Dissecting ALK-specific CD4 T Cell Responses for ALK-positive Anaplastic Large Cell Lymphoma Immunotherapy

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Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Place, Date

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Preliminary Remarks

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"Nothing in life is to be feared; it is only to be understood."

- Maria Skłodowska Curie

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Abstract

The aim of this thesis is to generate new insights into the immunogenicity of the anaplastic lymphoma kinase (ALK) by the first comprehensive investigation of ALK-specific CD4 T cell responses. ALK fusion proteins, resulting from chromosomal translocations, are the main oncogenic drivers in anaplastic large cell lymphoma (ALCL). The aberrant ALK expression in lymphoma cells is recognized by the immune system which is reflected by spontaneous ALK-specific humoral- and T cell responses. These spontaneous immune responses can be potentially promoted by immunotherapy to mediate tumor control and long-term protection. Previous efforts to dissect mechanisms of anti-ALK immunity have primarily focused on CD8 T cells and B cells. The presence and participation of CD4 T cells in the anti-ALK immune response remains poorly characterized.

The first aim of this study was the detection of ALK-specific CD4 T cells in humans. To address this, a combination of a peptide-based immunological approach with IFN-γ ELISPOT was used to analyze peripheral blood of uniformly treated ALK$^+$ALCL patients in clinical remission and healthy individuals for the presence of ALK-reactive CD4 T cells. In the next step, ALK-reactive CD4 T cell responses were dissected via single peptide screening to identify MHC class II restricted immunogenic ALK peptides in individual patients. The findings were integrated into immunization studies in mice to investigate ALK vaccine induced CD4 T cell responses *in vivo* and to explore the therapeutic efficacy of a selected ALK peptide vaccine in a syngeneic ALK-positive lymphoma model.

This study provides evidence for the presence of ALK-reactive CD4 T cell responses in human and mice and led to the identification of previously undescribed CD4 T cell epitopes within the ALK protein. Furthermore, the potential and efficacy of CD4-directed ALK peptide vaccines in inducing spontaneous and therapeutically relevant ALK-specific CD4 T cell responses were demonstrated.

These findings extent the knowledge on ALK immunity and provide essential information on ALK-reactive CD4 T cells, which can be potentially translated into the development of future immunotherapeutic strategies for the treatment of ALK-positive ALCL.

Zusammenfassung

Ziel dieser Arbeit ist es, durch die erste umfassende Untersuchung von ALK-spezifischen CD4-T-Zellantworten neue Einblicke in die Immunogenität der anaplastischen Lymphomkinase (ALK) zu gewinnen. ALK-Fusionsproteine, die aus chromosomalen Translokationen resultieren, sind die primären onkogenen Treiber beim anaplastischen großzelligen Lymphom (ALCL). Die aberrante ALK-Expression in Lymphomzellen wird vom Immunsystem erkannt, was sich in spontanen ALK-spezifischen humoralen und T-Zell-Antworten widerspiegelt. Diese spontan auftretenden Immunantworten können möglicherweise durch Immuntherapie gefördert werden, um Tumorkontrolle und Langzeitschutz zu vermitteln. Frühere Versuche, Mechanismen der Anti-ALK-Immunität zu untersuchen, konzentrierten sich hauptsächlich auf CD8-T-Zellen und B-Zellen. Das Vorhandensein und die Beteiligung von CD4-T-Zellen an der Anti-ALK-Immunantwort sind bisher schlecht charakterisiert.

Das erste Ziel dieser Studie war der Nachweis von ALK-spezifischen CD4-T-Zellen beim Menschen. Um dies zu untersuchen, wurde eine Kombination eines peptidbasierten immunologischen Ansatzes mit IFN-γ-ELISPOT verwendet, um peripheres Blut von einheitlich behandelten ALK$^+$ALCL-Patienten in klinischer Remission und gesunden Personen auf das Vorhandensein von ALK-reaktiven CD4-T-Zellen zu analysieren. Im nächsten Schritt wurden ALK-reaktive CD4-T-Zellantworten über ein Einzelpeptid-Screening analysiert, um MHC-Klasse-II-beschränkte immunogene ALK-Peptide bei einzelnen Patienten zu identifizieren. Die Ergebnisse wurden in Immunisierungsstudien an Mäusen integriert, um die durch ALK-Impfung induzierten CD4-T-Zellantworten *in vivo* zu untersuchen und die therapeutische Wirksamkeit einer ausgewählten ALK-Peptid-Impfung in einem ALK-positiven Lymphom-Modell zu demonstrieren.

Diese Studie liefert Hinweise auf das Vorhandensein von ALK-reaktiven CD4-T-Zellantworten bei Menschen und Mäusen und führte zur Identifizierung von zuvor nicht beschriebenen CD4-T-Zell-Epitopen innerhalb des ALK Proteins. Darüber hinaus wurden das Potenzial und die Wirksamkeit von CD4-gerichteten ALK-Peptid-Impfungen bei der Induktion spontaner und therapeutisch relevanter ALK-spezifischer CD4-T-Zellantworten gezeigt.

Diese Ergebnisse erweitern das Wissen über die ALK-Immunität und liefern wichtige Informationen zu ALK-reaktiven CD4-T-Zellen, die in die Entwicklung zukünftiger immuntherapeutischer Strategien zur Behandlung von ALK-positivem ALCL umgesetzt werden können.

Abbreviations

aa	Amino acids
Ab	Antibody
ALCL	Anaplastic large cell lymphoma
ALK	Anaplastic lymphoma kinase
APC	Antigen presenting cell
BCR	B cell receptor
B-NHL	B-Non-Hodgkin Lymphoma
BM	Bone marrow
BL	Burkitt lymphoma
CNS	Central nervous system
CAR T cells	Chimeric antigen receptor T cell
CTLC1	Clathrin heavy chain-like 1
CIR	Cumulative incidence or relapse
c-di GMP	Cyclic diguanylate monophosphate
CDN	Cyclic dinucleotide
CMV	Cytomegalovirus
CpG	Cytosine-phosphate-guanine
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
DLBCL	Diffuse large B cell lymphoma
ELISPOT	Enzyme-linked immunospot
EMA	Epithelial membrane antigen
EICNHL	European Inter-Group for Childhood Non-Hodgkin Lymphoma
EFS	Event-free survival
FDA	Food and Drug Administration
FOXP3	Forkhead box protein P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HL	Hodgkin Lymphoma
HLA	Human leukocyte antigen
ICB	Immune checkpoint blockade
ISCOM	Immunostimulatory complexes
IMT	Inflammatory myofibroblastic tumors
ILC3	Innate lymphoid cells
IFN-γ	Interferon gamma
IL	Interleukin
JAK3	Janus kinase 3
LTK	Leukocyte tyrosine kinase
B or T-LBL	Lymphoblastic B or T cell lymphoma
MACS	Magnetic-activated cell sorting
MHC	Major histocompartibility complex
MDD	Minimal disseminated disease
MRD	Minimal residual disease
NSCLC	Non-small cell lung cancer
NHL	Non-Hodgkin lymphoma
NHL-BFM	NHL-Berlin-Frankfurt-Münster
NK cells	Natural killer cells
NPM	Nucleophosmin
OR	Overall response
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
PTCL	Peripheral T cell lymphoma
PI3K	Phosphatidylinositol 3-kinase
PD-1	Programmed cell death-1
PD-L1	Programmed cell death-Ligand 1
PFS	Progression-free survival
ROR-γ	RAR-related orphan receptor gamma
ERK	Ras-extracellular signal-regulated kinase
RT-PCR	Real-time polymerase chain reaction
RTK	Receptor tyrosine kinase
Treg	Regulatory T cell
STAT3	Signal transducer-activator of transcription proteins 3
SCT	Stem cell transplantation
STING	Stimulator of interferon gene
SLP	Synthetic long peptide
TCR	T cell receptor
Th17 cells	T helper 17 cells
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
TAA	Tumor-associated antigen
WHO	World Health Organization

1. Introduction

1.1. Lymphoma

Lymphomas are clonal neoplasms originating from abnormal lymphoid cells that form tumors in lymph nodes and other parts of the body. The World Health Organization (WHO) classifies lymphomas according to the type of cell from which they are derived (immature and mature T cells, B cells, or natural killer (NK) cells) and their molecular, cytogenetic, and clinical characteristics (Swerdlow et al. 2017). Lymphomas are broadly categorized into two main groups: Non-Hodgkin Lymphoma (NHL) and Hodgkin Lymphoma (HL). NHL represent a highly heterogeneous group of B-, T-, or NK cell neoplasms accounting for approximately 70% of lymphoma cases worldwide (Siegel et al. 2019; Swerdlow et al. 2017). Compared to NHLs, HLs are less heterogeneous and of B-cell origin (Torre et al. 2015; Swerdlow et al. 2017).
NHL is the fourth most common diagnosed cancer among children in Germany and accounts for about 7% of childhood cancers in the developed world (Kaatsch et al. 2019; Kaatsch 2010). The most common NHL subtypes in children and adolescents are burkitt lymphoma (BL), diffuse large B cell lymphoma (DLBCL), lymphoblastic T cell- or B cell lymphoma (T-LBL, B-LBL), and anaplastic large cell lymphoma (ALCL) (Sandlund & Martin 2016; Sandlund 2015; Minard-Colin et al. 2015; Burkhardt et al. 2005).

1.2. ALK-positive Anaplastic Large Cell Lymphoma (ALK^+ALCL) in children and adolescents

1.2.1. Classification of ALCL

ALCLs represent a group of peripheral T cell lymphomas that have common immunophenotypical and morphological characteristics, including strong CD30 expression and loss of T cell markers, but differ in genetic characteristics, clinical presentation, and prognosis (Swerdlow et al. 2017; Montes-Mojarro et al. 2018). ALCL was first described in 1985 as anaplastic large CD30-positive NHL with morphologic similarity to malignant histiocytosis and characteristics overlapping with classic HL (Stein et al. 1985). Subsequent genotypic and immunophenotypic analysis revealed that these CD30-positive NHLs represent a heterogenous group of tumors derived from activated lymphoid cells of T cell origin, which lead to the categorization of ALCLs in the group of peripheral T cell lymphomas (PTCLs) (Stein et al. 1985; O'Connor et al. 1987; Herbst et al. 1989). In 1994, the discovery of the t(2;5)(p23;q35) chromosomal translocation, that fuses the nucleophosmin *(NPM1)* gene to the anaplastic lymphoma kinase (*ALK*) gene (NPM-ALK) in the majority of ALCLs, led to the distinction of two subtypes, ALK-positive and ALK-negative ALCL (Morris et al. 1994). ALK-positive ALCL was

subsequently incorporated in the 2008 WHO classification of hematopoietic and lymphoid tissue as distinct entity (Swerdlow et al. 2008). Based on new clinical and molecular findings, the revision of the WHO classification in 2016 now recognizes four different subgroups of ALCL: systemic ALK-positive ALCL (ALK^+ALCL), systemic ALK-negative ALCL (ALK^-ALCL), primary cutaneous ALCL (pcALCL), and, as a provisional entity, breast implant-associated ALCL (BI-ALCL) (Swerdlow et al. 2017).

1.2.2. Epidemiology and clinical characteristics

ALCL accounts for about 10-15% of pediatric and adolescent NHLs (Burkhardt et al. 2005) and affects approximately 20-25 patients in Germany each year (Seidemann et al. 2001). More than 90% of cases in children and adolescents express oncogenic ALK fusion proteins. The majority of those are characterized by the t(2;5)(p23;q35) chromosomal translocation that results in the expression of the NPM-ALK fusion protein. In about 10% of patients, other variant ALK fusion partners are observed (Perkins et al. 2005; Brugières, Le Deley, et al. 2009; Damm-Welk et al. 2009; Seidemann et al. 2001; Brugières et al. 1998). ALK^+ALCL shows a moderate male predominance with a male:female ratio of 1.8:1. (Burkhardt et al. 2005). Most patients present in advanced stages with peripheral, mediastinal or intra-abdominal lymph node involvement and B-symptoms (60%) including fever, weight loss, and night sweats. Even though the majority of cases manifest as nodal disease, extra-nodal involvement is common in about 60% of patients, particularly in skin, bone, lung, and soft tissue (Brugières et al. 1998; Seidemann et al. 2001; Brugières, Le Deley, et al. 2009; Reiter et al. 1994; Williams et al. 2002; Mori et al. 2003; Rosolen et al. 2005; Lowe et al. 2009; Alexander et al. 2014). Central nervous system involvement is rare (1-3%) (Nomura et al. 2013; Williams et al. 2013), so is leukemic manifestation which occurs in less than 1% of ALCLs (Onciu et al. 2003; Spiegel et al. 2014). The frequency of observed bone marrow involvement strongly depends on the detection method. Bone marrow infiltration is detected in less than 15% of cases by cytology and/or histology (Brugières, Le Deley, et al. 2009; Brugières et al. 1998; Seidemann et al. 2001; Williams et al. 2002; Rosolen et al. 2005; Lowe et al. 2009). The more sensitive method, reverse-transcription (RT)-PCR for *NPM-ALK*, allows for the detection of minimal disseminated disease (MDD) in bone marrow or peripheral blood in 50-60% of patients (Mussolin et al. 2005; Damm-Welk et al. 2007).

1.2.3. Morphological characteristics

ALCLs were originally described to consist of large anaplastic cells with abundant cytoplasm and pleomorphic nuclei that strongly express the CD30 antigen and grow within lymph node sinuses (Stein et al. 1985). Since then, the definition of ALCLs has evolved considerably and

studies provided evidence that ALK⁺ALCLs show a broad spectrum of morphological heterogeneity (Benharroch et al. 1998; Falini et al. 1998). The characteristic “hallmark cells”, defined as large neoplastic cells with abundant eosinophilic cytoplasm, a prominent Golgi structure and multiple eccentric horseshoe- or kidney-shaped nuclei, are detected in all subtypes (Benharroch et al. 1998; Falini et al. 1998; Stein et al. 2000). According to distinct morphological features, varying in small- and medium-sized cells to cases in which large anaplastic cells predominate as well as the presence of histiocytic bystander cells, the WHO classification recognizes five morphological patters of ALK⁺ALCLs: the common pattern (60-70%), the lymphohistiocytic pattern (10%), the small cell pattern (5-10%), the Hodgkin’s-like pattern (3%), and the composite pattern (15%) (Swerdlow et al. 2017; Benharroch et al. 1998; Stein et al. 2000; Leoncini et al. 1990; Bayle et al. 1999; Pileri et al. 1990).

1.2.4. Immunophenotype and cell of origin

ALK⁺ALCLs are classified as mature T cell neoplasms, however, the majority of ALCLs lack T cell receptor (TCR) expression and several pan T cell markers, resulting in a loss of T cell immunophenotype. More than 75% of ALK⁺ALCLs are negative for the T cell marker CD3. Other T cell markers including CD4, CD5 and CD2 are present in 40-70% of cases and CD45 and CD45RO are variably expressed (Savage et al. 2008; Bonzheim et al. 2004). Most neoplastic cells are negative for CD8 but express cytotoxic associated molecules such as granzyme B, perforin and TIA-1. Despite neoplastic cells usually lack the expression of TCRs and TCR signaling molecules, 90% of cases exhibit TCR rearrangements on the genomic level. (Bonzheim et al. 2004; Foss et al. 1996; Savage et al. 2008; Herbst et al. 1989). The loss of proximal TCR molecules has been shown to be induced by the constitutive ALK activity in neoplastic cells (Ambrogio et al. 2009). Indicative for an activated T cell phenotype is the expression of the epithelial membrane antigen EMA (mucin1, MUC1) (Ten Berge et al. 2001; Agrawal et al. 1998).

Due to the almost universal expression of cytotoxic-associated molecules, it has been considered that ALK⁺ALCL arises from activated cytotoxic T cells. Recent research, however, provided new insights that raises the possibility that ALCL arises in early thymocytes which, despite absent or incomplete TCR rearrangements, progress through T cell developmental stages driven by ALK-induced bypass mechanisms (Moti et al. 2014; Malcolm et al. 2016). Gene expression analysis of ALK⁺ALCLs revealed the expression of a set of genes associated with a T helper 17 (Th17) cell phenotype including IL-17A, IL-17F, IL-26, IL-22, and RAR-related orphan receptor gamma (ROR-γ) (Iqbal et al. 2010; Matsuyama et al. 2012) and IL-22 and IL-17 have also been detected in the circulation of ALK⁺ALCL patients (Savan et al. 2011; Mellgren et al. 2012; Knörr, Damm-Welk, et al. 2018). A recent comprehensive analysis of primary lymphoma data revealed that some ALCLs not only show a Th17 signature but are

also enriched for innate lymphoid cell (ILC3) overexpressed genes (Schleussner et al. 2018). ALK$^+$ALCL pathogenesis and maintenance is strongly dependent on ALK-signaling which also heavily influences the phenotype of tumor cells. In line, the expression of IL-6, IL-17A, IL-17F, perforin, and granzyme B in ALCL cells have been shown to be the consequence of NPM-ALK activity (Matsuyama et al. 2012; Pearson et al. 2011). Thus, the Th17/cytotoxic T cell screw might be rather attributed to the driving ALK fusion oncogenes than to the cell of origin. In any case, the identification of ALCL origin needs further investigation.

1.2.5. Molecular characteristics and pathogenesis

ALK fusion proteins resulting from chromosomal translocations have a central oncogenic role in ALK$^+$ALCL. ALK was first described in ALCL in 1994 and was later found to be rearranged, amplified, or mutated also in several other tumors including inflammatory myofibroblastic tumors (IMT), neuroblastoma, and non-small cell lung cancer (NSCLC) (Morris et al. 1994; Mariño-Enríquez & Dal Cin 2013; Lovly et al. 2014; De Brouwer et al. 2010; Rikova et al. 2007). Wildtype ALK is a highly conserved tyrosine kinase receptor (RTK) that is part of the insulin receptor superfamily and shares a high degree of homology with the leukocyte tyrosine kinase (LTK) (Iwahara et al. 1997). The human *ALK* gene is located on the 2p23 chromosomal segment and encodes a 1620 aa long peptide that undergoes post-translational modifications and gives rise to the 180-220 kDa ALK protein (Iwahara et al. 1997; Morris et al. 1997). As a classical RTK, ALK consists of an extracellular ligand-binding domain, a transmembrane domain and an intracellular catalytic tyrosine kinase domain. While the intracellular tyrosine kinase domain of ALK shares similarity with other insulin receptors, the extracellular domain is unique among RTKs (Morris et al. 1997; Iwahara et al. 1997; Palmer et al. 2009). In mammals, ALK is almost exclusively expressed in the central- and peripheral nervous system during development. ALK protein levels seem to almost fully diminish in all tissues after birth, but low mRNA expression has been detected in few restricted zones of the brain, the small intestine, and testis in adult mice (Iwahara et al. 1997; Hurley et al. 2006; Vernersson et al. 2006). In human, mRNA expression was found in the adult brain, small intestine, testis, prostate, and colon but no other tissues (Morris et al. 1997). The ALK expression patterns in mammals suggest a possible function of ALK in the development of the nervous system. However, the exact physiological role of full-length ALK in humans is still poorly characterized, as are its natural ligands.

In ALCL, the genomic breakpoint in the *ALK* gene is almost universally located in an intronic region flanked by exon 19 and 20 and involves the whole intracytoplasmic domain of ALK (Krumbholz et al. 2018; Luthra et al. 1998; Ladanyi & Cavalchire 1996). Each of the ALK translocations generates a different fusion protein consisting of a 5´-translocation partner fused to the ALK catalytic domain at the 3′ end. Depending on the ALK fusion partner, the chimeric

proteins localize to distinct subcellular compartments. In most cases, the 5′ partner provides dimerization domains allowing the formation of holo- and heterocomplexes which results in constitutive ALK tyrosine kinase activity and subsequent activation of prominent interconnected downstream signaling pathways. Constitutive ALK signaling induces cell transformation by controlling substantial cellular processes involved in cell proliferation, survival, cytoskeletal rearrangements, phenotypic changes, metabolism, and immune evasion (Palmer et al. 2009; Chiarle et al. 2008). The most prominent and best characterized ALK induced pathways are the Ras-extracellular signal-regulated kinase (ERK) pathway which mainly mediates increased proliferation of ALCL cells, and the janus kinase 3 (JAK3)–signal transducer and activator of transcription proteins 3 (STAT3) pathway and the phosphatidylinositol 3-kinase (PI3K)–Akt pathway that promote enhanced survival of ALCL cells by regulating anti-apoptotic and cell cycle molecules (Wasik et al. 2009; Crockett et al. 2004; Voena et al. 2007; Vega et al. 2006; Marzec et al. 2011; Martinengo et al. 2014; Hallberg & Palmer 2016; Chiarle et al. 2005).

The most prevalent t(2;5)(p23;q35) chromosomal translocation in ALCL fuses the *NPM1* gene on chromosome 5 to *ALK* (Morris et al. 1994). *NPM1* encodes the ubiquitous expressed protein nucleophosmin (NPM) which is implicated in several pathways including ribosomal biogenesis, mRNA processing, chromatin remodeling, apoptosis and DNA repair (Box et al. 2016). The fusion of the N-terminal promotor and proximal domain of NPM to the cytoplasmic catalytic region of ALK gives rise to an 80 kD NPM-ALK chimeric protein **(Figure 1)**. NPM-ALK forms homodimers which is mediated by the NPM oligomerization domain and leads to reciprocal ALK autophosphorylation and consequently to strong and constitutive tyrosine kinase activity. Alternatively, dimerization with wild type NPM targets ALK into nuclear and nucleolar regions, explaining the characteristic cytoplasmic, nuclear and nucleolar ALK staining pattern in tumor cells.

In cases of variant fusion partners, ALK expression is restricted to the cytoplasm and/or cell membrane. Variant ALK partners include tropomyosin 3 (TPM3), tropomyosin 4 (TPM4), TRK-fused gene (TFG), 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (ATIC), clathrin heavy chain-like 1 (CLTC1), moesin (MSN), non-muscle myosin heavy chain 9 (MYH9), and ALK lymphoma oligomerization partner on chromosome 17 (ALO17) (Lamant et al. 1999; Colleoni et al. 2000; Ma et al. 2000; Hernández et al. 2002; Touriol et al. 2000; Tort et al. 2001; Lamant et al. 2003; Cools et al. 2002).

Besides ALK translocations, only few ALK-independent secondary genetic alterations have been described (Salaverria et al. 2008; Youssif et al. 2009).

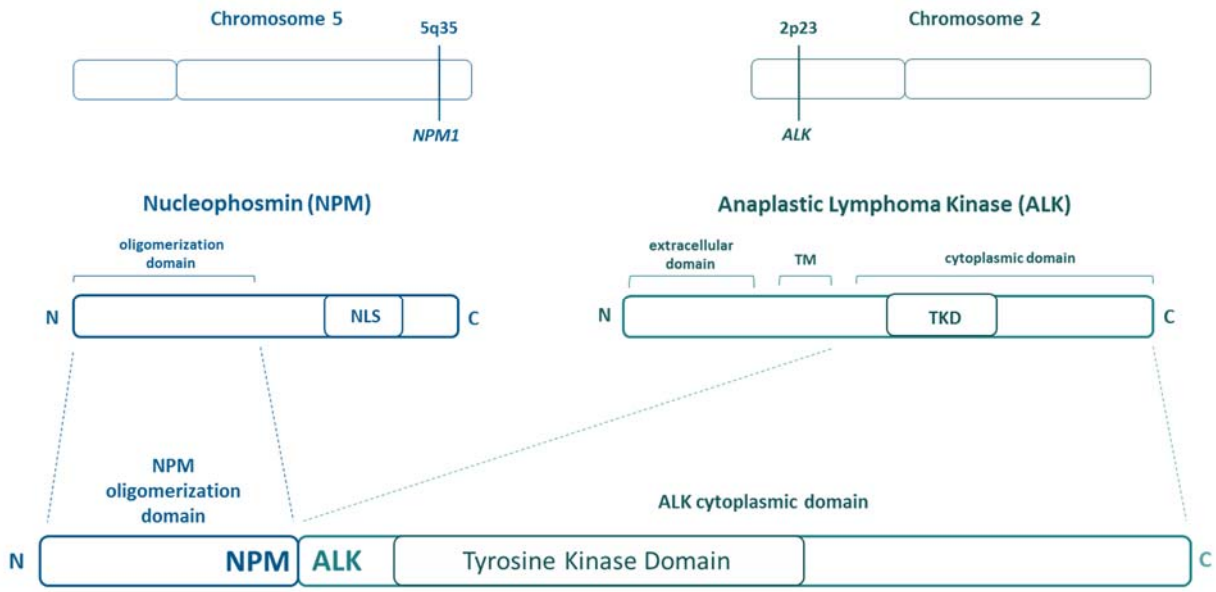


Figure 1: Schematic representation of the t(2;5)(p23;q35) translocation and the resulting oncogenic NPM-ALK fusion protein. The t(2;5)(p23;q35) chromosomal translocation leads to the fusion of the oligomerization domain of nucleophosmin (NPM) to the cytoplasmic catalytic domain of the anaplastic lymphoma kinase (ALK). The resulting NPM-ALK fusion protein plays a central oncogenic role in ALCL.

1.2.6. Treatment of ALK$^+$ALCL

Front-line therapy

ALK$^+$ALCL is chemo-sensitive with a high response rate to different chemotherapy regimens. A variety of treatment strategies have been used in clinical trials reaching from short intensive B-NHL-like regimens to protocols used to treat leukemia. Despite the differences in chemotherapy agents, therapy duration and dose intensity, the outcome of patients have largely remained unchanged with an 2-5 year event-free-survival (EFS) of 65-75% across trials (Seidemann et al. 2001; Le Deley et al. 2010; Brugières, Le Deley, et al. 2009; Pillon et al. 2012; Laver et al. 2005; Lowe et al. 2009; Alexander et al. 2014; Rosolen et al. 2005).

An overview of pediatric ALCL treatment is shown in **Table 1**.

The most recent European trial, the ALCL99 study, used a chemotherapy backbone with low cumulative drug doses and a short treatment duration of 4-5 months which lead to a 10 year progression-free survival (PFS) of 70% and overall survival (OS) of 90% (Le Deley et al. 2010; Brugières et al. 2009; Mussolin et al. 2020). The addition of vinblastine to the chemotherapy backbone and vinblastine maintenance resulted in a delay of relapse but after a 2-year follow-up, no significant differences on EFS were identified (Le Deley et al. 2010).

In contrast to the ALCL99 study, the American ANHL0131 trial used an anthracycline based chemotherapy backbone and involved induction therapy followed by 15 cycles of maintenance

therapy (Alexander et al. 2014). The three-year EFS and OS did not significantly differ from the ALCL99 trial and further intensification of chemotherapy did not decrease the relapse rate in children with ALCL in two studies (Le Deley et al. 2010; Alexander et al. 2014).

Chemotherapy is associated with toxic side effects that occur during treatment including hematological toxicity, infections, stomatitis, and liver toxicity and patients are in risk for late effects such as infertility and secondary malignancies (Wrobel et al. 2011; Moser et al. 2020). Together, these data suggest that chemotherapy has reached its limit for the treatment of ALCL and new therapeutic strategies are needed to improve patient's outcome.

Table 1: Historic overview of pediatric ALCL treatment

Study	Inclusion Criteria	N	Treatment	EFS	OS
NHL-BFM90 (1990–1995)	All stages <18 years	89	Duration: 2–5 months Maximum cumulative doses: HD MTX, cyclophosphamide 3.4 g/m^2, ifosfamide 12 g/m^2, etoposide 1,300 mg/m^2, doxorubicin 150 mg/m^2	76% (5 year)	ns
UKCCSG NHL 9000 protocols (1990–1998)	All stages <18 years	72	Duration: 2–5 months Maximum cumulative doses: HD MTX, cyclophosphamide 2.3 g/m^2, etoposide 350 mg/m^2, doxorubicin 120 mg/m^2	59% (5 year)	65% (5 year)
POG 9315 (1994–2000)	Stage III–IV <22 years	86	Randomization: APO maintenance versus APO maintenance alternating with IDM/HiDAC Duration: 12 months, Maximum cumulative doses: HD MTX, doxorubicin 600 mg/m^2	71.8% (4 year)	88.1% (4 year)
AIEOP LNH-92 (1993–1997)	All stages <15 years	34	Duration: 24 months Maximum cumulative doses: HD MTX, cyclophosphamide 7.5 g/m^2, etoposide 6.3 g/m^2 daunorubicin 60 mg/m^2	65% (10 year)	85% (10 year)
ALCL-99 (1999–2006)	All stages, except isolated skin disease, completely resected stage I or CNS involvement. <22 years	352	Randomization: MTX 1 mg/m^2 over 24 h + IT versus 3 mg/m^2 over 3 h and no IT Duration: 2–5 months Maximum cumulative doses: HD MTX, cyclophosphamide 3.4 g/m^2, ifosfamide 12g/m^2, etoposide 1,300 mg/m^2, doxorubicin 150 mg/m^2	74.1% (2 year)	92.5% (2 year)
		217	Randomization: +/- VBL to induction therapy and maintenance VBL Duration: 4–12 months Maximum cumulative doses: HD MTX, cyclophosphamide 3.4 g/m^2, ifosfamide 12 g/m^2, etoposide 1,300 mg/m^2, doxorubicin 150 mg/m^2	71% (2 year)	94% (2 year)
CCG-5941 (1996–2001)	Non-localized disease <21 years	86	Duration: 11 months Maximum cumulative doses: cyclophosphamide 8.4 g/m^2, etoposide 1,400 mg/m^2 , anthracycline 210 mg/m^2	68% (5 year)	80% (5 year)
AIEOP LNH-97 (1997–2000)	All stages <18 years	32	Duration: 2–5 months Maximum cumulative doses: HD MTX, cyclophosphamide 3.4 g/m^2, ifosfamide 12 g/m^2, etoposide 1,300 mg/m^2, doxorubicin 150 mg/m^2	68% (5 year)	87% (5 year)
ANHL0131 (2004–2008)	Stage III–IV <21 years	125	Randomization: APO maintenance versus APO with day 1 VBL instead of VCR on day 1, 8, and 15 Duration: 12 months Maximum cumulative doses: doxorubicin 300 mg/m^2	76% (3 year)	85% (3 year)

Adapted from (Tole et al. 2018), AIEOP= Italian association of pediatric hematology and oncology; ALCL=anaplastic large cell lymphoma; APO = doxorubicin/prednisone/vincristine/6-mercaptopurine and methotrexate; BFM = Berlin-Frankfurt-Münster; B-NHL=B-cell non-hodgkin lymphoma; CCG = Children's cancer group; CNS=central nervous system; EFS = event free survival; HD MTX = high-dose methotrexate; IDM/HiDAC=intermediate-dose methotrexate/high-dose cytarabine; IT = intrathecal; MTX = methotrexate; NHL-BFM = Non-hodgkin lymphoma-Berlin-Frankfurt-Münster; ns = not specified; OS = overall survival; POG = Pediatric oncology group; UKCCSG = UK Children's cancer study group; VBL = vinblastine; VCR = vincristine.

Treatment at relapse

Up to date, there is no standard therapy for the treatment of relapsed or refractory ALCL. Several retrospective studies demonstrated that relapsed patients still have a 50-60% survival chance. In these studies, a variety of therapeutic strategies have been used reaching from single-agent chemotherapy to multi-agent chemotherapy regimens consolidated by autologous or allogeneic hematopoietic stem cell transplantation (SCT) (Brugières et al. 2000; Woessmann et al. 2011; Mori et al. 2006; Woessmann et al. 2006; Gross et al. 2010; Fukano et al. 2015; Strullu et al. 2015; Brugières et al. 2009).

A good efficacy of weekly single-agent vinblastine in relapsed refractory ALCL was reported in a study of 36 patients (Brugières et al. 2009). In order to evaluate a risk-adapted strategy for the treatment of relapsed patients, the European Intergroup for Childhood non-Hodgkin Lymphoma (EICNHL) has implemented a prospective ALCL relapse trial (Ruf et al. 2015; Knörr et al. 2020). The results of the study confirmed the good efficacy of allogeneic SCT in high-risk relapse patients with a 3-year EFS of 64%. A very low relapse rate of 10–20% has been observed in patients with relapsed or progressive ALCL after allogeneic SCT, which is indicative for a graft versus ALCL effect (Woessmann et al. 2006). In addition, weekly administration of vinblastine in the low-risk group resulted in a 3-year EFS of 85%, suggesting that patients with low risk relapses might benefit from a vinblastine monotherapy (Ruf et al. 2015; Knörr et al. 2020). Notably, vinblastine was found to induce functional maturation of dendritic cells (DCs) *in vitro* and *in vivo* (Tanaka, Matsushima, Mizumoto, et al. 2009; Tanaka, Matsushima, Nishibu, et al. 2009). The efficacy of vinblastine might therefore not only be attributed to its cytotoxic effect but also to its immune stimulatory potential.

New therapeutic agents

Two new therapeutic agents, bretuximab vedotin and crizotinib are incorporated in the currently recruiting ANHL12P1 study. The study is based on the ALCL99 chemotherapy backbone and aims to assess the safety and feasibility of these two novel agents in front-line therapy.

Brentuximab vedotin is an anti-CD30 antibody drug conjugate that selectively distributes the anti-micotubule agent monomethylauristatin E (MMAE) to CD30-positive cells, which leads to cell death. A phase II clinical study in adults with relapsed or refractory ALCL reported an overall response (OR) of 86% (Pro et al. 2012). This study led to the approval of brentuximab vedotin by the US Food and Drug Administration (FDA) for the treatment of adult patients with relapsed ALCL after failure of multi-agent chemotherapy. The majority of patients received allogeneic SCT after brentuximab-vedotin treatment which lead to a 5-year progression free survival (PFS) of 39% and OS of 60% in the subsequent follow-up study (Pro et al. 2016). The

same observation was made in a study of single-agent brentuximab-vedotin in children with relapsed ALCL and HL, which demonstrated the efficacy of this drug as re-induction therapy for bridging to SCT (Locatelli et al. 2017).

ALCL tumor cell survival is strongly dependent on ALK signaling, which makes ALK-inhibitors a promising treatment option. Several ALK-inhibitors have been developed for the treatment of ALK-positive NSCLC, four of which are approved by the FDA and several others being under clinical investigation (reviewed in (Sharma et al. 2018). Crizotinib is an oral, first-generation small molecule ALK/MET inhibitor that induced a high response rate in a phase I/II trial of pediatric ALCL patients and in adults with refractory/relapsed ALCL with tolerable toxicity (Mossé et al. 2013; Gambacorti Passerini et al. 2014; Mossé et al. 2017). In contrast to ALK-positive NSCLC patients in whom crizotinib treatment leads to the emergence of drug resistance within only a few months, no clear indications for drug resistance development have been made in ALCL patients so far. However, early relapses after crizotinib discontinuation have been observed (Gambacorti-Passerini et al. 2016). Even though crizotinib induced complete remission (CR) in most patients, it has not been reported curative and may require continuous life-long treatment. For this reason, most patients to date have received crizotinib treatment to induce CR prior to SCT.

1.2.7. Prognostic factors

An analysis of cumulative data from European national studies from the 1990s by the European Intergroup Study for ALCL identified mediastinal, visceral, and skin involvement as poor clinical prognostic factors (Le Deley et al. 2008). In addition, the small cell variant and the lymphohistiocytic subtypes have been associated with a poor 3-year EFS and OS in several clinical trials (Alexander et al. 2014; Lamant et al. 2011; Brugières et al. 1998; Mussolin et al. 2020). The detection of minimal disseminated disease (MDD) and minimal residual disease (MRD) by RT-PCR for *NPM-ALK* transcripts in bone marrow or blood, has been demonstrated to be of prognostic value and allows the identification of high-risk patients (Damm-Welk et al. 2007; Damm-Welk et al. 2014; Mussolin et al. 2013).

The majority of ALK$^+$ALCL patients mount a humoral immune response to ALK which is reflected by detectable ALK autoantibodies in serum and plasma (Pulford et al. 2000; Ait-Tahar et al. 2006). ALK-antibody titers have been shown to inversely correlate with risk of relapse (Ait-Tahar et al. 2010). By combining ALK-antibody (AB) titers and MDD, Mussolin et al. described a risk stratification of patients into high risk (MDD-positive and low AB titer), low risk (MDD-negative and high AB titer), and intermediate risk (all other patients) groups with a progression free survival of 28%, 93%, and 68%, respectively (Mussolin et al. 2013).

In relapse, the most consistent poor prognostic factor is an early relapse within 12 months from diagnosis. In addition, the BFM study group reported CNS, BM involvement and CD3 positivity as risk factors in relapsed/refractory patients (Brugières et al. 2000; Woessmann et al. 2011).

1.3. Cancer immunology and immunotherapy

1.3.1. Cancer immunology

Cancer is characterized by accumulation of mutations and loss of normal regulatory mechanisms that give rise to the expression of potential tumor antigens that are visible to the immune system (Hanahan & Weinberg 2011; Dunn et al. 2004). Tumor-antigens presented by major histocompatibility (MHC) molecules on the surface of tumor cells can be recognized by T cells that spontaneously arise in cancer patients (Boon et al. 1994; Coulie et al. 2014). However, they rarely provide consistent immune protection. Tumor heterogeneity is strongly shaped by active immune responses and during cancer progression tumor cells develop mechanisms to avoid immune destruction (immune editing) and negative regulatory mechanisms in lymphoid organs or the tumor microenvironment (TME) contribute to the failure of protective immunity in many patients (Dunn et al. 2002; Dunn et al. 2004; Palucka & Coussens 2016; Motz & Coukos 2013).

An anti-tumor response that leads to effective killing of cancer cells and provides tumor control requires the initiation of a series of stepwise events **(Figure 2)** (Chen & Mellman 2013). In the first step, tumor antigens need to be released by dying neoplastic cells and captured by antigen presenting cells (APCs), especially dendritic cells (DCs), for presentation of processed peptides. Soluble antigens can be transported through the lymphatic vessels and are captured by lymph node resident DCs, while tissue resident DCs take up and present antigens at the tumor site. This step requires accompanying stimuli including inflammatory cytokines and other factors that guide and specify immunity and prevent the induction of peripheral tolerance (Dudek et al. 2013; Mildner & Jung 2014). In the presence of such “danger signals”, DCs undergo maturation, a process accompanied by phenotypic and functional changes. Mature DCs display reduced phagocytic activity, increased expression of MHC- and co-stimulatory molecules, and secrete high amounts of immune stimulatory cytokines (Dalod et al. 2014). During the maturation process, DCs also change chemokine receptor patterns to acquire the capacity to migrate to T cell areas in secondary lymphoid tissues where they actively present captured and processed antigens via MHC class I and II molecules to naïve CD8- and CD4 T cells, respectively (Steinman 2012; Dalod et al. 2014; Dudek et al. 2013). This critical step determines the nature of the immune response. Priming and activation of anti-tumor effector T cells requires the recognition of the tumor antigen as foreign, or the antigen needs to represent a protein to which central tolerance has been incomplete. Thus, the balance between activated

effector T cells and regulatory T cells (T_{reg}) that maintain tolerance to self-antigens, determines the outcome of the initiated immune response. Upon antigen recognition, naïve CD8 T cells differentiate into cytotoxic effector T cells (CTLs) (Zhang & Bevan 2011). CTLs are considered as the major anti-tumor effector cells as their strong cytotoxic activity leads to direct killing of cancer cells. Naïve CD4 T cells give rise to T helper cells with distinct cytokine profiles and effector functions or, in case of central tolerance, to T_{regs} with strong immunosuppressive capacity (Borst et al. 2018; Togashi et al. 2019). The quality of T cell priming is essential for the generation of potent anti-tumor T cells. Ideal endowed T cell properties include strong recognition of respective peptide-MHC (pMHC) complexes, expression of surface molecules allowing trafficking to the tumor side, and extended longevity and memory. Once primed and activated, T cells leave the lymph node and optimally migrate to the tumor where they recognize and kill cancer cells. The eradication of cancer cells causes the release of additional tumor antigens that, again can be captured by DCs and leads to the re-initiation of a self-sustaining anti-tumor immunity cycle (Chen & Mellman 2013).

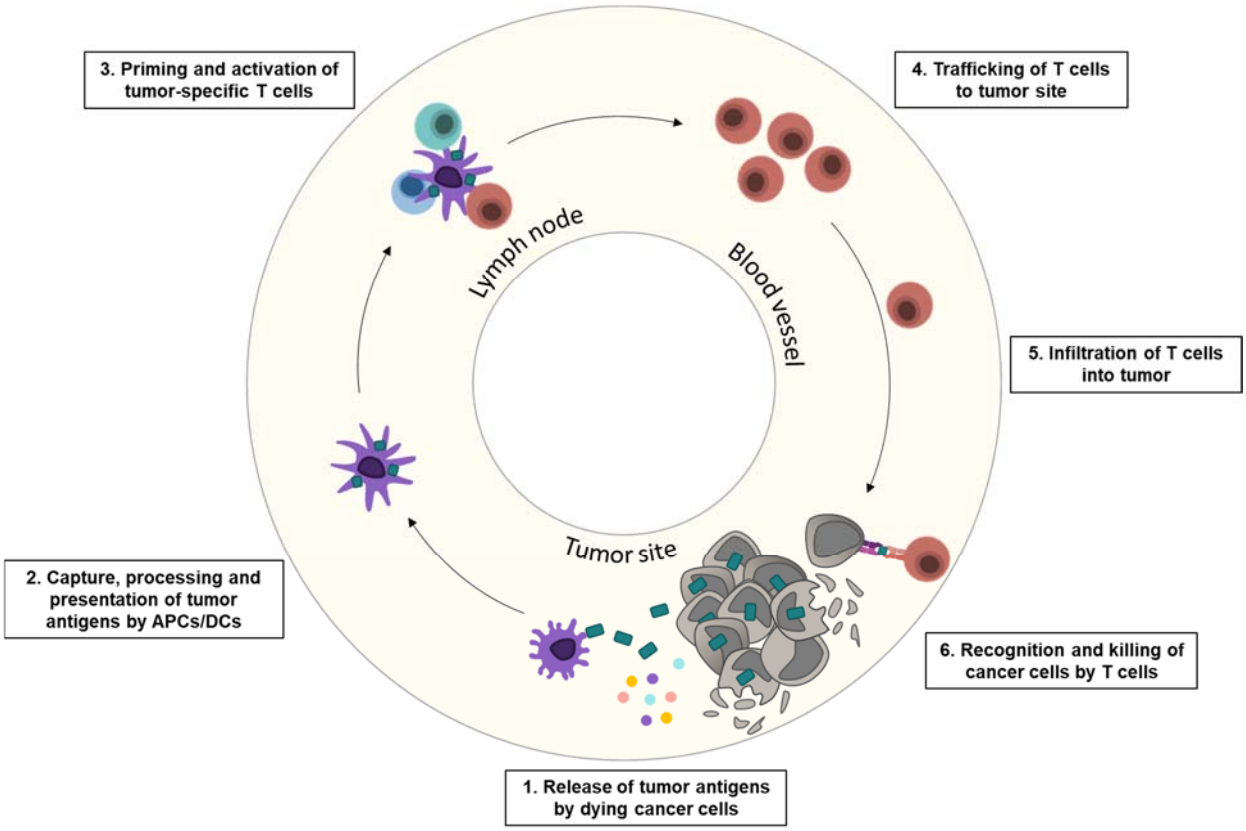


Figure 2: The cancer-immunity cycle (adapted from (Chen & Mellman 2013))
The initiation and maintenance of protective anti-tumor immunity requires a series of events and interplay between different subsets of immune cells which are schematically outlined. Tumor antigens (TAs) released by dying cancer cells are captured by antigen presenting cells (APCs), especially dendritic cells (DCs), for presentation of processed peptides which requires immunostimulatory factors (colored circles). DCs undergo a maturation process and migrate to lymph nodes to actively present TAs to naïve T cells. After priming, tumor-specific T cells leave the lymph node and travel to the tumor-side, where they infiltrate the tumor and recognize cancer cells via TCR-pMHC interactions which leads to tumor cell killing and release of additional tumor antigens. Inhibitory factors from tumor or tumor microenvironment and immune regulatory mechanisms can oppose the initiation or limit/halt the anti-tumor immune response.

1.3.3. Cancer immunotherapy

Cancer immunotherapies aim to promote natural occurring immune responses against tumors or to initiate cancer immunity by overcoming inhibitory mechanisms that oppose amplification or arresting the anti-tumor response. They mainly rely on two principal mechanisms of action: (1) passive immunotherapy via adoptive transfer of tumor-targeting T cells such as chimeric antigen receptor T cells (CAR T cells) or administration of anti-tumor antibodies or (2) active immunotherapy that promotes the patient´s immune system by provision of antibodies directed against immune regulatory checkpoint molecules or via vaccines that expand tumor-specific T cells (Galluzzi et al. 2014). Both, passive and active immunotherapy, mainly rely on the action of T cells and their ability to control cancers (Waldman et al. 2020).

Cancer immunotherapy has advanced rapidly in the clinic particularly due to the success of immune checkpoint inhibitors and CAR T cell therapy. Immune checkpoint blockade (ICB) by antibodies targeting programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) or cytotoxic T-lymphocyte antigen-4 (CTLA-4) can unleash the activity of natural occurring anti-tumor T cells by eliminating negative regulations of T cell priming and effector functions and are already integrated into standard of care regimens for the treatment of several cancers (Darvin et al. 2018). Autologous CD19-directed CAR T cells showed impressive efficacy in patients with hematological malignancies which led to the approval of axicabtagene ciloleucel (Yescarta, Kite Pharma) and tisagenlecleucel (Kymriah, Novartis) for the treatment of patients with relapsed/refractory large B cell lymphoma and B cell acute lymphoblastic leukemia (Maude et al. 2018; Neelapu et al. 2017). Despite the success of ICB and CAR T cell therapies, there remain significant limitations in therapeutic efficacy and safety for both approaches. Moreover, only a fraction of patients show durable responses and immune-related side effects are frequently observed during treatment (Martins et al. 2019; Brown & Mackall 2019).

Therapeutic cancer vaccines are designed to induce robust anti-tumor responses or to reinforce the immunological memory to specific tumor-antigens. In 2010, the first therapeutic DC-based vaccine, sipuleucel-T (Provenge ®), was approved by the FDA for the treatment of prostate cancer (Kantoff et al. 2010).

The choice of a potent tumor antigen is an important and determining component in vaccine design. Tumor antigens can be classified into two main categories: tumor-associated antigens (TAAs) and tumor-specific antigens or neoantigens. TAAs are self-proteins abnormally expressed by cancer cells such as overexpressed antigens (e.g. MUC-1, survivin), differentiation antigens (e.g. Mart1), and cancer testis antigens (e.g. MAGE-3, NY-ESO-1) (Hollingsworth & Jansen 2019). Although, TAAs can induce T cell responses (Romero et al. 2002; Gnjatic et al. 2006; Hobo et al. 2013), cancer vaccines targeting TAAs face the challenge to overcome tolerance as high-affinity T cells that recognize self-antigens are largely eliminated by central and peripheral tolerance mechanisms. In contrast, neoantigens arise from cancer-specific mutations and are recognized as foreign by the immune system. Neoantigen-directed strategies have shown promising results in several preclinical studies and have been tested in phase I human clinical trials with promising efficacy (Türeci et al. 2016; Ott et al. 2017; Sahin et al. 2017; Yarchoan et al. 2017; Guo et al. 2018).

Numerous efforts have been made to improve vaccination protocols and several vaccine designs, delivery systems and adjuvants are under clinical and preclinical investigation to enhance immunogenicity of cancer vaccines. These include cellular vaccines, virus vector vaccines, and molecular vaccines consisting of either peptides, DNA, or RNA (Hollingsworth & Jansen 2019). Also, diverse adjuvants including toll-like receptor (TLR) agonists such as unmethylated cytosine-phosphate-guanine (CpG) oligonucleotides, polycytidylic acid (Poly I:C), and stimulator of interferon gene (STING) as well as co-stimulatory biomolecules, immunostimulatory complexes (ISCOM), protein-conjugates, liposomes, hydrogels and self-assembling delivery systems e.g. amphiphilic peptides are being explored (He et al. 2018).

Cancer vaccines are generally well tolerated and have limited observed toxicity, however, their clinical efficacy as monotherapy remains modest compared to other immunotherapies, especially in advanced cancer patients (Baxevanis & Perez 2016; Zhao et al. 2019). Growing preclinical evidence suggest that combining cancer vaccines with other treatment modalities such as immune checkpoint inhibitors greatly enhance immunogenicity and efficacy. The combination of a cancer vaccines and checkpoint blockade inhibition is currently investigated in several ongoing phase I/II clinical trials (Mougel et al. 2019).

1.3.2. CD4 T cells in tumor immunity and immunotherapy

CD4 T cells represent a highly versatile and polyfunctional subset of lymphocytes that exhibit a large degree of plasticity by differentiating into various sub lineages in response to environmental stimuli. These sub lineages can exert a broad range of effector functions during the initiation, expansion and memory phase of immune responses **(Figure 3)** (Saravia et al. 2019). There is growing evidence that certain CD4 T cell subsets essentially participate in anti-tumor responses, are required for protective anti-tumor immunity and can be effectively used

for immunotherapeutic interventions (Kennedy & Celis 2008; Melssen & Slingluff 2017). CD4 T cells can, under certain conditions, directly eliminate tumor cells by cytolytic mechanisms, mediate anti-tumor immunity by the production of effector cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and IL-2, or direct anti-tumor responses by shaping the TME and by providing help to other immune cells (Kennedy & Celis 2008; Borst et al. 2018; Tay et al. 2020).

It is well established that the activation, proliferation and differentiation of B cells in response to many antigens requires the help of CD4 T cells (Crotty 2015). Internalized antigens that have been recognized by B cell receptors (BCR) are presented on MHC class II molecules to antigen specific CD4 helper T cells. Through CD40-CD40L interactions, other co-stimulators and directed release of cytokines, CD4 T cells consequently stimulate B cell proliferation and the differentiation of naïve B cells to antibody secreting plasma cells and memory cells. The accompanying processes of antibody isotype switching, somatic hypermutation and selection of high-affinity receptors are also guided by the help of CD4 T cells (Crotty 2015). Tumor-specific CD4 T cell responses have been correlated with the presence of serum antibodies against tumor antigens in cancer patients (Gnjatic et al. 2003; Reed et al. 2015) and are likely involved in initiating local humoral responses in tertiary lymphoid structures adjacent to solid tumors (Dieu-Nosjean et al. 2016; Sharonov et al. 2020).

Besides providing help to B cells, CD4 helper T cells also actively promote CD8 T cell priming, support both effector-and memory functions of CTLs and help CTLs to overcome negative regulations (Borst et al. 2018). Help for CTL responses is particularly delivered during a secondary T cell priming step, in which both CD4- and CD8 T cells recognize their respective antigens on the same DC. During this interaction, CD4 T cells enable DCs to optimize antigen presentation and specific cytokine and co-stimulatory signals for the priming, clonal expansion and differentiation into effector and memory CTLs (Bevan 2004; Bennett et al. 1997; Castellino & Germain 2006; Bedoui et al. 2016; Hor et al. 2015; Ridge et al. 1998; Joffre et al. 2012). CD4 T cell help has been shown to initiate a gene expression program in CD8 T cells that improves the magnitude, longevity and cytotoxic functions of primary and memory CTL responses (Ahrends et al. 2017; Ahrends et al. 2016; Provine et al. 2016; Janssen et al. 2005) This enables CTLs to overcome many recognized obstacles of anti-tumor responses and can lead to protective anti-tumor immunity (Borst et al. 2018; Kennedy & Celis 2008).

In contrast to the essential participation of CD4 T helper cells in anti-tumor responses, the immunosuppressive subset of T_{reg} cells can suppress cancer immunity (Togashi et al. 2019). T_{reg} cells are characterized by the expression of the transcription factor forkhead box protein P3 (FOXP3) and serve to maintain immune homeostasis and self-tolerance and to prevent autoimmunity (Hori et al. 2017). Depending on the side at which T_{reg} cells develop, they are classified into two subtypes. Thymus-derived (tT_{regs}) originate from self-reactive thymocytes

with high-affinity binding to self-antigens and develop to a functionally mature T cell subpopulation with strong immunosuppressive activity. Under certain conditions, conventional T cells (T_{conv}) in the periphery give rise to immunosuppressive FOXP3 expressing T_{reg} cells (induced-T_{regs} or iT_{regs}) that are involved in peripheral tolerance (Sakaguchi et al. 2010; Togashi & Nishikawa 2017). The immunosuppressive activity of T_{reg} cells is mediated by several mechanisms including consumption of IL-2, CTLA-4 mediated suppression of APCs, production of immunosuppressive cytokines such as IL-10, TGF-β and IL-35, conversion of ATP to adenosine, and secretion of perforin and granzyme B (Von Boehmer 2005).

It is becoming increasingly recognized that CD4 T cells are essential for developing and sustaining effective anti-tumor immunity even in cancer immunotherapies specifically designed to elicit CTL responses (Kreiter et al. 2015). Thus, efforts are made to specifically target CD4 T cells to generate potent and durable anti-tumor responses. In vaccine design, the inclusion of promiscuous MHC class II epitopes led to improved clinical outcomes in several studies (Melssen & Slingluff 2017). Also, long peptide vaccines represent a superior strategy to induce broad integrated anti-tumor immune responses due to the inclusion of both, CD8 and CD4 T cell epitopes (Slingluff 2011; Kenter et al. 2009; Tsuji et al. 2013). Preliminary observations in CAR T cell therapy suggest that a higher CD4:CD8 T cell ratio in leukapheresis products correlate with an improved clinical response and that CD4 CAR T cells exhibit polyfunctional anti-tumor properties with similar *in vivo* and *in vitro* efficacy as CD8 CAR T cells (Garfall et al. 2019; Xhangolli et al. 2019; Yang et al. 2017).
The presence of immunosuppressive T_{regs} in the TME is associated with poorer prognosis among several cancers (Sayour et al. 2015; Saito et al. 2016; Zhou et al. 2017). Therefore, several different therapeutic strategies have emerged to specifically target T_{regs}. These include T_{reg} depletion by chemotherapeutic agents (e.g. cyclophosphamide) or antibody-dependent targeting of highly expressed surface markers (e.g. CD25, CTLA-4), inhibition of T_{reg} trafficking to the tumor site (e.g. CCR4), or selectively targeting T_{reg} essential signalling molecules (e.g. PI3K, LCK) (Tanaka & Sakaguchi 2019). Since T_{regs} constitutively express high levels of surface receptors including CTLA-4 and PD-1, ICB therapy potentially affects T_{reg} mediated suppression of immune responses. In line, the therapeutic efficacy of the CTLA-4 inhibitor ipilimumab has been associated with depletion of T_{regs} in the TME (Vargas et al. 2018; Bulliard et al. 2013; Selby et al. 2013; Simpson et al. 2013). In contrary, there are indications that PD-1 blockage not only reactivates tumor-specific effector T cells but also potentiates the immunosuppressive function of T_{reg} cells (Togashi et al. 2018). To this end, the potential conversion of intratumoral T_{regs} into anti-tumor effector T cells is being investigated (Overacre-Delgoffe et al. 2017; Wang et al. 2018; Liu et al. 2013).

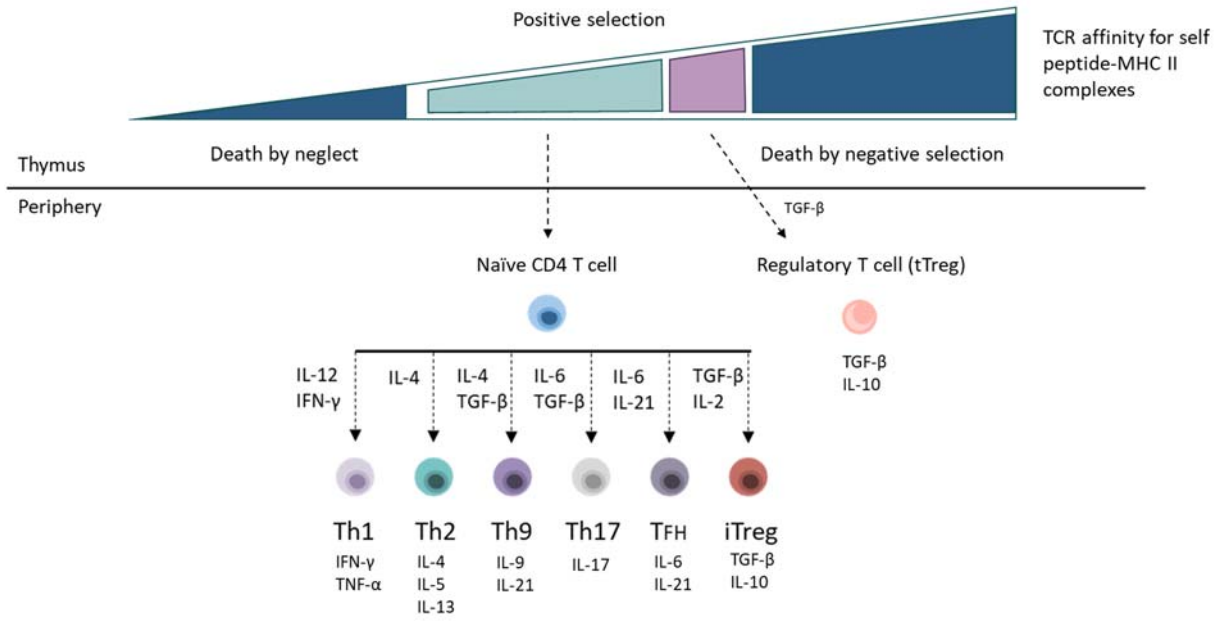


Figure 3: Thymic development of CD4 T cells and diversity of CD4 T cell subsets.
(adapted from (Tay et al. 2020))**.** During thymic development, the T cell fate is determined by the affinity of TCRs for self-peptide-MHC complexes. Thymocytes with no or low affinity to self-peptides die by neglect due to failure of initiated TCR signaling. Thymocytes with high self-reactivity are negatively selected and undergo apoptosis. Only thymocytes with intermediate TCR affinities receive activating TCR signaling and undergo positive selection and thymic maturation to naïve T cells. Some thymocytes harboring TCRs with moderately high affinities to self-proteins develop into regulatory T cells (T_{regs}). In the periphery, naïve CD4 T cells upregulate transcription factors involved in specific subset differentiation and the expression of effector cytokines in response to environmental cytokine milieu and TCR activation.

1.4. ALK as target for cancer immunotherapy

The ALK protein has many properties as a potent target for cancer immunotherapy. First, ALCL cells are completely dependent on ALK-signaling for proliferation and survival, thus, immune escape of ALK negative clones is unlikely. Second, the specific expression of ALK in ALCL cells with only limited expression in other tissues (despite some immune privileged sites) greatly minimizes the risk of autoimmune reactions, making it safe for application also in pediatric patients. And third, ALK$^+$ALCL patients have a natural occurring humoral and cellular response to ALK which could be promoted by immunotherapy to mediate long-term protection.

1.4.1. Immune response to ALK in ALK$^+$ALCL patients

There are several clinical and histopathological indications that ALK$^+$ALCL provokes a spontaneous immune response (reviewed in (Stadler et al. 2018). In some patients a “wax and wane” course of the disease has been observed. In these cases, the tumor spontaneously

disappears without therapeutic intervention but grows again within weeks or month to be finally diagnosed as ALK⁺ALCL. This observation suggests an initial control of tumor cells by the immune system, that, after a time of equilibrium finally fails and the tumor escapes. A fact that might also explain some unusual late relapses between 10 and even more than 20 years after diagnosis, that occurred within states of immunosuppression (Woessmann 2018).

The varying amount of detectable tumor cells and the presence of abundant reactive bystander cells in the different ALCL subtypes is an additional indicator for an ongoing immune reaction (Lamant et al. 2011; Brugières et al. 1998). Another supporting clinical factor is the high incidence of B symptoms in ALK⁺ALCL patients that suggests, at least, an unspecific stimulation of the immune system by ALCL (Seidemann et al. 2001; Brugières, Le Deley, et al. 2009). In line, pro-inflammatory cytokines such IL-17a, IL-9, sIL-2R, HGF and sCD30 have been detected in the serum of ALCL patients at diagnosis and serum concentrations of IFN-γ, IL-6, sIL-2R and IP-10 have been shown to correlate with clinical and biological characteristics as well as relapse risk among ALK⁺ALCL patients (Savan et al. 2011; Mellgren et al. 2012; Knörr et al. 2018).

Together, these observations indicate that the patient's immune system recognizes ALK⁺ALCL cells, influences disease progression, and might contributes to tumor control. A specific immune response to ALK in ALK⁺ALCL patients is reflected by the presence of humoral and T cell mediated immune reactions (outlined in **Figure 4** and reviewed in Stadler et al. 2018).

1.3.1.1. Humoral immune response to ALK

In 2000, Pulford et al. first described the presence of autologous anti-ALK antibodies in serum of ALK⁺ALCL patients (Pulford et al. 2000) which was confirmed by subsequent studies (Ait-Tahar et al. 2006; Mussolin et al. 2009). An extended analysis of antibodies to ALK in pediatric ALK⁺ALCL patients prior to treatment demonstrated that ALK antibody titers inversely correlated with the risk of relapse (Ait-Tahar et al. 2010). A correlation of persisting anti-ALK antibody titers during chemotherapy treatment and protection from relapse was further demonstrated in pediatric ALK⁺ALCL patients (Mussolin et al. 2017). Persisting antibody titers after the completion of therapy as well as a mild reduction compared to pretreatment titers predicted a protection against relapse.

Antibodies against ALK were also detected in patients with variant ALK fusion proteins and other ALK-expressing tumors such as NSCLC (Damm-Welk et al. 2016; Awad et al. 2017). Epitope mapping of ALK antibody binding sites in ALCL and NSCLC suggest a similar recognition pattern (Knörr et al. 2018; Awad et al. 2017). However, a possible association between epitope recognition and intensity of the patient's humoral response has not been studied so far. Also, the involvement of CD4 T cells in the humoral anti-ALK response remains, up to date, uncharacterized.

Although the humoral immune response to ALK has been proved to be a prognostic parameter (Ait-Tahar et al. 2010; Mussolin et al. 2017; Mussolin et al. 2013), it is unlikely that anti-ALK antibodies have a direct anti-tumor activity as ALK fusion proteins are exclusively expressed intracellularly. Therefore, the presence of antibodies against ALK may rather represent a surrogate marker for an ongoing cellular immune response in patients than contributing to direct tumor control.

1.3.1.2. CD8 T cell response to ALK

Passoni et al. first demonstrated the immunogenicity of ALK in initiating CD8 T cell responses by using a reverse immunological approach (Passoni et al. 2002). Two predicted synthetic ALK peptides were tested for their capacity to elicit ALK-specific CTL responses. The analysis led to the detection of functional anti-ALK CD8 T cells within the peripheral blood of healthy donors (Passoni et al. 2002). In a subsequent study, CD8 T cell responses to these ALK peptides were also detected in ALK$^+$ALCL patients in clinical remission. The study provided first indications for the presence of memory CD8 T cells in patients (Ait-Tahar et al. 2006). This observation was confirmed by flow cytometric analysis. A high frequency of circulating ALK-specific CD8 T cells was detected in peripheral blood of ALK$^+$ALCL patients and healthy donors (Passoni et al. 2006). However, in patients, effector and memory T cells were found whereas in healthy donors ALK-specific CD8 T cells showed a predominately naïve phenotype (Passoni et al. 2006).

The detection of ALK-specific CD8 T cells in ALK$^+$ALCL patients mainly relied on HLA matched *in silico* selected ALK peptides with no guarantee for physiological presentation on APCs and tumor cells. To ensure endogenous NPM-ALK peptide processing, Singh et al. investigated ALK-specific CD8 T cells responses in ALK$^+$ALCL patients by using autologous DCs transfected with *NPM-ALK* mRNA as APCs for T cell stimulation (Singh et al. 2016). This study revealed persisting CD8 T cell responses against ALK up to nine years after diagnosis. In a subsequent analysis, ALK-specific T cell responses were studied in a large cohort of uniformly treated ALK$^+$ALCL patients in clinical remission (Singh et al. 2019). Using two approaches, in which T cells were either stimulated with autologous DCs transfected with *NPM-ALK* mRNA or with DCs pulsed with ALK synthetic long peptides (SLPs), ALK-reactive T cells were detected in a large proportion of analyzed patients. The T cell responses were mainly directed against regions within the ALK tyrosine kinase domain, suggesting a prevalence of immunogenic epitopes in this area (Singh et al. 2019).

The natural occurring anti-ALK immune response that leads to the observed maintenance of memory CD8 T cells in patients provides a potential basis for immunotherapy.

Chiarle et al. demonstrated the protective and therapeutic potential of an ALK DNA vaccine in a ALK$^+$ALCL mouse model (Chiarle et al. 2008). Immunization with plasmids encoding for

cytoplasmic portions of ALK induced durable local and systemic lymphoma protection in vaccinated mice which were characterized by ALK-specific IFN-γ responses and CTL mediated cytotoxicity. In combination with chemotherapy, the ALK vaccine significantly enhanced the survival of mice (Chiarle et al. 2008). In addition, a DNA-based ALK vaccine also showed efficacy in mouse models of ALK-positive lung cancer (Voena et al. 2015). These studies provided the first evidence for the therapeutic potential of ALK-directed vaccines in preventing the growth of ALK-positive cancers *in vivo.*

1.2.1.3. CD4 T cell response to ALK

The presence of ALK-specific humoral and CTL cell responses in ALK$^+$ALCL patients suggests an involvement of CD4 T cells in ALK immunity. However, these responses remain poorly characterized. Only one study reported ALK-specific CD4 T cell responses against two *in silico* selected synthetic ALK peptides in a small cohort of ALK$^+$ALCL patients (Ait-tahar et al. 2007). The role and participation of ALK-directed CD4 T cells in ALK+ALCL has, however, not been investigated any further.

1.4.2. Immunotherapy in ALK$^+$ALCL

Up to date, there are only limited immunotherapeutic treatment options for patients with ALK$^+$ALCL.

Allogeneic SCT is the most common immunotherapy deployed in leukemia and lymphoma patients and its efficacy in the treatment of children with relapsed ALK$^+$ALCL is well established (Woessmann et al. 2006; Gross et al. 2010; Strullu et al. 2015; Fukano et al. 2015).

ICB therapy provides a potential therapeutic option for ALCL treatment. The PD-1 inhibitor nivolumab showed efficacy in two ALK$^+$ALCL patients with refractory disease (Hebart et al. 2016; Rigaud et al. 2018). In a phase I-II clinical trial in children and young adults with lymphoma and non-CNS solid tumours, nivolumab was generally well tolerated and showed clinical activity in lymphoma patients (Davis et al. 2020). CD30-targeting CAR T cells have been investigated in a preclinical- and a phase I dose escalation study in which one adult patient with relapsed ALK$^+$ALCL reached remission after therapy (Hombach et al. 2016; Ramos et al. 2017). Also, ALK-specific CAR T cells are under experimental investigation for possible therapeutic application in ALK-positive neuroblastoma (Walker et al. 2017). However, since ALK fusion proteins are exclusively expressed intracellularly, ALK-directed CAR T cell therapy is no suitable therapeutic option in ALCL.

Given the natural occurring spontaneous immune response to ALK in patients, the development of a therapeutic ALK vaccine that promotes the endogenous anti-ALK immune response provides a feasible strategy for ALK$^+$ALCL treatment.

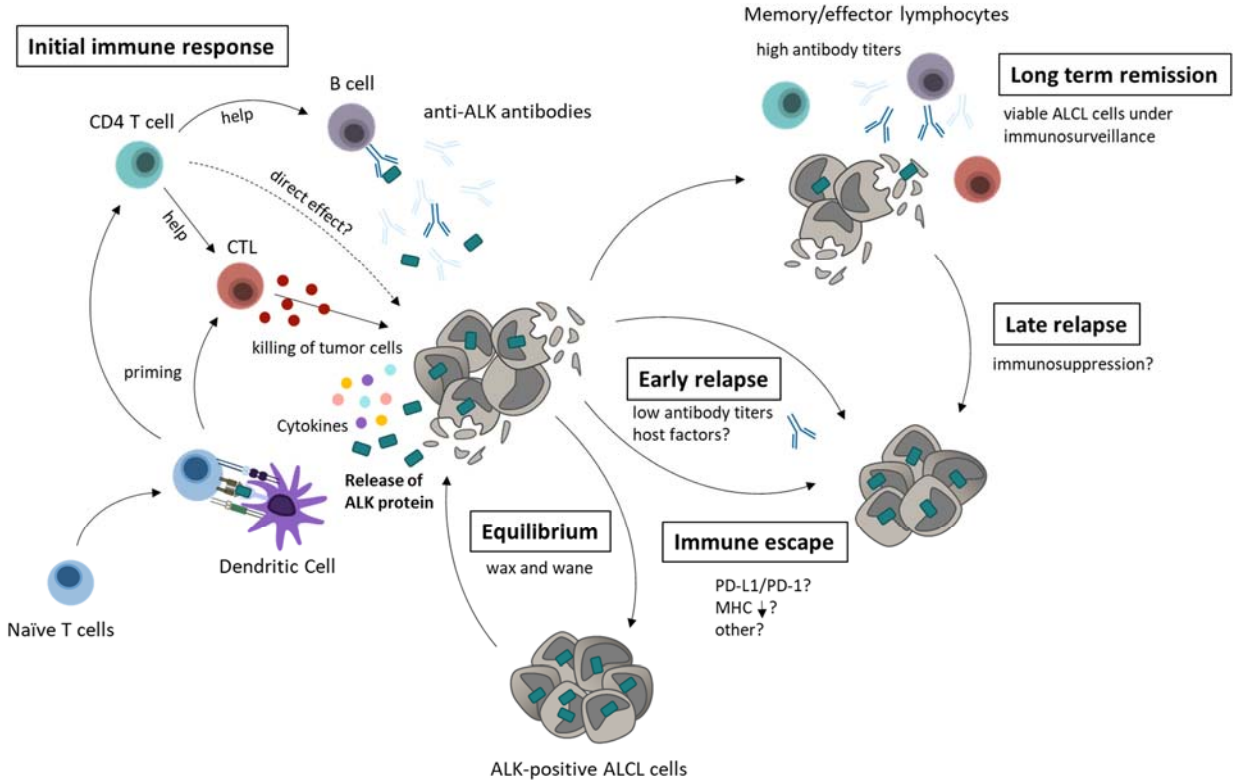


Figure 4: Current understanding of ALK-specific immune responses in patients with ALK$^+$ALCL. Adapted from (Stadler et al. 2018). The initial spontaneous anti-ALCL response is shown on the left. ALK protein (turquoise squares), released from dying cancer cells is captured and processed by dendritic cells and subsequently presented to cells of the adaptive immune system which leads to the priming and activation of ALK-specific T-and B cells. Cytokines (small colored circles) and other factors shape the type of the immune response. Long-term remission is characterized by the presence of an immunologic memory (shown on the upper right). Factors that could lead to immune escape and early relapses are indicated in the lower right.

2. Aim

Current treatment for children and adolescents with ALK$^+$ALCL is based on chemotherapeutic protocols which uniformly lead to an event-free survival of 70%. Therapy intensification did not reduce the relapse rate but increased toxicity. Therefore, new treatment strategies are needed. The aberrant ALK expression in ALK$^+$ALCL induces spontaneous humoral and cellular immune responses which can be potentially promoted by immunotherapy to mediate tumor control and long-term protection. The induction of protective anti-tumor immunity requires the participation of CD4 T cells which guide immune responses and provide essential help to CD8 T cells and B cells.
Whereas humoral and CD8 T cell responses against ALK have been extensively examined both experimentally and clinically, the involvement and characteristics of ALK-specific CD4 T cells has not been investigated so far.

This study aimed to detect and characterize ALK-specific CD4 T cell responses in children and adolescents with ALK$^+$ALCL, in healthy individuals, and in immunization studies in mice, to provide new information on ALK immunity and to set the basis for the development of a therapeutic ALK vaccine (outlined in **Figure 5**).

The main objectives were:

- The detection of ALK-reactive CD4 T cells in ALK$^+$ALCL patients in clinical remission and healthy donors by combining an HLA-independent peptide-based immunological approach and IFN-γ ELISPOT.

- The identification of MHC class II restricted immunogenic CD4 T cell peptides that can be potentially included in the design of an ALK-directed cancer vaccine by dissecting the ALK-specific CD4 T cell response in individual patients.

- The validation of findings in immunization studies in mice by investigating ALK peptide vaccine induced CD4 T cell responses and by evaluating the therapeutic efficacy of a selected ALK peptide vaccine.

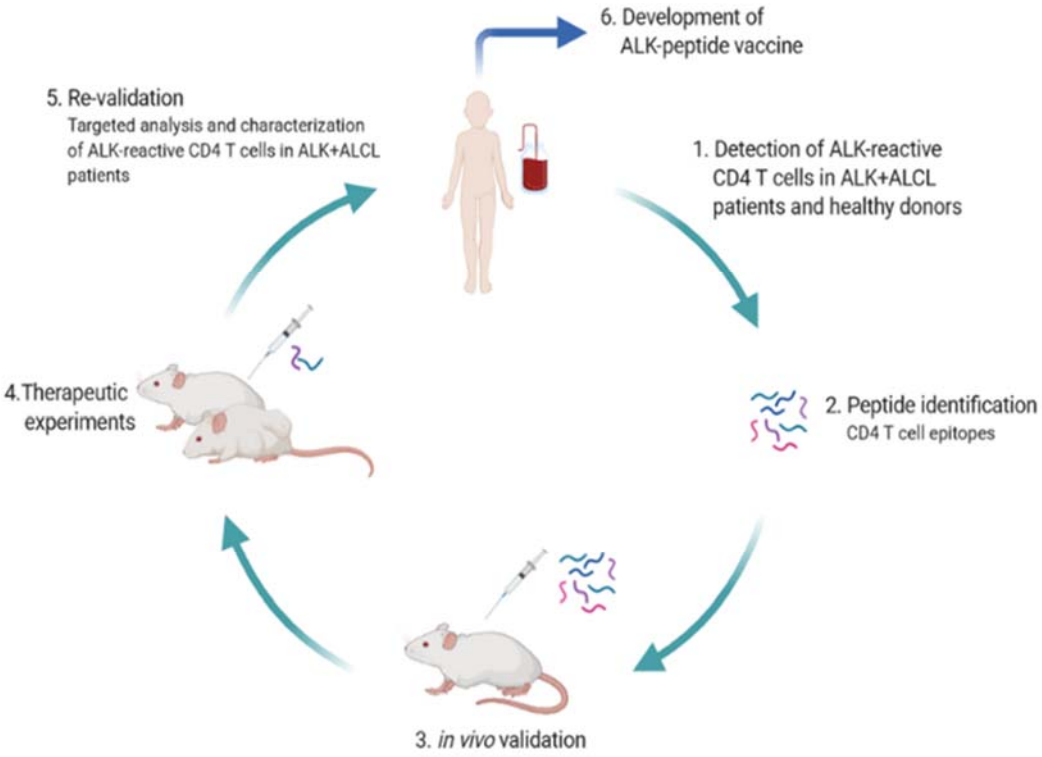


Figure 5: Outline of the main objectives and further steps of the study.
(created with Biorender.com)

3. Material and Methods

3.1. Material

Table 2: List of Material and Resources

Product	Company	Cat. Number
Media and supplements		
X-Vivo 15	Biozym	881026
AIM-V	Thermo Fisher Scientific	12055-083
CTS™ OpTimizer	Thermo Fisher Scientific	A10485-01
DMEM/F12	Thermo Fisher Scientific	11330057
IMDM	Thermo Fisher Scientific	12440053
RPMI 1640	Thermo Fisher Scientific	21870076
DMEM	Thermo Fisher Scientific	21969035
DPBS	Thermo Fisher Scientific	14190169
CTL-Test medium	Cellular Technology Ltd.	CTLT-005
CTL-Wash supplement (10x)	Cellular Technology Ltd.	CTLW-010
Ultrapure Water	Fisher Scientific	11538646
Fetal Bovine Serum (FBS)	Fisher Scientific	10-437-028
Human Serum (HS)	Pan Biotech	P40-2701
AB Human Serum (HS)	Merck	H5667
Penicillin/Streptomycin (PS)	Corning	30-002-CI
Gentamycin	Fisher Scientific	11500506
Geneticin (G-418)	Thermo Fisher Scientific	10131-035
HEPES	Fisher Scientific	11560496
Corning L-glutamine solution	Fisher Scientific	MT25005CI
Red Blood Cell Lysing Buffer	Merck	R7757
UltraPure™ 0.5M EDTA, pH 8.0	Thermo Fisher Scientific	15575020
Dimethyl sulfoxide (DMSO)	Merck	D2650
2-mercaptoethanol	Thermo Fisher Scientific	21985-023
Non-essential amino acid solution	Thermo Fisher Scientific	11140068
Human Transferrin	Lonza	CC-4205
Human insulin solution	Merck	I9278
Lymphoprep™	Stemcell	7861
Ficoll Paque Plus	Merck	GE17-1440-02

Trypan Blue, 0.4%	Thermo Fisher Scientific	15250061
Paraformaldehyde (PFA) 4 %	Morphisto	11762.00100
Cytokines and inflammatory mediators		
Interleukin-2 (IL-2)	PeproTech	200-02
Interleukin-4 (IL-4)	Miltenyi Biotec	130-093-922
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)	PeproTech	300-03
Interleukin-6 (IL-6)	Miltenyi Biotec	130-093-932
Interleukin-1β (IL-1β)	Miltenyi Biotec	130-093-898
Tumor necrosis factor α (TNF-α)	PeproTech	300-01A
Prostaglandin E2 (PGE_2)	PeproTech	3632464
Cyclosporin A	Merck	30024
Phytohemagglutinin-L solution (PHA)	Thermo Fisher Scientific	00-4977-03
eBioscience cell stimulation cocktail	Thermo Fisher Scientific	00-4970-03
Magnetic activated cell sorting		
OctoMACS	Miltenyi Biotec	130-042-108
MS Columns	Miltenyi Biotec	130-042-201
Pre-Separation Filters	Miltenyi Biotec	130-095-823
CD14 MicroBeads, human	Miltenyi Biotec	130-118-906
CD4 MicroBeads, human	Miltenyi Biotec	130-045-101
EasySep™ Mouse CD8a Positive Selection Kit II	Stemcell	18953
EasyStep™ Magnet	Stemcell	18000
ELISPOT		
Multiscreen ELISPOT Plates	Merck	MAIPS4510
Anti-human IFN-γ mAB 1-D1K	Mabtech	3420-3-1000
Anti-human IFN-γ mAB 7-B6-1 biotin	Mabtech	3420-6-1000
Streptavidin HRP	Mabtech	3310-9
TMB substrate	Mabtech	3651-10
Mouse IFN-γ ELISpotPLUS kit (HRP)	Mabtech	3321-4HPT
ALK-antibody titers		
pcDNA NPM-ALK expression vector	Kindly provided by Dr. Stephen Morris (St. Jude's Children Research Hospital, Memphis, TN, USA)	
Fugene® 6 Transfection Reagent	Promega	E2691

Invitrogen pcDNA™ 3.1 vector	Thermo Fisher Scientific	V79020
Dako HRP-conjugated anti-human IgG	CiteAB	P0214
SIGMAFAST™ 3,3′-Diaminobenzidine tablets	Merck	D4293
Antibodies		
Human		
DC and B cell phenotype		
HLA.DR.PerCP	BD Bioscience	347402
CD197.FITC	BD Bioscience	561271
CD40.APC	BD Bioscience	555591
BD Simultest™ CD3 FITC/CD19 PE	BD Bioscience	342404
CD80.FITC	BD Bioscience	557226
CD83.APC	BD Bioscience	551073
CD86.PE	BD Bioscience	557344
FcR Blocking Reagent, human	Miltenyi Biotec	130-059-901
Mouse		
Intracellular cytokine staining		
FcR Blocking Reagent, mouse	Miltenyi Biotec	130-092-575
APC Rat Anti-Mouse IFN-γ	BD Bioscience	554413
CD4.PE	Miltenyi Biotec	130-121-131
CD8.FITC	Miltenyi Biotec	130-118-329
Dextramer staining		
ALK-MHC Dextramer	Immudex	Costumer-designed product www.immudex.com
CD8.FITC		130-118-329
Zombie Aqua™	BioLegend	423101
Peptides		
PepTivator® CMV pp65	Miltenyi Biotec	130-093-435
CEFT MHC-II Pool	JPT	PM-CEFT-MHC-II
Synthetic ALK peptides	JPT or GenScript	custom synthesized
Commercial assays		
Fixation and Permeabilization Solution Kit with GolgiPlug™	BD Bioscience	555028

Cell lines		
COS-1	DSMZ (Braunschweig, Germany)	
NIH-3T3 hCD40L	Kindly provided by Dr. Gordon Freeman (Dana-Farber Cancer Institute, Boston, MA, USA)	
R37-ALK$^+$ transgenic mouse cell line	Chiarle Laboratory Turin, Italy	(Chiarle et al. 2003)
Mouse strains		
Balb/c	Charles River Laboratory (USA)	
C57BL/6	Charles River Laboratory (USA)	
Other products		
Cord Blood Collection Bag	Macopharma	MSC1200PU
c-di-GMP VacciGrade™	Invivogen	vac-nacdg
Hardware		
BD FACSVerse	BD Bioscience	bdbiosciences.com
BD FACSCelesta	BD Bioscience	bdbiosciences.com
MACS Quant Analyzer 10	Miltenyi Biotec	miltenyibiotec.com
Immunospot CTL Analyzer (5.1.36)	C.T.L.	immunospot.eu
AID ELISPOT Reader	AID	Elispot.com
Bio-Sys Bioreader® 4000	BIO-SYS GmbH	biosys.de
Software		
GraphPad Prism 6	GraphPad	http://www.graphpad.com
FlowJo software	FlowJo	http://www.flowjo.com
Flowlogic software	Miltenyi Biotec	https://www.miltenyibiotec.com
CTL Immunospot 5.1.36	C.T.L.	immunospot.eu
AID ELISPOT software version 7.0 (16577)	AID	Elispot.com
FACSDiva 8.0.1.1	BD Bioscience	bdbiosciences.com
BD FACSuite software	BD Bioscience	bdbiosciences.com

3.2. Methods

All *in vitro* experiments were conducted under sterile conditions. Working steps were performed in Laminar Flow Hoods and cells were incubated at 37°C in a humidified incubator with 5% CO_2. Commercial assays were performed according to manufacturer's protocols unless otherwise specified. Reconstitution of peptides and cytokines and preparation of other substances was performed under recommended guidelines.

3.2.1 Detection of ALK-reactive CD4 T cells and identification of immunogenic peptides in ALK$^+$ALCL patients and healthy donors

3.2.1.1. Overview of the workflow

To detect ALK-reactive CD4 T cells in peripheral blood of ALK$^+$ALCL patients and healthy donors, an autologous and peptide-based immunological approach which allows an HLA-independent analysis and additionally ensures endogenous processing of synthetic ALK peptides was used **(Figure 6)**. Peripheral blood mononuclear cells (PBMCs) from patients or healthy donors were isolated by gradient density centrifugation and subsequently sorted by a magnetic cell separation system using anti-CD14 magnetic microbeads. From the CD14-positive cell fraction, fast dendritic cells (fastDCs) were generated. CD14-negative cells were further purified to CD4 T cells. In a separate step, CD14$^-$/CD4$^-$ PBMCs were co-cultivated with NIH-3T3 hCD40L feeder cells to activate and expand autologous B cells. For the stimulation of CD4 T cells, synthetic long overlapping (NPM)-ALK peptides were synthesized, which were combined to ALK peptide pools. Pulsing of DCs with ALK peptide pools ensured the uptake, processing and presentation of the peptides by MHC class II molecules. Peptide loaded DCs were used to stimulate the autologous CD4 T cells once a week over three weeks to prime and enrich for possible peptide-reactive cells. ALK recognition was then tested by IFN-γ ELISPOT. To identify single immunogenic peptides within the ALK-peptide pools, patient´s ALK-reactive CD4 T cells were once re-stimulated with peptide pulsed autologous B cells and then further tested in an IFN-γ ELISPOT assay for single peptide recognition.

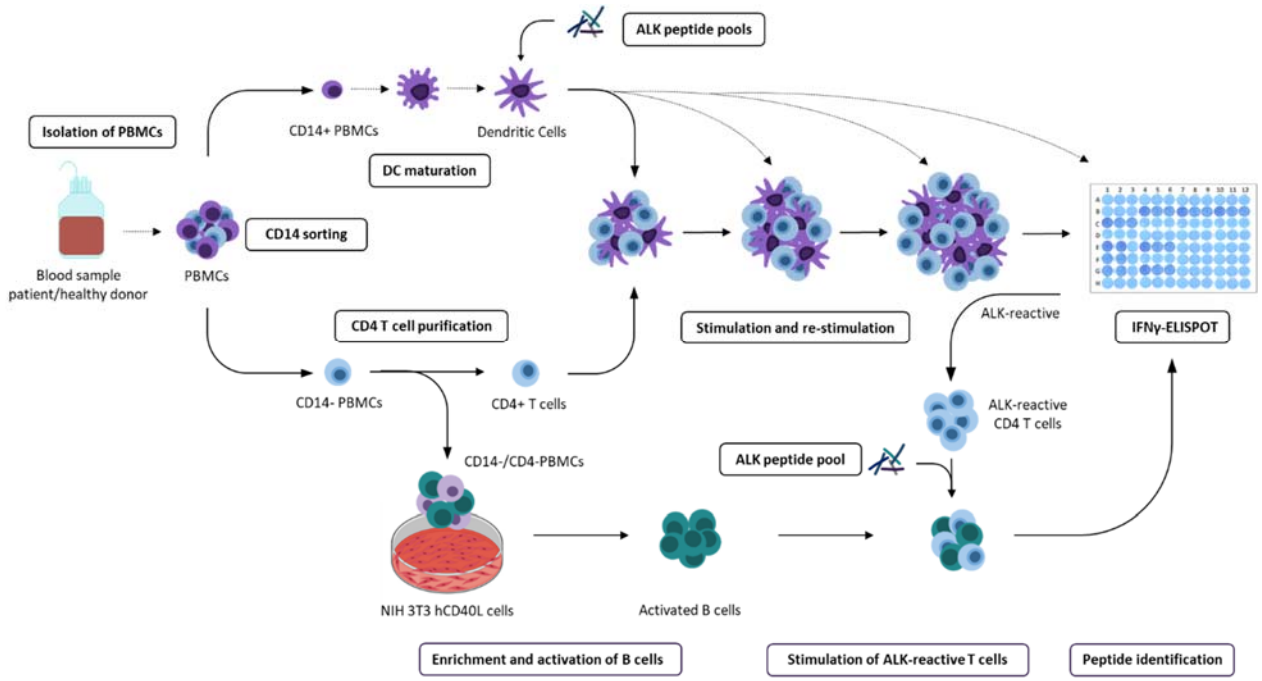


Figure 6: Overview of the workflow for the detection of ALK-reactive CD4 T cells and identification of single immunogenic peptides.

3.2.1.2. ALK$^+$ALCL patients and healthy donors

The study was approved by the ethical committee of the Medical Faculty of the Justus-Liebig-University, Giessen, Germany (193/11) and the University Hospital Hamburg-Eppendorf (UKE), Hamburg, Germany (PV7249).

Nine ALK$^+$ALCL patients harbouring NPM-ALK or variant ALK translocations **(Table 3)** and six healthy donors were analyzed for the presence of ALK-reactive CD4 T cell responses. ALK$^+$ALCL patients were included in the Non-Hodgkin Lymphoma Berlin-Frankfurt-Münster 95 (NHL-BFM 95) or ALCL 99 studies or the NHL-BFM Registry 2012. They received therapy with comparable BFM-type front-line protocols. One patient was analysed at time of diagnosis before start of the treatment and after therapy. All other patients were in clinical remission at least one year after standard front-line chemotherapy treatment and without relapse at time of analysis. All patients were above 13 years of age, had no current infection or immunosuppression and no medical condition prohibiting blood drawing. For all patients, a written informed consent for the study was obtained after the patients had been informed about the study orally and in writing by a physician of the NHL-BFM study center. The patients had been first informed about the current translational study by their treating physicians. In case of interest, they were contacted by a physician of the NHL-BFM study center for patient information and consent. After written informed consent, 100 ml peripheral blood was collected at a regular follow-up appointment at their local hospitals by an experienced physician of the study center, into citrate phosphate dextrose (CPD)-containing bags using a modified umbilical

cord blood collection system. The blood samples were immediately pseudonymized by the blood-drawing physician before reaching the research laboratory.

Anonymized leucocyte fractions (buffy coats) from cytomegalovirus (CMV)-seropositive healthy donors were included in this study as controls and were kindly provided by the Institute of Transfusion Medicine and Clinical Immunology of the University Hospital Giessen and Marburg (UKGM) and the Institute of Transfusion Medicine and Diagnostic Center of the University Hospital Hamburg-Eppendorf (UKE). The HLA alleles of patients and healthy donors were identified by high-resolution (4-digit) HLA sequencing.

Table 3: Characteristics of analyzed ALK$^+$ALCL patients

Patient	Gender	Age at analysis (years)	ALK-fusion	ALK Ab titer (Diagnosis)	ALK Ab titer (Analysis)	Time (years) from diagnosis to analysis	HLA Haplotype class I	HLA Haplotype class II
1	male	17.4	NPM-ALK	1:6750	1:6750	1.8	A 02:01, 11:01 B 35:57, 40:01 C 03:04, 04:01	DRB1 01:01, 07:01P DQB1 02:02, 05:01
2	male	16.2	NPM-ALK	1:20250	1:250	3.2	A 03:02, 24:02 B 07:02, 14:02 C 07:02, 08:02	DRB1 07:01P, 14:54 DQB1 03:03, 05:03
3	male	16.4	TPM3-ALK	1:250	negative	7.8	A 03:01, 03:01 B 07:02, 35:01 C 04:01, 07:02	DRB1 11:04, 15:01 DQB1 03:01, 06:02
4	male	13.5 15.6	NPM-ALK	1:6750	1:6750 negative	0 2.1	A 26:01, 26:01 B 13:02, 58:01 C 03:02, 06:02	DRB1 13:01, 13:02 DQB1 06:03, 06:09
5	female	17.11	NPM-ALK	1:6750	negative	2.8	A 02:01, 31:01 B 07:02, 40:01 C 03:04, 07:02	DRB1 15:01 DQB1 06:02
6	male	19.9	X-ALK	1:6750	1:750	2.1	A 01:01, 03:01 B 08:01, 15:03 C 02:10, 07:01	DRB1 13:01, 07:01P DQB1 02:01, 02:02
7	female	21.5	X-ALK	1:6750	1:750	3.11	A 03:01 B 07:02 C 07:02	DRB1 04:07, 13:01 DQB1 03:01, 06:03
8	male	16.1	NPM-ALK	1:6750	n/a	12.3	A 01:01, 03:01 B 08:01, 40:01 C 03:04, 07:01	DRB1 03:01, 13:02 DQB1 02:01, 06:04
9	male	18.7	NPM-ALK	1:6750	negative	3.5	n/a	n/a

X-ALK = variant ALK fusion partner: ALK Ab titer = ALK-antibody titer; n/a = not available

3.2.1.3. ALK-antibody titers

Anti-ALK antibody titers were determined in patient´s sera by an immunocytochemical approach, described in (Pulford et al. 2000). Briefly, COS-1 cells were transiently transfected with *pcDNA3-NPM-ALK* plasmids using Fugene6 transfection reagent. 72 h after transfection, cells were harvested and cytocentrifuge preparations were made. The cytocentrifuge preparations were incubated with patient´s plasma/sera in dilutions between 1:100 and 1:60750 for 30 minutes, washed with PBS, and then incubated with horseradish-peroxidase (HRP)–conjugated rabbit anti-human immunoglobulin G (IgG). ALK-antibody titers were determined by indirect immunoperoxidase staining and considered positive until the dilution of detectable staining.

3.2.1.4. Synthetic long-overlapping ALK peptides

41 long overlapping (NPM)-ALK peptides were synthesized and selectively combined to ALK peptide pools consisting of five to six single peptides. The ALK peptide pools were used to pulse autologous DCs or B cells for the stimulation of donor derived CD4 T cells. Based on previous studies and identified ALK-antibody binding sites in ALK$^+$ALCL patients, the selected peptides encompassed the fusion region of NPM-ALK, the N-terminal cytoplasmic region of ALK (Pool NT), the whole tyrosine kinase domain (Pools TK-A-D) with a juxta-kinase region (Pool JK-D) and the C-terminal end (Pool CT) of the ALK protein **(Figure 7)** (Singh et al. 2019; Singh et al. 2016; Knörr, Weber, et al. 2018). The peptides were 30 amino acids (aa) in length and had a sequence overlap of 16 aa to enable efficient processing and presentation and to include most of the possible CD4 T cell epitopes. Due to a possible patent filing, the exact sequences of the ALK peptides are not published in this thesis. The peptides were custom synthesized by JPT or GenScript, had a purity above 90% and a confirmed composition analyzed by mass spectrometry. Each lyophilized peptide was dissolved in dimethyl sulfoxide (DMSO) or ultrapure water according to the manufacture's recommendation to a final stock concentration of 5 mM. Peptide pools were prepared in a concentration of 500 µM, aliquoted to avoid multiple freezing thawing cycles, and stored at – 80°C until further use.
As experimental positive controls, two commercially available peptide pools were used to stimulate and detect virus specific CD4 T cells. The PepTivator® CMV pp65 pool consists of 15-mer peptides with an 11 aa overlap that cover the complete sequence of the pp65 protein of human CMV. The CEFT pool consists of 14 peptides, each corresponding to a defined MHC class II restricted CD4 T cell epitope from CMV, Epstein-Barr virus, Influenza virus and Clostridium tetani.

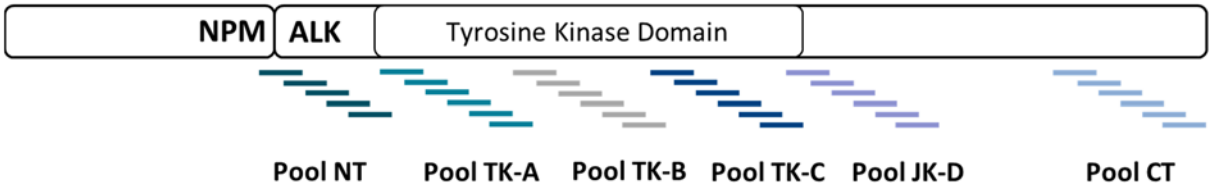


Figure 7: Schematic representation of synthetic long overlapping ALK peptides to study ALK reactive CD4 T cell responses.

3.2.1.5. Isolation of PBMCs from peripheral blood or leucocyte fractions

PBMCs from ALK$^+$ALCL patient´s peripheral blood or leukocyte fractions from healthy donors were isolated by density gradient centrifugation. The donor's blood samples were diluted 1:1 with PBS and overlaid on Lymphoprep in 50 ml tubes. Then, tubes were centrifuged at 800 x *g* for 20-30 min at room temperature (RT) without brakes. The upper plasma layer was carefully discarded, PBMCs on the interface were recovered into separate tubes and washed twice. After the washing steps, PBMCs were counted in addition of Trypan Blue staining to determine total viable cell numbers.

3.2.1.6. Magnetic-activated cell sorting (MACS)

Magnetic-activated cell sorting (MACS) was conducted using reagents from Miltenyi Biotec according to manufacturer's instructions. The principle of the separation method is outlined in **(Figure 8)**. For the generation of DCs, the purification of CD4 T cells, and the activation and expansion of B cells, PBMCs were first separated using CD14 microbeads. DCs were matured from the CD14$^+$ fraction and CD14$^-$ cells were further purified to CD4 T lymphocytes. CD14$^-$/CD4$^-$ cells were co-cultured with NIH-3T3 hCD40L feeder cells to activate and expand autologous B cells.

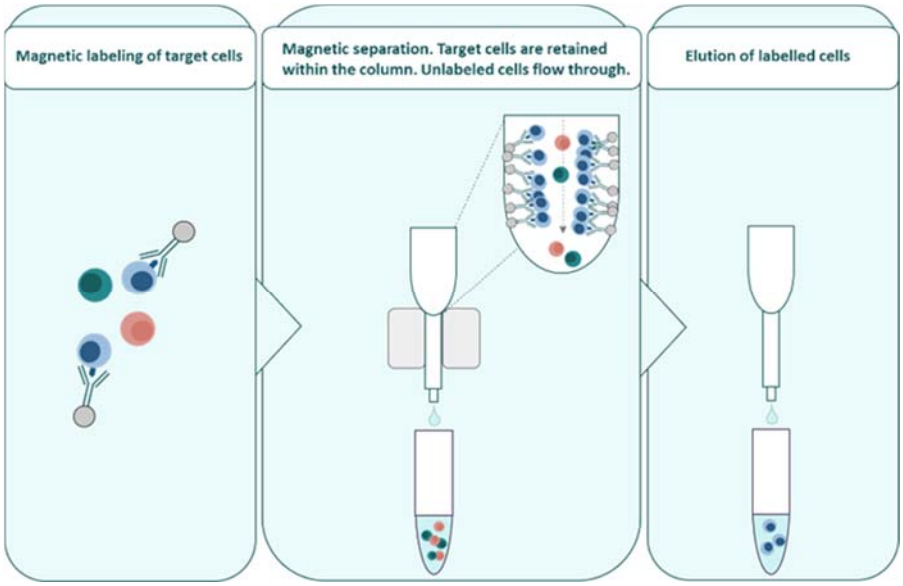


Figure 8: Principle of the magnetic-activated cell sorting (MACS) technique.

All steps were performed with pre-cooled solutions and cells were kept on ice to prevent cell death, capping of antibodies on the cell surface and non-specific cell labeling. PBMCs were resuspended in cold MACS buffer (PBS supplemented with 0.5% FBS and 2 mM EDTA) and centrifuged at 200 x *g* for 10 min to remove platelets. The supernatant was completely aspirated, and the cell pellet resuspended in 80 µl buffer per 10^7 cells. Then, 20 µl of CD14 microbeads were added per 10^7 cells and, after mixing, incubated for 15 min at 4°C in the refrigerator. After incubation, cells were washed once and resuspended in 500 µl buffer per 10^8 cells. In the meantime, MS columns were placed into the magnetic field of the separator and rinsed with buffer. The cell suspension was first passed through pre-separation filters to retain possible cell aggregates and subsequently applied onto the columns. The flow through containing unlabeled cells was collected in separate tubes. Columns were washed three times with buffer, removed from the separator and placed into collection tubes. The magnetically labeled $CD14^+$ cells were flushed out by applying buffer onto the column and firmly pushing the plunger. This step was repeated twice to recover all possible retained $CD14^+$ cells. The purified monocytes and macrophages were washed and subsequently matured into DCs as described in the next section.

Unlabeled $CD14^-$ cells from the flow-through were further purified to CD4 T cells using CD4 microbeads and by repeating the described steps of cell separation. After washing, a calculated cell number of CD4 T cells were resuspended in AIM-V supplemented with 5% human serum (HS) and seeded into 6 well plates. The cells were incubated at 37°C/5% CO_2 until first stimulation with peptide pulsed DCs. Remaining CD4 T cells were cryopreserved at -80°C for later testing of a possible memory response against ALK. In addition, CD4 T cell purity was confirmed by flow cytometry.

The $CD14^-/CD4^-$ cells, which are present in the flow through after CD4 T cell sorting, were washed and transferred onto NIH-3T3 CD40L feeder cells for activation and expansion of B lymphocytes described in 3.2.1.8.

3.2.1.7. Generation and maturation of monocyte derived dendritic cells

Many protocols for *in vitro* maturation of monocyte derived DCs have been established which commonly include the generation of immature DCs by incubation of monocytes for 5-7 days in the presence of GM-CSF and IL-4 followed by activation and maturation with proinflammatory, microbial and T cell derived stimuli for another 2-3 days (Castiello et al. 2011). To reduce this time consuming procedure, a 48h protocol of DC maturation, which has been proven to generate highly efficient mature DCs with full T cell stimulatory capacity, was adapted from (Dauer et al. 2003).
Monocytes and macrophages obtained from CD14 sorting of PBMCs (described in 3.2.1.6.) were washed, counted and resuspended in serum free X-Vivo 15 media supplemented with IL-4 (500U/ml) and GM-CSF (1000U/ml). A total of 1.5 - 3 x 10^6 cells were then seeded into each well of 6 well plates and incubated for 24h followed by incubation with the proinflammatory mediators IL-1ß (10ng/ml), TNF-α (200U/ml), IL-6 (1000U/ml) and PGE_2 (1 µM) for another 24 h. Then, matured DCs were carefully collected, washed, and counted. The immunophenotype of matured DCs was analyzed by flow cytometry. DCs were pulsed for 3h with ALK peptide pools or respective experimental controls for the stimulation of patient´s CD4 T cells (described in 3.2.9.). Remaining DCs were aliquoted and cryopreserved for further stimulations and IFN-γ ELISPOT analysis.

3.2.1.8. Generation and expansion of CD40 activated B cells

Due to the limitation of available autologous DCs in ALK^+ALCL patients and healthy donors, autologous CD40-activated B cells have been used as alternative source of APCs for the stimulation of donors ALK-reactive CD4 T cells **(Figure 9)**. CD40-activated B cells were generated from patients $CD14^-/CD4^-$ PBMCs using a previously described method (Schultze et al. 2002). CD40 stimulation was supplied by NIH-3T3 feeder cells expressing recombinant human CD40 ligand (NIH-3T3 hCD40L), kindly provided by Dr. Gordon J. Freeman, Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA. The resulting autologous B cells were pulsed with respective ALK peptide pools to once re-stimulate patients ALK-reactive CD4 T cells, which were further tested in an IFN-γ ELISPOT for single peptide recognition **(Figure 9)** (described in 3.2.1.9.).

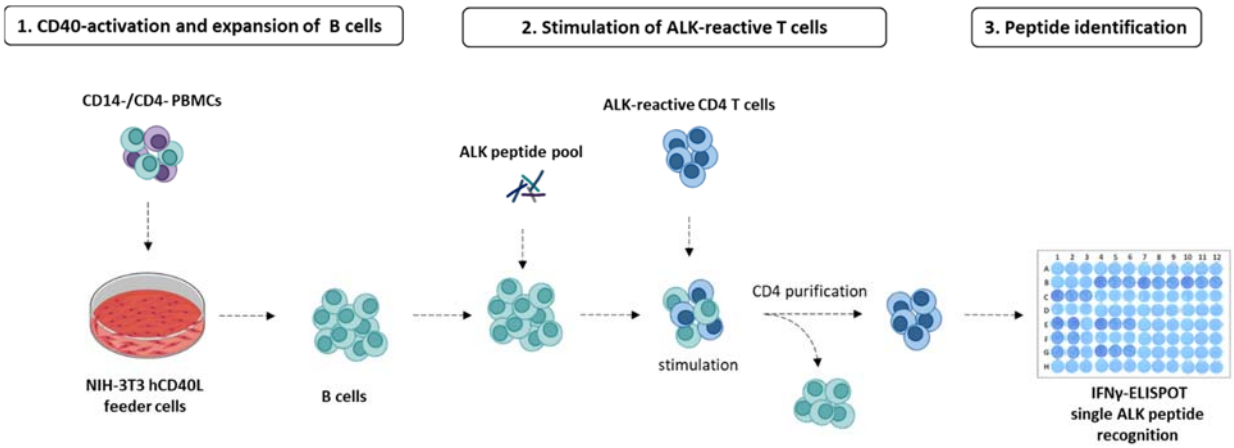


Figure 9: Workflow for single ALK peptide identification using autologous B cells as APCs

Table 4: Composition of NIH-3T3 hCD40L feeder cell and B cell media

Feeder cell media		**B cell media**	
DMEM/F12		IMDM	
HEPES	15 mM	HEPES	25 mM
FBS	10%	Human Serum	10%
L-Glutamine	2.5 mM	L-Glutamine	4 mM
Gentamycin	15 µg/ml	Gentamycin	15 µg/ml
Added for culturing:		**Freshly added:**	
G-418 (selection media)	200 µg/ml	rh Transferrin	50 µg/ml
		rh Insulin	5 µg/ml
		IL-4	100 U/ml
		Cyclosporin A	5.5 x 10^{-7} M

Maintenance and preparation of NIH-3T3 hCD40L feeder cells

NIH-3T3 hCD40L is an adherent murine fibroblast cell line and was maintained by passaging twice per week before reaching confluency. Cells were gently detached using PBS/2mM EDTA, collected, centrifuged at 225 x *g* for 5 min and, after counting, seeded in cell culture dishes in feeder cell media supplemented with the selection antibiotic G-418. For B cell culture, NIH-3T3 hCD40L cells were harvested and resuspended in feeder cell media without G-418 to a concentration of 1x10^6 cells/ml. Then, cells were irradiated using a cobalt source with a total dose of 96 Gy (9600 rad) and subsequently further diluted 1:4 in media to a final

concentration of $2.5x10^4$ cells/ml. 2 ml of the cell suspension was plated into each well of a 6 well plate and incubated over night at 37°C/5% CO_2. On the next day, the adherence of NIH-3T3 hCD40L cells was confirmed and co-culture with patient´s $CD14^-/CD4^-$ PBMCs was started (day 0).

CD40-B cell culture

Patient´s $CD14^-/CD4^-$ PBMCs were washed, counted and resuspended in B cell media supplemented with transferrin and insulin to a concentration of $2x10^6$ cells/ml. Then, cyclosporin A and IL-4 were freshly added at the indicated concentrations. NIH-3T3 hCD40L plates were washed and $CD14^-/CD4^-$ PBMCs were carefully transferred into each well in a concentration of $8x10^6$ cells/well. The co-cultures were incubated at 37°C/5% CO_2. On day 4, new plates of irradiated NIH-3T3 hCD40L cells were prepared as described above. On day 5, PBMCs were harvested, washed twice, and re-cultured with irradiated NIH-3T3 hCD40L feeder cells in cell numbers indicated in **Table 5**. After 15 days of re-culturing, CD40-B cell cultures consisted of more than 95% pure B cells, which was confirmed by flow cytometry. Aliquots of patient's pure B cells were regularly cryopreserved and stored at -80°C until further use. For CD4 T cell stimulation, B cells were first Ficoll-density centrifuged to remove cellular debris and dead cells from the co-culture before pulsing with ALK-peptides (described in 3.2.1.9.).

Table 5: Timetable for the expansion of CD40-activated B cells

Day -1	Preparation of CD40L feeder cell plates	
Day 0	Start of co-culture	$2x10^6$ PBMCs /ml
Day 4	Preparation of CD40L feeder cell plates	
Day 5	Re-culture of PBMCs	$2x10^6$ PBMCs /ml
Day 7	Preparation of CD40L feeder cell plates	
Day 8	Re-culture of B cells (≥85% $CD19^+$ cells)	$1.5x10^6$ PBMCs/ml
Day 11	Preparation of CD40L feeder cell plates	
Day 12	Re-culture of B cells (≥95% $CD19^+$ cells)	$1x10^6$ B cells /ml
Day 14	Preparation of CD40L feeder cell plates	
Day 15	Re-culture of B cells	$0.75x10^6$ B cells/ml
Day 18	Repeat re-culturing as desired	

3.2.1.9. CD4 T cell stimulation

For the stimulation of CD4 T cells and for the identification of single immunogenic ALK peptides in responding patients, ALK-peptide pulsed autologous DCs or B cells were used as APCs, respectively.
Autologous mature DCs were washed, counted and resuspended in serum free AIM-V media. For the starter culture, DCs were divided into separate tubes and pulsed for 3 h with 2.5 µM ALK peptide pools or 0.6 nM experimental controls (PepTivator® CMV pp65 or CEFT MHC-II Pool) in the incubator (37°C/5% CO_2). To ensure ventilation and resuspension during this time, caps were loosened, and cells were gently mixed by shaking every 30-60 min. In the meantime, purified CD4 T cells were washed, counted and resuspended in AIM-V supplemented with 5% HS and 20U/ml IL-2. After pulsing, CD4 T cells were stimulated in 48 well plates with the ALK peptide pool loaded DCs in a 10:1 ratio (effector:target). In addition to the experimental positive controls, unstimulated CD4 T cells were cultured under the same conditions without peptide and were used as controls. For each condition at least two separate starter cultures were initiated. CD4 T cells were re-stimulated twice every seven days with freshly thawed and rested DCs under the same conditions to prime and enrich for possible ALK-specific CD4 T cells. In every re-stimulation, the cell numbers were increased, and remaining cells were cryopreserved. In between, consumed media was replaced every 3-4 days in addition of 20 U/ml IL-2.
In case of ALK-peptide pool recognition, after stimulation with peptide pool loaded DCs, reactive CD4 T cells were once re-stimulated with freshly prepared autologous B cells. B cells were harvested, washed and Ficoll-Paque centrifuged to remove debris and non-viable cells. Similar to DC pulsing, B cells were counted, divided into separate tubes in 1 ml of CTS™OpTimizer media and pulsed for 3 h with 2.5 µM of the respective ALK-peptide pool. Patient´s ALK-reactive CD4 T cells were then stimulated with ALK pool loaded B cells in a target:effector ratio of 1:1 in CTS™OpTimizer media supplemented with 2% HS, 2 mM L-glutamine, 15 µg/ml gentamycin and 20U/ml IL-2. After seven days, cells were collected and CD4 T cells were positively selected using CD4 microbeads and the protocol described in 3.2.1.6. The ALK-reactive purified CD4 T cells were then analyzed for single ALK peptide recognition in an IFN-γ ELISPOT.

3.2.1.10. IFN-γ ELISPOT

The enzyme-linked immunospot (ELISPOT) assay is a very sensitive method to measure the frequency of cytokine secreting cells at a single cell level. Due to its high sensitivity and quantitative value, it is the most common cellular immunoassay to measure antigen-specific immune responses and is particularly useful for the detection of small populations of antigen

specific cells (detection limit is one out of $1x10^5$ cells) (Slota et al. 2011). The principle of the technique **(Figure 10)** is based on the culture of cells on specific antibody-coated surfaces in the absence or presence of stimuli. Secreted cytokines, released by antigen-specific cells, are captured by the surface-bound antibodies. After an optimized incubation time, cells are washed away, and secreted cytokines can be detected by biotinylated specific detection antibodies that are bound by a streptavidin-enzyme conjugate. The addition of a precipitating substrate such as tetramethylbenzidine (TMB) results in the formation of spots that each corresponds to an individual cytokine-secreting cell. IFN-γ is an abundant cytokine produced by Th1 cells, a population of CD4 helper T cells favorable for anti-tumor responses (Kim & Cantor 2014). Therefore, the IFN-γ ELISPOT was used to detect ALK-specific CD4 T cells in ALK^+ALCL patients and healthy donors.

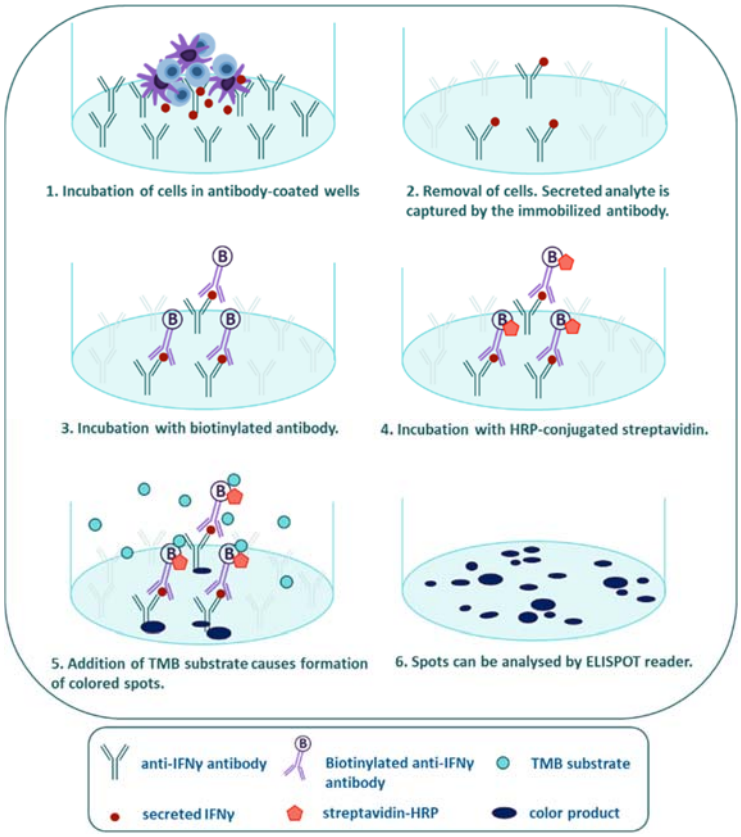


Figure 10: Principle of the enzyme-linked immunospot (ELISPOT)

Preparation and coating of ELISPOT plates

Plates were pre-treated with 35% ethanol in PBS for 1 min. Then, membranes were washed 5 times with PBS and subsequently coated with 100 µl/well of 10 µg/ml anti-human IFN-γ antibody. The plates were incubated overnight at 4°C in the dark.

Preparation and incubation of cells

Stimulated and enriched CD4 T cells were tested in the IFN-γ ELISPOT for the recognition of APCs pulsed with ALK peptide pools or single ALK peptides. Per well, a total of 3-6 x 10^3 DCs or 2-3 x10^4 B cells were incubated with stimulated CD4 T cells in a target:effector ratio of 1:5 or 1:1, respectively. In addition, purified unstimulated or single stimulated CD4 T cells were tested for ALK pool recognition to detect a possible memory response. Either serum-free AIM-V, AIM-V supplemented with 5% HS or CTL Test was used in the ELISPOT test. DCs or B cells were prepared as described in 3.2.1.9. and pulsed for 3 h with 20 µg/ml ALK peptide pools or single ALK peptides, and 0.6 nM experimental controls in separate tubes in serum free media. To identify MHC class II alleles responsible for ALK-peptide presentation in individual patients, APCs were additionally incubated with 20 µg/ml HLA-DR blocking antibodies. The stimulated and enriched CD4 T cells (two separate cultures per condition) were rested overnight in CTL Test medium, washed three times and afterwards resuspended in media. ELISPOT plates were conditioned with media for at least 30 min prior to use. After peptide pulsing, target cells were washed, resuspended in media and plated together with CD4 T cells at indicated cell numbers in the plates. Cells were tested in duplicates or triplicates for each condition. The testing conditions and respective controls are outlined in **Figure 11**. Plates were carefully wrapped in aluminum foil to avoid evaporation and incubated at 37°C in a humidified incubator with 5% CO_2 for 20h (DCs) or 48h (B cells).

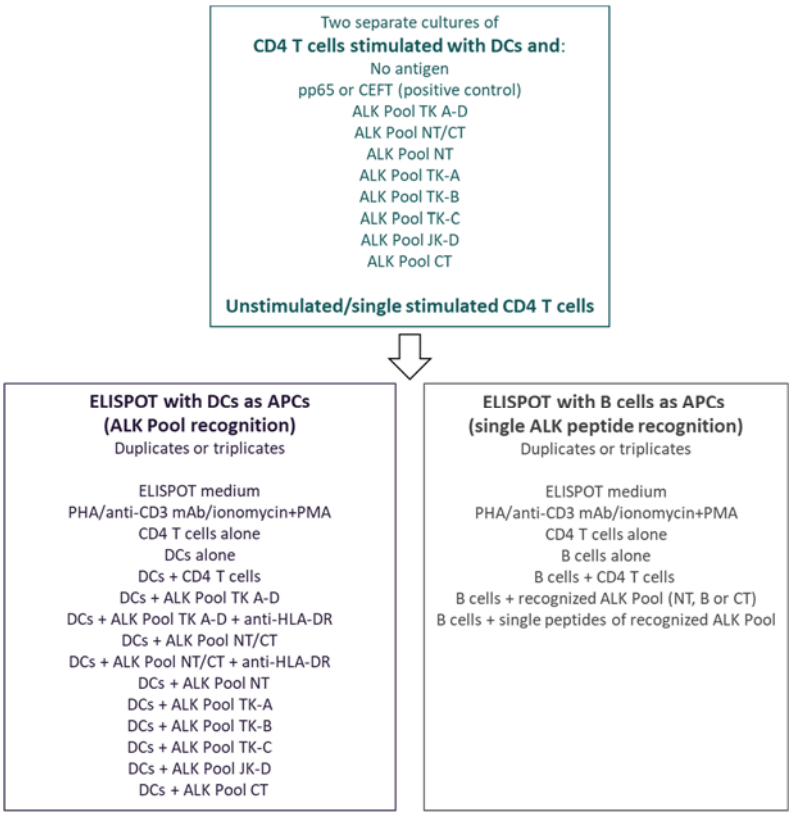


Figure 11: Testing conditions of ALK-reactive CD4 T cells in the IFN-γ ELISPOT

Development and detection of spots

After the incubation time, cells were removed, and plates were washed 5 times with PBS. The anti-IFN-γ biotin detection antibody was diluted to 1 µg/ml in PBS containing 0.5% FBS and 100 µl was added to each well. The plates were incubated for 2h at RT in the dark. After washing steps, a 1:1000 dilution of Streptavidin-HRP in PBS/0.5% FBS was added to each well. The plates were incubated for another hour at RT in the dark. The TMB substrate was passed through a 0.45 µm filter and, after washing the plates, added to each well. After distinct spots emerged, color development was stopped by extensively washing in deionized water. Plates were then dried and stored in the dark at RT until quantification of spots with ELISPOT analyzers. Responses in each microculture were defined as positive when the mean number of IFN-γ spots against the tested antigen was at least two-fold higher than the background reactivity (no antigen).

3.2.1.11. Flow cytometry

Flow cytometry was performed to analyze the immunophenotype of matured DCs and CD40 activated B cells and the purity of magnetic microbead sorted CD4 T cells. Representative results are shown in **Figure 12**.

Staining of cells for flow cytometric analysis was conducted using standard protocols for immunological studies. Briefly, cells were harvested, washed and resuspended in staining buffer (PBS supplemented with 2% FBS and 2mM EDTA). 100 µl of the cell suspension ($1x10^5$ – $1x10^6$ cells) was added to each polystyrene round-bottom 12 x 75 mm tube and, according to manufacturer's instructions, 0.1-10 µg/ml fluorescent conjugated antibodies were added. Cells were incubated for 20-30 min at 4°C in the dark. Then, cells were washed and subsequently fixed in 2% paraformaldehyde solution for 10 min at 4°C. After washing, cells were resuspended in appropriate amount of buffer and stored at 4°C in the dark until acquiring. Flow cytometry was performed on a BD FACSVerse (BD Biosciences) or MACSQuant10 (Miltenyi Biotec) analytical flow cytometer and data were analyzed using FlowJo or Flowlogic software.

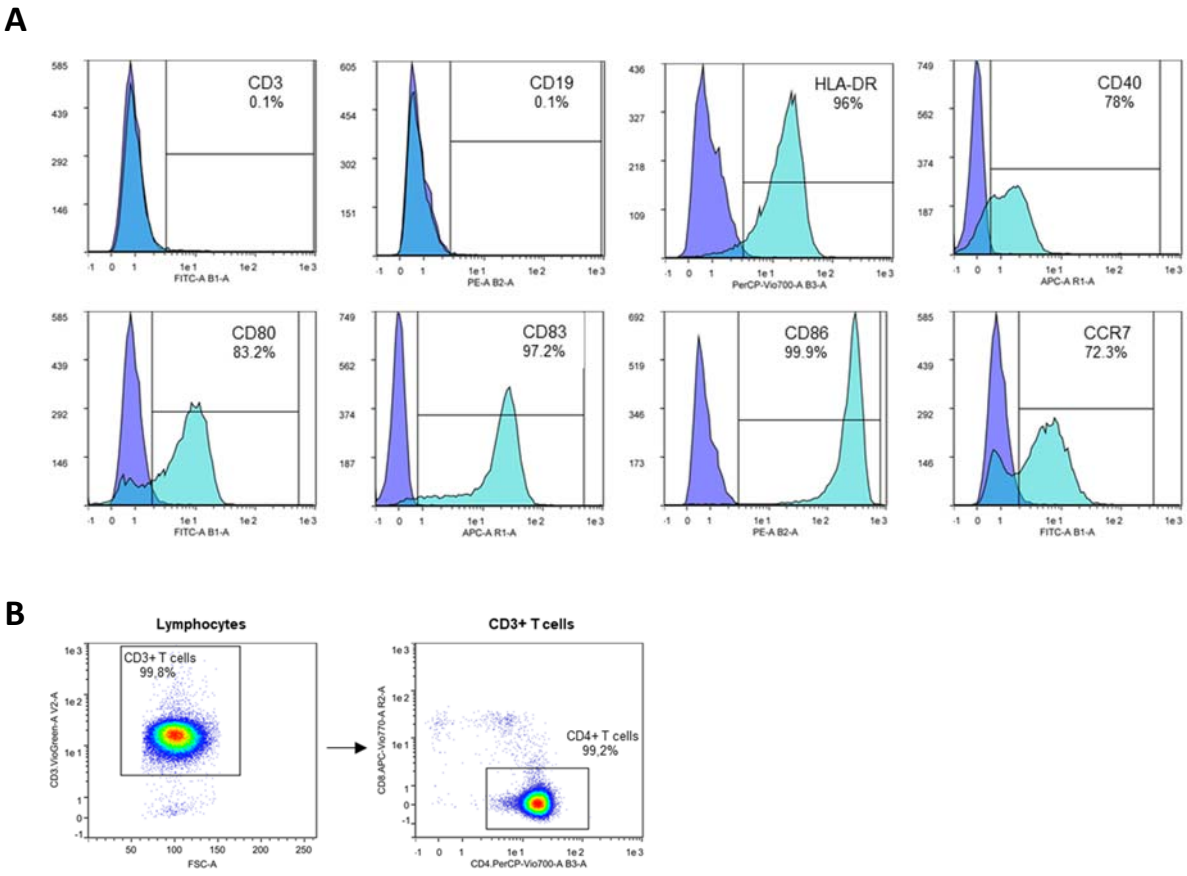


Figure 12: Flow cytometric analysis of DC phenotype and CD4 T cell purity
Cells were stained for 20 min at 4°C with indicated fluorescent-conjugated antibodies after blocking of Fcγ receptors. (**A**) Representative analysis (overlay histogram) of unstained DCs (purple) and DCs after maturation from CD14+ monocytes (aqua) by following specific conjugated antibodies: CD3.FITC, CD19.PE, HLA-DR.PerCP, CD40.APC, CD80.FITC, CD83.APC, CD86.PE, and CD197/CCR7.FITC. (**B**) Representative analysis of CD4 T cell purity after magnetic microbead sorting using fluorescent-conjugated antibodies specific for CD3, CD8, and CD4.

3.2.2 Validation of ALK vaccine induced T cell responses *in vivo*

3.2.2.1. Mice

All animal experiments were approved by the Boston Children's Hospital (BCH) Institutional Animal Care and Use Committee and were conducted in accordance with the standards outlined in the National Research Council's Guide for the Care and Use of Laboratory Animals and BCH's PHS Assurance.
6-8-week old, female Balb/c or C57BL/6 mice were purchased from Charles River Laboratory (USA) and were housed under specific pathogen free conditions in accordance with the animal care and use regulations of Boston Children´s Hospital.

3.2.2.2. Synthetic long-overlapping ALK peptides

Eight 36aa long ALK peptides were synthesized and selectively tested in vaccination experiments for immunogenicity and therapeutic efficacy in mice **(Figure 13)**. Due to a possible patent filing, the exact sequences of the peptides are not published in this thesis. The peptides were custom synthesized by Genscript, had a purity above 90% and a confirmed composition analyzed by mass spectrometry. Each lyophilized peptide was dissolved in DMSO or ultrapure water according to the manufacture's recommendation. After reconstitution, suitable aliquots were prepared, to avoid multiple freezing thawing cycles, and stored at –80°C until further use. The immunogenicity of the peptides in eliciting ALK-specific CD4 T cell responses in mice was tested in vaccination experiments in which mice were immunized with ALK peptide vaccines containing either single peptides or selective peptide combinations.

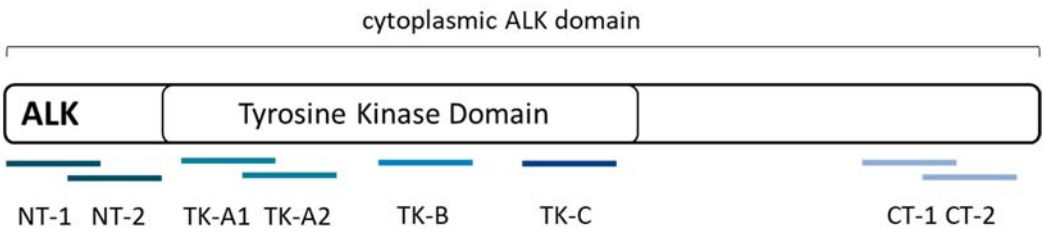


Figure 13: Schematic representation of synthetic long overlapping ALK peptides to study ALK reactive CD4 T cell responses *in vivo.*

3.2.2.3. Immunization of mice with ALK peptide vaccines

Balb/c or C57BL/6 mice were immunized three times in an interval of two weeks with 10 nmol of ALK peptide/s combined with 25 µg of the adjuvant cyclic diguanylate monophosphate (c-di-GMP), which is a cyclic dinucleotide (CDN) and STING agonist, in a final volume of 100 µl PBS. Mice were vaccinated under 2-4% isoflurane anesthesia by subcutaneous injection at the base of the tail. Seven days after the third vaccination, mice were sacrificed and splenocytes isolated for analysis of ALK peptide-specific T cell responses.

3.2.2.4. Therapeutic experiments

For the therapeutic evaluation of an ALK peptide vaccine in a local and systemic lymphoma setting, mice were challenged subcutaneous (s.c.) or intra venous (i.v.) with R37 ALK$^+$ lymphoma cells prior to immunization. The R37 cell line is derived from NPM-ALK transgenic mice on a Balb/c background in which human NPM-ALK transcription was targeted to T cells (Chiarle et al. 2003) and was kindly provided by Dr. Roberto Chiarle´s Laboratory, Department of Biomedical Sciences and Human Oncology, University of Torino, Italy.
One day after tumor cell injection, mice were randomized and divided into control (non-vaccinated) and experimental (vaccinated) cohorts. Mice in the experimental groups were vaccinated three times every 11 days with 10 nmol of the CT-s2 peptide (20 aa long peptide within the overlapping sequence of CT-1+2) and 25 µg c-di-GMP (CDN, adjuvant) in a final volume of 100 µl PBS as described above in 3.2.2.3.

Subcutaneous injection
1x10^6 R37 ALK$^+$ lymphoma cells were injected subcutaneously into the upper flank in a final volume of 100 µl PBS. After nodule formation, tumors size was measured bi-weekly using a caliper and tumor volume (mm^3) was calculated using the formula:

$$V = (W^2 \times L)\ /2$$

(V) tumor volume, (W) tumor width, (L) tumor length

Intravenous tail vein injection
Mice were shortly exposed to a heat lamp for vasodilatation of the tail vein. Then, mice were placed into a mouse restrain apparatus and the injection site was sterilized with alcohol. Using a 30-gauge syringe, 1x10^6 R37 ALK$^+$ lymphoma cells in 100 µl PBS were slowly injected into the tail vein. Mice were conscientiously monitored during and after the procedure.

3.2.2.5. Isolation of splenocytes

Spleens were collected from control mice and mice receiving ALK peptide vaccines at day 7 post-vaccination. All steps were performed with pre-cooled solutions and cells were kept on ice to prevent apoptosis. Mice were sacrificed and spleens harvested into cold RPMI supplemented with 10% FBS, 2 nM L-glutamine, 1% PS, 1x HEPES, 1x NEAA, and 2-mercaptoethanol. Then, spleens were transferred onto a 70 µm cell strainer and were gently disaggregated by pressing the spleen tissue through the strainer with a syringe plunger under continuous addition of medium. Splenocytes were washed and erythrocytes lysed by the addition of Red Blood Cell Lysing Buffer. Then, cells were resuspended in medium and counted in the presence of Trypan Blue to determine viable total cell numbers. Tubes were kept on ice until stimulation of splenocytes for flow cytometric analysis.

3.2.2.6. Stimulation of splenocytes

Splenocytes of control and vaccinated mice were stimulated with respective ALK peptides and controls and were subsequently analyzed by flow cytometry for antigen specific CD4 T cell responses. 5 x10^5 splenocytes were seeded into each well of a 96-well round bottom plate and stimulated with 10 µg/ml of respective 36aa long ALK peptides for 12h or for 2h in case of 9mer peptides and the PMA/ionomycin positive control. Unstimulated cells were included as control. After the incubation time, cytokine release was stopped by the addition of the protein transport inhibitor brefeldin A for 4h.

3.2.2.7. Intracellular cytokine staining (ICS)

For ICS, the BD Cytofix/Cytoperm™ Plus Fixation/Permiabilization Kit was used according to the manufacturer´s protocol. After incubation of splenocytes with brefeldin A, to allow cytokine accumulation, cells were washed with staining buffer and Fc receptors were blocked for 10 min prior to staining of surface antigens. After a washing step, cells were stained with the fluorochrome-conjugated antibody specific for CD4 or in combination with CD8 for 30 min at 4°C. Then, cells were washed twice and subsequently permeabilized for 20 min at 4°C. After, permeabilization, cells were washed twice with Perm/Wash buffer (which was maintained during all following washing steps) and intracellular cytokine staining was performed using an APC-conjugated antibody specific for IFN-γ. Then, cells were washed and resuspended in staining buffer prior to flow cytometric analysis.

3.2.2.8. Dextramer staining

MHC Dextramer® (Immudex) consists of a dextran polymer backbone carrying an optimized number of MHC molecules and fluorochromes for the detection of antigen-specific T cells via flow cytometry (www.immunodex.com). For the detection of ALK-specific CD8 T cells in vaccinated and control mice, a custom designed dextramer, containing a previously identified CD8 T cell epitope (TK-Bs) in the ALK protein (unpublished, Chiarle Lab, Boston, USA), was used. Dextramer staining of splenocytes was performed according to the recommended protocol. Briefly, cells were washed with staining buffer and afterwards incubated with 10 µl MHC dextramer in 50 µl buffer for 10 min at RT. Then, FITC-conjugated anti-CD8 antibody was added and cells were incubated for 20 min at 4°C. After several washing steps, cells were resuspended in FACS buffer and analyzed by flow cytometry. In addition, a live/dead cell staining with Aqua Zombie dye was performed.

3.2.2.9. Flow cytometry

After stimulation and staining of cells as described in 3.2.2.6, 3.2.2.7 and 3.2.2.8, ALK-vaccine induced T cell responses were analyzed by flow cytometry. The gating for the analysis of IFN-γ secreting CD4 and CD8 T cells is shown in **Figure 14**.
Flow cytometric analysis was performed on a BD Celesta (BD Biosciences) analytical flow cytometer.

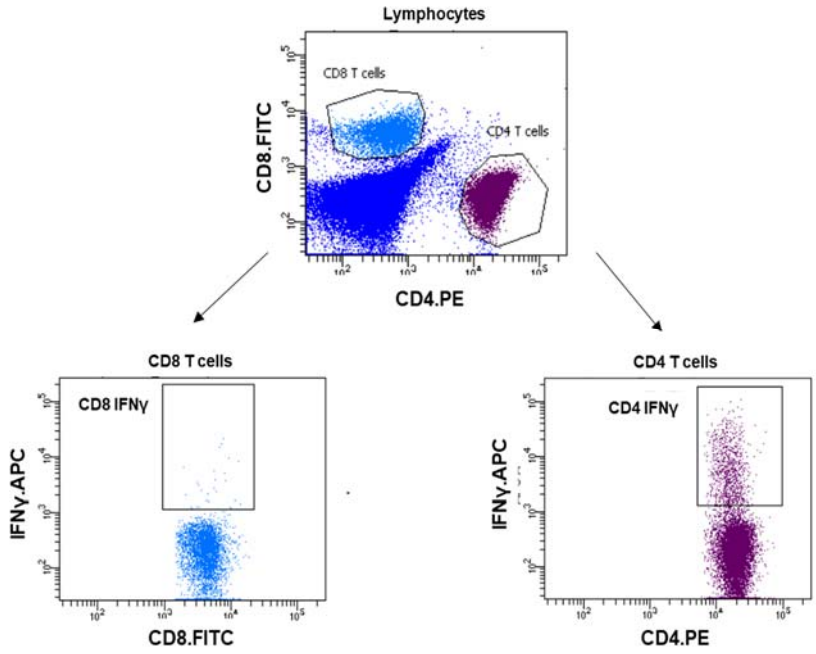


Figure 14: Gating strategy for the analysis of ALK vaccine induced T cell responses in mouse splenocytes

3.2.2.10 Statistical analysis

Kaplan-Meier survival curves were generated with GraphPad Prism 6 and P values were determined with the log-rank Mantel-Cox test. Statistical analysis of tumor growth was performed using student t test for paired data. P values of <0.05 were considered to be significant.

4. Results

4.1. ALK-reactive CD4 T cells are present in ALK$^+$ALCL patients and healthy donors and are directed against three main regions within the ALK protein

To detect ALK-reactive CD4 T cells in ALK$^+$ALCL patients and healthy donors, a synthetic long peptide (SLP)-based approach was established which allowed an HLA-independent analysis of CD4 T cell responses and additionally ensured individual processing and presentation of ALK peptides (**Figure 15A**). To include most of the possible CD4 T cell epitopes, 41 30 aa long synthetic ALK peptides with a sequence overlap of 16 aa were selectively combined to ALK peptide pools covering the fusion region of NPM-ALK and the N-terminal cytoplasmic region of ALK (Pool NT), the whole tyrosine kinase domain (Pools TK-A-C) with a juxta-kinase region (Pool JK-D) and the C-terminal end (Pool CT) of the ALK protein (**Figure 15B**). Autologous DCs were pulsed with the respective ALK peptide pools and used to stimulate and enrich possible ALK-reactive CD4 T cells, which were then tested in an IFN-γ ELISPOT for peptide pool recognition (**Figure 15A**). Using this method, nine ALK$^+$ALCL patients harboring NPM-ALK or x-ALK translocations and six healthy donors were analyzed for the presence of ALK reactive CD4 T cells. All patients were above 13 years of age and in clinical remission without relapse after standard front-line chemotherapy and were analyzed between 1.8 and 12.3 years after diagnosis. One patient (patient 4) was analyzed at diagnosis and 1.5 years after therapy.

Of the nine enrolled patients and six healthy donors, eight patients and all healthy donors exhibited ALK-reactive CD4 T cell responses to one or more ALK peptide pools.

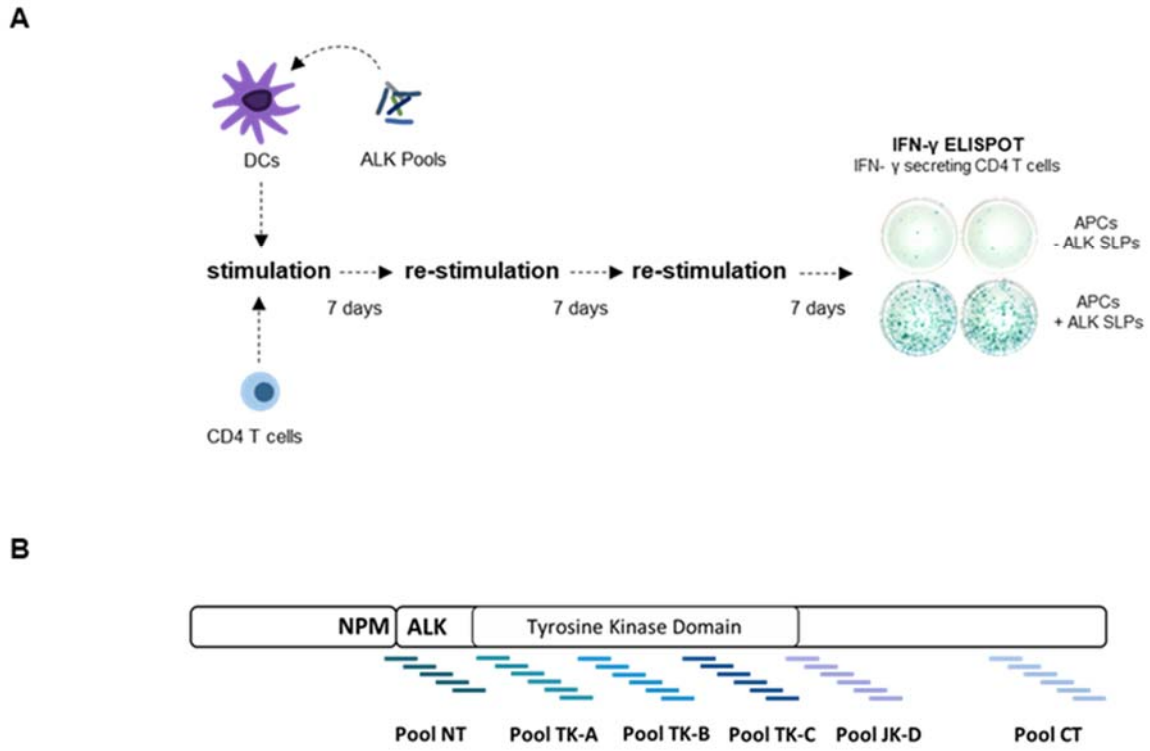
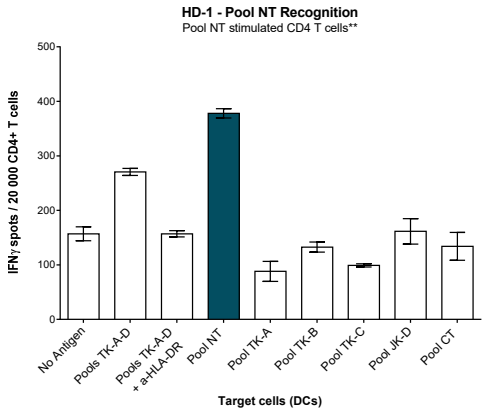


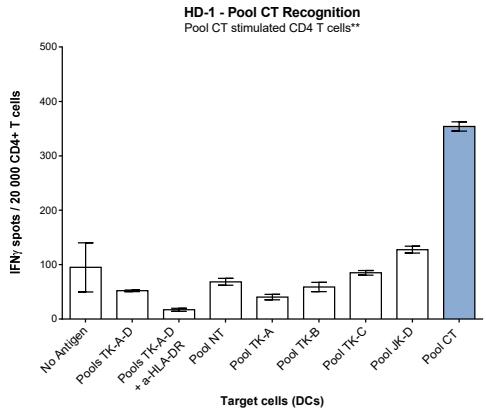
Figure 15: Experimental layout for the detection of ALK-reactive CD4 T cells in ALK$^+$ALCL patients and healthy donors. (A) Autologous DCs pulsed with ALK pools, consisting of synthetic long overlapping peptides (SLPs), were used to stimulate and enrich possible ALK-reactive CD4 T cells, which were then tested in an IFN-γ ELISPOT for ALK pool recognition. **(B)** Schematic representation of ALK pools used in this study.

Healthy Donors

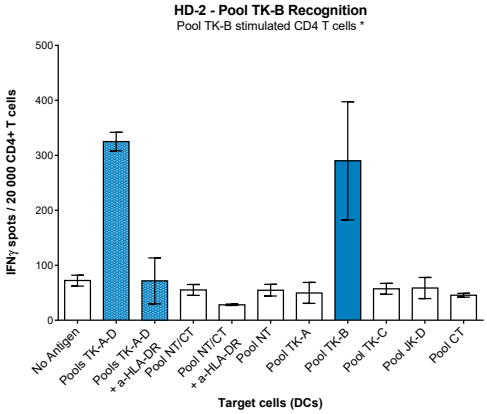
ALK recognition by CD4 T cells of healthy donors was uniformly directed against the N-terminal region, the Pool TK-B within the tyrosine kinase domain, and the C-terminal domain of ALK (**Figure 16**). Both, Pool NT and Pool CT were recognized by CD4 T cells of five healthy donors (**Figure 16A, C-F**) whereas Pool TK-B exhibited reactivity in four donors (**Figure 16B-D, F**). The ALK pool enriched CD4 T cells of all healthy donors recognized not only the corresponding pool but also the respective control pools confirming the specific recognition of ALK peptides **(Figure 16)**. To determine the HLA-restriction of the observed CD4 T cell responses, blocking experiments with antibodies specific for HLA-DR were performed. HLA-DR restriction of the response was observed in CD4 T cells reactive to Pool TK-B of HD-2 **(Figure 16B)** and in reactive cells to Pool NT of HD-5 **(Figure 16E)**. In all other healthy donors, the ALK pool specific CD4 T cell responses were either not, or only mildly diminished by HLA-DR blocking, indicating that ALK peptides were presented by other HLA alleles **(Figure 16 C,D,F)**.

A

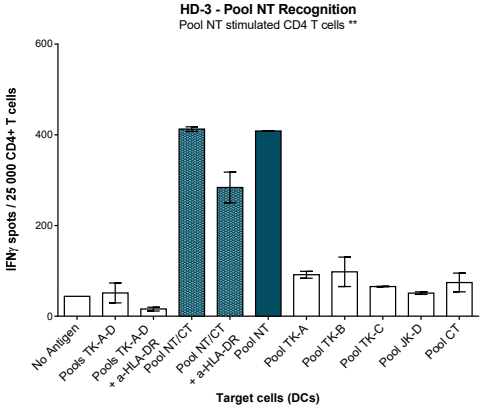

HD-1 - Pool NT Recognition
Pool NT stimulated CD4 T cells**
IFNγ spots / 20 000 CD4+ T cells
500
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

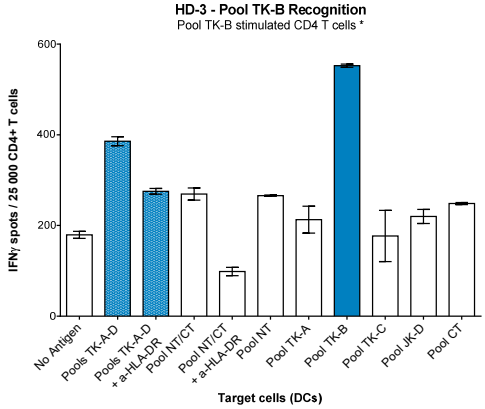

HD-1 - Pool CT Recognition
Pool CT stimulated CD4 T cells**
IFNγ spots / 20 000 CD4+ T cells
500
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

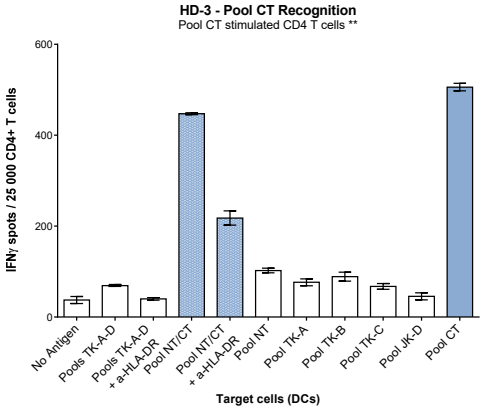
B


HD-2 - Pool TK-B Recognition
Pool TK-B stimulated CD4 T cells *
IFNγ spots / 20 000 CD4+ T cells
500
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pool NT/CT
Pool NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

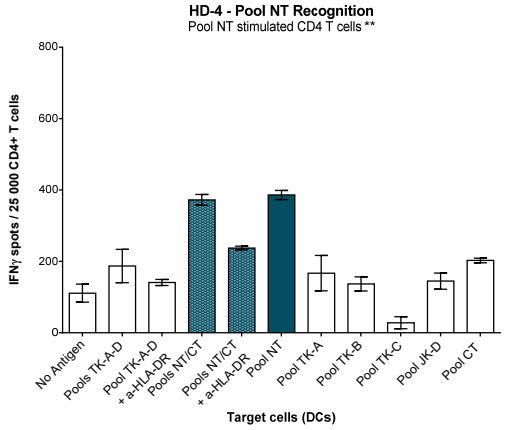
C

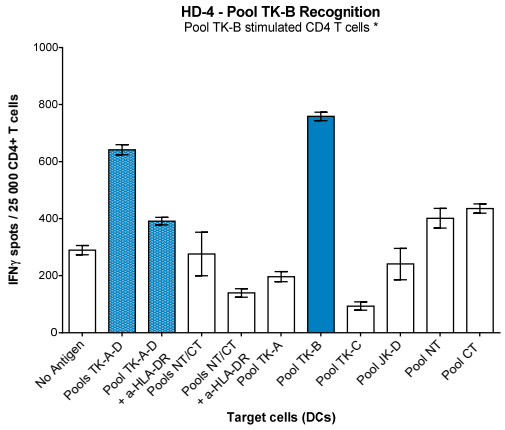

HD-3 - Pool NT Recognition
Pool NT stimulated CD4 T cells **
IFNγ spots / 25 000 CD4+ T cells
600
400
200
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pool NT/CT
Pool NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

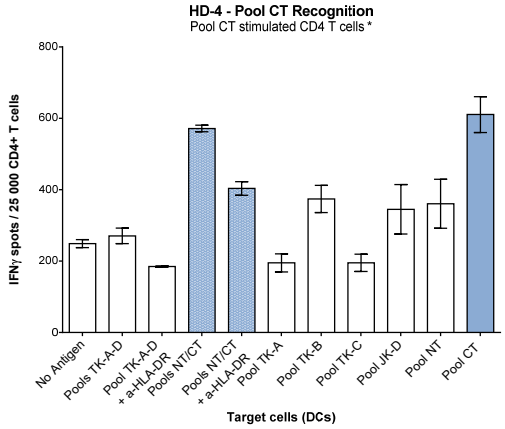

HD-3 - Pool TK-B Recognition
Pool TK-B stimulated CD4 T cells *
IFNγ spots / 25 000 CD4+ T cells
600
400
200
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pool NT/CT
Pool NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)


HD-3 - Pool CT Recognition
Pool CT stimulated CD4 T cells **
IFNγ spots / 25 000 CD4+ T cells
600
400
200
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pool NT/CT
Pool NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

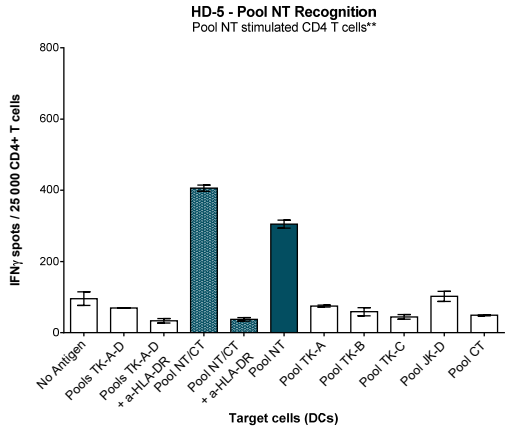
D

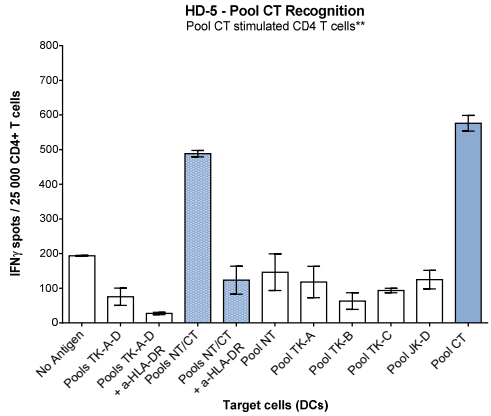

HD-4 - Pool NT Recognition
Pool NT stimulated CD4 T cells **
IFNγ spots / 25 000 CD4+ T cells
800
600
400
200
0
No Antigen
Pools TK-A-D
Pool TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)


HD-4 - Pool TK-B Recognition
Pool TK-B stimulated CD4 T cells *
IFNγ spots / 25 000 CD4+ T cells
1000
800
600
400
200
0
No Antigen
Pools TK-A-D
Pool TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool NT
Pool CT
Target cells (DCs)


HD-4 - Pool CT Recognition
Pool CT stimulated CD4 T cells *
IFNγ spots / 25 000 CD4+ T cells
800
600
400
200
0
No Antigen
Pools TK-A-D
Pool TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool NT
Pool CT
Target cells (DCs)

E


HD-5 - Pool NT Recognition
Pool NT stimulated CD4 T cells**
IFNγ spots / 25 000 CD4+ T cells
800
600
400
200
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pool NT/CT
Pool NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)


HD-5 - Pool CT Recognition
Pool CT stimulated CD4 T cells**
IFNγ spots / 25 000 CD4+ T cells
800
700
600
500
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

F

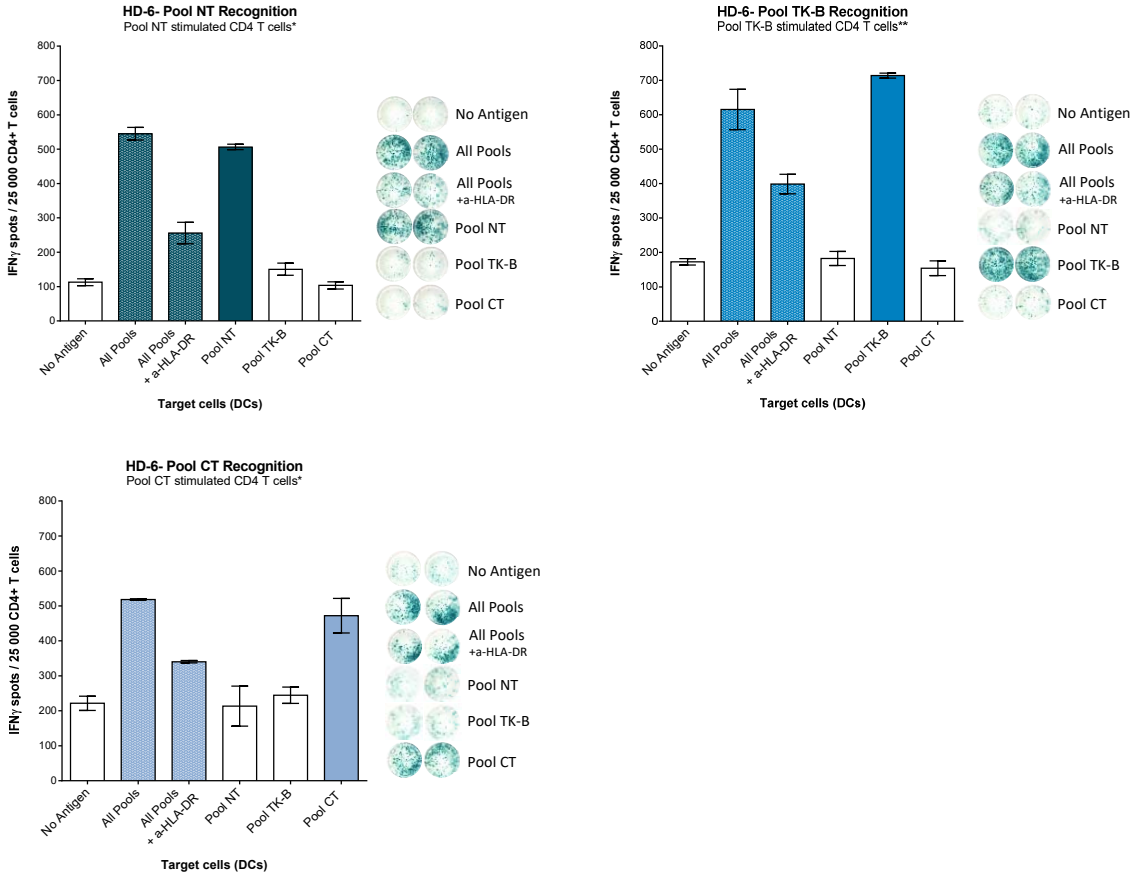


Figure 16: CD4 T cell responses against ALK peptide pools in healthy donors.
Autologous DCs were pulsed with ALK peptide pools and used to stimulate and enrich possible ALK-reactive CD4 T cells, which were then tested in an IFN-γ ELISPOT for ALK pool recognition. (**A-F**) Quantification of IFN-γ secreting CD4 T cells in response to DCs loaded with respective ALK pools in indicated healthy donors. Bar graphs show the mean number of spot counts (equal to the number of IFN- γ secreting CD4 T cells) +/- SD to the indicated conditions (x-axis; DCs pulsed with the respective ALK pool or without antigen). A response was defined as positive when the spot count was ≥ 2-fold higher than the mean of the negative control (no antigen). For each condition, two separate cultures were initiated. Asterisks in the heading indicate if one (*), or both (**) initiated cultures recognized the respective ALK pool. Control pool TK-A-D contained the single fusion peptide and Pools-TK-A-D; Control pool NT/CT consisted of Pool NT and Pool CT; All Pool control indicated all peptides from Pool NT, Pool TK-B, and Pool CT. HLA-DR blocking is indicated as Pool "X"+ a-HLA-DR.

ALK⁺ALCL patients

In contrast to healthy donors, ALK-specific CD4 T cell responses in ALK⁺ ALCL patients were also directed against other regions of the ALK protein.

CD4 T cells of patient 1 were reactive to Pool TK-A. The response was abrogated by HLA-DR blocking, indicating a restriction of the response to HLA-DR alleles (**Figure 17A**). Patient 2 exhibited CD4 T cell responses against Pool TK-A, TK-C, JK-D and Pool CT with variable restriction to HLA-DR molecules (**Figure 17B**). CD4 T cells of patient 3 recognized Pool TK-B **(Figure 17C)**. Due to limitations of DCs in this patient, the recognition was confirmed using CD14-CD3 depleted PBMCs as APCs in the IFN-γ ELISPOT.

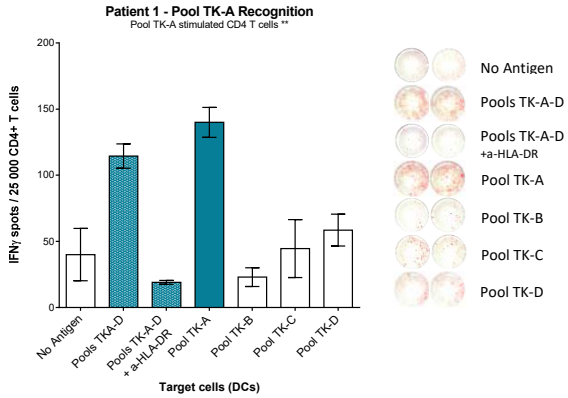
Patient 4 was analyzed at diagnosis and in remission after therapy. At diagnosis, an *ex vivo* CD4 T cell response to Pool NT was detected, which suggested already established memory cells (**Figure 17D**, CD4 memory response). Furthermore, the *ex vivo* recognition of the control Pools, TK-A-D and NT/CT, was the first indication of CD4 T cell reactivity to the NPM-ALK fusion peptide. This was confirmed by Pool NT stimulated CD4 T cells, that strongly recognized the N-terminal region and the respective controls. In addition, CD4 T cell responses to Pool TK-B and Pool CT were observed (**Figure 17D**). 1.5 years after therapy, CD4 memory T cells against the fusion peptide were still present. The strong reactivity to the NPM-ALK fusion peptide was confirmed after separate enrichment of CD4 T cells to the fusion peptide, to Pool NT, and to a control pool consisting of Pool NT, Pool TK-B and Pool CT (indicated as All Pools) (**Figure 17E**). The NPM-ALK fusion represents a cancer-specific neoepitope and a potential target for immunotherapy. This is the first report of CD4 T cells recognizing the NPM-ALK fusion region. The observed CD4 T cell response to Pool TK-B and Pool CT at diagnosis, was, however, lost after therapy.

Patient 5 exhibited ALK-reactive CD4 T cells against Pool NT and CT (**Figure 17F**) which were also recognized by patient´s 6 CD4 T cells (**Figure 17G**). In addition, in patient 6, CD4 T cells to Pool JK-D could be enriched, and the response abrogated by HLA-DR blocking (**Figure 17G**). Surprisingly, patient 7 harboured ALK-reactive CD4 T cells with TCRs recognizing peptides in all tested ALK pools (**Figure 17H**). Of note, this patient had an x-ALK translocation with an unknown ALK fusion partner. Furthermore, the recognition of Pool TK-C-enriched CD4 T cells of both, Pool TK-C and JK-D, and vice versa indicates a CD4 T cell epitope within the overlapping sequence of TK-C6 and JK-D1 (**Figure 17H**). In patient 9, peptides within Pool TK-B and Pool CT were recognized by CD4 T cells (**Figure 17I**).

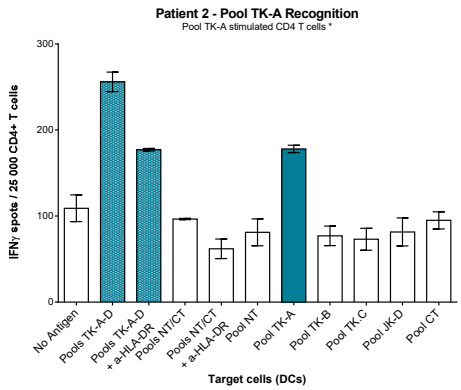
This data demonstrates the existence of a natural occurring CD4 T cell repertoire recognizing ALK in the peripheral blood of ALK$^+$ALCL patients after therapy and healthy donors, which can be primed and enriched *in vitro*. Specific regions within the N-terminal intracellular domain (Pool NT), the tyrosine kinase domain (Pool TK- B), and the C-terminal end (Pool CT) of the ALK protein seem to be naturally immunogenic and contain promiscuous CD4 T cell epitopes independent of the HLA haplotype across all donors. However, compared to healthy donors, the CD4 TCR repertoire against ALK in patients showed a broader regional diversity and was partly directed also to other ALK regions (**Figure 18**).

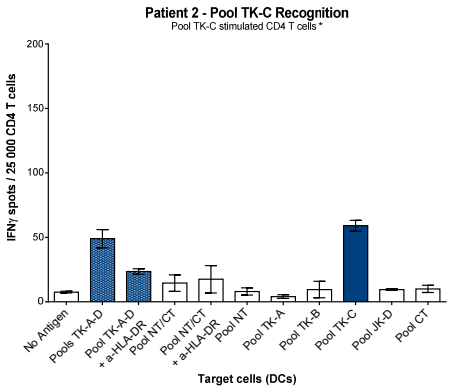
A summary of ALK pool recognition by CD4 T cells of ALK$^+$ALCL patients and healthy donors is shown in **Table 6**.

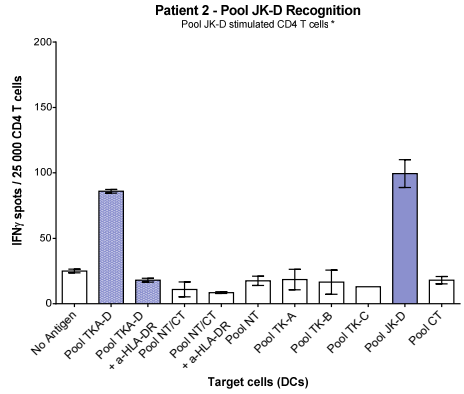
A

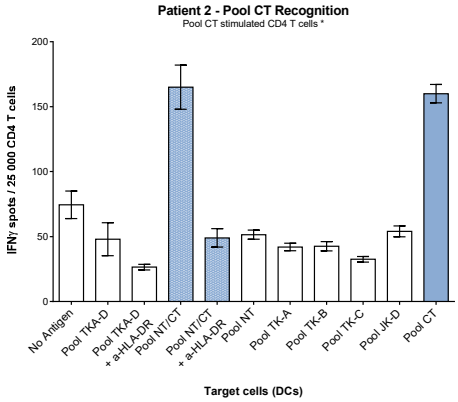

Patient 1 - Pool TK-A Recognition
Pool TK-A stimulated CD4 T cells **
IFNγ spots / 25 000 CD4+ T cells
200
150
100
50
0
No Antigen
Pools TKA-D
Pools TK-A-D + a-HLA-DR
Pool TK-A
Pool TK-B
Pool TK-C
Pool TK-D
Target cells (DCs)
No Antigen
Pools TK-A-D
Pools TK-A-D +a-HLA-DR
Pool TK-A
Pool TK-B
Pool TK-C
Pool TK-D

B

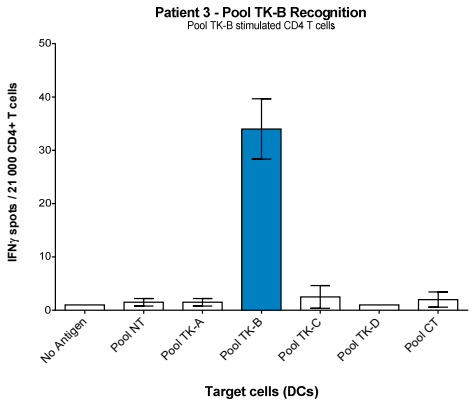

Patient 2 - Pool TK-A Recognition
Pool TK-A stimulated CD4 T cells *
IFNγ spots / 25 000 CD4+ T cells
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

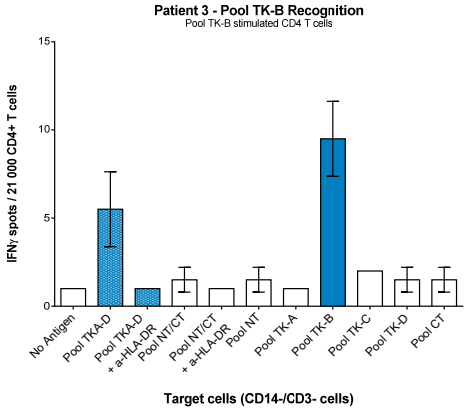

Patient 2 - Pool TK-C Recognition
Pool TK-C stimulated CD4 T cells *
IFNγ spots / 25 000 CD4 T cells
200
150
100
50
0
No Antigen
Pools TK-A-D
Pool TK-A-D + a-HLA-DR
Pool NT/CT
Pool NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)


Patient 2 - Pool JK-D Recognition
Pool JK-D stimulated CD4 T cells *
IFNγ spots / 25 000 CD4 T cells
200
150
100
50
0
No Antigen
Pool TKA-D
Pool TKA-D + a-HLA-DR
Pool NT/CT
Pool NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

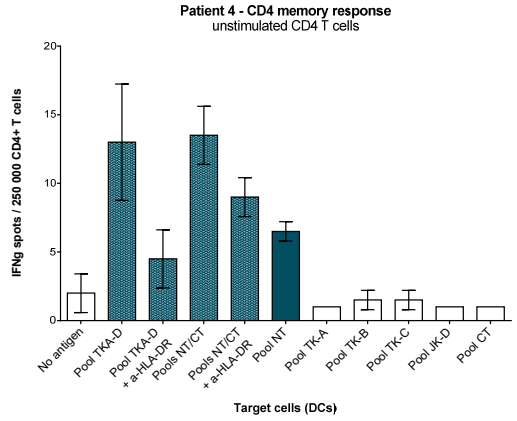

Patient 2 - Pool CT Recognition
Pool CT stimulated CD4 T cells *
IFNγ spots / 25 000 CD4 T cells
200
150
100
50
0
No Antigen
Pool TKA-D
Pool TKA-D + a-HLA-DR
Pool NT/CT
Pool NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

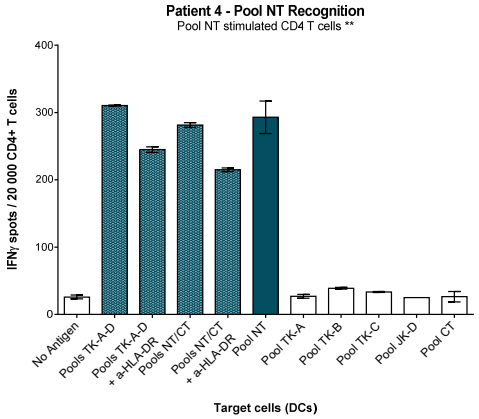
C

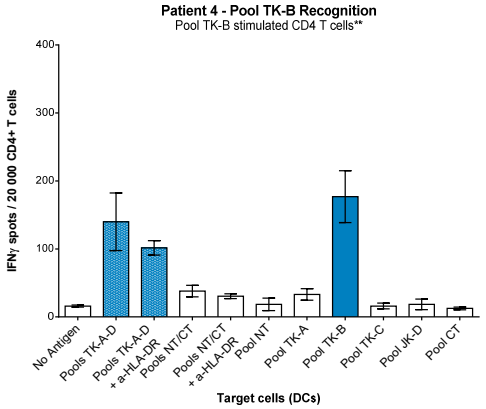

Patient 3 - Pool TK-B Recognition
Pool TK-B stimulated CD4 T cells
IFNγ spots / 21 000 CD4+ T cells
50
40
30
20
10
0
No Antigen
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool TK-D
Pool CT
Target cells (DCs)

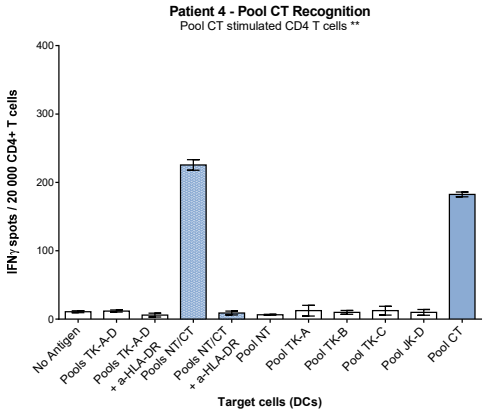

Patient 3 - Pool TK-B Recognition
Pool TK-B stimulated CD4 T cells
IFNγ spots / 21 000 CD4+ T cells
15
10
5
0
No Antigen
Pool TKA-D
Pool TKA-D + a-HLA-DR
Pool NT/CT
Pool NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool TK-D
Pool CT
Target cells (CD14-/CD3- cells)

D

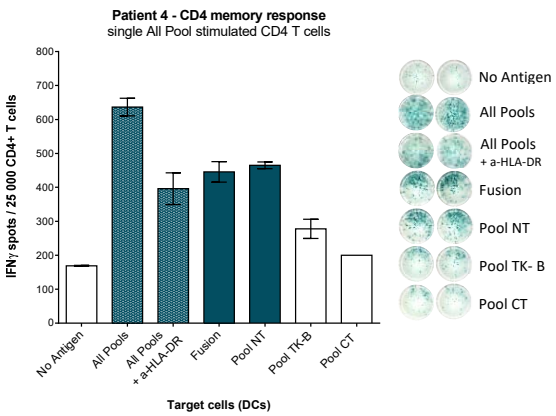

Patient 4 - CD4 memory response
unstimulated CD4 T cells
IFNg spots / 250 000 CD4+ T cells
Target cells (DCs)
No antigen
Pool TKA-D
Pool TKA-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT

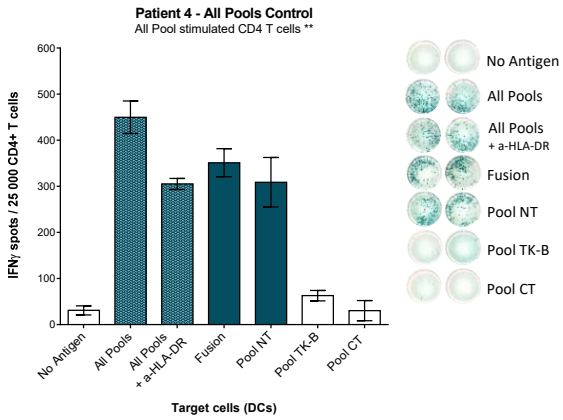

Patient 4 - Pool NT Recognition
Pool NT stimulated CD4 T cells **
IFNγ spots / 20 000 CD4+ T cells
Target cells (DCs)
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT

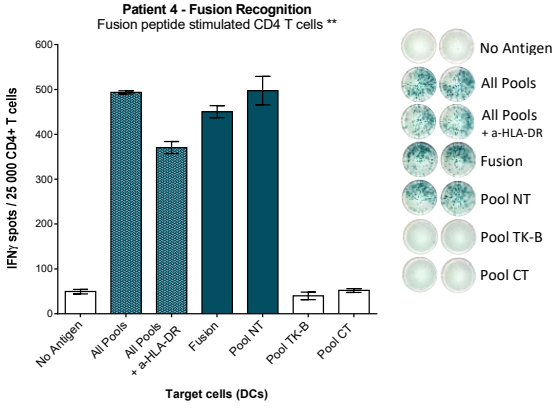

Patient 4 - Pool TK-B Recognition
Pool TK-B stimulated CD4 T cells**
IFNγ spots / 20 000 CD4+ T cells
Target cells (DCs)
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT

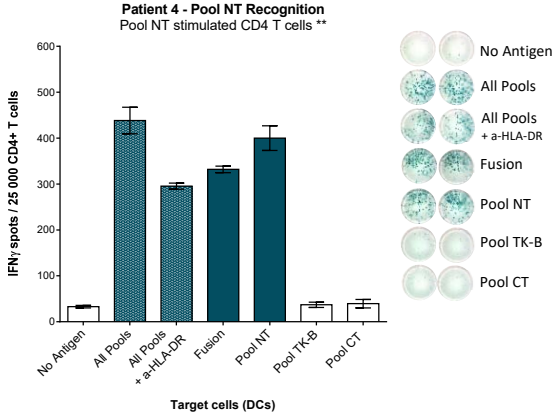

Patient 4 - Pool CT Recognition
Pool CT stimulated CD4 T cells **
IFNγ spots / 20 000 CD4+ T cells
Target cells (DCs)
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT

E

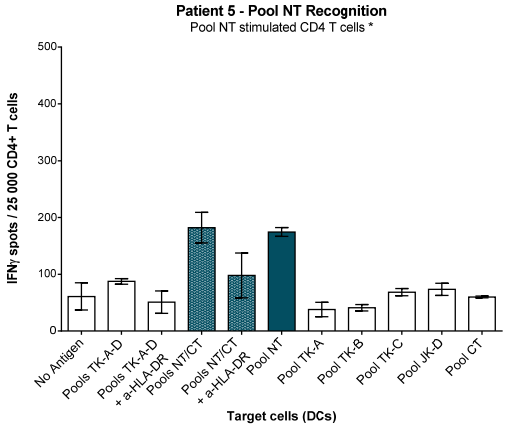

Patient 4 - CD4 memory response
single All Pool stimulated CD4 T cells
IFNγ spots / 25 000 CD4+ T cells
Target cells (DCs)
No Antigen
All Pools
All Pools + a-HLA-DR
Fusion
Pool NT
Pool TK-B
Pool CT
No Antigen
All Pools
All Pools + a-HLA-DR
Fusion
Pool NT
Pool TK- B
Pool CT

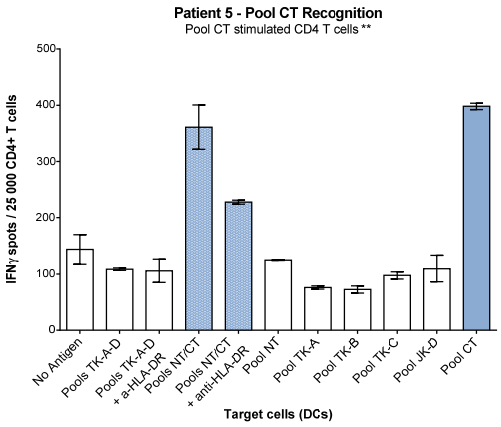

Patient 4 - All Pools Control
All Pool stimulated CD4 T cells **
IFNγ spots / 25 000 CD4+ T cells
Target cells (DCs)
No Antigen
All Pools
All Pools + a-HLA-DR
Fusion
Pool NT
Pool TK-B
Pool CT
No Antigen
All Pools
All Pools + a-HLA-DR
Fusion
Pool NT
Pool TK-B
Pool CT


Patient 4 - Fusion Recognition
Fusion peptide stimulated CD4 T cells **
IFNγ spots / 25 000 CD4+ T cells
Target cells (DCs)
No Antigen
All Pools
All Pools + a-HLA-DR
Fusion
Pool NT
Pool TK-B
Pool CT
No Antigen
All Pools
All Pools + a-HLA-DR
Fusion
Pool NT
Pool TK-B
Pool CT

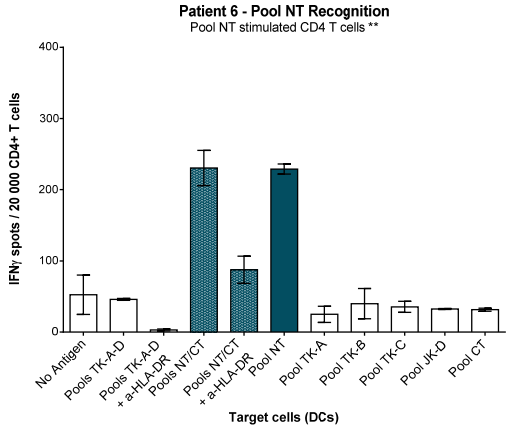

Patient 4 - Pool NT Recognition
Pool NT stimulated CD4 T cells **
IFNγ spots / 25 000 CD4+ T cells
Target cells (DCs)
No Antigen
All Pools
All Pools + a-HLA-DR
Fusion
Pool NT
Pool TK-B
Pool CT
No Antigen
All Pools
All Pools + a-HLA-DR
Fusion
Pool NT
Pool TK-B
Pool CT

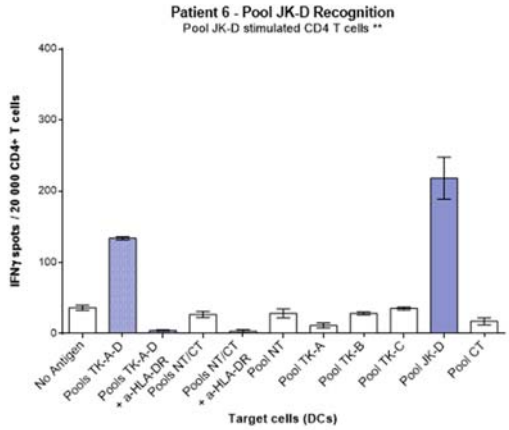
F

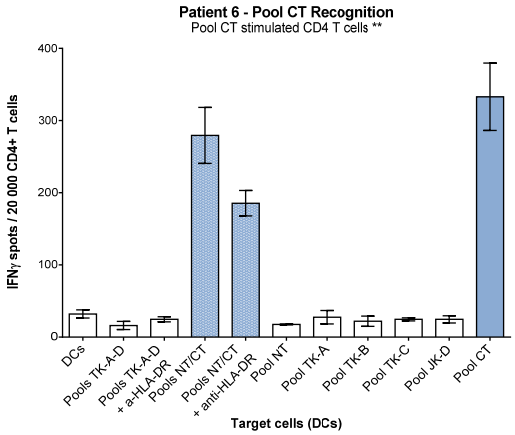

Patient 5 - Pool NT Recognition
Pool NT stimulated CD4 T cells *
IFNγ spots / 25 000 CD4+ T cells
500
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)


Patient 5 - Pool CT Recognition
Pool CT stimulated CD4 T cells **
IFNγ spots / 25 000 CD4+ T cells
500
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + anti-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

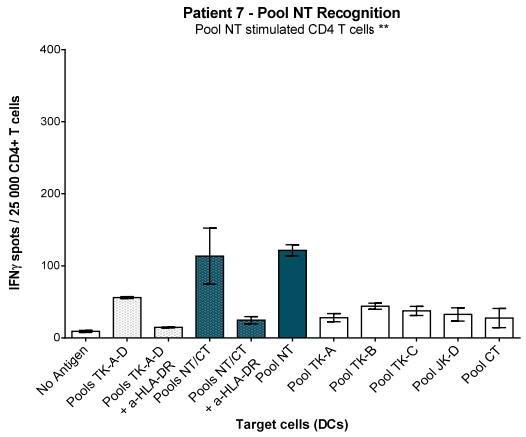
G

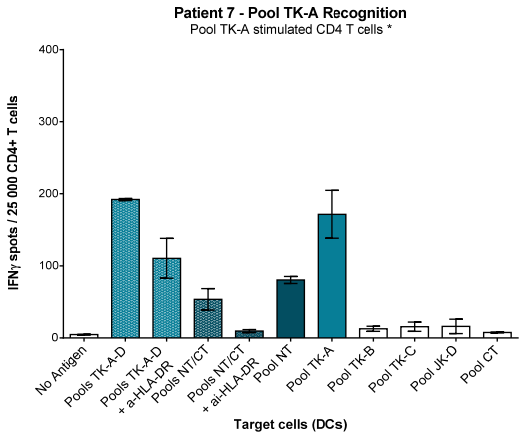

Patient 6 - Pool NT Recognition
Pool NT stimulated CD4 T cells **
IFNγ spots / 20 000 CD4+ T cells
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

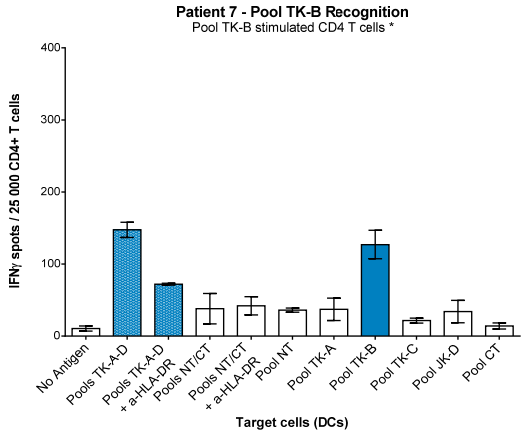

Patient 6 - Pool JK-D Recognition
Pool JK-D stimulated CD4 T cells **
IFNγ spots / 20 000 CD4+ T cells
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

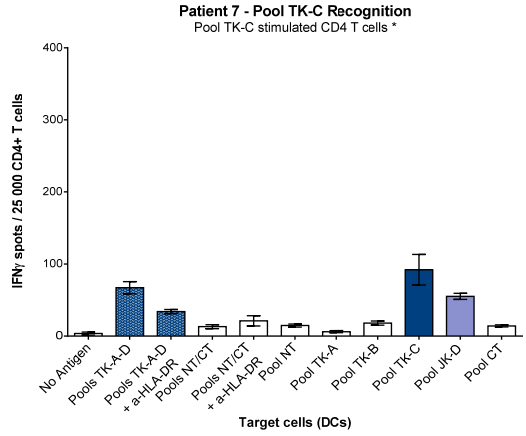

Patient 6 - Pool CT Recognition
Pool CT stimulated CD4 T cells **
IFNγ spots / 20 000 CD4+ T cells
400
300
200
100
0
DCs
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + anti-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

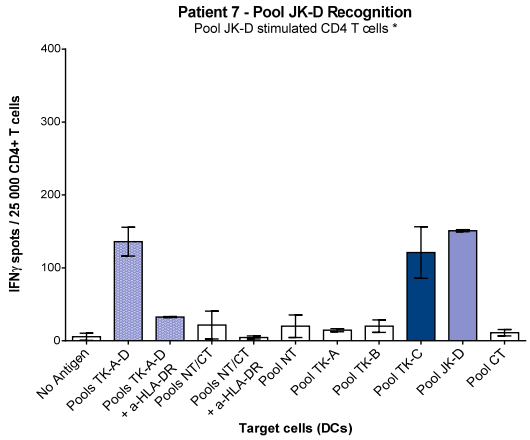
H

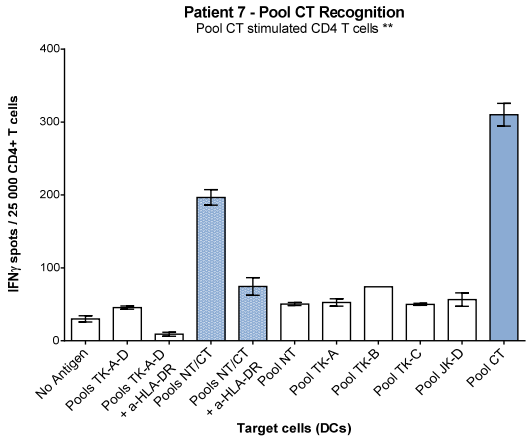

Patient 7 - Pool NT Recognition
Pool NT stimulated CD4 T cells **
IFNγ spots / 25 000 CD4+ T cells
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)


Patient 7 - Pool TK-A Recognition
Pool TK-A stimulated CD4 T cells *
IFNγ spots / 25 000 CD4+ T cells
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)


Patient 7 - Pool TK-B Recognition
Pool TK-B stimulated CD4 T cells *
IFNγ spots / 25 000 CD4+ T cells
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)


Patient 7 - Pool TK-C Recognition
Pool TK-C stimulated CD4 T cells *
IFNγ spots / 25 000 CD4+ T cells
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)


Patient 7 - Pool JK-D Recognition
Pool JK-D stimulated CD4 T cells *
IFNγ spots / 25 000 CD4+ T cells
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)


Patient 7 - Pool CT Recognition
Pool CT stimulated CD4 T cells **
IFNγ spots / 25 000 CD4+ T cells
400
300
200
100
0
No Antigen
Pools TK-A-D
Pools TK-A-D + a-HLA-DR
Pools NT/CT
Pools NT/CT + a-HLA-DR
Pool NT
Pool TK-A
Pool TK-B
Pool TK-C
Pool JK-D
Pool CT
Target cells (DCs)

I

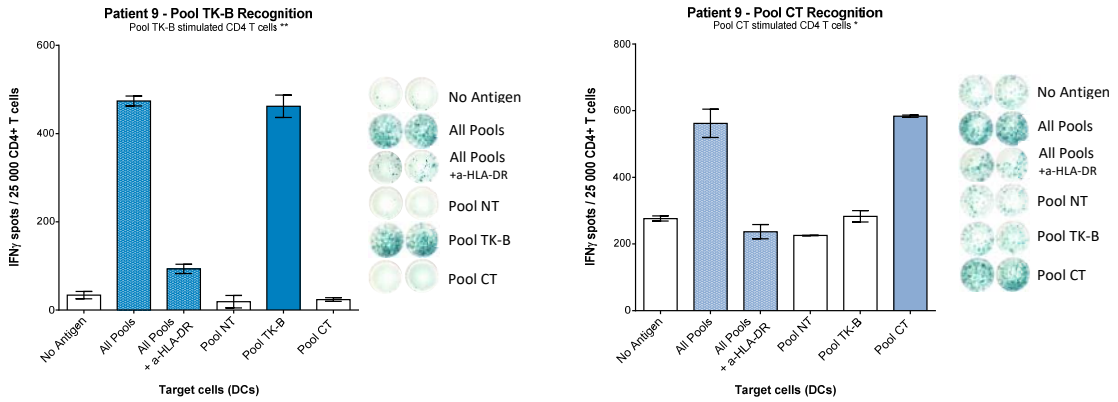


Figure 17: CD4 T cell responses against ALK peptide pools in ALK$^+$ALCL patients
(**A-I**) Quantification of IFN-γ secreting CD4 T cells in response to DCs loaded with respective ALK pools in indicated ALK$^+$ALCL patient´s. Graphs show the mean number of spot counts (equal to the number of IFN- γ secreting CD4 T cells) +/- SD to the indicated conditions (x-axis; DCs pulsed with the respective ALK pool or without antigen). A response was defined as positive when the spot count was ≥ 2-fold higher than the mean of the negative control (no antigen). For each condition, two separate cultures were initiated and were tested in duplicates in the IFN-γ ELISPOT. Asterisks in the heading indicate if one (*), or both (**) initiated cultures recognized the respective ALK pool. Control pool TK-A-D contained the single fusion peptide and Pools-TK-A-D; Control Pool NT/CT consisted of Pool NT and Pool CT; All Pool control indicates all peptides from Pool NT, Pool TK-B, and Pool CT. HLA-DR blocking is indicated as Pool “X”+ a-HLA-DR.

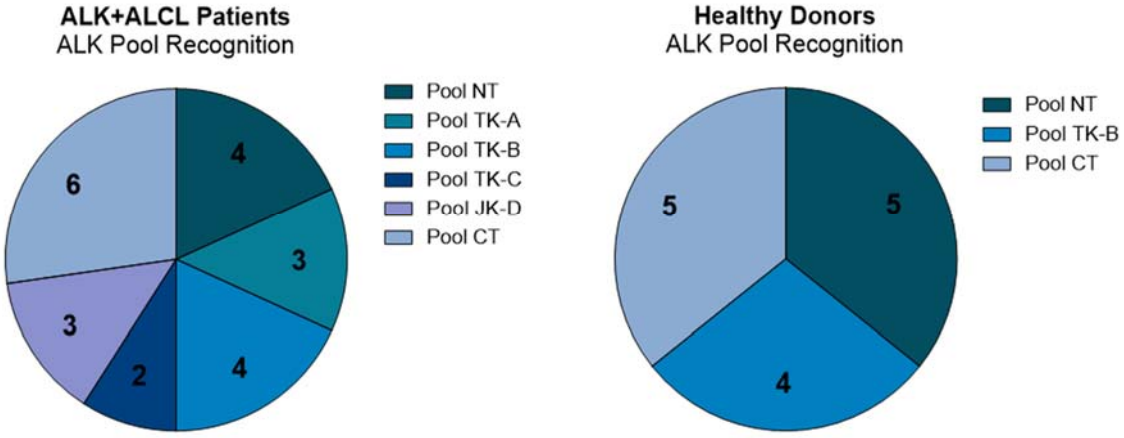


Figure 18: Distribution of ALK pool recognition in ALK$^+$ALCL patients and healthy donors.
Numbers indicate responding patients (left) or healthy donors (right) in whom ALK-reactive CD4 T cells to the indicated ALK pool were detected.

Table 6: ALK pool recognition by ALK$^+$ALCL patients and healthy donors

Patient	ALK Translocation	ALK Pool Recognition						Years after Diagnosis
		NT	TK-A	TK-B	TK-C	JK-D	CT	
1	NPM-ALK		+					1.8
2	NPM-ALK		+		+	+	+	3.2
3	TPM3-ALK			+				7.8
4	NPM-ALK	+		+			+	0
		+						2.0
5	NPM-ALK	+					+	2.8
6	X-ALK	+				+	+	2.1
7	X-ALK	+	+	+	+	+	+	3.11
8	NPM-ALK							12.3
9	NPM-ALK			+			+	3.5
Healthy Donor								
1	-	+					+	-
2	-			+				-
3	-	+		+			+	-
4	-	+		+			+	-
5	-	+					+	-
6	-	+		+			+	-

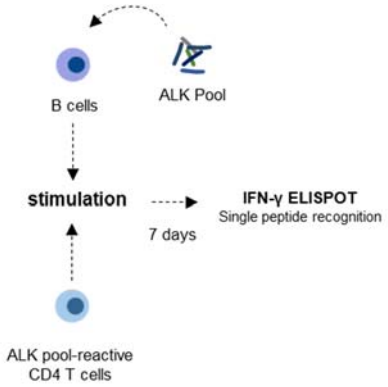
NPM=nucleophosmin; ALK=anaplastic lymphoma kinase; TPM3=tropomyosin3; X-ALK=variant/unknown fusion partner

4.2. Identification of single immunogenic ALK peptides in ALK$^+$ALCL patients

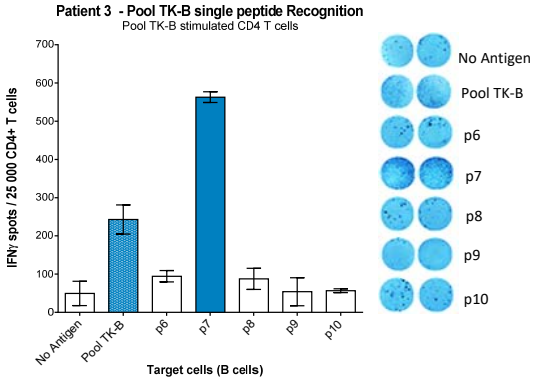
Current available algorithms to predict MHC class II epitopes are often inaccurate due to the variety in alleles and the open structure of the MHC class II binding groove that leads to substantial variation in epitope length. To identify single 30mer immunogenic ALK peptides, containing the respective CD4 T cell epitopes in ALK+ALCL patients, ALK Pool-reactive CD4 T cells were once re-stimulated with ALK peptide loaded autologous B cells and subsequently tested in an IFN-γ ELISPOT for single peptide recognition (**Figure 19A**).
Pool TK-B reactive CD4 T cells of patient 3 were tested against 36 aa long single ALK peptides with a 12 aa sequence overlap (p6-10) corresponding to the sequence of Pool TK-B. The recognized epitope was identified within peptide 7 (p7), which covers the sequence of the single peptides TK-B1+2 (**Figure 19B**).
To dissect the reactivity against the fusion region in patient 4, Pool NT enriched CD4 T cells were tested for recognition of the single Pool NT peptides (fusion peptide, NT-1, NT-2, NT-3, and NT-4). CD4 T cells strongly recognized the fusion peptide, confirming epitope recognition within the 30 aa long sequence (**Figure 19C**). To exclude NPM reactivity and to narrow down the exact epitope, the fusion-reactive CD4 T cells were further tested against 15mer peptides covering the sequence of NPM (F1), the NPM-ALK fusion (F2), and the adjacent part of ALK (F3), providing evidence for the exact CD4 T cell epitope in the NPM-ALK fusion (**Figure 19D**).
The CD4 T cell epitope in patient's 5 Pool CT reactive cells was identified within single peptide CT-3 (**Figure 19E**). In patient 6, Pool CT-reactive CD4 T cells recognized an epitope within the 16 aa overlapping sequence of CT-2 and CT-3 (**Figure 19F**) and in patient-7, a CD4 T cell epitope within the C-terminal ALK region was identified in the overlapping sequence of CT-1 and CT-1 (**Figure 19G**). In addition, in this patient, the 16 aa long sequence in the TK-C6-JK-D1 overlapping region contained another epitope (shown in **Figure 17H**).

This is the first description of human CD4 T cell epitopes within ALK. However, no correlation between epitope recognition and patient´s HLA haplotypes or ALK-antibody titers at diagnosis or analysis was identified.

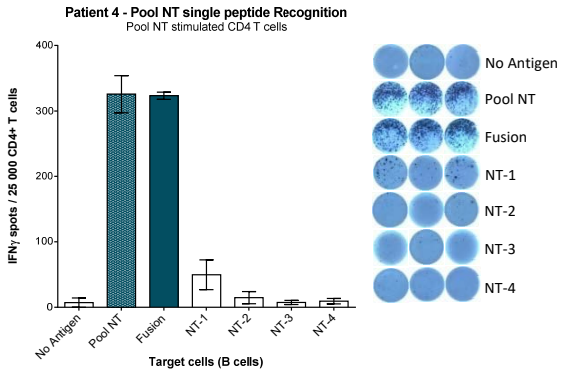
A


B cells
ALK Pool
stimulation
7 days
IFN-γ ELISPOT
Single peptide recognition
ALK pool-reactive
CD4 T cells

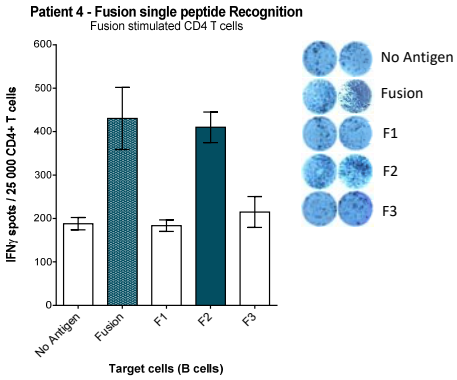
B


Patient 3 - Pool TK-B single peptide Recognition
Pool TK-B stimulated CD4 T cells
IFNγ spots / 25 000 CD4+ T cells
No Antigen
Pool TK-B
p6
p7
p8
p9
p10
Target cells (B cells)

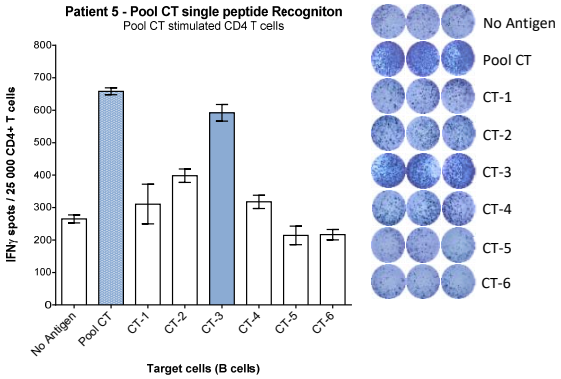
C


Patient 4 - Pool NT single peptide Recognition
Pool NT stimulated CD4 T cells
IFNγ spots / 25 000 CD4+ T cells
No Antigen
Pool NT
Fusion
NT-1
NT-2
NT-3
NT-4
Target cells (B cells)

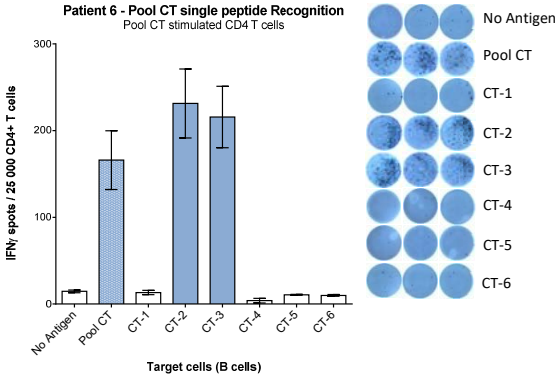
D


Patient 4 - Fusion single peptide Recognition
Fusion stimulated CD4 T cells
IFNγ spots / 25 000 CD4+ T cells
No Antigen
Fusion
F1
F2
F3
Target cells (B cells)

E


Patient 5 - Pool CT single peptide Recogniton
Pool CT stimulated CD4 T cells
IFNγ spots / 25 000 CD4+ T cells
No Antigen
Pool CT
CT-1
CT-2
CT-3
CT-4
CT-5
CT-6
Target cells (B cells)

F


Patient 6 - Pool CT single peptide Recognition
Pool CT stimulated CD4 T cells
IFNγ spots / 25 000 CD4+ T cells
No Antigen
Pool CT
CT-1
CT-2
CT-3
CT-4
CT-5
CT-6
Target cells (B cells)

G

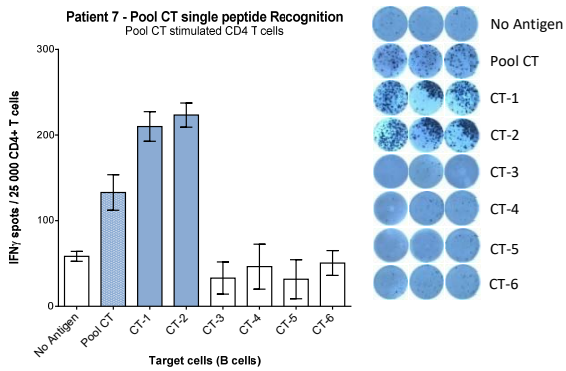

Patient 7 - Pool CT single peptide Recognition
Pool CT stimulated CD4 T cells
IFNγ spots / 25 000 CD4+ T cells
No Antigen
Pool CT
CT-1
CT-2
CT-3
CT-4
CT-5
CT-6
Target cells (B cells)

Figure 19: Single ALK peptide identification in ALK$^+$ALCL patients
(**A**) Experimental layout for the identification of single ALK peptides recognized by patient´s CD4 T cells. Autologous B cells were pulsed with the respective ALK pool and used to re-stimulate ALK-reactive CD4 T cells, which were then tested in an IFN-γ ELISPOT for single ALK peptide recognition. (**B-G**) Quantification of IFN-γ secreting CD4 T cells in response to B cells loaded with indicated ALK peptides in ALK$^+$ALCL patient´s. Graphs show the mean number of spot counts +/- SD to the indicated conditions (x-axis; B cells pulsed with the respective ALK peptides or without antigen). A response was defined as positive when the spot count was ≥ 2-fold higher than the mean of the negative control (no antigen).

4.3. ALK peptide vaccines induce spontaneous CD4 T cell responses in mice

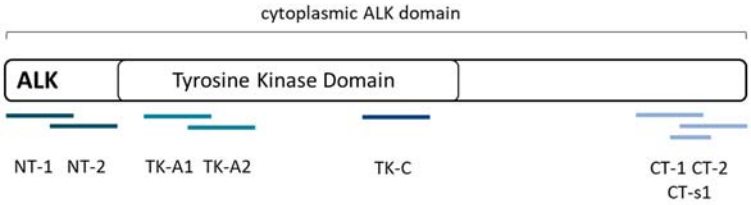
To investigate whether the immunogenic ALK regions also induce CD4 T cell responses *in vivo,* vaccination experiments in mice were performed. Preliminary immunization experiments in BALB/c mice with an DNA-based ALK vaccine that encodes for the entire intracellular portion of ALK translocations led to a strong T cell immune response with therapeutic efficacy in both, ALK-positive ALCL, and NSCLC models (Chiarle, Martinengo, et al. 2008; Voena et al. 2015). By dissecting these T cell responses via an array of 36 aa long overlapping synthetic peptides encompassing the cytoplasmic ALK portion, several immunogenic ALK peptides were identified (unpublished, data not shown, Dr. Chiarle Laboratory, Boston Children´s Hospital, MA, USA) that were determined to include CD4 T cell epitopes in the N-terminal part (NT-1 and NT-2), in the tyrosine kinase domain (TK-A1 and A2) and the C-terminus (CT-1 and CT-2) of ALK (**Figure 20A**). Based on this data and the findings in humans, in whom similar ALK regions induced CD4 T cell responses (described above, **Figure 16,17, 18, 19**), these peptides were selected to be included in individual ALK peptide vaccines to study ALK-induced CD4 T cell responses in mice. Despite high sequence homology between mouse and human ALK, peptides CT-1+2 are human-specific (i.e. composed of an amino acid sequence that differs between mouse and human ALK), whereas the sequences of peptides NT-1+2 and TK-A1+2 represent shared antigens with an identical sequence in both organisms.

To determine ALK-induced CD4 T cell responses, BALB/c mice were immunized with ALK vaccines composed of the individual ALK peptides (NT-1+2, TK-A1+2, or CT-1+2) and the adjuvant cyclic diguanylate monophosphate (c-di-GMP) which is a cyclic dinucleotide (CDN) and STING agonist known to increase vaccine potency (Dubensky et al. 2013) (**Figure 20B**). After three vaccinations in an interval of two weeks, splenocytes of vaccinated and control mice (non-vaccinated) were *in vitro* stimulated with the respective ALK peptides and the CD4 T cell response analyzed for ALK reactivity (IFN-γ secretion) via flow cytometry (**Figure 20B**). Surprisingly, all tested ALK vaccines induced strong and reproducible spontaneous CD4 T cell responses in BALB/c mice (**Figure 20C, D**). In none of the control mice, CD4 T cell responses to ALK were observed **(Figure 20C, D)**. Vaccines composed of NT-1+2 or TK-A1+2 elicited comparable CD4 T cell responses with up to 9% of analyzed CD4 T cells (10^4) secreting IFN-γ in response to the respective peptides (**Figure 20D**). In CT-1+2 immunized mice, CD4 T cell responses were even higher, with up to 13% of analyzed CD4 T cells responding to the ALK peptides (**Figure 20D**). In addition to the single CT-1 and CT-2 peptides, splenocytes of CT-1+2 vaccinated mice were tested for recognition of a 19 aa long peptide (CT-s1) encompassing an overlapping region of CT-1 and CT-2, which was equally recognized by CD4 T cells confirming an epitope within this sequence (**Figure 20D**). To validate the CD4 T cell epitope within CT-s1 and to evaluate if ALK immunogenicity and the observed vaccine induced CD4 T

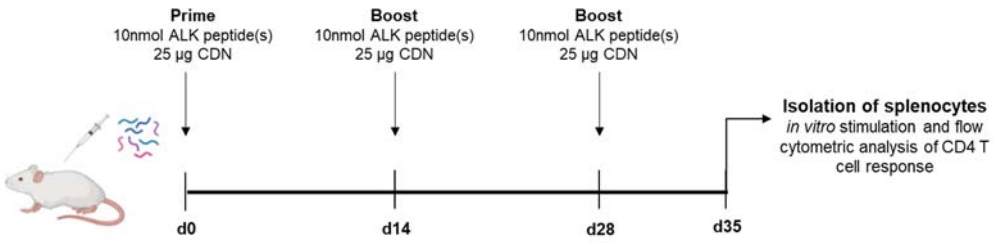
cell response is restricted to the MHC class II alleles, I-A^d and I-E^d, of BALB/c mice, BALB/c and C57BL/6 mice were immunized with the CT-s1 vaccine. Comparable spontaneous CD4 T cell responses to CT-s1 were found in both mouse strains confirming that immunogenic ALK peptides are also presented by I-A^b (C57BL/6 haplotype) molecules (**Figure 20E, F**).
It is, indeed, surprising, that all tested ALK vaccines induced almost equally strong CD4 T cell responses. Therefore, a 36 aa long ALK peptide (TK-C) with sequence homology in mice and humans, which showed no specific immunogenicity in the ALK DNA vaccine induced immune response, was selected for immunization (**Figure 20A**). Vaccine-induced CD4 T cell responses to TK-C could be detected in immunized mice (**Figure 20G, H**). Although, the responses were modest (mean around 3%) compared to the other vaccines, it is possible that the potent vaccine formulation combined with the overall observed immunogenicity of ALK induces an "background" reactivity to otherwise less immunogenic ALK peptides.

Together these data demonstrate that ALK vaccines composed of peptides comprising potential CD4 T cell epitopes, induce spontaneous and measurable ALK-specific CD4 T cell responses *in vivo*. These CD4 T cell responses can be potentially harnessed for therapeutic ALK vaccines to support ALK-specific CD8 T cells.

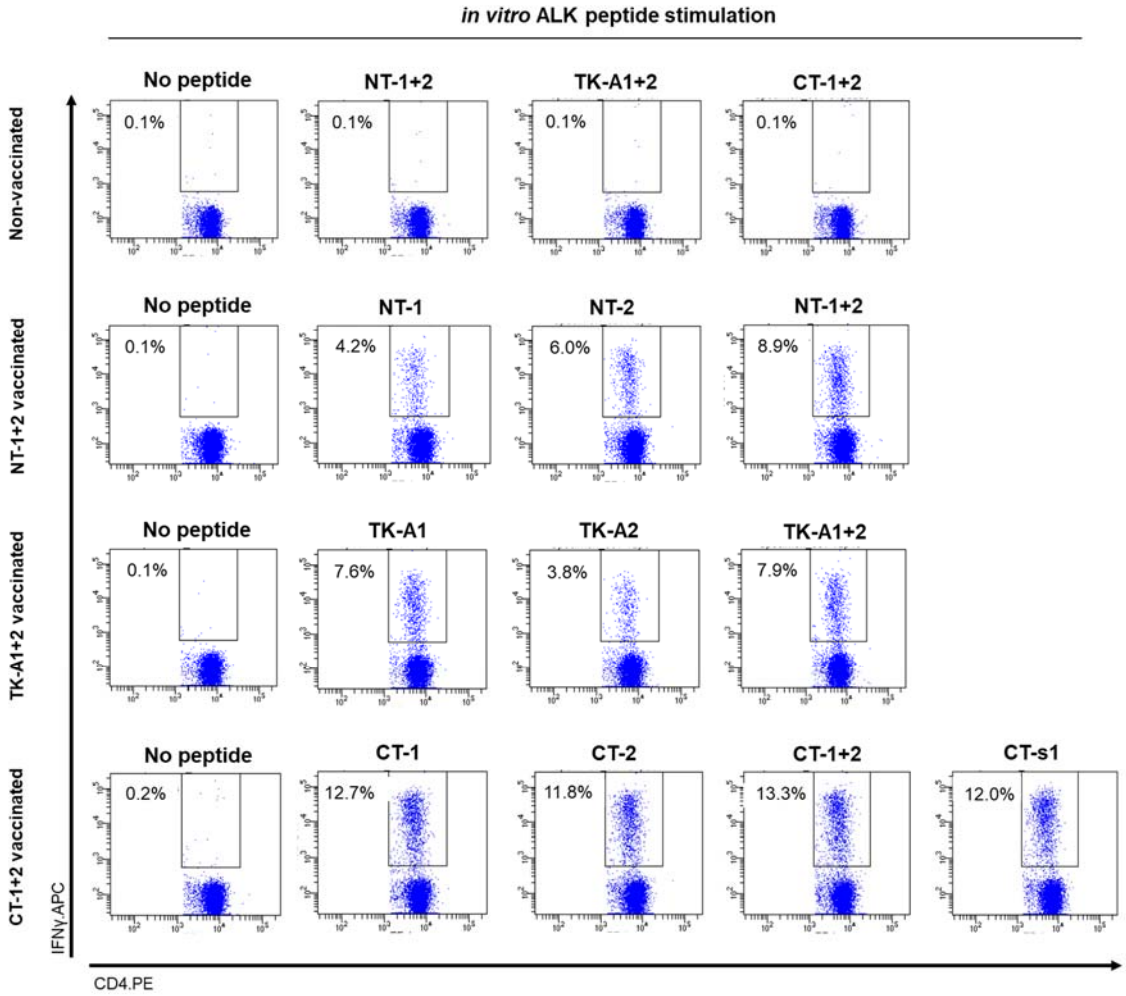
A


cytoplasmic ALK domain
ALK
Tyrosine Kinase Domain
NT-1 NT-2
TK-A1 TK-A2
TK-C
CT-1 CT-2
CT-s1

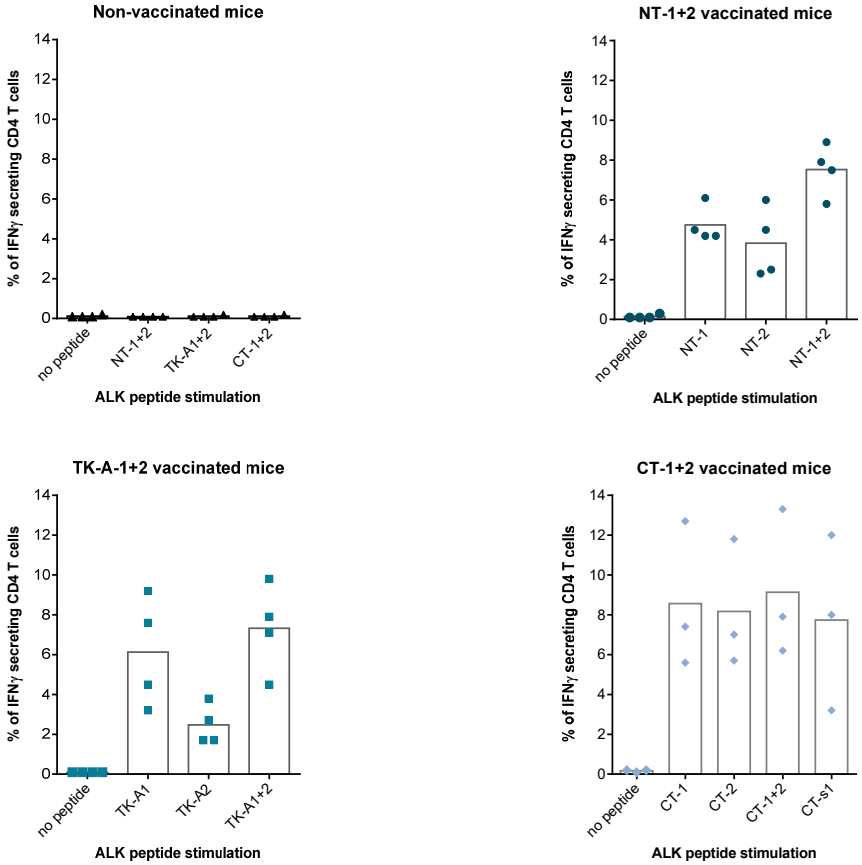
B


Prime
10nmol ALK peptide(s)
25 µg CDN
Boost
10nmol ALK peptide(s)
25 µg CDN
Boost
10nmol ALK peptide(s)
25 µg CDN
Isolation of splenocytes
in vitro stimulation and flow cytometric analysis of CD4 T cell response
d0
d14
d28
d35

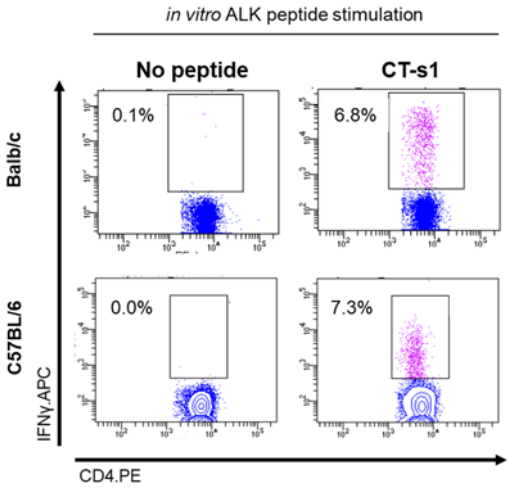
C


in vitro ALK peptide stimulation
Non-vaccinated
NT-1+2 vaccinated
TK-A1+2 vaccinated
CT-1+2 vaccinated
No peptide
NT-1+2
TK-A1+2
CT-1+2
0.1%
0.1%
0.1%
0.1%
No peptide
NT-1
NT-2
NT-1+2
0.1%
4.2%
6.0%
8.9%
No peptide
TK-A1
TK-A2
TK-A1+2
0.1%
7.6%
3.8%
7.9%
No peptide
CT-1
CT-2
CT-1+2
CT-s1
0.2%
12.7%
11.8%
13.3%
12.0%
IFNγ.APC
CD4.PE

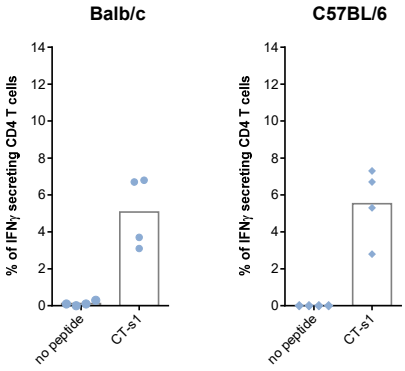
D


Non-vaccinated mice
NT-1+2 vaccinated mice
TK-A-1+2 vaccinated mice
CT-1+2 vaccinated mice
% of IFNγ secreting CD4 T cells
ALK peptide stimulation
no peptide
NT-1+2
TK-A1+2
CT-1+2
NT-1
NT-2
TK-A1
TK-A2
CT-1
CT-2
CT-s1

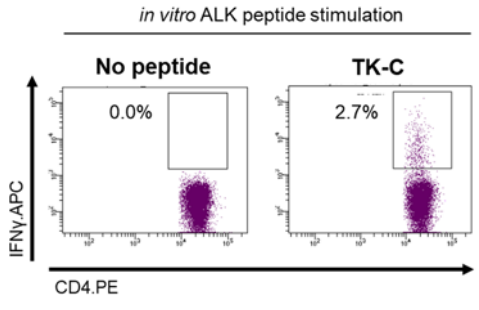
E


in vitro ALK peptide stimulation
No peptide
CT-s1
Balb/c
C57BL/6
0.1%
6.8%
0.0%
7.3%
IFNγ.APC
CD4.PE

F


Balb/c
C57BL/6
% of IFNγ secreting CD4 T cells
no peptide
CT-s1

G


in vitro ALK peptide stimulation
No peptide
TK-C
0.0%
2.7%
IFNγ.APC
CD4.PE

H

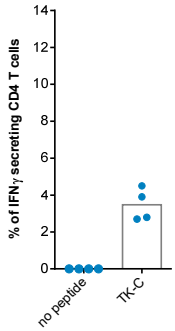

% of IFNγ secreting CD4 T cells
no peptide
TK-C

Figure 20: ALK vaccine induced spontaneous CD4 T cell responses in mice.
(**A**) Schematic representation of ALK peptides used in immunization experiments. (B) Experimental design for the analysis of ALK vaccine induced spontaneous CD4 T cell responses. Mice were immunized three times in an interval of two weeks with ALK vaccines consisting of 10nmol of each respective ALK peptide and 25 µg CDN. Seven days after the third vaccination, splenocytes were isolated and analyzed via flow cytometry for CD4 T cell mediated IFN-γ release in response to ALK peptides. (**C**) Representative ALK vaccine induced CD4 T cell responses in BALB/c mice determined by flow cytometry. (**D**) CD4 T cell mediated IFN-γ release in response to indicated ALK peptides in non-vaccinated mice (n=4), NT-1+2 vaccinated mice (n=4), TK-A1+2 vaccinated mice (n=4), and CT-1+2 vaccinated mice (n=4). (**E, F**) ALK-specific CD4 T cell response in CT-s1 vaccinated Balb/c or C57BL76 mice (both, n=4). (**G, H**) CD4 T cell response to TK-C in Balb/c mice vaccinated with the TK-C vaccine (n=4)

4.4. Identification of a CD4 T cell epitope within the tyrosine kinase domain of ALK

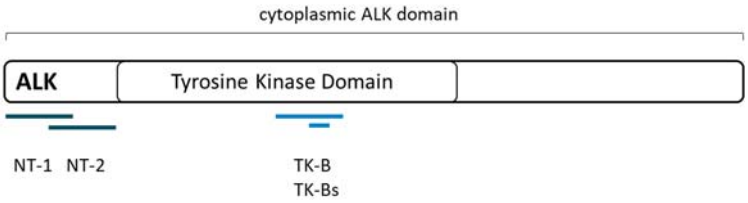
CD4 T cells provide help for CD8 T cells which is essential for protective anti-tumor immunity. In terms of vaccine design, long peptides (25-30 aa) centered around predicted CD8 epitopes are regarded to be superior to short peptides (9-10 aa) because the flanking peptide regions frequently provide CD4 antigens that can boost the CD8 T cell response.

To test whether the inclusion of CD4 T cell antigenic peptides (NT-1 and NT-2) positively affects ALK-specific CD8 T cell responses, combinatorial vaccines were designed for immunization experiments. Mice were either vaccinated with a 36 aa long ALK peptide (TK-B) which encompasses a pervious identified 9mer long CD8 T cell epitope in the tyrosine kinase domain (TK-Bs) (data not shown, unpublished, Dr. Chiarle Laboratory, Boston Children´s Hospital, MA, USA) or in combination with the CD4 T cell peptides NT-1+2 (peptides shown in **Figure 21A**). To monitor T cell responses in both compartments, splenocytes of vaccinated mice were analyzed for CD4-and CD8 T cell mediated IFN-γ secretion upon *in vitro* peptide stimulation and, in addition, stained for the presence of anti-ALK CD8 T cells by dextramers specific for the CD8 T cell epitope TK-Bs.

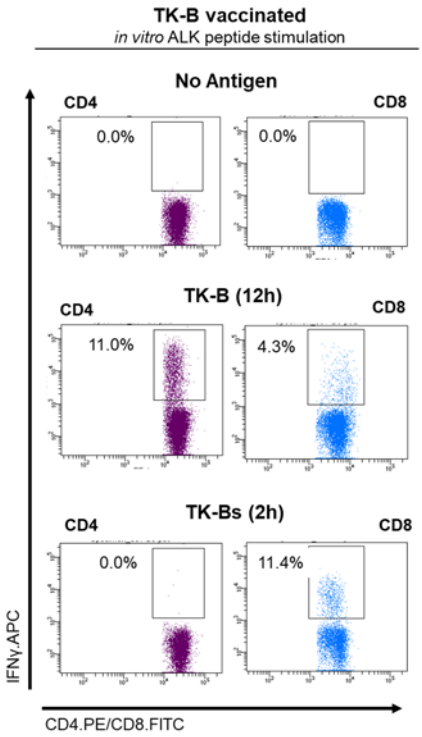
Unexpectedly, the TK-B vaccine, which was purposely included to induce ALK-specific CD8 T cells, induced also strong CD4 T cell responses in vaccinated mice (**Figure 21B, C**). The TK-B specific CD4 T cell response was completely abrogated in splenocytes stimulated with the exact 9mer CD8 T cell epitope TK-Bs, indicating that the newly identified CD4 epitope is located at the flanking regions centered around TK-Bs (**Figure 21B,C**). CD8 T cells responded with IFN-γ secretion, as expected, to TK-B, and even higher, to the exact epitope TK-Bs (**Figure 21B,C**). Furthermore, a high number of ALK-specific CD8 T cells were detected in spleens of vaccinated mice constituting to almost up to 24% of analyzed CD8 T cells (3.5×10^4) (**Figure 21D,E**). After *in vitro* stimulation, up to 11.4% of analyzed CD8 T cells in spleens were functionally producing IFN-γ in response to the TK-Bs peptide (**Figure 21E**). In mice immunized with the vaccine comprising of TK-B and NT-1+2 peptides, a strong CD4 T cell response was observed in response to the NT-1+2 peptides, to the TK-B peptide, and to the triple combination TK-B+NT-1+2, confirming the previous observations and the newly identified CD4 T cell epitope within the TK-B peptide (**Figure 21F, G**). However, compared to mice that were vaccinated only with the TK-B vaccine, CD8 T cell responses to the respective peptides in TK-B+NT-1+2 vaccinated mice were strongly reduced (≤ 2%) (**Figure 21F, G, I**). Also, the percentage of ALK-specific CD8 T cells in spleens was greatly diminished (**Figure 21H**).

These data provide evidence for a previously unknown CD4 T cell epitope in the ALK protein within the flanking region of an already identified CD8 epitope. Although, the inclusion of CD4 helper epitopes in cancer vaccine design has been shown to elicit potent anti-tumor responses, these data indicate that the choice, number, and possibly the location of helper epitopes play a crucial role in ALK vaccine induced CTL immune responses.

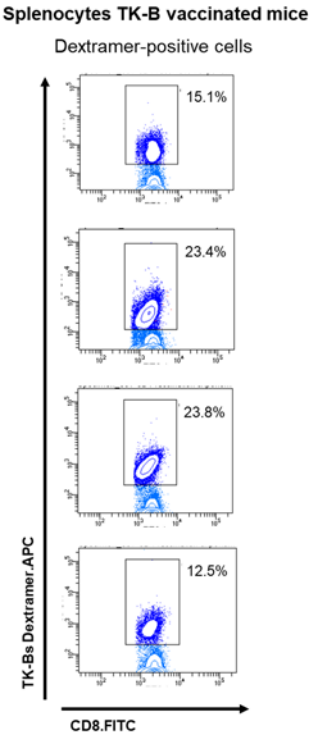
A


cytoplasmic ALK domain
ALK
Tyrosine Kinase Domain
NT-1 NT-2
TK-B
TK-Bs

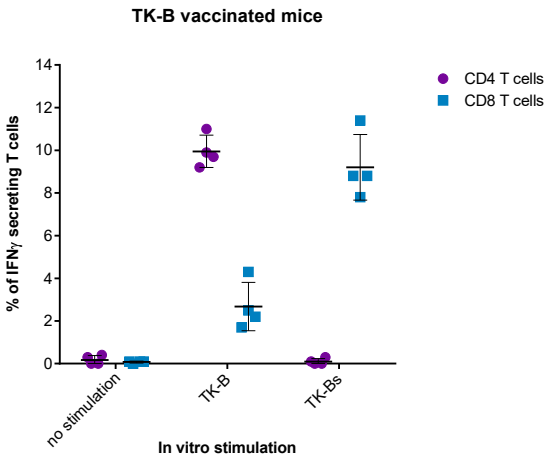
B


TK-B vaccinated
in vitro ALK peptide stimulation
No Antigen
CD4
CD8
0.0%
0.0%
TK-B (12h)
11.0%
4.3%
TK-Bs (2h)
0.0%
11.4%
IFNγ.APC
CD4.PE/CD8.FITC

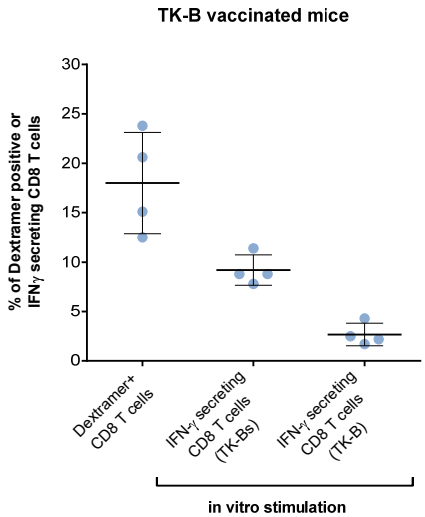
D


Splenocytes TK-B vaccinated mice
Dextramer-positive cells
15.1%
23.4%
23.8%
12.5%
TK-Bs Dextramer.APC
CD8.FITC

C


TK-B vaccinated mice
CD4 T cells
CD8 T cells
% of IFNγ secreting T cells
no stimulation
TK-B
TK-Bs
In vitro stimulation

E


TK-B vaccinated mice
% of Dextramer positive or IFNγ secreting CD8 T cells
Dextramer+ CD8 T cells
IFNγ secreting CD8 T cells (TK-Bs)
IFNγ secreting CD8 T cells (TK-B)
in vitro stimulation

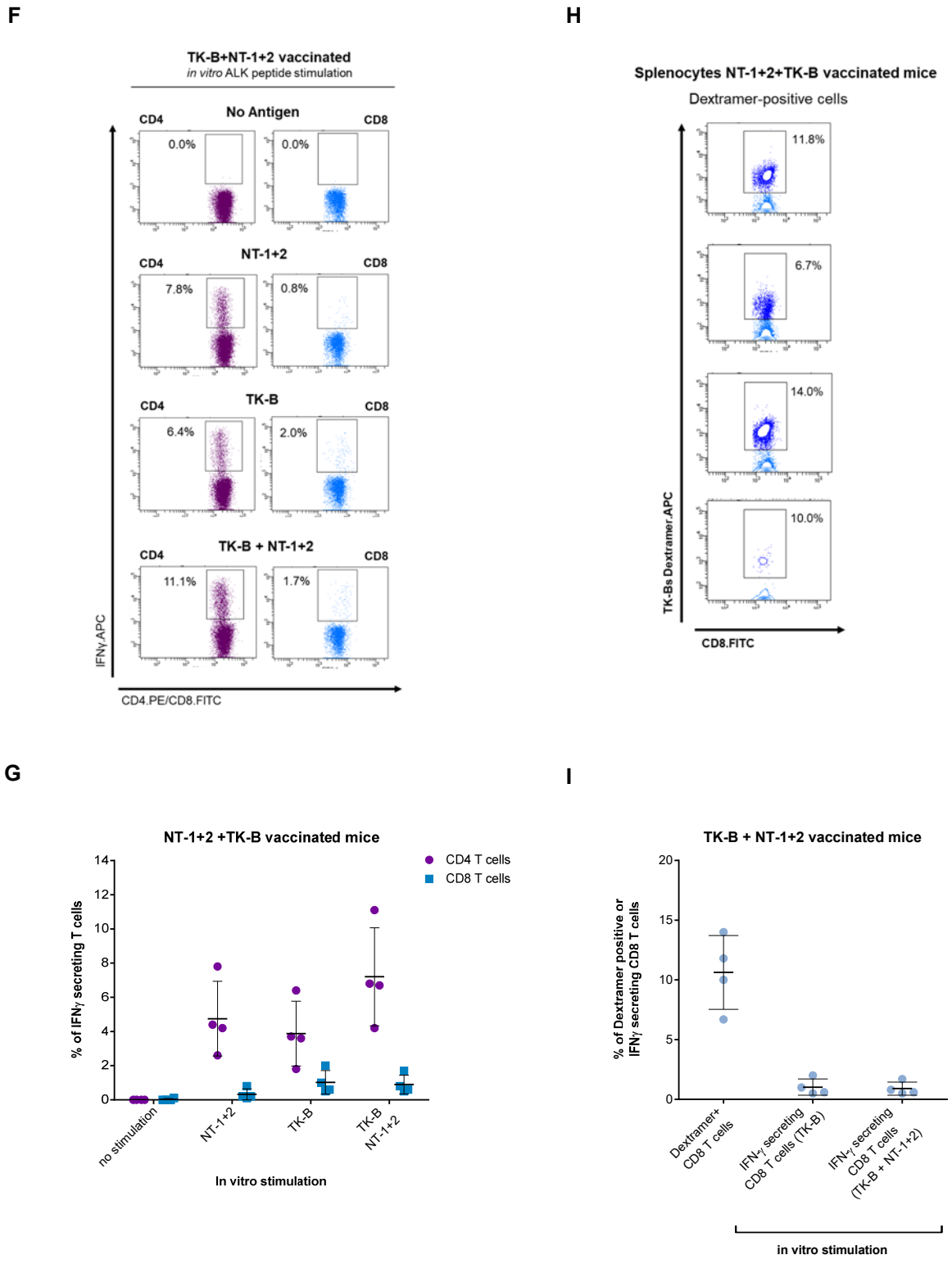


Figure 21: ALK peptide vaccine induced spontaneous CD4- and CD8 T cell responses in mice. Mice were immunized three times in an interval of two weeks with ALK vaccines consisting of 10nmol of each respective ALK peptide and 25 µg CDN. Seven days after the third vaccination, splenocytes were isolated and analyzed via flow cytometry for CD4- and CD8 T cell mediated IFN-γ release in response to ALK peptides. (**A**) Schematic representation of ALK peptides included in the vaccines. (**B, C, F, G**) Vaccine induced CD4- and CD8 T cell responses in TK-B vaccinated mice (n=4) (B,C) and TK-B+NT-1+2 vaccinated mice (n=4) (F, G). (**D, H**) ALK-specific CD8 T cells in splenocytes of TK-B (D) and TK-B+NT-1+2 (H) vaccinated mice determined by dextramer / CD8 staining. (**E, I**) Representation of ALK-specific CD8 T cells (dextramer-positive) and IFN-γ secreting CD8 T cells after *in vitro* peptide stimulation in splenocytes of vaccinated mice.

4.5. A therapeutic CD4 T cell targeting ALK peptide vaccine prolongs survival of mice in a systemic ALK$^+$ lymphoma model and reduces tumor growth of local tumors

Neoantigens are potent targets for cancer immunotherapy as they lack expression in healthy tissue and are recognized by T cells as foreign. Neoantigens are frequently recognized by CD4 T cells (Linnemann et al. 2015; Kreiter et al. 2015) and personalized SLP based cancer vaccines targeting neoantigens have shown promising efficacy in clinical studies (Ott et al. 2017; Sahin et al. 2017). Furthermore, the activity of tumor reactive CD4 T cells has been shown to be required for potent tumor rejection even in tumors that do not express MHC class II molecules (Alspach et al. 2019).

To test the therapeutic efficacy of a ALK-directed CD4 vaccine in local and systemic lymphoma setting, BALB/c mice were challenged sub cutaneous (s.c.) or intravenous (i.v.) with a syngeneic ALK$^+$ lymphoma cell line (R37), derived from BALB/c NPM-ALK transgenic mice and characterized by low I-A^d expression (Chiarle et al. 2003) **(Figure 22C)**, previous to immunization with a ALK vaccine. The vaccine was composed of a 20 aa long peptide (CT-s2) encompassing a sequence in the overlapping region of CT-1 and CT-2 (**Figure 22A,B)**. CT-s2 represents a neoepitope in mice, as the sequence is specific for the human ALK protein.

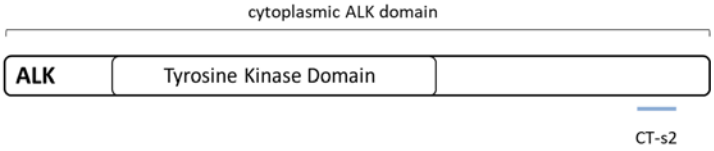
In all mice s.c. challenged with tumor cells, a localized lymphoma mass grew by day 11 after tumor injection (**Figure 22D**). However, compared to the control group (non-vaccinated, n=6), tumor growth was significantly attenuated in all vaccinated mice (n=7) leading to an overall reduced tumor mass by the endpoint at day 25 (**Figure 22D, E**).

In the systemic setting, all control mice (non-vaccinated, n=6) died of systemic disease involving the central nervous system, lymphoid tissues, soft tissues, liver, kidney, and ovaries between day 18 and 24 after tumor challenge (median survival 21 days) (**Figure 22F**). The vaccine significantly prolonged the survival of all vaccinated mice (n=6; p value 0.0007) reflected by a median survival of 35.5 days (**Figure 22F**).

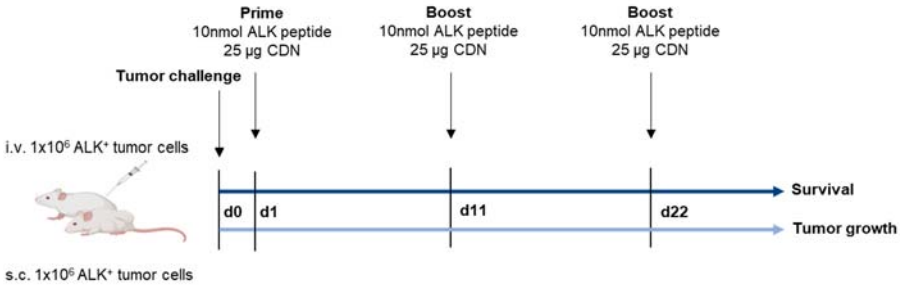
This is the first report of an ALK-directed CD4 vaccine with therapeutic efficacy in ALK$^+$ lymphoma. Although the exact mechanism of mode of action as not been investigated, it can be suggested that the vaccine induced ALK-specific CD4 T cells provided tumor-protection via mechanisms independent of direct recognition of tumor cells as they exhibited low to no MHC class II expression.

In line with previous reports, in which an ALK DNA vaccine showed protective and therapeutic potential in ALK$^+$ lymphoma and NSCLC models, this data provides additional evidence for therapeutic efficacy of a neoepitope directed CD4 vaccine in an ALK$^+$ lymphoma model.

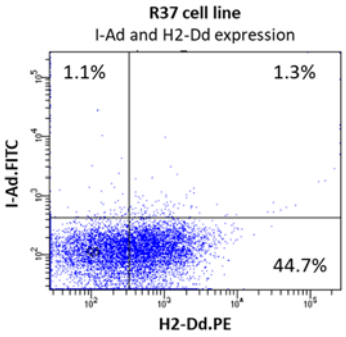
A


cytoplasmic ALK domain
ALK
Tyrosine Kinase Domain
CT-s2

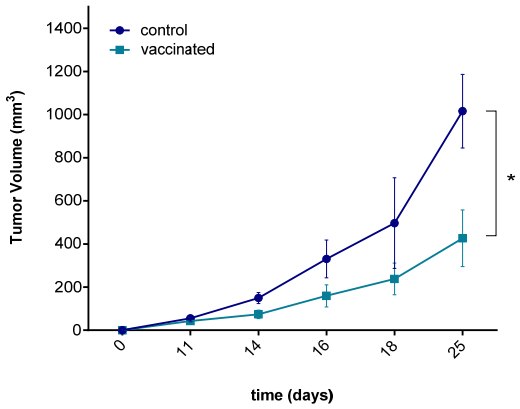
B


Prime
10nmol ALK peptide
25 µg CDN
Boost
10nmol ALK peptide
25 µg CDN
Boost
10nmol ALK peptide
25 µg CDN
Tumor challenge
i.v. 1x10⁶ ALK⁺ tumor cells
s.c. 1x10⁶ ALK⁺ tumor cells
d0
d1
d11
d22
Survival
Tumor growth

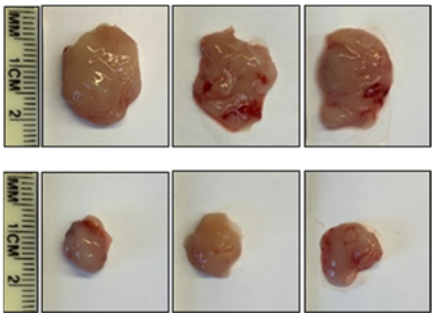
C


R37 cell line
I-Ad and H2-Dd expression
1.1%
1.3%
44.7%
I-Ad.FITC
H2-Dd.PE

D


control
vaccinated
Tumor Volume (mm³)
1400
1200
1000
800
600
400
200
0
0
11
14
16
18
25
time (days)
*

E



F

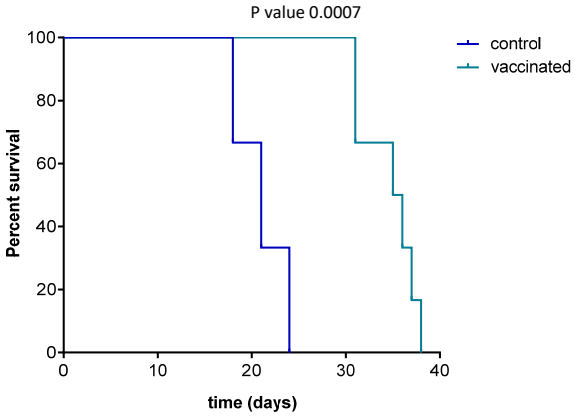

P value 0.0007
control
vaccinated
Percent survival
100
80
60
40
20
0
0
10
20
30
40
time (days)

Figure 22: Therapeutic efficacy of a CD4 T cell directed ALK peptide vaccine on local and systemic lymphoma growth. (**A**) Schematic representation of the ALK peptide (CT-s2) included in the studied vaccine. (**B**) Experimental design. Balb/c mice were challenged s.c. or i.v. with $1x10^6$ ALK^+ lymphoma cells (R37). One day after tumor challenge, mice were immunized with the 10 nmol of the ALK CT-s2 vaccine and 25µg CDN, followed by two boosts every 11 days. Tumor growth and survival of mice was monitored. (**C**) Flow cytometric analysis of I-Ad and H2-Dd expression in the ALK^+ lymphoma cell line R37. (**D**) Tumor growth over time in vaccinated (n=7) and control (n=6) mice. For each time point the median +/- SD is shown. p =0.0001 (t-test). (**E**) Representation of s.c. tumors of control and vaccinated mice. (**F**) Kaplan-Meier survival curve of vaccinated (n=6) and control (n=6) mice. p=0.0007 (log rank Mantel-Cox test)

5. Discussion

The induction of protective and durable anti-tumor responses requires a complex interplay between different subsets of immune cells (Chen & Mellman 2013). In ALK$^+$ALCL, the aberrant expression of oncogenic ALK fusion proteins is recognized by the immune system which is reflected by ALK-specific humoral- and T cell responses (Stadler et al. 2018). These spontaneous responses can be potentially harnessed by immunotherapy to mediate tumor control and long-term protection. Previous efforts to dissect mechanisms of anti-ALK immunity have primarily focused on CD8 T cells and B cells (Pulford et al. 2000; Ait-Tahar et al. 2006; Mussolin et al. 2009; Passoni et al. 2002; Passoni et al. 2006; Singh et al. 2016; Singh et al. 2019). The role of CD4 T cells remained largely neglected. However, it is becoming increasingly evident that CD4 T cells significantly contribute to protective tumor control and are likely involved in ALK-directed immune responses.

In this study, spontaneous CD4 T cell responses to the ALK protein were detected and investigated in uniformly treated ALK$^+$ALCL patients in clinical remission, in healthy individuals, and in vaccination studies in mice.

5.1. Detection of ALK-specific CD4 T cells in ALK$^+$ALCL patients and healthy donors

ALK-reactive CD4 T cells were detected using an approach in which autologous DCs were pulsed with ALK peptide pools consisting of single synthetic overlapping peptides that were combined to cover the main regions of the cytoplasmic portion of ALK. The peptide loaded DCs were used to stimulate and enrich possible ALK-reactive CD4 T cells, which were tested in an IFN-γ ELISPOT for ALK recognition. This method allowed a human leukocyte antigen (HLA)-independent analysis and ensured individual uptake, processing, and presentation of peptides by patient´s DCs, a scenario closely recapitulating physiological processes. This principle is standard of use in immunological studies and peptide-based vaccines are relying on the same mechanism.

The protocol for the detection of ALK-specific CD4 T cells involved an initial stimulation followed by two re-stimulations in an interval of seven days before the final ELISPOT read-out. This protocol potentially allows for the priming of both, naïve and memory CD4 T cells. Except for patient 4, who exhibited a detectable memory response (defined by testing of unstimulated CD4 T cells) to pool NT before and after therapy, no *ex vivo* responses were detected in any other donors, suggesting that ALK-reactive CD4 T cells needed priming to be activated and enriched in those cases. Moreover, for each condition, two separate cultures were initiated, and occasionally ALK-reactive CD4 T cells were enriched and detected only in one of those cultures. These observations indicate that a natural occurring CD4 TCR repertoire against ALK exists in the peripheral blood of patients and healthy donors with variable frequencies of cells

recognizing epitopes within different immunogenic ALK regions. The applied method allowed the enrichment and detection of ALK-reactive CD4 T cells (memory and/or naïve) in eight out of nine patients and all healthy donors, regardless of the frequency. All responding patients were between 13 and 18.4 years of age at disease onset and analyzed between 0 and 7.8 years after diagnosis. Only in one patient (patient 8), no clear ALK-reactive CD4 T cell responses were detected. This patient was the youngest in the cohort (3.8 years) when diagnosed with ALK$^+$ALCL and was analyzed more than 11 years after therapy. Several factors might have contributed to the absence of ALK-specific CD4 T cells in this patient. Besides possible host factors, such as the HLA haplotype, the young age at disease onset and the time of analysis might have impacted the detection of ALK-reactive CD4 T cells. Memory T cells are predominantly generated during infancy, youth and young adulthood in response to antigen exposure (Farber et al. 2014). Thus, the generation of memory CD4 T cells to ALK in this patient might have been incomplete and/or possible established ALK-specific memory T cells might have been depleted or affected by chemotherapeutic treatment. Also, the CD4 TCR repertoire could have been altered by treatment. Studies on long-term effects of chemotherapy on the adaptive immune system demonstrated universal changes and incomplete recovery of patient´s CD4 T cells, especially in memory subpopulations, that persisted for years after treatment (Gustafson et al. 2020; Van Tilburg et al. 2011). A negative impact of chemotherapy on the immunological memory to ALK in this young patient is therefore conceivable. To identify and evaluate possible risk factors contributing to the absence of ALK-specific CD4 T cells in patients, further studies comprising a larger cohort of individuals are needed.

In this study, several CD4 T cell epitopes within ALK were identified by dissecting the ALK-specific CD4 T cell responses in individual patients via screening of single ALK peptides. In this screening, autologous CD40-activated B cells were used as APCs for the stimulation of ALK-reactive CD4 T cells. B cells primarily capture antigens via highly epitope-specific BCRs and present processed peptides through MHC class II molecules to CD4 T cells (Yuseff et al. 2013). CD40-activated B cells have been demonstrated to efficiently stimulate and expand naïve and memory T cells (Von Bergwelt-Baildon et al. 2004). Here, the capacity and efficiency of autologous B cells in the stimulation of ALK-reactive CD4 T cells was demonstrated, which led to the identification of single immunogenic CD4 T cell peptides in the ALK protein. Whether B cell recognition of the respective ALK peptides led to improved uptake and stimulation of CD4 T cells should be further investigated.

The current analysis primarily focused on the detection and identification of ALK-specific CD4 T cells and epitopes by measuring IFN-γ secretion in response to ALK peptides. Although, this analysis provided previously undescribed information on ALK-specific CD4 T cells and

epitopes, it doesn´t provide a comprehensive picture on the overall CD4 T cell response. CD4 T cells are highly versatile and polyfunctional cells that exhibit a large degree of plasticity and effector functions in response to antigens and environmental stimuli (Saravia et al. 2019). IFN-γ is a cytokine primarily secreted by the Th1 subset of CD4 T cells, which have been associated with favorable anti-tumor activity (Kim & Cantor 2014). However, whether other possible CD4 T cell subsets are involved in the anti-ALK response has not been investigated in this study and cannot be excluded. Therefore, a broader flow cytometry-based analysis of ALK-reactive CD4 T cell subsets should be conducted to define the different ALK responding cell populations. Such an analysis also allows for the characterization of possible differences between ALK-specific CD4 T cell subsets in ALK+ALCL patients and healthy donors e.g. whether ALK-specific CD4 T cells in patients derive from memory cells as indicated for CD8 T cells (Passoni et al. 2006). As memory T cells have been shown to mediate potent and durable anti-tumor immunity (Liu et al. 2020), the presence of ALK-specific memory CD4-and CD8 T cells could possibly enhance immunotherapeutic strategies.

5.2. ALK immunogenicity and CD4 T cell epitopes

In line with previous reports in which ALK-specific T cells were observed in healthy individuals (Singh et al. 2019; Passoni et al. 2002; Passoni et al. 2006), ALK-specific CD4 T cells were detected not only in ALK$^+$ALCL patients, but also in healthy donors. The identified CD4 T cell responses were directed against specific regions within the N-terminal intracellular domain, the tyrosine kinase domain, and the C-terminal end of the ALK protein. These regions seem to be naturally immunogenic and contain promiscuous CD4 T cell epitopes independent of the HLA haplotype across all donors. Compared to healthy donors, the CD4 T cell responses against ALK in patients showed a broader regional diversity and were partly directed also to other regions within the tyrosine kinase domain of ALK. This is not surprising, as the TCR repertoire dynamically evolves and changes during lifetime and in response to challenges for the immune system, such as infections and neoplastic cells (Aversa et al. 2020). The former presence of ALK expressing ALCL cells in patients, thus, potentially explains a broader repertoire of T cell specificities.

It is remarkable, that the same immunogenic ALK regions observed in humans also induced CD4 T cell responses in mice. The intracellular N-terminal region and large parts of the ALK tyrosine kinase domain share sequence homology in human and mice. Therefore, the presence of ALK-directed CD4 T cell responses to these ALK regions in both species suggests that universal CD4 T cell epitopes are located within these sequences. This observation not only highlights the potent immunogenicity of the ALK protein but also makes mice ideal model organisms to study anti-tumor responses to ALK *in vivo*.

The observed immunogenicity of the ALK tyrosine kinase domain is in accordance with former studies. CD8 T cell responses primarily directed against sequences covered by peptides of the TK-B pool have been described in analysis of ALK$^+$ALCL patients (Singh et al. 2019). Also, a truncated ALK DNA vaccine covering the kinase domain induced protective CD8 T cell mediated responses in an ALK$^+$ lymphoma mouse model (Chiarle, Martinengo, et al. 2008). The presence of CD4 T cell epitopes within the intracellular N-terminal, and C-terminal domain of ALK has, however, not been described before. Strikingly, these ALK regions have been identified to contain high affinity ALK-antibody binding sites in ALK-positive ALCL and NSCLC patients (Knörr, Weber, et al. 2018; Awad et al. 2017). Given that CD4 T cell help is required for B cell activation, proliferation and differentiation into antibody secreting plasma cells in response to many protein antigens (Sharonov et al. 2020), this observation suggests that immunogenic ALK peptides within these regions are recognized by both, CD4 T cells and B cells, and that ALK-specific CD4 T cells mediate antibody production in ALK$^+$ALCL patients. This is in line with reports of tumor-specific CD4 T cell responses that correlated with the presence of serum antibodies to tumor antigens in cancer patients (Gnjatic et al. 2003; Reed et al. 2015) and with the presumption that anti-ALK antibodies represent a surrogate marker for an overall ALK-directed immune response. In turn, since anti-ALK antibodies in patients are found to be of the IgG isotype, which can bind to Fcγ receptors on DCs and macrophages, ALCL-specific antibodies potentially contribute to the activation of ALK-specific T cells (Pulford et al. 2000; Ait-Tahar et al. 2006; Carmi et al. 2015). Further studies are needed to investigate whether CD4 T cells and B cells share the same epitopes. If this is the case, the identification of antibody binding sites would allow to discover patient specific CD4 T cell epitopes which could be potentially included into a personalized immunotherapy.

Neoepitopes are potent targets for immunotherapy as they arise from cancer-specific mutations and are recognized by the immune system as foreign thereby circumventing tolerance mechanisms (Karpanen & Olweus 2017). The clinical significance of T cells recognizing neoepitopes is becoming increasingly evident especially due to the efficacy of ICB therapy in cancers with a high mutational load (Darvin et al. 2018; Robert 2020). Experimental and clinical studies demonstrated that personalized neoepitope vaccines can induce substantial neoantigen-specific T cell responses (Kreiter et al. 2015; Sahin et al. 2017; Ott et al. 2017). However, current efforts to identify neoepitopes with the potential to generate broad integrated and clinically relevant anti-tumor responses in patients relies on time consuming personalized approaches as very few neoantigens (average about 1-2% of identified neoepitopes in many cancers) spontaneously elicit T cell responses (Hu et al. 2018; Karpanen & Olweus 2017).

The present study provides evidence for the NPM-ALK fusion as potent neoepitope for ALCL by the first description of NPM-ALK fusion reactive CD4 T cells in one patient. The NPM-ALK fusion directed CD4 T cell response was already present at time of diagnosis and characterized by established memory cells after the patient achieved clinical remission. Neoepitopes are frequently and predominantly recognized by CD4 T cells which can be partially explained by the less restrictive binding properties of peptides to MHC class II molecules compared to MHC class I (Linnemann et al. 2015; Kreiter et al. 2015; Sahin et al. 2017; Ott et al. 2017). Neoepitope specific CD4 T cells can potentially exert direct anti-tumor activity and/or provide essential help to CD8 T cells to mediate tumor rejective responses (Overwijk 2015). In addition, a study in mouse models of cancer suggests that multimer vaccines targeting CD4 neoepitopes can act by enhancing antigen spreading and thereby unmasking CD8 T cell epitopes previously not sufficiently visible to the immune system (Kreiter et al. 2015). The NPM-ALK fusion neoepitope, therefore provides a potent target for vaccine design that could enforce the priming and activation of both, ALCL specific CD4-and CD8 T cells to provide protective anti-tumor immunity.

5.3. ALK and immunological tolerance

ALK is a highly conserved receptor tyrosine kinase that shares sequence homology with the leukocyte tyrosine kinase (Iwahara et al. 1997). Therefore, it could be assumed that at least some parts of ALK might be target of central tolerance or induce peripheral tolerance. Indeed, ALK DNA vaccination in an ALK-positive lung cancer model led not only to an increase of effector T cells but also of intratumoral regulatory T cells (Voena et al. 2015). However, the overall observed immunogenicity of ALK and the presence of ALK-directed T cell responses in ALK^{+}ALCL patients, in healthy individuals, and in mice raises the question whether ALK represents a protein to which central tolerance is incomplete. Central tolerance is facilitated by negative selection of self-reactive thymocytes during T cell development which is mediated by ectopic thymic expression of tissue-specific genes, and maintained by regulatory T cells (Peterson et al. 2008; Sakaguchi et al. 2010). Since wild-type ALK is almost exclusively expressed in the central- and peripheral nervous system during development and protein levels nearly fully diminish in all tissues after birth, except for some immune privileged sites in restricted zones of the brain and testis (Morris et al. 1997), one can speculate whether the establishment of central tolerance to ALK might be absent or incomplete. Interestingly, NPM-ALK-positive cells have been detected in peripheral blood and NPM-ALK-negative tissues of healthy adults and in cord blood of healthy newborns (Maes et al. 2001; Trümper et al. 1998; Laurent et al. 2012), suggesting that chromosomal ALK translocations spontaneously appear in healthy individuals. In addition, a study on the humoral immune response to ALK reported

the detection of anti-ALK antibodies in a healthy donor (Pulford et al. 2000). Together, these observations hint towards an incomplete immunological tolerance for ALK.
As central and peripheral tolerance is maintained by regulatory T cells that express high-affinity TCRs to self-antigens, they are primarily activated and expanded in the TME (Togashi et al. 2019). In this regard, a targeted analysis of possible ALK-specific T_{reg} cells should be conducted *in vitro* and *in vivo* to identify potential immunosuppressive epitopes which might oppose immunotherapeutic approaches.

5.4. CD4 T cell responses to ALK vaccines in mice

The present study confirms the potential of ALK as ideal target for tumor vaccination and provides the first evidence of direct involvement of ALK-specific CD4 T cell responses in lymphoma control. Apart from being highly tumor specific and immunogenic, ALK is absolute essential for ALCL maintenance. As mentioned, the physiological expression of ALK is limited mostly to fetal development and immunological suppressed compartments, which greatly minimized the risk of vaccine induced autoimmune reactions (Vernersson et al. 2006). In line with previous reports, mice immunized with ALK vaccines overcame tolerance but remained healthy and without signs of acute inflammation (data not shown) (Chiarle, Martinengo, et al. 2008; Voena et al. 2015).
In this study, mice were immunized with selected human ALK peptides together with the STING-activating CDN cyclic diguanylate monophosphate (c-di-GMP). This vaccine formulation led to the induction of strong ALK-specific CD4 T cell responses in two mouse strains and even to an ALK peptide that previously showed no specific immunogenicity (TK-C), highlighting the potency of the immune stimulatory adjuvant.
STING activating CDNs represent pathogen-associated molecular patterns (PAMPs) that activate cytosolic surveillance pathways and thereby stimulate the innate immune system (McWhirter et al. 2009; Burdette & Vance 2013). Immunizations with CDNs in combination with antigens have been demonstrated to induce distinct adaptive T cell and humoral responses and are under investigation for applications as adjuvants in cancer vaccines (Dubensky et al. 2013; Ebensen et al. 2011; Chandra et al. 2014; Woo et al. 2014; Hanson et al. 2015). c-di-GMP acts through the recruitment of granulocytes and monocytes and induces the maturation of DCs (Karaolis et al. 2007). Considering that immunization with peptide vaccines rely on the uptake of the target antigens by DCs, that require immune stimulatory signals to mature and effectively present the processed peptides to T cells in lymph nodes, the action of c-di-GMP on this process might have significantly supported the activation of ALK-specific CD4 T cells in mice.

CD8 T cells are considered as the main effector cells in tumor immunity as their strong cytolytic function leads to direct tumor cell eradication (Zhang & Bevan 2011). The presence of ALK-specific CD8 T cells in ALK$^+$ALCL patients, therefore, provides an essential advantage for immunotherapeutic approaches. A previous analysis demonstrated that patient´s CD8 T cells mainly recognized epitopes within the ALK tyrosine kinase domain (Singh et al. 2019) to which also CD4 T cell responses were detected in the present study. The essential role of CD4 T cells in maintaining and supporting potent anti-tumor responses by mediating help to CTLs is becoming increasingly recognized and the last past 5 years provided essential findings on the requirement of CD4 T cells also in immunotherapy (reviewed in (Tay et al. 2020; Borst et al. 2018)). Different approaches emerged to specifically target CD4 T cells for immunotherapy. In cancer vaccine research, the effect of specific (i.e. tumor derived) and non-specific MHC class II helper epitopes have been explored and the clinical benefit of using long peptides (potentially including CD8 and CD4 T cell epitopes) over short peptides was demonstrated in several studies (reviewed in (Slingluff 2011; Melssen & Slingluff 2017; Hollingsworth & Jansen 2019). Here, it was addressed whether ALK vaccines containing CD4 T cell peptides positively influence ALK-specific CD8 T cell responses. Taking advantage of a known CD8 T cell epitope (TK-Bs) within the 36 aa long TK-B peptide, allowed to specifically monitor ALK-reactive CD8 T cells in vaccinated mice. The analysis led to the identification of a previously undescribed CD4 T cell epitope at the flanking region of the known ALK CD8 antigen in mice. This observation is in line with findings that describe the frequent presence of MHC class II epitopes in the vicinity of predicted CD8 T cell epitopes (Borst et al. 2018). Whether the induced CD4 T cells conferred help to ALK-specific CD8 T cells remains an open question. However, the TK-B peptide vaccine generated potent ALK-specific immune responses in both compartments, making it attractive for further vaccination studies. Surprisingly, the additional inclusion of the NT-2 and NT-2 peptides to TK-B negatively impacted ALK-specific CD8 T cell responses. This observation suggests that the number and/or location of CD4 T cell epitopes play a crucial role in ALK vaccine induced CTL responses which should be considered in vaccine design and needs to be further investigated in combinatorial immunization experiments. A negative impact of helper peptides on CD8 T cell responses was also reported in multi-peptide vaccines consisting of 12 MHC class I- and 6 MHC class II-restricted peptides for melanoma patients. The authors suggested the induction of regulatory T cells or the modulation of homing receptors as possible explanations for the negative effect on CD8 T cells (Slingluff et al. 2011; Slingluff et al. 2013). These observations indicate that the development of an ALK vaccine requires a careful selection of ALK peptides to avoid the induction of immunosuppressive responses, and additionally argue against the use of the entire (NPM)-ALK protein for immunization.

5.5. Therapeutic ALK vaccine

Current treatment for children and adolescents with ALK$^+$ALCL is based on multi-agent chemotherapy which reaches cure only in 70% of children and is associated with acute toxicity and risk for late effects such as secondary malignancies, heart failure, and obesity/metabolic syndrome (Brugières, Le Deley, et al. 2009; Alexander et al. 2014; Wrobel et al. 2011; Mussolin et al. 2020). An ALK vaccine as consolidation after modified induction therapy could mediate long-term tumor control and prevent relapse or, when applied after relapse, reduce the risks and late effects associated with otherwise necessary allogeneic SCT.

In this study, the therapeutic efficacy of a CD4 directed ALK peptide vaccine was evaluated in a syngeneic ALK+ lymphoma model. The vaccine significantly attenuated the growth of local tumors and prolonged the survival of mice in a systemic lymphoma setting, demonstrating for the first time the therapeutic potential of a CD4 directed ALK peptide vaccine. Of note, the vaccine comprised a peptide which represents a neoantigen in mice. As mentioned, T cell responses to neoepitopes in cancer patients have been associated with effective anti-tumor immunity and are frequently and predominantly recognized by CD4 T cells (Linnemann et al. 2015; Kreiter et al. 2015; Ott et al. 2017; Sahin et al. 2017). Although these data are preliminary, it suggests that ALK-specific CD4 T cells have therapeutic significance and are capable to, at least partly, control ALK$^+$ lymphoma. However, the exact mechanism of action has not been studied and remains open for speculations. Since the syngeneic ALK$^+$ lymphoma cell line exhibited low to no MHC class II expression, it can be suggested that the vaccine-induced ALK-specific CD4 T cells provided tumor-protection via mechanisms independent of direct recognition of tumor cells (e.g. by providing help to CD8 T cells). An important aspect, which clearly needs to be further addressed, is the immune stimulatory effect of c-di-GMP, which was used as a vaccine adjuvant. Indeed, the potency of CDNs as adjuvants for systemic immunity is not fully elucidated. While mucosal administration of STING agonists has been demonstrated to significantly promote immunity to vaccines (Karaolis et al. 2007; Ebensen et al. 2007; Yan et al. 2009), the adjuvant effects of parenteral application is not well understood. Low doses of adjuvant c-di-GMP (5 µg) have been reported to elicit substantial humoral responses to highly immunogenic antigens (OVA and ß-galactosidase) (Libanova et al. 2010; Ebensen et al. 2007) whereas the same dose conferred no immunogenicity when administered with the influenza antigen hemagglutinin (Madhun et al. 2011). In contrary, high doses between 70-290 µg c-di-GMP were needed as adjuvant for eliciting robust immune responses to hepatitis B- or *Staphylococcus aureus* antigens (Hu et al. 2009; Gray et al. 2012). These responses were, however, accompanied by substantial systemic inflammation (Gray et al. 2012). The limited potency of low dose CDNs, when administered parenterally, has been associated with of rapid clearing from tissue and, hence, poor lymphatic uptake (Hanson et al.

2015). It seems therefore unlikely, that the moderate dose of 25 µg of c-di-GMP adjuvant used in this study induced unspecific systemic immune reactions that impacted lymphoma growth, at least not in the local setting. This is in line with the observation that vaccinated mice showed no signs of acute inflammation. Therefore, it can be reasoned that the efficacy of the ALK vaccine is attributed to the induced ALK-specific CD4 T cells, which might have been primed more efficiently due to the adjuvant.

Follow-up studies should include a cohort of mice vaccinated with c-di-GMP alone in the presence of ALK^+ lymphoma cells to closely study the effect of this adjuvant. In addition, a more comprehensive analysis on the ALK vaccine induced immune response, including intratumoral immune cells, should be conducted to uncover the function of ALK specific CD4 T cells in therapeutic vaccines.

A possible combination with other treatment modalities such as ALK inhibitors (to decrease tumor burden and enhance the release of tumor antigens) or checkpoint inhibitors (to unleash the activity of ALCL specific T cells) in association with ALK vaccination might potentiate anti-ALK immune responses and mediate durable long-term protection.

6. Summary and Outlook

This study generated new insights into the immunogenicity of the ALK protein by the first comprehensive description of ALK-specific CD4 T cell responses in human and mice. The analysis led to the identification of previously undescribed CD4 T cell epitopes within ALK. Furthermore, the potential and efficacy of CD4 T cell directed ALK peptide vaccines in inducing spontaneous and therapeutically relevant ALK-specific CD4 T cell responses were demonstrated. The generated data provide an essential basis for further vaccination studies and will thereby contribute to the development of a less-toxic treatment for children and adolescents with ALK+ALCL in the future.

Some open questions remain including the possible involvement of regulatory T cells and immunosuppressive epitopes in the anti-ALK response which demands for a targeted analysis of spontaneous and ALK peptide vaccine induced T cell responses. Such an analysis will help to select and validate immunogenic CD4 and CD8 T cell epitopes that can be eventually included into the design of a potential vaccine for ALK$^+$ALCL patients.

Immune monitoring during treatment should be included into current and future clinical trials, to survey treatment response and to provide additional information on the interplay between the immune system and ALK$^+$ALCL. A vaccine for ALCL could mediate a long-term immunosurveillance and could be decisive in preventing lymphoma recurrence and in assuring long term remission. One of the major challenges in the future will be the choice of the most suitable immunotherapy for an individual patient. It is essential to determine which patients benefit from vaccination, unspecific immune stimulation (e.g. vinblastine or ICB), or a combination of both for the induction of protective immunity and which patients require allogeneic blood stem cell transplantation for cure, which could be followed by ALK vaccination. A better understanding of host and tumor factors which determine the presence and nature of immune responses in ALK$^+$ALCL will help with these decisions. A therapeutic ALK vaccine likely requires a pre-existing spontaneous immune response to be effective, making low-risk patients' suitable candidates for initial vaccination studies. It is reasonable to suggest that the design of vaccination studies should involve a combination with other treatment modalities such as ALK inhibitors or immune checkpoint inhibitors. Crizotinib and nivolumab have been already tested in clinical trials for safety and are available for further studies. The focus for high-risk or relapsed ALCL patients might additionally include the optimization of allogeneic SCT and checkpoint inhibitors.

References

Agrawal, B., Krantz, M.J., Parker, J. & Longenecker, B.M., 1998. Expression of MUC1 mucin on activated human T cells: Implications for a role of MUC1 in normal immune regulation. *Cancer Research*, 58(18), pp.4079–4081.

Ahrends, T., Babała, N., Xiao, Y., Yagita, H., Van Eenennaam, H. & Borst, J., 2016. CD27 Agonism Plus PD-1 Blockade Recapitulates CD4+ T-cell Help in Therapeutic Anticancer Vaccination. *Cancer Research*, 76(10), pp.2921–2931.

Ahrends, T., Spanjaard, A., Pilzecker, B., Bąbała, N., Bovens, A., Xiao, Y., Jacobs, H. & Borst, J., 2017. CD4+ T Cell Help Confers a Cytotoxic T Cell Effector Program Including Coinhibitory Receptor Downregulation and Increased Tissue Invasiveness. *Immunity*, 47(5), pp.848-861.e5.

Ait-tahar, K., Barnardo, M.C.N. & Pulford, K., 2007. CD4 T-Helper Responses to the Anaplastic Lymphoma Kinase (ALK) Protein in Patients with ALK-Positive Anaplastic Large-Cell Lymphoma. , (5), pp.1898–1901.

Ait-Tahar, K., Cerundolo, V., Banham, A.H., Hatton, C., Blanchard, T., Kusec, R., Becker, M., Smith, G.L. & Pulford, K., 2006. B and CTL responses to the ALK protein in patients with ALK-positive ALCL. *International Journal of Cancer*, 118(3), pp.688–695.

Ait-Tahar, K., Damm-Welk, C., Burkhardt, B., Zimmermann, M., Klapper, W., Reiter, A., Pulford, K. & Woessmann, W., 2010. Correlation of the autoantibody response to the ALK oncoantigen in pediatric anaplastic lymphoma kinase-positive anaplastic large cell lymphoma with tumor dissemination and relapse risk. *Blood*, 115(16), pp.3314–3319.

Alexander, S., Kraveka, J.M., Weitzman, S., Lowe, E., Smith, L., Lynch, J.C., Chang, M., Kinney, M.C., Perkins, S.L., Laver, J., Gross, T.G. & Weinstein, H., 2014. Advanced stage anaplastic large cell lymphoma in children and adolescents: Results of ANHL0131, a randomized phase III trial of APO versus a modified regimen with vinblastine: A report from the children's oncology group. *Pediatric Blood and Cancer*, 61(12), pp.2236–2242.

Alspach, E., Lussier, D.M., Miceli, A.P., Kizhvatov, I., DuPage, M., Luoma, A.M., Meng, W., Lichti, C.F., Esaulova, E., Vomund, A.N., Runci, D., Ward, J.P., Gubin, M.M., Medrano, R.F.V., Arthur, C.D., White, J.M., Sheehan, K.C.F., Chen, A., Wucherpfennig, K.W., Jacks, T., Unanue, E.R., Artyomov, M.N. & Schreiber, R.D., 2019. MHC-II neoantigens shape tumour immunity and response to immunotherapy. *Nature*, 574(7780), pp.696–701.

Ambrogio, C., Martinengo, C., Voena, C., Tondat, F., Riera, L., Di Celle, P.F., Inghirami, G. & Chiarle, R., 2009. NPM-ALK oncogenic tyrosine kinase controls T-cell identity by transcriptional regulation and epigenetic silencing in lymphoma cells. *Cancer Research*, 69(22), pp.8611–8619.

Aversa, I., Malanga, D., Fiume, G. & Palmieri, C., 2020. Molecular T-cell repertoire analysis as source of prognostic and predictive biomarkers for checkpoint blockade immunotherapy. *International Journal of Molecular Sciences*, 21(7).

Awad, M.M., Mastini, C., Blasco, R.B., Mologni, L., Voena, C., Mussolin, L., Mach, S.L., Adeni, A.E., Lydon, C.A., Sholl, L.M., Jänne, P.A. & Chiarle, R., 2017. Epitope mapping of spontaneous autoantibodies to anaplastic lymphoma kinase (ALK) in non-small cell lung cancer. *Oncotarget*, 8(54), pp.92265–92274.

Baxevanis, C.N. & Perez, S.A., 2016. Cancer vaccines: limited success but the research should remain viable. *Expert Review of Vaccines*, 15(6), pp.677–680.

Bayle, C., Charpentier, A., Duchayne, E., Manel, A.M., Pages, M.P., Robert, A., Lamant, L., Dastugue, N., Bertrand, Y., Dijoud, F., Emile, J.F., Machover, D., Brugières, L. & Delsol, G., 1999. Leukaemic presentation of small cell variant anaplastic large cell lymphoma: Report of four cases. *British Journal of Haematology*, 104(4), pp.680–688.

Bedoui, S., Heath, W.R. & Mueller, S.N., 2016. CD4+ T-cell help amplifies innate signals for primary CD8+ T-cell immunity. *Immunological Reviews*, 272(1), pp.52–64.

Benharroch, D., Meguerian-Bedoyan, Z., Lamant, L., Amin, C., Brugières, L., Terrier-Lacombe, M.J., Haralambieva, E., Pulford, K., Pileri, S., Morris, S.W., Mason, D.Y. & Delsol, G., 1998. ALK-positive lymphoma: A single disease with a broad spectrum of morphology. *Blood*, 91(6), pp.2076–2084.

Bennett, S.R.M., Carbone, F.R., Karamalis, F., Miller, J.F.A.P. & Heath, W.R., 1997. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *Journal of Experimental Medicine*, 186(1), pp.65–70.

Ten Berge, R.L., Snijdewint, F.G.M., Von Mensdorff-Pouilly, S., Poort-Keesom, R.J.J., Oudejans, J.J., Meijer, J.W.R., Willemze, R., Hilgers, J. & Meijer, C.J.L.M., 2001. MUC1 (EMA) is preferentially expressed by ALK positive anaplastic large cell lymphoma, in the normally glycosylated or only partly hypoglycosylated form. *Journal of Clinical Pathology*, 54(12), pp.933–939.

Von Bergwelt-Baildon, M., Maecker, B., Menezes, I., Nadler, L.M. & Schultze, J.L., 2004. Correspondence re R. Lapointe et al., CD40-stimulated B lymphocytes pulsed with tumor antigens are effective antigen-presenting cells that can generate specific T cells (Cancer Research (2004) 64 (4055-4057)). *Cancer Research*, 64(19), p.7181.

Bevan, M.J., 2004. Helping the CD8+ T-cell response. *Nature Reviews Immunology*, 4(8), pp.595–602.

Von Boehmer, H., 2005. Mechanisms of suppression by suppressor T cells. *Nature Immunology*, 6(4), pp.338–344.

Bonzheim, I., Geissinger, E., Roth, S., Zettl, A., Marx, A., Rosenwald, A., Müller-Hermelink, H.K. & Rüdiger, T., 2004. Anaplastic large cell lymphomas lack the expression of T-cell receptor molecules or molecules of proximal T-cell receptor signaling. *Blood*, 104(10), pp.3358–3360.

Boon, T., Cerottini, J.-C., Van den Eynde, B., van der Bruggen, P. & Van Pel, A., 1994. Tumor Antigens Recognized by T Lymphocytes. *Annual Review of Immunology*, 12(1), pp.337–365.

Borst, J., Ahrends, T., Bąbała, N., Melief, C.J.M. & Kastenmüller, W., 2018. CD4+ T cell help in cancer immunology and immunotherapy. *Nature Reviews Immunology*, 18(10), pp.635–647.

Box, J.K., Paquet, N., Adams, M.N., Boucher, D., Bolderson, E., O'Byrne, K.J. & Richard, D.J., 2016. Nucleophosmin: From structure and function to disease development. *BMC Molecular Biology*, 17(1), pp.1–12.

De Brouwer, S., De Preter, K., Kumps, C., Zabrocki, P., Porcu, M., Westerhout, E.M., Lakeman, A., Vandesompele, J., Hoebeeck, J., Van Maerken, T., De Paepe, A., Laureys, G., Schulte, J.H., Schramm, A., Van Den Broecke, C., Vermeulen, J., Van Roy, N., Beiske, K., Renard, M., Noguera, R., Delattre, O., Janoueix-Lerosey, I., Kogner, P., Martinsson, T., Nakagawara, A., Ohira, M., Caron, H., Eggert, A., Cools, J., Versteeg, R. & Speleman, F., 2010. Meta-analysis of neuroblastomas reveals a skewed ALK mutation spectrum in tumors with MYCN amplification.

Clinical Cancer Research, 16(17), pp.4353–4362.

Brown, C.E. & Mackall, C.L., 2019. CAR T cell therapy: inroads to response and resistance. *Nature Reviews Immunology*, 19(2), pp.73–74.

Brugières, L., Le Deley, M.C., Pacquement, H., Meguerian-Bedoyan, Z., Terrier-Lacombe, M.J., Robert, A., Pondarré, C., Leverger, G., Devalck, C., Rodary, C., Delsol, G. & Hartmann, O., 1998. CD30+ anaplastic large-cell lymphoma in children: Analysis of 82 patients enrolled in two consecutive studies of the French Society of Pediatric Oncology. *Blood*, 92(10), pp.3591–3598.

Brugières, L., Quartier, P., Le Deley, M.C., Pacquement, H., Perel, Y., Bergeron, C., Schmitt, C., Landmann, J., Patte, C., Terrier-Lacombe, M.J., Delsol, G. & Hartmann, O., 2000. Relapses of childhood anaplastic large-cell lymphoma: Treatment results in a series of 41 children - A report from the French Society of Pediatric Oncology. *Annals of Oncology*, 11(1), pp.53–58.

Brugières, L., Pacquement, H., Le Deley, M.C., Leverger, G., Lutz, P., Paillard, C., Baruchel, A., Frappaz, D., Nelken, B., Lamant, L. & Patte, C., 2009. Single-drug vinblastine as salvage treatment for refractory or relapsed anaplastic large-cell lymphoma: A report from the french society of pediatric oncology. *Journal of Clinical Oncology*, 27(30), pp.5056–5061.

Brugières, L., Le Deley, M.C., Rosolen, A., Williams, D., Horibe, K., Wrobel, G., Mann, G., Zsiros, J., Uyttebroeck, A., Marky, I., Lamant, L. & Reiter, A., 2009. Impact of the methotrexate administration dose on the need for intrathecal treatment in children and adolescents with anaplastic large-cell lymphoma: Results of a randomized trial of the EICNHL group. *Journal of Clinical Oncology*, 27(6), pp.897–903.

Bulliard, Y., Jolicoeur, R., Windman, M., Rue, S.M., Ettenberg, S., Knee, D.A., Wilson, N.S., Dranoff, G. & Brogdon, J.L., 2013. Activating fc γ receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. *Journal of Experimental Medicine*, 210(9), pp.1685–1693.

Burdette, D.L. & Vance, R.E., 2013. STING and the innate immune response to nucleic acids in the cytosol. *Nature Immunology*, 14(1), pp.19–26.

Burkhardt, B., Zimmermann, M., Oschlies, I., Niggli, F., Mann, G., Parwaresch, R., Riehm, H., Schrappe, M. & Reiter, A., 2005. The impact of age and gender on biology, clinical features and treatment outcome of non-Hodgkin lymphoma in childhood and adolescence. *British Journal of Haematology*, 131(1), pp.39–49.

Carmi, Y., Spitzer, M.H., Linde, I.L., Burt, B.M., Prestwood, T.R., Perlman, N., Davidson, M.G., Kenkel, J.A., Segal, E., Pusapati, G. V., Bhattacharya, N. & Engleman, E.G., 2015. Allogeneic IgG combined with dendritic cell stimuli induce antitumour T-cell immunity. *Nature*, 521(7550), pp.99–104.

Castellino, F. & Germain, R.N., 2006. Cooperation between CD4+ and CD8+ T cells: When, where, and how. *Annual Review of Immunology*, 24(1), pp.519–540.

Castiello, L., Sabatino, M., Jin, P., Clayberger, C., Marincola, F.M., Krensky, A.M. & Stroncek, D.F., 2011. Monocyte-derived DC maturation strategies and related pathways: A transcriptional view. *Cancer Immunology, Immunotherapy*, 60(4), pp.457–466.

Chandra, D., Quispe-Tintaya, W., Jahangir, A., Asafu-Adjei, D., Ramos, I., Sintim, H.O., Zhou, J., Hayakawa, Y., Karaolis, D.K.R. & Gravekamp, C., 2014. STING Ligand c-di-GMP Improves

Cancer Vaccination against Metastatic Breast Cancer. *Cancer Immunology Research*, 2(9), pp.901–910.

Chen, D.S. & Mellman, I., 2013. Oncology meets immunology: The cancer-immunity cycle. *Immunity*, 39(1), pp.1–10.

Chiarle, R., Gong, J.Z., Guasparri, I., Pesci, A., Cai, J., Liu, J., Simmons, W.J., Dhall, G., Howes, J., Piva, R. & Inghirami, G., 2003. NPM-ALK transgenic mice spontaneously develop T-cell lymphomas and plasma cell tumors. *Blood*, 101(5), pp.1919–1927.

Chiarle, R., Simmons, W.J., Cai, H., Dhall, G., Zamo, A., Raz, R., Karras, J.G., Levy, D.E. & Inghirami, G., 2005. Stat3 is required for ALK-mediated lymphomagenesis and provides a possible therapeutic target. *Nature Medicine*, 11(6), pp.623–629.

Chiarle, R., Voena, C., Ambrogio, C., Piva, R. & Inghirami, G., 2008. The anaplastic lymphoma kinase in the pathogenesis of cancer. *Nature Reviews Cancer*, 8(1), pp.11–23.

Chiarle, R., Martinengo, C., Mastini, C., Ambrogio, C., Escamard, V.D., Forni, G. & Inghirami, G., 2008. The anaplastic lymphoma kinase is an effective oncoantigen for lymphoma vaccination. *Nature Medicine*, 14(6), pp.676–680.

Colleoni, G.W.B., Bridge, J.A., Garicochea, B., Liu, J., Filippa, D.A. & Ladanyi, M., 2000. ATIC-ALK: A novel variant ALK gene fusion in anaplastic large cell lymphoma resulting from the recurrent cryptic chromosomal inversion, inv(2)(p23q35). *American Journal of Pathology*, 156(3), pp.781–789.

Cools, J., Wlodarska, I., Somers, R., Mentens, N., Pedeutour, F., Maes, B., De Wolf-Peeters, C., Pauwels, P., Hagemeijer, A. & Marynen, P., 2002. Identification of novel fusion partners of ALK, the anaplastic lymphoma kinase, in anaplastic large-cell lymphoma and inflammatory myofibroblastic tumor. *Genes Chromosomes and Cancer*, 34(4), pp.354–362.

Coulie, P.G., Van Den Eynde, B.J., Van Der Bruggen, P. & Boon, T., 2014. Tumour antigens recognized by T lymphocytes: At the core of cancer immunotherapy. *Nature Reviews Cancer*, 14(2), pp.135–146.

Crockett, D.K., Lin, Z., Elenitoba-Johnson, K.S.J. & Lim, M.S., 2004. Identification of NPM-ALK interacting proteins by tandem mass spectrometry. *Oncogene*, 23(15), pp.2617–2629.

Crotty, S., 2015. A brief history of T cell help to B cells. *Nature reviews. Immunology*, 15(3), pp.185–9.

Dalod, M., Chelbi, R., Malissen, B. & Lawrence, T., 2014. Dendritic cell maturation: Functional specialization through signaling specificity and transcriptional programming. *EMBO Journal*, 33(10), pp.1104–1116.

Damm-Welk, C., Mussolin, L., Zimmermann, M., Pillon, M., Klapper, W., Oschlies, I., D'Amore, E.S.G., Reiter, A., Woessmann, W. & Rosolen, A., 2014. Early assessment of minimal residual disease identifies patients at very high relapse risk in NPM-ALK-positive anaplastic large-cell lymphoma. *Blood*, 123(3), pp.334–337.

Damm-Welk, C., Siddiqi, F., Fischer, M., Hero, B., Narayanan, V., Camidge, D.R., Harris, M., Burke, A., Lehrnbecher, T., Pulford, K., Oschlies, I., Siebert, R., Turner, S. & Woessmann, W., 2016. Anti-ALK antibodies in patients with ALK-positive malignancies not expressing NPM-ALK. *Journal of Cancer*, 7(11), pp.1383–1387.

Damm-Welk, C., Klapper, W., Oschlies, I., Gesk, S., Röttgers, S., Bradtke, J., Siebert, R., Reiter, A. & Woessmann, W., 2009. Distribution of NPM1-ALK and X-ALK fusion transcripts in paediatric

anaplastic large cell lymphoma: A molecular-histological correlation. *British Journal of Haematology*, 146(3), pp.306–309.

Damm-Welk, C., Busch, K., Burkhardt, B., Schieferstein, J., Viehmann, S., Oschlies, I., Klapper, W., Zimmermann, M., Harbott, J., Reiter, A. & Woessmann, W., 2007. Prognostic significance of circulating tumor cells in bone marrow or peripheral blood as detected by qualitative and quantitative PCR in pediatric NPM-ALK-positive anaplastic large-cell lymphoma. *Blood*, 110(2), pp.670–677.

Darvin, P., Toor, S.M., Sasidharan Nair, V. & Elkord, E., 2018. Immune checkpoint inhibitors: recent progress and potential biomarkers. *Experimental and Molecular Medicine*, 50(12), p.165.

Dauer, M., Obermaier, B., Herten, J., Haerle, C., Pohl, K., Rothenfusser, S., Schnurr, M., Endres, S. & Eigler, A., 2003. Mature Dendritic Cells Derived from Human Monocytes Within 48 Hours: A Novel Strategy for Dendritic Cell Differentiation from Blood Precursors. *The Journal of Immunology*, 170(8), pp.4069–4076.

Davis, K.L., Fox, E., Merchant, M.S., Reid, J.M., Kudgus, R.A., Liu, X., Minard, C.G., Voss, S., Berg, S.L., Weigel, B.J. & Mackall, C.L., 2020. Nivolumab in children and young adults with relapsed or refractory solid tumours or lymphoma (ADVL1412): a multicentre, open-label, single-arm, phase 1–2 trial. *The Lancet Oncology*, 21(4), pp.541–550.

Le Deley, M.C., Reiter, A., Williams, D., Delsol, G., Oschlies, I., McCarthy, K., Zimmermann, M. & Brugières, L., 2008. Prognostic factors in childhood anaplastic large cell lymphoma: Results of a large European intergroup study. *Blood*, 111(3), pp.1560–1566.

Le Deley, M.C., Rosolen, A., Williams, D.M., Horibe, K., Wrobel, G., Attarbaschi, A., Zsiros, J., Uyttebroeck, A., Marky, I.M., Lamant, L., Woessmann, W., Pillon, M., Hobson, R., Mauguen, A., Reiter, A. & Brugières, L., 2010. Vinblastine in children and adolescents with high-risk anaplastic large-cell lymphoma: Results of the randomized ALCL99-vinblastine trial. *Journal of Clinical Oncology*, 28(25), pp.3987–3993.

Dieu-Nosjean, M.C., Giraldo, N.A., Kaplon, H., Germain, C., Fridman, W.H. & Sautès-Fridman, C., 2016. Tertiary lymphoid structures, drivers of the anti-tumor responses in human cancers. *Immunological Reviews*, 271(1), pp.260–275.

Dubensky, T.W., Kanne, D.B. & Leong, M.L., 2013. Rationale, progress and development of vaccines utilizing STING-activating cyclic dinucleotide adjuvants. *Therapeutic Advances in Vaccines*, 1(4), pp.131–143.

Dudek, A.M., Martin, S., Garg, A.D. & Agostinis, P., 2013. Immature, semi-mature, and fully mature dendritic cells: Toward a DC-cancer cells interface that augments anticancer immunity. *Frontiers in Immunology*, 4(DEC), pp.1–14.

Dunn, G.P., Old, L.J. & Schreiber, R.D., 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity*, 21(2), pp.137–148.

Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J. & Schreiber, R.D., 2002. Cancer immunoediting: From immunosurveillance to tumor escape. *Nature Immunology*, 3(11), pp.991–998.

Ebensen, T., Libanova, R., Schulze, K., Yevsa, T., Morr, M. & Guzmán, C.A., 2011. Bis-(3',5')-cyclic dimeric adenosine monophosphate: Strong Th1/Th2/Th17 promoting mucosal adjuvant. *Vaccine*, 29(32), pp.5210–5220.

Ebensen, T., Schulze, K., Riese, P., Morr, M. & Guzmán, C.A., 2007. The bacterial second messenger cdiGMP exhibits promising activity as a mucosal adjuvant. *Clinical and Vaccine Immunology*, 14(8), pp.952–958.

Falini, B., Bigerna, B., Fizzotti, M., Pulford, K., Pileri, S.A., Delsol, G., Carbone, A., Paulli, M., Magrini, U., Menestrina, F., Giardini, R., Pilotti, S., Mezzelani, A., Ugolini, B., Billi, M., Pucciarini, A., Pacini, R., Pelicci, P.G. & Flenghi, L., 1998. ALK expression defines a distinct group of T/null lymphomas ('ALK lymphomas') with a wide morphological spectrum. *American Journal of Pathology*, 153(3), pp.875–886.

Farber, D.L., Yudanin, N.A. & Restifo, N.P., 2014. Human memory T cells: Generation, compartmentalization and homeostasis. *Nature Reviews Immunology*, 14(1), pp.24–35.

Foss, H.D., Anagnostopoulos, I., Araujo, I., Assaf, C., Demel, G., Kummer, J.A., Hummel, M. & Stein, H., 1996. Anaplastic large-cell lymphomas of T-cell and null-cell phenotype express cytotoxic molecules. *Blood*, 88(10), pp.4005–11.

Fukano, R., Mori, T., Kobayashi, R., Mitsui, T., Fujita, N., Iwasaki, F., Suzumiya, J., Chin, M., Goto, H., Takahashi, Y., Hara, J., Park, Y.-D., Inoue, M., Koga, Y., Inagaki, J., Sakamaki, H., Adachi, S., Kawa, K., Kato, K. & Suzuki, R., 2015. Haematopoietic stem cell transplantation for relapsed or refractory anaplastic large cell lymphoma: a study of children and adolescents in Japan. *British Journal of Haematology*, 168(4), pp.557–563.

Galluzzi, L., Vacchelli, E., Bravo-San Pedro, J.M., Buqué, A., Senovilla, L., Baracco, E.E., Bloy, N., Castoldi, F., Abastado, J.P., Agostinis, P., … Kroemer, G., 2014. Classification of current anticancer immunotherapies. *Oncotarget*, 5(24), pp.12472–12508.

Gambacorti-Passerini, C., Mussolin, L. & Brugieres, L., 2016. Abrupt relapse of ALK-positive lymphoma after discontinuation of crizotinib. *New England Journal of Medicine*, 374(1), pp.95–96.

Gambacorti Passerini, C., Farina, F., Stasia, A., Redaelli, S., Ceccon, M., Mologni, L., Messa, C., Guerra, L., Giudici, G., Sala, E., Mussolin, L., Deeren, D., King, M.H., Steurer, M., Ordemann, R., Cohen, A.M., Grube, M., Bernard, L., Chiriano, G., Antolini, L. & Piazza, R., 2014. Crizotinib in advanced, chemoresistant anaplastic lymphoma kinase-positive lymphoma patients. *Journal of the National Cancer Institute*, 106(2), p.djt378.

Garfall, A.L., Dancy, E.K., Cohen, A.D., Hwang, W.T., Fraietta, J.A., Davis, M.M., Levine, B.L., Siegel, D.L., Stadtmauer, E.A., Vogl, D.T., Waxman, A., Rapoport, A.P., Milone, M.C., June, C.H. & Melenhorst, J.J., 2019. T-cell phenotypes associated with effective CAR T-cell therapy in postinduction vs relapsed multiple myeloma. *Blood Advances*, 3(19), pp.2812–2815.

Gnjatic, S., Nishikawa, H., Jungbluth, A.A., Güre, A.O., Ritter, G., Jäger, E., Knuth, A., Chen, Y.T. & Old, L.J., 2006. NY-ESO-1: Review of an Immunogenic Tumor Antigen. *Advances in Cancer Research*, 95, pp.1–30.

Gnjatic, S., Atanackovict, D., Jäger, E., Matsuo, M., Selvakumar, A., Altorki, N.K., Maki, R.G., Dupont, B., Ritter, G., Chen, Y.T., Knuth, A. & Old, L.J., 2003. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: Correlation with antibody responses. *Proceedings of the National Academy of Sciences of the United States of America*, 100(15), pp.8862–8867.

Gray, P.M., Forrest, G., Wisniewski, T., Porter, G., Freed, D.C., DeMartino, J.A., Zaller, D.M., Guo, Z.,

Leone, J., Fu, T.M. & Vora, K.A., 2012. Evidence for cyclic diguanylate as a vaccine adjuvant with novel immunostimulatory activities. *Cellular Immunology*, 278(1–2), pp.113–119.

Gross, T.G., Hale, G.A., He, W., Camitta, B.M., Sanders, J.E., Cairo, M.S., Hayashi, R.J., Termuhlen, A.M., Zhang, M.J., Davies, S.M. & Eapen, M., 2010. Hematopoietic Stem Cell Transplantation for Refractory or Recurrent Non-Hodgkin Lymphoma in Children and Adolescents. *Biology of Blood and Marrow Transplantation*, 16(2), pp.223–230.

Guo, Y., Lei, K. & Tang, L., 2018. Neoantigen vaccine delivery for personalized anticancer immunotherapy. *Frontiers in Immunology*, 9(JUL).

Gustafson, C.E., Jadhav, R., Cao, W., Qi, Q., Pegram, M., Tian, L., Weyand, C.M. & Goronzy, J.J., 2020. Immune cell repertoires in breast cancer patients after adjuvant chemotherapy. *JCI Insight*, 5(4).

Hallberg, B. & Palmer, R.H., 2016. The role of the ALK receptor in cancer biology. *Annals of Oncology*, 27(Supplement 3), pp.iii4–iii15.

Hanahan, D. & Weinberg, R. a, 2011. Hallmarks of cancer: the next generation. *Cell*, 144(5), pp.646–74.

Hanson, M.C., Crespo, M.P., Abraham, W., Moynihan, K.D., Szeto, G.L., Chen, S.H., Melo, M.B., Mueller, S. & Irvine, D.J., 2015. Nanoparticulate STING agonists are potent lymph node-targeted vaccine adjuvants. *Journal of Clinical Investigation*, 125(6), pp.2532–2546.

He, X., Abrams, S.I. & Lovell, J.F., 2018. Peptide Delivery Systems for Cancer Vaccines. *Advanced Therapeutics*, 1(5), p.1800060.

Hebart, H., Lang, P. & Woessmann, W., 2016. Nivolumab for refractory anaplastic large cell lymphoma: A case report. *Annals of Internal Medicine*, 165(8), pp.607–608.

Herbst, H., Tippelmann, G., Anagnostopoulos, I., Gerdes, J., Schwarting, R., Boehm, T., Pileri, S., Jones, D.B. & Stein, H., 1989. Immunoglobulin and T-cell receptor gene rearrangements in Hodgkin's disease and Ki-1-positive anaplastic large cell lymphoma: Dissociation between phenotype and genotype. *Leukemia Research*, 13(2).

Hernández, L., Beà, S., Bellosillo, B., Pinyol, M., Falini, B., Carbone, A., Ott, G., Rosenwald, A., Fernández, A., Pulford, K., Mason, D., Morris, S.W., Santos, E. & Campo, E., 2002. Diversity of genomic breakpoints in TFG-ALK translocations in anaplastic large cell lymphomas: Identification of a new TFG-ALKXL chimeric gene with transforming activity. *American Journal of Pathology*, 160(4), pp.1487–1494.

Hobo, W., Strobbe, L., Maas, F., Fredrix, H., Greupink-Draaisma, A., Esendam, B., De Witte, T., Preijers, F., Levenga, H., Van Rees, B., Raymakers, R., Schaap, N. & Dolstra, H., 2013. Immunogenicity of dendritic cells pulsed with MAGE3, Survivin and B-cell maturation antigen mRNA for vaccination of multiple myeloma patients. *Cancer Immunology, Immunotherapy*, 62(8), pp.1381–1392.

Hollingsworth, R.E. & Jansen, K., 2019. Turning the corner on therapeutic cancer vaccines. *npj Vaccines*, 4(1), pp.1–10.

Hombach, A.A., Görgens, A., Chmielewski, M., Murke, F., Kimpel, J., Giebel, B. & Abken, H., 2016. Superior Therapeutic Index in Lymphoma Therapy: CD30+ CD34+ Hematopoietic Stem Cells Resist a Chimeric Antigen Receptor T-cell Attack. *Molecular Therapy*, 24(8), pp.1423–1434.

Hor, J.L., Whitney, P.G., Zaid, A., Brooks, A.G., Heath, W.R. & Mueller, S.N., 2015. Spatiotemporally

Distinct Interactions with Dendritic Cell Subsets Facilitates CD4+ and CD8+ T Cell Activation to Localized Viral Infection. *Immunity*, 43(3), pp.554–565.

Hori, S., Nomura, T. & Sakaguchi, S., 2017. Control of regulatory T cell development by the transcription factor Foxp3. *Journal of Immunology*, 198(3), pp.981–985.

Hu, D.L., Narita, K., Hyodo, M., Hayakawa, Y., Nakane, A. & Karaolis, D.K.R., 2009. c-di-GMP as a vaccine adjuvant enhances protection against systemic methicillin-resistant Staphylococcus aureus (MRSA) infection. *Vaccine*, 27(35), pp.4867–4873.

Hu, Z., Ott, P.A. & Wu, C.J., 2018. Towards personalized, tumour-specific, therapeutic vaccines for cancer. *Nature Reviews Immunology*, 18(3), pp.168–182.

Hurley, S.P., Clary, D.O., Copie, V. & Lefcort, F., 2006. Anaplastic lymphoma kinase is dynamically expressed on subsets of motor neurons and in the peripheral nervous system. *Journal of Comparative Neurology*, 495(2), pp.202–212.

Iqbal, J., Weisenburger, D.D., Greiner, T.C., Vose, J.M., McKeithan, T., Kucuk, C., Geng, H., Deffenbacher, K., Smith, L., Dybkaer, K., Nakamura, S., Seto, M., Delabie, J., Berger, F., Loong, F., Au, W.Y., Ko, Y.H., Sng, I., Armitage, J.O. & Chan, W.C., 2010. Molecular signatures to improve diagnosis in peripheral T-cell lymphoma and prognostication in angioimmunoblastic T-cell lymphoma. *Blood*, 115(5), pp.1026–1036.

Iwahara, T., Fujimoto, J., Wen, D., Cupples, R., Bucay, N., Arakawa, T., Mori, S., Ratzkin, B. & Yamamoto, T., 1997. Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene*, 14(4), pp.439–449.

Janssen, E.M., Droin, N.M., Lemmens, E.E., Pinkoski, M.J., Bensinger, S.J., Ehst, B.D., Griffith, T.S., Green, D.R. & Schoenberger, S.P., 2005. CD4+ T-cell help controls CD8+ T-cell memory via TRAIL-mediated activation-induced cell death. *Nature*, 434(7029), pp.88–93.

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

Kaatsch, P., 2010. Epidemiology of childhood cancer. *Cancer Treatment Reviews*, 36(4), pp.277–285.

Kaatsch, P., Grabow, D. & Spix, C., 2019. Deutsches Kinderkrebsregister Jahresbericht / Annual Report 2018 German Childhood Cancer Registry. , pp.1–176.

Kantoff, P.W., Higano, C.S., Shore, N.D., Berger, E.R., Small, E.J., Penson, D.F., Redfern, C.H., Ferrari, A.C., Dreicer, R., Sims, R.B., Xu, Y., Frohlich, M.W. & Schellhammer, P.F., 2010. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *New England Journal of Medicine*, 363(5), pp.411–422.

Karaolis, D.K.R., Means, T.K., Yang, D., Takahashi, M., Yoshimura, T., Muraille, E., Philpott, D., Schroeder, J.T., Hyodo, M., Hayakawa, Y., Talbot, B.G., Brouillette, E. & Malouin, F., 2007. Bacterial c-di-GMP Is an Immunostimulatory Molecule. *The Journal of Immunology*, 178(4), pp.2171–2181.

Karpanen, T. & Olweus, J., 2017. The potential of donor T-cell repertoires in neoantigen-targeted cancer immunotherapy. *Frontiers in Immunology*, 8(DEC), p.1718.

Kennedy, R. & Celis, E., 2008. Multiple roles for CD4+ T cells in anti-tumor immune responses. *Immunological Reviews*, 222(1), pp.129–144.

Kenter, G.G., Welters, M.J.P., Valentijn, A.R.P.M., Lowik, M.J.G., Berends-van Der Meer, D.M.A., Vloon,

A.P.G., Essahsah, F., Fathers, L.M., Offringa, R., Drijfhout, J.W., Wafelman, A.R., Oostendorp, J., Fleuren, G.J., Van Der Burg, S.H. & Melief, C.J.M., 2009. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *New England Journal of Medicine*, 361(19), pp.1838–1847.

Kim, H.J. & Cantor, H., 2014. CD4 T-cell subsets and tumor immunity: the helpful and the not-so-helpful. *Cancer immunology research*, 2(2), pp.91–98.

Knörr, F., Weber, S., Singh, V.K., Pulford, K., Reiter, A., Woessmann, W. & Damm-Welk, C., 2018. Epitope mapping of anti-ALK antibodies in children with anaplastic large cell lymphoma. *Clinical Immunology*, 195, pp.77–81.

Knörr, F., Brugières, L., Pillon, M., Zimmermann, M., Ruf, S., Attarbaschi, A., Mellgren, K., Burke, G.A.A., Uyttebroeck, A., Wróbel, G., Beishuizen, A., Aladjidi, N., Reiter, A., Woessmann, W. & European Inter-Group for Childhood Non-Hodgkin Lymphoma, 2020. Stem Cell Transplantation and Vinblastine Monotherapy for Relapsed Pediatric Anaplastic Large Cell Lymphoma: Results of the International, Prospective ALCL-Relapse Trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*, p.JCO2000157.

Knörr, F., Damm-Welk, C., Ruf, S., Singh, V.K., Zimmermann, M., Reiter, A. & Woessmann, W., 2018. Blood cytokine concentrations in pediatric patients with anaplastic lymphoma kinase-positive anaplastic large cell lymphoma. *Haematologica*, 103(3), pp.477–485.

Kreiter, S., Vormehr, M., Van De Roemer, N., Diken, M., Löwer, M., Diekmann, J., Boegel, S., Schrörs, B., Vascotto, F., Castle, J.C., Tadmor, A.D., Schoenberger, S.P., Huber, C., Türeci, O. & Sahin, U., 2015. Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature*, 520(7549), pp.692–696.

Krumbholz, M., Woessmann, W., Zierk, J., Seniuk, D., Zimmermann, M., Singh, V.K. & Metzler, M., 2018. Characterization and diagnostic application of genomic NPM- ALK fusion sequences in anaplastic large-cell lymphoma. , 9(41), pp.26543–26555.

Ladanyi, M. & Cavalchire, G., 1996. Molecular variant of the NPMALK rearrangement of Ki□l lymphoma involving a cryptic ALK splice site. *Genes, Chromosomes and Cancer*, 15(3), pp.173–177.

Lamant, L., McCarthy, K., D'Amore, E., Klapper, W., Nakagawa, A., Fraga, M., Maldyk, J., Simonitsch-Klupp, I., Oschlies, I., Delsol, G., Mauguen, A., Brugières, L. & Le Deley, M.C., 2011. Prognostic impact of morphologic and phenotypic features of childhood ALK-positive anaplastic large-cell lymphoma: Results of the ALCL99 Study. *Journal of Clinical Oncology*, 29(35), pp.4669–4676.

Lamant, L., Gascoyne, R.D., Duplantier, M.M., Armstrong, F., Raghab, A., Chhanabhai, M., Rajcan-Separovic, E., Raghab, J., Delsol, G. & Espinos, E., 2003. Non-muscle myosin heavy chain (MYH9): A new partner fused to ALK in anaplastic large cell lymphoma. *Genes Chromosomes and Cancer*, 37(4), pp.427–432.

Lamant, L., Dastugue, N., Pulford, K., Delsol, G. & Mariamé, B., 1999. A new fusion gene TPM3-ALK in anaplastic large cell lymphoma created by a (1;2)(q25;p23) translocation. *Blood*, 93(9), pp.3088–3095.

Laurent, C., Lopez, C., Desjobert, C., Berrebi, A., Damm-Welk, C., Delsol, G., Brousset, P. & Lamant, L., 2012. Circulating t(2;5)-positive cells can be detected in cord blood of healthy newborns. *Leukemia*, 26(1), pp.188–190.

Laver, J.H., Kraveka, J.M., Hutchison, R.E., Chang, M., Kepner, J., Schwenn, M., Tarbell, N., Desai, S., Weitzman, S., Weinstein, H.J. & Murphy, S.B., 2005. Advanced-stage large-cell lymphoma in children and adolescents: Results of a randomized trial incorporating intermediate-dose methotrexate and high-dose cytarabine in the maintenance phase of the APO regimen: A pediatric oncology group phase III trial. *Journal of Clinical Oncology*, 23(3), pp.541–547.

Leoncini, L., Del Vecchio, M.T., Kraft, R., Megha, T., Barbini, P., Cevenini, G., Poggi, S., Pileri, S., Tosi, P. & Cottier, H., 1990. Hodgkin's disease and CD30-positive anaplastic large cell lymphomas--a continuous spectrum of malignant disorders. A quantitative morphometric and immunohistologic study. *The American journal of pathology*, 137(5), pp.1047–57.

Libanova, R., Ebensen, T., Schulze, K., Bruhn, D., Nörder, M., Yevsa, T., Morr, M. & Guzmán, C.A., 2010. The member of the cyclic di-nucleotide family bis-(3′, 5′)-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant. *Vaccine*, 28(10), pp.2249–2258.

Linnemann, C., Van Buuren, M.M., Bies, L., Verdegaal, E.M.E., Schotte, R., Calis, J.J.A., Behjati, S., Velds, A., Hilkmann, H., Atmioui, D. El, Visser, M., Stratton, M.R., Haanen, J.B.A.G., Spits, H., Van Der Burg, S.H. & Schumacher, T.N.M., 2015. High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. *Nature Medicine*, 21(1), pp.81–85.

Liu, Q., Sun, Z. & Chen, L., 2020. Memory T cells: strategies for optimizing tumor immunotherapy. *Protein and Cell*, 11(8), pp.549–564.

Liu, Y., Wang, L., Predina, J., Han, R., Beier, U.H., Wang, L.C.S., Kapoor, V., Bhatti, T.R., Akimova, T., Singhal, S., Brindle, P.K., Cole, P.A., Albelda, S.M. & Hancock, W.W., 2013. Inhibition of p300 impairs Foxp3+ T regulatory cell function and promotes antitumor immunity. *Nature Medicine*, 19(9), pp.1173–1177.

Locatelli, F., Mauz-Koerholz, C., Neville, K., Llort, A., Beishuizen, A., Daw, S., Pillon, M., Aladjidi, N., Klingebiel, T., Landman-Parker, J., Medina-Sanson, A., August, K., Huebner, D., Sachs, J., Hoffman, K., Kinley, J., Song, S., Song, G., Zhang, S. & Gore, L., 2017. A phase 1/2 study of Brentuximab Vedotin in pediatric patients with relapsed/refractory (R/R) systemic Anaplastic Large-Cell Lymphoma (SALCL) or R/R Godgkin Lymphoma (HL). *Hematological Oncology*, 35, pp.248–249.

Lovly, C.M., Gupta, A., Lipson, D., Otto, G., Brennan, T., Chung, C.T., Borinstein, S.C., Ross, J.S., Stephens, P.J., Miller, V.A. & Coffin, C.M., 2014. Inflammatory myofibroblastic tumors harbor multiple potentially actionable Kinase fusions. *Cancer Discovery*, 4(8), pp.889–895.

Lowe, E.J., Sposto, R., Perkins, S.L., Gross, T.G., Finlay, J., Zwick, D. & Abromowitch, M., 2009. Intensive chemotherapy for systemic anaplastic large cell lymphoma in children and adolescents: Final results of children's cancer group study 5941. *Pediatric Blood and Cancer*, 52(3), pp.335–339.

Luthra, R., Pugh, W.C., Waasdorp, M., Morris, W., Cabanillas, F., Chan, P.K. & Sarris, A.H., 1998. Mapping of genomic t(2;5)(p23;q35) break points in patients with anaplastic large cell lymphoma by sequencing long-range PCR products. *Hematopathology and molecular hematology*, 11(3–4), pp.173–83.

Ma, Z., Cools, J., Marynen, P., Cui, X., Siebert, R., Gesk, S., Schlegelberger, B., Peeters, B., De Wolf-

Peeters, C., Wlodarska, I. & Morris, S.W., 2000. Inv(2)(p23q35) in anaplastic large-cell lymphoma induces constitutive anaplastic lymphoma kinase (ALK) tyrosine kinase activation by fusion to ATIC, an enzyme involved in purine nucleotide biosynthesis. *Blood*, 95(6), pp.2144–2149.

Madhun, A.S., Haaheim, L.R., Nøstbakken, J.K., Ebensen, T., Chichester, J., Yusibov, V., Guzman, C.A. & Cox, R.J., 2011. Intranasal c-di-GMP-adjuvanted plant-derived H5 influenza vaccine induces multifunctional Th1 CD4+ cells and strong mucosal and systemic antibody responses in mice. *Vaccine*, 29(31), pp.4973–4982.

Maes, B., Vanhentenrijk, V., Wlodarska, I., Cools, J., Peeters, B., Marynen, P. & De Wolf-Peeters, C., 2001. The NPM-ALK and the ATIK-ALK fusion genes can be detected in non-neoplastic cells. *American Journal of Pathology*, 158(6), pp.2185–2193.

Malcolm, T.I.M., Villarese, P., Fairbairn, C.J., Lamant, L., Trinquand, A., Hook, C.E., Burke, G.A.A., Brugières, L., Hughes, K., Payet, D., Merkel, O., Schiefer, A.I., Ashankyty, I., Mian, S., Wasik, M., Turner, M., Kenner, L., Asnafi, V., Macintyre, E. & Turner, S.D., 2016. Anaplastic large cell lymphoma arises in thymocytes and requires transient TCR expression for thymic egress. *Nature Communications*, 7, p.10087.

Mariño-Enríquez, A. & Dal Cin, P., 2013. ALK as a paradigm of oncogenic promiscuity: Different mechanisms of activation and different fusion partners drive tumors of different lineages. *Cancer Genetics*, 206(11), pp.357–373.

Martinengo, C., Poggio, T., Menotti, M., Scalzo, M.S., Mastini, C., Ambrogio, C., Pellegrino, E., Riera, L., Piva, R., Ribatti, D., Pastorino, F., Perri, P., Ponzoni, M., Wang, Q., Voena, C. & Chiarle, R., 2014. ALK-dependent control of hypoxia-inducible factors mediates tumor growth and metastasis. *Cancer Research*, 74(21), pp.6094–6106.

Martins, F., Sofiya, L., Sykiotis, G.P., Lamine, F., Maillard, M., Fraga, M., Shabafrouz, K., Ribi, C., Cairoli, A., Guex-Crosier, Y., Kuntzer, T., Michielin, O., Peters, S., Coukos, G., Spertini, F., Thompson, J.A. & Obeid, M., 2019. Adverse effects of immune-checkpoint inhibitors: epidemiology, management and surveillance. *Nature Reviews Clinical Oncology*, 16(9), pp.563–580.

Marzec, M., Liu, X., Wong, W., Yang, Y., Pasha, T., Kantekure, K., Zhang, P., Woetmann, A., Cheng, M., Odum, N. & Wasik, M.A., 2011. Oncogenic kinase NPM/ALK induces expression of HIF1α mRNA. *Oncogene*, 30(11), pp.1372–1378.

Matsuyama, H., Suzuki, H.I., Nishimori, H., Noguchi, M., Yao, T., Mano, H., Sugimoto, K., Miyazono, K., Dc, W., Matsuyama, H., Suzuki, H.I., Nishimori, H., Noguchi, M., Yao, T. & Komatsu, N., 2012. miR-135b mediates NPM-ALK – driven oncogenicity and renders IL-17 – producing immunophenotype to anaplastic large cell lymphoma. , 118(26), pp.6881–6892.

Maude, S.L., Laetsch, T.W., Buechner, J., Rives, S., Boyer, M., Bittencourt, H., Bader, P., Verneris, M.R., Stefanski, H.E., Myers, G.D., Qayed, M., De Moerloose, B., Hiramatsu, H., Schlis, K., Davis, K.L., Martin, P.L., Nemecek, E.R., Yanik, G.A., Peters, C., Baruchel, A., Boissel, N., Mechinaud, F., Balduzzi, A., Krueger, J., June, C.H., Levine, B.L., Wood, P., Taran, T., Leung, M., Mueller, K.T., Zhang, Y., Sen, K., Lebwohl, D., Pulsipher, M.A. & Grupp, S.A., 2018. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *New England Journal of Medicine*, 378(5), pp.439–448.

McWhirter, S.M., Barbalat, R., Monroe, K.M., Fontana, M.F., Hyodo, M., Joncker, N.T., Ishii, K.J., Akira, S., Colonna, M., Chen, Z.J., Fitzgerald, K.A., Hayakawa, Y. & Vance, R.E., 2009. A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. *Journal of Experimental Medicine*, 206(9), pp.1899–1911.

Mellgren, K., Hedegaard, C.J., Schmiegelow, K. & Müller, K., 2012. Plasma cytokine profiles at diagnosis in pediatric patients with non-hodgkin lymphoma. *Journal of Pediatric Hematology/Oncology*, 34(4), pp.271–275.

Melssen, M. & Slingluff, C.L., 2017. Vaccines targeting helper T cells for cancer immunotherapy. *Current Opinion in Immunology*, 47, pp.85–92.

Mildner, A. & Jung, S., 2014. Development and function of dendritic cell subsets. *Immunity*, 40(5), pp.642–656.

Minard-Colin, V., Brugières, L., Reiter, A., Cairo, M.S., Gross, T.G., Woessmann, W., Burkhardt, B., Sandlund, J.T., Williams, D., Pillon, M., Horibe, K., Auperin, A., Le Deley, M.C., Zimmerman, M., Perkins, S.L., Raphael, M., Lamant, L., Klapper, W., Mussolin, L., Poirel, H.A., Macintyre, E., Damm-Welk, C., Rosolen, A. & Patte, C., 2015. Non-hodgkin lymphoma in children and adolescents: Progress through effective collaboration, current knowledge, and challenges ahead. *Journal of Clinical Oncology*, 33(27), pp.2963–2974.

Montes-Mojarro, I.A., Steinhilber, J., Bonzheim, I., Quintanilla-Martinez, L. & Fend, F., 2018. The pathological spectrum of systemic anaplastic large cell lymphoma (ALCL). *Cancers*, 10(4), pp.1–22.

Mori, T., Kiyokawa, N., Shimada, H., Miyauchi, J. & Fujimoto, J., 2003. Anaplastic large cell lymphoma in Japanese children: Retrospective analysis of 34 patients diagnosed at the National Research Institute for Child Health and Development. *British Journal of Haematology*, 121(1), pp.94–96.

Mori, T., Takimoto, T., Katano, N., Kikuchi, A., Tabuchi, K., Kobayashi, R., Ayukawa, H., Kumagai, M., Horibe, K. & Tsurusawa, M., 2006. Recurrent childhood anaplastic large cell lymphoma: a retrospective analysis of registered cases in Japan. *British Journal of Haematology*, 132(5), pp.594–597.

Morris, S.W., Kirstein, M.N., Valentine, M.B., Dittmer, K.G., Shapiro, D.N., Saltman, D.L. & Look, A.T., 1994. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. *Science*, 263(5151), pp.1281–1284.

Morris, S.W., Naeve, C., Mathew, P., James, P.L., Kirstein, M.N., Cui, X. & Witte, D.P., 1997. ALK the chromosome 2 gene locus altered by the t(2;5) in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK). *Oncogene*, 14(18), pp.2175–2188.

Moser, O., Zimmermann, M., Meyer, U., Klapper, W., Oschlies, I., Schrappe, M., Attarbaschi, A., Mann, G., Niggli, F., Spix, C., Kontny, U., Klingebiel, T., Reiter, A., Burkhardt, B. & Woessmann, W., 2020. Second malignancies after treatment of childhood non-Hodgkin lymphoma: a report of the Berlin-Frankfurt-Muenster study group. *Haematologica*, p.haematol.2019.244780.

Mossé, Y.P., Lim, M.S., Voss, S.D., Wilner, K., Ruffner, K., Laliberte, J., Rolland, D., Balis, F.M., Maris, J.M., Weigel, B.J., Ingle, A.M., Ahern, C., Adamson, P.C. & Blaney, S.M., 2013. Safety and activity of crizotinib for paediatric patients with refractory solid tumours or anaplastic large-cell lymphoma:

A Children's Oncology Group phase 1 consortium study. *The Lancet Oncology*, 14(6), pp.472–480.

Mossé, Y.P., Voss, S.D., Lim, M.S., Rolland, D., Minard, C.G., Fox, E., Adamson, P., Wilner, K., Blaney, S.M. & Weigel, B.J., 2017. Targeting ALK with crizotinib in pediatric anaplastic large cell lymphoma and inflammatory myofibroblastic tumor: A Children's Oncology Group study. *Journal of Clinical Oncology*, 35(28), pp.3215–3221.

Moti, N., Malcolm, T., Hamoudi, R., Mian, S., Garland, G., Hook, C.E., Burke, G.A.A., Wasik, M.A., Merkel, O., Kenner, L., Laurenti, E., Dick, J.E. & Turner, S.D., 2014. Anaplastic large cell lymphoma-propagating cells are detectable by side population analysis and possess an expression profile reflective of a primitive origin. *Oncogene*, 34(14), pp.1843–1852.

Motz, G.T. & Coukos, G., 2013. Deciphering and Reversing Tumor Immune Suppression. *Immunity*, 39(1), pp.61–73.

Mougel, A., Terme, M. & Tanchot, C., 2019. Therapeutic cancer vaccine and combinations with antiangiogenic therapies and immune checkpoint blockade. *Frontiers in Immunology*, 10(MAR), p.467.

Mussolin, L., Pillon, M., Zimmermann, M., Carraro, E., Basso, G., Knoerr, F., Woessmann, W. & Damm-Welk, C., 2017. Course of anti-ALK antibody titres during chemotherapy in children with anaplastic large cell lymphoma. *British Journal of Haematology*, 182(5), pp.733–735.

Mussolin, L., Le Deley, M.-C., Carraro, E., Damm-Welk, C., Attarbaschi, A., Williams, D., Burke, A., Horibe, K., Nakazawa, A., Wrobel, G., Mann, G., Csóka, M., Uyttebroeck, A., Fernández-Delgado Cerdá, R., Beishuizen, A., Mellgren, K., Burkhardt, B., Klapper, W., Turner, S.D., D, E.S., Lamant, L., Reiter, A., Woessmann, W., Brugières, L. & Pillon, M., 2020. Prognostic Factors in Childhood Anaplastic Large Cell Lymphoma: Long Term Results of the International ALCL99 Trial on behalf of the European Inter-Group for Childhood Non-Hodgkin lymphoma (EICNHL). *Cancers*, 12, p.2747.

Mussolin, L., Bonvini, P., Ait-Tahar, K., Pillon, M., Tridello, G., Buffardi, S., Lombardi, A., Pulford, K. & Rosolen, A., 2009. Kinetics of humoral response to ALK and its relationship with minimal residual disease in pediatric ALCL. *Leukemia*, 23(2), pp.400–402.

Mussolin, L., Pillon, M., D'Amore, E.S., Santoro, N., Lombardi, A., Fagioli, F., Zanesco, L. & Rosolen, A., 2005. Prevalence and clinical implications of bone marrow involvement in pediatric anaplastic large cell lymphoma. *Leukemia*, 19(9), pp.1643–1647.

Mussolin, L., Damm-Welk, C., Pillon, M., Zimmermann, M., Franceschetto, G., Pulford, K., Reiter, A., Rosolen, A. & Woessmann, W., 2013. Use of minimal disseminated disease and immunity to NPM-ALK antigen to stratify ALK-positive ALCL patients with different prognosis. *Leukemia*, 27(2), pp.416–422.

Neelapu, S.S., Locke, F.L., Bartlett, N.L., Lekakis, L.J., Miklos, D.B., Jacobson, C.A., Braunschweig, I., Oluwole, O.O., Siddiqi, T., Lin, Y., Timmerman, J.M., Stiff, P.J., Friedberg, J.W., Flinn, I.W., Goy, A., Hill, B.T., Smith, M.R., Deol, A., Farooq, U., McSweeney, P., Munoz, J., Avivi, I., Castro, J.E., Westin, J.R., Chavez, J.C., Ghobadi, A., Komanduri, K. V., Levy, R., Jacobsen, E.D., Witzig, T.E., Reagan, P., Bot, A., Rossi, J., Navale, L., Jiang, Y., Aycock, J., Elias, M., Chang, D., Wiezorek, J. & Go, W.Y., 2017. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-Cell lymphoma.

New England Journal of Medicine, 377(26), pp.2531–2544.

Nomura, A., Narita, Y., Miyakita, Y., Ohno, M., Fukishima, S., Maruyama, T., Muragaki, Y. & Shibui, S., 2013. Clinical presentation of anaplastic large-cell lymphoma in the central nervous system. *Molecular and Clinical Oncology*, 1(4), pp.655–660.

O'Connor, N.T.J., Stein, H., Gatter, K.C., Wainscoat, J.S., Crick, J., Al Saati, T., Falini, B., Delsol, G. & Manson, D.Y., 1987. Genotypic analysis of large cell lymphomas which express the Ki□1 antigen. *Histopathology*, 11(7), pp.733–740.

Onciu, M., Behm, F.G., Raimondi, S.C., Moore, S., Harwood, E.L., Pui, C.-H. & Sandlund, J.T., 2003. ALK-positive anaplastic large cell lymphoma with leukemic peripheral blood involvement is a clinicopathologic entity with an unfavorable prognosis. Report of three cases and review of the literature. *American journal of clinical pathology*, 120(4), pp.617–25.

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., 2017. An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature*, 547(7662), pp.217–221.

Overacre-Delgoffe, A.E., Chikina, M., Dadey, R.E., Yano, H., Brunazzi, E.A., Shayan, G., Horne, W., Moskovitz, J.M., Kolls, J.K., Sander, C., Shuai, Y., Normolle, D.P., Kirkwood, J.M., Ferris, R.L., Delgoffe, G.M., Bruno, T.C., Workman, C.J. & Vignali, D.A.A., 2017. Interferon-γ Drives Treg Fragility to Promote Anti-tumor Immunity. *Cell*, 169(6), pp.1130-1141.e11.

Overwijk, W.W., 2015. Human CD4+ T cells spontaneously detect somatic mutations in cancer cells. *Nature Medicine*, 21(1), pp.12–14.

Palmer, R.H., Vernersson, E., Grabbe, C. & Hallberg, B., 2009. Anaplastic lymphoma kinase: signalling in development and disease. *Biochemical Journal*, 420(3), pp.345–361.

Palucka, A.K. & Coussens, L.M., 2016. The Basis of Oncoimmunology. *Cell*, 164(6), pp.1233–1247.

Passoni, L., Scardino, A., Bertazzoli, C., Gallo, B., Coluccia, A.M.L., Lemonnier, F.A., Kosmatopoulos, K. & Gambacorti-Passerini, C., 2002. ALK as a novel lymphoma-associated tumor antigen: Identification of 2 HLA-A2.1-restricted CD8+T-cell epitopes. *Blood*, 99(6), pp.2100–2106.

Passoni, L., Gallo, B., Biganzoli, E., Stefanoni, R., Massimino, M., Di Nicola, M., Gianni, A.M. & Gambacorti-Passerini, C., 2006. In vivo T-cell immune response against anaplastic lymphoma kinase in patients with anaplastic large cell lymphomas. *Haematologica*, (91), pp.48–55.

Pearson, J.D., Lee, J.K.H., Bacani, J.T.C., Lai, R. & Ingham, R.J., 2011. NPM-ALK and the junb transcription factor regulate the expression of cytotoxic molecules in ALK-positive, anaplastic large cell lymphoma. *International Journal of Clinical and Experimental Pathology*, 4(2), pp.124–133.

Perkins, S.L., Pickering, D., Lowe, E.J., Zwick, D., Abromowitch, M., Davenport, G., Cairo, M.S. & Sanger, W.G., 2005. Childhood anaplastic large cell lymphoma has a high incidence of ALK gene rearrangement as determined by immunohistochemical staining and fluorescent in situ hybridisation: A genetic and pathological correlation. *British Journal of Haematology*, 131(5), pp.624–627.

Peterson, P., Org, T. & Rebane, A., 2008. Transcriptional regulation by AIRE: Molecular mechanisms of central tolerance. *Nature Reviews Immunology*, 8(12), pp.948–957.

Pileri, S., Falini, B., Delsol, G., Stein, H., Baglioni, P., Poggi, S., Martelli, M.F., Rivano, M.T., Mason, D.Y. & Stansfeld, A.G., 1990. Lymphohistiocytic T□cell lymphoma (anaplastic large cell lymphoma CD30+/Ki□1 + with a high content of reactive histiocytes). *Histopathology*, 16(4), pp.383–391.

Pillon, M., Gregucci, F., Lombardi, A., Santoro, N., Piglione, M., Sala, A., D'Amore, E.S.G., De Santis, R., Casale, F., Zecca, M., Mussolin, L. & Rosolen, A., 2012. Results of AIEOP LNH-97 protocol for the treatment of anaplastic large cell lymphoma of childhood. *Pediatric Blood and Cancer*, 59(5), pp.828–833.

Pro, B., Advani, R., Brice, P., Bartlett, N.L., Rosenblatt, J.D., Illidge, T., Matous, J., Ramchandren, R., Fanale, M., Connors, J.M., Yang, Y., Sievers, E.L., Kennedy, D.A. & Shustov, A., 2012. Brentuximab vedotin (SGN-35) in patients with relapsed or refractory systemic anaplastic large-cell lymphoma: Results of a phase II study. *Journal of Clinical Oncology*, 30(18), pp.2190–2196.

Pro, B., Advani, R.H., Brice, P., Bartlett, N.L., Rosenblatt, J.D., Illidge, T., Matous, J., Ramchandren, R., Fanale, M.A., Connors, J.M., Fenton, K., Huebner, D., Pinelli, J. & Shustov, A.R., 2016. Five-Year Survival Data from a Pivotal Phase 2 Study of Brentuximab Vedotin in Patients with Relapsed or Refractory Systemic Anaplastic Large Cell Lymphoma. *Blood*, 128(22), pp.4144–4144.

Provine, N.M., Larocca, R.A., Aid, M., Penaloza-MacMaster, P., Badamchi-Zadeh, A., Borducchi, E.N., Yates, K.B., Abbink, P., Kirilova, M., Ng'ang'a, D., Bramson, J., Haining, W.N. & Barouch, D.H., 2016. Immediate Dysfunction of Vaccine-Elicited CD8 + T Cells Primed in the Absence of CD4 + T Cells. *The Journal of Immunology*, 197(5), pp.1809–1822.

Pulford, K., Falini, B., Banham, A.H., Codrington, D., Roberton, H., Mason, D.Y., Dc, W., Pulford, K., Falini, B., Banham, A.H., Codrington, D., Roberton, H., Hatton, C. & Mason, D.Y., 2000. Immune response to the ALK oncogenic tyrosine kinase in patients with anaplastic large-cell lymphoma. *Blood*, 96(4), pp.1605–1607.

Ramos, C.A., Ballard, B., Zhang, H., Dakhova, O., Gee, A.P., Mei, Z., Bilgi, M., Wu, M., Liu, H., Grilley, B., Bollard, C.M., Chang, B.H., Rooney, C.M., Brenner, M.K., Heslop, H.E., Dotti, G. & Savoldo, B., 2017. Clinical and immunological responses after CD30-specific chimeric antigen receptor – redirected lymphocytes. , 127(9), pp.1–10.

Reed, C.M., Cresce, N.D., Mauldin, I.S., Slingluff, C.L. & Olson, W.C., 2015. Vaccination with melanoma helper peptides induces antibody responses associated with improved overall survival. *Clinical Cancer Research*, 21(17), pp.3879–3887.

Reiter, A., Schrappe, M., Tiemann, M., Parwaresch, R., Zimmermann, M., Yakisan, E., Dopfer, R., Bucsky, P., Mann, G., Gadner, H. & Riehm, H., 1994. Successful treatment strategy for Ki-1 anaplastic large-cell lymphoma of childhood: A prospective analysis of 62 patients enrolled in three consecutive Berlin-Frankfurt-Munster group studies. *Journal of Clinical Oncology*, 12(5), pp.899–908.

Ridge, J.P., Di Rosa, F. & Matzinger, P., 1998. A conditioned dendritic cell can be a temporal bridge between a CD4 + T-helper and a T-killer cell. *Nature*, 393(6684), pp.474–478.

Rigaud, C., Abbou, S., Minard-Colin, V., Geoerger, B., Scoazec, J.Y., Vassal, G., Jaff, N., Heuberger, L., Valteau-Couanet, D. & Brugieres, L., 2018. Efficacy of nivolumab in a patient with systemic

refractory ALK+ anaplastic large cell lymphoma. *Pediatric Blood and Cancer*, 65(4).

Rikova, K., Guo, A., Zeng, Q., Possemato, A., Yu, J., Haack, H., Nardone, J., Lee, K., Reeves, C., Li, Y., Hu, Y., Tan, Z., Stokes, M., Sullivan, L., Mitchell, J., Wetzel, R., MacNeill, J., Ren, J.M., Yuan, J., Bakalarski, C.E., Villen, J., Kornhauser, J.M., Smith, B., Li, D., Zhou, X., Gygi, S.P., Gu, T.L., Polakiewicz, R.D., Rush, J. & Comb, M.J., 2007. Global Survey of Phosphotyrosine Signaling Identifies Oncogenic Kinases in Lung Cancer. *Cell*, 131(6), pp.1190–1203.

Robert, C., 2020. A decade of immune-checkpoint inhibitors in cancer therapy. *Nature Communications*, 11(1), pp.1–3.

Romero, P., Valmori, D., Pittet, M.J., Zippelius, A., Rimoldi, D., Lévy, F., Dutoit, V., Ayyoub, M., Rubio-Godoy, V., Michielin, O., Guillaume, P., Batard, P., Luescher, I.F., Lejeune, F., Liénard, D., Rufer, N., Dietrich, P.Y., Speiser, D.E. & Cerottini, J.C., 2002. Antigenicity and immunogenicity of Melan-A/MART-1 derived peptides as targets for tumor reactive CTL in human melanoma. *Immunological Reviews*, 188, pp.81–96.

Rosolen, A., Pillon, M., Garaventa, A., Burnelli, R., D'Amore, E.S., Giuliano, M., Comis, M., Cesaro, S., Tettoni, K., Moleti, M.L., Tamaro, P., Visintin, G. & Zanesco, L., 2005. Anaplastic large cell lymphoma treated with a leukemia-like therapy: Report of the Italian Association of Pediatric Hematology and Oncology (AIEOP) LNH-92 protocol. *Cancer*, 104(10), pp.2133–2140.

Ruf, S., Brugieres, L., Pillon, M., Zimmermann, M., Attarbaschi, A., Mellgren, K., Williams, D., Uyttebroeck, A., Wrobel, G., Reiter, A. & Woessmann, W., 2015. Risk-adapted Therapy for Patients with Relapsed or Refractory ALCL – final report of the Prospective ALCL-Relapse Trial of the EICNHL. *British Journal of Haematology 171:45-45*.

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., 2017. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature*, 547(7662), pp.222–226.

Saito, T., Nishikawa, H., Wada, H., Nagano, Y., Sugiyama, D., Atarashi, K., Maeda, Y., Hamaguchi, M., Ohkura, N., Sato, E., Nagase, H., Nishimura, J., Yamamoto, H., Takiguchi, S., Tanoue, T., Suda, W., Morita, H., Hattori, M., Honda, K., Mori, M., Doki, Y. & Sakaguchi, S., 2016. Two FOXP3 + CD4 + T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nature Medicine*, 22(6), pp.679–684.

Sakaguchi, S., Miyara, M., Costantino, C.M. & Hafler, D.A., 2010. FOXP3 + regulatory T cells in the human immune system. *Nature Reviews Immunology*, 10(7), pp.490–500.

Salaverria, I., Beà, S., Lopez-Guillermo, A., Lespinet, V., Pinyol, M., Burkhardt, B., Lamant, L., Zettl, A., Horsman, D., Gascoyne, R., Ott, G., Siebert, R., Delsol, G. & Campo, E., 2008. Genomic profiling reveals different genetic aberrations in systemic ALK-positive and ALK-negative anaplastic large cell lymphomas. *British Journal of Haematology*, 140(5), pp.516–526.

Sandlund, J.T., 2015. Non-Hodgkin Lymphoma in Children. *Current Hematologic Malignancy Reports*,

10(3), pp.237–243.

Sandlund, J.T. & Martin, M.G., 2016. Non-Hodgkin lymphoma across the pediatric and adolescent and young adult age spectrum. *Hematology*, 2016(1), pp.589–597.

Saravia, J., Chapman, N.M. & Chi, H., 2019. Helper T cell differentiation. *Cellular and Molecular Immunology*, 16(7), pp.634–643.

Savage, K.J., Harris, N.L., Vose, J.M., Ullrich, F., Jaffe, E.S., Connors, J.M., Rimsza, L., Pileri, S.A., Chhanabhai, M., Gascoyne, R.D., Armitage, J.O. & Weisenburger, D.D., 2008. ALK - anaplastic large-cell lymphoma is clinically and immunophenotypically different from both ALK + ALCL and peripheral T-cell lymphoma, not otherwise specified: Report from the International Peripheral T-Cell Lymphoma Project. *Blood*, 111(12), pp.5496–5504.

Savan, R., McFarland, A.P., Reynolds, D.A., Feigenbaum, L., Ramakrishnan, K., Karwan, M., Shirota, H., Klinman, D.M., Dunleavy, K., Pittaluga, S., Anderson, S.K., Donnelly, R.P., Wilson, W.H. & Young, H.A., 2011. A novel role for IL-22R1 as a driver of inflammation. *Blood*, 117(2), pp.575–584.

Sayour, E.J., McLendon, P., McLendon, R., De Leon, G., Reynolds, R., Kresak, J., Sampson, J.H. & Mitchell, D.A., 2015. Increased proportion of FoxP3+ regulatory T cells in tumor infiltrating lymphocytes is associated with tumor recurrence and reduced survival in patients with glioblastoma. *Cancer Immunology, Immunotherapy*, 64(4), pp.419–427.

Schleussner, N., Merkel, O., Costanza, M., Liang, H.C., Hummel, F., Romagnani, C., Durek, P., Anagnostopoulos, I., Hummel, M., Jöhrens, K., Niedobitek, A., Griffin, P.R., Piva, R., Sczakiel, H.L., Woessmann, W., Damm-Welk, C., Hinze, C., Stoiber, D., Gillissen, B., Turner, S.D., Kaergel, E., von Hoff, L., Grau, M., Lenz, G., Dörken, B., Scheidereit, C., Kenner, L., Janz, M. & Mathas, S., 2018. The AP-1-BATF and -BATF3 module is essential for growth, survival and TH17/ILC3 skewing of anaplastic large cell lymphoma. *Leukemia*, 32(9), pp.1994–2007.

Schultze, J.L., Freeman, G.J., Gribben, J.G. & Nadler, L.M., 2002. Method of promoting B-cell proliferation and activation with CD40 ligand and cyclosporin. United States Patent 6465251.

Seidemann, K., Tiemann, M., Schrappe, M., Yakisan, E., Simonitsch, I., Janka-Schaub, G., Dörffel, W., Zimmermann, M., Mann, G., Gadner, H., Parwaresch, R., Riehm, H. & Reiter, A., 2001. Short-pulse B-non-Hodgkin lymphoma-type chemotherapy is efficacious treatment for pediatric anaplastic large cell lymphoma: A report of the Berlin-Frankfurt-Münster Group Trial NHL-BFM 90. *Blood*, 97(12), pp.3699–3706.

Selby, M.J., Engelhardt, J.J., Quigley, M., Henning, K.A., Chen, T., Srinivasan, M. & Korman, A.J., 2013. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer immunology research*, 1(1), pp.32–42.

Sharma, G.G., Mota, I., Mologni, L., Patrucco, E., Gambacorti-Passerini, C. & Chiarle, R., 2018. Tumor resistance against ALK targeted therapy-Where it comes from and where it goes. *Cancers*, 10(3).

Sharonov, G. V., Serebrovskaya, E.O., Yuzhakova, D. V., Britanova, O. V. & Chudakov, D.M., 2020. B cells, plasma cells and antibody repertoires in the tumour microenvironment. *Nature Reviews Immunology*, 20(5), pp.294–307.

Siegel, R.L., Miller, K.D. & Jemal, A., 2019. Cancer statistics, 2019. *CA: A Cancer Journal for Clinicians*, 69(1), pp.7–34.

Simpson, T.R., Li, F., Montalvo-Ortiz, W., Sepulveda, M.A., Bergerhoff, K., Arce, F., Roddie, C., Henry, J.Y., Yagita, H., Wolchok, J.D., Peggs, K.S., Ravetch, J. V., Allison, J.P. & Quezada, S.A., 2013. Fc-dependent depletion of tumor-infiltrating regulatory t cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *Journal of Experimental Medicine*, 210(9), pp.1695–1710.

Singh, V.K., Werner, S., Hackstein, H., Lennerz, V., Reiter, A., Wölfel, T., Damm-Welk, C. & Woessmann, W., 2016. Analysis of nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)-reactive CD8 + T cell responses in children with NPM-ALK + anaplastic large cell lymphoma. *Clinical & Experimental Immunology*, 186(1), pp.96–105.

Singh, V.K., Werner, S., Schwalm, S., Lennerz, V., Ruf, S., Stadler, S., Hackstein, H., Reiter, A., Wölfel, T., Damm-Welk, C. & Woessmann, W., 2019. NPM-ALK-reactive T-cell responses in children and adolescents with NPM-ALK positive anaplastic large cell lymphoma. *OncoImmunology*, 8(9), pp.1–13.

Slingluff, C.L., 2011. The present and future of peptide vaccines for cancer: Single or multiple, long or short, alone or in combination? *Cancer Journal*, 17(5), pp.343–350.

Slingluff, C.L., Petroni, G.R., Chianese-Bullock, K.A., Smolkin, M.E., Ross, M.I., Haas, N.B., Von Mehren, M. & Grosh, W.W., 2011. Randomized multicenter trial of the effects of melanoma-associated helper peptides and cyclophosphamide on the immunogenicity of a multipeptide melanoma vaccine. *Journal of Clinical Oncology*, 29(21), pp.2924–2932.

Slingluff, C.L., Lee, S., Zhao, F., Chianese-Bullock, K.A., Olson, W.C., Butterfield, L.H., Whiteside, T.L., Leming, P.D. & Kirkwood, J.M., 2013. A randomized phase II trial of multiepitope vaccination with melanoma peptides for cytotoxic T cells and helper T cells for patients with metastatic melanoma (E1602). *Clinical Cancer Research*, 19(15), pp.4228–4238.

Slota, M., Lim, J.B., Dang, Y. & Disis, M.L., 2011. ELISpot for measuring human immune responses to vaccines. *Expert Review of Vaccines*, 10(3), pp.299–306.

Spiegel, A., Paillard, C., Ducassou, S., Perel, Y., Plantaz, D., Strullu, M., Eischen, A., Lutz, P., Lamant, L., Le Deley, M.C. & Brugières, L., 2014. Paediatric anaplastic large cell lymphoma with leukaemic presentation in children: A report of nine French cases. *British Journal of Haematology*, 165(4), pp.545–551.

Stadler, S., Singh, V.K., Knörr, F., Damm-Welk, C. & Woessmann, W., 2018. Immune response against ALK in children with ALK-positive anaplastic large cell lymphoma. *Cancers*, 10(4).

Stein, H., Foss, H.D., Durkop, H., Marafioti, T., Delsol, G., Pulford, K., Pileri, S. & Falini, B., 2000. CD30+ anaplastic large cell lymphoma: A review of its histopathologic, genetic, and clinical features. *Blood*, 96(12), pp.3681–3695.

Stein, H., Mason, D.Y., Gerdes, J., O'Connor, N., Wainscoat, J., Pallesen, G., Gatter, K., Falini, B., Delsol, G. & Lemke, H., 1985. The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic lymphoid tissue: Evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. *Blood*, 66(4), pp.848–858.

Steinman, R.M., 2012. Decisions about dendritic cells: Past, present, and future. *Annual Review of Immunology*, 30, pp.1–22.

Strullu, M., Thomas, C., Le Deley, M.C., Chevance, A., Kanold, J., Bertrand, Y., Jubert, C., Dalle, J.H., Paillard, C., Baruchel, A., Lamant, L., Michel, G. & Brugières, L., 2015. Hematopoietic stem cell

transplantation in relapsed ALK+ anaplastic large cell lymphoma in children and adolescents: A study on behalf of the SFCE and SFGM-TC. *Bone Marrow Transplantation*, 50(6), pp.795–801.

Swerdlow, S.H., Campo, E., Pileri, S.A., Harris, N.L., Stein, H., Siebert, R., Advani, R., Ghielmini, M., Salles, G.A., Zelenetz, A.D. & Jaffe, E.S., 2017. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *The blood journal*, 127(20), pp.453–462.

Swerdlow, S.H., Campo, E., Harris, N.L., Jaffe, E.S., Pileri, S.A., Stein, H., Thiele, J. & Vardiman, J.W., 2008. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition*, Lyon, France: IARC Press.

Tanaka, A. & Sakaguchi, S., 2019. Targeting Treg cells in cancer immunotherapy. *European Journal of Immunology*, 49(8), pp.1140–1146.

Tanaka, H., Matsushima, H., Mizumoto, N. & Takashima, A., 2009. Classification of chemotherapeutic agents based on their differential in vitro effects on dendritic cells. *Cancer Research*, 69(17), pp.6978–6986.

Tanaka, H., Matsushima, H., Nishibu, A., Clausen, B.E. & Takashima, A., 2009. Dual therapeutic efficacy of vinblastine as a unique chemotherapeutic agent capable of inducing dendritic cell maturation. *Cancer Research*, 69(17), pp.6987–6994.

Tay, R.E., Richardson, E.K. & Toh, H.C., 2020. Revisiting the role of CD4+ T cells in cancer immunotherapy—new insights into old paradigms. *Cancer Gene Therapy*, pp.1–13.

Van Tilburg, C.M., Van Gent, R., Bierings, M.B., Otto, S.A., Sanders, E.A.M., Nibbelke, E.E., Gaiser, J.F., Janssens-Korpela, P.L., Wolfs, T.F.W., Bloem, A.C., Borghans, J.A.M. & Tesselaar, K., 2011. Immune reconstitution in children following chemotherapy for haematological malignancies: A long-term follow-up. *British Journal of Haematology*, 152(2), pp.201–210.

Togashi, Y., Kamada, T., Sasaki, A., Nakamura, Y., Fukuoka, S., Tada, Y., Kawazoe, A., Shitara, K. & Nishikawa, H., 2018. Clinicopathological, genomic and immunological features of hyperprogressive disease during PD-1 blockade in gastric cancer patients. *Journal of Clinical Oncology*, 36(15_suppl), pp.4106–4106.

Togashi, Y. & Nishikawa, H., 2017. Regulatory T cells: Molecular and cellular basis for immunoregulation. *Current Topics in Microbiology and Immunology*, 410, pp.3–27.

Togashi, Y., Shitara, K. & Nishikawa, H., 2019. Regulatory T cells in cancer immunosuppression — implications for anticancer therapy. *Nature Reviews Clinical Oncology*, 16(6), pp.356–371.

Tole, S., Wheaton, L. & Alexander, S., 2018. Pediatric Anaplastic Large Cell Lymphoma—A Review. *Oncology & Hematology Review (US)*, 14(1), p.21.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-tieulent, J. & Jemal, A., 2015. Global Cancer Statistics, 2012. *CA: a cancer journal of clinicians.*, 65(2), pp.87–108.

Tort, F., Pinyol, M., Pulford, K., Roncador, G., Hernandez, L., Nayach, I., Kluin-nelemans, H.C., Kluin, P., Touriol, C., Delsol, G., Mason, D. & Campo, E., 2001. Molecular Characterization of a New ALK Translocation Involving Moesin (MSN-ALK) in Anaplastic Large Cell Lymphoma. , 81(3), pp.419–426.

Touriol, C., Greenland, C., Lamant, L., Pulford, K., Bernard, F., Rousset, T., Mason, D.Y. & Delsol, G., 2000. Further demonstration of the diversity of chromosomal changes involving 2p23 in ALK-positive lymphoma: 2 cases expressing ALK kinase fused to CLTCL (clathrin chain polypeptide-

like). *Blood*, 95(10), pp.3204–3207.

Trümper, L., Pfreundschuh, M., Bonin, F. V. & Daus, H., 1998. Detection of the t(2;5)-associated NPM/ALK fusion cDNA in peripheral blood cells of healthy individuals. *British Journal of Haematology*, 103(4), pp.1138–1144.

Tsuji, T., Sabbatini, P., Jungbluth, A.A., Ritter, E., Pan, L., Ritter, G., Ferran, L., Spriggs, D., Salazar, A.M. & Gnjatic, S., 2013. Effect of Montanide and poly-ICLC adjuvant on human self/tumor antigen-specific CD4+ T cells in phase I overlapping long peptide vaccine trial. *Cancer immunology research*, 1(5), pp.340–350.

Türeci, Ö., Vormehr, M., Diken, M., Kreiter, S., Huber, C. & Sahin, U., 2016. Targeting the heterogeneity of cancer with individualized neoepitope vaccines. *Clinical Cancer Research*, 22(8), pp.1885–1896.

Vargas, F.A., Furness, A.J.S., Litchfield, K., Joshi, K., Rosenthal, R., Ghorani, E., Solomon, I., Lesko, M.H., Ruef, N., Roddie, C., … Kirk, A., 2018. Fc Effector Function Contributes to the Activity of Human Anti-CTLA-4 Antibodies. *Cancer Cell*, 33(4), pp.649-663.e4.

Vega, F., Medeiros, L.J., Leventaki, V., Atwell, C., Cho-Vega, J.H., Tian, L., Claret, F.X. & Rassidakis, G.Z., 2006. Activation of mammalian target of rapamycin signaling pathway contributes to tumor cell survival in anaplastic lymphoma kinase-positive anaplastic large cell lymphoma. *Cancer Research*, 66(13), pp.6589–6597.

Vernersson, E., Khoo, N.K.S., Henriksson, M.L., Roos, G., Palmer, R.H. & Hallberg, B., 2006. Characterization of the expression of the ALK receptor tyrosine kinase in mice. *Gene Expression Patterns*, 6(5), pp.448–461.

Voena, C., Menotti, M., Mastini, C., Di Giacomo, F., Longo, D.L., Castella, B., Merlo, M.E.B., Ambrogio, C., Wang, Q., Minero, V.G., Poggio, T., Martinengo, C., D'Amico, L., Panizza, E., Mologni, L., Cavallo, F., Altruda, F., Butaney, M., Capelletti, M., Inghirami, G., Janne, P.A. & Chiarle, R., 2015. Efficacy of a Cancer Vaccine against ALK-Rearranged Lung Tumors. *Cancer Immunology Research*, 3(12), pp.1333–1343.

Voena, C., Conte, C., Ambrogio, C., Erba, E.B., Boccalatte, F., Mohammed, S., Jensen, O.N., Palestro, G., Inghirami, G. & Chiarle, R., 2007. The tyrosine phosphatase Shp2 interacts with NPM-ALK and regulates anaplastic lymphoma cell growth and migration. *Cancer Research*, 67(9), pp.4278–4286.

Waldman, A.D., Fritz, J.M. & Lenardo, M.J., 2020. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nature Reviews Immunology*, pp.1–18.

Walker, A.J., Majzner, R.G., Zhang, L., Wanhainen, K., Long, A.H., Nguyen, S.M., Lopomo, P., Vigny, M., Fry, T.J., Orentas, R.J. & Mackall, C.L., 2017. Tumor Antigen and Receptor Densities Regulate Efficacy of a Chimeric Antigen Receptor Targeting Anaplastic Lymphoma Kinase. *Molecular Therapy*, 25(9), pp.2189–2201.

Wang, D., Quiros, J., Mahuron, K., Pai, C.C., Ranzani, V., Young, A., Silveria, S., Harwin, T., Abnousian, A., Pagani, M., Rosenblum, M.D., Van Gool, F., Fong, L., Bluestone, J.A. & DuPage, M., 2018. Targeting EZH2 Reprograms Intratumoral Regulatory T Cells to Enhance Cancer Immunity. *Cell Reports*, 23(11), pp.3262–3274.

Wasik, M.A., Zhang, Q., Marzec, M., Kasprzycka, M., Wang, H.Y. & Liu, X., 2009. Anaplastic Lymphoma Kinase (ALK)-Induced Malignancies: Novel Mechanisms of Cell Transformation and Potential

Therapeutic Approaches. *Seminars in Oncology*, 36(SUPPL. 1), pp.S27-35.

Williams, D., Mori, T., Reiter, A., Woessman, W., Rosolen, A., Wrobel, G., Zsiros, J., Uyttebroeck, A., Marky, I., Le Deley, M.C. & Brugières, L., 2013. Central nervous system involvement in anaplastic large cell lymphoma in childhood: Results from a multicentre European and Japanese study. *Pediatric Blood and Cancer*, 60(10), pp.E118–E121.

Williams, D.M., Hobson, R., Imeson, J., Gerrard, M., McCarthy, K. & Pinkerton, C.R., 2002. Anaplastic large cell lymphoma in childhood: Analysis of 72 patients treated on The United Kingdom Children's Cancer Study Group chemotherapy regimens. *British Journal of Haematology*, 117(4), pp.812–820.

Woessmann, W., 2018. *unpublished work*, Department of Pediatric Hematology and Oncology, University Hospital Hamburg-Eppendorf, Hamburg, Germany.

Woessmann, W., Peters, C., Lenhard, M., Burkhardt, B., Sykora, K.W., Dilloo, D., Kremens, B., Lang, P., Führer, M., Kühne, T., Parwaresch, R., Ebell, W. & Reiter, A., 2006. Allogeneic haematopoietic stem cell transplantation in relapsed or refractory anaplastic large cell lymphoma of children and adolescents - A Berlin-Frankfurt-Münster group report. *British Journal of Haematology*, 133(2), pp.176–182.

Woessmann, W., Zimmermann, M., Lenhard, M., Burkhardt, B., Rossig, C., Kremens, B., Lang, P., Attarbaschi, A., Mann, G., Oschlies, I., Klapper, W. & Reiter, A., 2011. Relapsed or refractory anaplastic large-cell lymphoma in children and adolescents after Berlin-Frankfurt-Muenster (BFM)-type first-line therapy: A BFM-Group study. *Journal of Clinical Oncology*, 29(22), pp.3065–3071.

Woo, S.R., Fuertes, M.B., Corrales, L., Spranger, S., Furdyna, M.J., Leung, M.Y.K., Duggan, R., Wang, Y., Barber, G.N., Fitzgerald, K.A., Alegre, M.L. & Gajewski, T.F., 2014. STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity*, 41(5), pp.830–842.

Wrobel, G., Mauguen, A., Rosolen, A., Reiter, A., Williams, D., Horibe, K., Brugières, L. & Le Deley, M.C., 2011. Safety assessment of intensive induction therapy in childhood anaplastic large cell lymphoma: Report of the ALCL99 randomised trial. *Pediatric Blood and Cancer*, 56(7), pp.1071–1077.

Xhangolli, I., Dura, B., Lee, G.H., Kim, D., Xiao, Y. & Fan, R., 2019. Single-cell Analysis of CAR-T Cell Activation Reveals A Mixed TH1/TH2 Response Independent of Differentiation. *Genomics, Proteomics and Bioinformatics*, 17(2), pp.129–139.

Yan, H., KuoLee, R., Tram, K., Qiu, H., Zhang, J., Patel, G.B. & Chen, W., 2009. 3′,5′-Cyclic diguanylic acid elicits mucosal immunity against bacterial infection. *Biochemical and Biophysical Research Communications*, 387(3), pp.581–584.

Yang, Y., Kohler, M.E., Chien, C.D., Sauter, C.T., Jacoby, E., Yan, C., Hu, Y., Wanhainen, K., Qin, H. & Fry, T.J., 2017. TCR engagement negatively affects CD8 but not CD4 CAR T cell expansion and leukemic clearance. *Science Translational Medicine*, 9(417).

Yarchoan, M., Johnson, B.A., Lutz, E.R., Laheru, D.A. & Jaffee, E.M., 2017. Targeting neoantigens to augment antitumour immunity. *Nature Reviews Cancer*, 17(4), pp.209–222.

Youssif, C., Goldenbogen, J., Hamoudi, R., Carreras, J., Viskaduraki, M., Cui, Y.X., Bacon, C.M., Burke, G.A.A. & Turner, S.D., 2009. Genomic profiling of pediatric ALK-positive anaplastic large cell

lymphoma: A children's cancer and leukaemia group study. *Genes Chromosomes and Cancer*, 48(11), pp.1018–1026.

Yuseff, M.I., Pierobon, P., Reversat, A. & Lennon-Duménil, A.M., 2013. How B cells capture, process and present antigens: A crucial role for cell polarity. *Nature Reviews Immunology*, 13(7), pp.475–486.

Zhang, N. & Bevan, M.J., 2011. CD8+ T Cells: Foot Soldiers of the Immune System. *Immunity*, 35(2), pp.161–168.

Zhao, J., Chen, Y., Ding, Z.Y. & Liu, J.Y., 2019. Safety and efficacy of therapeutic cancer vaccines alone or in combination with immune checkpoint inhibitors in cancer treatment. *Frontiers in Pharmacology*, 10.

Zhou, Y., Shao, N., Aierken, N., Xie, C., Ye, R., Qian, X., Hu, Z., Zhang, J. & Lin, Y., 2017. Prognostic value of tumor-infiltrating Foxp3+ regulatory T cells in patients with breast cancer: A meta-analysis. *Journal of Cancer*, 8(19), pp.4098–4105.

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Publications

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Stadler S, Singh VK, Knörr F, Damm-Welk C, Woessmann W. Immune Response against ALK in Children with ALK-Positive Anaplastic Large Cell Lymphoma. Cancers (Basel). 2018 Apr 10;10(4). pii: E114.

Stadler S, Nguyen CH, Schachner, Milovanovic D, Holzner S, Brenner S, Eichsteininger J, Stadler M, Senfter D, Krenn L, Schmidt WM, Huttary N, Krieger S, Koperek O, Bago-Horvath Z, Brendel KA, Marian B, de Wever O, Mader RM, Giessrigl B, Jäger W, Dolznig H, Krupitza G. Colon cancer cell-derived 12(S)-HETE induces the retraction of cancer-associated fibroblasts via MLC2, RHO/ROCK and Ca2+ signalling. Cell Mol Life Sci. 2017 May;74(10):1907-1921.

Holzner S, Brenner S, Atanasov AG, Senfter D, **Stadler S**, Nguyen CH, Fristiohady A, Milovanovic D, Huttary N, Krieger S, Bago-Horvath Z, de Wever O, Tentes I, Özmen A, Jäger W, Dolznig H, Dirsch VM, Mader RM, Krenn L, Krupitza G. Intravasation of SW620 colon cancer cell spheroids through the blood endothelial barrier is inhibited by clinical drugs and flavonoids in vitro. Food Chem Toxicol. 2018 Jan; 111:114-124.

Nguyen CH, Huttary N, Atanasov AG, Chatuphonprasert W, Brenner S, Fristiohady A, Hong J, **Stadler S**, Holzner S, Milovanovic D, Dirsch VM, Kopp B, Saiko P, Krenn L, Jäger W, Krupitza G. Fenofibrate inhibits tumour intravasation by several independent mechanisms in a 3-dimensional co-culture model. Int J Oncol. 2017 May;50(5):1879-1888.

Nguyen CH, Brenner S, Huttary N, Atanasov AG, Dirsch VM, Chatuphonprasert W, Holzner S, **Stadler S**, Riha J, Krieger S, de Martin R, Bago-Horvath Z, Krupitza G, Jäger W. AHR/CYP1A1 interplay triggers lymphatic barrier breaching in breast cancer spheroids by inducing 12(S)-HETE synthesis. Hum Mol Genet. 2016 Nov 15;25(22):5006-5016.

Holzner S, Senfter D, **Stadler S**, Staribacher A, Nguyen CH, Gaggl A, Geleff S, Huttary N, Krieger S, Jäger W, Dolznig H, Mader RM, Krupitza G. Colorectal cancer cell-derived microRNA200 modulates the resistance of adjacent blood endothelial barriers in vitro. Oncol Rep. 2016 Nov;36(5):3065-3071.

Nguyen CH, Brenner S, Huttary N, Li Y, Atanasov AG, Dirsch VM, Holzner S, **Stadler S**, Riha J, Krieger S, Milovanovic D, Fristiohardy A, Simonitsch-Klupp I, Dolznig H, Saiko P, Szekeres T, Giessrigl B, Jäger W, Krupitza G. 12(S)-HETE increases intracellular Ca(2+) in lymph-endothelial cells disrupting their barrier function in vitro; stabilization by clinical drugs impairing calcium supply. Cancer Lett. 2016 Sep 28;380(1):174-83.

Nguyen CH, **Stadler S**, Brenner S, Huttary N, Krieger S, Jäger W, Dolznig H, Krupitza G. Cancer cell-derived 12(S)-HETE signals via 12-HETE receptor, RHO, ROCK and MLC2 to induce lymph endothelial barrier breaching. Br J Cancer. 2016 Jul 26;115(3):364-70.

Nguyen CH, Senfter D, Basilio J, Holzner S, **Stadler S**, Krieger S, Huttary N, Milovanovic D, Viola K, Simonitsch-Klupp I, Jäger W, Martin R, Krupitza G. NF-κB contributes to MMP1 expression in breast cancer spheroids causing paracrine PAR1 activation and disintegrations in the lymph endothelial barrier in vitro. Oncotarget. 2015