Salomon, B.; Lenschow, D. J.; Rhee, L.; Ashourian, N.; Singh, B.; Sharpe, A.; Bluestone, J. A., B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* **2000,** *12* (4), 431-40.

279. Zheng, Y.; Manzotti, C. N.; Liu, M.; Burke, F.; Mead, K. I.; Sansom, D. M., CD86 and CD80 differentially modulate the suppressive function of human regulatory T cells. *J Immunol* **2004,** *172* (5), 2778-84.

280. Yamazaki, S.; Iyoda, T.; Tarbell, K.; Olson, K.; Velinzon, K.; Inaba, K.; Steinman, R. M., Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* **2003,** *198* (2), 235-47.

281. Schwiebs, A.; Friesen, O.; Katzy, E.; Ferreirós, N.; Pfeilschifter, J. M.; Radeke, H. H., Activation-Induced Cell Death of Dendritic Cells Is Dependent on Sphingosine Kinase 1. *Front Pharmacol* **2016,** *7*, 94-94.

282. Chen, M.; Wang, J., Programmed cell death of dendritic cells in immune regulation. *Immunol Rev* **2010,** *236*, 11-27.

283. Cohn, L.; Chatterjee, B.; Esselborn, F.; Smed-Sörensen, A.; Nakamura, N.; Chalouni, C.; Lee, B. C.; Vandlen, R.; Keler, T.; Lauer, P.; Brockstedt, D.; Mellman, I.; Delamarre, L., Antigen delivery to early endosomes eliminates the superiority of human blood BDCA3+ dendritic cells at cross presentation. *J Exp Med* **2013,** *210* (5), 1049-63.

284. Yin, W.; Gorvel, L.; Zurawski, S.; Li, D.; Ni, L.; Duluc, D.; Upchurch, K.; Kim, J.; Gu, C.; Ouedraogo, R.; Wang, Z.; Xue, Y.; Joo, H.; Gorvel, J.-P.; Zurawski, G.; Oh, S., Functional Specialty of CD40 and Dendritic Cell Surface Lectins for Exogenous Antigen Presentation to CD8+ and CD4+ T Cells. *EBioMedicine* **2016,** *5*, 46-58.

285. Vonderheide, R. H.; Burg, J. M.; Mick, R.; Trosko, J. A.; Li, D.; Shaik, M. N.; Tolcher, A. W.; Hamid, O., Phase I study of the CD40 agonist antibody CP-870,893 combined with carboplatin and paclitaxel in patients with advanced solid tumors. *Oncoimmunology* **2013,** *2* (1), e23033-e23033.

286. Johnson, P.; Challis, R.; Chowdhury, F.; Gao, Y.; Harvey, M.; Geldart, T.; Kerr, P.; Chan, C.; Smith, A.; Steven, N.; Edwards, C.; Ashton-Key, M.; Hodges, E.; Tutt, A.; Ottensmeier, C.; Glennie, M.; Williams, A., Clinical and Biological Effects of an Agonist Anti-CD40 Antibody: A Cancer Research UK Phase I Study. *Clinical Cancer Research* **2015,** *21* (6), 1321-1328.

287. Hassan, S. B.; Sørensen, J. F.; Olsen, B. N.; Pedersen, A. E., Anti-CD40-mediated cancer immunotherapy: an update of recent and ongoing clinical trials. *Immunopharmacol Immunotoxicol* **2014,** *36* (2), 96-104.

288. Berner, V.; Liu, H.; Zhou, Q.; Alderson, K. L.; Sun, K.; Weiss, J. M.; Back, T. C.; Longo, D. L.; Blazar, B. R.; Wiltrout, R. H.; Welniak, L. A.; Redelman, D.; Murphy, W. J., IFN-gamma mediates CD4+ T-cell loss and impairs secondary antitumor responses after successful initial immunotherapy. *Nat Med* **2007,** *13* (3), 354-60.

289. Kedl, R. M.; Jordan, M.; Potter, T.; Kappler, J.; Marrack, P.; Dow, S., CD40 stimulation accelerates deletion of tumor-specific CD8(+) T cells in the absence of tumor-antigen vaccination. *Proc Natl Acad Sci U S A* **2001,** *98* (19), 10811-6.

290. Rüter, J.; Antonia, S. J.; Burris, H. A.; Huhn, R. D.; Vonderheide, R. H., Immune modulation with weekly dosing of an agonist CD40 antibody in a phase I study of patients with advanced solid tumors. *Cancer Biol Ther* **2010,** *10* (10), 983-93.

291. Bourgeois, C.; Rocha, B.; Tanchot, C., A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science* **2002,** *297* (5589), 2060-3.

292. de Gruijl, T. D.; Luykx-de Bakker, S. A.; Tillman, B. W.; van den Eertwegh, A. J. M.; Buter, J.; Lougheed, S. M.; van der Bij, G. J.; Safer, A. M.; Haisma, H. J.; Curiel, D. T.; Scheper, R. J.; Pinedo, H. M.; Gerritsen, W. R., Prolonged Maturation and Enhanced Transduction of Dendritic Cells Migrated from Human Skin Explants After In Situ Delivery of CD40-Targeted Adenoviral Vectors. *The Journal of Immunology* **2002,** *169* (9), 5322-5331.

293. Hangalapura, B. N.; Oosterhoff, D.; Aggarwal, S.; Wijnands, P. G.; van de Ven, R.; Santegoets, S. J.; van den Tol, M. P.; Hooijberg, E.; Pereboev, A.; van den Eertwegh, A. J.; Curiel, D. T.; Scheper, R. J.; de Gruijl, T. D., Selective transduction of dendritic cells in human lymph nodes and superior induction of high-avidity melanoma-reactive cytotoxic T cells by a CD40-targeted adenovirus. *J Immunother* **2010,** *33* (7), 706-15.

294. Dullforce, P.; Sutton, D. C.; Heath, A. W., Enhancement of T cell-independent immune responses in vivo by CD40 antibodies. *Nat Med* **1998,** *4* (1), 88-91.

295. García de Vinuesa, C.; MacLennan, I. C.; Holman, M.; Klaus, G. G., Anti-CD40 antibody enhances responses to polysaccharide without mimicking T cell help. *Eur J Immunol* **1999,** *29* (10), 3216-24.

296. López-Yglesias, A. H.; Zhao, X.; Quarles, E. K.; Lai, M. A.; VandenBos, T.; Strong, R. K.; Smith, K. D., Flagellin induces antibody responses through a TLR5- and inflammasome-independent pathway. *J Immunol* **2014,** *192* (4), 1587-96.

297. Zhao, Y.; Yang, J.; Shi, J.; Gong, Y.-N.; Lu, Q.; Xu, H.; Liu, L.; Shao, F., The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* **2011,** *477* (7366), 596-600.

298. Treanor, J. J.; Taylor, D. N.; Tussey, L.; Hay, C.; Nolan, C.; Fitzgerald, T.; Liu, G.; Kavita, U.; Song, L.; Dark, I.; Shaw, A., Safety and immunogenicity of a recombinant hemagglutinin influenza-flagellin fusion vaccine (VAX125) in healthy young adults. *Vaccine* **2010,** *28* (52), 8268-74.

299. Turley, C. B.; Rupp, R. E.; Johnson, C.; Taylor, D. N.; Wolfson, J.; Tussey, L.; Kavita, U.; Stanberry, L.; Shaw, A., Safety and immunogenicity of a recombinant M2e-flagellin influenza vaccine (STF2.4xM2e) in healthy adults. *Vaccine* **2011,** *29* (32), 5145-52.

300. Vicente-Suarez, I.; Brayer, J.; Villagra, A.; Cheng, F.; Sotomayor, E. M., TLR5 ligation by flagellin converts tolerogenic dendritic cells into activating antigen-presenting cells that preferentially induce T-helper 1 responses. *Immunology letters* **2009,** *125* (2), 114-118.

301. Nguyen, C. T.; Hong, S. H.; Sin, J. I.; Vu, H. V.; Jeong, K.; Cho, K. O.; Uematsu, S.; Akira, S.; Lee, S. E.; Rhee, J. H., Flagellin enhances tumor-specific $CD8^+$ T cell immune responses through TLR5 stimulation in a therapeutic cancer vaccine model. *Vaccine* **2013,** *31* (37), 3879-87.

302. Machluf, N.; Arnon, R., Therapeutic MUC1-Based Cancer Vaccine Expressed in Flagella—Efficacy in an Aggressive Model of Breast Cancer. *World Journal of Vaccines* **2012,** *02*.

303. Engelhardt, R.; Mackensen, A.; Galanos, C., Phase I trial of intravenously administered endotoxin (Salmonella abortus equi) in cancer patients. *Cancer Res* **1991,** *51* (10), 2524-30.

304. Mackensen, A.; Galanos, C.; Wehr, U.; Engelhardt, R., Endotoxin tolerance: regulation of cytokine production and cellular changes in response to endotoxin application in cancer patients. *Eur Cytokine Netw* **1992,** *3* (6), 571-9.

305. Goto, S.; Sakai, S.; Kera, J.; Suma, Y.; Soma, G.-I.; Takeuchi, S., Intradermal administration of lipopolysaccharide in treatment of human cancer. *Cancer Immunology, Immunotherapy* **1996,** *42* (4), 255-261.

306. Suffredini, A. F.; Hochstein, H. D.; McMahon, F. G., Dose-Related Inflammatory Effects of Intravenous Endotoxin in Humans: Evaluation of a New Clinical Lot of Escherichia coli O:113 Endotoxin. *The Journal of Infectious Diseases* **1999,** *179* (5), 1278-1282.

307. HUGH L. PREAS, I.; JUBRAN, A.; VANDIVIER, R. W.; REDA, D.; GODIN, P. J.; BANKS, S. M.; TOBIN, M. J.; SUFFREDINI, A. F., Effect of Endotoxin on Ventilation and Breath Variability. *American Journal of Respiratory and Critical Care Medicine* **2001,** *164* (4), 620-626.

308. Vermaelen, K., Vaccine Strategies to Improve Anti-cancer Cellular Immune Responses. *Front Immunol* **2019,** *10*, 8-8.

309. Schau, I.; Michen, S.; Hagstotz, A.; Janke, A.; Schackert, G.; Appelhans, D.; Temme, A., Targeted delivery of TLR3 agonist to tumor cells with single chain antibody fragment-conjugated nanoparticles induces type I-interferon response and apoptosis. *Scientific Reports* **2019,** *9* (1), 3299.

310. Foldvari, M.; Chen, D. W.; Nafissi, N.; Calderon, D.; Narsineni, L.; Rafiee, A., Non-viral gene therapy: Gains and challenges of non-invasive administration methods. *Journal of Controlled Release* **2016,** *240*, 165-190.

311. Wille-Reece, U.; Flynn, B.; Loré, K.; Koup, R.; Kedl, R.; Mattapallil, J.; Weiss, W.; Roederer, M.; Seder, R., HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. *Proceedings of the National Academy of Sciences of the United States of America* **2005,** *102*, 15190-4.

312. Wille-Reece, U.; Wu, C.-Y.; Flynn, B.; Kedl, R.; Seder, R., Immunization with HIV-1 Gag protein conjugated to a TLR7/8 agonist results in the generation of HIV-1 Gag-specific Th1 and CD8+ T cell responses. *Journal of immunology (Baltimore, Md. : 1950)* **2005,** *174*, 7676-83.

313. Butterfield, L. H.; Vujanovic, L.; Santos, P. M.; Maurer, D. M.; Gambotto, A.; Lohr, J.; Li, C.; Waldman, J.; Chandran, U.; Lin, Y.; Lin, H.; Tawbi, H. A.; Tarhini, A. A.; Kirkwood, J. M., Multiple antigen-engineered DC vaccines with or without IFNα to promote antitumor immunity in melanoma. *Journal for ImmunoTherapy of Cancer* **2019,** *7* (1), 113.

314. Zhou, C.; Zhu, C.; Liu, Q., Toward in silico Identification of Tumor Neoantigens in Immunotherapy. *Trends in Molecular Medicine* **2019,** *25* (11), 980-992.

315. Accolla, R. S.; Tosi, G., Optimal MHC-II-restricted tumor antigen presentation to CD4+ T helper cells: the key issue for development of anti-tumor vaccines. *J Transl Med* **2012,** *10*, 154.

316. Mougel, A.; Terme, M.; Tanchot, C., Therapeutic Cancer Vaccine and Combinations With Antiangiogenic Therapies and Immune Checkpoint Blockade. *Front Immunol* **2019,** *10*, 467-467.

317. Gatti-Mays, M. E.; Redman, J. M.; Collins, J. M.; Bilusic, M., Cancer vaccines: Enhanced immunogenic modulation through therapeutic combinations. *Hum Vaccin Immunother* **2017,** *13* (11), 2561-2574.

318. Bohnenkamp, H. R.; Papazisis, K. T.; Burchell, J. M.; Taylor-Papadimitriou, J., Synergism of Toll-like receptor-induced interleukin-12p70 secretion by monocyte-derived dendritic cells is mediated through p38 MAPK and lowers the threshold of T-helper cell type I responses. *Cellular Immunology* **2007,** *247* (2), 72-84.

319. Tsujimoto, H.; Efron, P.; Matsumoto, T.; Ungaro, R.; Abouhamze, A.; Ono, S.; Mochizuki, H.; Moldawer, L., Maturation of murine bone marrow-derived dendritic cells with poly(I:C) produces altered TLR-9 expression and response to CpG DNA. *Immunology letters* **2006,** *107*, 155-62.

320. Zhu, M.; Xu, W.; Su, H.; Huang, Q.; Wang, B., Addition of CpG ODN and Poly (I:C) to a standard maturation cocktail generates monocyte-derived dendritic cells and induces a potent Th1 polarization with migratory capacity. *Hum Vaccin Immunother* **2015,** *11* (7), 1596-1605.

321. Fu, J.; Kanne, D. B.; Leong, M.; Glickman, L. H.; McWhirter, S. M.; Lemmens, E.; Mechette, K.; Leong, J. J.; Lauer, P.; Liu, W.; Sivick, K. E.; Zeng, Q.; Soares, K. C.; Zheng, L.; Portnoy, D. A.; Woodward, J. J.; Pardoll, D. M.; Dubensky, T. W., Jr.; Kim, Y., STING agonist formulated cancer vaccines can cure established tumors resistant to PD-1 blockade. *Science translational medicine* **2015,** *7* (283), 283ra52-283ra52.

322. Feng, Y.; Forsell, M. N.; Flynn, B.; Adams, W.; Loré, K.; Seder, R.; Wyatt, R. T.; Karlsson Hedestam, G. B., Chemical cross-linking of HIV-1 Env for direct TLR7/8 ligand conjugation compromises recognition of conserved antigenic determinants. *Virology* **2013,** *446* (1-2), 56-65.

323. Bargh, J. D.; Isidro-Llobet, A.; Parker, J. S.; Spring, D. R., Cleavable linkers in antibody-drug conjugates. *Chem Soc Rev* **2019,** *48* (16), 4361-4374.

324. Melero, I.; Berman, D. M.; Aznar, M. A.; Korman, A. J.; Pérez Gracia, J. L.; Haanen, J., Evolving synergistic combinations of targeted immunotherapies to combat cancer. *Nat Rev Cancer* **2015,** *15* (8), 457-72.

325. Durgeau, A.; Virk, Y.; Corgnac, S.; Mami-Chouaib, F., Recent Advances in Targeting CD8 T-Cell Immunity for More Effective Cancer Immunotherapy. *Front Immunol* **2018,** *9*, 14-14.

326. Poschke, I.; Lövgren, T.; Adamson, L.; Nyström, M.; Andersson, E.; Hansson, J.; Tell, R.; Masucci, G. V.; Kiessling, R., A phase I clinical trial combining dendritic cell vaccination with adoptive T cell transfer in patients with stage IV melanoma. *Cancer Immunol Immunother* **2014,** *63* (10), 1061-71.

327. Chodon, T.; Comin-Anduix, B.; Chmielowski, B.; Koya, R. C.; Wu, Z.; Auerbach, M.; Ng, C.; Avramis, E.; Seja, E.; Villanueva, A.; McCannel, T. A.; Ishiyama, A.; Czernin, J.; Radu, C. G.; Wang, X.; Gjertson, D. W.; Cochran, A. J.; Cornetta, K.; Wong, D. J.; Kaplan-Lefko, P.; Hamid, O.; Samlowski, W.; Cohen, P. A.; Daniels, G. A.; Mukherji, B.; Yang, L.; Zack, J. A.; Kohn, D. B.; Heath, J. R.; Glaspy, J. A.; Witte, O. N.; Baltimore, D.; Economou, J. S.; Ribas, A., Adoptive transfer of MART-1 T-cell receptor transgenic lymphocytes and dendritic cell vaccination in patients with metastatic melanoma. *Clin Cancer Res* **2014,** *20* (9), 2457-65.

7. List of abbreviations

Acronym	Definition
α	Anti-
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
AICD	Activation-induced cell death
ALR	AIM2-like receptors
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
BCG	Bacillus Calmette-Guérin
BCL-2	B cell lymphoma 2
BiTE	T cell engaging bispecific antibody
CAR	Chimeric antigen receptor
CARD	Caspase activation and recruitment domain
CD	Clusters of differentiation
CD40L	CD40 ligand
cDC	myeloid/conventional DC
CDC	Complement-dependent cytotoxicity
C_H	Constant domain of the heavy chain
cGAS	cyclic GMP-AMP synthase
C_L	Constant domain of the light chain
CLR	C-type lectin receptor family
CpG	Deoxycytidyl-phosphate-deoxyguanosine
CPP	Cell-penetrating peptide
CRD	Carbohydrate recognition domains
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte antigen 4
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
DDX41	DEAD box polypeptide 41 (DDX41)
Dec205	205 kD membrane protein
DNA	Deoxyribonucleic acid
DNGR-1	dendritic cell natural killer lectin group receptor-1
DPBS	Dulbecco's phosphate buffered saline
E.coli	Echerichia coli
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
Fab	Antigen-binding fragment
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FcR	Fc receptors
FDA	U.S. Food and Drug Administration
FITC	Fluorescein isothiocyanate
Flg	Flagellin
Flt3L	Fms-like tyrosine kinase 3 ligand

GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Good manufacturing practice
GO	Gemtuzumab ozogamycin
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HSC	Hematopoietic stem cell
i.d.	Intradermal
ICI	Immune checkpoint inhibition
iDC	Immature DC
IDH	Isocitrate dehydrogenase
IFI16	interferon-γ inducible protein 16
IFN	Interferon
Ig	Immunoglobulin
Igκ leader	Ig kappa leader
IL	Interleukin
i.t.	Intratumoral
ITAM	Immune-receptor tyrosin-based activation motifs
ITIM	Immune-receptor tyrosine based inhibitory motifs
K_D	Dissociation constant
KMT2A	Histone-lysine N-methyltransferase 2A
LAA	Leukemia-associated antigen
LB	Lysogeny broth
LC	Langerhans cell
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
LSC	Leukemic stem cell
LSP	long synthetic peptides
m	mutated
mAb	Monoclonal antibody
MAC	Multifunctional antibody construct
M-CSF	Macrophage colony-stimulating factor
mDC	Mature DCs
MDS	Myelodisplastic syndrome
MFI	Mean fluorescence intensity
mFlg	Mutated flagellin
MHC	Major histocompatibility complex
MLL	Mixed lineage leukemia
mNPM1	Mutated nucleophosmin
moDC	Monocyte-derived DC
MPL	Monophosphoryl lipid A
MR	Mannose receptor
MyD88	Myeloid differentiation factor 88
NAIP	Neuronal apoptosis inhibitory protein
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK cell	Natural killer cell
NLR	Nod-like receptors
NLRC4	NLR-containing a CARD domain 4
NPM1	Nucleophosmin
NY-ESO-1	New York esophageal squamous cell carcinoma-1

ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PGLALA	P329G, L234A and L235A mutations
p-MHC	Peptide-MHC complex
PRAME	Preferentially expressed antigen in melanoma
PRR	Pattern recognition receptor
p-value	Probability value
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
RU	Response unit
scFv	Single chain variable fragment
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SEM	Standard error of the mean
SMCC	Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate
ssRNA	Single-stranded RNA
STING	Stimulator of interferon genes
TAA	Tumor-associated antigen
TAP	Transporters associated with antigen processing
TCR	T cell receptor
Th cell	T helper cell
T_m	Melting temperature
TIR	Toll/IL-1R homology
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIF	TIR domain containing adapter-inducing interferon
TSA	Tumor-specific antigen
WT1	Wilm’s tumor protein 1

8. Acknowledgements

First of all, I want to thank my supervisor Prof. Dr. Karl-Peter Hopfner for the opportunity to work on the exciting field of cancer immunotherapy and for the continuous support during my PhD project. I am especially grateful for the freedom to establish my experiments and to design my research, which allowed me to develop my own ideas.

I would like to express my gratitude to my “second supervisor” Prof. Dr. Marion Subklewe for the great collaboration and the helpful scientific input. I especially appreciate her effort to initiate the NPM1 project in collaboration with Dr. Marieke Griffioen, which strengthened the impact of my work. Moreover, I want to thank Dr. Katrin Deiser for scientific discussions at the very beginning of my work and Alina Lohner for collaborating in the NPM1 project and performing lots of experiments. Thanks also to the other members of the AG Subklewe for drawing blood or borrowing reagents that run out. In this line, I’m grateful that people were always willing to donate blood, especially my limited number of “Saskia only” donors that bleeded regularly.

I also want to thank the additional members of my Thesis Advisory Committee, namely Prof. Dr. Simon Rothenfußer and Prof. Dr. Thomas Brocker, for valuable advice and my graduate school “Immunotargeting of Cancer (i-Target)” for their great support. In addition, I want to thank Prof. Dr. Christine Josenhans and her team members for the contribution to my project and Andreas Maiser from the group of Prof. Dr. Heinrich Leonhardt for a nice time at the microscope and amazing results.

Special thanks go to Nadja for her infinite scientific support and useful advices, project-wise, but also personally. I am especially thankful that she took the time to not only look over all of my posters and abstracts but to also proof-read my complete thesis, which clearly improved the quality by removing lots of “both”s, “also”s and too long sentences. I also thank Lorenza, Björn and Markus to proof-read parts of it.

I am very grateful to the former and current members of the antibody subgroup Siret, Anja, Alex, Lorenza, Szilvia, Björn, Enrico, Markus, Anna R., Moni, Laia, Claudia, Anna K. and Simon for their help and great working atmosphere. I also thank all the other members of the Hopfner lab. I really enjoyed not only working with you, but also the lab retreats (including the one where I almost drowned, thanks Matthias!), walks to Mensa, coffee times and funny conversations. I had a great time and hope that my future colleagues will be as great as you are!

I also like to thank my friends from Munich, especially Sophia, Kathi and Peter, for sharing good moments and continuous support also during exhausting times, and also my friends from home, especially Lisa. I am very happy that I have Manu at my side. I can always count on you and I’m looking forward to our shared future!

Ganz besonders möchte ich mich bei meinen Eltern und Großeltern bedanken, die mich in jeglicher Situation unterstützt haben und mir stets mit Frankenwein-Survivalpaketen die Heimat nach München gebracht haben. Ohne euch wäre diese Arbeit nicht möglich gewesen und ich wäre heute nicht da, wo ich bin.