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Generation of FLT3-ITD-reactive T-cell populations from different sources

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Summary

Approximately 25% of acute myeloid leukemias (AMLs) carry internal tandem duplications (*ITD*) of various lengths within the gene encoding the FMS-like tyrosine kinase receptor 3 (FLT3). Although varying duplication sites exist, most of these length mutations affect the protein´s juxtamembrane domain. FLT3-ITDs support leukemic transformation by constitutive phosphorylation resulting in uncontrolled activation, and their presence is associated with worse prognosis. As known form previous work, they represent leukemia- and patient-specific neoantigens that can be recognized by autologous AML-reactive $CD8^+$ T cells (Graf *et al.,* 2007; Graf *et al.,* unpublished). Herein, in patient **FL**, diagnosed with FLT3-ITD^+ AML and in first complete remission after induction chemotherapy, T cells against her leukemia´s individual FLT3-ITD were detected at a frequency up to $1.7x10^{-3}$ among peripheral blood $CD8^+$ T lymphocytes. This rather high frequency suggested, that FLT3-ITD-reactive T cells had been expanded *in vivo* due to the induction of an anti-leukemia response.

Cell material from AML patients is limited, and the patients´ anti-leukemia T-cell repertoire might be skewed, e.g. due to complex previous leukemia-host interactions and chemotherapy. Therefore, allogeneic sources, i.e. buffy coats (BCs) from health donors and umbilical cord blood (UCB) donations, were exploited for the presence and the expansion of FLT3-ITD-reactive T-cell populations. BC- and UCB-derived $CD8^+$ T cells, were distributed at 10^5 cells per well on microtiter plates and, were stimulated with antigen-presenting cells (APCs) transfected with *in vitro*-transcribed mRNA (IVT-mRNA) encoding selected FTL3-ITDs. APCs were autologous $CD8^-$ blood mononuclear cells, monocytes or FastDCs.

Buffy coat lymphocytes from 19 healthy individuals were analyzed for $CD8^+$ T-cell reactivity against three immunogenic FLT3-ITDs previously identified in patients **VE**, **IN** and **QQ** and designated as **VE_**, **IN_** and **QQ_**FLT3-ITD, respectively. These healthy donors carried at least one of the HLA I alleles known to present an ITD-derived peptide from one of these FLT3-ITDs. Reactivities against single ITDs were observed in 8/19 donors. In 4 donors the frequencies of ITD-reactive T cells were determined and were estimated to be in the range of $1.25x10^{-6}$ to $2.83x10^{-7}$ $CD8^+$ T cells. These frequencies were 1,000- to 10,000-fold lower than the frequency of autologous FLT3-ITD-reactive T cells observed in patient **FL.** Restricting HLA I molecules were identified in two donors. In one of them, the recognition of **VE_**FLT3-ITD was found to be restricted by HLA-C*07:02, which is different from the HLA allele restricting the anti-ITD T cells of patient **VE**. In another donor, the recognition of **IN_**FLT3-ITD was restricted by HLA-B*35:01, which also had been observed in patient **IN** (Graf *et al.,* unpublished). By gradual 3´-fragmentation of the **IN_***FLT3-ITD* cDNA, the 10-mer peptide **CPSDNEYFYV** was identified as the target of allogeneic T cells against **IN_**FLT3-ITD.

Lymphocytes in umbilical cord blood predominantly exhibit a naïve phenotype. Seven UCB donations were analyzed for T-cell responses against the FLT3-ITDs of patients **VE**, **IN**, **QQ**, **JC** and **FL** irrespective of their HLA phenotype. ITD-reactive responses against all stimulatory FLT3-ITDs were observed in 5/7 UCB donations. The frequencies of T cells against single FLT3-ITDs in $CD8^+$ lymphocytes were estimated to be in the range of $1.8x10^{-5}$ to $3.6x10^{-6}$, which is nearly 15-fold higher than the frequencies observed in BCs. Restricting HLA I molecules were identified in 4 of these 5 positive UCB donations. They were mostly different from those observed in the respective patients. But in one UCB donation T cells against the **JC**_FLT3-ITD had exactly the same peptide specificity and HLA restriction as seen before in patient **JC** (Graf *et al.,* 2007). Analyses of UCB responder lymphocytes led to the identification of the 10-mer peptide **YESDNEYFYV**, encoded by **FL**_FLT3-ITD, that was recognized in association with the frequent allele HLA-A*02:01. This peptide was able to stimulate and enrich ITD-reactive T cells from UCB lymphocytes *in vitro*. Peptide responders not only recognized the peptide, but also COS-7 cells co-transfected with **FL**_*FLT3-ITD* and *HLA-A*02:01*.

In conclusion, T cells against AML- and individual-specific FLT3-ITDs were successfully generated not only from patient-derived blood, but also from allogeneic sources. Thereby, ITD-reactive T cells were detected more readily and at higher frequencies in umbilical cord blood than in buffy coat lymphocytes. It occurred that peptide specificity and HLA restriction of allogeneic, ITD-reactive T cells were identical to autologous patient-derived T cells. As shown herein, allogeneic, FLT3-ITD-reactive T cells can be used for the identification of FLT3-ITD-encoded peptides, e.g. for future therapeutic vaccination studies. In addition, these T cells or their receptors can be applied to adoptive transfer.

Zusammenfassung

Bei etwa einem Viertel akuter myeloischer Leukämien (AML) lassen sich im Gen des *FMS-like tyrosine kinase receptor 3* (*FLT3*) interne Tandemduplikationen (ITD) unterschiedlicher Länge nachweisen. Diese Längenmutationen treten meistens in der Juxtamembrandomäne auf. ITD führen zur konstitutiven Phosphorylierung und Aktivierung des Proteins. Ihre Präsenz ist mit schlechterer Prognose assoziiert. Sie repräsentieren Leukämie- und Patienten-spezifische Neoantigene, die von $CD8^+$ T-Zellen auf AML-Zellen erkannt werden können (Graf *et al.*, 2007; Graf *et al.*, unveröffentlicht). Im Blut der AML-Patientin **FL** wurden in erster kompletter Remission nach Induktionschemotherapie $CD8^+$ FLT3-ITD-reaktive Lymphozyten in einer Frequenz von $1{,}7\times10^{-3}$ gefunden. Diese hohe Frequenz legte nahe, dass bei der Patientin infolge einer Leukämie-Wirt-Interaktion *in vivo* eine Expansion ITD-reaktiver T-Zellen induziert worden war.

Zellmaterial von AML-Patienten steht nur in begrenzter Menge zur Verfügung. Darüber hinaus ist anzunehmen, dass das AML-reaktive T-Zellrepertoire von Patienten durch komplexe Leukämie-Wirt-Interaktionen und durch Chemotherapie kompromittiert ist. Deshalb wurde untersucht, inwieweit FLT3-ITD-reaktive T-Zellen in *buffy coat* (BC)-Lymphozyten gesunder Spender und in Nabelschnurblut (*umbilical cord blood*, UCB) nachweisbar sind und daraus expandiert werden können. Zur *In vitro*-Stimulation wurden in Mikrotiterplatten $CD8^+$ T-Zellen (10^5/Kultureinheit) gegen Antigen-präsentierende Zellen (APC) stimuliert, die mit *in vitro* transkribierter RNA (IVT-RNA) für verschiedene FLT3-ITD transfiziert waren. Als APC wurden autologe $CD8^-$ mononukleäre Blutzellen eingesetzt sowie daraus isolierte Monozyten und FastDC.

In BC von 19 gesunden Spendern wurde nach $CD8^+$ T-Zellreaktivität gegen die immuno-genen FLT3-ITD der Patienten **VE**, **IN** und **QQ** gesucht, bezeichnet als **VE_**, **IN_**, bzw. **QQ_**FLT3-ITD. Die BC-Spender trugen mindestens eines der HLA I-Allele, von denen bekannt war, dass sie Peptide aus diesen FLT3-ITD präsentierten. Reaktivität gegen einzelne ITD wurde bei 8/19 Spendern beobachtet. Bei vier Spendern wurden die Frequenzen ITD-reaktiver T-Zellen in $CD8^+$ Lymphozyten ermittelt. Sie lagen in einem Bereich von $1{,}25\text{x}10^{-6}$ bis $2{,}83\text{x}10^{-7}$ und damit etwa 3-4 Größenordnungen unter der Frequenz ITD-reaktiver T-Zellen bei der Patientin **FL**. Bei zwei BC-Spendern wurden restringierende HLA-Allele identifiziert, nämlich HLA-C*07:02 für **VE_**FLT3-ITD und HLA-B*35:01 für **IN_**FLT3-ITD. Während die ITD-reaktiven T-Zellen des Patienten **VE** durch ein anderes HLA-Molekül restringiert waren, war HLA-B*35:01 auch Restriktionsmolekül der ITD-reaktiven T-Zellen des Patienten **IN** (Graf *et al.*, unveröffentlicht). Durch 3´-Fragmentierung der **IN_**FLT3-ITD-cDNA wurde das Dekamer **CPSDNEYFYV** als

Zielpeptidantigen allogener T-Zellen gegen die ITD des Patienten **IN** identifiziert.

UCB-Lymphozyten tragen überwiegend einen naiven Phänotyp. Sieben UCB-Spenden wurden ungeachtet ihres HLA-Typs auf $CD8^+$ T-Zellantworten gegen die ITD der Patienten **VE**, **IN**, **QQ**, **JC** und **FL** untersucht. In fünf der sieben UCB-Spenden wurden ITD-spezifische Immunantworten gegen alle zur Stimulation eingesetzten ITD beobachtet. Die T-Zellfrequenzen gegen einzelne ITD in $CD8^+$ Lymphozyten lagen bei $8x10^{-5}$ bis $3,6x10^{-6}$ und waren damit fast 15-fach höher als in BC-Lymphozyten. Restringierende HLA I-Moleküle wurden in 4/5 positiven UCB-Spenden ermittelt. Sie unterschieden sich mit einer Ausnahme von den Restriktionslelementen der patienteneigenen Immunantworten. In einer UCB-Spende wiesen T-Zellen gegen **JC**_FLT3-ITD die gleiche Peptidspezifität und HLA-Restriktion auf wie für autologe T-Zellen beschrieben (Graf *et al.*, 2007). Weitere Analysen von ITD-reaktiven UCB-Lymphocyten führten zur Identifizierung des Dekamers **YESDNEYFYV** aus der FLT3-ITD der Patientin **FL**, das in Assoziation mit dem häufig vorkommenden HLA-A*02:01 erkannt wurde. Mit diesem Peptid ließen sich *in vitro* ITD-reaktive $CD8^+$ T-Zellen aus UCB-Lymphozyten stimulieren und anreichern. Die peptidreaktiven T-Zellen erkannten nicht nur das ITD-Peptid, sondern auch mit ***FL**_FLT3-ITD* und *HLA-A*02:01* ko-tranfizierte COS-7-Zellen.

In der vorliegenden Arbeit wurden T-Zellen gegen individuelle FLT3-ITD nicht nur aus Patientenblut, sondern auch aus allogenen Quellen generiert. Dabei wurden ITD-reaktive T-Zellen in Nabelschnurblut häufiger und in höheren Frequenzen nachgewiesen als in *buffy coat*-Lymphozyten. Es kam vor, dass allogene, ITD-reaktive T-Zellen die gleiche Peptidspezifität und HLA-Restriktion aufwiesen wie patienteneigene T-Zellen. Allogene, ITD-reaktive T-Zellen eignen sich, wie hier gezeigt, für die Identifizierung FLT3-ITD-kodierter Peptidantigene, beispielsweise zum künftigen Einsatz in therapeutischen Impfungstudien. Darüber hinaus können solche T-Zellen bzw. ihre Rezeptoren für den adoptiven Transfer verwendet werden.

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Abbreviations

aa	Amino acid
AD	Aqua distilled water (see dH2O)
ADCC	Antibody-dependent cellular cytotoxicity
Ag	Antigen
AEC	3-amino-9-ethylcarbazole
AICD	Activation induced cell death
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BC	Buffy coats
BSA	Bovine serum albumin
BTLA	B and T lymphocyte attenuator
CD	Cluster of differentiation
cDNA	Complementary DNA
CMV	Cytomegalovirus
CR	Complete remission
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DEPC	Di-ethyl pyro-carbonat
dH2O	Distilled H2O
DLI	Donor lymphocyte infusion
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
E:T	Effector to target ratio
EBV	Epstein Barr-Virus
EDTA	Ethylenediamine tetra-acetic acid
EGFP	Enhanced green fluorescent protein
ELISPOT	Enzyme-linked immunosorbent assay
EP	Electroporation
ER	Endoplasmic reticulum
ERAPI	ER aminopeptidase I
FAB	French American British Cooperative Group
FACS	Fluorescence activated cell sorting
FastDC	Fast generated dendritic cells
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLT3	Fibroblast-macrophage stimulating factor receptor (FMS)-like tyrosine-kinase receptor 3
FSC	Forward scatter
GM-CSF	Granulocyte-monocyte colony-stimulating factor
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
HS	Human serum
HSCT	Hematopoietic stem cell transplantation
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukine
ITD	Internal tandem duplication

IU	International Unit
IVS	*In vitro*-stimulation
IVT	*In vitro* transcription
IVT-mRNA	*In vitro* transcribed mRNA
Kb	Kilobase
KDa	Kilodalton
LB medium	Lysogeny broth medium
LAAs	Leukemia-specific antigens
LSAs	Leukemia-specific antigens
LDA	Limiting dilution assay
mAb	Monoclonal antibody
MACS	Magnetic cell sorting
MFI	Mean fluorescence intensity
mHAg	Minor histocompatibility antigen
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MLPC	Mixed lymphocytes peptide culture
MOCK	Same treatment but without reagent
NK	Natural killer cell
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed death-1
PGE-2	Prostaglandin E2
PHA	Phytohemagglutinin
PI	Propidium iodide
rATP	Riboadenosine tri-phosphate
rCTP	Ribocytosine tri-phosphate
rGTP	Riboguanosine tri-phosphate
RNA	Ribonucleic acid
rNTP	Ribonucleotide tri-phosphate
rpm	Round per minute
RPMI medium	Roswell Park Memorial Institute medium
RT	Room temperature
RT-PCR	Reverse-transcription PCR
rUTP	Ribouridine tri-phosphate
SEREX	Serological screening of cDNA expression libraries
SSC	Side scatter
TAA	Tumor associated antigen
TAP	Transporter associated with antigen processing
TBE	Tris-borate-EDTA
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
TCR	T-cell receptor
T_H	T-helper cell
TM	TransMessenger™
TNF	Tumor necrosis factor
Treg	Regulatory T cell
Tris	Tris (hydroxymethyl)-aminomethane
UV	Ultraviolet
WHO	World Health Organization
WT1	Wilms' tumor antigen 1

List of tables and figures

1. Introduction

Over 150 years before Rudolf Virchow (1845) coined the term ‘‘leukemia’’, which means cancers of white blood cells. Leukemia is one of the top 15 most frequently occurring types of cancer and the 11th most common cause of cancer-related death (Mathers *et al.,* 2001). Leukemia refers to a group of neoplastic disorders characterized by malignant transformation of hematopoietic or lymphatic cells. These transformed cells are characterized by an increased rate of self-renewal and an aberrant differentiation. The underlying process of development of leukemia is called leukemogenesis. Leukemias are classified based upon whether the leukemia is acute versus chronic and myeloid versus lymphoid that is: Acute myeloid leukemia (AML) - predominantly in adults, Chronic myeloid leukemia (CML) -in middle-aged and elderly, Acute lymphoblastic leukemia (ALL) ~80% of childhood leukemia and Chronic lymphoblastic leukemia (CLL) -after the age of 50 years.

1.1 Acute meyloid leukemia

‘‘Acute myeloid leukemia’’ refers to a group of hematopoietic neoplasms-derived from cells committed to the myeloid lineage of cellular development with myeloblasts as its unipotent stem-cell. AML is a primarily clonal disorder with proliferation of myeloid precursors with reduced or lacking capacity to differentiate into more mature descendants. AML is characterized by an increase in the number of myeloid cells in the bone marrow, peripheral blood, and other tissues frequently, resulting in hematopoietic insufficiency with reduced cell counts of erythrocytes, platelets, and neutrophils. This in consequence leads to anemia, bleeding, and a growing risk of infection (Abu-Duhier *et al.,* 2000). The pathogenesis of acute myelogenous leukemia is a multi-step process driven by multiple genetic alterations, affecting regulation of cell proliferation, differentiation, self-renewal, survival, cell cycle checkpoint control, DNA repair and chromatin stability, and cell dissemination. Studies in mouse models, using genetic alterations found in AML-patients, have shown that a minimum of two genetic alterations (genetic hits) is required for pre-leukemic myeloproliferation to initiate AML (Kelly and Gilliland, 2002; Schessl *et al.,* 2005; Kim *et al.,* 2008). Class I mutations confer increased survival and proliferative advantages to the cells, e.g. FLT3-ITD, N-RAS or K-RAS, and constitutively active c-kit, while, class II mutations block differentiation, e.g. AML1-ETO, PML-RARα, inv(16) or transcription factors like C/EBPα. The combination of such a differentiation arrest together with mutations affecting genes that control proliferation, results in an uncontrolled growth of immature cell clones, leading to the clinical entity of AML (Steffen *et al.,* 2005). Moreover, the differentiation of normal blasts into mature progeny cells is inhibited by AML cells, which is probably mediated by different chemokines produced by AML cells (Youn *et al.,* 2000).

The two most commonly used classification systems for AML are the French-American-British (FAB) classification system and the World Health Organization (WHO) system. The FAB system (Bennett *et al.,* 1985; Bene *et al.,* 1995; De Vita (editor), 1997) divides AML into eight groups based on maturation stage and the type of cell from which the leukemia originates [**Table 1.1.1,** FAB (Bene et al., 1995) and WHO classification (Fauci *et al.,* 2008) of AML].

Table 1.1.1: FAB classification and WHO classification of AML.

FAB classification		**WHO classification**	
Type	**Name**	**% of AML**	
M0	undifferentiated leukemia	5	**I. AML with recurrent genetic abnormalities** - AML with t(8;21)(q22;q22);*RUNX1/RUNX1T1* - AML with abnormal bone eosinophils [inv(16)(p13q22) or t(16;16)(p13;q22); *CBFB/MYH11*] - Acute promyelocytic leukemia [AML with t(15;17)(q22;q12))(*PML/RARA*) and variants] - AML with 11q23 (*MLL*) abnormalities
M1	myeloblastic leukemia without maturation	20	
M2	myeloblastic leukemia with maturation	30	
M3 **M3v**	promyelocytic leukemia variant microgranular APL	10	**II. AML with multilineage dysplasia** - following a MDS or myeloproliferative disorder - without antecedent MDS
M4M4eo	myelomonocytic leukemia Myelomoncytic leukemia with eosinophilia	20 5	**III. AML and MDS, therapy-related** - alkylating agent-related - Topoisomerase type II inhibitor-related - other types
M5 **M5a** **M5b**	monocytic leukemia monoblastic, undifferentiated monocytic, differentiated	5 5	**IV. AML not otherwise categorized** - AML minimally differentiated - AML without maturation - AML with maturation - acute myelomonocytic leukemia - acute monoblastic and monocytic leukemia - acute erythroid leukemia - acute megakaryoblastic leukemia - acute basophilic leukemia - acute panmyelosis with myelofibrosis - myeloid sarcoma
M6	Erythroleukemia (DiGuglielmo□s disease)	5	
M7	megakaryoblastic leukemia	1	

According to this classification, AML is confirmed when the bone marrow contains more than 30% blasts. More recently the FAB classification has been replaced by the WHO (World Health Organization) system emphasizing genetic features (Harris *et al.,* 1999; Arber, 2001; Vardiman *et al.,* 2002; Vardiman *et al.,* 2009). The WHO also lowers the threshold for the diagnosis of AML to 20% myeloid blasts of the cells identified in the blood or bone marrow. In Germany approximately 9,100 persons are newly diagnosed with AML each year, which corresponds to 2.1% of all newly diagnosed malignant diseases. The age-specific incidence of AML increases from 3.5/100,000 under the age of 45 then ascends to 15/100,000 in persons older than 70 years and to 35/100,000 over the age of 90. Only 15-20% of AML patients are children (Robert-Koch-Institut and Gesellschaft der epidemilogischen Krebsregister in Deutschland e.V., 2008). AML is slightly more common in males than in females. Besides this, geographical variations

have been shown: the highest incidence of AML is found in North America and Europe, whereas, the lowest incidence in Asia and Latin America (Fuchs, 2002).

Established therapeutic regimens in AML consist of poly-chemotherapy (Bishop, 1997), divided into two phases: induction and consolidation. Induction chemotherapy shall achieve a complete remission according to clinical criteria by reducing the amount of leukemic cells to an undetectable level (<5% leukemic cells in the BM). The aim of post-remission consolidation chemotherapy is to eliminate any residual undetectable disease. Although the complete remission can be achieved in about 80% of AML-patients by intensive chemotherapy-based treatments, long-term survival is rather low (5-year overall survival 20-25%) due to the persistence of minimal residual disease in about 80% of AML-patients (Venditti *et al.,* 2000; Li *et al.,* 2003; Houtenbos *et al.,* 2006). For patients with a high risk of relapse, or when relapse occurred, despite maintenance chemotherapy, the only proven potentially curative therapy is allogeneic hematopoietic stem cell transplantation (allo-HSCT) (Kolb *et al.,* 1995; Copelan, 2006) associated with a graft-versus-leukemia (GvL) immune effect. However, allo-HSCT is associated with a considerable morbidity and mortality. General complications are graft-verus-host disease (GvHD) and infectious complications such as cytomegalovirus (CMV) and Epstein Barr-Virus (EBV) reactivations and others, leading to a treatment-related mortality of 10-25% (Kolitz, 2006). Despite these drawbacks, allo-HSCT is the only curative therapeutic option for AML patients in a refractory or relapsing disease. Current efforts to improve the allo-HSCT concept, aim at reducing the GvHD and enhancing GvL effect. Progress made during the recent years, relates to identify in the basis of genetic alterations subgroups at particular risk for relapse and/or resistance to conventional treatment and new drugs for successful combination in high-risk patients (Bacher *et al.,* 2010; Metzelder *et al.,* 2012).

1.2 Leukemia associated antigens

During the last two decades a considerable number of AML-related antigens have been identified after the discovery of the first tumor-associated antigen (TAA) in melanoma (van der Bruggen *et al.,* 1991). These leukemia antigens have been identified by methods such as T-cell driven expression cloning, serological analysis of recombinant cDNA expression libraries (SEREX), mass spectrometry (MS), single nucleotide polymorphisms (SNPs), serial analysis of gene expression (SAGE) and 2-dimensional gel electrophoresis (Guinn *et al.,* 2007). The functional role of leukemia antigens in tumor cells is very much diverse and controversial. Some increase malignant cell growth; also provide targets for effective anti-leukemia immune responses. It has become increasingly apparent that leukemia antigens play a multifaceted role: not only as potential targets for immunotherapy, but also as biomarkers of disease stage (leukemia cell proliferation), response to treatment and survival. Most of these antigens show a differential mRNA and protein expression in malignant cells compared to normal tissue.

Leukemia antigens can be categorized into four groups on the basis of their expression patterns in nonmalignant and leukemic cells (Anguille *et al.,* 2012). Leukemia-specific antigens (LSAs) are generated by mutations, in particular by chromosomal translocations, such as BCR-ABL and ETV6-AML1 (Yotnda *et al.,* 1998; Yun *et al.,* 1999; listed in http://cancerimmunity.org/peptide), but also by length mutations, such as FLT3-ITD (Graf *et al.,* 2007) and point mutations, such as NPM1 (Falini and Martelli, 2011; Greiner *et al.,* 2012). Leukemia-associated antigens (LAAs) are expressed also by normal cells, but are over-expressed in leukemia cells (e.g. Bcl-2, G250, hTERT, MPP11, MUC1, PR3, RHAMM, Survivin, and WT1) (listed in http://cancerimmunity.org/peptide; Greiner *et al.,* 2008; Anguille *et al.,* 2012). Hence, autoimmunity could be a logical consequence of targeting LAAs. Therefore, it is of crucial importance to define their tissue distribution and expression patterns. Cancer-testis (CT) or cancer-germline (CG) antigens are a large group of immunogenic antigens that are normally expressed only in germ cells of the testes and, to a lesser extent, in ovaries and placental trophoblasts. Some CT/CG antigens are also reported in AML (e.g. Cyclin A1, PRAME and RAGE-1) (listed in http://cancerimmunity.org/peptide; Anguille *et al.,* 2012). Ubiquitous antigens are expressed also in a wide variety of normal tissues (Anguille *et al.,* 2012). Due to their promiscuous and non-leukemia-specific expression patterns, they are considered as irrelevant targets for AML immunotherapy.

Antigens that can induce anti-leukemia T-cell responses in AML patients are truly tumor-specific (e.g. FLT3-ITD, BCR-ABL, and ETV6-AML1 fusion protein), shared tumor-specific (e.g. hTERT, BAGE-1, RHAMM), tissue or cell-type-specific (i.e. differentiation antigens) or overexpressed in leukemic blasts (e.g. G250, RAGE-1, PRAME, PR-3, BCL-2, WT1 and Survivin) (listed in http://cancerimmunity.org/peptide; Anguille *et al.,* 2012). CTLs against LAAs have been shown to induce cell lysis of autologous leukemic blasts (Greiner *et al.,* 2002, 2005 and 2008; Anguille *et al.,* 2012). The LAAs RHAMM, PR-3, and WT1 were tested in peptide vaccination trials and immunological as well clinical responses were detected in patients with different hematologic malignancies including AML (Oka *et al.,* 2004; Rezvani *et al.,* 2008; Schmitt *et al.,* 2008). As mentioned above, some leukemia antigens are critically involved in mechanisms of tumor growth such as proliferation (RHAMM, AurA, MPP11 and SSX2IP), inhibition of apoptosis (Bcl-2, survivin, and PR-3), differentiation (PRAME, MUC1 and WT1), telomerase activity (hTERT) and demethylation (Greiner *et al.,* 2008) (**Figure 1.2.1**). Mutated antigens, such as $FLT3^{mut}$, $NPM1^{mut}$, $CEBPA^{mut}$, AML1-ETO and BCR-ABL, are of particular interest in AML immunotherapy, because they can be expected to be stably expressed in malignant cells throughout the disease course. Many studies have shown that AML patients with *FLT3-ITD* mutations have poor cure rates due to a lower complete remission and a higher relapse rate compared to those patients not bearing this mutation (Tallman *et al.,* 2005; Dohner *et al.,* 2005). FLT3-ITDs are not only immunogenic by themselves, but also induce a potentially immunogenic

phenotype by enhancing the expression of LAAs such as PR-3, RHAMM, Survivin, WT1 and PRAME (Brackertz *et al.,* 2011). Some of the LAAs have been proposed to be of clinical relevance on the basis of early-phase trials in AML (Greiner *et al.,* 2008; Schmitt *et al.,* 2008; Rezavani *et al.,* 2008; Van Driessche *et al.,* 2012).

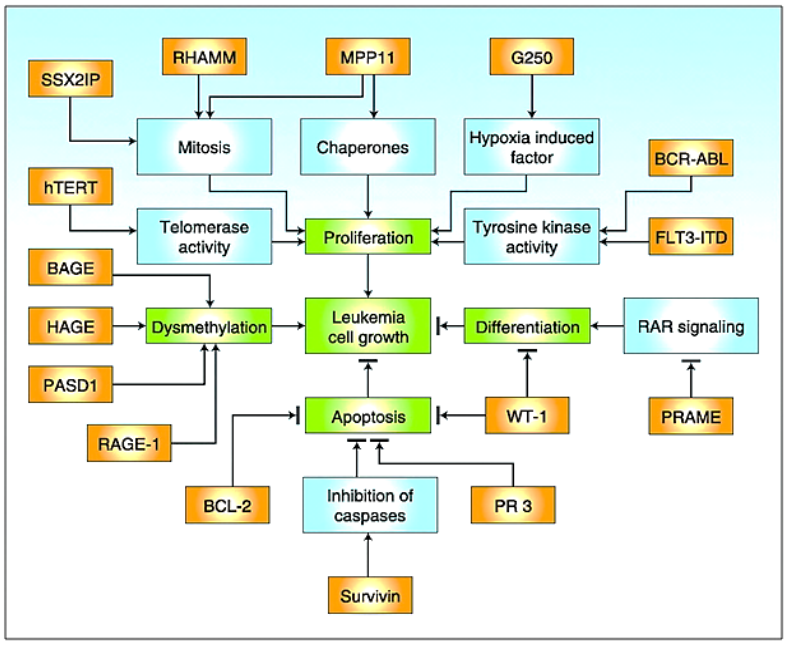


Figure 1.3.1: Leukemia-associated antigens. Immunogenic LAA marked in orange might exercise a positive effect on the proliferation of leukemia cells or hamper the apoptosis of leukemia cells, thus increasing the tumor burden in leukemia patients. (Adopted from: Greiner *et al.,* 2008)

The identification and characterization of T-cell-defined leukemia antigens fueled the development of antigen-specific immunotherapy approaches for AML, which can be divided into two main strategies: 'active' immunotherapy (i.e. antigen-specific vaccination) and 'passive' immunotherapy (i.e. adoptive transfer of antigen-specific T cells). According to the Translational Research Working Group of the National Cancer Institute, the success of any antigen-specific immunotherapeutic strategy depends critically on the choice of an "ideal" target antigen (Cheever *et al.,* 2009). The "ideal" candidate target antigen for AML immunotherapy should have the following features: (1) leukemia-specific, (2) display high and homogeneous expression in most leukemic blasts including leukemic stem cells, (3) play a defined oncogenic role, which supports stable and homogenous expression and (4) immunogenic. Because of their truly leukemia-restricted expression pattern, LSAs are considered to be the most appropriate targets for immunotherapeutic intervention. As opposed to LAAs, FLT3-ITD (Spiekermann *et al.,* 2003) as well as fusion proteins ETV6-AML1, AML1-ETO and BCR-ABL (Stams *et al.,* 2005; Yuan *et al.,* 2001; Schmidt-Arras *et al.,* 2005; Yotnda *et al.,* 1998), are specifically expressed in leukemia cells. It can be expected that immune responses against these LSAs are less prone to tolerance control, because of that LSAs are supposed to induce stronger and more persistent immune responses. The internal tandem duplications (ITD) of the *FLT3* gene is the most frequent alteration observed in AML and it contributes to leukemic transformation (Choi *et al*., 2005; Stirewalt and Radich, 2003; Gilliland and Griffin, 2002a). It has been shown that in *FLT3-ITD*$^{+}$ AML, FLT3-ITDs are in general already present in the leukemic stem cells (Levis *et al.*,

2005). These stem cells are the driving source for relapse. Scholl and colleagues, demonstrated the potency of FLT3-ITD-encoded peptides and related mimotopes to bind to HLA class-I alleles (Scholl *et al.*, 2006). And it has been clearly demonstrated by Graf *et al.,* (2007) that FLT3-ITDs can generate neoepitopes endogenously processed and presented to autologous AML-reactive $CD8^+$ T cells.

1.3 FLT3-receptor

The FLT3 (fibroblast-macrophage stimulating factor receptor (FMS)-like tyrosine-kinase receptor 3), also known as FLK-2 (fetal liver kinase 2) and STK-1 (stem-cell kinase 1), belongs to a family of type III receptor tyrosine kinases (RTKs) (Gilliland and Griffin, 2002a and 2002b; Kottaridis *et al.,* 2003a; Stirewalt and Radich, 2003). Two groups independently reported the cloning of the *FLT3* gene (Matthews *et al.,* 1991; Rosnet *et al.,* 1991). Its structure resembles c-KIT, c-FMS and platelet-derived growth factor receptors α and β (Naoe *et al.,* 2004). Wild-type FLT3-receptor is expressed on the cell surface as a monomer structure shown in **Figure 1.3.1**.

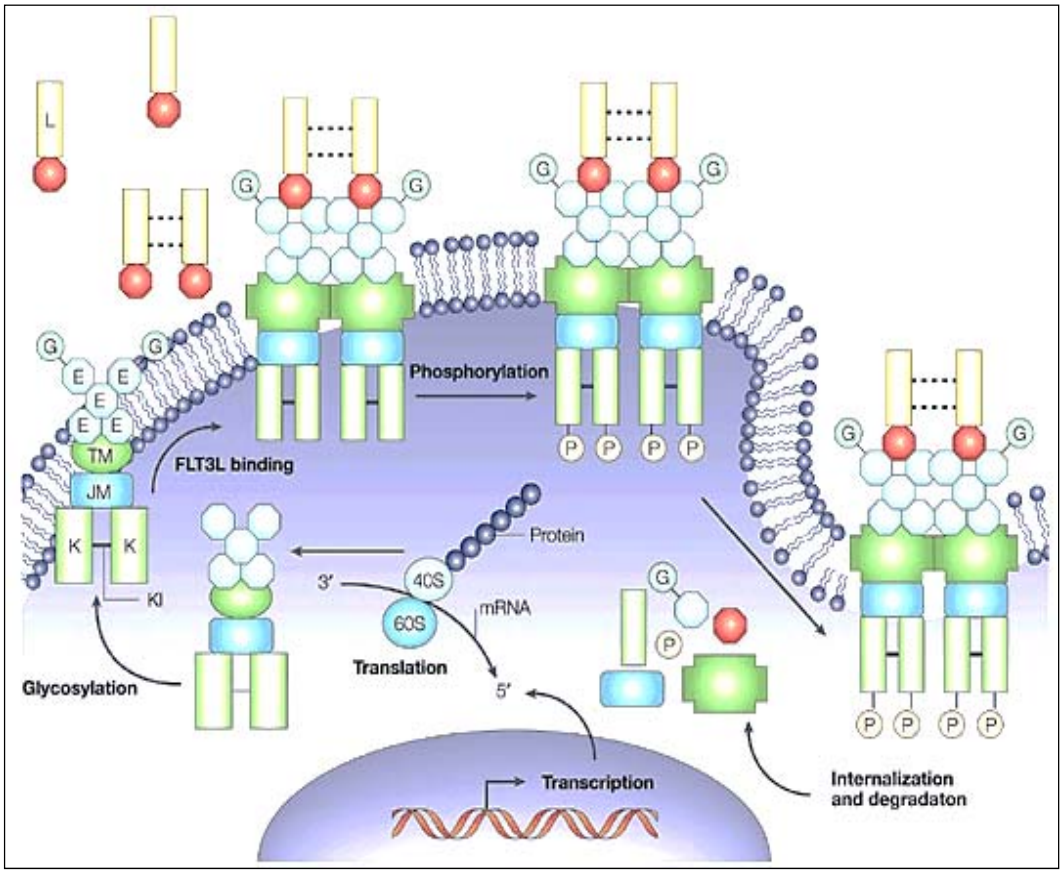


Figure 1.3.1: Structure and activation of wild-type (wt) FLT3.
Transcription of the FLT3 gene produces FLT3 mRNA, which is translated to the FLT3 protein. FLT3 contains five extracellular immunoglobulin-like domains (E), a transmembrane domain (TM), a juxtamembrane domain (JM) and two tyrosine-kinase domains (K) that are linked through the tyrosine-kinase insert (KI). Cytoplasmic FLT3 undergoes glycosylation (G), which promotes localization of the receptor to the membrane. Wt-FLT3 remains as a monomeric, inactivated protein on the cell surface until FLT3 ligand (L) binds the receptor and induces receptor dimerization. FLT3 dimerization promotes phosphorylation (P) of the tyrosine-kinase domains. The dimerized receptors are quickly internalized and degraded. (Adopted from: Stirewalt and Radich, 2003)

Two forms of FLT3 exist: a 158-160 kDa membrane-bound form, which has a N-linked glycosylation; and a cytoplasmic 130-143 kDa form that is not glycosylated (Lyman, 1995). The FLT3-receptor is predominantly expressed on the cell surface of hematopoietic progenitors in the bone marrow, thymus and lymph nodes (Rosnet *et al.,* 1993; deLapeyriere *et al.,* 1995), but it is also found in the placenta, brain and gonads (Maroc *et al*., 1993). In normal bone marrow, the expression seems to be restricted to early progenitors, including $CD34^+$ cells with high levels of expression of c-KIT (Gilliland and Griffin, 2002b). Under pathological conditions FLT3 expression or overexpression can be detected in a wide range of hematopoietic malignancies including 70–100% of AML, ALL and CML (Birg *et al*., 1992; Drexler, 1996; Rosnet *et al.,* 1996).

1.3.1 FLT3-receptor mutations in AML

The *FLT3* gene is located on chromosome 13q12 and contains 24 exons encoding a 993-amino acid protein (Abu-Duhier *et al*., 2001; Gilliland and Griffin, 2002b). Amino acid residues 572–603 and 604-958 represent the juxtamembrane and tyrosine kinase domains, respectively (Rosnet *et al.,* 1993). Activating mutations of *FLT3* are present in about one third of AML cases, making *FLT3* one of the most frequently mutated gene found in AML (Nakao *et al.,* 1996; Gilliland and Griffin, 2002a; Schnittger *et al.,* 2002; Kottaridis *et al.,* 2003b; Stirewalt and Radich, 2003) (**Figure 1.3.1.1**).

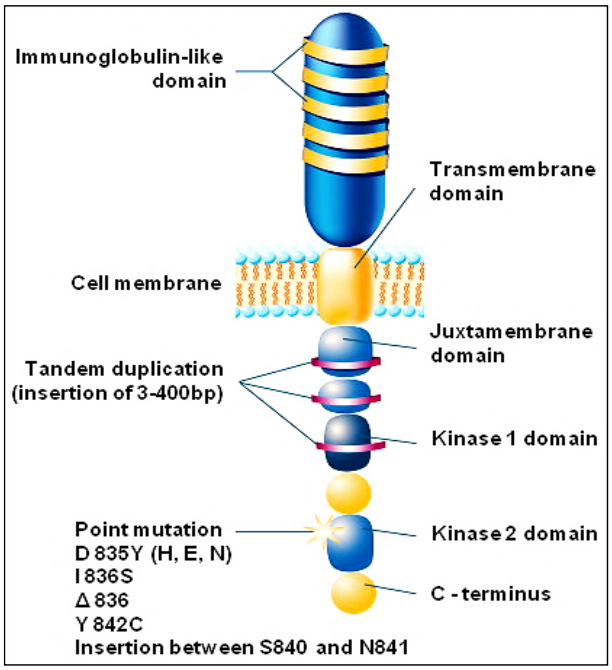


Figure 1.3.1.1: FLT3-receptor with prominent mutations.
FLT3-receptor showing the location of the internal tandem duplication of gene within the juxtamembrane domain as well as the point mutations and gene insertions in the second kinase domain. (Adopted from: Litzow *et al.,* 2005, illustration by Kenneth Probst)

Two major classes of *FLT3* mutations have been described in AML, leading to constitutive activation of the FLT3-receptor and therefore uncontrolled cell proliferation (Choi *et al*., 2005). First, internal tandem duplications (ITD) in exon 14 and/or exon 15 of the receptor were observed in about 25% of AML patients, lead to the insertion of several amino acids in the

juxtamembrane domain (Nakao *et al.,* 1996; Thiede *et al.,* 2002; Steudel *et al.,* 2003; Stirewalt and Radich, 2003). Second, point mutations within a certain part of the tyrosine-kinase domain (TK), the so-called activation loop, make up one important alteration (*FLT3-TKD*) (Gilliland and Griffin, 2002a and 2002b). They have been observed in about 7% of all AML and preferentially affect the codon 835 (Yamamoto *et al*., 2001; Abu-Duhier *et al.,* 2001; Thiede *et al.,* 2002). The most common substitution is Asp835Tyr, but other substitutions, including Asp835Val, Asp835His, Asp835Glu, and Asp835Asn, have also been reported (Yamamoto *et al.,* 2001).

1.3.2 Functional role of FLT3-receptor in normal hematopoiesis and malignancies

The wild-type FLT3-receptor is expressed on the cell surface as a monomer and is inactive in the absence of its ligand (Weiss and Schlessinger, 1998). The FLT3 ligand (FL) is a type I transmembrane protein. It exists in two forms, a membrane bound and a soluble form. Both forms are expressed by bone marrow stroma cells (Lyman, 1995) as well as by cells of the myeloid, B- and T-cell lineage (Brasel *et al*., 1995). Although, FL is a weak stimulator of proliferation and mainly functions *via* synergism with the stem-cell factor (the ligand of c-KIT) or other cytokines (Hannum *et al*., 1994; Lyman and Jacobsen, 1998), FL stimulation is a prerequisite for the activation of the wild-type FLT3-receptor, which subsequently activates several signaling pathways, including JAK/STAT5, Ras/mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3 kinase (PI3K)/AKT pathways (Drexler and Quentmeier, 2004; Small*,* 2008) (**Figure 1.3.2.1**). These pathways lead to increased proliferation and inhibition of apoptosis. Binding of FL induces receptor dimerization, leading to the autophosphorylation of the receptor. Ligand-induced dimerization of receptors is thought to expose tyrosine autophosphorylation sites and to stabilize the active conformational state, which further enhances the activation of the receptors (Turner *et al.,* 1996; Weiss and Schlessinger, 1998). The crystal structure of the autoinhibited form of FLT3 has been published by Griffith *et al.,* (2004). The normal juxtamembrane domain interacts with the kinase domain, buries the catalytic centre, and thus stabilizes the inactive kinase conformation. Ligand-induced dimerization and conformational changes lead to phosphorylation of two key JM tyrosine residues at positions 589 and 591. This event destabilizes the conformation of the JM domain and allows it to excavate the active center of the kinase, leading to its activation. The FLT3 has been shown to serve important functions in early hematopoietic progenitor proliferation and survival as well as in macrophage and dendritic cell differentiation (Gilliland and Griffin, 2002b; Stirewalt and Radich, 2003). Mice with a homozygous deletion of the *FLT3-receptor* gene were healthy and showed normal peripheral blood counts. However, they had reduced numbers of early B-cell precursors and defects in primitive cells in the bone marrow, resulting in a reduced ability to reconstitute B-cell, T-cell and myeloid lineages when transplanted into irradiated mice (Mackarehtschian *et al.,* 1995). Similar to this, targeted disruption of the *FL* gene in mice led to a significant reduction in the amount of leukocytes, myeloid and lymphoid progenitors of the

bone marrow. In addition, stem cells isolated from *FLT3* knockout mice are deficient in their ability to repopulate lymphoid and myeloid compartments when introduced into lethally irradiated animals (Mackarehtschian *et al.,* 1995). In contrast, mice overexpressing FL developed leukemia after a long latency period (Hawley *et al*., 1998).

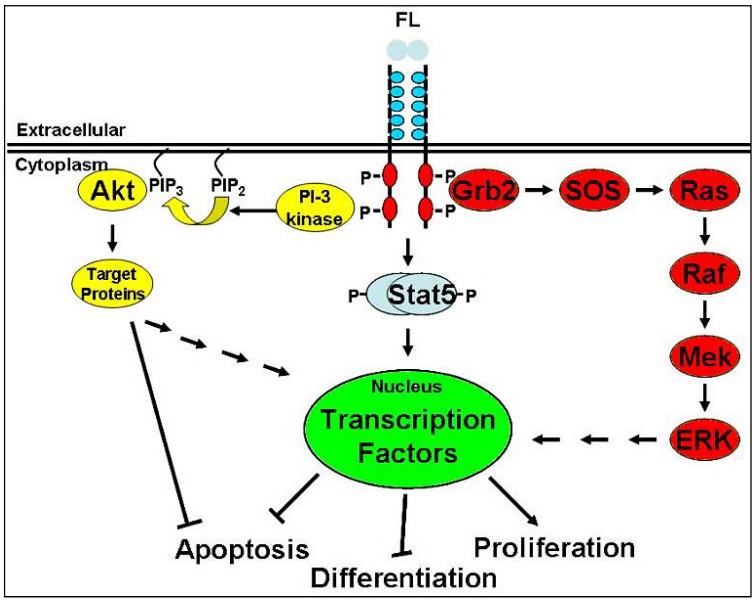


Figure 1.3.2.1: FLT3 signals via activation of multiple downstream pathways.
The binding of FLT3 ligand causes the FLT3 protein to dimerize, initiating auto-phosphorylation and kinase activity. The kinase, which is always active in constitutively activated FLT3 mutation, activates numerous pathways, including the PI3 kinase/AKT pathway, the ras/MAP kinase pathway, and the STAT5 phosphorylation pathway. All of these pathways interrupt the processes of apoptosis, differentiation, and proliferation. (Adopted from: Small, 2008)

FLT3-ITD length mutations have been detected in all FAB subtypes of AML, with the highest reported frequency in FAB M3, M1 and M5 subtypes and less frequently in the M2 subtype (Gilliland and Griffin, 2002a; Schnittger *et al.,* 2002; Thiede *et al.,* 2002; Stirewalt and Radich, 2003). The prevalence of *FLT3-ITD* in patients with AML increases with age, ranging from 5-15% in pediatric patients to 25-35% in adults. The ITDs are always in frame, but revealed to be highly individual with regard to size and length, ranging from 3-400 base pair (Schnittger *et al*., 2002). The preferential initiation site for *FLT3-ITDs* ranges from codon 573 to 620, and about 60% of all *ITDs* occur between codons 591 and 601 (Yokota *et al*., 1997; Thiede *et al*., 2002). Recently, Breitenbuecher *et al.,* (2009) have demonstrated that 28.7% of ITDs integrate in the TKD1 and not, as previously assumed, in the JM domain of *FLT3.* These repeat sequences may disrupt the autoinhibitory activity of the JM domain by destroying the conformational integrity of the JM domain, whereby the JM domain can no longer inhibit the kinase and allows the ligand-independent activation of the receptor (Griffith *et al.,* 2004; Hubbard, 2004). However, not only *ITDs* but also insertions or the substitution of particular amino acid residues and even shortening causes constitutive phosphorylation of FLT3 independent of FL binding to the receptor (Kiyoi and Naoe, 2002). Moreover, clones harboring *FLT3-ITDs* of different lengths have been observed in AML cells of the same patient and an almost complete loss of the wild-type *FLT3* allele was found in 6% to 10% of all samples (Whitman *et al*., 2001; Thiede *et al*., 2002; Schnittger *et al*., 2002). Most studies in patients with AML have found that *FLT3-ITD* is a strong, independent predictor of poor clinical outcome compared to *FLT3-TKD* mutations (Iwai

et al., 1999; Kiyoi *et al.,* 1999; Kondo *et al.,* 1999; Abu-Duhier *et al.,* 2001; Schnittger *et al.,* 2002; Thiede *et al.,* 2002).

1.3.3 FLT3 Inhibitors

It is well known that patients harboring *FLT3-ITDs* have a poor prognosis (Abu-Duhier *et al.,* 2000). The presence of *FLT3-ITDs* correlates with higher leukocyte and blast counts, with decreased remission induction rates, and decreased disease-free and overall survival (Kottaridis *et al.,* 2001), compared to *FLT3-ITD*-negative AMLs. Because of a profoundly negative prognostic impact on the clinical outcome of patients with AML, researchers have sought to find effective small-molecule inhibitors of this receptor tyrosine kinase (TKIs). Over the past 10 years, well over a dozen different compounds have been examined *in vitro* as well as clinically and reported on as potential FLT3 inhibitors such as midostaurin (PKC-412), lestaurtinib (CEP-701), sorafenib (BAY43-9006), sunitinib (SU11248), KW-2449, AC220, AP-24534, SB1518, ITR-260 and many more. All of the agents were capable of inhibiting both wild-type and FLT3-ITD autophosphorylation, although generally less inhibition was observed against the wild-type receptor. These agents can be broadly classified as highly selective (eg, AC220, sorafenib), intermediate (eg, sunitinib, KW-2449), and less selective (eg, lestaurtinib, midostaurin). They have been tested in AML patients as single agents or in combination with chemotherapy (Pratz and Levis, 2010). The initial series of FLT3 inhibitors were limited by suboptimal pharmacokinetics and inadequate specificity. However, some agents have shown promise in clinical trials with transient responses in patients with *FLT3*-mutant AML. Newer compounds, such as AC220, appear more promising. Preclinical studies of this agent have demonstrated significant potency and specificity, and an early-phase trial has reported a number of complete remissions (Fathi and Levis, 2011). Although, the sorafenib responses in *FLT3-ITD*$^+$ AML are usually transient (Zhang *et al.,* 2008; Crump *et al.,* 2010; Scholl *et al.,* 2011), but, in various cases with relapsed *FLT3-ITD*$^+$ AML showed prolonged remissions under sorafenib monotherapy (Metzelder *et al.,* 2009; Safaian *et al.,* 2009; Metzelder *et al.,* 2010; Mohan *et al.,* 2011; Mori and Sprague, 2012). Recently, Metzelder *et al.,* (2012) have retrospectively evaluated the outcome of 65 *FLT3-ITD*$^+$ AML patients treated with sorafenib. This study confirmed that sorafenib monotherapy has significant activity in *FLT3-ITD*$^+$ AML, and provided evidence that sorafenib synergizes with allogeneic immune effects of allo-HSCT in inducing durable remissions. Also under development is an anti-FLT3 monoclonal antibody, IMC-EB10 (Youssoufian *et al.,* 2010), which blocks signaling by binding to the receptor and also induces antibody-dependent cell-mediated cytotoxicity (ADCC). Preclinical studies have confirmed the anti-proliferative effects of IMC-EB10 against both wild-type *FLT3* and *FLT3* mutant AML models (Youssoufian *et al.,* 2010).

1.4 Immune system

During the course of evolution, nature developed a complex system of cells and molecules to defend organisms in a concerted action against multiple pathogens like bacteria, viruses, parasites as well as certain tumors. To achieve this challenging task, the human immune system has developed two main protective systems: the innate (antigen non-specific) and the adaptive (antigen-specific humoral and cellular) immune system (Janeway, 2001; Murphy *et al.,* 2008; Abbas *et al.,* 2007). However, the innate and adaptive responses are highly cooperative systems, thus potentiating the efficiency of immune responsiveness. The innate immune system is evolutionarily more ancient and represents the first immediate line of defense against invading pathogens. This includes physical, chemical and microbiological barriers, but also cellular and molecular factors (neutrophils, monocytes, macrophages, natural killer cells, dendritic cells, complement, cytokines, and acute phase proteins) that provide the immediate host defense. The different mechanisms of innate immunity to fight infections- phagocytosis, opsonization, and complement-mediated lysis -need the exposure of innate immune cells to the surface of pathogens. Adaptive (specific, acquired) immunity is characterized by targeted effector responses using antigen-specific receptors on lymphocytes, which provide humoral and cellular immunity (Janeway, 2001; Murphy *et al.,* 2008). Due to the tremendous diversity of their receptors, cells of the adaptive immune system are able to generate immune reactions specifically directed against non-self molecules (so called antigens) of virtually any foreign pathogen. The antigens are presented to and recognized by the antigen-specific T and B cells (via T-cell receptor- TCR or B-cell receptor- BCR, respectively). This process leads to cell priming, activation, and differentiation, usually occurring within lymphoid tissues. The B cells recognize antigens directly via a membrane-bound BCR and activated B cells start to proliferate and differentiate in the B-cell regions of secondary lymphatic organs into antibody (Ab)-producing plasma cells. Secreted antibodies can directly bind to antigens, and Ab/antigen complexes may subsequently activate the complement system, neutralize bacterial toxins or opsonize pathogens to facilitate their recognition and elimination by various effector mechanisms, e.g. promotion of phagocytosis. Elimination or control of infections with intracellular pathogens is often strictly dependent on T cells which recognize short pathogen-derived peptides that are presented on the surface of infected host cells (Janeway, 2001; Abbas *et al.,* 2007).

1.4.1 Fundamentals of T-cell immunology

T cells recognize their cognate antigenic peptides presented by professional antigen-presenting cells (pAPCs) via a heterodimeric TCR usually consisting of an α and a β chain. Antigen recognition requires the interaction of the T-cell receptor with the fitting HLA/peptide complex as well as the co-ligation of the CD8 or CD4 co-receptor (Saizawa *et al*., 1987) with the presenting HLA class I or II molecule, respectively. This in turn induces the activation and phosphorylation

of intracellular domains and enzymes (Murphy *et al.,* 2008) within the T cell, resulting in the switch to an effector T-cell phenotype. The quality of a T-cell response is described by the terms affinity and avidity (Sette *et al*., 1994; Deng *et al*., 1997). Affinity means the strength with which the TCR binds to the HLA/peptide complex. However, a high affinity does not automatically coincide with immune-dominance and *vice versa* (Müllbacher *et al*., 1999). Avidity describes in a simplified way the summation of all interactions between all receptors and ligands on the effector and target cells (Murphy *et al.,* 2008). Studies concerning adoptive T-cell transfer showed that high-avidity T cells are more efficient than low-avidity T lymphocytes in the control of early immune responses due to a more stable binding to APCs *via* costimulatory molecules (Gray *et al.*, 2003). The differences in the avidity of different T cells are explained by the necessity of the effector cells to recognize their antigen on APCs independently of whether the latter express costimulatory molecules or not (Murphy *et al.,* 2008).

1.4.2 T-cell maturation

Thymocytes evolve from common lymphoid progenitors (CLP), which receive stimuli from thymic epithelial cells (TEC) to develop into thymocyte progenitors (Schlenner *et al.,* 2010). Progenitor double-negative ($CD4^-CD8^-$) T cells from the bone marrow enter in the thymus, and rearrange the TCR genes to become $TCR\alpha\beta^+$ $CD4^+CD8^-$ or $CD4^-CD8^+$ T cells (the majority) or $TCR\gamma\delta^+$ $CD4^+CD8^-$ or $CD4^-CD8^+$ T cells (the minority) (Immunology, Goldsby *et al.,* 2003). The selection processes in the thymus are accompanied by loss of one of the co-receptors, CD4 and CD8, resulting in single-positive CD4 or CD8 T cells that recognize peptides presented by only HLA class II or class I molecules, respectively. Positive selection in the thymus eliminates T cells, which are unable to recognize self-HLA and negative selection eliminates thymocytes with high-affinity receptors for self-HLA molecules alone or self-antigen plus self-HLA in order to prevent autoimmunity, a process called *clonal deletion* (Goldsby *et al.,* 2003; Murphy *et al.,* 2008). Mature single-positive T lymphocytes leave the thymus and populate in the peripheral lymphoid tissues. The immune system has developed further mechanisms of *peripheral tolerance* leading to the elimination of self-reactive T cells outside the thymus. These mechanisms are mainly based on the lack of costimulatory signals to T cells that encounter self-antigens, leading to anergy (unresponsiveness) or apoptosis (Delves and Roitt, 2000a, 2000b; Parkin and Cohen, 2001; Abbas *et al.,* 2007). Besides αβ-T cells, γδ-T cells represent only about 5% of T lymphocytes in the blood, recognize protein as well as non-protein antigens and are not HLA-restricted.

1.4.3 Antigen processing and presentation

The presentation of protein antigens to T cells in association with or restricted by HLA molecules requires intracellular processing pathways, which are different for $CD4^+$ and $CD8^+$ T

cells. $CD8^+$ T lymphocytes recognize peptides that are processed *via* the endogenous pathway and presented by HLA class I molecules. Such peptides result from proteins (endogenous antigens) that have been newly synthesized in the cell, such as viral or tumor proteins (Brode and Macary, 2004). In the cytosol, those ubiquitinylated endogenous proteins undergo a first proteolytic degradation by proteasomes. The resulting protein fragments are transported back into the endoplasmatic reticulum (ER) by the transporter associated with antigen processing (TAP) (Lobigs and Müllbacher, 1993). The N-terminus of protein is further trimmed by aminopeptidases within the ER (e.g. the IFN-γ-inducible aminopeptidase ERAPI; Saric *et al.,* 2002) to a peptide length of eight to nine amino acid residues. In a next step, a part of those peptides are non-covalently associated with a newly synthesized HLA class I heavy chain and a β2-microglobulin light chain by the HLA class I loading complex. This HLA class I peptide complex dissociates from the loading complex and is transported through the Golgi apparatus to the cell surface where it can be detected by $CD8^+$ cytotoxic T cells (**Figure 1.4.3.1**).

$CD4^+$ T cells recognize peptides, derived from extracellular pathogens that are presented by HLA class II molecules. Uptake of exogenous proteins by APCs is accomplished by phagocytosis or endocytosis. Protein antigens are enclosed in endosomes, which subsequently fuse with HLA class II-containing acidic lysosomes, causing protein degradation. The assembly of the HLA class II molecule takes place in the endoplasmic reticulum. An HLA class II molecule consists of an α and a β chain that are non-covalently linked and synthesized in the ER. To prevent peptide binding already in the ER, newly synthesized HLA class II molecules assemble with an HLA class II-associated protein, called invariant chain (Ii), blocking the peptide binding groove. This trimeric complex trimerizes and is released from the ER as a nine subunit complex. A second function of invariant chain is the direction of HLA class II molecules to a low-pH endosomal compartment where peptide loading takes place. There, the invariant chain is cleaved by proteases, leaving a small fragment (CLIP, class II associated invariant peptide) bound to the HLA class II molecule. For antigen binding, the CLIP fragment is replaced by the peptide. This process is supported by an HLA-DM molecule that acts as a molecular chaperone and hinders the aggregation of HLA class II molecules. Peptides bound to the HLA class II molecules result from extracellular pathogens or proteins that are taken up into endocytic vesicles. The HLA II/peptide complexes are finally translocated to the cell surface (Murphy *et al.,* 2008; McMichael *et al*., 2002) and can be recognized by antigen-specific $CD4^+$ T cells. Peptides presented by HLA class II molecules are in general longer than those bound to HLA class I molecules (**Figure 1.4.3.1**).

MHC class I pathway **MHC class II pathway**

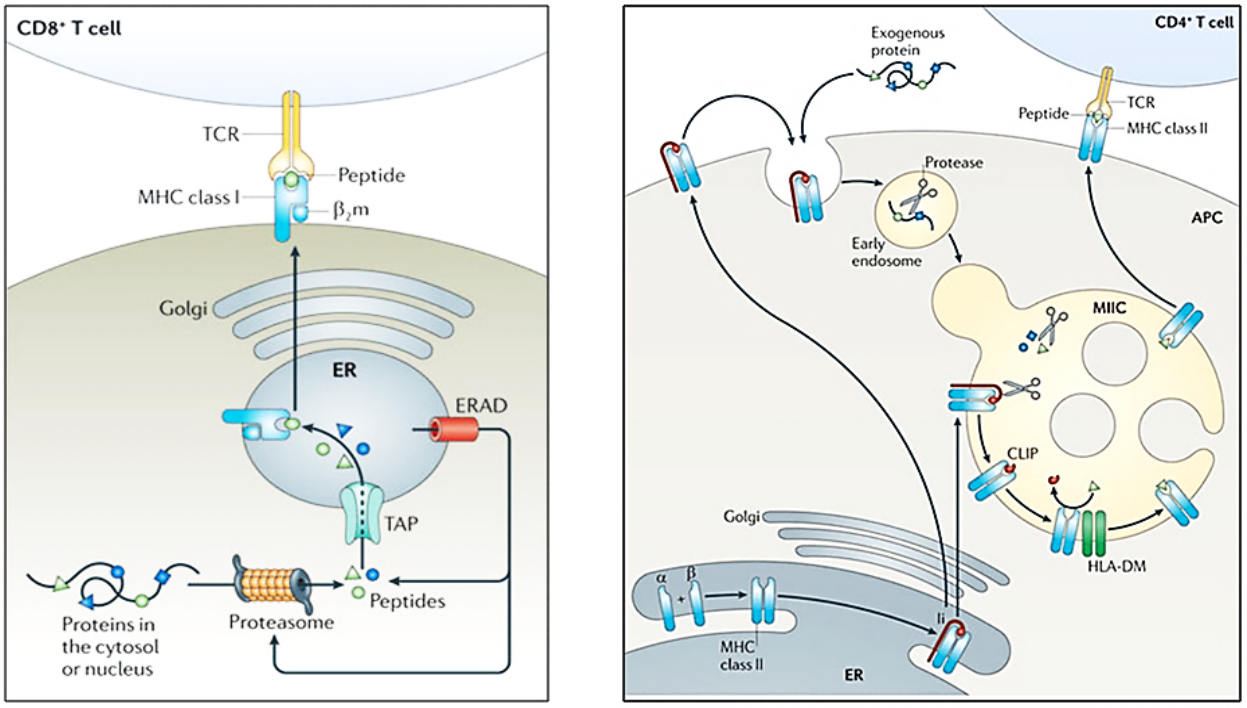


Figure 1.4.3.1: The basic HLA (MHC) class I and class II antigen presentation pathway.
(Adopted from: Neefjes *et al.,* 2011)

1.4.4 CD8$^+$ T cells

Immunity towards a variety of intracellular viral, bacterial and protozoan pathogens as well as tumors is strongly dependent on the generation of robust CD8$^+$ T-cell responses. The major tasks of specific CD8$^+$ immunity comprise the generation of effector cells that are responsible for definitive elimination of the pathogen as well as the generation of memory cells that survive long term and continuously patrol various tissues in search for evidence of re-attack (Williams and Bevan, 2006; Reiner *et al.,* 2007). The course of a CD8$^+$ T-cell response can generally be divided into four phases: the “priming phase” (I) during which naïve CD8$^+$ T cells are primed, followed by an “effector phase” (II) which is terminated by a dramatic “contraction phase” (III) leaving behind only 5-10% of the original burst size. The remaining cells are often maintained at relatively stable numbers during the “memory phase” (IV).

1.4.4.1 Role of APCs differentiation in CD8$^+$ T-cell activation (priming)

Dendritic cells (DCs) are the most potent professional APCs and are found in most tissues. Their HLA expression, for example, is ten to one hundred times higher than that found in other APCs, like B cells or monocytes. Only few DCs are required to generate an effective T-cell response. Single mature DCs are sufficient to stimulate one hundred to three thousand T cells (Bhardwaj *et al.,* 1992; Blauvelt *et al.,* 1995). Different developmental states of DCs are also accompanied by different functions. Immature DCs (iDCs), for instance, are attracted to

peripheral tissues by danger signals secreted by damaged cells (Matzinger *et al*., 2002). Thereby, iDCs get in contact with antigens. Immature DCs express only low levels of MHC (major histocompatibility complex) or HLA (human leukocyte antigen) molecules, which are indispensable for antigen presentation to and recognition by T cells. For the initiation of T-cell immune responses, $CD8^+$ T cells have to interact with professional APCs presenting cognate antigens (**Figure 1.4.4.1.1**). However, for full T-cell activation, the engagement of TCR and CD8 co-receptors (by HLA-peptide complexes) (**''signal 1''**) is not sufficient. Another signal (**''signal 2''**), termed as co-stimulation, is provided by the engagement of costimulatory molecules (*e.g.* by the interaction of CD28 with -CD80 and CD28 with -CD86). This can result in the activation ("priming") of naïve T cells. Other important co-stimulators for T cells comprise other members of the TNFR family such as interactions between CD40L-CD40 or others (Croft *et al.,* 2003; Watts, 2005; Lipscomb and Masten, 2002). CTLA-4 and PD-1 are expressed by T cells as a consequence of their activation via the TCR and via CD28. CTLA-4 interacts with CD80 and CD86, PD-1 with PD-L1 and PD-L2. These interactions provide inhibitory signals and lead to down regulation of the immune response (Norde *et al.,* 2012; Thaventhiran *et al.,* 2012). Also, BTLA (CD272) is a negative regulator of T cells upon activation (Derré *et al.,* 2010).

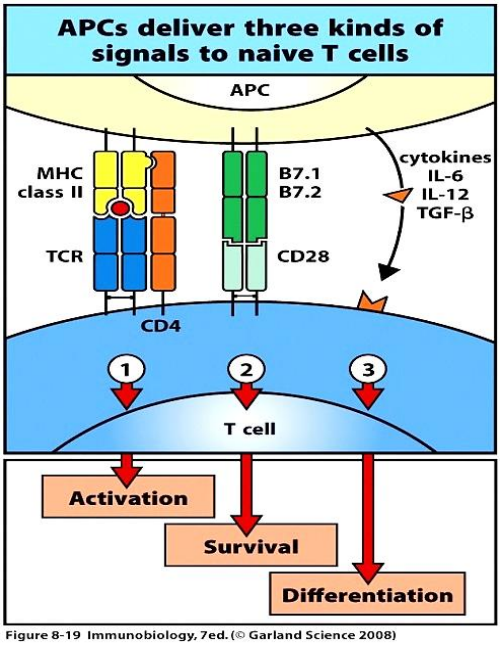


Figure 1.4.4.1.1: Signals required for T-cell activation. **''Signal 1''** is mediated by the interaction of antigen peptides presented by MHC class II molecules with the antigen-specific TCR. **''Signal 2''** results from the stabilization of the synapse through adhesion molecules and the generation of signals via costimulatory molecules present on the surface of APCs and T cells. B7.1/B7.2 (CD80/CD86) on APCs interact with their common receptor, CD28, on T cells to generate activatory signals, while interaction with cytotoxic T lymphocyte–associated protein 4 (CTLA4) generates inhibitory signals (not shown). **''Signal 3''** is produced by the secretion of cytokines by APCs, which act via cytokine receptors on T cells in order to polarize them toward an effector phenotype. (Adopted from: Janeway's Immunobiology, 7th edition, Murphy *et al.,* 2008)

If antigen presentation to a naïve T-cell *via* HLA/peptide complexes takes place without the engagement of costimulatory molecules, that might induce T-cell anergy and apoptosis (Delves and Roitt, 2000a and 2000b; Parkin and Cohen, 2001; Murphy *et al.,* 2008). Therefore, for example iDCs are considered to be less suitable for the activation of naïve T cells, as they rarely express costimulatory molecules. The main task of iDCs consists of the recognition and uptake of pathogens (Murphy *et al.,* 2008). Their ingestion triggers the migration of the iDCs into the regional lymph nodes. In consequence, iDCs lose their ability for antigen uptake and processing, but increase their HLA expression. Upon arrival in the lymph nodes they have

become mature DCs (mDCs) that express high amounts of costimulatory molecules on the cell surface (Sallusto *et al*., 1999; Murphy *et al.,* 2008). Although both TCR signals and co-stimulation play important roles in priming and clonal expansion of T cells, most $CD8^+$ T cell responses require further signals (**"signal 3"**) to improve their efficacy (Mescher *et al.,* 2006). These signals can be provided by different cytokines like IL-2, IL-12, IL-15, IFN-γ and TGF-β. In addition, also $CD4^+$ T cells contribute in delivering "signal 3". Whether $CD4^+$ T-cell help acts directly on the responding $CD8^+$ T cells or indirectly through APCs is still controversially discussed (Huster *et al.,* 2004; Kolumam *et al.,* 2005; Whitmire *et al.,* 2005; Mescher *et al.,* 2006; Castellino and Germain, 2006).

1.4.4.2 Priming and differentiation of $CD8^+$ T cells

Prior to first antigen contact, the precursor frequency of naïve $CD8^+$ (as well as $CD4^+$) T cells for different epitope specificities is very low and has been estimated to lie very constantly in the range of 50-200 cells per individual (Arstila *et al.,* 1999; Blattman *et al.,* 2002; Moon *et al.,* 2007). In order to run across their antigen, naïve $CD8^+$ T cells need to constitutively migrate through secondary lymphoid tissues, where they eventually encounter their cognate antigen (*e.g.* a processed pathogen-derived peptide) presented by APCs. Priming takes place in the secondary lymphoid organs, e.g. the lymph nodes, to which DCs migrate from the periphery after antigen-uptake (**Figure 1.4.4.2.1**). For homing to these organs, naïve T cells as well as DCs express the chemokine receptor CCR7. Adhesion molecules and chemokines (e.g. CCL21 or SLC and CCL19) allow the cells to patrol tissues in search. Upon encounter of the respective antigen, the T-cell is activated to an effector cell by interaction with the APC *via* several receptor-ligand interactions. Crosslinking of the TCR/CD3 complex after antigen binding causes phosphorylation of tyrosines within the cytoplasmic tail of CD3. This leads to the activation of intracellular signal transduction pathways and activation of cytokine genes responsible for T-cell proliferation. The primary immune response leads to the clonal expansion of antigen-specific T cells, generating both effector and memory cells. After a subsequent encounter with the same antigen, memory cells enable a superior secondary immune response (Delves and Roitt, 2000a and 2000b; Parkin and cohen, 2001), differentiate into $CCR7^-$ effector cells and proliferate, which is sustained by an autocrine IL-2 secretion. Efficient $CD8^+$ T-cell priming initiates vigorous expansion of few selected precursor cells through which the overall population size of antigen-reactive T cells gets enormously enhanced. In addition, proliferation and expansion is accompanied by the generation of T cells with distinct phenotypical characteristics that correlate with functionally discrete $CD8^+$ T-cell subsets (**Figure 1.4.4.2.1**).

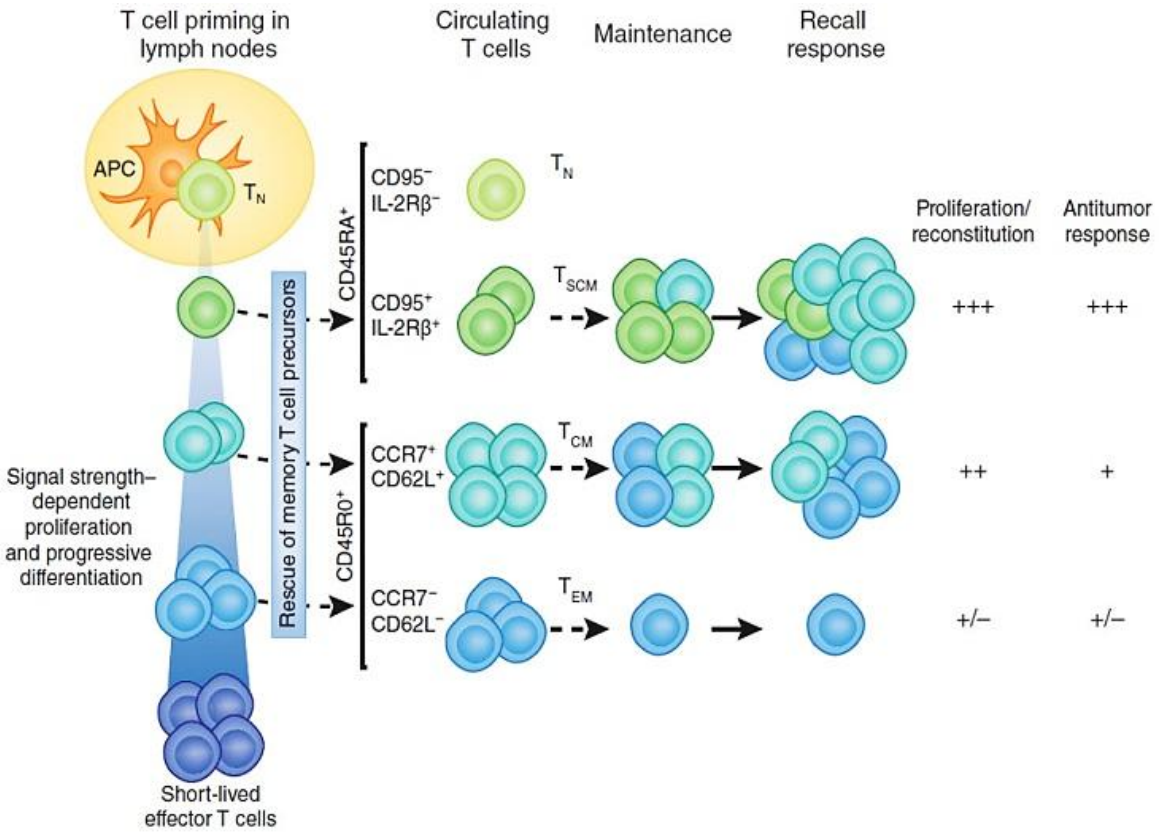


Figure 1.4.4.2.1: Naïve T cells are primed by antigen-presenting cells in secondary lymphoid organs.
After priming phase, proliferating T cells progress along a differentiation pathway, losing naïve and acquiring effector properties. Gattinoni *et al.,* (2011) have identified a previously unknown memory T-cell type i.e. distinct from the previously characterized the T_{CM} and T_{EM} cells. T_{SCM} cells are less differentiated and share several markers, including CD45RA, with the naïve T cells. T_{SCM} cells self-renew and differentiate in response to cytokines (dotted black arrows) or antigen (solid black arrows) and, in adoptive transfer experiments, have the highest reconstitution and antitumor capacity. (Adopted from: Sallusto and Lanzavecchia, 2011)

1.4.4.3 Effector mechanisms of $CD8^+$ T lymphocytes

Two major subtypes of effector cells are $CD4^+$ T cells with a predominant helper function via cytokine-secretion cells (Th) and $CD8^+$ T cells with their characteristic cytolytic capability (CTLs) (Murphy *et al.,* 2008). The principal mechanism of CTL-mediated cytolysis is the delivery of cytotoxic granule proteins granzyme B and perforin to target cells. Perforin is a pore-forming protein, which is present as a monomer in the cytotoxic granules of CTLs. It undergoes polymerization in the lipid bilayer of the target-cell plasma membrane and forms an aqueous channel. Through these channels, granzymes are being passed. Granzyme B, a most important member of granule serine proteases found specifically in the cytotoxic granules, proteolytically cleaves and thereby activates caspases within the target cell. Activation of caspases and further downstream molecules induces DNA fragmentation resulting in apoptosis. Another mechanism of CTL killing is mediated by binding of Fas ligand (FasL) on the CTL to the “death receptor” Fas, which is expressed on many target-cell types. The Fas/FasL interaction also induces apoptosis of the target-cell by activation of caspases. Cytokines produced by $CD8^+$ T cells upon

antigen encounter include IFN-γ, TNF-α, and lymphotoxin, function to activate macrophages and induce inflammation (Murphy *et al.,* 2008; Abbas *et al.,* 2007).

1.5 Adoptive T-cell Therapy

Besides vaccination, another powerful tool in the repertoire of immunotherapeutic strategies is adoptive T-cell therapy. Adoptive T-cell cancer immunotherapy is based on the premise that T cells have the capacity to specifically kill tumor cells as well as to proliferate and persist after transfer and, therefore, can eliminate residual or newly emerging malignant cells (Rosenberg *et al.,* 2008). In general, adoptive T-cell therapy can be subdivided into the usage of primary T cells or genetically modified lymphocytes. Primary T cells can be of various origins and comprise patient-derived tumor-infiltrating T cells (TILs), *ex vivo* primed T-cell bulk cultures of autologous or allogeneic origin or even T-cell clones. Tumor-specific adoptive T-cell therapy was initially developed by expanding tumor-infiltrating lymphocytes from melanoma lesions *in vitro* (Rosenberg *et al.,* 1994). These procedures are associated with high costs, laborious individual preparation and a non-predictable efficacy. To overcome these drawbacks, adoptive T-cell therapies using genetically engineered T cells have recently been prevailing (Restifo *et al.,* 2012). Thereby $CD8^+$ T cells are either transduced with genes encoding a T-cell receptor (TCR) that recognizes the complex of a malignoma-associated or-specific peptide and a particular MHC class I molecule (Morgan *et al.,* 2006; Johnson *et al.,* 2009; Restifo *et al.,* 2012), or T cells are transfected with genes encoding a chimeric-antigen receptor (CAR) that is composed of an antibody recognition domain and a cytoplasmic domain of the CD3 molecule (Eshhar, 2008; Kalos *et al.*, 2011; Liu *et al.,* 2012; Restifo *et al.,* 2012). Modifications comprise e.g. the provision of co-stimulatory molecules, beneficial cytokines or survival factors (Gattinoni *et al.,* 2012). Vaccination studies using peptides, from some of the leukemia-associated antigen, revealed a reduction of blasts in the bone marrow at least for a while (Schmitt *et al.*, 2008). The therapeutic potential of adoptive T-cell transfer to eradicate malignant cells has been impressively demonstrated and intensively studied in patients with melanoma, EBV-associated tumors and malignancies of the hematopoietic system (Kolb *et al.*, 2004; Kahl *et al.*, 2007; Moosmann *et al.*, 2010; Dudley *et al.*, 2010; Schmid *et al.*, 2012; Parmar *et al.*, 2011; Parkhurst *et al.*, 2011). Still, adoptive T-cell immunotherapy of leukemia with leukemia antigen-specific T cells is largely unexplored.

1.6 Allogeneic T-cell response against Leukemia antigens

In hematologic malignancies, allo-HSCT is considered as a most successful method of adoptive immunotherapy. Its therapeutic effect relies on the induction of a potent GvL effect mediated by donor-derived T cells. The clinical success of donor lymphocyte infusion (DLI) convincingly demonstrated the existence of GvL activity. DLI for the treatment of leukemic relapse after allo-

HSCT was introduced in the early 1990s (Kolb *et al.,* 1990; Collins and Fernandez, 1994; Porter *et al.,* 1994). Being extremely effective in chronic myeloid leukemia, however, clinical benefit was shown to be limited in AML patients, possibly as a result of great tumor burden during relapse (Kolb *et al*., 1995; Porter *et al.,* 1996; Schmid *et al.,* 2007). Responses to conventional DLI occur in 15-30% of patients with AML, but remissions are often transient and long-term survival and cure occurs in approximately 20% of patients. Nevertheless, patients treated with DLI appear to have better outcomes than patients who never receive DLI, with an estimated overall survival of 21% versus 9% at 2 years, respectively (Schmid *et al.,* 2007). Interestingly, haploidentical T-lymphocyte transfusion applied to non-transplanted AML patients resulted in positive clinical effects (Colvin *et al.,* 2009).

It is assumed that the therapeutic effect of allo-HSCT is mediated by T cells against minor histocompatibility antigens (mHAgs) that are preferentially expressed on hematopoietic cells (Smits *et al.,* 2009; Norde *et al.,* 2009). mHAgs are, alloantigens capable of eliciting an allogeneic T-cell response after allo-tranplantation even when donor and recipient are HLA-matched. mHAgs are polymorphic proteins. Peptides harboring polymorphic amino acids are processed, presented in association with certain HLA molecules and recognized by allo-reactive, donor-derived T cells on recipient cells. Although hematopoiesis-restricted mHAgs are promising target antigens, the practical use of mHAgs for specific immunotherapy is hampered due to the fact that only a limited number of mHAg has been identified so far. This explains why only a very much limited number of patients undergoing allo-HSCT would potentially profit from mHAg-directed immunotherapy (Spierings and Goulmy, 2005). This means that it is currently very difficult to improve the therapeutic effect of allo-HSCT on the basis of these antigens. The emergence of leukemia antigen-specific immune responses after allo-HSCT or DLI has been shown to coincide with the induction of clinical responses, suggesting a prominent role of Leukemia antigens in mediating GvL (Melenhorst *et al.,* 2009). GvL reactions may also be directed against LAAs or LSAs provided the donor-derived immune cells are able to mount a response against them. This motivates to focus on those leukemia targets that are more frequently and specifically expressed on leukemia cells either by vaccinating patients and perhaps also donors or to adoptively transfer T cells specifically recognizing these antigens.

It appears more likely that T cells against leukemia-specific antigens can be expanded from patients´ blood as compared to healthy donors'. However, blood donations from patients are limited and the patients´ anti-leukemia T-cell repertoire might be skewed due to complex previous leukemia/host interactions, chemotherapy and post-allo-HSCT immunosuppressive treatment (Ersvaer *et al.,* 2010). This assumption is supported by comparative data obtained with tumor-reactive CTL clones derived from individuals with and without cancer (Karanikas *et al.,* 2010). It can be supposed that healthy individuals have a more diversified T-cell repertoire

(Hakim *et al.,* 2005) with better proliferation behavior than cancer patients. Reports concerning the detection of naïve/memory $CD8^+$ T-cell responses against LAAs in healthy individuals' peripheral blood lymphocytes have been published. The LAAs were PR1 (Scheibenbogen *et al.,* 2002; Rezvani *et al.,* 2003, and 2007), WT1 (Rezvani *et al.,* 2003 and 2007; Weber *et al.,* 2009), PRAME (Griffioen *et al.,* 2006; Rezvani *et al.,* 2009; Quintarelli *et al.,* 2008), RHAMM (Greiner *et al.,* 2004; Casalegno-Gardu˜no *et al.,* 2012), survivin (Grube *et al.,* 2007), and Cyclin-A1 (Ochsenreither *et al.,* 2012). However, the precursor frequency of leukemia-reactive $CD8^+$ T cells in these healthy individuals was rather low (Delain *et al.,* 1994; Smit *et al.,* 1998; Rezvani *et al.,* 2003 and 2009; Westermann *et al.,* 2004; Barrett and Rezvani, 2007; Brackertz *et al.,* 2011). Herein, healthy individuals were analyzed for the presence of T-cell reactivity against AML-specific FLT3-ITDs.

2. Aim of the study

The aim of the presented work was to generate FLT3-ITD-reactive, stable CTL populations and clones from allogeneic lymphocyte sources. It was planned to generate via stimulation with *in vitro*-transcribed RNA (IVT-RNA) encoding FLT3-ITDs:

- stable $CD8^+$ T-cell clones against FLT3-ITD from a patient with *FLT3-ITD*$^+$ AML (providing a reference model system).

- stable $CD8^+$ T-cell populations and clones against known immunogenic FLT3-ITDs
    - from the peripheral blood lymphocytes of healthy individuals, who carry the HLA I alleles presenting the immunogenic neoepitopes derived from these FLT3-ITDs, and, possibly,
    - from umbilical cord blood lymphocytes as a rich source of naïve T cells.

Furthermore, it was planned

- to characterize FLT3-ITD-reactive allogeneic $CD8^+$ T cells with respect to
    - their HLA class I restriction,
    - their peptide specificity,
    - their TCR usage, and, if possible,

to compare these characteristics with the characteristics of $CD8^+$ T-cell responses against the respective FLT3-ITDs in autologous systems.

3. Materials

3.1 Chemicals and reagents

Acetic acid 96%	Merck, Darmstadt
3-Amino-9Ethyl-Carbazol (AEC) tablets	Sigma, Steinheim
Aqua B. Braun	Braun, Melsungen
Agarose	Starlab, Ahrensburg
Agarose, ultrapure	Gibco, Karlsruhe
Big Dye Premix 3.1	Genterprise, Mainz
Bromo-phenol blue	Merck, Darmstadt
β-mercaptoethanol	Sigma, Steinheim
Chloroform	Roth, Karlsruhe
Diethyl Pyrocarbonat (DEPC)	Sigma, Steinheim
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
DMF (N,N-dimethylformamid)	Roth, Karlsruhe
100 bp DNA ladder	peqlab, Erlangen; Fermmentas, Leon-Rot
1 kb DNA ladder	peqlab, Erlangen; New-England Biolab, Frankfurt
DNA Blue Run 6x	Fermmentas, Leon-Rot
Ethanol, 99%	Applichem, Darmstadt
ethanol absolute	Sigma, Steinheim
Ethidiumbromide 10 mg/ml	Sigma, Steinheim
Ethylene diamine tetra acetate (EDTA)	Sigma, Steinheim
Ficoll Lymphoprep	PAA Lab, Pasching; Biochrom KG, Berlin
Formaldehyde 37%	Merck, Darmstadt; Applichem, Darmstadt
Formamide	Merck, Darmstadt
Gel electrophoresis QA-Agarose TM	Q Biogene, Heidelberg
Gene pulser cuvette 0.4 cm gap	BIO-Rad München; VWR (Int.), Darmstadt
Glycerol	Sigma, Steinheim
GelRed	Biotium, Darmstadt
H2O2 30%	Merck, Darmstadt
Isoamyl alcohol	Merck, Darmstadt
Isopropanol	Fisher Scientific, Schwerte
L-glutamine 200mM (100x)	Gibco, Karlsruhe
Lipofectamine 2000™ Transfection Reagent	Invitrogen, Karlsruhe
$M^7G(5')ppp(5')G$ RNA Capping Analog	Invitrogen, Karlsruhe
Mopholinopropansulfonsäure (MOPS)	Roth, Karlsruhe; USB, Cleveland, Ohio
NaCl Salt	Sigma, Steinheim; Roth, Karlsruhe
OPTIMEM with Glutamax-I	Gibco, Karlsruhe
Phosphat-buffered saline (PBS)	Biochrom, Berlin; Life technologies, Darmstadt
Poly(A) polymerase reaction buffer 5x	USB, Cleveland, Ohio

Roti® Phenol:Chloroform	Roth, Karlsruhe
rATP 10 mM	Promega, Mannheim
0.5-10 kb RNA ladder	Invitrogen, Karlsruhe
Sodium acetate	Merck, Darmstadt
TBE buffer 10x	Serva Electrophoresis, Heidelberg
TransMessenger™ Transfection Reagent	QIAGEN, Hilden
Tris-HCl	Sigma, Steinheim
Trypan blue	Merck, Darmstadt
Trypan blue 0.4%	Sigma, Steinheim
Trypsine-EDTA	Gibco, Karlsruhe
Tween 20	Sigma, Steinheim

The m7G(5')ppp(5')G RNA-Capping analog was dissolved in 13,7 µl DEPC-H2O to prepare a 100 mM solution, and stored at -20°C. DNA marker was stored at 4°C. RNA marker was stored at -80°C.

3.2 Buffers and solutions

Buffer/solution	Components	Amounts
Acetic acid 0.2N	Acetic acid 96% H2O	11.33 ml ad 1,000 ml
Sodium acetate 0.2N pH 8.0	Sodium acetate H2O	16.4 g ad 1,000 ml
Sodium acetate 3M pH 7.0	sodium acetate H2O-DEPC	24.6 g ad 100 ml
EDTA 0.5M pH 8.0	EDTA H2O-DEPC	18.6 g ad 100 ml
NaCl 0.15M	NaCl H2O	4.5 g ad 100 ml
AEC-staining solution Filtered with 0.45 µm	Aminoethylcarbazol Dimethylformamide ELISPOT-acetate buffer 30% H2O2	1 tablet (2.5 g) 2.5 ml 47.5 ml 25 µl
ELISPOT-staining buffer	0.2 N acetic acid 0.2 N sodium acetate H2O	46 ml 110 ml 469 ml
PBS for ELISPOT development	PBS H2O	95.5 g ad 10 liters
0.5x TBE buffer	10x TBE buffer H2O	100 ml ad 2,000 ml
Agarose gel 1%	Agarose powder 0.5x TBE buffer	1 g ad 100 ml
H2O –DEPC	DEPC H2O	1 ml ad 1,000 ml
10x MOPS pH 7.0	MOPS 3M sodium acetate EDTA 0.5M H2O	41.6 g 16.7 ml 10 ml ad 1,000 ml
RNA gel	Agarose DEPC- H2O 37% formaldehyde 10x MOPS	0.56 g 56 ml 7 ml 7 ml
RNA sample buffer	Formamide 37% formaldehyde	100 µl 70 µl

	10x MOPS Bromo-phenol blue 1% Ethidium bromide 1% H2O-DEPC	50 µl 20 µl 1 µl ad 500 µl
Trypan blue stock solution	Trypan blue H2O	1 g ad 1,000 ml
Trypan blue staining solution	Trypan blue stock solution NaCl 0.15M	75 ml 25 ml
MACS buffer	BSA EDTA 0.5M PBS	2.5 g 2 ml ad 500 ml
FACS buffer	BSA PBS	0.5 g ad 500 ml
FACS fixing buffer	37% formaldehyde PBS	1.35 ml ad 50 ml
Erythrocytes lysis buffer	Ammonium chloride Sodium bicarbonate EDTA H2O	8.3 g 0.84 g 29.3 g ad 1,000 ml

Table 3.2.1: Buffers and solutions

3.3 Materials for bacterial culture

3.3.1 Plasmids

All plasmids, encoding HLA class I molecules used in this work, were available in our group (AG T. Wölfel), except pcDNA3.1.HLA-C*07:01, which was kindly provided by Dr. P. van der Bruggen (LICR, Brussels, Belgium) and pcDNA3.1.HLA-C*0702, which was kindly provided by Dr. H. Wang (Japanese Red Cross, Central Blood Center, Tokyo, Japan). Plasmid pcDNA6/V5-His.pp65 encoding pp65 of Human CMV was kindly provided by Dr. B. Plachter (Institute of Virology, University of Mainz). If required, the inserts were controlled by DNA sequencing. Below, is a list of plasmids encoding different FLT3-ITDs and control antigens used herein.

Name of the insert	**pcDNA3.1**	**pcDNA3.1/V5-His TOPO**	**pcDNA3**	**pIRES*hyg*3**	**pcDNA6/V5-His TOPO**
EGFP	x				
CMV pp65					x
CMV pp65		**x**			
Sig_pp65_DC.LAMP			x		
wtFLT3$_{\text{Full Length}}$				x	
JC_FLT3-ITD$_{\text{Full Length}}$				x	
wtFLT3$_{\text{Full Length}}$		**x**			
IN_FLT3-ITD$_{\text{Full Length}}$		**x**			
FL_FLT3-ITD$_{\text{Full Length}}$		**x**			
wtFLT3$_{\text{Exon13-16}}$			x		
IN_FLT3 -ITD$_{\text{Exon13-16}}$			x		
VE_FLT3 -ITD$_{\text{Exon13-16}}$			x		
QQ_FLT3 -ITD$_{\text{Exon13-16}}$			x		

FL_FLT3 -ITD$_{Exon13-16}$		**x**			
Sig_wtFLT3$_{Exon13-16}$_DC.LAMP			x		
Sig_IN_FLT3 -ITD$_{Exon13-16}$_DC.LAMP			x		
Sig_VE_FLT3 -ITD$_{Exon13-16}$_DC.LAMP			x		
Sig_QQ_FLT3 -ITD$_{Exon13-16}$_DC.LAMP			x		
IN_FLT3-ITD$_{91}$		**x**			
IN_FLT3-ITD$_{55}$		**x**			
IN_FLT3-ITD$_{56}$		**x**			
IN_FLT3-ITD$_{57}$		**x**			
IN_FLT3-ITD$_{59}$		**x**			
IN_FLT3-ITD$_{60}$		**x**			
IN_FLT3-ITD$_{62}$		**x**			
IN_FLT3-ITD$_{63}$		**x**			
IN_FLT3-ITD$_{64}$		**x**			
IN_FLT3-ITD$_{65}$		**x**			

Table 3.3.1.1: Plasmids.
The table shows all the antigenic plasmids used herein. Plasmids in bold and italics were cloned for this project; the others clones were already available in the lab. Sig/pcDNA_DC.LAMP; Sig/pcDNA3 was constructed by ligation of signal sequences of the human *lamp1* and *dc.lamp* genes into the multiple cloning site of pcDNA3.

3.3.2 Bacterial strains

TOP 10 bacteria *one-shot* E. coli (Invitrogen, Karlsruhe) were used to propagate all recombinant plasmids. Genotype of TOP 10 bacteria is: F^- *mcr*A Δ(*mrr-hsd* RMS-*mcr*BC) □80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 *nup*G.

3.3.3 Substances and media for bacterial culture and conservation

Bacto Yeast Extract DIFCO	Becton Dickinson, Heidelberg
Bitek Agar DIFCO	Becton Dickinson, Heidelberg
Tryptone Peptone DIFCO	Becton Dickinson, Heidelberg
S.O.C. Medium	Invitrogen, Karlsruhe

LB (Luria-Bertani) medium:	10 g Bacto Tryptone, 5 g yeast extract, 10 g NaCl, 1 liter H2O
LB-ampicilline:	1 liter LB medium, 100 µg amplicilline
LB agar-ampicilline:	1 liter LB medium, 15 g Bacto Agar, 100 µg amplicilline
Glycerol stocks:	500 µl bacterial culture (in LB medium) and 500 µl 60% glycerol

All media were stored at 4°C.

3.4 Antibiotics

Ampicilline	Sigma, Steinheim
Geneticine (G418) 50 mg/ml	Gibco, Karlsruhe
Penicilline-streptomycine	Gibco, Karlsruhe

Antibiotics were stored at -20.C in 10 ml alliquots.

3.5 Primers

Primer ID	Primer sequence 5`-3` direction	Annealing Temperature
$FLT3_{Full}$ Forward BamH1	5- TCTAAAGGATCCGCCATGCCGGCGTTGGCGCGCGACGGC -3	69.3 °C
$FLT3_{Full}$ Reverse Xba I	5- GGGTCTAGACCCGAATCTTCGACCTGAGCCTGCGG -3	68.5 °C
$FLT3_{exon13}$ Forward BamH1	5- AAAGGATCCGCCATGGTCGTTTTAACCCTGCTAATT -3	65.1 °C
$FLT3_{exon16}$ Reverse Xba I	5- GGATCTAGAGACATGAGTGCCTCACTTTCAGAGC -3	63.4 °C
pp65 Forward BamH1	5- GGGGGATCCGAGTCGCGCGGTCGCCGTTGTCCC -3	66.0 °C
pp65 Reverse Xba I	5- GGGATCTAGACCTCGGTGCTTTTTGGGCGTCG -3	64.2 °C
$FLT3_{exon13}$ Forward	5- GCCACCATGGTCGTTTTAACCCTGCTAATTTGTCACAAG -3	60.0 °C
$FLT3_{exon16}$ Reverse	5- GGGTCTAGACATGAGTGCCTCTCTTTCAGA -3	63.0 °C
IN_FLT3-ITD_{273} Reverse	5- TCATGATCCTAGTACCTTCCCAAACTC -3	60.4 °C
IN_FLT3-ITD_{165} Reverse	5- CTAATTTTCTCTTGGAAACTCCCATTT -3	58.9 °C
IN_FLT3-ITD_{168} Reverse	5- CTAGCAATTTTCTCTTGGAAACTCCCA -3	61.9°C
IN_FLT3-ITD_{171} Reverse	5- CTAGGGGCAATTTTCTCTTGGAAACTC -3	63.4 °C
IN_FLT3-ITD_{177} Reverse	5- CTAATCTGAGGGGCAATTTTCTCTTGG -3	61.6 °C
IN_FLT3-ITD_{180} Reverse	5- GTAGAAGTACTAATTATCTGAGGGGCAATTTTCTCT -3	66.1 °C
IN_FLT3-ITD_{186} Reverse	5- AACGTACTAGTACTCATTATCTGAGGGGCAATTTTC -3	67.2 °C
IN_FLT3-ITD_{189} Reverse	5- ATCAACCTAGAAGTACTCATTATCTGAGGGGCAATTTTC -3	68.4 °C
IN_FLT3-ITD_{192} Reverse	5- ATCTTAGTAGAAGTACTCATTATCTGAGGGGCAATTTTC -3	67.0 °C
IN_FLT3-ITD_{195} Reverse	5- TTAAACGTAGAAGTACTCATTATCTGAGGGGCAATTTTC -3	67.4 °C

Table 3.5.1: List of primers, used in the cloning of indicated antigens.
This table specifies all the primers applied for amplification of PCR products used in the cloning of indicated FLT3-ITDs and other control antigens into pcDNA3.1 V5/His expression vector used in this study (e.g. 3' fragmentation of *FLT3-ITD* $_{Exon13-16}$ cDNA of patient **IN**).

3.6 Enzymes

DNAse I	Roche, Mannheim
Yeast poly (A) polymerase	USB, Cleveland, Ohio
Easy-A polymerase	Stratagene, Heidelberg
PCR-Polymerase (VWR)	VWR International, Darmstadt
Perfect Taq plus	5 Prime, Hamburg
T4 DNA ligase	Roche, Mannheim
Apa I	Promega, Mannheim
*Bam*H I	Promega, Mannheim
*Eco*R I	Promega, Mannheim
*Hin*d III	Promega, Mannheim
Xba I	Promega, Mannheim
Calf Intestine Phosphatase (CIP)	NEB Biolabs, Germany

All enzymes were stored at -20°C.

3.7 Molecular biology kits

High Pure PCR Purification Kit	Roche, Mannheim
HiSpeed Plasmid Maxi kit	Qiagen, Hilden

pcDNA3.1/V5 His TOPO TA Expression kit	Qiagen, Hilden
QIAprep Spin Miniprep kit	Qiagen, Hilden
QIAquick Gel Extraktion kit	Qiagen, Hilden
T7 Production System Ribomax Large Scale RNA	Promega, Mannheim
mMESSAGE mMACHINE® T7 Kit	Ambion, Life Technologies, Karlsruhe
RNase free DNase Set	Qiagen, Hilden
RNeasy Mini kit	Qiagen, Hilden
Superscript III 1st strand kit	Invitrogen, Karlsruhe
Taq PCR core kit	Qiagen, Hilden
Taq PCR kit	peqlab, Erlangen
Vectastain Elite ABC PK-6100	Linaris, Wertheim-Bettingen
perfect Taq-Master PCR kit	5 PRIME, Hamburg

3.8 Materials for cell culture and cellular assays

3.8.1 Cell lines

COS-7 cells: The COS-7 cell line is transformed African green monkey kidney fibroblast cells, developed from CV-1 line by Yakov Gluzman in the early 1980s (Gluzman *et al.,* 1981). This line contains large T-antigen, retains complete permissiveness for lytic growth of SV40 and supports the replication of SV40 mutants.

293T cells: HEK 293T, was generated in the early 70s by Graham and his colleagues, derived from the human embryonic kidney 293 cell line (Graham *et al.,* 1977). 293T is a highly transfectable derivative of the 293 cell line into which the temperature sensitive gene for SV40 T-antigen, was inserted (DuBridge *et al.,* 1987), which allows the episomal replication of transfected plasmids containing the SV40 origin of replication.

EBV-transformed B-cells: The B-cell line AK-EBV-B cells is a suspension lymphoblastoid cell line (LCL) was immortalized by the transformation with the Epstein Barr virus (Knuth *et al.*, 1989).

3.8.2 Substances and media for cell culture

AIM V® culture medium Gibco	Gibco, Karlsruhe
BSA (bovine serum albumine)	Sigma, Steinheim
RPMI 1640 Medium	Gibco, Karlsruhe
OPTIMEM	Gibco, Karlsruhe
Trypsin-EDTA	Gibco, Karlsruhe
FCS-fetal calf serum (heat inactivated)	PAA Lab., Pasching; Biochrom, Berlin

HS- human serum	Healthy donors' blood donations (heat inactivated HS)
RPMI complete:	RPMI 1640 + 10% FCS + 1% Penicillin-Streptomycin
AIM V complete:	AIM V + 10% HS
FastDCs medium:	RPMI 1640 + 2% HS
FastDCs d0-medium:	RPMI 1640 + 2% HS + GM-CSF (1000 U/ml) + IL-4 (500 U/ml)
FastDCs d1-medium:	RPMI 1640 + 2% HS + IL-1β (10 ng/ml) + IL-6 (1000 U/ml) + TNF-α (10 ng/ml) + PGE_2 (1 µg/ml)
T-cell medium:	AIM V + 10% HS + IL-2 (20 U/ml) + IL-4 (10 ng/ml) + IL-7 (5 ng/ml)
Freezing medium:	FCS + 10% DMSO and AIM V + 40% HS + 10% DMSO

(If any medium of this list was not used then specified in the method and result section)
All media were stored at 4°C

3.8.3 Cytokines

rhGM-CSF (Leukine Sagramostim)	Berlex, Richmond, CA, USA
rhIL-1β	BD Biosiences, Heidelberg
Prostaglandin E2	Sigma, Taufkirchen
rhTNF-α	Sigma, Taufkirchen; Promokine, Heidelberg
rhIL-4	Miltenyi Biotec, Bergisch Gladbach
rhIL-6	Promokine, Heidelberg
rhIL-2	Proleukin, Chiron behring, Marburg
rhIL-7	Miltenyi Biotec, Bergisch Gladbach
rhIL-12	Miltenyi Biotec, Bergisch Gladbach
rhIL-15	Miltenyi Biotec, Bergisch Gladbach

All cytokines were stored either at -80°C or at -20°C.

3.9 Donors and blood samples

3.9.1 AML Patient

Leukemic cells, of patient **FL** suffering from AML, were routinely checked for FLT3-ITD expression by the local laboratory for clinical diagnostics (BTA/MTA: K. Busch and B. Schuch; Head: PD Dr. Georg Heß). All blood samples were drawn with informed consent from patient or their relatives according to institutional policies and approval by the institutional review board. From patient **FL** (**Table 3.9.1.1**), 50 ml of leukemic blood were taken at the time of diagnosis. Peripheral blood mononuclear cells (PBMCs) were collected at different time points after reconstitution before or after chemotherapy cycles.

Patient ID	Age at diagnosis	Gender	FAB type	Leukocyte count ($x10^3$/µl)*	AML blast fraction*	CMV status	HLA class I		
							-A	-B	-C
FL	27 years	♀	M1	113.6	94%	positive	*01:01	*07:01 *08:01	*07:01 *07:02

Table 3.9.1.1: AML patient.
This table gives an overview of patient **FL** at the time of AML diagnosis.

3.9.2 Healthy donors

After informed consent to blood donors, buffy coats collected from $HCMV^+$ healthy donors were received from the Transfusion Centre (Dr. R. Conradi), University Medical Center, Johannes Gutenberg-University, Mainz. These donors were serotyped for HLA class I alleles (2-digit serotyping). Out of twenty three donors, nineteen $HCMV^+$ donors were investigated for anti-FLT3-ITD response against selected FLT3-ITDs. Donors, according to routine serotyping, expressed at least one of the HLA class I alleles i.e. HLA-A*32, HLA-B*27 and HLA-A*11, which presents the FLT3-ITD epitope to $CD8^+$ T cells (**Table 3.9.2.1**). PBMCs were isolated by FICOLL-density gradient centrifugation method.

Healthy donor	HLA -A	HLA -B	HLA -C	against FLT3-ITD of patient
# 6798	*03, *32	*07, *61	*02, *07	**IN** (IN/583-CP-25)
# 2655	*02, *32	*07, *61	*02, *07	**IN** (IN/583-CP-25)
# 2673	*03, *32	*35, *57	*04, *06	**IN** (IN/583-CP-25)
# 6647	*26, *32	*35, *49	*04, *07	**IN** (IN/583-CP-25)
# 2567	*02, *32	*44, *62	*03, *05	**IN** (IN/583-CP-25)
# 1066	*28, *32	*14, *62	*03, *08	**IN** (IN/583-CP-25)
# 1853	*03, *32	*35, *57	*04, *06	**IN** (IN/583-CP-25)
# 7300	*07, *32	*04, *15	*02, *07	**IN** (IN/583-CP-25)
# 1546	*11, *30	*13, *44	*05, *06	**QQ** (QQ/591-D-7)
# 0147	*02, *11	*07, *15	*03, *07	**QQ** (QQ/591-D-7)
# 2662	*11, *30	*39, *51	*04	**QQ** (QQ/591-D-7)
# 7986	*11, *32	*07	*07	**QQ** (QQ/591-D-7)
# 1035	*02, *11	*08, *51	-	**QQ** (QQ/591-D-7)
# 3501	*03, *11	*07, *10	*01, *07	**QQ** (QQ/591-D-7)
# 1605	*31, *32	*18, *27	*07	**VE** (VE/585-RA-14)
# 7926	*30, *68	*18, *27	*05, *07	**VE** (VE/585-RA-14)
# 1158	*11, *26	*27, *38	*01	**VE** (VE/585-RA-14)
# 0189	*02, *23	*27, *44	*03, *04	**VE** (VE/585-RA-14)
# 1798	*03, *24	*07, *27	*02, *07	**VE** (VE/585-RA-14)

Table 3.9.2.1: List of healthy donors analyzed against three selected immunogenic FLT3-ITDs previously identified as in patients IN, QQ and VE.
Buffy coats collected from the healthy individuals were received from Transfusion Centre, University Medical Center, Mainz. As mentioned here, these donors were serotyped for HLA class I alleles (2-digit serotyping). Healthy donor buffy coats shown in yellow, turquoise and purple colors were analyzed against **IN_**, **QQ_** and **VE_**FLT3-ITD, respectively.

3.9.3 Umbilical cord blood donations

After informed consent to the parents, umbilical cord blood donations, were received from Klinik für Geburtshilfe und Frauenheilkunde (Dr. A. Pohl), University Medical Center of the Johannes Gutenberg-University, Mainz, Germany. In this study, eight umbilical cord blood donors (**Table 3.9.3.1**) were analyzed for anti-FLT3-ITD response.

Umbilical cord blood donor	Amount of blood received (in ml)	UBMCs obtained (in million)
MZUCB#1	40	154
MZUCB#2	36	120
MZUCB#3	42	135
MZUCB#4	29	103
MZUCB#5	40	140
MZUCB#12	45	165
MZUCB#15	28	116
MZUCB#23	31	129

Table 3.9.3.1: List of Umbilical cord blood donors.
Given amount of cord blood was received from Klinik für Geburtshilfe und Frauenheilkunde, University Medical Center, Johannes Gutenberg-University, Mainz, Germany. Umbilical cord blood mononuclear cells (UBMCs) were isolated by Ficoll-density gradient centrifugation method described in **Method section 4.2**.

3.10 Antibodies

Antibody	Clone	Isotype	Company, Location
IgG1-FITC/IgG1-PE	H2	IgG2a mouse	Beckman Coulter, Krefeld
CD3-FITC/CD3-PE	UCHT1	IgG1 mouse	Beckman Coulter, Krefeld
CD3-FITC/CD8-PE	UCHT1/B9.11	IgG1 mouse	Beckman Coulter, Krefeld
CD4-PE/FITC	13B8.2	IgG1 mouse	Beckman Coulter, Krefeld
CD8-PE/FITC	B9.11	IgG1 mouse	Beckman Coulter, Krefeld
CD11c-PE	BU15	IgG1 mouse	Beckman Coulter, Krefeld
CD14-PE/FITC	116	IgM mouse	Beckman Coulter, Krefeld
CD16-FITC	3G8	IgG1 mouse	Beckman Coulter, Krefeld
CD19-FITC	89B	IgG1 mouse	Beckman Coulter, Krefeld
CD25-FITC	1HT44H3	IgG2a mouse	Beckman Coulter, Krefeld
CD27-PE	1A4CD27	IgG1 mouse	Beckman Coulter, Krefeld
CD45RA-PE	2H4LDH11LDB9	IgG1 mouse	Beckman Coulter, Krefeld
CD45RO-PE	UCHL1	IgG2a mouse	Beckman Coulter, Krefeld
CD56-PE	N901	IgG1 mouse	Beckman Coulter, Krefeld
CD62L-PE	DREG56	IgG1 mouse	Beckman Coulter, Krefeld
CD80-FITC	MAB104	IgG1 mouse	Beckman Coulter, Krefeld
CD83-PE	HB15a	IgG2b mouse	Beckman Coulter, Krefeld
CD86-FITC	2331 (Fun.1)	IgG1 mouse	Pharmingen, Heidelberg
CD94-FITC	HP-3D9	IgG1 mouse	Pharmingen, Heidelberg
CD127-PE	R34.34	IgG1 mouse	Beckman Coulter, Krefeld
CD135	polyclonal	IgG rabbit	Santa Cruz, Heidelberg
CCR7-FITC	FAB197F	IgG2a mouse	R&D Systems, Wiesbaden
Mab anti-IFN-γ clone	1-D1K	IgG1 mouse	Mabtech, Nacka, Schweden
Mab anti-IFN-γ clone	7-B6-1	IgG1 mouse	Mabtech, Nacka, Schweden
Mab anti-HLA I, w6/32	hybridoma supernatant	IgG2a mouse	Parham, 1979 HB95 (ATCC)
Mab anti-HLA-A*02, PA2.1	hybridoma supernatant	IgG1 mouse	Parham, 1978 HB117 (ATCC)
Mab anti-HLA class-I	clone MEM-81	IgG1 mouse	Novus Biologicals, Littleton
Goat-anti-mouse (GAM)-FITC	polyclonal	None	Novus Biologicals, Littleton

CD8 Microbeads	BW135/80	IgG2a mouse	Miltenyi Biotec, Bergisch Gladbach
CD8 Microbeads	TÜK4	IgG2a mouse	Miltenyi Biotec, Bergisch Gladbach

Table 3.10.1: Antibodies.

3.11 Synthetic peptides

The peptides used herein (**Table 3.11.1**) were either synthesized by Dr. Drijfhout (LUMC, Leiden, Netherlands), or kindly provided by Prof. Dr. S. Stevanović (Eberhard Karls University, Tübingen, Germany). Peptides encoded by *FLT3-ITDs* were predicted by public domain algorithms using computer-based epitope prediction [BMAS (Parker *et al.,* 1994), SYFPEITHI (Rammensee *et al.,* 1999) and NetMHC 3.2 (Lundegard *et al.,* 2008a and 2008b); Larsen *et al.,* 2005; Tenzer *et al.,* 2005]. The purity of the peptides after HPLC analysis was >80%. Their masses were checked by mass-spectrometry. Lyophilised peptides were solubilized in DMSO at a concentration of 40 mg/ml, further diluted with PBS to 2 mg/ml and frozen in aliquots at -20°C. If not otherwise indicated, the peptides were used at a concentration of 5 µg/ml in the mixed lymphocyte peptide culture and 10 µg/ml in IFN-γ ELISPOT assays.

Peptide-Name	**Amino acid sequence**	**Amino acid position**	**Derived from**	**HLA-restriction**	**Synthesized by**
$pp65_{495-503}$	NLVPMVATV	495 – 503	CMV pp65	HLA-A*02:01	Dr. Drijfhout Leiden, Netherlands)
$pp65_{417-426}$	TPRVTGGGAM	417 – 426	CMV pp65	HLA-A*07:01	
VE_FLT3-ITD$_{591-599}$ /A3	**YVDFREYER**	**591 – 599**	**VE_FLT3-ITD**	**HLA-A*03:01**	
FL_FLT3-ITD$_{597-606}$ /A2	**YESDNEYFYV**	**597 – 606**	**FL_FLT3-ITD**	**HLA-A*02:01**	
IN_FLT3-ITD$_{610-619}$ /B35	**CPSDNEYFYV**	**610 – 619**	**IN_FLT3-ITD**	**HLA-B*35:01**	
IN_FLT3-ITD$_{610-618}$ /B35	CPSDNEYFY	610 – 618		HLA-B*35:01	Prof. Dr. S. Stevanović Tübingen, Germany

Table 3.11.1: Peptides.
The table shows all the peptides used herein. Peptides in bold were predicted for this project using computer-based epitope prediction; the others peptides were already available in the lab.

3.12 Glass and plastic equipment

Beaker	Schott, Mainz
Duran glass flasks	Schott, Mainz
Cell counting chamber (Fuchs-Rosenthal)	Schreck, Hofheim
Cell strainer	Becton Dickinson, Heidelberg
Centrifugation tube 15 ml, 50 ml	Greiner, Frickenhausen
Centrifugation tube Leucosep ®	Greiner, Frickenhausen
Cryobox system 100	VWR international, Darmstadt
Cryobox nalgene	VWR international, Darmstadt
Cryotubes	NUNC, Wiesbaden

FACS tubes 5 ml, 75x12 mm	Sarstedt
Filter 0.45 and 0.22 µm	Schleicher
Cell culture 96-flat, -U or V bottom plates;	Greiner, Frickenhausen; Costar, USA
Cell and tissue culture 48-, 24-, 6-well plates	Greiner, Frickenhausen; Costar, USA
MACS cell suspension filter	Miltenyi Biotec, Bergisch Gladbach
MACS MS+ separation columns	Miltenyi Biotec, Bergisch Gladbach
Multiscreen ELISPOT plates MSIP	Millipore, Werner, Bergisch Gladbach
PCR tubes 0.2 ml	MBP, San Diego, CA
Petri dishes (9 cm, 13 cm)	Greiner, Frickenhausen
Pipette tips (0.5-10 µl, 1-200 µl, 200-1000 µl)	
TipOne Pipette Tips, Graduated Filter Tips,	Starlab GmbH Ahrensburg,
Pipette tips 1, 2, 5, 10, 25, 50ml	Costar, USA; Greiner, Frickenhausen
Reaction tubes (1.5 ml)	Eppendorf, Hamburg
Syringes10 ml, 20ml (DISCARDIT™II,	Becton Dickinson, Heidelberg
Sterile-cup filter units 0.22 µm /0.45 µm	Millipore, Werner, Bergisch Gladbach
Tissue culture units 30, 80, 175 cm²	Greiner, Frickenhausen

3.13 Laboratory instruments

Instrument	**Description**	**Company and location**
Analytical balance	Precisa	PAG Oerlikan AG, Zürich
Aluminium foil	0.1mm	Carl Roth, Karlsurhe
Bacteria incubator	Heraeus B6200	Heraeus, Hanau
Balance	M3P Sartorius	Sartorius, Göttingen
Centrifuges	Heraeus B6200 Galaxy Mini 5417, 5415 R	Heraeus, Hanau Merck, Darmstadt Eppendorf, Hamburg
Cell culture tube recks	-	VWR, Darmstadt
Centrifuge	Megafuge 3.0 R	Heraeus, Hanau
Experimental gloves	Small and middle	Semperet, Wien
Hettich	Rotixa BP	Labotec, Wiesbaden
Cuvette for photometry	Ultrospec Type 4000 8454	Pharmacia Biotech, Freiburg
Electroporation equipment	Gene Pulser® II	Gene Pulser, BioRad, Munich
Electrophoresis chamber	Easy-Cast™ Minigel Systems	AGS, Heidelberg
Electrophoresis power supply	Gene power supply GPS 200/400	Pharmacia, Freiburg
Electrophoresis Power Pac	Pac 300	BioRad, München
ELISPOT image analyser	Axio-Imager M1 microscope; Axio-Cam MR camera; KS ELISPOT software 4.8/4.9	Carl Zeiss, Stuttgart Carl Zeiss, Jena

Flow cytometry Instrument FACS analysis software	FACS Canto and FACS Aria; FlowJo v7.6.5, 32-bits	Becton Dickinson, Heidelberg Becton Dickinson, Heidelberg
Cell Culture Incubator	CB210, CO2 aerated	Binder, Tuttlingen
Incubator for bacteria	Biometra OV 5	Biometra, Göttingen
Magnet for cell separation	MPC magnetic holder; MiniMACS	Miltenyi, Bergisch Gladbach
Magnetic stirrer with heating plate	IKAMAG, REC-G	Janke & Hunkel, Staufen Staufen
Microwave oven	MWS 2819	Bauknecht, neunkirchen
N2 bank	XLC 1370 and MVE 1400 series	MVE Europe, Solingen
N2 Tank	Taylor, Wharton -180	Tec Lab Königstein,
PCR thermocycler	Trio-Thermoblock, T1 Thermocycler; T Gradient	Biometra, Göttingen
Phase contrast microscope	Axiovert 25 C	Zeiss, Jena
pH-meter	Digital CG 837	Schott, Mainz
Photometer	Ultrospec 3000 UV/Visible	Pharmacia Biotech, Freiburg
Pipettes (adjustable pipetman)	0,5-10 µl	Eppendorf, Hamburg
	P20, P100, P200, P1000	Gilson, Bad Camberg
Multi-channel pipettes	m100 and m300	BIOHIT, Helsinki, Finland
Pipetting aid	ACCU-JET® PIPETTE AID, BRAND	VWR international, Darmstadt
Laminar flow clean bench	Herasafe K518	Kendro, Langenselbold
Shaker for bacteria	Certomat® R/H (shaker/box)	Braun Biotech, Melsungen
Thermo-mixer	Thermomixer Comfort	Eppendorf, Hamburg
Deionisated water installation	Milli-Q plus	Millipore, Eschborn
Water bath	Type 1013	GFL, Cambridge, England
Drying hood	UT 6420	Heraeus, Hanau
Vortexer	VF2	Janke und Kunkel, Staufen
UV illuminator	BioDoc Analyze Transilluminator	Biometra, Göttingen
Gel analysis software	BioDocAnalyse	Biometra, Göttingen
Hemacytometer	Fuchs-Rosenthal	Marienfeld, Lauda-Königshofen
Ice generator	UBE50/35	Ziegra, Isernhagen
Microscope	Model TMS	Nikon, Japan
Refrigerator, freezer	Bosch Economic Ökosuper, Sikafrost comfort Thermo scientific	Robert Bosch GmbH, Stuttgart; Liebherr, Ochsenhausen; Siemens, Berlin
Autoclave Steriliser	KSG sterilisator	KSG, Olching

Table 3.13.1: Laboratory instruments.

4. Methods

4.1 Cell culture

To prevent bacterial and fungal contaminations, cells were manipulated under a laminar flow sterile bench, taking standard precautions to maintain a sterile environment (Freshney, 2000). COS-7 and HEK 293T cells were used as host cells for transient transfections with plasmids encoding genes of interest, e.g. to serve as antigen-presenting cells for functional assays (e.g. IFN-γ ELISPOT assay). COS-7 and 293T cells were cultivated at 37°C with 5% CO_2 in RPMI +10% Fetal Calf Serum + 1% Penicillin-Streptomycin (RPMI Complete Medium). COS-7 cells and 293T cells grow as plastic adherent monolayer cell cultures. They were splited twice in a week either by trypsin/EDTA treatment at 37°C for 5 minutes (COS-7) or by gently taping and shaking the flask (293T cells). After a washing step, cells were seeded again in 25 ml fresh medium. Suspension cells like LCL (lymphoblastoid cell line) cells were also maintained in RPMI complete medium and they were subcultured every 3-4 days at a cell density of $2.0x10^5$ cells/ml. Medium was replaced every 2-3 weeks. Centrifugation steps were always performed at 1500 rpm for 7 minutes at 20°C, if not, otherwise specified. For adherent cells medium was replaced with fresh medium one day before use in functional assays.

4.2 Isolation of PBMCs from whole blood or buffy coats by density gradient centrifugation

Peripheral Blood Mononuclear Cells (PBMCs) are the single nucleated cell populations circulating in the blood (mainly comprising the lymphocyte and monocyte population). Donors' and patient's blood was obtained as either concentrated "Buffy coats" from the local blood bank or whole blood from University Medical Center, Mainz. PBMCs were isolated by centrifugation on a Ficoll-Hypaque gradient. 15 ml FICOLL were given onto the porous filter disc of a 50-ml Leucosep® centrifugation tube, and centrifuged at 1,500 rpm, room temperature (RT) for 1 min. Heparinised blood was diluted in a 1:2 ratio with PBS and given in portions of 35 ml per tube onto the filter. Cells were centrifuged for 15 min at 2,500 rpm at RT without a break. To harvest the mononuclear cells, the leukocyte ring was collected in a fresh 50-ml centrifugation tube and washed twice with PBS. Centrifugations were performed for 10 min at 1,800 rpm and another 7 min at 1,500 rpm to remove platelets. If the pellet still appeared too red after the washing steps, indicating too many red blood cells, erythrocytes were lysed by incubation with 5 ml of erythrocytes lysis buffer for 5 min on ice and the cells were washed again. The resulting PBMCs were either cryopreserved in aliquots in FCS + 10% DMSO or directly applied to cell culture.

4.3 Immunomagnetic cell separation of $CD8^+$ or $CD14^+$ cells from PBMCs

For immunomagnetic cell separation, the MACS® technology was used. The method is based on the use of antibody-coated magnetic MACS microbeads binding to cell surface proteins of the cell types of interest which are withheld when passing through MACS columns placed in the magnetic field of MACS separators. Cells could be purified in less than 30 minutes. Freshly isolated PBMCs were used for the isolation of either $CD8^+$ cells or $CD14^+$ cells, to maximize the yield. In case of cryopreserved PBMCs, cell pellets were pretreated with 1 mg/ml DNase I (QIAGEN) for 5 minutes at RT, a procedure which efficiently eliminates cell clump formation caused by DNA released by dead cells. Positive selection of $CD8^+$ or $CD14^+$ cells was performed following the manufacturer's instructions. In brief, PBMCs were washed with MACS buffer and then magnetically labelled with 15 µl microbeads per $1.0x10^7$ cells for 15 min at 4°C. After incubation, 1.5 ml MACS buffer was added to the cell suspension, gently mixed and 500 µl aliquots applied slowly to the pre-equilibrated separation column. After separation, the column was washed three times with 500 µl MACS buffer and removed from the magnetic separator. During the whole process unlabeled cells passed through and could be collected for further purposes while the labeled cells were first withheld, then collected, washed and processed as required. MACS microbeads are super-paramagnetic particles of approximately 50 nanometers in diameter. Due to the particles' biodegradable matrix composed of iron oxide and polysaccharide, they usually disappear within a few days in culture.

4.4 Generation of FastDCs from PBMCs

Dendritic cells (DCs) are the most potent professional APC for inducing primary T-cell responses. Besides "traditional" protocols for generating monocyte-derived DCs, many studies have also described the use of mature DCs generated from monocytes in only 48 hours ("FastDCs") (Dauer *et al.,* 2003a; Obermaier *et al.,* 2003). Dendritic cells were generated as described by Dauer *et al.*, (2003 and 2005). FastDCs were generated from $CD14^+$ cells of PBMCs (in the case of patient FL) or from adherent PBMCs (in case of healthy donors or umbilical cord blood donations (UCB donations). In brief, for the generation of FastDCs either freshly FICOLL-separated PBMCs or cryopreserved PBMCs were used. The PBMCs were washed and adjusted to a density of $5.0x10^6$/ml in RPMI medium. The cell suspension was split into portions of 3 ml per well in 6-well-plates and incubated for 1 hour at 37°C. Later, the non-adherent cells were removed by gently rinsing the wells 3 times with prewarmed PBS. The plastic-adherent cells were cultivated for one day in medium B, supplemented with rhGM-CSF (1000 U/ml) and rhIL-4 (500 U/ml). On d2, rhIL-6, rhTNF-α, PGE-2 and rhIL-1β were added at concentrations of 1000 U/ml, 10 ng/ml, 1 µg/ml and 10 ng/ml, respectively. Cells were harvested as mature FastDCs on d3 after washing the wells with PBS. Afterwards, FastDCs were either directly used for electroporation/transfection with antigen-encoding IVT-mRNA or cryopreserved in aliquots. FastDCs generated according to this protocol showed, similar maturation markers as

classical DCs, down-regulation of CD14 and up-regulation of HLA-DR, CD11c, CD80, CD86 and CD83 (**Figure 4.4.1**). The yield of FastDCs obtained with this method was higher than with the classical method (Kvistborg *et al.,* 2009). At maximum, FastDCs yield reached up to 5% of starting PBMCs number.

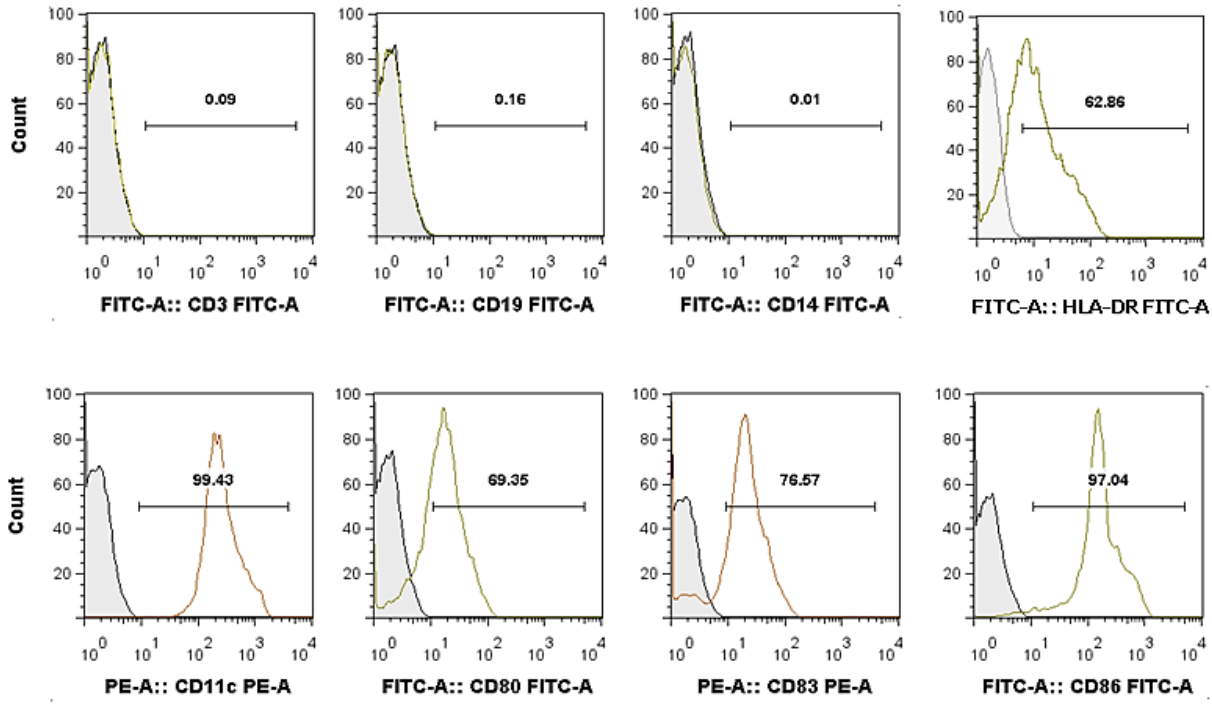


Figure 4.4.1: Phenotypic analysis of UCB-FastDCs.
FastDCs were generated from adherent cells (monocytes) of MZUCB #15 according to Dauer *et al.,* (2003). FastDCs were harvested on d3 and after washing; they were stained with FITC- or PE-labeled antibodies directed against surface molecules. FastDCs were measured by flow cytometry, for expression of CD3, CD19, CD14, HLA-DR, CD11c, CD80, CD83 and CD86, for maturation and purity of FastDCs. Isotype control IgG1-stained populations in grey.

4.5 Cryopreservation and thawing of cells

For cryopreservation, cells were centrifuged; pellet was resuspended in 1 ml freezing medium [10% DMSO either with Human Serum (HS) or with Fetal Calf Serum (FCS)], and transferred into cryotubes. For cryopreservation, cryoboxes filled with isopropanol, were used to guarantee a linear freezing rate of approximately 1°C per minute. After 24 to 48 hours, aliquots were transferred into a liquid nitrogen (N_2) bank for long term storage Thawing was performed by incubating the tubes in a 37°C water-bath until defrosting of the cells was visible. Cells were then immediately transferred into 10 ml of the respective medium, and washed twice before use.

4.6 Flow cytometry analysis

Expression of certain cell surface molecules was analyzed by flow cytometry analysis. Cells of interest were distributed into polypropylene tubes (BD Biosciences) at 1.0-2.0 x10^5 cells/tube and washed with FACS buffer. Centrifugation steps were always performed at 1500 rpm for 7

minutes at 20°C. For surface staining, cells were incubated for 20 min at 4°C with the respective fluorescence-labeled or unlabeled primary antibodies according to the manufacturer's instructions. Cells were also labeled with corresponding isotype-matched control antibodies as negative controls. Afterwards, cells were washed twice to get rid of unbound antibody. If the primary antibody was unconjugated, staining with a second FITC-conjugated goat anti-mouse (GAM) or goat anti-rabbit (H+L) IgG-F(ab)$_2$-antibody was performed under the same conditions. This second antibody binds to the anti-mouse-Fab-fragment of the primary antibody. Cells were directly analyzed using the BD FACS Canto II system if the cells had to be alive during analysis e.g. for single cell sorting using the BD FACS Aria cell sorter or for cell enrichment assay using magnetic microbeads. Otherwise, surface-stained cells were fixed with 0.3 to 0.5 ml PBS/1% para-formaldehyde solution (FACS-fixing buffer) and analyzed within 24h. Fluorescence measurement was performed in the FITC or PE channel. The relative fluorescence of labeled cells to background fluorescence was determined and depicted in a histogram using FlowJo version 7.5.5 software.

4.7 Electroporation of APCs with IVT-mRNA

It has been previously demonstrated that transfection of mRNA by electroporation is a safe, highly efficient and clinically applicable method for antigen expression (Van Tendeloo *et al.,* 2001; Van Tendeloo *et al.,* 2007; Van Driessche *et al.,* 2009). Several studies have previously reported on the electroporation of PBMC with mRNA (Teufel *et al.,* 2005; Johnson *et al.,* 2006; Van Camp *et al.,* 2010) to stimulate and to monitor tumor-specific and CMV-specific T cells. Antigen-presenting cells ($CD8^-$ PBMCs, monocytes or FastDCs) were washed with OPTIMEM and adjusted to a cell density of $2.0x10^6$ cells/100 µl. 100-400 µl of these cell suspensions were transferred into 0.4 cm-gap electroporation cuvette and placed on ice for 1 minute. 15 µg IVT-mRNA/10^6 cells were added and mixed well. Cells were immediately pulsed with the Gene Pulser II (BioRad) using an optimized program that applied 350 V and 300 µF square wave condition. After electroporation, cells were placed on ice for a minute. Electroporated cells were then washed twice and transferred into prewarmed medium until the use as APCs for stimulating antigen-specific $CD8^+$ T cells. Washing steps were always performed by centrifugation at 1500 rpm for 7 minutes at 20°C.

4.8 Transfection of FastDCs with IVT-mRNA

Transfection of FastDCs with IVT-mRNA was performed with the TransMessenger™ Transfection reagent (QIAGEN) using the manufacturer's guidelines. For each new batch of TransMessenger™ transfection reagent, the transfection conditions had to be tested to find out the optimal amount of IVT-mRNA (between 200 to 800 ng) and the best RNA:TransMessenger™ ratio (between 1:2 to 1:8) to be applied according to the

manufacturer's instructions. For optimization purposes, combinations of these conditions were applied to the transfection of FastDCs using *EGFP*-encoding IVT-mRNA as a marker. For most TransMessenger batches 0.8 µg IVT-mRNA and a 1:8 (w:v) ratio of RNA:TransMessenger™ gave the best results and these conditions were used for transfections. For the formation of transfection complexes, 100 µl ECR buffer was mixed with 1.6 µl enhancer-R and 0.8 µg IVT-mRNA was added; the mixture was incubated 5 minutes at room temperature to enable compaction of RNA. 6.4 µl TransMessenger™ reagent was added and the transfection solution was then incubated for 20 minutes at RT to enable the formation of transfection complexes. These cells were then diluted 1:2 in FastDC-medium. In parallel, $2.0x10^5$ FastDCs per transfection reaction were spin down in 15 ml plastic tube by centrifugation. Supernatants were completely discarded and the cell pellets were gently resuspended in transfection complexes. Cells were incubated for 3 hours at 37°C to enable the RNA transfection process. Following the transfection, FastDCs were centrifuged and transfection complexes were removed carefully. Transfected FastDCs were directly used as APCs for stimulating antigen-specific $CD8^+$ T cells. Centrifugation steps were always performed at 1500 rpm for 7 minutes at 20°C.

4.9 *In vitro* stimulation (IVS) of $CD8^+$ T cells with IVT-mRNA electroporated $CD8^-$ PBMCs or monocytes

For *in vitro* stimulation of $CD8^+$ T cells, either $CD8^-$ PBMCs or monocytes were used as antigen-presenting cells (APCs). $CD8^+$ T cells were isolated and purified as described in **Method section 4.3**. Purified T cells were washed and resuspended in T-cell medium. The culture medium was AIM-V supplemented with 5% HS, IL-2 (20 U/ml), IL-7 (5 ng/ml) and IL-4 (10 ng/ml) (AIM-V^{stim}). $CD8^+$ T cells were stimulated with IVT-mRNA electroporated APCs in two different culture formats, "bulk"-stimulation (see **Method section 4.9.1**) or "micro"-stimulation in the 96-well microculture format (see **Method section 4.9.2**). APCs were irradiated with 100Gy and electroporated with respective antigen-mRNAs as described in **Method section 4.7**. After washing, APCs were counted and added to the T cells at a target-to-lymphocyte ratio of 1:1. Restimulations were performed in 7 day intervals by replacing 100 µl supernatant from each well with equal volumes of fresh medium containing the same cytokine cocktail and stimulator cells. IVS-responder populations were tested by IFN-γ ELISPOT assays.

4.9.1 *In vitro* stimulation – "Bulk culture"

In the first attempt to detect anti-FLT3-ITD responses in healthy donors, isolated $CD8^+$ T cells were seeded in 24-well plate (10^6/well) in AIM-V^{stim}. T cells were stimulated at a 1:1 ratio with autologous $CD8^-$ PBMCs electroporated with *Sig-wtFLT3*-, *Sig-FLT3-ITD*- and *Sig-pp65*- IVT-mRNA, respectively. All responder T cells were restimulated on d7 under the same conditions and tested on day 12 in an IFN-γ ELISPOT assay.

4.9.2 *In vitro* stimulation – ''96-well Microcultures"

In a second attempt, stimulations of $CD8^+$ cells in a 96-well microculture format were performed. Isolated $CD8^+$ T cells were seeded in 96-well U-bottom plates (10^5/well) in AIM-V^{stim} and stimulated at a 1:1 ratio with autologous $CD8^-$ PBMCs electroporated with IVT- *Sig-FLT3-ITD* and *Sig-pp65* IVT-mRNA, respectively. As negative control, $CD8^+$ T cells were stimulated with mock-$CD8^-$ PBMCs (without IVT-mRNA). Responder T cells of each microculture were restimulated on d7 under the same culture conditions and tested on d12 in an IFN-γ ELISPOT assay. Only FLT3-ITD-reactive microcultures were restimulated two more times in similar culture conditions except that the IL-2 concentration was changed (100 U/ml rather than 20 U/ml). FLT3-ITD-reactive microcultures were restimulated using either $CD8^-$ PBMCs or monocytes as APCs depending on the availability of donors' material. IVS-responders were retested on d26 or later time point to confirm the FLT3-ITD reactivity and also to detect the HLA alleles presenting FLT3-ITD peptides recognized by the T cells. *In vitro* stimulation of UCB-$CD8^+$ T cells was also performed under similar culture conditions except for changes applied to the cytokine cocktail which are specified in the **Method section 4.10** or in **Result section**. Using this approach, either $9.6x10^6$ $CD8^+$ T cells (per healthy individual- 96 wells) or $1.2x10^6$ $CD8^+$ T cells (per umbilical cord blood donation- 12 wells) were analyzed for FLT3-ITD-reactivity.

4.10 *In vitro* stimulation of $CD8^+$ T cells with IVT-mRNA transfected FastDCs

In experiments testing umbilical cord blood, magnetic microbead-isolated $CD8^+$ T cells were seeded in 96-well U-bottom plate (10^5/well) in AIM-V/10% HS supplemented with 1 ng/ml IL-12, 5 ng/ml IL-7 and 5 ng/ml IL-15. Antigen IVT-mRNA was transfected into autologous FastDCs using the TransMessenger™ transfection protocol described in **Method section 4.8**. $CD8^+$ T cells were stimulated in a ratio of 1:10 with autologous FastDCs transfected with Sig-*FLT3-ITD* and Sig-*pp65* IVT-mRNA. Transfected FastDCs were irradiated at 10,000 rad prior to co-culture. Autologous $CD8^-$ non-adherent cells irradiated at 10,000 rad to prevent them from further growth were used as feeder cells at a density of $2.0x10^5$ cells/well. Using this approach, $1.6x10^6$ $CD8^+$ T cells per UCB donation (16 wells) were analyzed against FLT3-ITD. As negative control, $CD8^+$ T cells were stimulated with mock- (without IVT-mRNA) transfected FastDCs. All IVS-responder T cells were restimulated on d7 in a similar condition except IL-12 was replaced by IL-2 (100 IU/ml) and tested on d12 in an IFN-γ ELISPOT assay.

4.11 Ex vivo analysis of FLT3-ITD-reactive T cells in patient FL

$CD8^+$ T lymphocytes of patient **FL** (FL/585-14) were purified from blood PBMCs using $CD8^+$ microbeads. Autologous $CD8^-$ PBMCs or HLA-matched allogeneic FastDCs were used as target cells after they were electroporated with the patients' *FLT3-ITD* IVT-mRNA. These targets were added to a previously coated and blocked ELISPOT plate in 50 µl AIM-V + 5% HS. In an *ex vivo*

analysis of FLT3-ITD-reactivity, the $CD8^+$ T cells were directly applied to a 20-hour IFN-γ ELISPOT assay.

4.12 IFN-γ secretion assay (ISA)

The IFN-γ secretion assay is a reliable tool to study IFN-γ production at the single cell level (Desombere *et al.,* 2004). This technique allows to identify and to isolate IFN-γ secreting T cells in a mixed population of cells by selective enrichment of IFN-γ^+ cells labeled with PE-conjugated anti- IFN-γ antibody using anti-PE magnetic microbeads (Brosterhus *et al.,* 1999). The major advantage to this technique is that live IFN-γ producing T cells can be stained, analyzed and used for further experimentation. The disadvantage of this assay is the potential for cross binding of IFN-γ produced by T cells on neighbor non-secreting cells. By diluting the cells, the cross contamination can be reduced as well as mixing is also required throughout the capture incubation in order to minimize it further. Using this technique one can detect antigen-specific T cells down to frequencies as low as 0.01%. IFN-γ-secreting cells were detected using the IFN-γ secretion assay kit according to the manufacturer's instructions. *Ex vivo* $CD8^+$ T cells were seeded in a 24 well plate ($2.5x10^6$/well) in AIM-V^{stim} and stimulated at a 1:10 ratio with irradiated autologous FastDCs electroporated with **FL_***FLT3-ITD* IVT-mRNA. After 12 hours incubation with or without antigen, $CD8^+$ T cells from each 24-well were transferred to 15 ml tubes and washed with 10 ml cold buffer (PBS, 0.5% BSA, 2 mM EDTA) at1500 rpm, 10 min, 4°C. The cell pellet was suspended in 80 µl cold medium (AIM-V) and 20 µl IFN-γ Catch Reagent (a bispecific MAb directed against CD45 and IFN-γ) were added. After 5 min of incubation at 4°C, 10 ml of prewarmed (37°C) AIM-V medium was added. The cells were placed at 37°C on a slowly rotating platform to allow cytokine secretion for 45 min. The cells were then washed with cold buffer (1500 rpm, 10 min, 4°C) and suspended in 80 µl cold buffer. The secreted IFN-γ, bound to the catch reagent, was stained with 20 µl PE-conjugated IFN-γ-specific antibody (IFN-γ Detection Reagent) and additionally, anti-CD8-FITC antibody was used to stain the IFN-γ-positive cells. After an incubation period of 10 min at 4°C, cells were washed with cold buffer, spin down (1500 rpm, 10 min, 4°C) and resuspended in the FACS-buffer. IFN-γ secreting $CD8^+$ cells were immediately used for single T-cell sorting by the FACS Aria system (BD).

4.13 Cloning of FLT3-ITD-reactive T cells by FACS sorting

Flow cytometry sorted IFN-γ secreting $CD8^+$ cells were seeded at an estimated concentration of one T-cell per well in 96-well V-bottom plates in 100 µl AIM-V supplemented with 10% HS and IL-2 (100 U/ml). Clonal T cells were stimulated non-specifically with OKT3 (30 ng/ml) and irradiated allogeneic, HLA class-I matched, PBMCs ($5.0x10^4$/well) as feeder cells in 100 µl medium as above. Restimulations were performed in 14-day intervals by replacing 100 µl

supernatant per well with stimulator cells in 100 µl of fresh T-cell medium. The rest of the IFN-γ secreting $CD8^+$ T cells were also stimulated together in U-bottom plates in 100 µl medium. Sorted cells were stimulated non-specifically with OKT3 (30 ng/ml) and irradiated allogeneic, HLA class-I matched, PBMCs ($2.5x10^5$/well) as feeder cells in 100 µl medium. T-cell clones and microcultures were tested for FLT3-ITD recognition on d14 and d26 in an IFN-γ ELISPOT assay.

4.14 *In vitro* stimulation of T cells by peptide-loaded FastDCs

It is very well known that peptide-loaded dendritic cells prime and activate MHC-class I-restricted T cells more efficiently than any other cross-presenting DCs (Met *et al.,* 2003). To achieve, the improved priming of naïve T cells and rapid enrichment of FLT3-ITD-specific $CD8^+$ T cells, *in vitro* stimulation of UCB-$CD8^+$ T cells by peptide-loaded FastDCs was performed as follows: on d0, $1.0x10^5$ $CD8^+$ T cells were seeded per well in a 96-well- U-bottom plate in 100 µl AIM-V/10% HS containing 1 ng/ml IL-12, 5 ng/ml IL-7, and 5 ng/ml IL-15. T cells were stimulated with FastDCs pulsed with 5 µg/ml peptides in 10:1 ratio (effector:target). Restimulations were performed in 14-day intervals in a similar condition except IL-12 was replaced by IL-2 (100 IU/ml). Only growing microcultures were further stimulated with FastDCs pulsed with peptides in 10:1 ratio either in 96-well-flat-bottom, in 48-well or in 24-well-plate. Decision of plate selection for further stimulation was based on number of T cells taken for stimulation in 96-well flat-bottom plate at $2.0x10^5$ cells per 200 µl, in 48-well plate at $5.0x10^5$ cells per 1 ml or in 24-well-plate at $1.0x10^6$ cells per 2 ml medium. For restimulation half of the supernatant volume was discarded from each well and replaced with fresh medium containing the cytokine cocktail and peptide at concentrations as on d0. Responder T cells were tested for recognition of the respective antigen on d37 and d52 by IFN- γ ELISPOT assays (see **Method section 4.15**).

4.15 IFN-γ ELISPOT assay

The PVDF membrane of 96 well-Multiscreen ELISPOT plate MSIP S4510 was pre-wetted with 20 µl of 35% ethanol per well, washed three times with PBS and coated over night at 4°C with Mab anti-IFN-γ clone 1-D1K (0.5 µg/50 µl/well). To remove unbound antibodies, the membranes were washed three times with PBS. To block the PVDF membrane for non-specific binding of IFN-γ antibody, 50 µl AIM-V/10% HS medium was added to each well of the ELISPOT plate. After 30 minutes plates were washed with PBS and antigen-presenting cells, expressing FLT3-ITD antigen, were added to the plate. To test FLT3-ITD reactivity, effector T cells were added to ELISPOT plate and incubated for 20 hours at 37°C. By this time IFN-γ released by T cells was captured by the coating antibody. Afterwards, the ELISPOT plates were washed 6 times with PBS/0.05% Tween20 and detection antibody Mab anti- IFN-γ clone 7-B6-1 (0.12 µg/well),

diluted in PBS/0.5% BSA, were added. After 2 hours incubation at 37°C, unbound antibody was removed by washing the ELISPOT plates 3 times with PBS/0.05% Tween20. Avidin-biotin-peroxidase complex was diluted with PBS/0.01% Tween20, interacting with the detection antibody, was added for 1 hour at RT. ELISPOT plates were further washed 6 times and the antibody-peroxidase-complexes were visualized by pipetting 100 µl/well AEC staining solution into each well. The staining reaction was stopped under running tap-water. Spots were evaluated with computer-assisted video image analysis (Herr *et al.,* 1997) with a 3.15x magnification at a resolution of 1388x1040 pixels. Image acquisition was performed with an AxioCam MRC camera attached to a Zeiss Axio Imager M1 microscope via a 0.63x adapter (KS ELISPOT version 4.8, Zeiss, Jena/Germany). A response was defined as positive when the mean number of spots formed by T cells against individual FLT3-ITDs was at least 2-fold higher than the background reactivity or higher than the mean background plus two-fold standard deviations.

To test anti-FLT3-ITD response in autologous systems either $CD8^-$ PBMCs (4.0-6.0x10^4/well), monocytes (1.0-2.0x10^4/well), or FastDCs (5.0-10.0x10^3/well) were used as antigen-presenting cells (APCs). To asses HLA restriction element, responsible for FLT3-ITD or control antigen presentation, either COS-7 cells or HEK 293T cells (2.0x10^4/well), were co-transfected with plasmids-encoding HLA-I alleles and individual FLT3-ITDs or control antigens, and were used as APCs. Autologous APCs were transfected with IVT-mRNA either by electroporation or by TransMessenger transfection (see **Method section 4.7** and **4.8**). COS-7 cells or HEK 293T cells were transfected with Lipofectamin™ 2000 for antigen presentation. The APCs were washed and resuspended either in AIM-V/10% HS or in RPMI + 10% FCS and given into ELISPOT plate as indicated. Responder T cells (cell number/well specified in the result section) were added, either 3 hours after electroporation/Transmessenger™ or 24 hours after Lipofection™ 2000 transfection.

4.16 *In vitro* transcription and polyadenylation of RNA

In vitro transcription of antigen-encoding sequence was performed from recombinant plasmids carrying the antigens of interest using the T7 Polymerase (either RiboMAX™ Large Scale RNA Production T7 System from promega or mMESSAGE mMACHINE® T7 Ultra Kit from Applied Biosystems). To protect the IVT-mRNA from rapid extra- and intracellular degradation the IVT-mRNA was polyadenylated aiming at a synthesis of poly-A tails of about 100 to 200 rATPs (Yeast Poly(A) polymerase from USB, Cleveland, USA or mMESSAGE mMACHINE® T7 Ultra Kit from Applied Biosystems). The transcription start was enabled by the presence of the T7 promoter at 5' of the insert on all the plasmids used. For *in vitro* transcription, plasmids were linearized using restriction enzymes (Britten *et al.,* 2005) and complete digestion was verified by

gel electrophoresis. The digested plasmids were purified resuspended in RNase-free DEPC-H_2O and stored at -20°C.

4.16.1 IVT-mRNA preparation using the RiboMAX™ T7 System from promega

To generate IVT-mRNA following components were incubated for 4 hours at 37°C within a total volume of 100 µl.

Linearized template DNA (5 µg)	xx µl
T7 buffer (5x)	20 µl
rATP (100 mM)	7.5 µl
rCTP (100 mM)	7.5 µl
rUTP (100 mM)	7.5 µl
rGTP (100 mM)	1.5 µl
m^7G(5')ppp(5')G RNA capping analog (100 mM)	4.0 µl
T7 enzyme mix (Polymerase, phosphatase, RNase-Inhibitor)	10 µl
RNase free H_2O add up to 100 µl	xx µl

After the incubation period, phenol/chloroform/isoamylalcohol extraction was performed to purify IVT-mRNA as described below. The quality of the IVT-mRNA was checked in a denaturing RNA gel. Purified IVT-mRNA was stored at -80°C in aliquots of 150 µg and 300 µg IVT-mRNA. Polyadenylation of IVT-RNA was performed using following components in a total volume of 100 µl and incubated for 2 hours at 37°C.

IVT-mRNA (150 µg)	15 µl
Poly (A) polymerase buffer (5x)	20 µl
rATP (10 mM)	xx µl (according to IVT-RNA length)
Poly (A) polymerase (600 IU/µl)	xx µl (according to IVT-RNA length)
RNase free H_2O add up to 100 µl	xx µl

The rATP need was calculated to generate a poly(A) tail of approximately 120 As.

rATP (pmol) = nRNA mol (pmol)x120

1 IU of Poly(A) polymerase is the quantity of enzyme adding 1 A to one substrate molecule in 1 minute. Quantity of Poly(A) polymerase necessary to add 120 As to the RNA substrate was calculated as follows:

Poly(A) (IU) = [nRNA mol (pmol)x120]/incubation time (120 min).

The reaction was stopped, by phenol/chloroform/isoamylalcohol extraction and purified as described below in **Method section 4.16.3**. The quality of the IVT-mRNA as well as the success of polyadenylation was checked against the non-polyadenylated IVT-mRNA on a denaturing RNA gel described in **Method section 4.27.2**.

4.16.2 IVT-mRNA preparation using the mMESSAGE mMACHINE® T7 Ultra Kit

Ambion mMESSAGE mMACHINE® T7 Ultra Kit is designed for the efficient *in vitro* synthesis of large amounts of efficiently and correctly capped RNA. This kit includes a cap analog called Anti-Reverse Cap Analog (ARCA) (Stepinski *et al.,* 2001; Peng *et al.,* 2002). Inside ARCA, one of the 3' OH groups (closer to 7MG) is eliminated from the cap analog and is substituted with –OCH3. This modification allows T7 RNA polymerase to initiate transcription only with the remaining –OH group and thus synthesis of RNAs capped exclusively in the correct orientation with 100% functionality. To generate polyadenylated IVT-mRNA, following protocol steps were followed.

Amount	Component
10 µl	T7 2X NTP/ARCA
2 µl	10x T7 Reaction buffer
1 µg	Linear template DNA
2 µl	T7 Enzyme mix
Up to 20 µl	Nuclease-free Water

After 2 hours of incubation, 1 µl TURBO DNase was added and further incubated for 15 min at 37°C. Polyadenylation was performed in a total volume of 100 µl.

Amount	Component
21 µl	IVT-RNA reaction
20 µl	5x EPAP buffer
10 µl	25 mM $MnCl_2$
10 µl	ATP solution
37.5 µl	Nuclease-free Water

2.5 µl of this mixture was saved to be run on a gel next to the poly A-tailed IVT-mRNA. 4 µl *E*-PAP enzyme was added to the reaction and incubated for 45 minutes at 37°C.

The Polyadenylation reaction was stopped by adding 10 µl Ammonium Acetate stop solution. Phenol/chloroform/isoamylalcohol extraction was performed to purify the RNA as described below in **Method section 4.16.3**. The quality of the IVT-mRNA as well as the success of polyadenylation was checked against the non-polyadenylated IVT-mRNA on a denaturing RNA gel described in **Method section 4.27.2**. Due to the polyA-tail the IVT-mRNA migrated slower in the gel (**Figure 4.16.2.1**). The purified polyadenylated IVT-mRNA was stored at -80°C in aliquots.

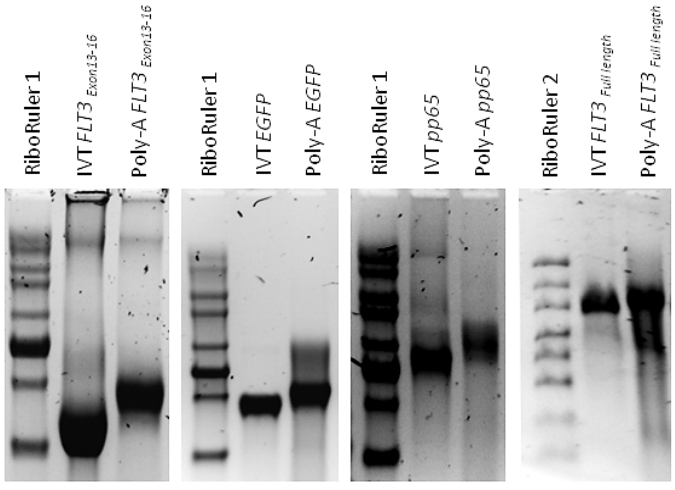


Figure 4.16.2.1: Polyadenylation of In vitro transcribed RNA.
Polyadenylation of IVT-mRNA induces a clear shift in length as seen on RNA gels. Quality and length of enzymatically polyadenylated IVT-mRNAs of FLT3-ITD $_{Exon13-16}$ (~500 bp), EGFP (900 bp), pp65 (1700 bp) and FLT3 $_{Full\ length}$ (~3000 bp) were controlled on a 1% denaturing RNA gel. The amounts of IVT-mRNAs and polyadenylated IVT-mRNAs loaded on the gels were 5 µg. RiboRuler 1: 0.5-10 Kb RNA Ladder, Invitrogen (2 µg) and RiboRuler 2: 0.2-6.0 Kb RNA Ladder, Fermentas (1 µg).

4.16.3 IVT-mRNA extraction and purification

Phenol/Chloroform/Isoamylalcohol (25:1:24) was added to the RNA sample in a 1:1 (v/v) ratio and vortexed. After centrifugation at 15,000 rpm for 3 minutes, the upper aqueous phase was transferred into a clean eppendorf tube. An equal volume of Chloroform/Isoamylalcohol (1:24) was added, the sample vortexed and centrifuged for 3 minutes at 15,000 rpm. The upper phase was again transferred into a new tube and shortly centrifuged at 15,000 rpm. Residual Chloroform/Isoamylalcohol was removed. The IVT-mRNA was precipitated overnight with 0.1 volume of 3M sodium acetate and 2.5 volume ethanol at -20°C. The sample was centrifuged for 30 minutes at 15,000 rpm and washed twice by centrifugation for 10 minutes at 15,000 rpm with 70% ethanol. The pellet was air-dried in the Laminar flow workbench and resuspended in 50 to 100 µl DEPC-H_2O. Concentration of IVT-mRNA was determined by spectrophotometer and the quality of IVT-mRNA was checked in denaturing RNA gels (see in **Method section 4.27.2**).

4.17 Extraction of total RNA

Isolation of total RNA from PBMCs or leukemia cells was performed using the QIAGEN RNeasy Mini kit according to the manufacturer's instructions. Total RNA was extracted from 5.0-10.0x10^6 cells per sample. The total RNA was eluted with RNase-free H_2O (30 µl per sample). Concentration was determined by spectrophotometer using a 1:20 dilution in water, and 2 µg was used for the quality check on an RNA gel. Total RNA was stored at -80°C until reverse transcription.

4.18 Reverse transcription (RT) for cDNA synthesis

In contrast to double stranded DNA, single-stranded RNA cannot be cloned into a plasmid. To transfer the genetic information encoded by RNA into dsDNA, RNA was transcribed into cDNA by reverse transcription PCR. RT-PCR was performed using the "SuperScript III First-Strand Synthesis System" (from Invitrogen) according to the manufacturer's instructions. The resulting cDNA was directly used for PCR or stored at -20°C.

4.19 Polymerase chain reaction (PCR)

Patient's FLT3-receptor gene/or internal tandem duplicated FLT3-receptor gene and Umbilical cord blood donors' HLA class I types were determined by specific amplification of the gene of interest from cDNA using polymerase chain reaction. PCR was performed to get the double-stranded DNA sequences from cDNA sequence. PCR reactions were carried out in a final volume of 50 µl in 0.2ml tubes. The following table shows the general composition of a PCR reaction:

10x reaction buffer	5 µl
dNTP Mix (10 mM)	1 µl
5' Primer -sense (5 µM)	2 µl
3' Primer -antisense (5 µM)	2 µl
Easy A polymerase (5U/µl)	0.5 µl
Template (dsDNA or cDNA)	1 µl
Nuclease free H_2O up to	50 µl

In parallel, a negative control with 1 µl PCR H_20 instead of the diluted DNA template was run. The PCR amplification was carried out over 30 cycles of steps 2-4 according to the following protocol:

1:	95 ° C,	3	minutes,
2:	95 ° C,	45	seconds,
3:	52-64 ° C,	30-45	seconds,
4:	72 ° C,	1-3	minutes,
5:	72 ° C,	10	minutes and
6:	4 ° C	pause	

The lengths and concentrations of the PCR products were analyzed by gel electrophoresis by applying 5 µl of the PCR products to 1% agarose gel as described in **Method section 4.27.1.**

4.20 Sequencing of DNA

Before sequencing, PCR products were purified using the High Pure PCR Purification Kit, according to the manufacturer's instructions. The purified product was eluted in 30-50 µl elution buffer, according to the concentration appreciated on the electrophoresis gel. Sequencing of DNA was performed using Big Dye Version 3.1 in combination with the BYOS-K sequencing service of Genterprise GENOMICS (Mainz). Samples were run in a total volume of 5 µl consisting of 1 µl of 5x Big Dye version 3.1 buffer, 1 µl Big Dye premix, 1 µl primer (5 pmol/µl) and 50-100 ng PCR product or 200-500 ng plasmid DNA adjusted to 5 µl with H_2O. Sequences were edited with the Chromas version 2.31 software. Analysis of sequences was performed with programs EditSeq 3.9.10 and SeqMan 3.61 (DNASTAR Inc.). For HLA subtype identification, sequence was run against standard HLA databases (IMGT/HLA database- http://www.ebi.ac.uk/imgt/hla/blast.html; and NCBI-BLAST database- http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides).

4.21 Purification of DNA by gel electrophoresis

In case of fragments from digested vector or PCR product from multiple PCR products, e.g. *wt-FLT3* and *FLT3-ITD*, a gel extraction was performed, using the QIAquick Gel Extraction kit. The piece of gel containing the DNA of interest was excised under weak UV light and gel extraction

was performed according to the manufacturer's instructions. The purified DNA was either directly used for further purposes, e.g. Ligation and TA cloning, or stored at -20°C.

4.22 Ligation of PCR products into expression vectors

The pcDNA3.1/V5-His-TOPO® TA expression kit was used which offers one-step cloning of *Taq*-amplified PCR products into a high-level expression vector. Topoisomerase activation of this vector allows PCR products to be ligated in short time and results in 90% recombinants. 1 µl vector, 1 µl 5x salt solution and 3 µl PCR product were incubated for 20 minutes at RT. The reaction mix was then ready for transformation into bacteria. For directional ligation, the PCR products to be cloned were amplified with primers containing restriction sites (**Table. 3.5.1**). PCR products were digested with the same restriction enzymes as the vector pcDNA3.1/V5-His. PCR products were purified with the High Pure PCR product purification kit according to the manufacturer's instruction. The digested vector was purified by gel electrophoresis and subsequent gel extraction (see **Method section 4.21**). Afterwards, the digested vector was dephosphorylated with 40 IU alkaline phosphatase (AP) for 2 hours at 37°C in a total volume of 100 µl containing 10 µl 10x dephosphatase buffer (i.e. 1x NEBuffer 3). The digested, dephosphorylated vector was purified with the PCR High Pure kit according to the manufacturer's instruction and then ready to use for cloning by ligation. Ligation was performed overnight at 4°C in a total volume of 10 µl containing 1 µl of 10x T4 DNA ligase buffer, 20 IU T4 DNA ligase and the respective volumes of vector and PCR product to reach a molar vector to insert-ratio of 3:1. The ligation mix was transformed into bacteria (see **Method section 4.23**). Bacteria were grown on $Agar^{Amp+}$ plates.

4.23 Transformation of bacteria

Recombinant plasmids resulting from TOPO TA-cloning reactions or ligations were transformed into bacteria to isolate positive clones. For transformation, the chemically-competent TOP10 E. coli were thawed on ice. For the transformation reaction, 2 µl cloning reaction was added to 50 µl bacteria and gently mixed. After incubation on ice for 30 minutes, cells were heat-shocked for 30 seconds at 42°C in a water bath. Following a short incubation on ice and addition of 250 µl SOC medium (warmed up to RT) the bacteria were shaken for 1 hour at 37°C. The transformed bacteria were plated on $Agar_{Amp+}$ plates in two volumes (20 µl and 200 µl) and colonies were enabled to grow overnight at 37°C. Colonies were picked the next day and transferred into LB medium.

4.24 Control of recombinant clones for the orientation of inserts by PCR

Five to ten Amp-selected single colonies were picked and transferred into 200 µl LB medium supplemented with ampicillin (100 µl/ml), and allowed to grow for 3 hours in a thermomixer at

37°C and 1200 rpm rotation. PCR to assess the orientation of inserts was performed using the insert-specific forward primer (sense), binding to the insert and BGH reverse primer (antisense), binding to the BGH polyadenylation sequence of the pcDNA3.1/TOPO TA cloning vector. Per sample 30 µl of bacterial suspension were centrifuged in 0.2 ml PCR tubes, resuspended in 8 µl H_2O and denatured at 99.9°C for 5 minutes. Per sample 12 µl master mix, containing 10 µl Perfect Taq Plus (5 PRIME GmbH, Hamburg), 1 µl sense primer (5 µM) and 1 µl antisense primer (5 µM), was added. PCR conditions were: 3 minutes at 95°C followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 56°C and 2 minutes at 72°C. After a final extension for 10 minutes at 72°C, the PCR was terminated at 4°C. From each PCR reaction 5 µl PCR products were analyzed by gel electrophoresis. Plasmids with correctly oriented inserts could be identified by the presence of PCR bands which were absent in plasmids with no or incorrectly oriented inserts. Bacteria carrying the plasmids containing the insert in the correct orientation were stored as glycerol stocks.

4.25 Plasmid isolation

Single colonies picked from transformed bacteria were cultured in either 5 ml or 200 ml LB medium supplemented with ampicillin. Depending on the culture volume plasmid DNA was isolated using the QIAprep Spin Miniprep kit or the HiSpeed Maxiprep kit, according to the manufacturer's instructions. In both cases DNA was eluted with "Elution Buffer" (or nuclease free water) and the concentration was determined by spectrophotometer. Plasmids were submitted for sequencing with 500-700 ng for each reaction, as described before (**Method section 4.20**). The DNA was stored at -20°C until further use.

4.26 Cloning of IN_FLT3-ITD cDNA fragments to find out the immunogenic peptide

In the model system of AML patient **IN**, HLA-A*32:01-restricted T cells recognized the autologous FLT3-ITD. To narrow down the peptide-coding region of the **IN**_*FLT3-ITD*$_{\text{Exon 13-16}}$ - cDNA recognized by the #1853/1H4 IVS-microculture, cDNA-fragmentation was performed by PCR from the C -terminal end of the **IN**_*FLT3-ITD*$_{\text{Exon 13-16}}$ cDNA. A *FLT3-ITD*$_{\text{Exon13}}$ specific sense primer (containing the natural ATG translation start codon) was used together with different antisense primers (see **Materials section 3.6**) which contained a stop codon at the C -terminus for the amplification and cloning of different C-terminally truncated fragments. The protocol for PCR amplifications of the gene fragments were as follows:

Perfect Tag plus 2x master mix	25 µl
5' primer (5 µM) in *FLT3-ITD*$_{\text{Exon13}}$ fragments	2 µl
3' primer (5 µM) in *FLT3-ITD*$_{\text{Exon16}}$ fragments	2 µl
dNTP Mix (10 mM)	1 µl
IN_FLT3-ITD DNA clone #5 (10ng)	1 µl
PCR H_2O add upto 50	19 µl

The amplification was carried out over 25 cycles of steps 2-4 according to the following protocol:

1:	95 ° C,	3	minutes,	
2:	95 ° C,	45	seconds,	
3:	52-64 ° C,	45	seconds,	
4:	72 ° C,	60	seconds,	
5:	72 ° C,	10	minutes	and
6:	4 ° C	pause		

The PCR products were purified and checked by agarose gel electrophoresis described below. Purified **IN**_*FLT3-ITD*$_{Exon13-16}$ gene fragments were cloned into the expression vector pcDNA3.1/V5-His using a TOPO TA Cloning system from Invitrogen. After transformation, recombinant TOP 10 bacteria were selected on LB agar plates (with 100 mg ampicillin/ml) and incubated overnight in an 37°C incubator. Next day, 8-12 colonies were inoculated in 100 µl LB medium for 1 hour and orientations of inserts were controlled via PCR using the *FLT3-ITD*$_{Exon13}$-specific forward primer and the vector-specific BGH reverse-primer. Clones encoding correctly oriented fragments were inoculated in 5 ml LB/Amp medium for 24 hours. The plasmids were isolated using the Qiagen plasmid miniprep kit. Recombinant clones were further analyzed by restriction digestion using the enzymes *BamHI* (present in *FLT3-ITD*$_{Exon13}$ primer) and *XbaI* (present in pcDNA3.1/V5-His vector) as well as sequencing (see **Method section 4.20**). The cDNA fragments generated from **IN**_*FLT3-ITD*$_{Exon\ 13-16}$ are listed below in **table 4.26.1. IN**_*FLT3-ITD*$_{Exon\ 13-16}$ cDNA fragments were co-transfected with HLA -B*35:01in COS-7 cells and tested for recognition by **IN**_FLT3-ITD-reactive #1853/1H4 responders to identify the precise cDNA fragment encoding the antigenic peptide in an IFN-γ ELISPOT assay as described in **Method section 4.15.**

Gene fragments	Position of fragments in entire FLT3 protein	DNA fragment length
IN_Wt-FLT3 $_{exon\ 13-16}$	554 – 637 (83 aa)	249 bp
IN_FLT3-ITD $_{exon\ 13-16}$	554 – 664 (110 aa)	330 bp
IN_FLT3-ITD $_{91}$	554 - 645 (91 aa)	273 bp
IN_FLT3-ITD $_{55}$	554 – 609 (55 aa)	165 bp
IN_FLT3-ITD $_{56}$	554 – 610 (56 aa)	168 bp
IN_FLT3-ITD $_{57}$	554 – 611 (57 aa)	171 bp
IN_FLT3-ITD $_{59}$	554 – 613 (59 aa)	177 bp
IN_FLT3-ITD $_{60}$	554 – 614 (60 aa)	180 bp
IN_FLT3-ITD $_{62}$	554 – 616 (62 aa)	186 bp
IN_FLT3-ITD $_{63}$	554 – 617 (63 aa)	189 bp
IN_FLT3-ITD $_{64}$	554 – 618 (64 aa)	192 bp
IN_FLT3-ITD $_{65}$	554 – 619 (65 aa)	195 bp

Table 4.26.1: List of FLT3-ITDExon13-16 cDNA fragments generated in the IN patient.
Fragments of FLT3-ITD$_{Exon13-16}$ were generated from clone #5 of **IN**_FLT3-ITD$_{Exon13-16}$. Number mentioned at the end of each fragments name represents the size of fragments in amino acid. Start and end position of each fragment is also mentioned.

4.27 Analysis of nucleic acids by gel electrophoresis

4.27.1 DNA gel electrophoresis

DNA gel electrophoresis was performed to check the qualities of plasmids, linearized DNA templates and PCR products as well as the sizes of inserts after digestion of recombinant

plasmids. Depending on the expected lengths of DNA molecules, gels with different percentages of agarose (w/v) were used as matrix. For 1% agarose solutions, 1.0 g agarose was dissolved in 100 ml 0.5 x TBE buffer by heating in a microwave. The GelRed solution was used as DNA staining dye, which intercalates between DNA bases and enables fluorescent visualization of DNA under UV light. The agarose gel solution was poured into a gel tray to prepare the desired size gel. The gel was placed in an electrophoresis tank previously filled with 0.5xTBE buffer. DNA samples were prepared by mixing 0.25-1 µg DNA with DNA probe buffer (6x, blue dye). DNA samples were loaded into the preformed wells of the gel and allowed to migrate at 10V/cm voltage. To enable analysis of DNA fragment size, 1 µg Molecular weight marker 1 kb ladder or 100 bp gene-ruler were run in parallel on the gel. Visualization of DNA bands was performed by illumination of the gel under UV lights (254-366 nm) and images were recorded using the Bio-Doc Analyse gel documentation system.

4.27.2 RNA gel electrophoresis

RNA gel electrophoresis was performed to check the quality of total RNA, quality and length of IVT-mRNA before and after polyadenylation. In the first case, quality was assessed by visualization of 28S and 18S ribosomal bands which appeared sharp if RNA was intact. In the second case, as mono-specific RNA was analyzed and the presence of a single sharp band indicated that RNA was not degraded. A shift in length became apparent when polyadenylated IVT-mRNA was analyzed parallel to non-polyadenylated IVT-mRNA. As matrix, 0.8% agarose denaturing gels were used. Denaturing conditions are important for RNA visualization. Due to the high prevalence of secondary structures of RNA, RNA migration could be altered. These structures are disrupted in the gel by Formaldehyde, making migration of RNA dependent on the charge and not on secondary structures. For the agarose solution, 0.56 g agarose was dissolved in 56 ml DEPC-H_2O by heating in a microwave. The solution was enabled to cool down to 50-65°C, and supplemented with 10x MOPS (7 ml) and 37% Formaldehyde (7 ml). The resulting solution was used to prepare the RNA gel and has been kept into an electrophoresis tank, previously filled with 1x MOPS buffer. RNA samples were prepared by mixing 1-2 µg RNA with 7.5 µl RNA probe buffer (Ambion kit) and denaturing at 72°C for 5 min. After cooling on ice for 5 min, samples were loaded onto the gel, which had been run for equilibration for 10 min at 6V/cm before. RNA samples were run for 90 min at 8V/cm. Higher voltages could have caused trailing and smearing of RNA bands. 3 µg of 1 kb Molecular weight markers (Invitrogen) was run in parallel on the gel to evaluate RNA lengths. Visualization of RNA bands was performed as described for DNA.

5. Results

5.1 *In vitro* stimulation of CD8$^+$ T cells using antigen-presenting cells transfected with IVT-mRNA

PBMCs directly isolated from peripheral blood have been demonstrated to be potent stimulators of antigen-specific T lymphocytes *in vitro* (Teufel *et al.,* 2005; Johnson *et al.,* 2006; Kreiter *et al.,* 2007; Van Camp *et al.,* 2010). Following IVT-mRNA electroporation, mainly the CD14$^+$ subpopulation of PBMCs was found to be highly transfected **(personal communication C. Graf)**. For CD8$^+$ T-cell stimulations to be performed herein, the use of CD8$^-$ PBMCs electroporated with IVT-mRNA was anticipated to be the most feasible and economic APC. The decision to use IVT-mRNA as an antigen format instead of peptides was based on the fact that IVT-mRNA covers all possible antigenic peptides restricted by any of the donors' HLA molecules. In contrast, peptide prediction algorithms are not available for all HLA alleles and predicted peptides might not be endogenously processed. Britten *et al.,* (2004) have demonstrated that electroporation of IVT-mRNA equals peptide-loading in its ability to detect even rare antigen-experienced T cells. An additional benefit of IVT-mRNA electroporation is that it can be relatively easily performed under good clinical practice conditions because the expression of mRNA is only transient and does not lead to insertional mutagenesis, as opposed to DNA as well as viral and retroviral vectors (Ponsaerts *et al.,* 2003).

5.1.1 Electroporation conditions for CD8$^-$ PBMCs/monocytes

Electroporation parameters for CD8$^-$ PBMCs have been already optimized in our lab by Claudine Graf. She has confirmed that EGFP expression could be detected prominently in monocytes (CD14$^+$) as well as in a small fraction of lymphocyte subsets within PBMCs. In both of the cases, electroporation with 350 V/300 µF yielded a higher percentage of EGFP expressing cells compared with electroporation at 250V/300 µF. A maximum of about 75% EGFP$^+$ monocytes and 20% EGFP$^+$ B lymphocytes could be detected already 12 hours after electroporation. EGFP expression stayed constant for at least 44 hours (**personal communication C. Graf**). It was confirmed, herein, that these parameters work very well when the technique was applied either to CD8$^-$ PBMCs or to monocytes **(Figure 5.1.1a)**. EGFP expression improved when 15 µg *EGFP* IVT-mRNA was used for the electroporation of 1.0x10^6 cells instead of lower amounts (**Figure 5.1.1b**).

Thereafter, in further electroporation experiments 1.0x10^6 cells were electroporated using 15 µg antigen-encoding IVT-mRNA at 350 V/300 µF (as described in **Method section 4.7**). Electroporation efficiency (*EGFP* as a readout) was measured by flow cytometry analysis performed 18 to 24 hours after electroporation (see **Method section 4.6**).

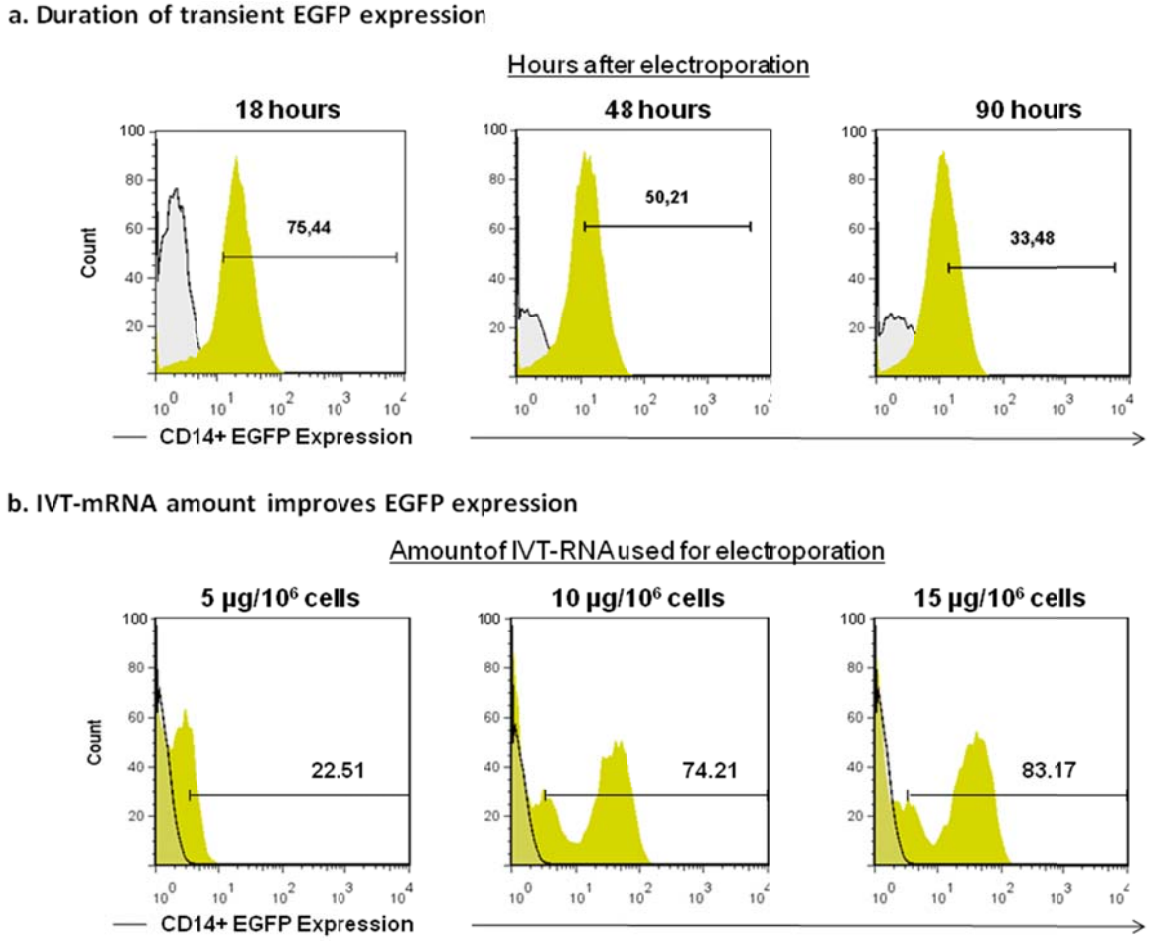


Figure 5.1.1.1: EGFP expression by CD14$^+$ cells (monocytes) after electroporation with EGFP IVT-mRNA.
CD8$^-$ PBMCs were washed with OPTIMEM and pipetted into an electroporation cuvette. These cells were electroporated with *EGFP* IVT-mRNA at previously optimized conditions (350 V/300 µF). After electroporation, cells were washed and resuspended in AIM-V/10%HS. **(a) Duration of transient expression of EGFP.** 2.0x10^6 CD8$^-$ PBMCs contained in 200 µl OPTIMEM were electroporated with 20 µg *EGFP* IVT-mRNA. 2.0x10^5 cells were analyzed by flow cytometry at indicated time points after electroporation. **(b) IVT-mRNA amount improves EGFP expression.** 1.0x10^6 CD8$^-$ PBMCs contained in 100 µl OPTIMEM were electroporated with different amounts of *EGFP* IVT-mRNA (5 µg, 10 µg and 15 µg). 2.0x10^5 cells were taken into FACS tubes for flow cytometry analysis after 18 hours of electroporation. To analyze these samples, MOCK and EGFP electroporated cells were stained with CD14-PE. After staining, the cells were washed with FACS buffer and fixed with PBS FACS fixation buffer (see **Method section 4.6**). Cells were gated for CD14 expression. These gating parameters were further used for the determination of EGFP expression by CD14$^+$ monocytes at the indicated time point.

5.1.2 Robustness of CD8$^-$ PBMCs as antigen-presenting cells

In order to investigate whether CD8$^-$ PBMCs transfected with IVT-mRNA can be used as efficient antigen-presenting cells for the stimulation of CD8$^+$ T-cell responses. The CMV antigen pp65 was used as a model antigen. HCMV$^+$ donor BC #1676, who carried *HLA-A*02:01* and -*B*07:02*, was analyzed for anti-pp65 reactivity by *in vitro* stimulation of CD8$^+$ T cells with *pp65* IVT-mRNA. CD8$^+$ T cells were stimulated with irradiated CD8$^-$ PBMCs electroporated with *pp65* IVT-mRNA at a 1:1 ratio. Restimulation was performed on d7 under identical stimulation conditions. The resulting responder populations were tested on d12 in an IFN-γ ELISPOT assay

(as schematically explained later in **Figure 5.3.1.1**). In this assay autologous CD8$^-$ PBMCs electroporated with *pp65* IVT-mRNA were used as APCs to guarantee the expression of all HLA I molecules of the donor and all potential pp65 epitopes. In addition, the responders of *pp65* RNA stimulation were tested for recognition of a known pp65 peptide restricted by HLA-A*02:01. As a result, the stimulation of CD8$^+$ T cells with CD8$^-$ PBMCs expressing pp65 after transfection of RNA-encoding pp65 could at least fourfold enrich anti-pp65 CD8$^+$ T cells. The control stimulation performed with mock-transfected CD8$^-$ PBMCs did not lead to an expansion of pp65-specific CD8$^+$ T cells. A strong response was observed against the HL-A*02:01-restricted peptide (**Figure 5.1.2.1**).

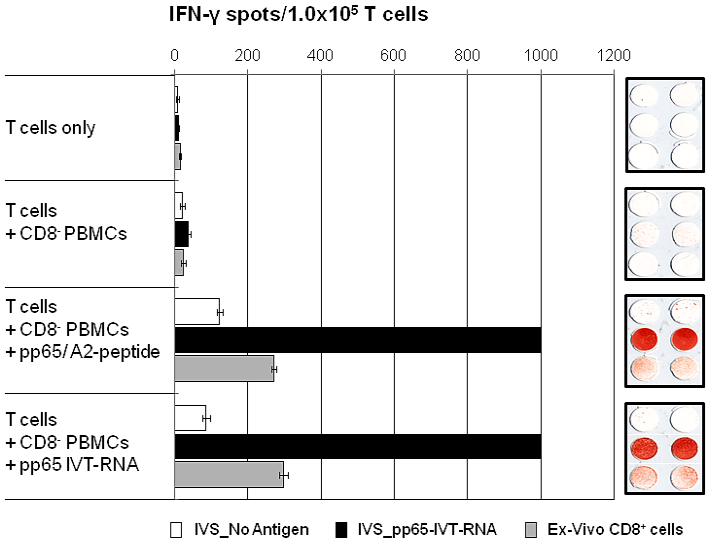


Figure 5.1.2.1: Stimulation of CD8$^+$ T cells with pp65 IVT-mRNA.
CD8$^+$ T cells isolated with microbeads from BC #1676 were stimulated on d0 and d7 with irradiated autologous CD8$^-$ PBMCs electroporated with pp65 IVT-mRNA. On d12, responder T cells (1.0x10^5/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of CD8$^-$ PBMCs (1.0x10^5/well) either electroporated without/with pp65 IVT-mRNA or loaded with a pp65/A2-peptide (NLVPMVATV, 1 µg/ml). Ex vivo CD8$^+$ T cells (1.0x10^5/well) were thawn and tested in parallel to demonstrate the ex vivo response against pp65. Bars represent the means of duplicates ±SD. 1000 spots/well are assumed number because this is innumerable by the ELISPOT reader.

Since HLA-A*02:01 and HLA-B*07:02 are known to be relevant HLA-restriction molecules for anti-pp65 T cells (Wills *et al.,* 1996; Solache *et al.,* 1999; Longmate *et al.,* 2001). The anti-pp65 responder lymphocytes were also tested for recognition of COS-7 cells transfected with plasmids-encoding these two HLA I alleles and either co-transfected with a pp65-encoding plasmid or loaded with peptides known to be presented by HLA-A*02:01 and HLA-B*07:02, respectively. It was observed that the T cells responding to RNA stimulation were restricted by HLA-A*02:01 or -B*07:02 and strongly reacted with known dominant synthetic pp65 peptides restricted by these two alleles (**Figure 5.1.2.2**). Again CD8$^-$ PBMCs electroporated with antigen-encoding IVT-mRNA proved to be suitable for the stimulation of CD8$^+$ T lymphocytes. In contrast to pp65, the frequency of FLT3-ITD-reactive T-cell in the peripheral blood of allogeneic sources was expected to be rather low. However, several studies had demonstrated that CD8$^-$ PBMCs electroporated with IVT-mRNA were also able to detect T-cell responses below the *ex vivo* detection threshold (e.g. Teufel *et al.,* 2005). So, this stimulation method fulfilled the prerequisites that were required for the analysis of autologous as well as allogeneic sources for the presence of CD8$^+$ T-cell responses against FLT3-ITD.

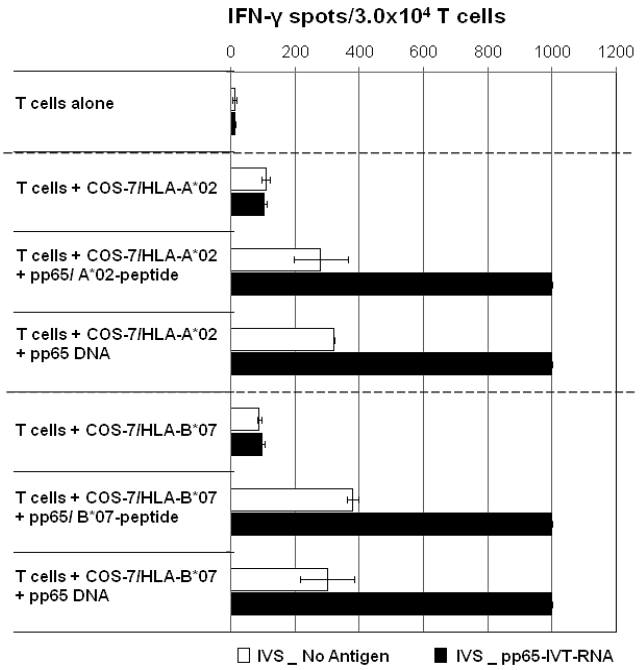


Figure 5.1.2.2: Peptide reactivity of CD8$^+$ T cells stimulated with pp65 IVT-mRNA.
CD8$^+$ T cells isolated with microbeads from BC #1676 were stimulated on d0 and d7 with autologous irradiated CD8$^-$ PBMCs electroporated with pp65 IVT-mRNA. IVS-microculture responder T cells ($3.0x10^4$/well) were tested on d19 in a 20-hour IFN-γ ELISPOT assay. Targets were either COS-7 cells ($2.0x10^4$/well) co-transfected with HLA-A*02:01 or -B*07:02 plasmid and a plasmid-encoding pp65 or COS-7 cells transfected with HLA-A*02:01 or –B*07:02 plasmid and loaded with synthetic pp65 peptides (1 µg/ml) restricted by HLA-A*02:01 (NLVPMVATV) –or HLA-B*07:02 (TPRVTGGGAM), respectively. Bars represent the means of duplicates ±SD. 1000 spots/well are assumed number because this is innumerable by the ELISPOT reader.

5.1.3 Transfection of FastDCs with the TransMessenger™ reagent

Thus far, electroporation has been the preferential method to transfect DCs with mRNA (Van Tendeloo *et al.,* 2001; Saeboe-Larssen *et al.,* 2002; Strobel *et al.,* 2000). Optimization of electroporation protocols have led to transfection efficiencies reaching 90% in a reproducible way, even in mature DCs (Schaft *et al.,* 2005). Electroporation is based on the physical deterioration of the plasma membrane integrity and, therefore, decreasing the viability of the treated cells (Canatella *et al.,* 2001). This limits the use of electroporation for transfecting DCs when a high yield of antigen-expressing DCs is required. To overcome this problem, it was decided to use TransMessenger™ transfection, a lipid-based formulation specially developed for mRNA transfection. This transfection procedure permits to transfect DCs with no or very limited cell death. It was also reported that antigen expression (mean fluorescence intensity) on per cell basis is higher using TransMessenger™ reagent compared to electroporation (**personal communication M. Fatho**).

5.1.3.1 Optimization of transfection efficiency

By means of EGFP expression as a readout, transfection efficiencies of FastDCs with TransMessenger™ were optimized. For this purpose, varying amounts of mRNA (200 to 800 ng) and RNA:TransMessenger™ ratios (1:2 to 1:8) were used according to the manufacturer's instructions. Combinations of these conditions were applied for the transfection of FastDCs with *EGFP* IVT-mRNA (see **Method section 4.8**). The best transfection efficiency (51%) was obtained with 400 ng *EGFP* IVT-mRNA at a RNA:TransMessenger™ ratio of 1:8 (**Figure 5.1.3.1.1**).

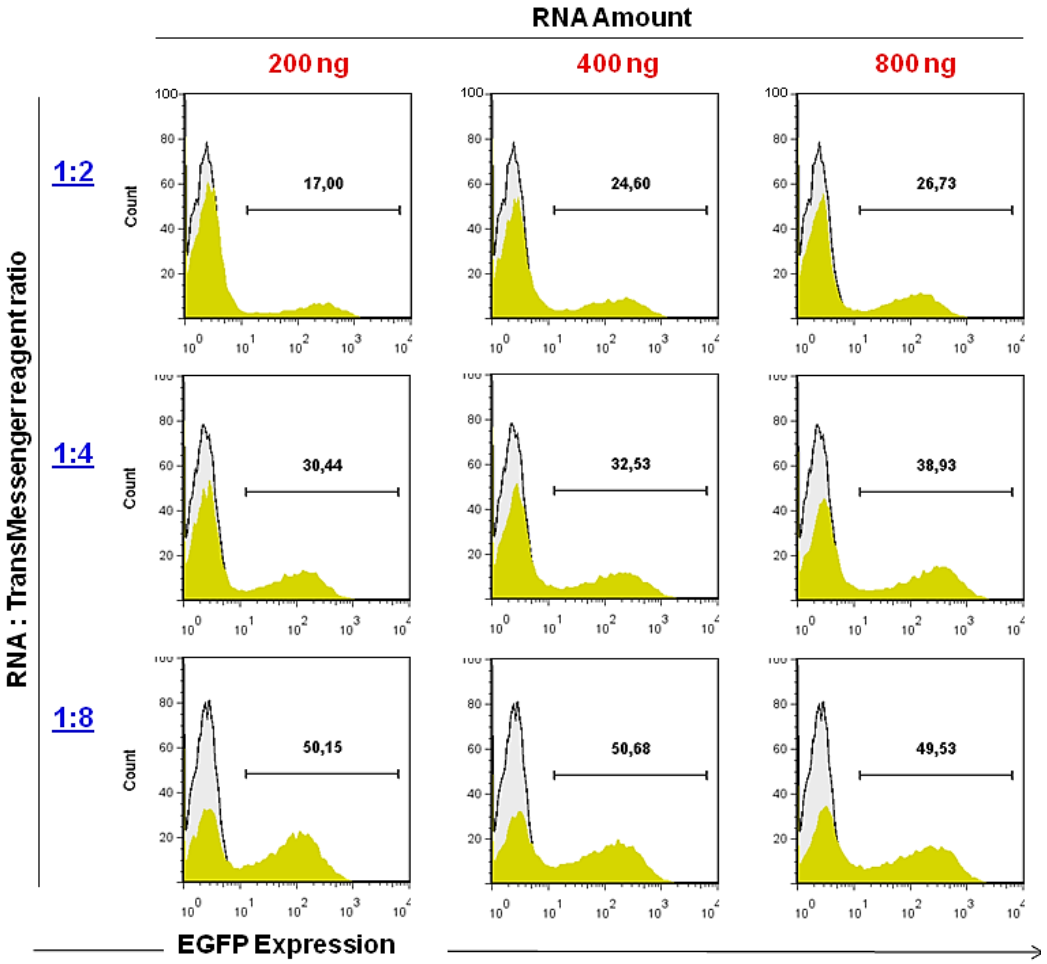


Figure 5.1.3.1.1: Optimization of FastDCs transfection using the TransMessenger™ reagent (TM). FastDCs were generated from monocytes of healthy donor BC #0934 using a protocol described before (Dauer *et al.,* 2003). FastDCs ($2.0x10^5$ cells/well) were transfected with varying amounts (200-800 ng) of *EGFP* IVT-mRNA, at varying RNA:TM ratios (1:2 to 1:8) according to the manufacturer's instructions (see **Method section 4.8**). Mock-transfected FastDCs served as a negative control. EGFP fluorescence intensity in transfected cells was measured by flow cytometry 24 hours after transfection. Values shown are the percentages of cells expressing EGFP protein.

Transfection of dendritic cells with TransMessenger™ leads to a higher level of antigen expression level on per cell basis than electroporation (Landi *et al.,* 2007; Yu *et al.,* 2007; **personal communication D. Eberts** and **M. Fatho**). Notably, EGFP expression on a per cell basis did not increase significantly with increasing RNA amounts above 400 ng. With the above-mentioned parameters, transfection efficiencies were ranging in between 10% to 30% in UCB donations and in between 20% to 50% in different healthy donors (**Figure 5.1.3.1.2**). It is also known from the PhD work of Dr. Emmanuelle Wesarg (2008) that the long-term survival of FastDCs transfected with TransMessenger™ is high, whereas, the physical damages caused by electroporation lead to the loss of most of the cells within 72 hours after transfection. Indeed, FastDCs transfected with TransMessenger™ remained viable according to propidium iodide (PI) exclusion and expressed EGFP up to 72 hours after transfection. Thus, TransMessenger™ appears considerably less toxic for transfected cells in comparison to electroporation. For those reasons, the TransMessenger™ method was used in those systems where amounts of antigen-presenting cells were very much limited (in AML patient **FL** and in UCB donations).

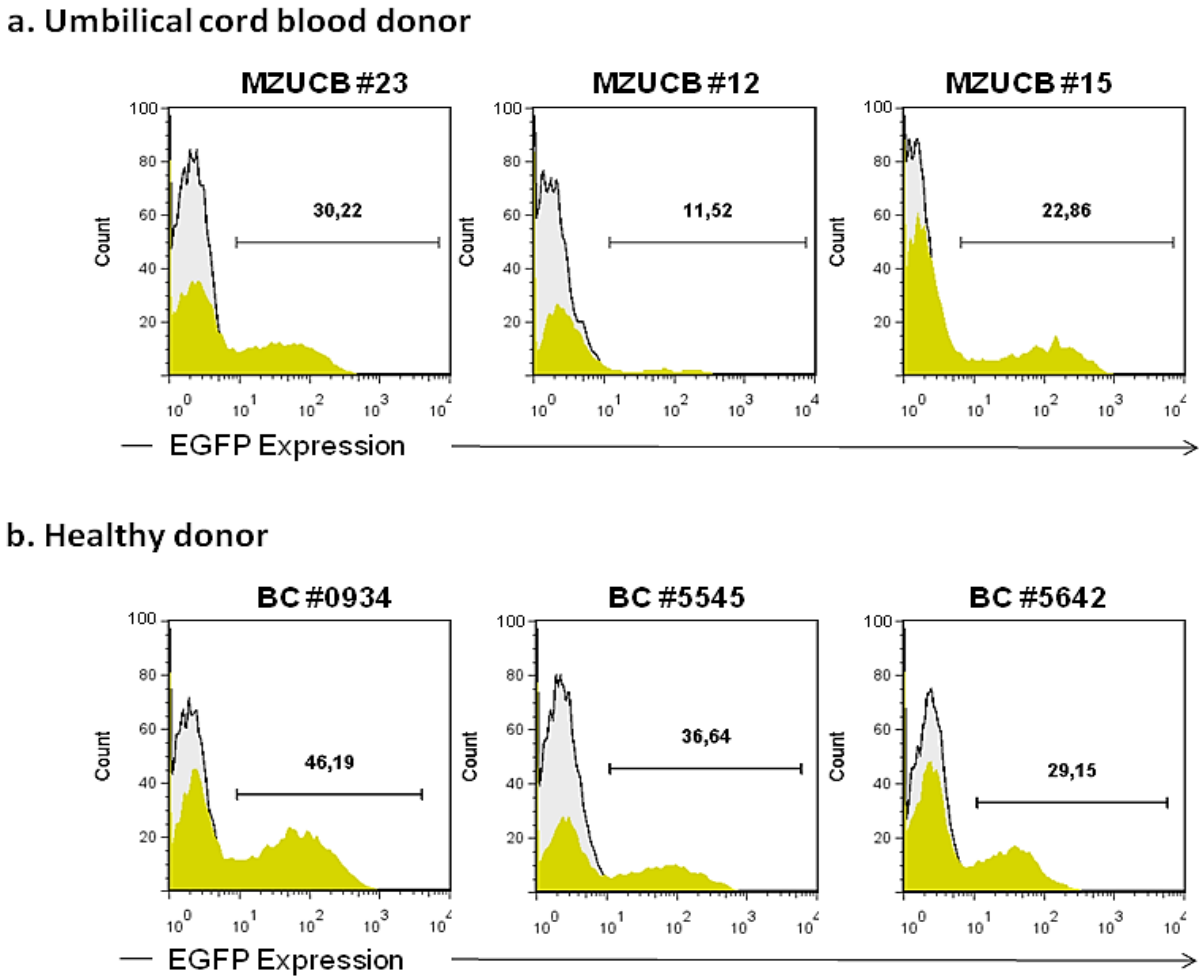


Figure 5.1.3.1.2: EGFP expression of FastDCs from (a) Umbilical cord blood donations and (b) Healthy individuals' transfected with the TransMessenger™ reagent.
FastDCs ($2.0x10^5$ cells/well) were transfected with 400 ng RNA at a 1:8 ratio of RNA:TM according to the manufacturer's instructions (see **Method section 4.8**). Mock-transfected FastDCs served as a negative control. EGFP fluorescence intensity in transfected cells was measured by flow cytometry 24 hours after transfection. Values shown are the percentages of cells expressing the EGFP protein.

5.1.3.2 Comparison of TransMessenger™ transfection with electroporation

FastDCs from $HCMV^+$ donor BC #7758 were transfected with *pp65* IVT-mRNA by electroporation as well as with the TransMessenger™ method. $CD8^+$ T cells isolated from peripheral blood of $HCMV^+$ donor were stimulated separately with both kinds of *pp65*-transfected FastDCs to stimulate pp65-specific T cells. As negative controls, $CD8^+$ T cells were stimulated with mock-transfected APCs. In order to provide equivalent conditions for all stimulated populations, stimulations were performed at a 1:10 APC-to-$CD8^+$ T-cell ratio. Responder lymphocytes were tested in an IFN-γ ELISPOT assay for recognition of COS-7 cells co-transfected with plasmids-encoding HLA-A*02:01 and pp65. Expansion of pp65-specific $CD8^+$ T cells was comparable for both procedures (**Figure 5.1.3.2.1**). This analysis clearly identified FastDCs transfected with IVT-mRNA using TransMessenger™ as potent antigen-presenting cells to stimulate anti-pp65 T-cell responses.

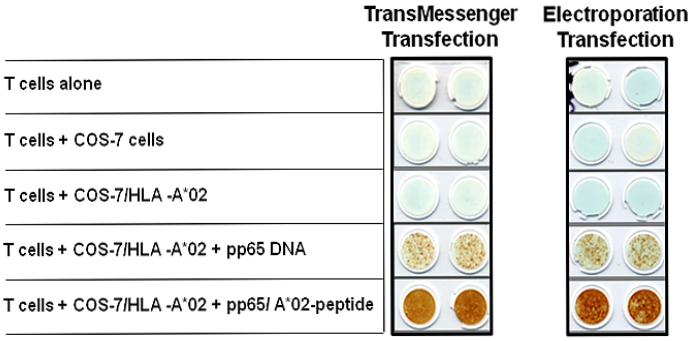


Figure 5.1.3.2.1: Comparative stimulation of pp65-reactive T cells with FastDCs transfected either with TransMessenger™ or by electroporation.
$CD8^+$ T cells isolated with microbeads from BC #7758 were stimulated separately with both kinds of *pp65* transfected FastDCs as described before (see **Method section 4.7** and **4.8**). On d19, responder T cells ($3.0x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay. Targets were COS-7 cells- ($2.0x10^4$/well) transfected with *HLA-A*02:01* and either co-transfected with a *pp65* plasmid or loaded with pp65 A2/peptide NLVPMVATV (1 µg/ml, see **Figures 5.1.2.2** and **5.1.2.3**).

5.1.4 *In vitro* stimulation of $CD8^+$ T cells under limiting dilution-like conditions improves the detection of T-cell responses against HCMV pp65

The frequencies of FLT3-ITD-reactive T cells in blood lymphocytes of healthy individuals were supposed to be very low, if detectable at all. E.g. T cells against the MAGE-3/A2 peptide were found in the $CD8^+$ $CD45RA^+$ subpopulation of blood lymphocytes of healthy individuals at frequencies ranging from 4.0 to $17.0x10^{-7}$ (Chaux *et al.,* 1998). Therefore, techniques to measure such a low frequency need to be highly sensitive. To tackle this, a method had to be set up with which large numbers of $CD8^+$ T cells could be analyzed for reactivity against FLT3-ITDs.

In a first approach, $CD8^+$ T cells were isolated from PBMCs of $HCMV^+$ seropositive healthy donors and seeded in 24-well-plates ($1.0x10^6$/well). They were stimulated at a 1:1 ratio with autologous $CD8^-$ PBMCs electroporated with *pp65* IVT-mRNA. All responder T cells were restimulated on d7 under the same culture conditions. On d12, responder T cells were tested in an IFN-γ ELISPOT assay for the recognition of COS-7 cells co-transfected with plasmids-encoding the respective *HLA*/*pp65* combinations. Thereby anti-HCMV pp65 responses were detectable (**Figure 5.1.4.1**), but they appeared rather weak considering the fact that anti-pp65 T cells are readily detected *ex vivo* at rather high frequencies in seropositive individuals. Bulk T-cell cultures do not provide any information about the frequency of T cells with a given specificity. In addition, the detection sensitivity of bulk cultures might be compromised by suppressive effects mediated by regulatory T cells or non-specifically proliferating T cells.

In a second approach, limiting dilution-like microculture conditions (modified from Chaux *et al.,* 1998; Coulie *et al.,* 2001) were used to enrich antigen-specific T cells. $CD8^+$ T cells from the same donor as above were seeded on a 96-well U-bottom plate at $1.0x10^5$/well. They were stimulated at a 1:1 ratio with autologous irradiated $CD8^-$ PBMCs electroporated with *pp65* IVT-mRNA. Responder T cells were restimulated on d7 under identical culture conditions and tested

also on d12 in an IFN-γ ELISPOT assay. Anti-pp65 responses were observed in every microculture**.** Spot numbers were up to three-fold higher than in bulk cultures (**Figure 5.1.4.2**, for comparison see **Figure 5.1.4.1**), indicating advantageous culture conditions in microcultures. Therefore, it was decided to use the microculture approach for in *vitro* stimulation to enrich, detect and quantitate FLT3-ITD-specific $CD8^+$ T cells in autologous as well as allogeneic sources.

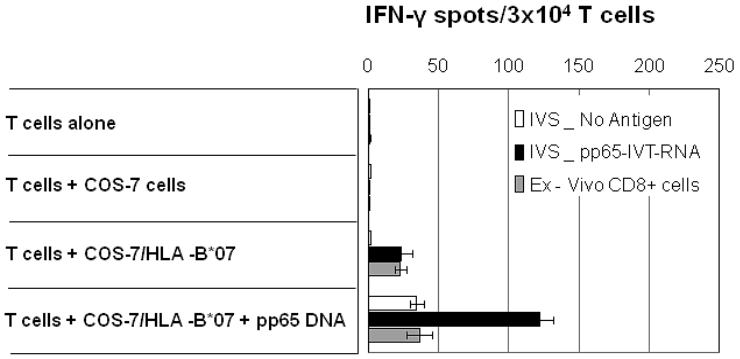


Figure 5.1.4.1: Bulk-stimulation of $CD8^+$ T cells against pp65 antigen.
On d12, responders of bulk T-cell stimulation ($3.0x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay. COS-7 cells ($2.0x10^4$/well) were co-transfected with *HLA-B*07:02* and -/+ *pp65* plasmid DNA. *Ex vivo* $CD8^+$ T cells ($1.0x10^5$/well) were also tested to see the spontaneous response against pp65. Bars represent the means of duplicates ±SD.

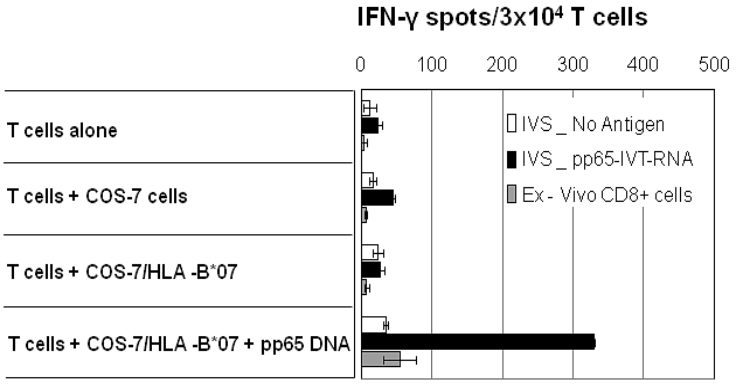


Figure 5.1.4.2:.Stimulation of $CD8^+$ T cells in quasi-limiting dilution improves antigen-specific response.
On d12, responders of microculture T-cell stimulation ($3.0x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay. COS-7 cells ($2.0x10^4$/well) were co-transfected with *HLA-B*07:02 -/+ pp65* plasmid DNA. *Ex vivo* $CD8^+$ T cells ($1.0x10^5$/well) were also tested to see the spontaneous response against pp65. Bars represent the means of duplicates ±SD.

5.2 $CD8^+$ T-cell response against FLT3-ITD in the autologous FL leukemia model

5.2.1 Short-term *in vitro* stimulation of autologous $CD8^+$ T cells against FL_FLT3-ITD IVT-mRNA

To extend the anti-FLT3-ITD response analyses previously performed in Mainz (patient-specific anti-FLT3-ITD responses have been observed in AML model systems **JC, IN, QQ, EI** and **VE**) (Graf *et al.,* 2007; Graf *et al.,* unpublished), it was planned to analyze the T-cell responses in the AML model derived from patient **FL**. **FL**-AML cells carry a 14 amino acid-long internal tandem duplication in the juxtamembrane domain of the FLT3-receptor starting at amino acid position 585 (**FL/585-14**). In this model the FLT3-ITD-encoding region ranging from the end of Exon 13 to the beginning of Exon 16 was amplified with primers 5' *FLT3* Exon 13 or 5' *FLT3*-Exon 13_{BamH1}/and 3' *FLT3* Exon $16_{Xba\ I}$ (see **Materials section 3.6**). The resulting PCR fragment was cloned into the pcDNA3.0 vector as described in **Method section 4.22**. This **FL**_*FLT3*-

ITD$_{Exon13-16}$-pcDNA3.0 construct was used to produce **FL**_*FLT3-ITD* IVT-mRNA for the *in vitro* stimulation of CD8$^+$ T cells from the patient´s blood. Microculture stimulation of CD8$^+$ T cells was performed using short-term *in vitro* stimulation procedure described in **Method section 4.9.2** and **4.10** (and schematically explained in **Figure 5.3.1.1)**. Either autologous CD8$^-$ PBMCs or allogeneic HLA I-matched mature FastDCs were used as APCs after electroporation with **FL**_*FLT3-ITD* IVT-mRNA. With autologous CD8$^-$ PBMCs as APCs, d19-proliferation rates of microcultures ranged from 5- to 12-fold. With allogeneic HLA I-matched mature FastDCs as APCs, d19-proliferation rates of microcultures ranged from 11- to 26-fold. The results obtained in the IFN-γ ELISPOT assay demonstrated that stimulation of CD8$^+$ T cells with either autologous CD8$^-$ PBMCs or allogeneic HLA I-matched mature FastDCs showed a clear response against the patient´s FLT3-ITD (**Figure 5.2.1.1a** and **5.2.1.1b**).

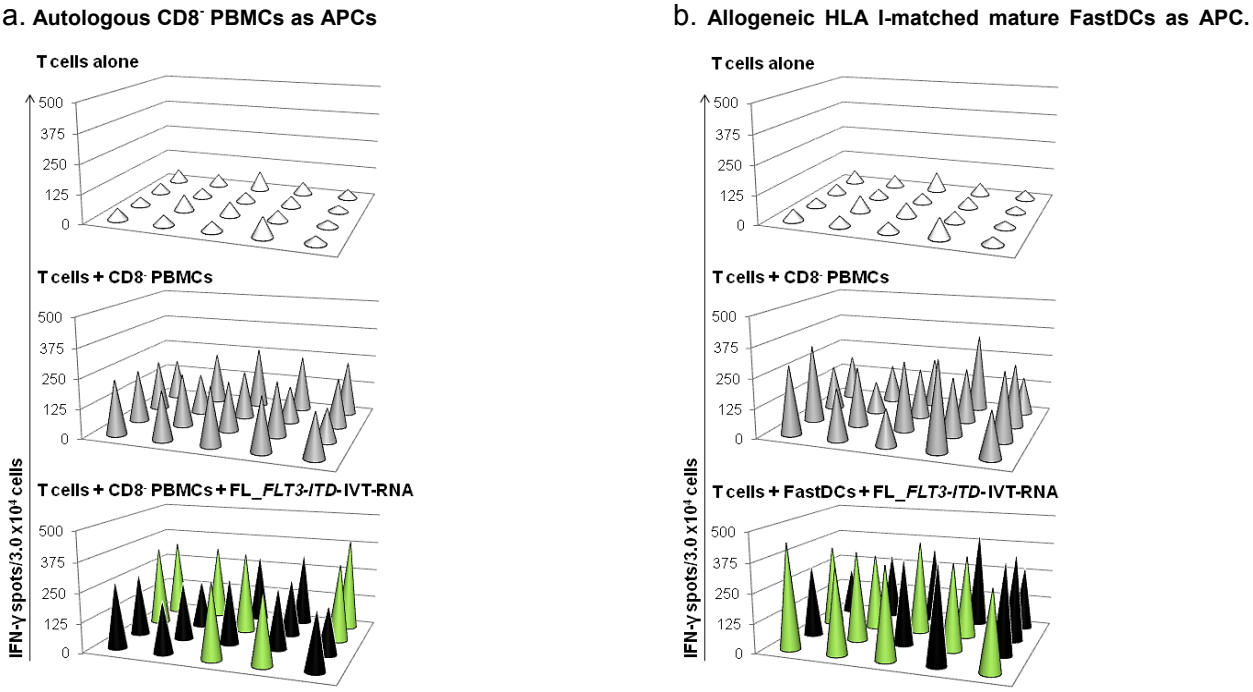
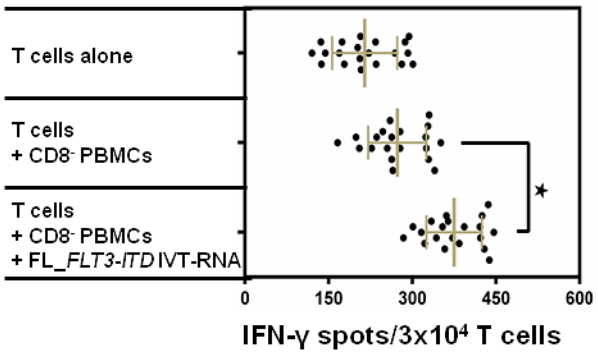


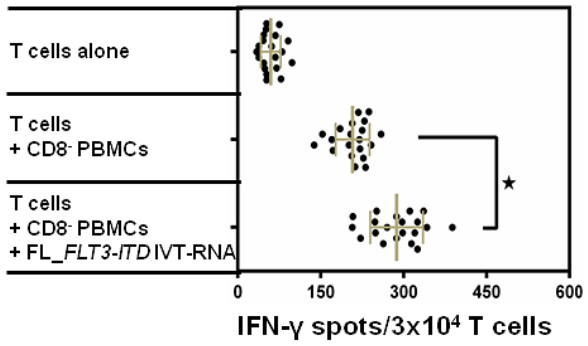
Figure 5.2.1.1.: *In vitro* stimulated CD8$^+$ T cells show responses against FLT3-ITD of patient FL.
CD8$^+$ T cells of patient **FL** were stimulated either with autologous CD8$^-$ PBMCs or with allogeneic HLA I-matched FastDCs as APCs after electroporation with **FL**_*FLT3-ITD* IVT-mRNA (see **Method section 4.9.2** and **4.10**). On d19, responder T cells (3.0x10^4/well) were tested in an IFN-γ ELISPOT assay. **(a) FLT3-ITD response using CD8$^-$ PBMCs as APCs:** CD8$^-$ PBMCs (6.0x10^4/well) were electroporated with IVT-mRNA-encoding **FL**_FLT3-ITD and used as target cells. **(b) FLT3-ITD response using allogeneic FastDCs as APCs:** allogeneic HLA I-matched mature FastDCs (5.0x10^3/well) were electroporated with IVT-mRNA-encoding **FL**_FLT3-ITD and used as target cells. The IFN-γ ELISPOT assay was developed after a 20-hour of incubation. FLT3-ITD-reactive microcultures were shown in green cones. Response was defined as ‘‘positive’’ when the number of IFN-γ spots against **FL**_FLT3-ITD was higher than the mean background plus two-fold standard deviations.

During the course of treatment, blood samples were collected from patient **FL** at different time points [before chemotherapy and after chemotherapy when patient was in first clinical remission (CR)], from which CD8$^+$ T lymphocytes were isolated. In each of the samples statistically significant CD8$^+$ T-cell responses were detected against **FL**_FLT3-ITD via short-term RNA stimulation using both APC types (**Figure 5.2.1.2**).

a. Response before chemotherapy: with autologous APC.



b. Response after chemotherapy /first CR: with autologous APC.



c. Response after chemotherapy /first CR: with allogeneic APC.

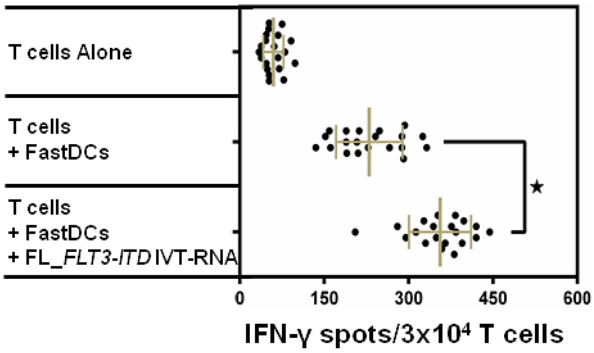


Figure 5.2.1.2: Summary of anti-FLT3-ITD responses exhibited by CD8$^+$ T cells from the blood of patient FL collected at different time points.

CD8$^+$ cells from PBMCs isolated at indicated time points from the blood of patient were stimulated, using same stimulation protocol, either by autologous CD8$^-$ PBMCs or by allogeneic HLA I-matched FastDCs electroporated with IVT-mRNA-encoding **FL**_FLT3-ITD (see **Figure 5.2.1.1**). Responder T cells (3.0x10^4/well) were tested in an IFN-γ ELISPOT assay. **(a) Pre-chemotherapy with autologous APCs:** d26-response using CD8$^-$ PBMCs as stimulator and target cells (6.0x10^4/well). **(b) after chemotherapy (in first clinical remission) with autologous APCs:** d19 response using CD8$^-$ PBMCs as stimulator and target cells (6.0x10^4/well). **(c) After chemotherapy (in first clinical remission) with allogeneic APCs:** d19 response using allogeneic HLA I-matched FastDCs as stimulator and target cells (5.0x10^3/well). Each dot represents the spot number induced by a single microculture and the green line indicates the means ±SD of twenty *in vitro* microcultures stimulated against **FL**_FLT3-ITD. The data were analyzed for statistical difference determined by two-tailed, unpaired Student´s t-test and the * p-values (<0.01) indicate the significant CD8$^+$ T-cell response against **FL**_FLT3-ITD.

5.2.2 Ex vivo T-cell analysis

When patient **FL** was in first complete remission after conventional chemotherapy, she donated blood samples for *ex vivo* analyses. CD8$^+$ T cells were directly applied to a 20-hour IFN-γ ELISPOT assay and tested against autologous CD8$^-$ PBMCs electroporated with **FL**_*FLT3-ITD* IVT-mRNA (see **Method section 4.11**). *Ex vivo* T-cell responses against **FL**_FLT3-ITD were detected repeatedly with fresh and frozen CD8$^+$ T cells (**Figure 5.2.2.1a** and **5.2.2.1b**). At its maximum, the *ex vivo* response against the **FL**_FLT3-ITD comprised 0.17% of CD8$^+$ T lymphocytes. It was decided to perform T-cell cloning to isolate, from the patient´s blood lymphocytes, **FL**_FLT3-ITD-specific clonal T cells using the IFN-γ secretion assay.

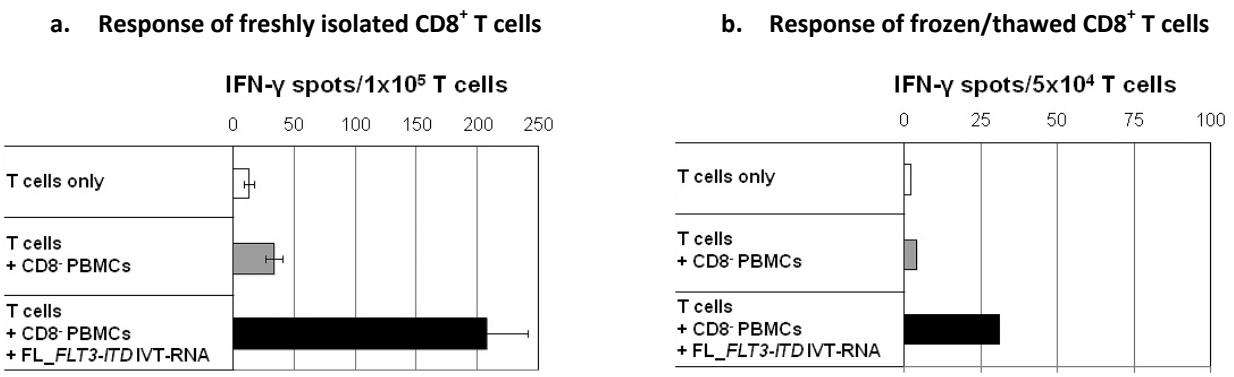


Figure 5.2.2.1: *Ex vivo* CD8$^+$ T-cell responses against the FLT3-ITD of patient FL.
(a) freshly isolated CD8$^+$ T cells (1.0x10^5/well) from the peripheral blood of the patient and **(b) frozen CD8$^+$ T cells** (thawed and kept overnight at rest) (5.0x10^4/well), were tested in a 20-hour IFN-γ ELISPOT assay. CD8$^-$ PBMCs were electroporated with IVT-mRNA expressing **FL**_FLT3-ITD, used as APCs (6.0x10^4/well). The bars represent means of duplicates ±SD. Shown are the representative *ex vivo* anti-FLT3-ITD-specific T-cell response in patient **FL.**

5.2.3 Cloning of FLT3-ITD-reactive CD8$^+$ T cells directly from blood lymphocytes using the interferon-γ secretion assay

To clone the **FL_**FLT3-ITD-reactive T cells, **FL_**FLT3-ITD-specific T cells were isolated directly from *ex vivo* CD8$^+$ T cells. The IFN-γ secretion assay was performed as described in **Method section 4.12,** unless IVT-mRNA was used as an antigen format. In brief, CD8$^+$ T cells were stimulated for 12 hours at a 1:10 ratio with irradiated autologous FastDCs electroporated with **FL**_*FLT3-ITD* IVT-mRNA. After 12 hours, T cells were labeled with a bispecific antibody conjugate directed against CD45 and IFN-γ and incubated at 37°C for 45 min permitting IFN-γ secretion. T cells were washed and stained with anti-IFN-γ PE mAb and anti-CD8 FITC mAb. CD8$^+$ IFN-γ-secreting **FL_**FLT3-ITD T cells were isolated and cloned by single cell sorting using FACS flow cytometry (**Figure 5.2.3.1**).

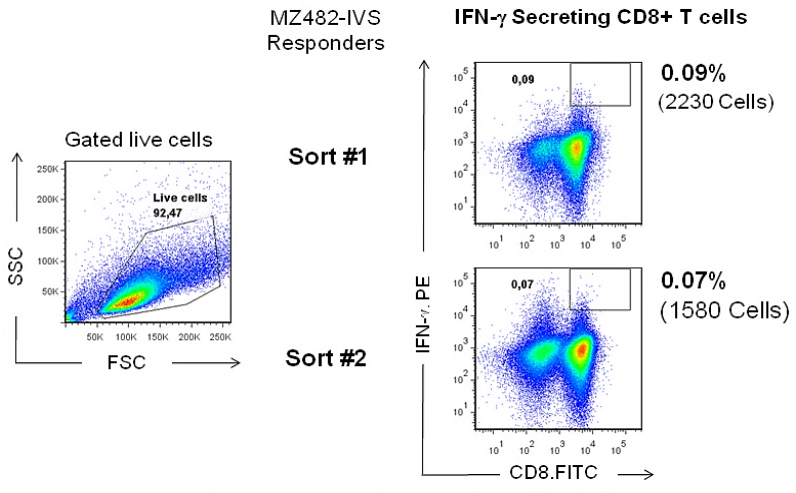


Figure 5.2.3.1: FACS sorting of *ex vivo* CD8$^+$ FLT3-ITD-reactive T cells.
Ex vivo CD8$^+$ T cells were stimulated for 12 hours at a 1:10 ratio with irradiated autologous FastDCs electroporated with **FL**_*FLT3-ITD* IVT-mRNA. After 12 hours, the IFN-γ secretion assay was performed (see **Method section 4.12**) to isolate FLT3-ITD-reactive T cells. FACS sorting of stimulated *ex vivo* CD8$^+$ cells resulted in 2230 cells (0.09%) in **Sort #1** (from 2,5x10^6 cells) and in1580 cells (0.07%) in **Sort #2** (from 2.25x10^6 cells).

The supposedly clonal T cells were stimulated non-specifically with OKT3 as described in **Method section 4.13**. Rest of the shorted IFN-γ-secreting CD8$^+$ T cells, from FACS Sort#1,

were also stimulated non-specifically (microculture stimulation) and on d14, these T cells demonstrated a weak reactivity against the patient's FLT3-ITD. It was also observed that these T cells weakly recognize AML blasts of patient **FL** (**Figure 5.2.3.2**).

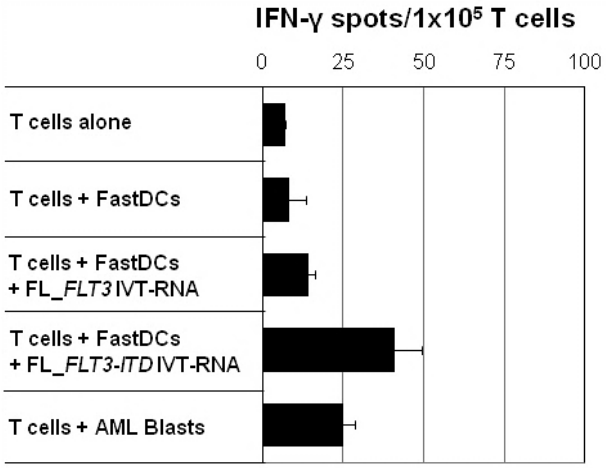


Figure 5.2.3.2: FACS sorted microculture T cells weakly recognize patient's FLT3-ITD and AML blasts.
FACS-sorted $CD8^+$ IFN-γ-secreting **FL_**FLT3-ITD-specific microculture T cells were non-specifically stimulated with OKT3/IL-2 method. d14 microculture responder T cells ($5.0x10^3$/well) were tested in a 20-hour IFN-γ ELISPOT assay for recognition of FastDCs ($5.0x10^3$/well) electroporated with the patients' *wt-FLT3/FLT3-ITD* IVT-mRNA as well as against the patient's AML cells ($5.0x10^4$/well). There is no spontaneous screation of IFN-γ by AML cells (thwan one night before test). The bars represent means of duplicates ±SD.

On d14 of non-specific stimulation, 16 out of 44 expandable T-cell clones were found to recognize **FL**_FLT3-ITD, which could be confirmed on d26 (**Figure 5.2.3.3**). **FL**_FLT3-ITD recognition was restricted only via HLA-A*01:01 (**Figure 5.2.3.4** for three representative T-cell clones)**.** In addition, three T-cell clones were also able to recognize autologous **FL**_AML cells (**Figure 5.2.3.5**). However, the T-cell clones were not able to maintain their specificity against the patient's FLT3-ITD during further rounds of non-specific stimulations under the same conditions (**Figure 5.2.3.6**).

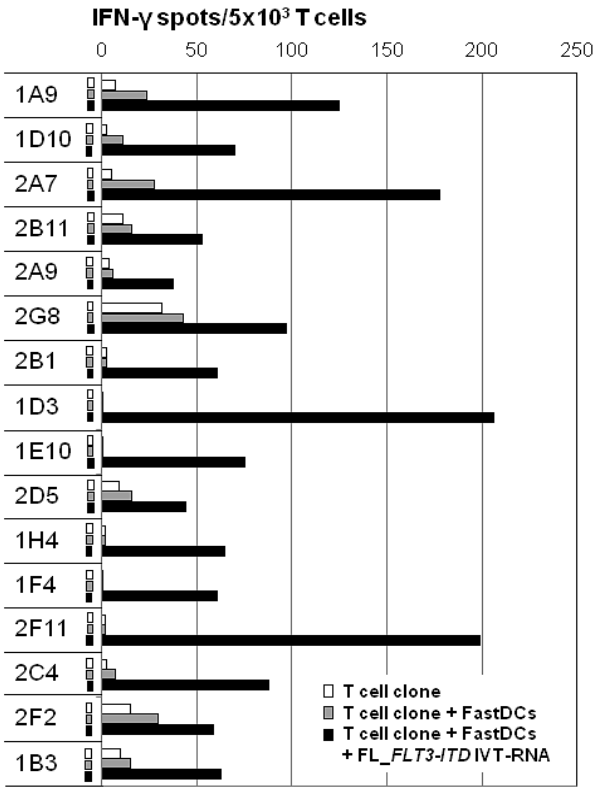


Figure 5.2.3.3: Non-specifically expanded T-cell clones respond to the FLT3-ITD of patient FL.
Using an IFN-γ secretion assay, FLT3-ITD-reactive T cells were isolated from stimulated *ex vivo* $CD8^+$ T cells. The resulting $CD8^+$ IFN-γ^+ T cells were sorted by flow cytometry and non-specifically stimulated twice with a OKT3/IL-2 rapid expansion protocol (see **Method section 4.13**). On d26, clonal T-cell cultures were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of autologous FastDCs ($5.0x10^3$/well) electroporated with the patient's *FLT3-ITD* IVT-mRNA. Shown are the results of 16 out of 44 T-cell clones expanded non-specifically. The bars represent single values.

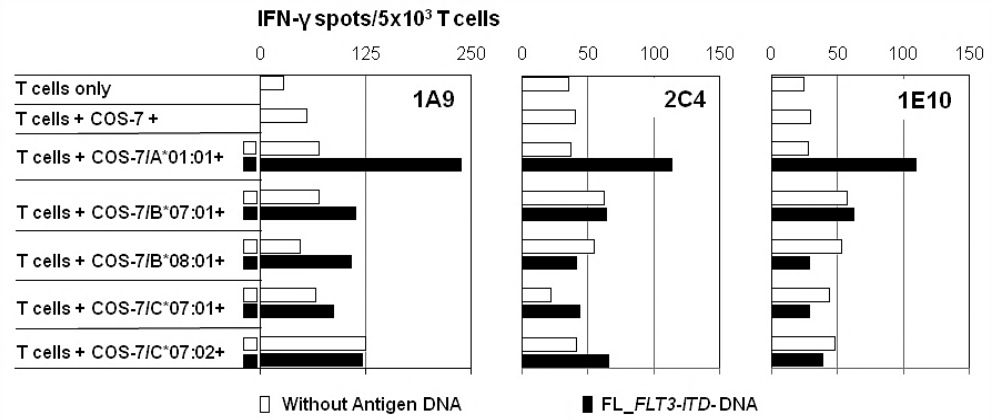


Figure 5.2.3.4: T-cell clones recognize the FLT3-ITD of patient FL in association with HLA-A*01:01.
Clonal T cells (see **Figure 5.2.3.3**) were tested in an IFN-γ ELISPOT assay against COS-7 cells ($2.0x10^4$/well) co-transfected with the patient's *HLA I* alleles and **FL**_*FLT3-ITD.* The T cells ($5.0x10^3$/well) were added after 24 hours, and the ELISPOT assay was developed after 20 hours of incubation. Shown are the results of three representative T-cell clones (1A9, 2C4 and 1E10). The bars represent single values.

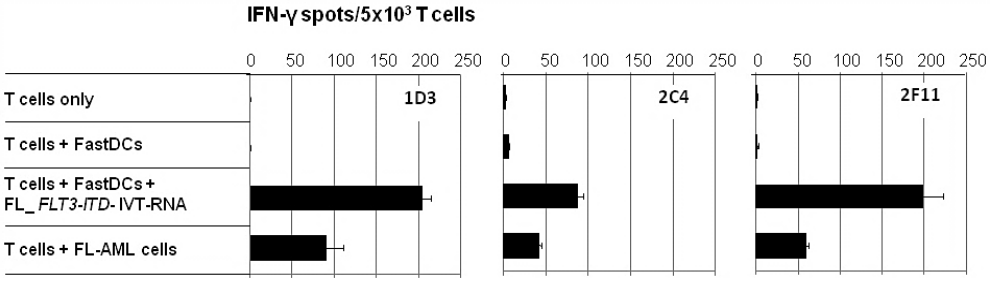


Figure 5.2.3.5: Clonal T cells also recognize the patient's AML blasts.
Expanded clonal T cells ($5.0x10^3$/well) were tested in a 20-hour IFN-γ ELISPOT assay against patient's AML blast cells ($5.0x10^4$/well). These T cells were also able to recognize autologous FastDCs ($5.0x10^3$/well) electroporated with the patient's *FLT3-ITD* IVT-mRNA. Shown are three T-cell clones (1D3, 2C4 and 2F11) that recognized autologous AML blasts. The bars represent means of duplicates ±SD.

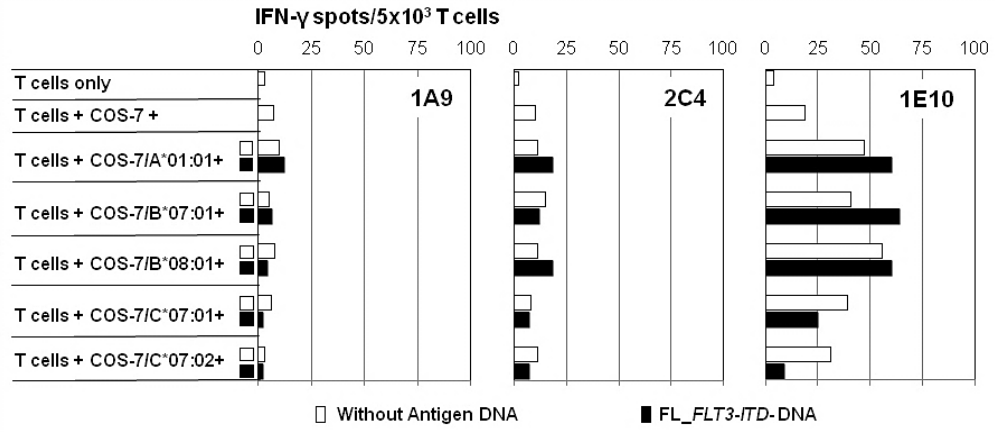


Figure 5.2.3.6: Non-specifically expanded T-cell clones do not maintain their specificity against the patient's FLT3-ITD.
During further rounds of non-specific expansion (OKT3/IL-2 method), T-cell clones lost their specificity for FLT3-ITD. On d47, clonal T cells were tested in an IFN-γ ELISPOT assay against COS-7 cells ($2.0x10^4$/well) co-transfected with the patient's *HLA I* alleles and **FL**_*FLT3-ITD*. The clonal T cells ($5.0x10^3$/well) were added after 24 hours, and the ELISPOT assay was developed after 20 hours of incubation. Shown are the results of three representative T-cell clones (1A9, 2C4 and 1E10). The bars represent single values.

5.3 *In vitro* stimulation of CD8$^+$ T cells from the peripheral blood of healthy buffy coat donors against three FLT3-ITDs (VE, IN and QQ) known to be immunogenic in autologous settings

5.3.1 Short-term *in vitro* stimulation with FLT3-ITD IVT-mRNA

So far, six immunogenic FLT3-ITDs associated with distinct HLA I alleles have been identified in autologous systems (**Table 5.3.1.1**). Three immunogenic FLT3-ITDs, identified in patients **IN** (IN/583-CP-25)**, VE** (VE/585/RA-14) and **QQ** (QQ/591-D-7)**,** were chosen to analyze healthy donors for anti-FLT3-ITD responses. These FLT3-ITDs were known to be restricted by HLA-A*32:01, -B*27:05 and -A*11:01, respectively.

Patient model	FLT3-ITD sequence	HLA-
JC/591-9 **(JC)**	. VQVTGSSDNEYFYVDFREYEYYVDFREYEYDLKWEFPRENLE.	A*01:01
IN/583-CP-25 **(IN)**	. VQVTGSSDNEYFYVDFREYEYDLKWEFPRENC# #PSDNEYFYVDFREYEYDLKWEFPRENLE.	A*32:01 B*35:01†
VE/585-RA-14 **(VE)**	. VQVTGSSDNEYFYVDFREYER# #ASDNEYFYVDFREYEYDLKWEFPRENLE.	B*27:05
EI/586-QP-9 **(EI)**	. VQVTGSSDNEYFYVDFQPDNEYFYVDFREYEYDLKWEFPRENLE.	A*32:01
QQ/591-D-7 **(QQ)**	. VQVTGSSDNEYFYVDFREYDYVDFREYEYDLKWEFPRENLE.	A*11:01
FL/585-14 **(FL)**	. VQVTGSSDNEYFYVDFREYESDNEYFYVDFREYEYDLKWEFPRENLE.	A*01:01

Table 5.3.1.1: Immunogenic FLT3-ITDs and their restricting HLA I alleles as identified so far in autologous systems.
Duplications are marked in red and green. Four of the ITDs contained insertions of 3 to 6 base pairs (encoded amino acids in black) preceding the duplication. underlined: HLA I-restricted peptide sequences, †: Restriction allele observed in one sample of patient **IN** (Graf *et al.,* 2007; Graf and Singh *et al.,* unpublished.

In total, nineteen HCMV-seropositive donors were investigated for anti-FLT3-ITD response (**Table 3.9.2.1**). Out of nineteen donors, eight donors carrying HLA-A*32:01 were stimulated against **IN_**FLT3-ITD, six donors carrying HLA-A*11:01 were stimulated against **QQ_**FLT3-ITD, and five donors carrying HLA-B*27:05 were stimulated against **VE_**FLT3-ITD. For each healthy individual, 96 IVS-microcultures were generated in 96-well U-bottom plates. Microbead-isolated CD8$^+$ T cells (total 9.6x10^6 CD8$^+$ T cells/donor; 1.0x10^5 CD8$^+$ T cells/microculture) were stimulated at a 1:1 ratio with autologous irradiated CD8$^-$ PBMCs electroporated with the respective *Sig-FLT3-ITD* IVT-mRNA (for *FLT3-ITD* construct see **Materials section 3.3.1**). The *in vitro* stimulation procedure is schematically described in **Figure 5.3.1.1.** In each case, stimulations with *Sig-pp65* IVT-mRNA were performed in parallel. Since no antibody was available for protein encoded by FLT3$_{\text{Exon 13-16}}$ (~500 bp), which was used for *in vitro* stimulation, therefore, EGFP expression was used as a readout for the transfection efficiency in each electroporation of CD8$^-$ PBMCs. In seventeen out of nineteen donors, the mRNA transfection efficiencies were in between 70-98% (of CD14$^+$ cells expressing EGFP) (**Table 5.3.1.2**). In remaining two donors (#1066 and #7300) transfection efficiencies were less (in between 25-60%), this might be donor dependent.

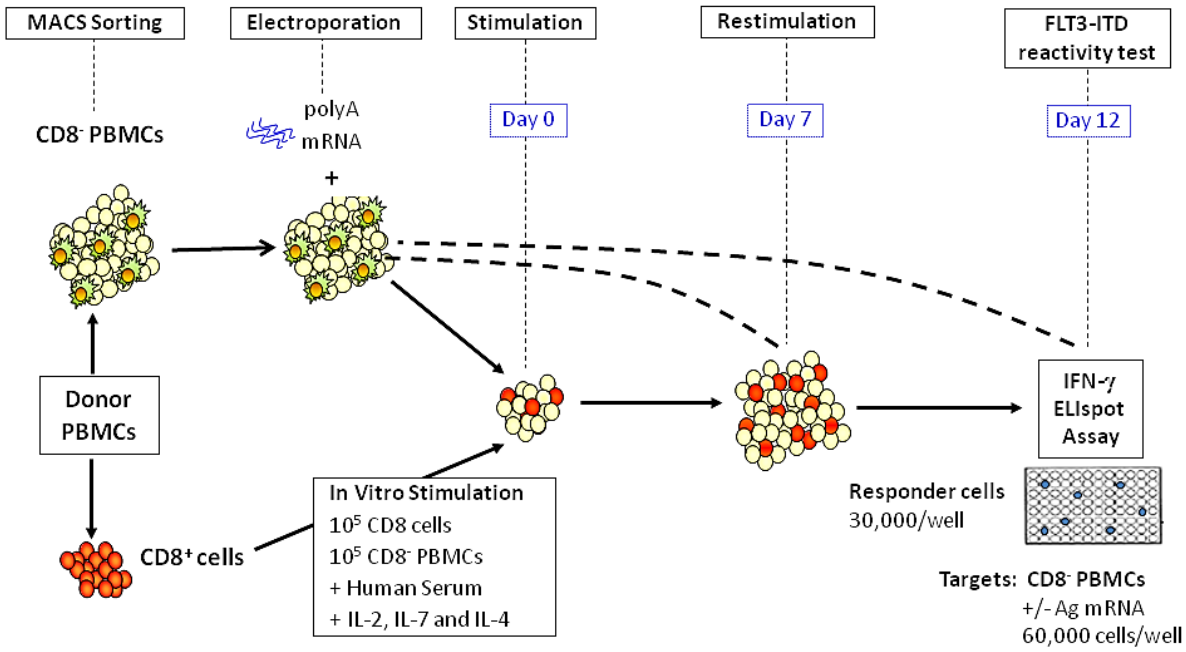


Figure 5.3.1.1: *In vitro* stimulation with FLT3-ITD-encoding IVT-mRNA.
Each donor carried the HLA I allele known to present a peptide from the respective stimulatory FLT3-ITD. In each donor $CD8^+$ T cells were stimulated with autologous irradiated $CD8^-$ PBMCs electroporated with an individual FLT3-ITD-encoding IVT-mRNA. *In vitro* stimulation was performed on d0 under given culture conditions and restimulated on d7 under same culture conditions (see **Method section 4.9.2**). On d12, responder cells ($3.0x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay for recognition of autologous $CD8^-$ PBMCs ($6.0x10^4$/well) electroporated with IVT-mRNA-encoding the respective stimulatory FLT3-ITD (see **Method section 4.15**). Stimulations with *pp65* IVT-mRNA and mock stimulations were performed in parallel.

Healthy donor	Electroporation efficiency: Percentages of $CD14^+$ cells expressing EGFP		
	1st stimulation (d0)	2nd stimulation (d7)	Screen test (d12)
# 6798	40.00	92.33	93.31
# 2655	94.53	89.91	93.99
# 2673	81.84	95.63	94.75
# 6647	94.19	90.61	89.35
# 2567	80.82	81.40	68.75
# 1066	33.40	26.10	47.80
# 1853	75.08	90.49	92.37
# 7300	25.30	30.10	57.60
# 1546	90.81	94.27	91.36
# 0147	93.68	90.77	97.04
# 2662	88.22	89.26	82.55
# 7986	93.82	86.92	88.76
# 1035	91.18	93.19	78.10
# 3501	71.38	97.65	97.65
# 1605	76.79	76.38	98.09
# 7926	94.86	90.12	87.03
# 1158	88.51	82.58	88.26
# 0189	69.89	94.23	87.96
# 1798	81.20	93.62	78.94

Table 5.3.1.2: IVT-mRNA transfection efficiencies of $CD8^-$ PBMCs.
During *in vitro* stimulation, donors $CD8^+$ T cells were stimulated with autologous $CD8^-$ PBMCs electroporated with selected *FLT3-ITD* IVT-mRNA. In each electroporation, $CD8^-$ PBMCs were also electroporated with *EGFP* IVT-mRNA (see **Method section 4.7**) and its expression was measured 18-24 hours after electroporation by flow cytometry (see **Method section 4.6**). In each donor, electroporation of $CD8^-$ PBMCs was performed on d0 and d7 and d12. Values represent the percentages of $CD14^+$ cells expressing EGFP. Yellow, turquoise and purple color donors were analyzed against **IN_**, **QQ_** and **VE_**FLT3-ITD, respectively.

Compared to EGFP (~900 bp) expression, few donors (#7926, #2662 and #0189) were also analyzed for expression of $FLT3_{full\text{-}length}$ after electroporation with IVT-mRNA-encoding full-length **IN**_*FLT3* (~3000 bp). It was observed that full-length FLT3 was expressed by $CD14^+$ cells of all three tested donors. The expression differences seen after EGFP and FLT3 electroporation might have been caused by the transfection conditions. The cells were transfected with equal amounts of IVT-mRNA (15 µg/10^6 cells). But it has to be considered that IVT-mRNA-encoding EGFP was three times smaller than IVT-mRNA for FLT3. As a consequence, it can be expected that transfected cells carried significantly more EGFP than FLT3 transcripts, which in turn resulted in a seemingly high transfection efficiency (**Figure 5.3.1.2**).

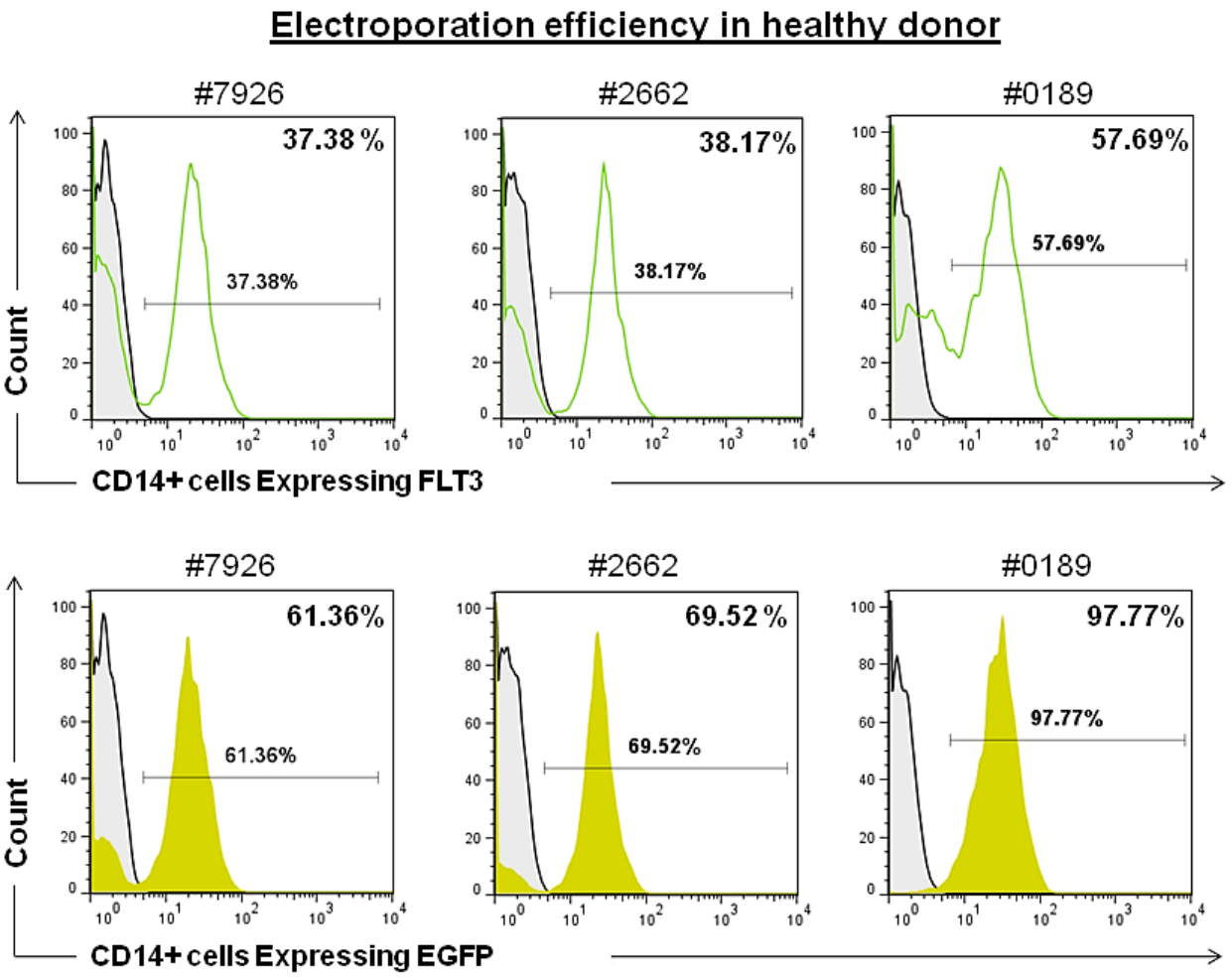
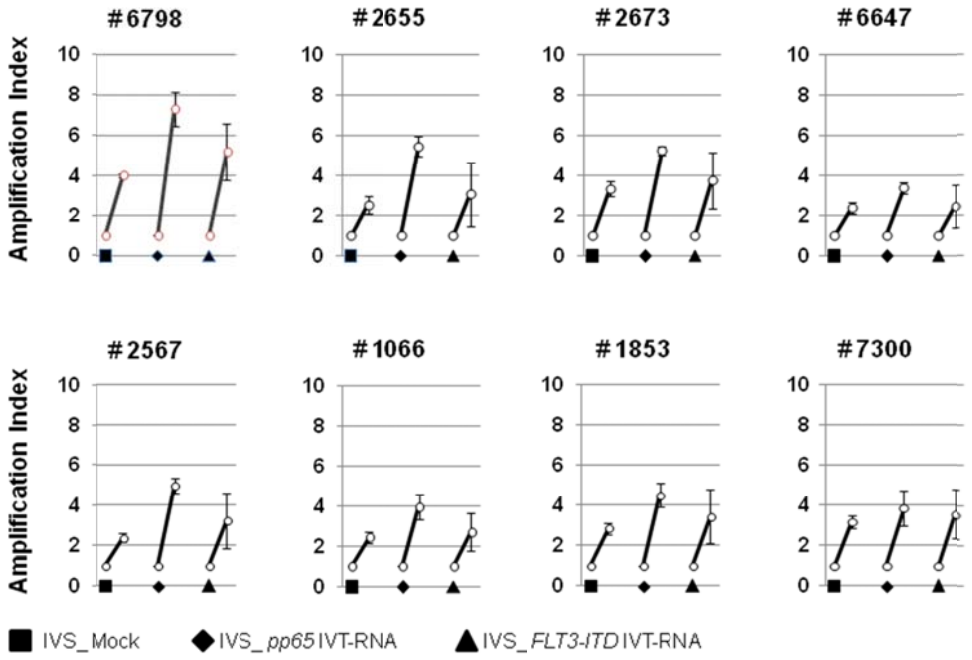


Figure 5.3.1.2: Efficient electroporation of IVT-mRNA-encoding full-length FLT3-ITD.
$CD8^-$ PBMCs, from healthy donors, #7926, #2662 and #0189, were electroporated with full-length *FLT3* IVT-mRNA (from patient **IN**) and *EGFP* IVT-mRNA at previously optimized condition (see **Method section 4.7**). 2.0x10^5 cells were taken into FACS tubes for flow cytometry analysis after 18 hours of electroporation. FLT3 electroporated cells were stained with anti-FLT3 antibody (sc480) and goat anti-rabbit IgG-$F(ab)_2$-antibody-FITC. Both EGFP- and FLT3-electroporated cells were also stained with CD14-PE. After staining, the cells were washed with FACS buffer and fixed with FACS fixation buffer. Cells were gated for CD14 expression.

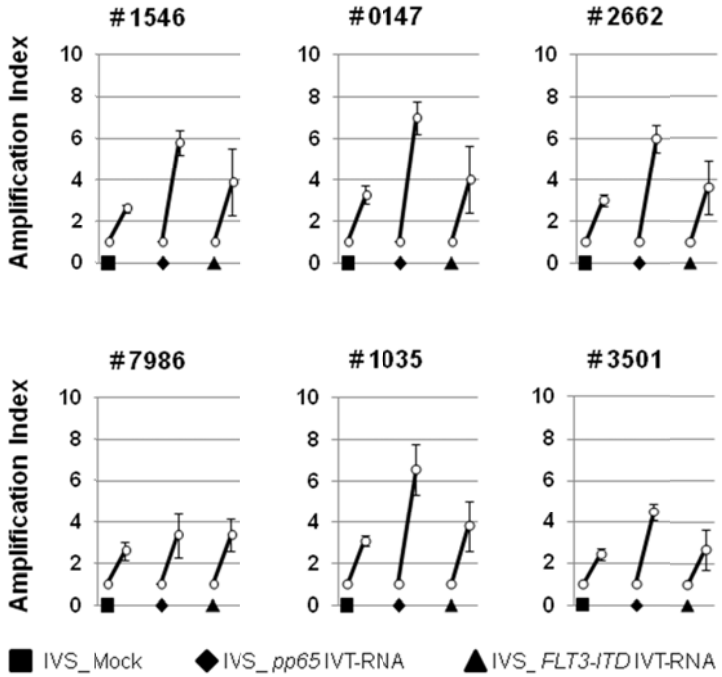
The proliferation behavior of *in vitro*-stimulated T-cell microcultures varied strongly (**Figure 5.3.1.3a, b** and **c**) even within the same donor. $CD8^+$ microcultures stimulated with *pp65* IVT-mRNA in most case revealed a somewhat better proliferation rate than the responders of

microcultures stimulated with *FLT3-ITD* IVT-mRNAs or responders of mock stimulation (APCs without RNA). In most of the donors, not much difference was observed in the proliferation rate of microculture responders stimulated against FLT3-ITD (mean of 96 microcultures) or responders of mock stimulation (mean of 2 or 4 microcultures).

a. HLA-A*32+ donors stimulated with IN_FLT3-ITD



b. HLA-A*11+ donors stimulated with QQ_FLT3-ITD



(*see figure legend on **page 68***)

c. HLA-B*27+ donors stimulated with VE_FLT3-ITD

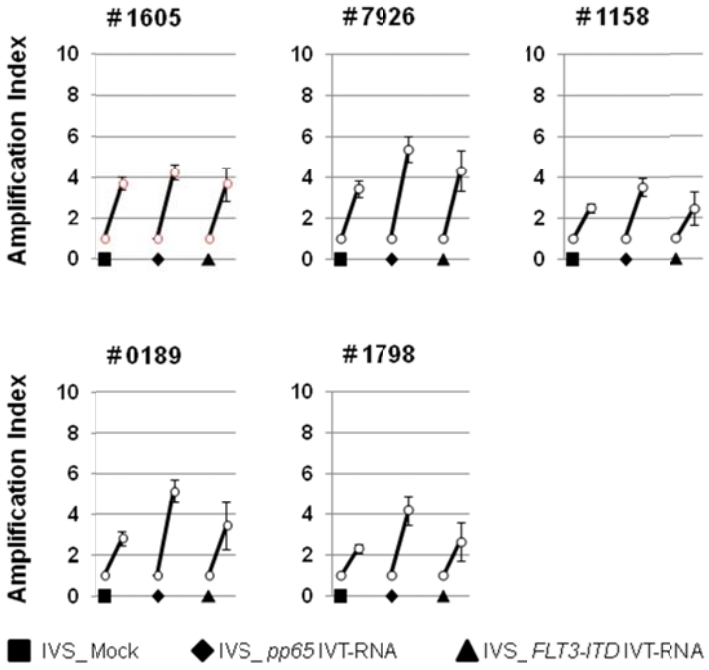


Figure 5.3.1.3: Proliferation of CD8$^+$ T cells after stimulation with *pp65* IVT-mRNA, with *FLT3-ITD* IVT-mRNA and after mock stimulation.
Healthy donor-derived CD8$^+$ T cells (1.0x10^5/well) were stimulated with CD8$^-$ PBMCs (1.0x10^5/well) electroporated with no IVT-mRNA, with *pp65* IVT-mRNA and with their respective *FLT3-ITD* IVT-mRNA (see **Method section 4.7, 4.9.2** and **Figure 5.3.1.1**). T cells were counted on d0, d7 and d12 of IVS-culture. The ratio of T-cell numbers at d12 to T-cell numbers at d0 represents the amplification index. These analyses were performed in **(a)** all eight donors stimulated against the FLT3-ITD of patient **IN**, **(b)** all six donors stimulated with the FLT3-ITD of patient **QQ** and **(c)** all five donors stimulated with FLT3-ITD of patient **VE**. Data represents the means of 2 or 4 microcultures of mock/pp65 stimulation and means of 96 microcultures of FLT3-ITD stimulations. ±SD.

On d12, the responder T cells were tested in an IFN-γ ELISPOT assay for recognition of autologous CD8$^-$ PBMCs electroporated with IVT-mRNA-encoding the respective *Sig-FLT3-ITD* against which they had been stimulated. FLT3-ITD-reactivity was observed, in few microcultures, of eight different donors against all the three selected FLT3-ITDs. Responders in a given microculture were defined as ‘‘positive’’ when the mean number of spot-forming T cells against the respective FLT3-ITD was either 2-fold higher than the background reactivity (baseline response against antigen-negative target cells only) or higher than the mean background plus two-fold standard deviations. In eight donors, IFN-γ spot numbers in few positive microcultures varied from 2- to 4-fold above background reactivity (**Table 5.3.1.3**). As a control for successful *in vitro* stimulation conditions, anti-pp65-reactivity was observed in all the healthy donors tested (**Table 5.3.1.3**).

Healthy donor	FLT3-ITD model	IFN-γ ELISpot assay (Day 12 screen test)	
		Anti-pp65	Anti- FLT3-ITD
# 6798	IN	+	+
# 2673	IN	+	+
# 1853	IN	+	+
# 0147	QQ	+	+
# 2662	QQ	+	+
# 1035	QQ	+	+
# 7926	VE	+	+
# 0189	VE	+	+

Table 5.3.3.1: Healthy donor-derived CD8$^+$ T cells respond to FLT3-ITDs and pp65/CMV.
CD8$^+$ T cells of healthy donors were stimulated with autologous CD8$^-$ PBMCs electroporated with IVT-mRNA-encoding the selected FLT3-ITDs or with *pp65* IVT-mRNA (see **Method section 4.9.2** and **Figure 5.3.1.1**). On d12, eight out of nineteen donors showed CD8$^+$ T-cell responses against **IN_**, **QQ_** and **VE_**FLT3-ITD. Anti-pp65 responses were observed in all of the donors.

When reactivity against FLT3-ITD was observed in healthy donors, only few out of 96 microcultures were reactive against the respective stimulatory FLT3-ITD. Best positive microcultures with reactivity against **IN_**FLT3-ITD were observed in donors #6798 (~4-fold above background), #2673 (~3-fold above background) and #1853 (~2-fold above background) (**Figure 5.3.1.4**). **QQ_**FLT3-ITD-specific responses were detected in donors #0147 (~3-fold above background), #2662 (~3-fold above background) and #1035 (~2-fold above background) (**Figure 5.3.1.5**). **VE_**FLT3-ITD-specific responses were observed in donor #7926 (~2-fold above background) and #0189 (~2-fold above background) (**Figure 5.3.1.6**).

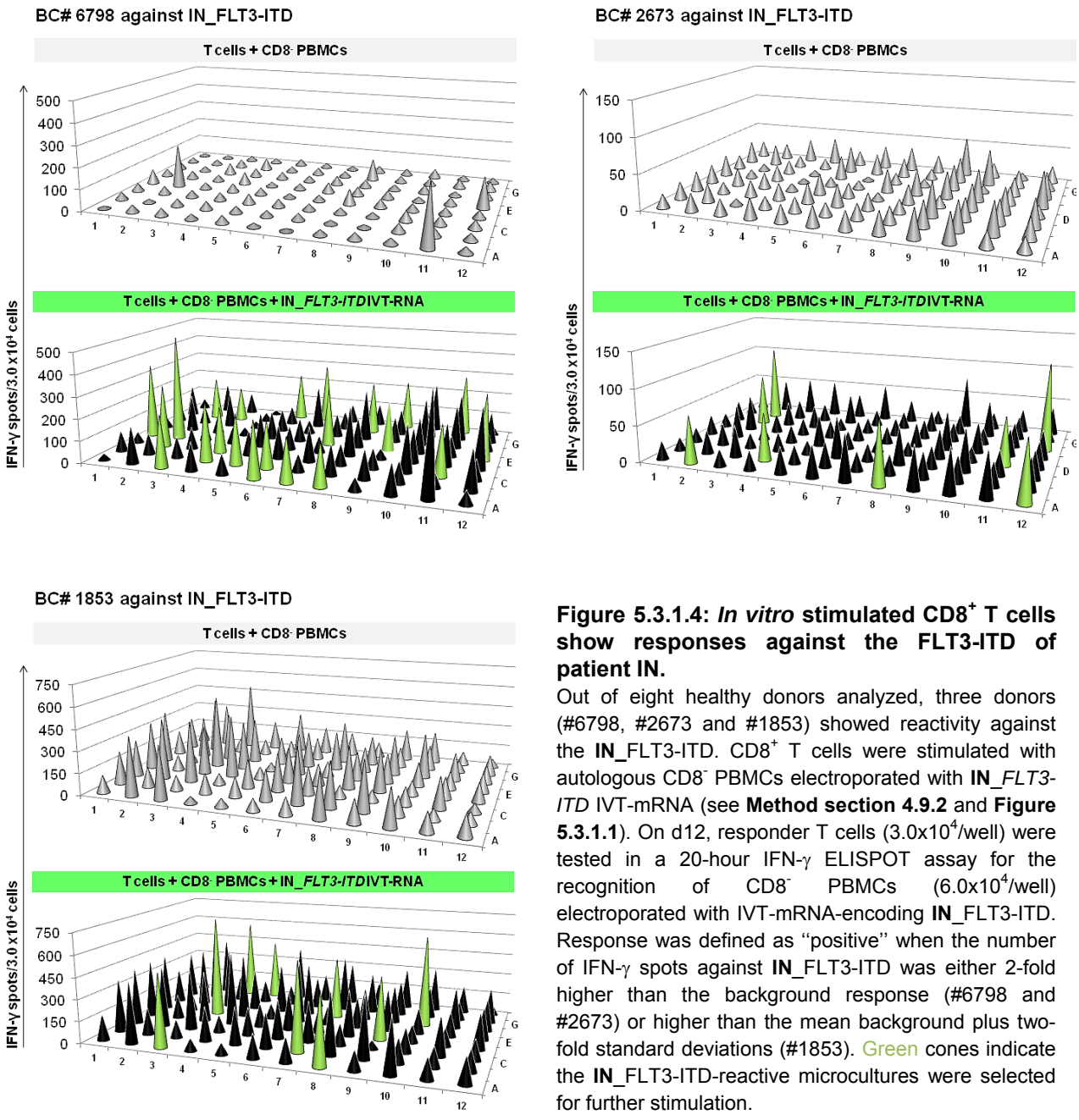


Figure 5.3.1.4: *In vitro* stimulated CD8$^+$ T cells show responses against the FLT3-ITD of patient IN.
Out of eight healthy donors analyzed, three donors (#6798, #2673 and #1853) showed reactivity against the **IN_**FLT3-ITD. CD8$^+$ T cells were stimulated with autologous CD8$^-$ PBMCs electroporated with **IN_***FLT3-ITD* IVT-mRNA (see **Method section 4.9.2** and **Figure 5.3.1.1**). On d12, responder T cells (3.0x10^4/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of CD8$^-$ PBMCs (6.0x10^4/well) electroporated with IVT-mRNA-encoding **IN_**FLT3-ITD. Response was defined as ‘‘positive’’ when the number of IFN-γ spots against **IN_**FLT3-ITD was either 2-fold higher than the background response (#6798 and #2673) or higher than the mean background plus two-fold standard deviations (#1853). Green cones indicate the **IN_**FLT3-ITD-reactive microcultures were selected for further stimulation.

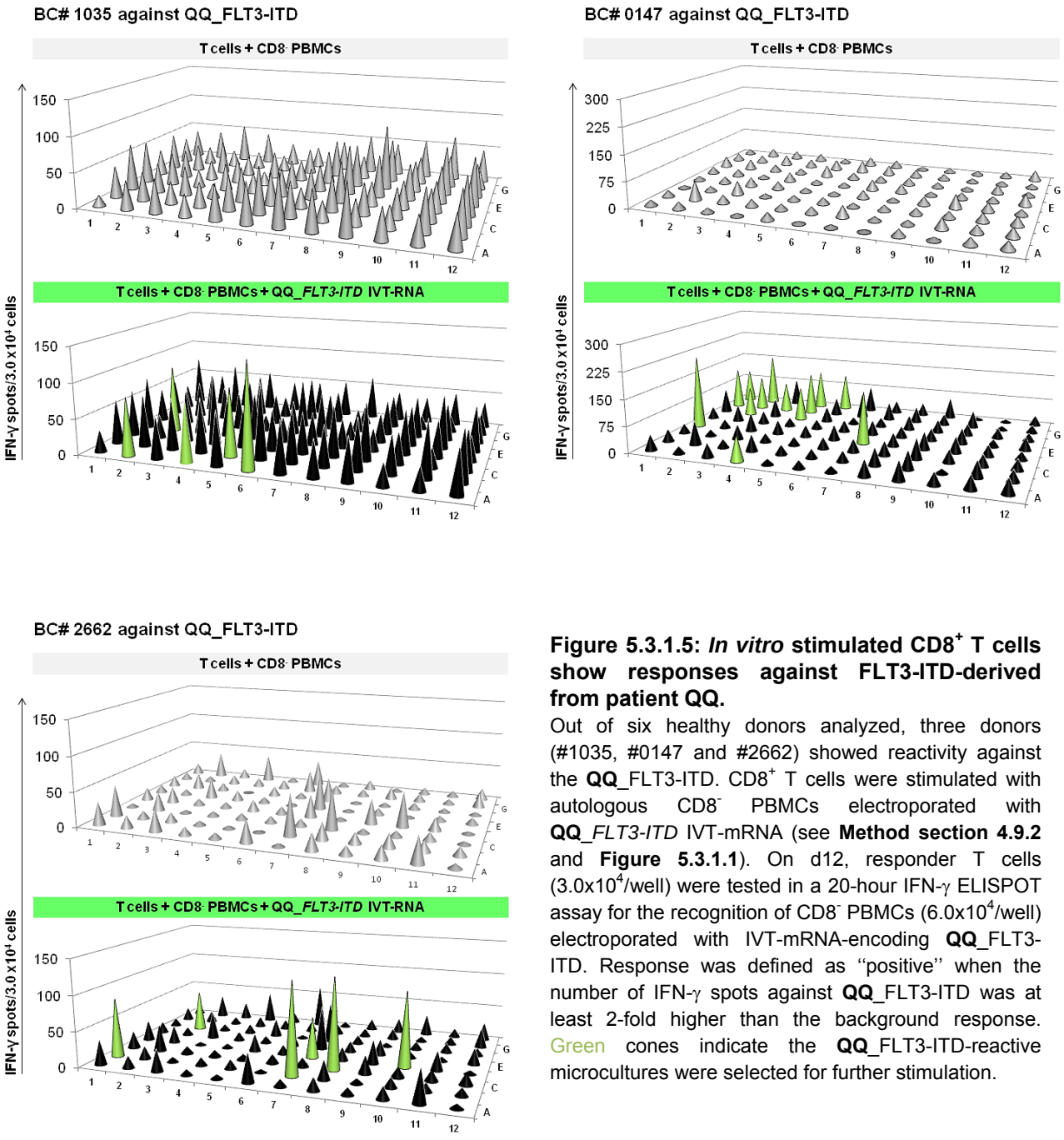


Figure 5.3.1.5: *In vitro* stimulated CD8$^+$ T cells show responses against FLT3-ITD-derived from patient QQ.

Out of six healthy donors analyzed, three donors (#1035, #0147 and #2662) showed reactivity against the **QQ**_FLT3-ITD. CD8$^+$ T cells were stimulated with autologous CD8$^-$ PBMCs electroporated with **QQ**_*FLT3-ITD* IVT-mRNA (see **Method section 4.9.2** and **Figure 5.3.1.1**). On d12, responder T cells ($3.0x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of CD8$^-$ PBMCs ($6.0x10^4$/well) electroporated with IVT-mRNA-encoding **QQ**_FLT3-ITD. Response was defined as ''positive'' when the number of IFN-γ spots against **QQ**_FLT3-ITD was at least 2-fold higher than the background response. Green cones indicate the **QQ**_FLT3-ITD-reactive microcultures were selected for further stimulation.

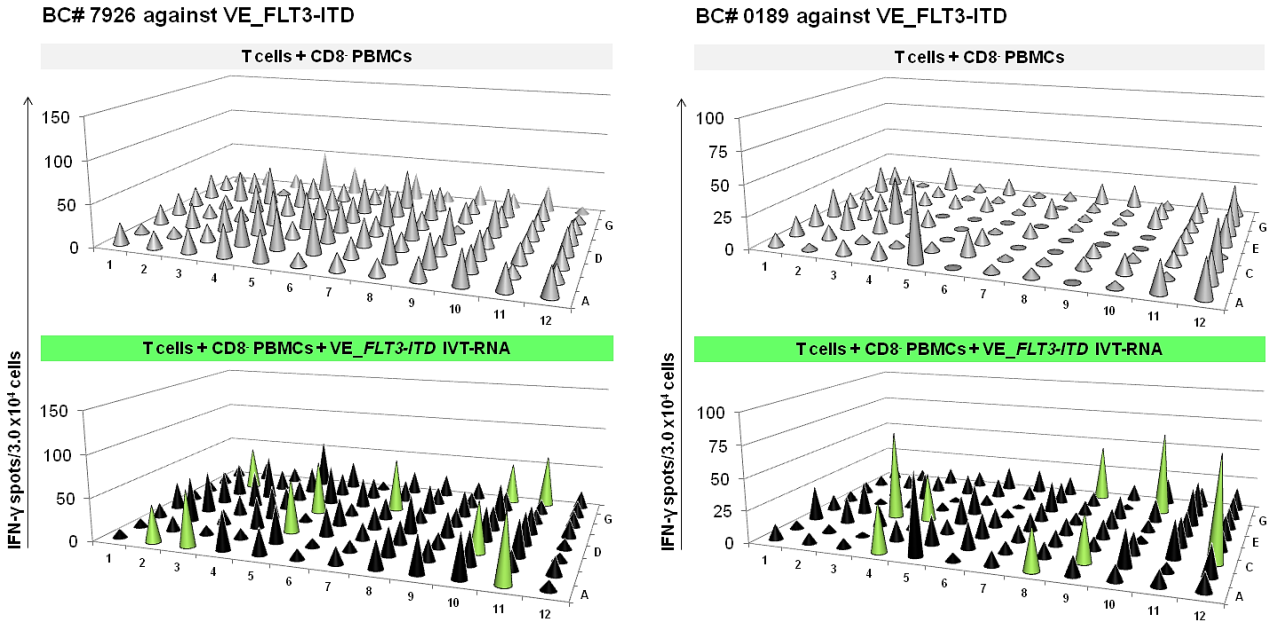
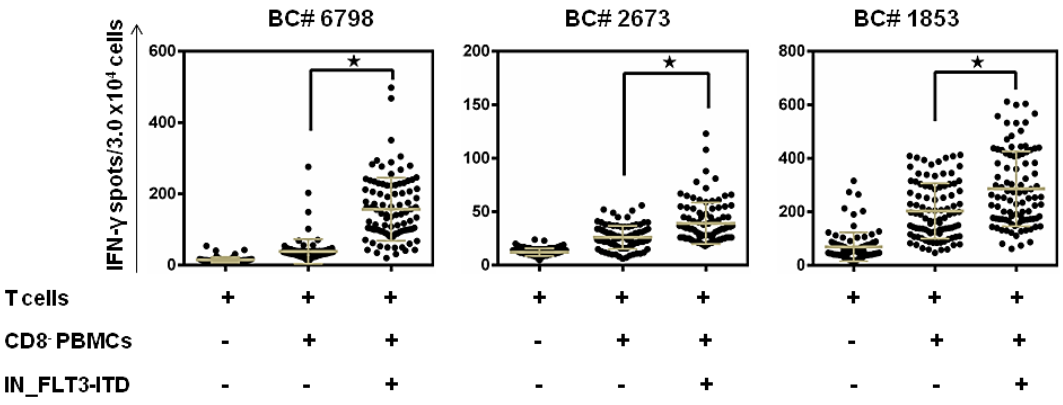


Figure 5.3.1.6: *In vitro* stimulated CD8$^+$ T cells show responses against FLT3-ITD-derived from patient VE.
Out of five healthy donors analyzed, two donors (#7926 and #0189) showed reactivity against the **VE**_FLT3-ITD. CD8$^+$ T cells were stimulated with autologous CD8$^-$ PBMCs electroporated with **VE**_*FLT3-ITD* IVT-mRNA (see **Method section 4.9.2** and **Figure 5.3.1.1**). On d12, responder T cells (3.0x10^4/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of CD8$^-$ PBMCs (6.0x10^4/well) electroporated with IVT-mRNA-encoding **VE**_FLT3-ITD. Response was defined as ''positive'' when the number of IFN-γ spots against **VE**_FLT3-ITD was at least 2-fold higher than the background response. Green cones indicate the **VE**_FLT3-ITD-reactive microcultures were selected for further stimulation.

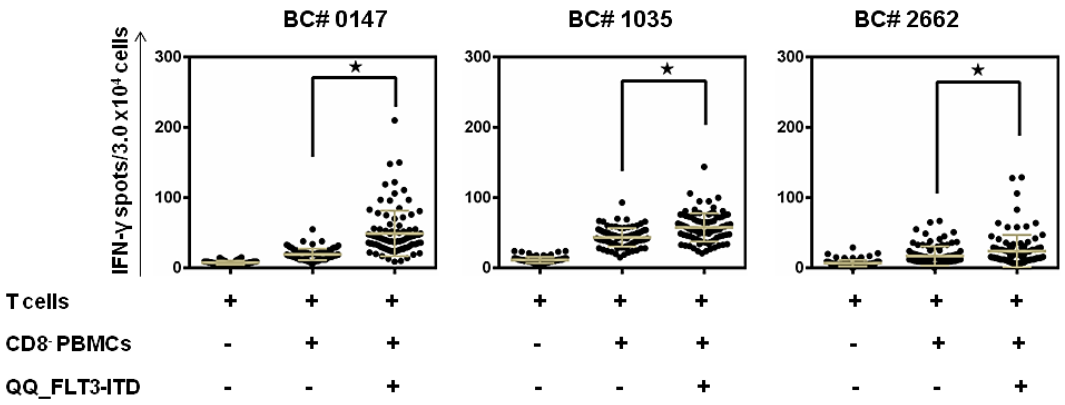
The probable frequency of precursor T cells in the original sample was estimated. Estimation is based on the lowest possible dilution (i.e. one FLT3-ITD-specific T-cell per reactive microculture) from which FLT3-ITD-reactive T cells could be enriched after mRNA stimulation. The frequencies of ITD-reactive T cells were then estimated to be in the range of 1.25x10^{-6} to 2.83x10^{-7} (**Table 5.3.2.1**), whereby a total of 9.6x10^6 CD8$^+$ T cells from each healthy donor have been analyzed (**Figure 5.3.1.7**).

a. HLA-A*32+ donors stimulated with IN_FLT3-ITD



(*see figure legend on **page 72***)

b. HLA-A*11+ donors stimulated with QQ_FLT3-ITD



c. HLA-B*27+ donors stimulated with VE_FLT3-ITD

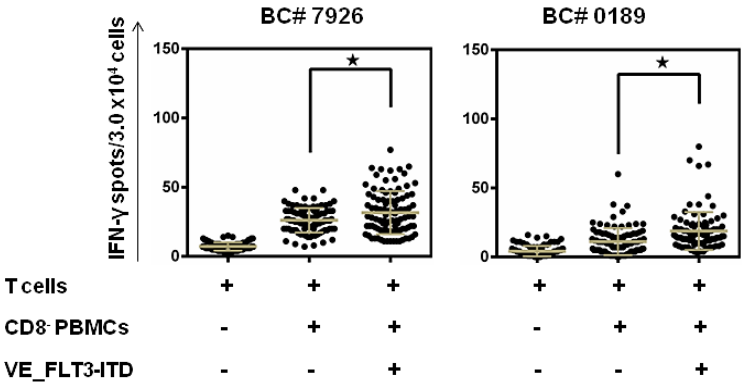


Figure 5.3.1.7: Anti-FLT3-ITD responses in healthy donors (shown are 8 healthy donors with FLT3-ITD-reactivity).
$CD8^+$ T cells from indicated healthy buffy coat donors were stimulated with autologous $CD8^-$ PBMCs electroporated with IVT-mRNA-encoding selected FLT3-ITDs (**IN, QQ** and **VE**) (see **Method section 4.9.2** and **Figure 5.3.1.1**). On d12, responder T cells ($3.0x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of $CD8^-$ PBMCs ($6.0x10^4$/well) expressing the respective stimulatory FLT3-ITDs after transfection with IVT-mRNA. Each dot represents the spot number induced by a single microculture. The green lines indicate the means of 96 IVS-microcultures ±SD. These data were analyzed for statistical difference determined by a two-tailed, unpaired Student´s t-test and the * p-values (<0.05) indicate the significant $CD8^+$ T-cell response against stimulatory FLT3-ITDs.

5.3.2 Long-term stimulation with FLT3-ITD IVT-mRNA

When buffy coat cells were available in sufficient quantities, further restimulations were performed for FLT3-ITD-reactive microcultures. They were restimulated at least twice using either autologous $CD8^-$ PBMCs or monocytes (adherent cells) electroporated with *FLT3-ITD* IVT-mRNA as described in **Method section 4.9.2**. Responding microcultures were retested on d26 or at later time points in an IFN-γ ELISPOT assay as described in **Method section 4.15**. In four out of eight donors ITD-reactive T cells could be propagated over two or more stimulation periods (**Table 5.3.2.1**). **IN_**FLT3-ITD-specific responses were further enriched with RNA-electroporated $CD8^-$ PBMCs and monocytes in donor #1853 (**Figure 5.3.2.1**) and **VE_**FLT3-ITD-specific responses were further enriched with RNA-electroporated monocytes in donor #7926 (**Figure 5.3.2.2**). **QQ_**FLT3-ITD-specific responses were further enriched in donor #2662 and #0147 using monocytes and $CD8^-$ PBMCs, respectively (**Figure 5.3.2.3a** and **5.3.2.3b**). As a

control, anti-pp65-reactivity could also be propagated using the same APCs as used for restimulation of FLT3-ITD-reactive microcultures in all of the four FLT3-ITD-reactive healthy donors tested (**Table 5.3.2.1** and **Figure 5.3.2.4**).

Healthy Donor (BC)	FLT3-ITD From patient	Screening test (Day 12)		Retest of ITD-reactivity (Day 26 or later)		Estimated frequencies of FLT3-ITD-reactive CD8+ T cells
		pp65	FLT3-ITD	pp65	FLT3-ITD	
#6798	IN	+	+	Not tested	Not tested	Not tested
#2673	IN	+	+	+	-	Not propagated
#1853	IN	+	+	+	+	2.83×10^{-7}
#0147	QQ	+	+	+	+	6.25×10^{-7}
#2662	QQ	+	+	+	+	8.33×10^{-7}
#1035	QQ	+	+	+	-	Not propagated
#7926	VE	+	+	+	+	1.25×10^{-6}
#0189	VE	+	+	+	-	Not propagated

Table 5.3.2.1: FLT3-ITD-reactive T cells can be enriched by further restimulations.
In eight donors with reactivity against **IN_**, **QQ_** and **VE_**FLT3-ITD, respectively (**Table 5.3.1.3**), FLT3-ITD-reactive microcultures were restimulated at least twice using either autologous $CD8^-$ PBMCs or monocytes (adherent cells) electroporated with *FLT3-ITD* IVT-mRNA as described in **Method section 4.9.2**. Responding microcultures were retested on d26 or at later time points in an IFN-γ ELISPOT assay. In four out of eight donors (#1853, #0147, #2662 and #7926), FLT3-ITD-reactive T cells could be propagated. Given values are estimated anti-FLT3-ITD T-cell frequencies calculated and described as in **Result section 5.3.1**. Yellow, turquoise and purple colored donors were positive against **IN_**, **QQ_** and **VE_**FLT3-ITD, respectively.

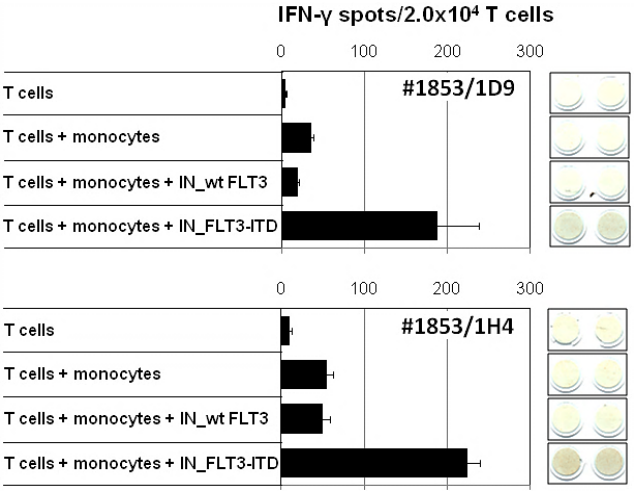


Figure 5.3.2.1: Confirmation of IN_FLT3-ITD-reactivity.
IN_FLT3-ITD-reactive microcultures 1D9 and 1H4 derived from donor #1853 (see **Figure 5.3.1.4**) were further restimulated twice with $CD8^-$ PBMCs and once with monocytes electroporated with IVT-mRNA-encoding **IN_**FLT3-ITD. On d40, responder T cells (2.0×10^4/well) were tested in a 20-hour IFN-γ ELISPOT assay. Monocytes (1.0×10^4/well), electroporated with IVT-mRNA-encoding **IN_**FLT3-ITD or wt-FLT3, were used as target cells. These two were the representative microcultures (from #1853) from which ITD-reactive T cells can be further enriched. The bars represent means of duplicates ±SD.

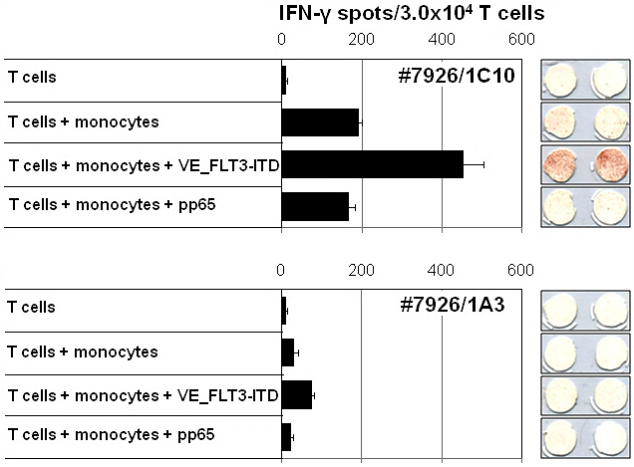


Figure 5.3.2.2: Confirmation of VE_FLT3-ITD-reactivity.
VE_FLT3-ITD-reactive microcultures 1A3 and 1C10 derived from donor #7926, (see **Figure 5.3.1.6**) were further restimulated twice with monocytes electroporated with IVT-mRNA-encoding **VE_**FLT3-ITD. On d26, responder T cells (3.0×10^4/well) were tested in a 20-hour IFN-γ ELISPOT assay. Monocytes (1.0×10^4/well) electroporated with IVT-mRNA-encoding **VE_**FLT3-ITD, were used as target cells. These two were the representative microcultures (from #7926) from which ITD-reactive T cells can be further enriched. The bars represent means of duplicates ±SD.

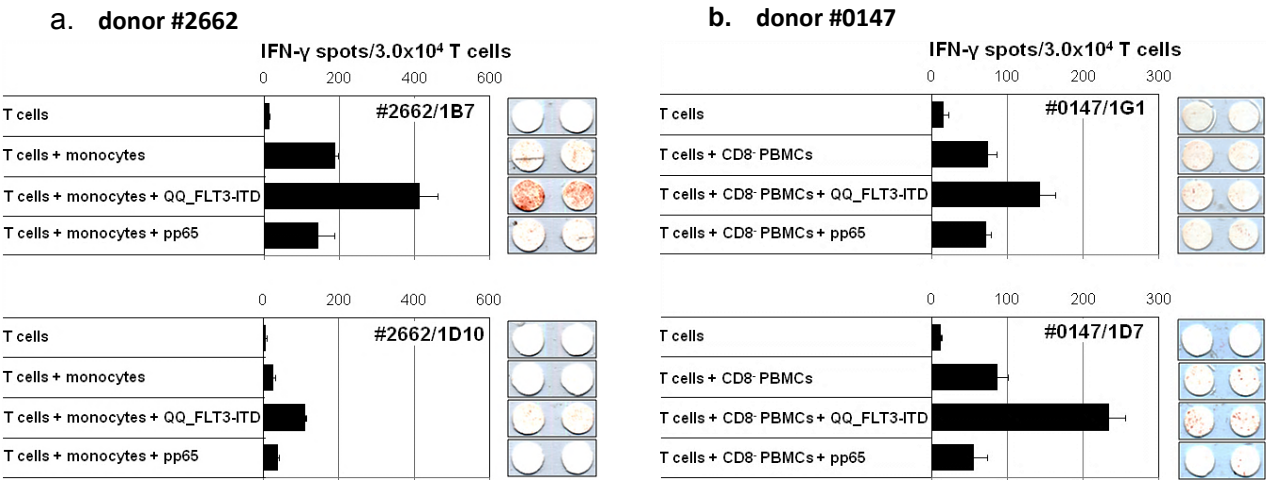


Figure 5.3.2.3: Confirmation of QQ_FLT3-ITD-reactivity.
(a) QQ_FLT3-ITD-reactive microcultures 1B7 and 1D10 derived from donor #2662 (see **Figure 5.3.1.5)** were further restimulated twice with monocytes electroporated with IVT-mRNA-encoding **QQ**_FLT3-ITD. On d26, responder T cells ($3.0x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay. Monocytes ($1.5x10^4$/well) electroporated with IVT-mRNA-encoding **QQ**_FLT3-ITD, were used as target cells. **(b) QQ**_FLT3-ITD-reactive microcultures 1D7 and 1G1 derived from donor #0147 (see **Figure 5.3.1.5**) were further restimulated twice with $CD8^-$ PBMCs electroporated with IVT-mRNA-encoding **QQ**_FLT3-ITD. On d26, responder T cells ($3.0x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay. $CD8^-$ PBMCs ($6.0x10^4$/well), were electroporated with IVT-mRNA-encoding **QQ**_FLT3-ITD, were used as target cells. Shown are the **QQ**_FLT3-ITD-reactive representative T-cell microcultures, from donor #2662 (1B7 and 1D10) and, from donor #0147 (1G1 and 1D7). The bars represent means of duplicates ±SD.

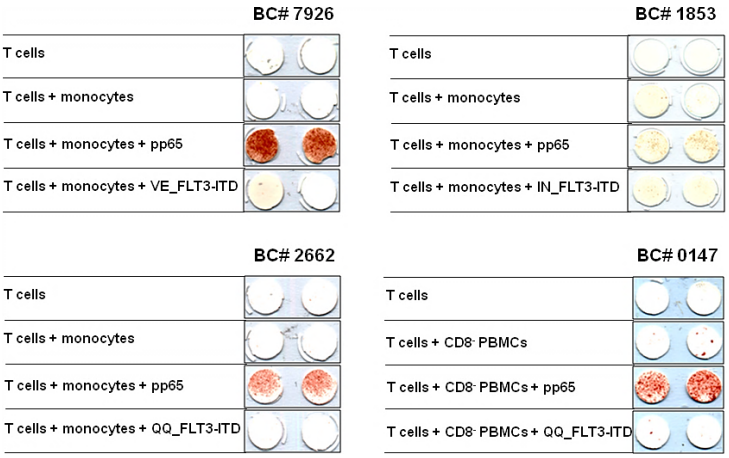


Figure 5.3.2.4: Confirmation of pp65/CMV-reactivity.
pp65-reactive microcultures from indicated FLT3-ITD-reactive donors were restimulated using same APCs as used for restimulation of FLT3-ITD-reactive microcultures (see **Table 5.3.2.1**) that were electroporated with pp65-encoding IVT-mRNA. Responding T cells ($2.0\text{-}3.0x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of either monocytes ($1.0\text{-}1.5x10^4$/well) or $CD8^-$ PBMCs ($6.0x10^4$/well) electroporated with IVT-mRNA-encoding pp65 or the respective FLT3-ITD

Shown are the representative pp65-reactive microcultures from FLT3-ITD-reactive donors #7926, #1853, #2662 and #0147 (**Table 5.3.1.3** and **Table 5.3.2.1**).

To assess their HLA I–restriction, responder T cells from d26 or later were tested for the recognition of COS-7 cells, co-transfected with the respective FLT3-ITD and all of the respective donor´s HLA I alleles, chosen according to the serotyping and to the frequencies of HLA suballeles in the German population. Restricting HLA I alleles were identified for **IN**_FLT3-ITD and **VE**_FLT3-ITD, but not for **QQ**_FLT3-ITD. In donor #1853, the **IN**_FLT3-ITD-specific T-cell population of microculture 1H4 was restricted via HLA-B*35:01 (**Figure 5.3.2.5**). HLA-B*35:01-restricted T-cell responses could be further enriched after two more restimulations with autologous monocytes electroporated with IVT-mRNA encoding **IN**_FLT3-ITD. On d40 and d47 1H4 responders were also tested for the recognition of the 9-mer peptide **CPSDNEYFY,** that

was available in the lab, was predicted to bind to HLA-B*35:01 and had been used in the analysis of autologous T cells in patient **IN.** Recognition of 9-mer peptide was clearly inferior compared to recognition of **IN**_*FLT3-ITD* fragment (**Figure 5.3.2.6**). This is why; cDNA fragmentation was performed to identify the true peptide (see **Result section 5.3.4**). In donor #7926, the **VE_**FLT3-ITD-specific T cells were restricted via HLA-C*07:02 (**Figure 5.3.2.7**).

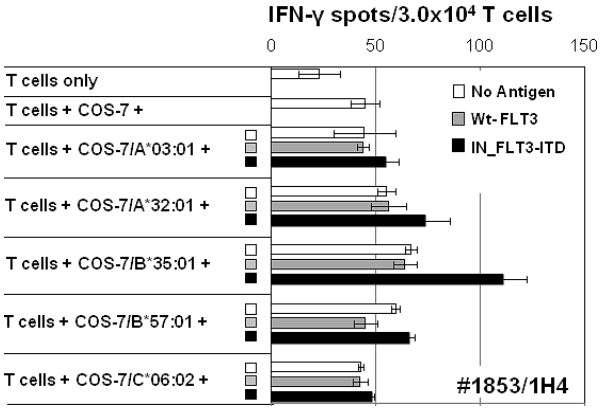


Figure 5.3.2.5: Microculture #1853/1H4 responders recognize IN_FLT3-ITD via HLA-B*35:01.
On d26, 1H4 responder T cells ($3.0x10^4$/well) from donor #1853 were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of COS-7 cells ($2.0x10^4$/well) co-transfected with the donor's *HLA I* alleles (predicted by serotyping) and with *wt-FLT3* or **IN**_*FLT3-ITD* cDNA, respectively. The bars represent means of duplicates ±SD.

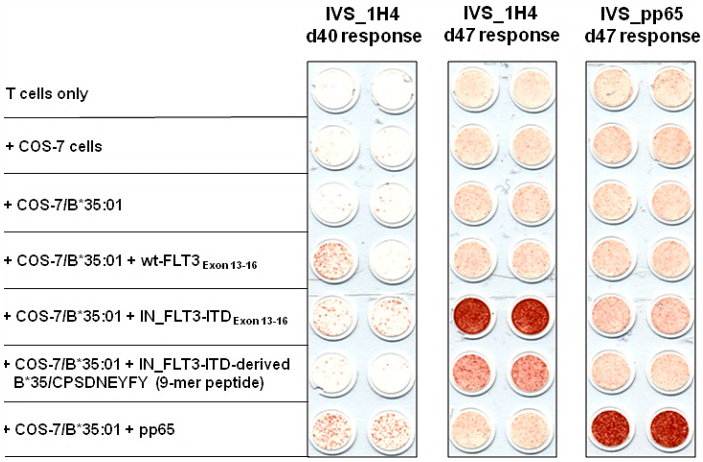


Figure 5.3.2.6: Enrichment of IN_FLT3-ITD-specific T cells restricted by HLA-B*35:01.
#1853/1H4 responders were further restimulated twice with monocytes expressing **IN**_FLT3-ITD. On d40 and d47, responders were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of COS-7 cells co-transfected with *HLA-B*35:01* cDNA ($2.0x10^4$/well) and wt-*FLT3*, **IN**_*FLT3-ITD* or *pp65* cDNA. Alternatively, COS-7 cells transfected with *HLA-B*35:01* cDNA were loaded with peptide CPSDNEYFY (10 µg/ml) encoded by the **IN**-FLT3-ITD.

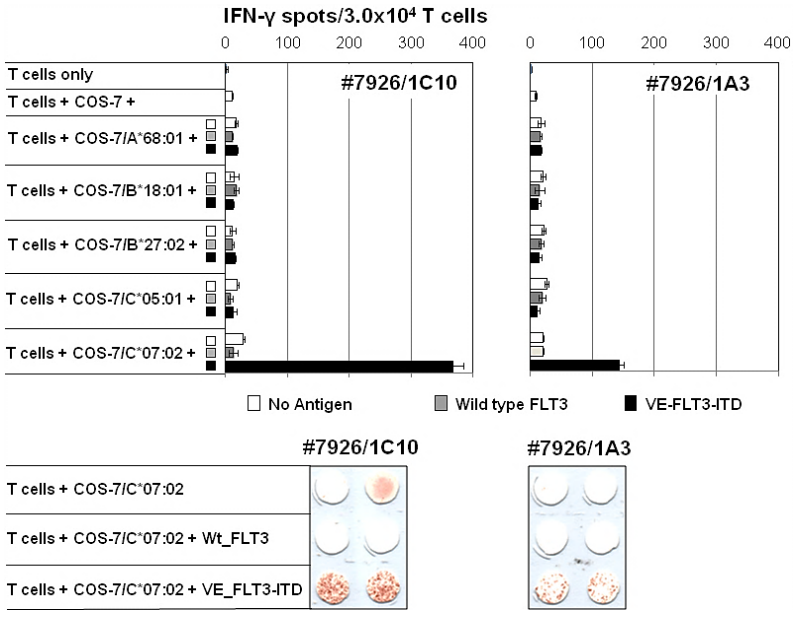


Figure 5.3.2.7: Microcultures from donor #7926 recognize VE_FLT3-ITD via HLA-C*07:02.
On d26, 1C10 and 1A3 responder T cells ($3.0x10^4$/well) from donor #7926 (see **Figure 5.3.2.2**) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of COS-7 cells ($2.0x10^4$/well) co-transfected with the donors' *HLA I* alleles and either with *wt-FLT3* or with **VE**_*FLT3-ITD*. Equivalent data were obtained with 2 further microcultures of the same model system. The bars represent means of duplicates ±SD. In the lower part ELISPOT filter scans are shown.

5.3.3 Efforts to generate a stable FLT3-ITD-reactive T-cell population from healthy donor #7926

Microcultures 1A3 and 1C10 generated from BC donor #7926 showed clear reactivity against **VE_**FLT3-ITD (**Figure 5.3.2.7**). These two microcultures were frozen on d33 for further studies. It was planned to use them for the generation of stable **VE_**FLT3-ITD-reactive T-cell populations. Fortunately, 8 months after the initial analysis, it was possible to get again a buffy coat from donor #7926. At first, it seemed preferable to expand the **VE_**FLT3-ITD-reactive T cells to have enough T cells for FACS sorting of ITD-specific T cells. For that purpose, microcultures 1A3 and 1C10 were thawed and pooled for the *in vitro* stimulation and expansion performed as before (see **Method section 4.9.2** and **Result section 5.3.1**). In each microculture, $1.0x10^5$ responder T cells were restimulated twice at a ratio of 1:1 with autologous irradiated $CD8^-$ PBMCs electroporated with IVT-mRNA-encoding **VE_**FLT3-ITD. Responder T cells were tested on d45 (d33+12) in an IFN-γ ELISPOT assay for the recognition of autologous $CD8^-$ PBMCs expressing **VE_**FLT3-ITD. Undoubtedly, a clear enrichment of **VE_**FLT3-ITD-specific T cells was observed (**Figure 5.3.3.1**).

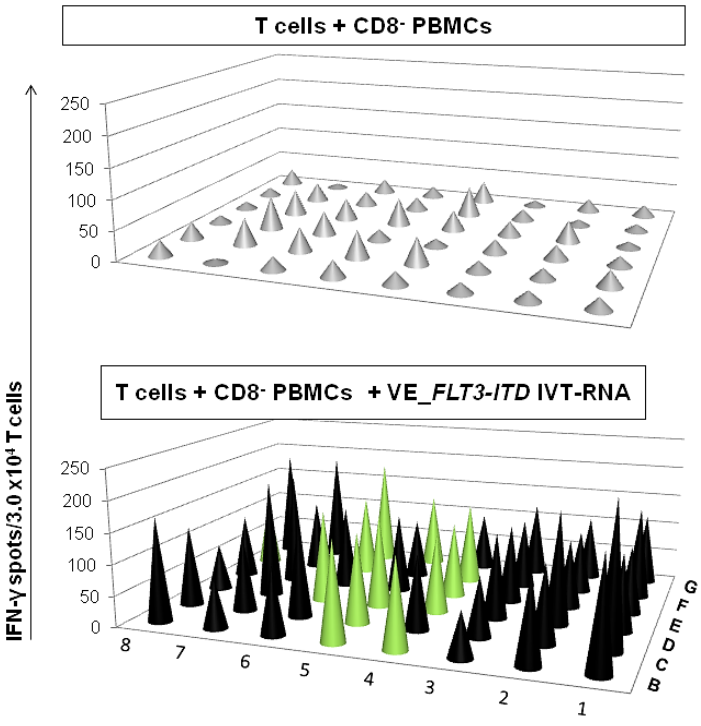


Figure 5.3.3.1: Enrichment of VE_FLT3-ITD-specific responses after additional stimulations
Pooled responders of d33 microcultures #7926/1A3 and /1C10 were restimulated twice using autologous $CD8^-$ PBMCs as APCs after electroporation with **VE**_*FLT3-ITD* IVT-mRNA. On d45 (d33+12) responder T cells ($3.0x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of autologous $CD8^-$ PBMCs ($6.0x10^4$/well) expressing the **VE_**FLT3-ITD antigen after RNA electroporation. Microcultures shown in green bars were selected for further *in vitro* stimulation.

Out of 48 microcultures, 12 microcultures showing the strong reactivity (**Figure 5.3.3.1**), were restimulated once more under identical culture conditions. On d52 responders were tested again for their reactivity against **VE_**FLT3-ITD. Out of 12 microcultures, three were found to be strongly ITD-reactive with microculture 1B5 showing the strongest enrichment (~20-fold enrichment) (**Figure 5.3.3.2**).

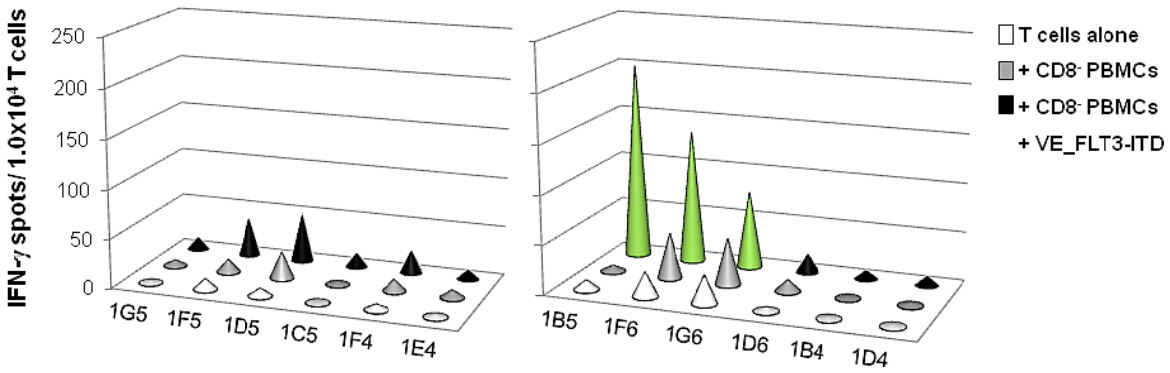


Figure 5.3.3.2: Further stimulation of microcultures descending from pooled #7926/1A3 and /1C10 (see Figure 5.3.3.1).
d52 (d33+19) responder T cells (1.0x10^4/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of autologous CD8$^-$ PBMCs (6.0x10^4/well) expressing **VE**_FLT3-ITD after RNA electroporation. Microcultures shown in green bars were used for the IFN-γ secretion assay.

Microcultures 1B5, 1F6 and 1G6 (**Figure 5.3.3.2**) were selected for the IFN-γ secretion assay (see **Method section 4.12**) and to purify **VE_**FLT3-ITD-specific T cells by FACS sorting. 1.0%, 0.5% and 0.4% IFN-γ-secreting CD8$^+$ **VE_**FLT3-ITD-reactive T cells were isolated from microculture 1B5, 1F6 and 1G6, respectively (**Figure 5.3.3.3**).

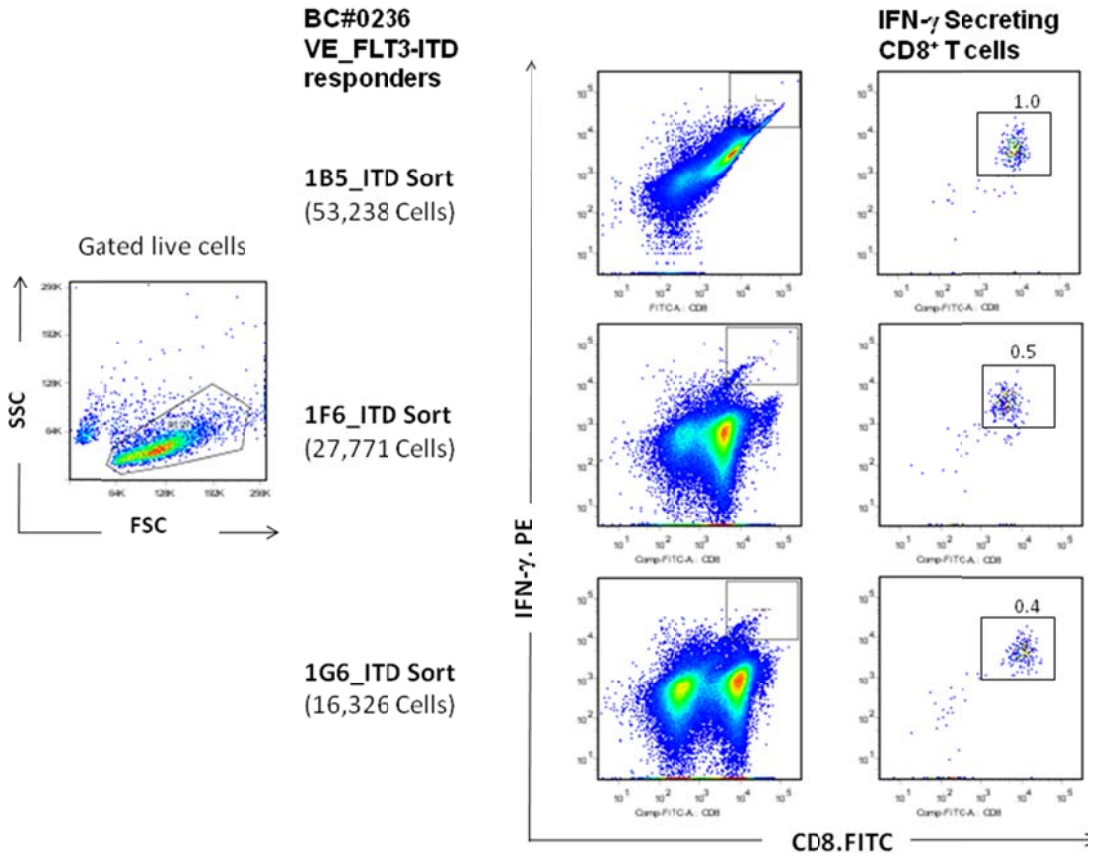


Figure 5.3.3.3: FACS sorting of VE_FLT3-ITD-reactive T cells using IFN-γ secretion assay.
In vitro stimulated d54 responder T cells from microcultures 1B5, 1F6 and 1G6 were stimulated for 12 hours at a 1:1 ratio with irradiated autologous CD8$^-$ PBMCs electroporated with IVT-mRNA-encoding **VE**_FLT3-ITD. After 12 hours, the IFN-γ secretion assay was performed to stain the IFN-γ$^+$ **VE**_FLT3-ITD-specific CD8$^+$ T cells. FACS sorting of stimulated T cells resulted in 53,238 putative FLT3-ITD-specific T cells (~1.0%) from **1B5 (**5.3x10^6 cells), 27,771 cells (~0.5%) from **1F6** (5.5x10^6 cells) and 16,326 cells (~0.4%) from **1G6** (4.1x10^6 cells).

FACS-sorted IFN-γ-secreting T cells were stimulated twice at a ratio of 1:1 with autologous irradiated $CD8^-$ PBMCs electroporated with IVT-mRNA-encoding **VE_**FLT3-ITD. After two stimulations, responder T cells were stimulated further either specifically with $CD8^-$ PBMCs electroporated with IVT-mRNA-encoding **VE_**FLT3-ITD or non-specifically with OKT3 as described in **Method section 4.13.** The responder T cells were again tested in an IFN-γ ELISPOT assay. However, none of these T-cell lines was specific for **VE_**FLT3-ITD.

5.3.4 Identification of an FLT3-ITD-encoded peptide antigen recognized by $CD8^+$ T cells derived from a healthy donor

In donor #1853 it was observed that T cells against the **IN_**FLT3-ITD were restricted via HLA-B*35:01 (**Figure 5.3.2.5** and **5.3.2.6**). **IN_**FLT3-ITD-specific responders, of microculture 1H4, were frozen on d33 and d47 for further studies. To identify the C-terminus of the antigenic peptide presented by HLA-B*35:01, a 3´-fragmentation of the **IN_***FLT3-ITD* cDNA was performed. The *FLT3-ITD* cDNA fragments (**Figure 5.3.4.1**) were generated and cloned into pcDNA3.1/V5-His6 as described in **Method section 4.26**. The correct orientation and sequence of the inserts were verified for all of the fragments before they were applied to T-cell recognition assays (**Figures 5.3.4.2** and **5.3.4.3**).

Figure 5.3.4.1: 3´-fragmentation of *FLT3-ITD*$_{Exon13-16}$ cDNA from patient IN.
The **IN_**FLT3-ITD amino acid sequence, as predicted from its cDNA sequence is shown in **Table 5.3.1.1**. Duplications are marked in red and green. The **IN_***FLT3-ITD* contains *a* 6 base pairs insertion (encode amino acids **CP** in black) preceding the duplication. 3´-fragments of the **IN_***FLT3-ITD* cDNA were generated and cloned into pcDNA3.1/V5-His6 as described in **Method section 4.26**. STOP codons integrated in the antisense primers (see **Material section 3.6**) guaranteed a translational stop. The predicted amino acid sequences encoded by the wild type and mutated **IN_***FLT3* cDNA comprising exons 13-16 and by the 3´-fragments generated from **IN_***FLT3-ITD.*

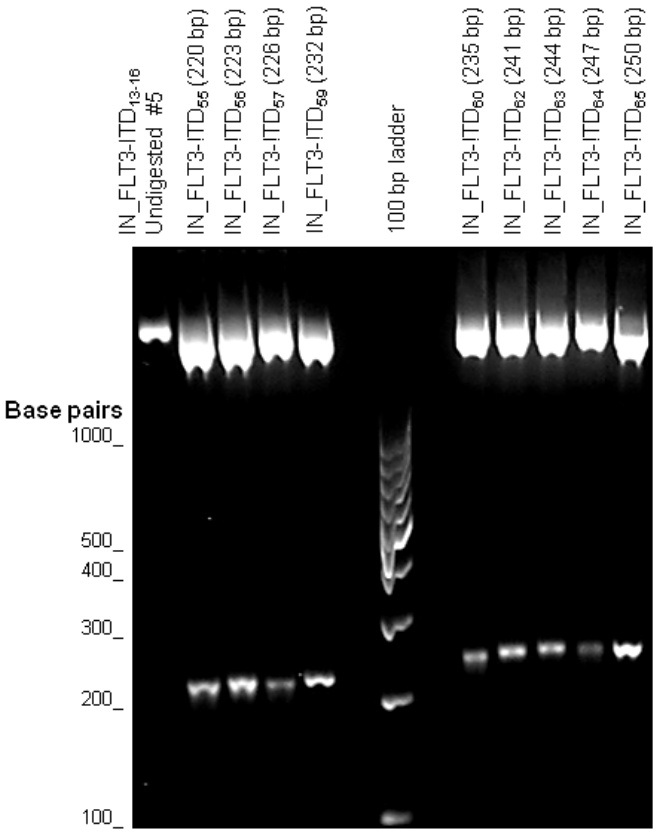


Figure 5.3.4.2: Restriction digestion of correctly oriented IN_*FLT3-ITD* fragments.

IN_*FLT3-ITD*$_{Exon\ 13\text{-}16}$ cDNA fragments were generated by PCR from **IN**_*FLT3-ITD* cDNA as described in **Method section 4.26** and cloned into the expression vector pcDNA3.1/V5-His using the TOPO TA-Cloning system. Plasmids containing their inserts in correct orientation (as verified by BGH-orientation PCR) were digested with restriction enzymes *BamHI* (present in the 5' *FLT3-ITD*$_{Exon13}$ primer) and *XbaI* (present in the 3' pcDNA3.1/V5-His vector). Digested plasmid fragments were analyzed on a 2.5% agarose gel at 100 Volt. A 100-base pair DNA marker was used to estimate the size of the digested DNA. For the designation of fragments see **Figure 5.3.4.1.**

Validated **IN**_*FLT3-ITD* cDNA fragments were co-transfected into COS-7 cells together with *HLA-B*35:01*. **IN**_FLT3-ITD-reactive T cells of microculture 1H4 (d47) were thawn, kept overnight in AIM-V + 10% HS with IL-2 (20 U/ml) and were tested for the recognition of COS-7 transfectants. Fragment **IN**_*ITD_64aa* ending with tyrosine at amino acid position 618 of the full-length FLT3-ITD was the shortest fragment still recognized by the T cells. But its recognition was inferior to the fragment **IN**_*ITD_65aa* ending with amino acid 619, which was valine. These data indicated that the peptide to be identified ended either with tyrosine 618 or valine 619 at its C-terminus (**Figure 5.3.4.4**). Two peptides, the 9-mer peptide **CPSDNEYFY** ending at amino acid position 618, and the 10-mer peptide **CPSDNEYFYV** ending at amino acid 619 were synthesized and tested for recognition by the d33 anti-FLT3-ITD T cells. In accordance with the testing of cDNA fragments (see **Figure 5.3.4.4**), the 10-mer peptide **CPSDNEYFYV** ending with amino acid 619 (V) was much better recognized than the 9-mer peptide **CPSDNEYFY** ending with amino acid 618 (Y) (**Figure 5.3.4.5** and **Figure 5.3.4.6**).

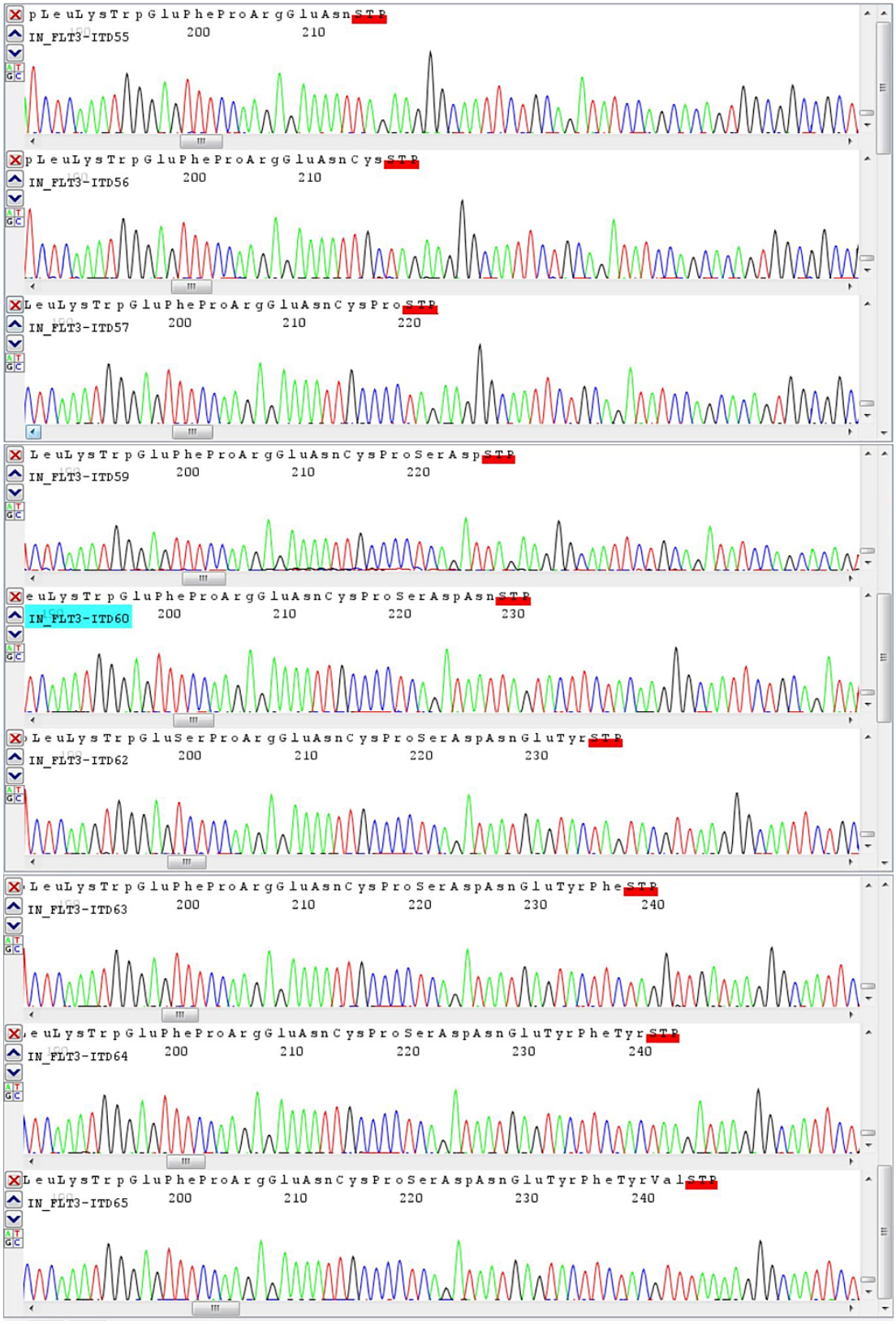


Figure 5.3.4.3: Sequencing of IN_FLT3-ITD$_{Exon\ 13\text{-}16}$ cDNA fragments.
Sequencing of the **IN_*FLT3-ITD*$_{Exon\ 13\text{-}16}$** cDNA fragments (for explanation and designation see **Figure 5.3.4.1**) was performed as described in **Method section 4.20**. Sequences were edited with the Chromas version 2.31 software. Analysis of the sequence was performed with programs EditSeq 3.9.10 and SeqMan 3.61 (DNASTAR Inc.). Alignment of the fragments was obtained with Codoncode Aligner software (http://www.codoncode.com/aligner/). The sequences comprise the complete ORFs. Stop codons inserted via antisense primer design are highlighted in red.

Figure 5.3.4.4: Testing of IN_*FLT3-ITD*$_{Exon13-16}$ cDNA fragments for recognition by T cells.
#1853/1H4 responders (d47; see **Figure 5.3.2.6**) were thawn, kept overnight in T-cell medium and IL-2 (20 U/ml) and were tested at 2.0x10^4/well in a 20-hour IFN-γ ELISPOT assay for the recognition of COS-7 cells (2.0x10^4/well) co-transfected with plasmids-encoding *HLA-B*35:01* and different **IN**_*FLT3-ITD*$_{Exon\ 13-16}$ fragments. Negative and positive controls were *wt-FLT3*$_{Exon13-16}$ and **IN**_*FLT3-ITD*$_{Exon13-16}$, respectively.

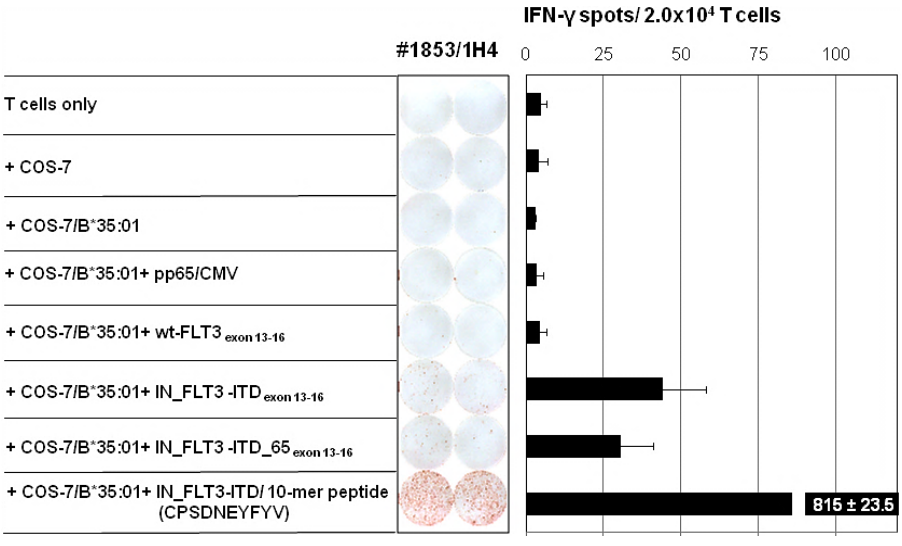


Figure 5.3.4.5: Microculture #1853/1H4 responder T cells recognize the 10-mer peptide encoded by fragment IN_*ITD*$_{65}$.
#1853/1H4 (d33) responder T cells (2.0x10^4/well; see **Figure 5.3.2.1**) were thawn and tested in a 20-hour IFN-γ ELISPOT assay for the recognition of the 10-mer peptide **CPSDNEYFYV** encoded by the **IN**_FLT3-ITD and loaded on COS-7 cells transfected with HLA-B*35:01 (2.0x10^4/well). The peptide concentration was 10 ug/ml. COS-7/B*35:01 cells (2.0x10^4/well) co-transfected with cDNA-encoding wt-FLT3, **IN**_FLT3-ITD or the **IN**_FLT3-ITD$_{65}$ fragment (see **Figure 5.3.4.4**) served as controls. The bars represent means of duplicates ±SD.

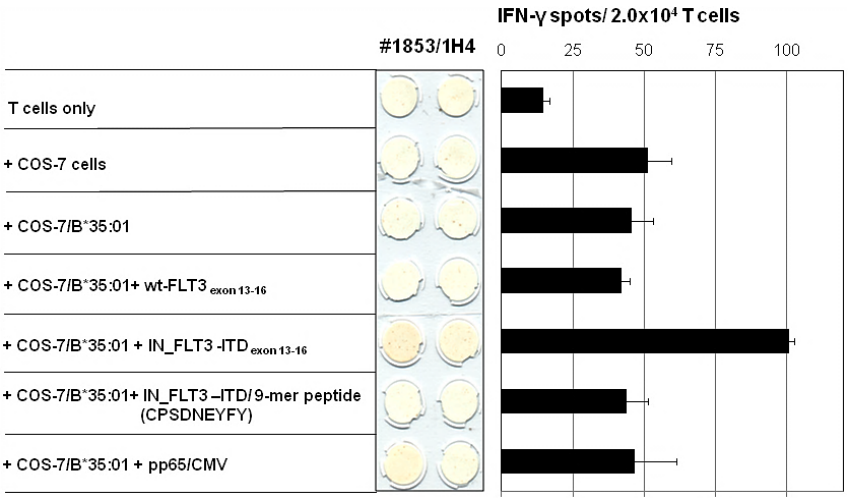


Figure 5.3.4.6: Microculture #1853/1H4 responders do not recognize the 9-mer peptide CPSDNEYFY.
#1853/1H4 (d33) responder T cells (2.0x10^4/well) were tested (before they were cryopreserved) in a 20-hour IFN-γ ELISPOT assay for the recognition of the 9-mer peptide **CPSDNEYFY** encoded by the **IN**_*FLT3-ITD* and loaded on COS-7 cells (2.0x10^4/well) transfected with *HLA-B*35:01* (COS-7/B*35:01). The peptide concentration was 10 ug/ml. COS-7/B*35:01 cells (2.0x10^4/well) co-transfected with cDNA-encoding pp65 (HCMV), wt-FLT3 and **IN**_FLT3-ITD served as controls. The bars represent means of duplicates ±SD.

5.4 *In vitro* stimulation of CD8$^+$ T cells from umbilical cord blood (UCB) against immunogenic FLT3-ITDs identified in patients VE, IN, QQ, JC and FL

5.4.1 Phenotyping of CD8$^+$ lymphocytes derived from umbilical cord blood (UCB)

T lymphocytes in umbilical cord blood predominantly exhibit a naïve phenotype associated with a high proliferative capacity (Gattinoni *et al.,* 2012). To test the hypothesis that they contain FLT3-ITD-reactive T cells at higher frequencies than buffy coats prepared from adult healthy donors, UCB lymphocytes were stimulated against five immunogenic FLT3-ITDs identified in leukemia patients **VE, IN, QQ, FL** and **JC**. For this purpose, eight umbilical cord blood donations (**Table 3.9.3.1**), were chosen and genotyped for their HLA I alleles (**Table 5.4.1.1**).

Table 5.4.1.1: Summary of UCB donations, their HLA I phenotypes and the FLT3-ITDs against which they were tested.

Umbilical cord blood donor	HLA -I alleles			CD8+ T cells were tested for reactivity against FLT3-ITD(s) of patient(s)
	-A	-B	-C	
MZUCB#1	*68:02, *01:01	*08:01	*08:02	VE, IN, QQ and FL
MZUCB#2	*02:01, *03:01	*40:01, *13:02	*08:02	VE, IN, QQ and FL
MZUCB#3	*02:01	*18:01, *51:01	*07, *15:01	VE, IN, QQ and FL
MZUCB#4	*02:01, *26:01	*44:02, *49:01	*05:01	VE, IN, QQ and FL
MZUCB#5	*03:01	*13:02, *5101	*06:02, *14:02	QQ and FL
MZUCB#12	*02:01, *03:01	*07:02, *51:01	*14:02, *07:02	FL
MZUCB#15	*01:01, *25:01	*07:02, *08:01	*07:01, *07:02	JC
MZUCB#23	*26:01, *68:02	*39:10, *41:02	*12:03, *17:03	QQ

To confirm the naïve phenotype of UCB lymphocytes, $CD8^+$ T cells from five UCB donations were purified with magnetic beads and analyzed by flow cytometry with antibodies against **CCR7, CD27, CD45RA** and **CD62L**. The proportion of naïve T cells was in the range of 85-99% of $CD8^+$ T cells (detailed in **Table 5.4.1.2**). As an example, the flow cytometric analysis of MZUCB #4 $CD8^+$ T cells is shown in **Figure 5.4.1.1**. Three UCB donations were in addition tested for the presence of CD4/CD8 double-positive T cells, regulatory T cells (Tregs) and natural killer (NK) cells (**Table 5.4.1.3** and **Figure 5.4.1.2**).

Umbilical cord blood donor	CD3/CD8	CCR7	CD27	CD45RA	CD62L
MZUCB#1	87.91/95.71	67.52	85.49	88.52	86.32
MZUCB#2	97.59/99.15	89.80	97.86	99.43	98.57
MZUCB#3	92.16/98.66	83.51	97.64	99.64	96.66
MZUCB#4	97.55/99.49	86.68	99.63	99.36	98.91
MZUCB#23	96.29/98.95	91.80	96.72	97.68	90.87

Table 5.4.1.2: Summary of the phenotyping of UCB-derived $CD8^+$ T cells.
$CD8^+$ T cells purified with magnetic beads from the indicated UCB donations were stained with antibodies against CCR7, CD27, CD45RA and CD62L (as detailed in **Figure 5.4.1.1**). Values represent the percentage of $CD8^+$ T cells expressing the surface markers.

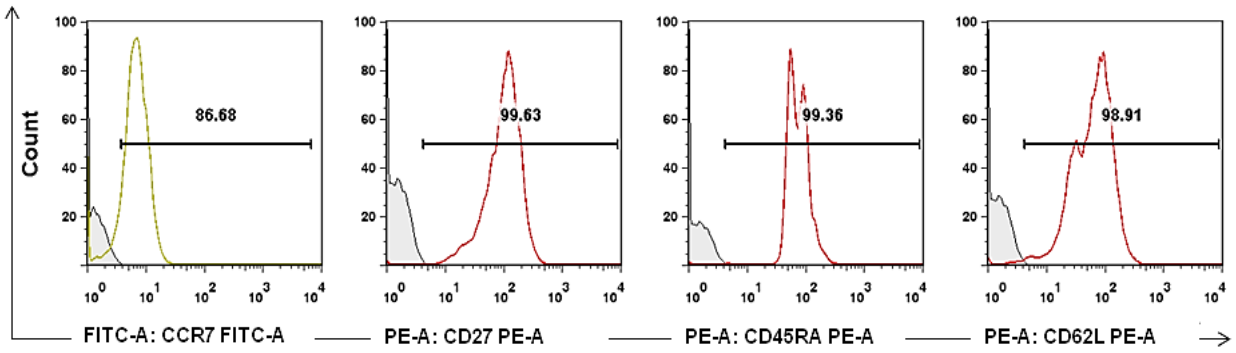


Figure 5.4.1.1: Flow cytometric analysis of MZUCB #4-derived $CD8^+$ T cells.
$CD8^+$ T cells sorted with magnetic beads were washed and stained with FITC- or PE-labeled antibodies directed against cell surface molecules CCR7, CD27, CD45RA and CD62L defining the naïve immune-phenotype. After staining, T cells were washed with FACS buffer and fixed with FACS fixation solution. The stained T cells were analyzed with flow cytometry (see **Method section 4.6**). Isotype control IgG1-stained populations are shown in filled histograms

CD8 subsets	Markers	MZUCB#23	MZUCB#15	MZUCB#5
Double positive cells	CD4+/CD8+	4.68	6.31	9.71
Tregs	CD4+/CD25+	1.04	1.03	3.66
	CD4+/CD127-	0.82	3.49	4.69
NK cells	CD8+/CD16+	2.92	6.06	-
	CD16+/CD56+	3.17	5.88	-

Table 5.4.1.2: Testing of UCB-derived $CD8^+$ cells for the presence of CD4/CD8 double-positive T cells, Tregs and NK cells.
Values represent % of $CD8^+$ T cells expressing the indicated markers. Flow cytometry data of MZUCB #15 are exemplarily shown in **Figure 5.4.1.2.**

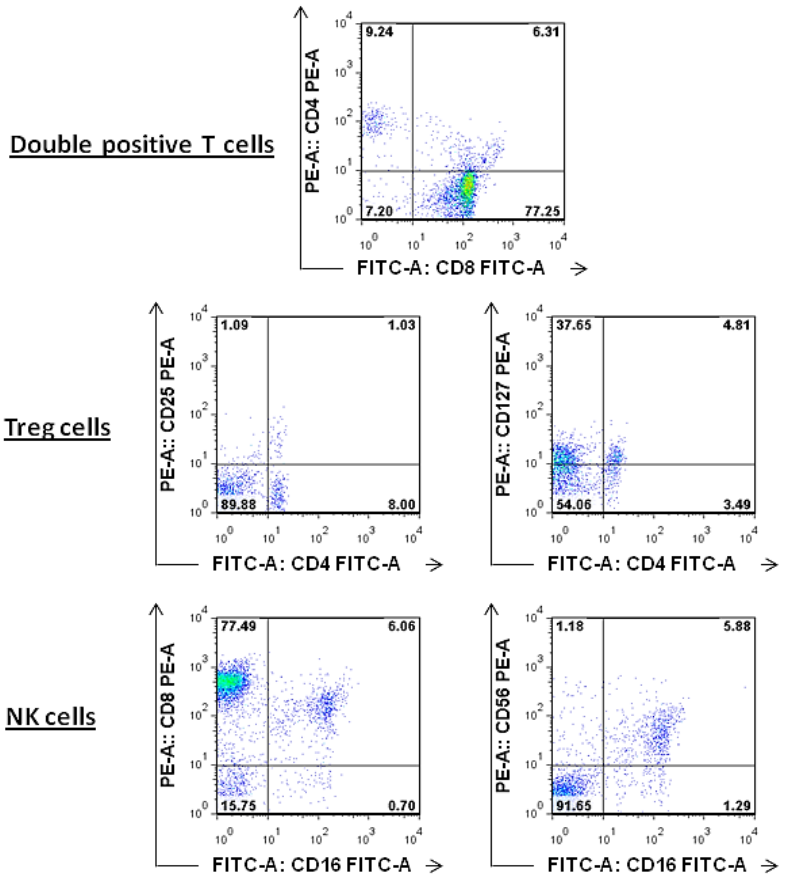


Figure 5.4.1.2: Flow cytometric analysis of CD8$^+$ cells purified from MZUCB #15.
CD8$^+$ T cells sorted with magnetic beads from MZUCB #15 were washed and stained with FITC- or PE-labeled antibodies directed against cell surface molecules defining double positive T cells (CD4$^+$/CD8$^+$), Tregs (CD4$^+$, CD25$^+$ and CD127$^-$) and NK cells (CD16$^+$ and CD56$^+$). After staining, T cells were washed with FACS buffer and fixed with FACS fixation solution (see **Method section 4.6**). CD8$^+$ T cells were measured with FACS flow cytometry and gated for the expression of the corresponding cell surface molecules.

CD8$^+$ T lymphocytes of MZUCB #23, were analyzed for the effect of different cytokine cocktails on the proliferation and differentiation of different subsets of CD8$^+$ T cells during OKT3 (anti-CD3 antibody)-based stimulation (**Figure 5.4.1.3**). In seven days, UCB T cells achieved a maximum proliferation (26-fold) in the presence of cytokines IL-12, IL-7 and IL-15 whereas in absence of cytokines UCB lymphocytes did not proliferate (not shown).

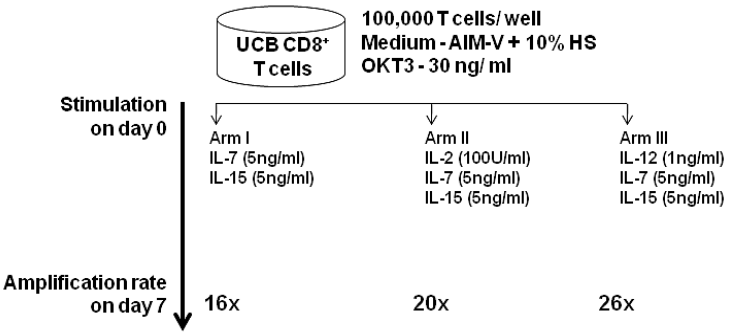


Figure 5.4.1.3: Experimental setup: Role of different cytokine cocktails on proliferation of UCB CD8$^+$ T cells.
T cells were stimulated nonspecifically for seven days using OKT3. In Arms I, II and III different cytokine cocktails were applied. x represents fold amplification of UCB-CD8$^+$ T cells in 7 days.

Naïve T cells attained a maximum differentiation towards the effector memory phenotype (CD8$^+$ T_{EM}: CD45RA^{low} CD45RO^{high} CCR7^{low}) with cytokine cocktails containing IL-12, IL-7 and IL-15. It was observed that, in general, the frequency of CD4/CD8 double-positive T cells, Tregs and NK cells decreased with all cytokine cocktails. However, the frequency of CD4/CD8 double-positive T cells increased in the presence of cytokines IL-12, IL-7 and IL-15 (**Table 5.4.1.4**).

CD8 subsets	Markers	Ex-vivo	Arm I	Arm II	Arm III
Naive T cells	CCR7	91.80	86.10	80.08	65.08
	CD27	96.72	99.64	99.29	98.42
	CD45RA	97.68	96.48	95.91	74.96
	CD45RO	25.03	72.18	74.82	86.26
	CD62L	90.87	73.54	70.06	88.55
Double positive cells	CD4+/CD8+	4.68	2.78	1.86	8.22
Tregs	CD4+/CD25+	0.26	0.10	0.12	0.24
	CD4+/CD127-	0.82	1.57	0.04	0.08
NK cells	CD8+/CD16+	2.92	0.03	0.04	0.08
	CD16+/CD56+	3.17	0.05	0.02	0.03

Table 5.4.1.4: The use of distinct cytokine cocktails impacts on the differentiation of UCB-CD8$^+$ T cells.
T cells expanded *in vitro* with different cytokine cocktails (see Arm I-III in **Figure 5.4.1.3**) were stained with antibodies directed against cell surface molecules defining naïve, effector and memory T cells, double positive T cells, Tregs and NK cells. After staining, washing and fixation (see **Method section 4.6**); T cells were measured with flow cytometry and gated for the expression of the corresponding cell surface molecules.

5.4.2 Stimulation with IVT-mRNA-encoding FLT3-ITDs

In total, eight UCB donations were analyzed for T-cell responses against the FLT3-ITDs derived from patients **JC, VE, IN, QQ** and **FL** either in consideration or irrespective of their HLA I haplotype (**Table 5.4.1.1**). Autologous T-cell responses in patients against these FLT3-ITDs had been restricted via HLA-A*01:01, -B*27:05, -A*32:01, -A*11:01 and -A*01:01, respectively (**Table 5.3.1.1**). Seven UCB donations were analyzed for anti-FLT3-ITD responses by stimulating CD8$^+$ T cells with FLT3-ITD mRNA under quasi-limiting dilution conditions as described in **Method section 4.9.2,** and schematically explained in **Figure 5.4.2.1**.

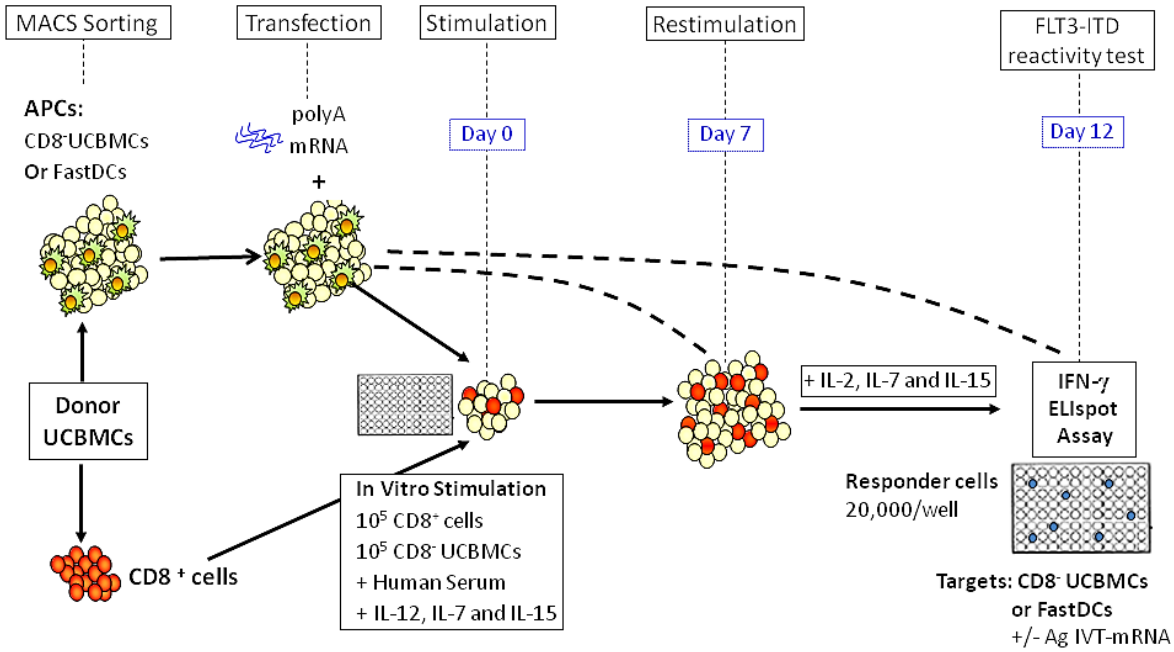
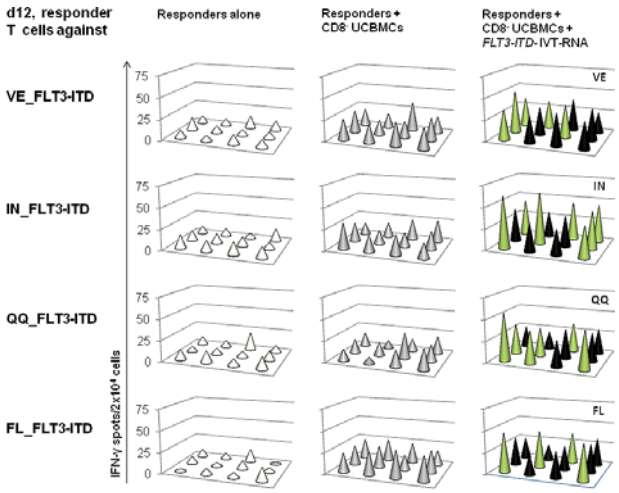


Figure 5.4.2.1: *In vitro* stimulation of UCB-CD8$^+$ T cells with FLT3-ITD-encoding IVT-mRNA.
On d0 1.0x10^5 CD8$^+$ T cells isolated with microbeads were seeded per well on a 96-well-U-bottom plate in 100 µl AIM-V/10% HS + 1 ng/ml IL-12, 5 ng/ml IL-7, and 5 ng/ml IL-15. These cells were stimulated with irradiated autologous CD8$^-$ UCBMCs or FastDCs transfected with IVT-mRNA-encoding FLT3-ITD at a 10:1 ratio (effector:target). For each FLT3-ITD, 12 microcultures were initiated. On d7, responder lymphocytes were restimulated under similar conditions except that IL-12 was replaced by IL-2 (100 IU/ml). On d12, responder cells (2.0x10^4/well) were tested in a 20-hour IFN-γ ELISPOT assay for recognition of autologous CD8$^-$ UCBMCs (4.0x10^4/well) or FastDCs (5.0x10^3/well) expressing the respective stimulatory FLT3-ITD. Stimulations with *pp65* IVT-mRNA and mock IVT-mRNA were performed in parallel depending on the availability of UCB cells.

Five UCB donations were analyzed for anti-FLT3-ITD responses using autologous CD8$^-$ UCB mononuclear cells (UCBMCs) as antigen-presenting cells as detailed in **Figure 5.4.2.1**. In four UCB donations (MZUCB #1, #2, #3 and #4) anti-FLT3-ITD responses were observed against all four FLT3-ITDs (**VE, IN, QQ** and **FL**) used for stimulation (**Figure 5.4.2.2a** and **b)**, whereas no reactivity was observed in MZUCB #5 stimulated against the FLT3-ITDs of patients **QQ** and **FL.** Anti-pp65/CMV responses were observed in 2 out of 5 UCB donations (MZUCB #1 and #2) (**Figure 5.4.2.3**).

MZUCB #1



MZUCB #2

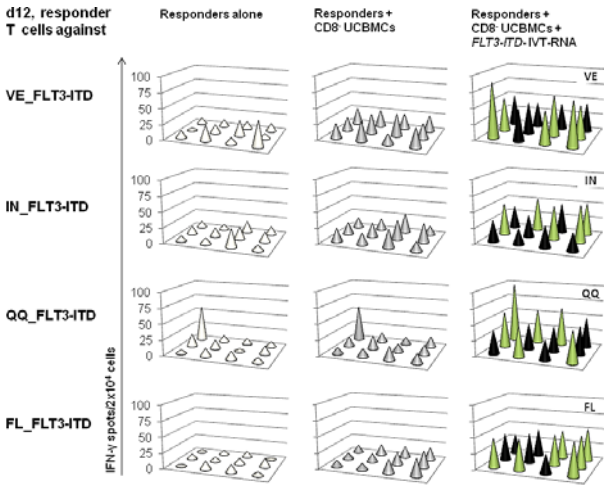
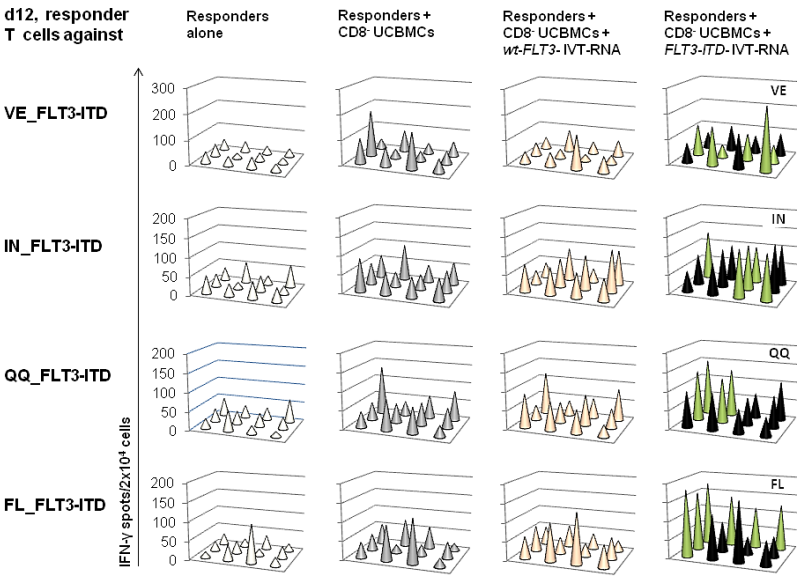


Figure 5.4.2.2a: *In vitro* stimulation of MZUCB #1- and MZUCB #2-CD8$^+$ T cells against FLT3-ITDs of patients VE, IN, QQ and FL.
UCB-derived CD8$^+$ T cells were stimulated twice with autologous CD8$^-$ UCBMCs transfected with IVT-mRNA-encoding **VE_, IN_, QQ_** or **FL**_FLT3-ITD, respectively, and responders ($2.0x10^4$/well) were analyzed for anti-FLT3-ITD reactivity on d12 (**Figure 5.4.2.1**). Targets were CD8$^-$ UCBMCs ($4.0x10^4$/well) electroporated with IVT-mRNA-encoding the respective stimulatory FLT3-ITD. A response was defined as ''positive'' when the number of IFN-γ spots against an FLT3-ITD was at least 2-fold higher than the baseline recognition (targets alone). Microcultures shown in green cones were selected for further studies.

MZUCB #3



*(see figure legend on **page 87**)*

MZUCB #4

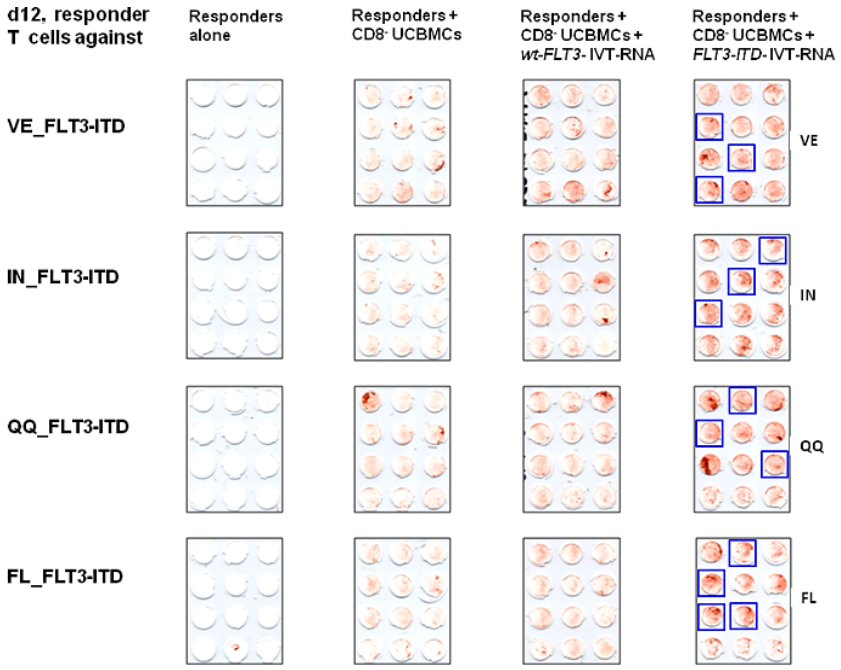


Figure 5.4.2.2b: *In vitro* stimulation of MZUCB #3- and MZUCB #4-CD8$^+$ T cells against FLT3-ITDs of patients VE, IN, QQ and FL.
Stimulations and analysis of CD8$^+$ T cells stimulated with autologous CD8$^-$ UCBMCs transfected with IVT-mRNA-encoding **VE_, IN_, QQ_** or **FL**_FLT3-ITD, respectively. Targets were CD8$^-$ UCBMCs (4.0x10^4/well) electroporated with IVT-mRNA-encoding the respective stimulatory FLT3-ITD. A response was defined as ''positive'' when the number of IFN-γ spots against an FLT3-ITD was at least 2-fold higher than the background response (targets + wt-FLT3). Microcultures shown in green cones or in blue boxes were selected for further studies.

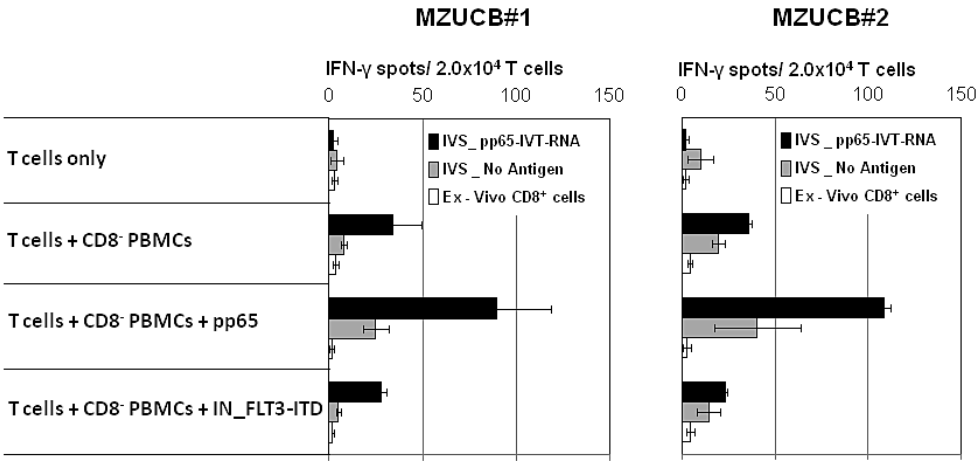


Figure 5.4.2.3: *In vitro* stimulation of MZUCB #1- and MZUCB #2-CD8$^+$ T cells against HCMV pp65.
Stimulations were performed as described in **Figure 5.4.2.1.** On d12, responders (2.0x10^4/well) were tested in a 20-hour IFN-γ ELISPOT assays for the recognition of autologous CD8$^-$ UCBMCs (4.0x10^4/well) expressing pp65 antigen after electroporation with IVT-mRNA. The bars represent the means of duplicates ±SD.

Two UCB donations were analyzed for anti-FLT3-ITD responses using autologous FastDCs transfected with *FLT3-ITD* IVT-mRNA, using the TransMessenger^{TM} method as described in **Method section 4.8** and **section 4.10**. HLA-A*01:01-positive donor MZUCB #15, was tested for reactivity against the FLT3-ITD of patient **JC** known to be immunogenic in the autologous system and restricted by HLA-A*01:01 (Graf *et al.,* 2007). CD8$^+$ T cells were stimulated under

two different culture conditions, i.e. ARM I (IL-2, IL-7 and IL-15) and ARM II (IL-12, IL-7 and IL-15) (see **Figure 5.4.1.3**). On d12 of stimulation, anti-FLT3-ITD responses were observed with both stimulation conditions (**Figure 5.4.2.4**). It was noticed that the ARM II cytokine cocktail had a better ability to prime FLT3-ITD-specific T cells than the ARM I cytokine cocktail. Remarkably, the responders in **JC**_FLT3-ITD-reactive microcultures could also recognize autologous FastDCs loaded with the previously identified HLA-A*01:01-restricted 9-mer peptide **YVDFREYEYY** (YVD/A1, see Graf *et al.,* 2007) (**Figure 5.4.2.4**). In donor MZUCB #23 no response was observed against the FLT3-ITD of patient **QQ.** The UCB lymphocytes were not analyzed in parallel for their reactivity against pp65/CMV due to limited cell material.

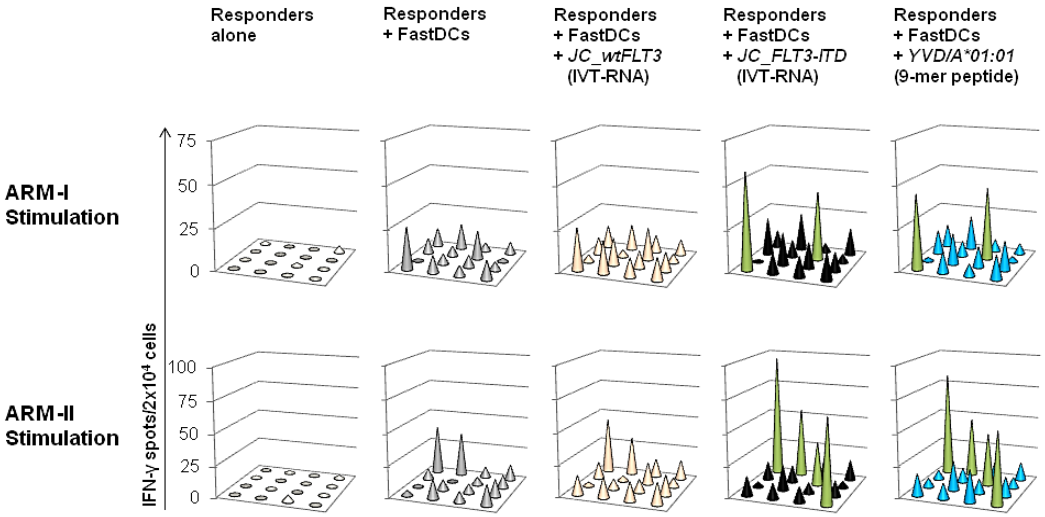


Figure 5.4.2.4: UCB-derived CD8$^+$ T cells recognize the FLT3-ITD of patient JC with an identical peptide specificity.
CD8$^+$ T cells from UCB of donor MZUCB #15 were stimulated with autologous FastDCs expressing *FLT3-ITD*$_{full\text{-}length}$ of patient **JC**. For cytokine cocktails used in Arms I and II see **Figure 5.4.1.3**. On d12, responder T cells (2.0x10^4/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of FastDCs (5.0x10^3/well) either transfected with IVT-mRNA-encoding wt-FLT3 or FLT3-ITD or loaded the peptide YVD/A1 (10 µg/ml). Responses were defined as ‘‘positive’’ when the number of IFN-γ spots against FLT3-ITD was at least 2-fold higher than the background (FastDCs + wt-FLT3). Microcultures shown in green are the FLT3-ITD-reactive microcultures. The cones represent single values.

The frequencies of FLT3-ITD-reactive T cells in the original UCB samples were calculated as described in **Result section 5.3.1**. They were estimated to be in the range of 3.6x10^{-6} to 1.8x10^{-5}, which is nearly 15-fold higher than the frequencies observed in adult healthy buffy coat donors (2.83x10^{-7} to 1.25x10^{-6}, see **Result section 5.3**). ITD-reactive microcultures (marked in green) were frozen from each of the donors for further studies.

5.4.3 Analysis of HLA restriction

HLA I genotyping (see **Method section 4.17- 4.20**) was performed on UCB donations, from which anti-FLT3-ITD T cells had been generated (see **Result section 5.4.2**). Four-digit typing results are shown in **Table 5.4.1.1**. In a first attempt, ITD-reactive individual microcultures (see

Figure 5.4.2.2a and **b**) generated from MZUCB #1 and #4, for which no $CD8^-$ UCBMCs were still available for further restimulation and expansion, were thawn and directly tested after an overnight resting phase (schematically shown in **Figure 5.4.3.1a)**. In a second attempt, ITD-reactive individual microcultures generated from MZUCB #2, #3 (see **Figure 5.4.2.2a** and **b**) and MZUCB #5 (not shown), were thawn and further restimulated one more time with $CD8^-$ UCBMCs still available as stimulators. Restimulation was performed as before (see **Method section 4.9.2** and **Figure 5.4.2.1**) and responders were tested on d19 (schematically summarized in **Figure 5.4.3.1b**). In both attempts responder T cells were tested for the recognition of 293T or COS-7 cells co-transfected with all of the HLA I alleles of the respective UCB donation and the stimulatory FLT3-ITD.

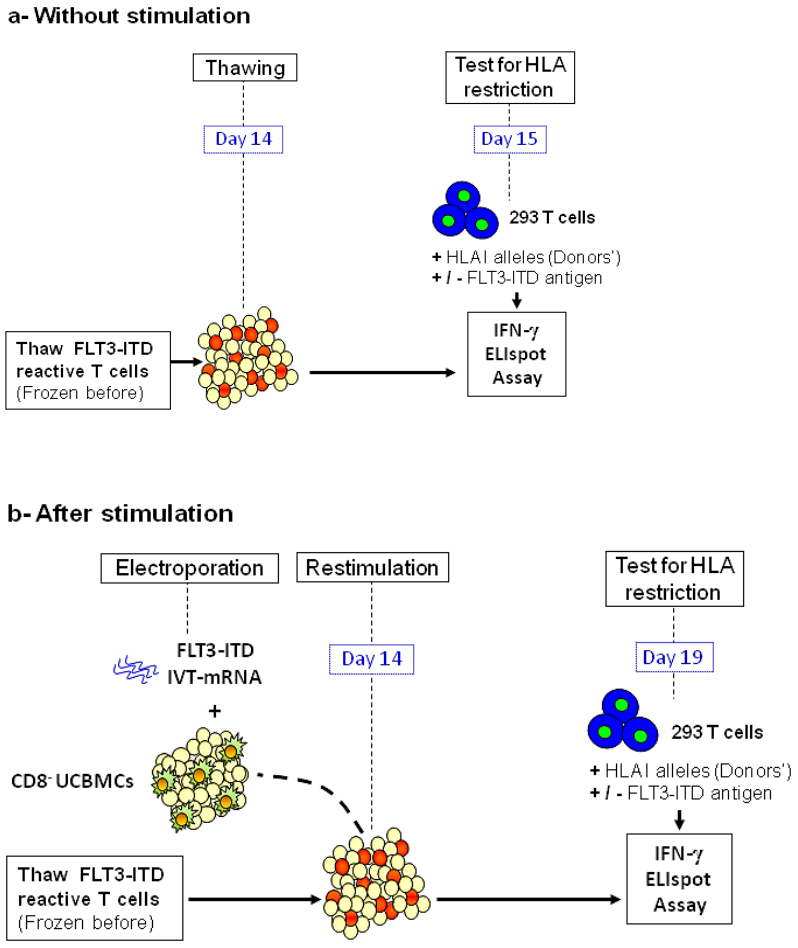


Figure 5.4.3.1: FLT3-ITD-reactive microcultures were tested for their HLA restriction either (a) without further stimulation or (b) after additional stimulation.
Depending on the availability of $CD8^-$ UBMCs, d14 frozen FLT3-ITD-reactive microcultures were thawn and tested on d15 after an overnight resting phase in T-cell medium (AIM-V with 10% HS with a cytokine cocktail containing IL-2, IL-7 and IL-15; concentrations detailed in **Figure 5.4.2.1**) (**a**) or on d19 after one further round of stimulation (stimulation conditions as detailed in **Figure 5.4.2.1**) (**b**) for the recognition of 293T or COS-7 cells co-transfected with all *HLA I* alleles of the respective UCB donation and with plasmids-encoding wt-FLT3 or the respective stimulatory FLT3-ITD.

The HLA I restriction of FLT3-ITD-specific responses was clearly determined in four UCB donations (MZUCB #2, MZUCB #3, MZUCB #4 and MZUCB #5), whereas, for MZUCB #1

responders, no restricting HLA I allele could be identified. The restricting alleles are listed in **Table 5.4.3.1**. Aside from the observation that, from single donations, $CD8^+$ T cells directed against different FLT3-ITDs were expanded, it was also seen that in some instances the recognition of single FLT3-ITDs was restricted by different HLA I alleles (**Figure 5.4.3.2**). The restricting HLA I molecules were different from those observed in the autologous patient-derived systems (see **Table 5.3.1.1**). Only in MZUCB #15 a T-cell response against the same peptide/HLA complex as in the original patient model was found (see **Figure 5.4.2.4** for **JC**_FLT3-ITD), which was proved in HLA-restriction test that **JC**_FLT3-ITD derived epitopes were endogenously processed and presented to T-cell via HLA-A*01:01 (**Figure 5.4.3.3**).

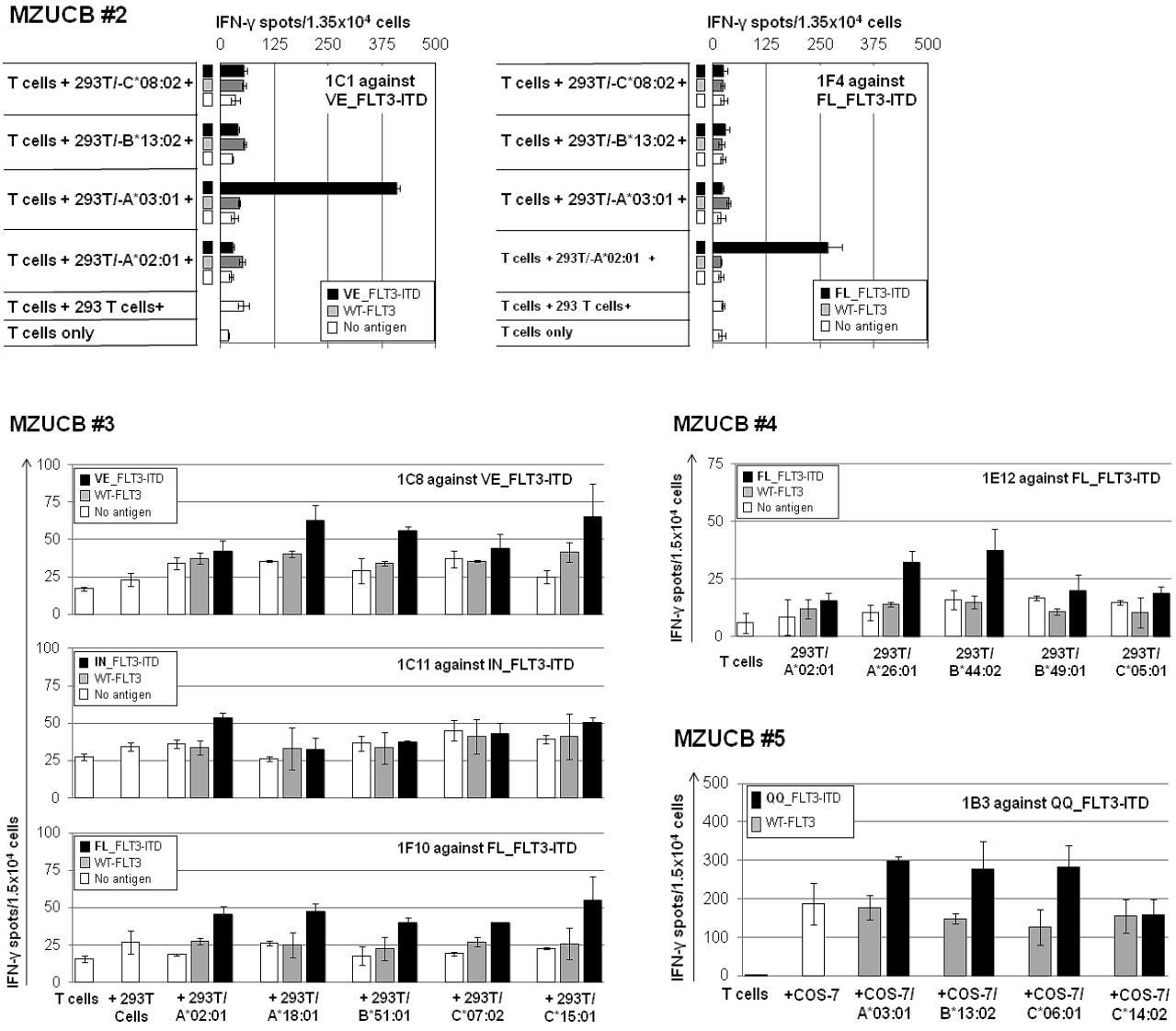


Figure 5.4.3.2: HLA restriction of FLT3-ITD-reactive microcultures generated from different UCB donations.

ITD-reactive microculture T cells from MZUCB #4 ($1.5x10^4$/well) were tested on d15 or microcultures from MZUCB #2 ($1.35x10^4$/well), MZUCB #3 and #5 ($1.5x10^4$/well) were tested on d19 in a 20-hour IFN-γ ELISPOT assay for the recognition of either 293T cells or COS-7 cells co-transfected with donors' individual *HLA I alleles* and the respective stimulatory *FLT3-ITD*$_{Exon\ 13\text{-}16}$ cDNA. As a control transfectants expressing wt-FLT3$_{Exon\ 13\text{-}16}$ were included. Shown are means of duplicates ±SD. Reactivities with certain HLA transfectants were considered positive, when the number of IFN-γ spots against FLT3-ITD was higher than the mean background plus two-fold standard deviations. Restricting HLA I alleles defined on this basis are listed in **Table 5.4.3.1**).

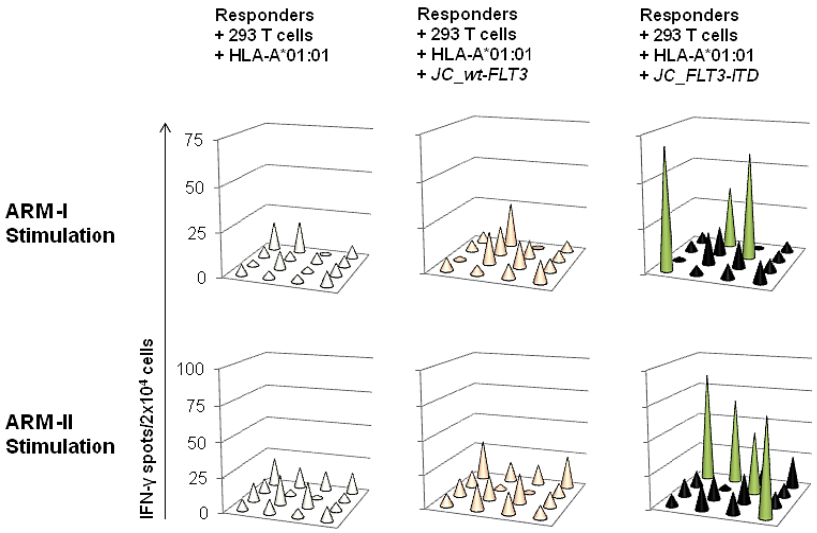


Figure 5.4.3.3: Response against JC_FLT3-ITD in MZUCB #15 was restricted via HLA-A*01:01.
d12 responder T cells ($2.0x10^4$/well) (see also **Figure 5.4.2.4**) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of 293T cells expressing HLA-A*01:01 ($2.0x10^4$/well) and transfected with plasmids-encoding wt-FLT3 or **JC**_FLT3-ITD. Shown are the results of both arms of stimulation. Responses were defined as "positive" when the number of IFN-γ spots against FLT3-ITD was at least 2-fold higher than the background (wt-FLT3). Microcultures shown in green are the FLT3-ITD-reactive microcultures. The cones represent single values.

Umbilical Cord Blood	FLT3-ITDs				
	VE	IN	QQ	FL	JC
MZUCB # 1	–	–	–	–	*
MZUCB # 2	A*03:01	–	–	A*02:01	*
MZUCB # 3	A*18:01 B*51:01 C*15:01	A*02:01	–	A*02:01, A*18:01 B*51:01, C*07:02 C*15:01	*
MZUCB # 4	–	–	–	A*26:01, B*44:02	*
MZUCB # 5	*	*	A*03:01 B*13:02 C*06:01	–	*
MZUCB # 15	*	*	*	*	A*01:01
MZUCB # 23	*	*	–	*	*

- not detected * not analyzed

Table 5.4.3.1: Summary of HLA I alleles restricting anti-FLT3-ITD responses by $CD8^+$ T cells derived from UCB donations (see original data in Figures 5.4.3.2 and 5.4.2.5).

5.4.4 Identification of an FLT3-ITD-derived peptide antigen recognized by $CD8^+$ UCB T cells

In MZUCB #2, FLT3-ITD-specific $CD8^+$ T cell responses were observed against ITDs derived from **FL** and **VE**. Restricting HLA I alleles were HLA-A*02:01 for **FL**_FLT3-ITD and -A*03:01 for **VE**_FLT3-ITD (**Figure 5.4.3.2**). Using available peptide algorithms BMAS (Parker *et al.,* 1994), SYFPEITHI (Rammensee *et al.,* 1999) and NetMHC 3.2 (Lundegard *et al.,* 2008a and 2008b), a 10-mer peptide for **FL**_FLT3-ITD/HLA-A*02:01 and 9-mer peptide for **VE**_FLT3-ITD/HLA-

A*03:01 were selected on the basis of high combined prediction scores and synthesized for recognition assays (**Figure 5.4.4.1**). **FL**_FLT3-ITD-specific microculture MZUCB #2/1H4 responders and **VE**_FLT3-ITD-specific microculture MZUCB #2/1A1 responders (see microcultures shown in green in **Figure 5.4.2.2a**) were tested for recognition of the **FL**_FLT3-ITD-derived peptide **YESDNEYFYV** (designated as **YES/A2**) and the **VE**_FLT3-ITD-derived peptide **YVDFREYER** (designated as **YVD/A3**) on 293T cells transfected with HLA-A*02:01 or HLA-A*03:01, respectively. **YES/A2** was recognized by **FL**_FLT3-ITD-reactive T cells (**Figure 5.4.4.2**), while T cells against **VE**_FLT3-ITD did not react with **YVD/A3** (**Figure 5.4.4.3**).

FL_FLT3-ITD VQVTGSSDNEYFYVDFREYESDNEYFYVDFREYEYDLKWEF

Length	Predicted peptide for HLA-A*02:01	Score (rank)		
		BMAS	SYFPEITHI	NetMHC 3.2
10-mer	**YESDNEYFYV**	**1220.475**	**13**	**0.480**
10-mer	REYESDNEYF	0.051	3	0.054
9-mer	ESDNEYFYV	0.652	7	0.158
9-mer	YESDNEYFY	0.150	4	0.068

VE_FLT3-ITD VQVTGSSDNEYFYVDFREYE*RA*SDNEYFYVDFREYEYDLKWEF

Length	Predicted peptide for HLA-A*03:01	Score (rank)		
		BMAS	SYFPEITHI	NetMHC 3.2
10-mer	YVDFREYERA	0.020	10	0.055
10-mer	FYVDFREYER	0.018	5	0.75
9-mer	**YVDFREYER**	**1.200**	**16**	**0.100**
9-mer	YERASDNEY	0.012	11	0.074

Figure 5.4.4.1: Prediction of HLA I binding peptides encoded by the FLT3-ITDs from AML patients FL and VE (see Table 5.3.1.1).
Peptides encoded by the *ITD* regions of **FL**_FLT3-ITD and **VE**_FLT3-ITD (predicted duplicated amino acid sequence in **red** and **green**) binding to HLA-A*02:01 and HLA-A*03:01 were predicted with the indicated public domain algorithms combining prediction for proteosomal processing, transporter associated with antigen processing (TAP), and binding affinity to one of both HLA alleles. The 10-mer **FL** peptide **YESDNEYFYV** and the 9-mer **VE** peptide **YVDFREYER** reached the highest scores with all algorithms and were, therefore, selected for further testing.

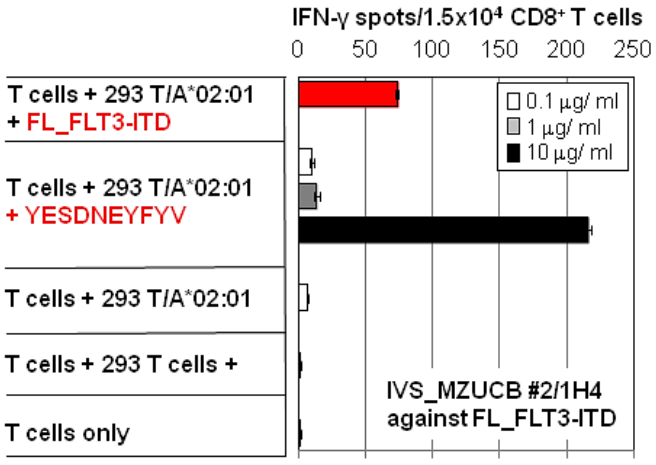


Figure 5.4.4.2: Recognition assay for the FL_FLT3-ITD-derived peptide YESDNEYFYV predicted to bind to HLA-A*02:01 (see Figure 5.4.4.1).
d14 microculture 1H4, generated from MZUCB #2 against **FL_FLT3-ITD**, was thawn and kept overnight in AIM-V+10%HS with IL-2 (20 U/ml) and IL-7 (5 ng/ml). T cells ($1.5x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of 293T/A*02:01 ($2.0x10^4$/well) transfectants loaded with peptide **YESDNEYFYV** (**YES/A2**) (at indicated concentrations). 293T/A*02:01 cells co-transfected with a plasmid-encoding **FL_FLT3-ITD** served as a positive control. The bars represent the means of duplicates ±SD.

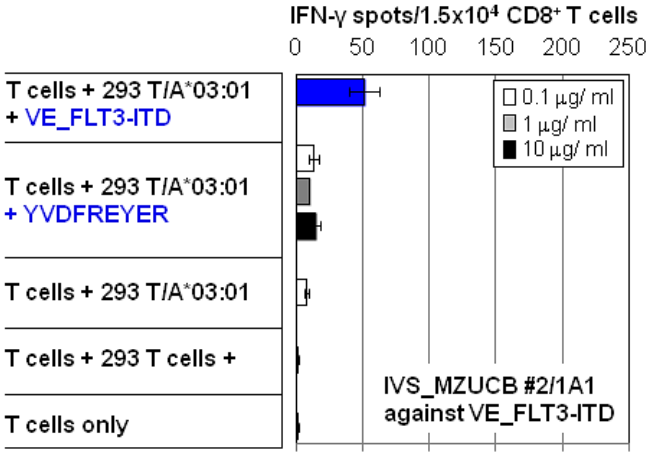


Figure 5.4.4.3: Recognition assay for the VE_FLT3-ITD-derived peptide YVDFREYER predicted to bind to HLA-A*03:01 (see Figure 5.4.4.1).
d14 microculture 1A1, generated from MZUCB #2 against **VE_FLT3-ITD**, was thawn and kept overnight in AIM-V+10%HS with IL-2 (20 U/ml) and IL-7 (5 ng/ml). T cells ($1.5x10^4$/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of 293T/A*03:01 ($2.0x10^4$/well) transfectants loaded with peptide **YVDFREYER (YVD/A3)** (at indicated concentrations). 293T/A*03:01 cells co-transfected with a plasmid-encoding **VE_FLT3-ITD** served as a positive control. The bars represent the means of duplicates ±SD.

To validate the antigenic capability of **YES/A2** peptide derived from **FL**_FLT3-ITD. Mixed Lymphocyte Peptide Culture (MLPC) was performed with lymphocytes from another HLA-A*02:01-positive UCB donation referred to as MZUCB #12. $CD8^+$ T cells isolated with microbeads were stimulated with **YES/A2** loaded on autologous FastDCs as described in **Method section 4.14** and schematically explained in **Figure 5.4.4.4**.

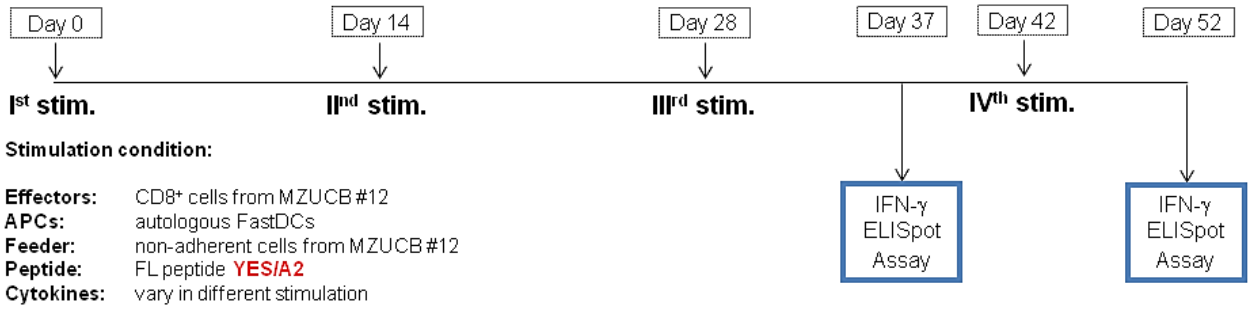


Figure 5.4.4.4: Schematic experimental setup for the *in vitro* stimulation of UCB-$CD8^+$ T cells with peptide YES/A2.
$CD8^+$ T cells from the HLA-A*02:01-positive donation MZUCB #12 were stimulated with **YES/A2 peptide** (at a concentration of 5 µg/ml) loaded on FastDCs at a 10:1 ratio (E:T) in a medium containing 1 ng/ml IL-12, 5 ng/ml -7, and 5 ng/ml IL-15 (see **Method section 4.14**). Restimulation of responding T cells was performed in 2-weeks intervals in a similar condition except IL-12 was replaced by 100 IU/ml IL-2. Responder T cells were tested on d37 and d52 in a 20-hour IFN-γ ELISPOT assay.

On d37, T cells stimulated with YES/A2 were able to recognize HLA-A*02:01-transfected 293T cells, either loaded with **YES/A2,** or co-transfected with **FL**_*FLT3-ITD*-encoding plasmid DNA. Recognition of peptide-loaded targets was completely abrogated by an antibody against HLA-A2 (**Figure 5.4.4.5A**). Two selected *in vitro* stimulation microcultures (IVS #1 and IVS #3) were further restimulated with **YES/A2** on d42 under identical culture conditions and tested on d52. Recognition of both the synthetic peptide and of transfectants endogenously expressing the natural peptide was confirmed. In addition, it was demonstrated that the T cells stimulated with **YES/A2** recognized targets transfected with either full-length **FL**_*FLT3-ITD* cDNA or the

FL_*FLT3-ITD*$_{Exon\ 13\text{-}16}$ fragment equally well (**Figure 5.4.4.5B**). **Figure 5.4.4.5C** demonstrates that **YES/A2**-specific UCB-CD8$^+$ T cells were specific for the ITD of patient FL and did not react with ITDs from patients **VE, IN** and **QQ.**

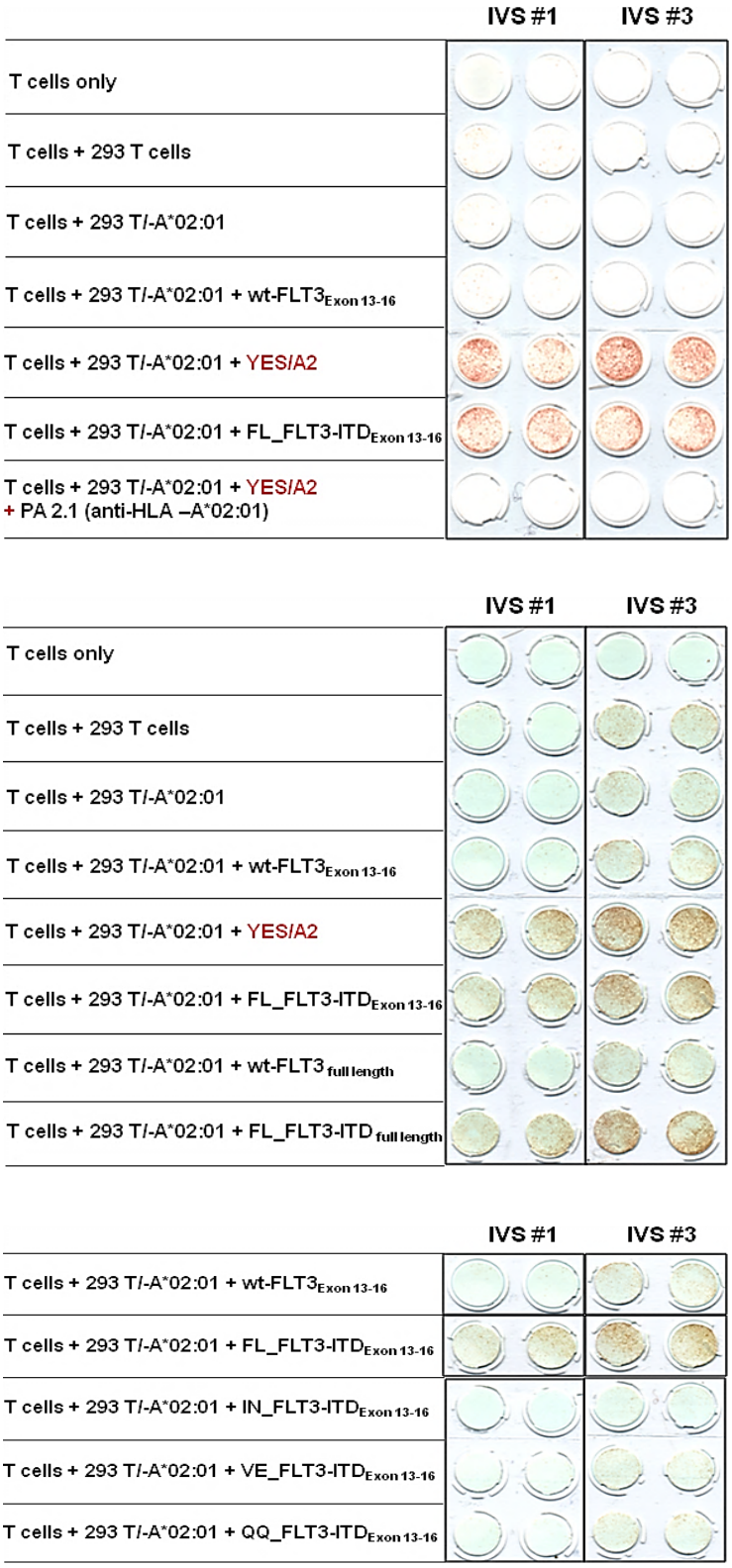


Figure 5.4.4.5: Generation of FL_FLT3-ITD-reactive T cells from MZUCB #12 by stimulation with the FL-peptide YES/A2.
CD8$^+$ T cells from MZUCB #12 (HLA-A*02:01-positive) were stimulated with the peptide **YES/A2** as scheduled in **Figure 5.4.4.4**. **(A)** On d37, responders (2.0x10^4/well) were tested in a 20-hour IFN-γ ELISPOT assay for the recognition of 293T/A*02:01 transfectants (2.0x10^4/well) exogenously loaded with **YES/A2** (10 µg/ml), or co-transfected with plasmids-encoding **FL**_FLT3-ITD$_{Exon\ 13\text{-}16}$ or wt-FLT3$_{Exon\ 13\text{-}16}$. The recognition of peptide-loaded transfectants was inhibited with a monoclonal antibody against HLA-A*02:01 (PA2.1). **(B)** On d52, responders (2.0x10^4/well) were tested again in a 20-hour IFN-γ ELISPOT assay for the recognition of 293T/A*02:01 transfectants (2.0x10^4/well) expressing **FL**_FLT3-ITD$_{Exon\ 13\text{-}16}$ as well **FL**_FLT3-ITD$_{full\ length}$. Controls were 293T/A*02:01 transfectants loaded with **YES/A2** (10 µg/ml) or co-transfected with *wt-FLT3*$_{Exon\ 13\text{-}16}$ as well as *wt-FLT3*$_{full\text{-}length}$ cDNA. (**C**) On d52, responders (2.0x10^4/well) were also tested for the recognition of 293T/A*02:01 transfectants (2.0x10^4/well) expressing FLT3-ITD$_{Exon\ 13\text{-}16}$ fragments derived from patients **IN, VE** and **QQ**. Controls were 293T/A*02:01 transfectants expressing wt-FLT3$_{Exon\ 13\text{-}16}$ and **FL**_FLT3-ITD$_{Exon\ 13\text{-}16}$. IVS#1 and IVS #3 are *in vitro* stimulation microculture #1 and microculture #3.

6. Discussion

Acute Myeloid Leukemia is one of the deadlier versions of leukemia, which is notoriously difficult and painful to treat. Cytotoxic chemotherapy and allo-HSCT are practically only two modalities to treat and cure AML. Except for patients with favorable karyotypes, chemotherapy has inevitable limitations of effectiveness due to chemo-resistance in a significant proportion of AML patients. Allo-HSCT is inherently accompanied by a variety of life-threatening complications related to GVHD, which limits candidates to younger and fitter patients. Given such situations, it is necessary to develop novel therapies (e.g. immunotherapy) that are in particular applicable to high-risk AML subtypes and can complement established treatments in a favourable way. Although our knowledge of the role of the immune system in the control of AML is still evolving, it is well established that AML cells, like many other cancer cells, display antigens that can trigger anti-leukemia immune responses (Anguille *et al.,* 2011a and 2011b). The GVL effect of allo-HSCT has shown that the immune system has an exquisite ability to specifically eradicate the leukemic cells that express particular antigens. This specificity is the heart of immunotherapy that at its best eliminates tumor cells without damaging normal cells. The identification of a series of promising AML-specific or -associated target antigens (Greiner *et al.*, 2008; Kadowaki and Kitawaki, 2011; Anguille *et al.,* 2012), and recent advances in other areas of immunology research, such as T-cell regulation including checkpoint blockade, immune barrier research and adoptive T-cell therapy (Gattinoni *et al.,* 2012), may enable antigen-specific immunotherapy to be established as a viable choice of therapy for AML. Evidently, the immunotherapeutic utility of any tumor antigen ultimately depends on its capacity to confer clinical benefit (Cheever *et al.,* 2009). Although phase III trial evidence is not yet available, several leukemia antigens have already demonstrated their potential clinical value in early-phase trials of active specific immunotherapy for AML (Anguille *et al.,* 2012) even in older and refractory patients (Greiner *et al.*, 2006; Guinn *et al*., 2007). Over the past years, an increasing number of AML antigens have been implicated in the GVL effect associated with allo-HSCT or DLI, indicating their therapeutic relevance in the context of passive immunotherapy (Smits *et al.,* 2009).

It is well known, that T cells are most important effectors of cellular cancer immunity, and carry long lasting memory. Therefore, recent cancer research has focused on the development and improvement of T-cell based immunotherapies (Barrett and Le Blanc, 2010; Smits *et al.,* 2011). The success of any antigen-specific immunotherapeutic strategy may critically depend on the choice of an “ideal” target antigen. In this context truly leukemia-specific mutated antigens are considered to be highly relevant targets for immunotherapeutic interventions (see **Chapter 1.2**). As an example, internal tandem duplications (ITD) of the *FLT3* gene represent one example of

a driving and immunogenic occurring in approximately 25% of AMLs (Choi *et al*., 2005; Gilliland and Griffin, 2002a). The allelic ratio of wt-FLT3 to FLT3-ITD in AML cells has been found to vary, but most commonly the mutant is present in a heterozygous form (Kottaridis *et al*., 2001; Whitman *et al.*, 2001; Thiede *et al.*, 2002). It has been shown that in FLT3-ITD$^+$ AMLs, FLT3-ITDs are in general already present in the leukemic stem cells (Levis *et al.*, 2005). Scholl and colleagues have demonstrated the potency of FLT3-ITD-encoded peptides and related mimotopes to bind to HLA class I alleles (Scholl *et al.*, 2006). The immunogenicity of an individual FLT3-ITD has been demonstrated for the first time by C. Graf *et al.,* (2007). Beyond that, individual FLT3-ITDs from five more AML patients were found to be immunogenic (**C. Graf, personal communication**, for ITDs from patients **IN, VE, EI** and **QQ**). Due to limited cell material from AML patients, it was difficult to perform more detailed analyses on ITD-reactive T cells. Therefore the main aim of the this work was to explore allogeneic lymphocyte sources, i.e. buffy coats from health donors and umbilical cord blood donations for the presence of ITD-reactive T cells, to generate from them stable T cell populations and clones and to analyze their fine specificity. The *FLT3-ITDs* used herein, were cloned by C. Graf and C. Wölfel from leukemic cell samples of patients **VE, IN, QQ, FL** and **JC.** They carried duplications of various lengths ranging from eight to twenty five amino acids (**Table 5.3.1.1**). All of them had their initiation site within codons 573-620, known to be the preferential mutation site for FLT3 length mutations (Thiede *et al*., 2002). Identical *ITDs* are rarely detected in leukemic samples of different patients, which is in line with the rather limited location of *FLT3-ITD* occurrence (Yokota *et al*., 1997; Thiede *et al*., 2002).

Herein, for the most part of *in vitro*-stimulations autologous CD8$^-$ PBMCs/UCBMCs or monocytes were used as APCs after electroporation with antigen-encoding IVT-mRNA. CD8$^-$ PBMCs/UCBMCs or monocytes instead of DCs were chosen, because the generation of autologous DCs was generally not feasible due to limited amount of available cell material especially in the AML patient and in UCB samples. DCs constitute only 0.1–0.5% of human PBMCs (Cavanagh and Von Andrian, 2002). The suitability of mRNA-electroporated PBMCs for the *in vitro*-stimulation of recall T-cell responses has been demonstrated first by Teufel and colleagues (2005). PBMCs isolated from peripheral blood were proven to be potent *in vitro*-stimulators of antigen-specific T lymphocytes (Johnson *et al.,* 2006; Kreiter *et al.,* 2007; Hiura *et al.,* 2007; Van Camp *et al.,* 2010) and can also be used as targets for readout purposes in immune-monitoring assays (Kreiter *et al*., 2007; Van Camp *et al.,* 2010). Under certain electroporation conditions, among PBMC the CD14$^+$ monocyte subpopulation is preferentially transfected upon electroporation with IVT-mRNA (**Table 5.3.1.2**) (**personal communication C. Graf**), which is highly desirable due to the superior T-cell stimulatory capacity attributed to monocytes (Ponsaerts *et al.,* 2002; Milano *et al.,* 2007).

The decision to use IVT-mRNA as an antigen format instead of peptides was based on the fact that the use of full-length mRNA allows to cover all epitopes of a given protein. The use of mRNA not only circumvented the need to predict and synthesize peptides, but guaranteed that stimulatory peptides were endogenously processed. $CD8^+$ T cells against pp65, directly tested *ex vivo* or stimulated *in vitro* prior to testing, were detected with similar efficiency, with $CD8^-$ PBMCs either electroporated with *pp65* IVT-mRNA or loaded with pp65/A*02 peptide (NLVPMVATV) (**Figure 5.1.2.2**). This observation confirmed the findings of Britten *et al.,* (2004), who have shown that electroporation of IVT-mRNA equals peptide-loading in its ability to detect even rare antigen-experienced T cells. An additional benefit of IVT-mRNA electroporation is that it can be relatively easily performed under good clinical practice conditions because the expression of mRNA is only transient and bears no risk for insertional mutagenesis (Ponsaerts *et al.,* 2003).

It is currently well accepted that the frequency of T cells specific for single MHC/peptide complexes in antigen-primed/unprimed circulating lymphocyte populations can be lower than 1 in 10^6 lymphocytes. E.g. for MAGE-encoded antigens this frequency appeared to be around $4.0x10^{-7}$ of $CD8^+$ T cells (Chaux *et al.,* 1998). Further on, it has also been demonstrated that the frequency of precursor T cells recognizing LAAs in healthy individuals is highly inconsistent (Delain *et al.,* 1994; Salomo et al., 1995; Smit *et al.,* 1998; Rezvani *et al.,* 2003 and 2009; Griffioen *et al.,* 2006; Ho *et al.,* 2006; Brackertz *et al.,* 2011). When already frequencies against shared tumor antigens, that are expressed on non-malignant tissues to various extent, are rather low in healthy individuals, it was reasonable to assume that frequencies against mutations specifically occurring in malignant cells are even lower. Therefore, techniques to expand and detect T cells against FLT3-ITDs needed to be highly sensitive and allow the majority of T cells with a given specificity to proliferate. To tackle this, a method had to be set up, with which large numbers of $CD8^+$ T cells can be analyzed for reactivity against FLT3-ITDs.

Conventional approaches for the *in vitro*-stimulation of leukemia-specific T-cell responses are traditionally based on the enrichment of leukemia-reactive CTLs in bulk culture before cloning them by limiting dilution (Bleakley and Riddell, 2004). Herein, in initial experiments it was attempted to enrich FLT3-ITD-reactive T cells by this procedure, but this proved to be inefficient (data not shown). After having proven that antigen-specific were efficiently expanded under microculture conditions on 96-well plates (**Figures 5.1.4.1** and **5.1.4.2**), LD-like conditions were chosen to screen for the presence of FLT3-ITD-reactive T cells (see **chapter 5.1.4**). The major advantage of this strategy was that it allowed for the estimation of precursor T cell frequencies.

Especially patients in first remission after conventional chemotherapy are prime candidates for FLT3-ITD-specific immunotherapy aiming at the extinction of minimal residual disease. In theory

this can be achieved by inducing or strengthening anti-FLT3-ITD-specific T-cell responses via therapeutic vaccination or by the adoptive transfer of FLT3-ITD-specific T cells. For the latter approach it would be obvious to apply autologous T cells. The adoptive transfer of autologous tumor-reactive T cells with defined specificity has been most extensively studied in melanoma it has been successfully performed in melanoma (see **Chapter 1.5** in the Introduction). The expansion of autologous FLT3-ITD-specific clonal T cells was exercised in detail in patient **FL** diagnosed with FLT3-ITD-positive AML, who was in first complete clinical remission and exhibited rather high frequencies of $CD8^+$ T cells against her individual FLT3-ITD (**Figure 5.2.2.1** and **Table 5.3.1.1**).

The data previously obtained by Graf *et al.,* (2007) were supported by the analysis of patient **FL**. Anti-**FL_**FLT3-ITD responses were detected using the limiting dilution-like microculture stimulation approach both with autologous $CD8^-$ PBMCs and with allogeneic HLA class I-matched FastDCs electroporated with IVT-mRNA encoding **FL_**FLT3-ITD (**Figure 5.2.1.1**). FLT3-ITD-reactivity was observed in several independent stimulations from the patient's PBMCs collected at different time points before and after chemotherapy (**Figure 5.2.1.2**)**.** These data document the persistence and detectability of FLT3-ITD-specific T cells *in vivo* over a period of at least 12 months. In this patient T cells against **FL_**FLT3-ITD were even detectable directly *ex vivo* without pre-stimulation both in fresh and in frozen $CD8^+$ T cells (**Figure 5.2.2.1**). IFN-γ-secreting **FL_**FLT3-ITD-reactive T cells were isolated directly from *ex vivo* $CD8^+$ T cells with an IFN-γ secretion assay and cloned by single cell sorting using flow cytometry. The clonal T cells were stimulated non-specifically with OKT3/IL-2 using a modified rapid expansion protocol (Dudley *et al.*, 2003). Sixteen growing T-cell clones were able to recognize **FL_**FLT3-ITD (**Figure 5.2.3.3**) and recognition was restricted via HLA-A*01:01 (**Figure 5.2.3.4**). In addition, three of the T-cell clones also recognized autologous AML cells of patient **FL** (**Figure 5.2.3.5**). However, it was repeatedly observed that the T-cell clones lost their specificity upon further stimulation with OKT3/IL-2. Therefore, this stimulation procedure is not considered as a satisfactory way in establishing stable FLT3-ITD-reactive T cell clones.

Clearly, limited availability of peripheral blood donations and altered proliferation behavior of T cells in patients requiring rather aggressive chemotherapy set narrow boundaries to all efforts in generating FLT3-ITD-specific T cells from autologous sources. It might be advantageous to expand leukemia antigen-specific T cells from *ex vivo* generated allogeneic T cells that have not been subjected to immune-editing *in vivo* (Awong *et al.*, 2011). Recently, it has been shown that TAA-reactive CTL clones derived from cancer patients, although occurring at higher frequencies, appeared to exhibit reduced proliferative capacity, a lower intensity of T-cell receptor expression and weaker lytic capacity as compared to T cells from healthy donors with the same fine specificity (Karanikas *et al.*, 2010). It may, therefore, be advantageous to choose

allogeneic sources for the generation of ITD-specific T cells. Healthy donors have not been subjected to high-dose chemotherapy, are supposed to have a superior thymic function and a more diversified T-cell repertoire (Hakim *et al.*, 2005), with better proliferation behavior. Donor-derived lymphocytes have impressively demonstrated that immunotherapy can work in leukemia (Kolb *et al*., 1995). However, the precursor frequency of leukemia-reactive CTLs in healthy individuals is rather low. But, it has been reported that T-cell responses against several LAAs can be expanded from the peripheral blood of healthy donors. The antigens were PR1 (Scheibenbogen *et al.,* 2002; Rezvani *et al.,* 2003 and 2007), WT1 (Rezvani *et al.,* 2003 and 2007; Weber *et al.,* 2009), PRAME (Griffioen *et al.,* 2006; Rezvani *et al.,* 2009; Quintarelli *et al.,* 2008), RHAMM (Greiner *et al.,* 2004; Casalegno-Gardu˜no *et al.,* 2012), Survivin (Grube *et al.,* 2007), BCR-ABL (Butt *et al.,* 2005) and Cyclin-A1 (Ochsenreither *et al.,* 2012). Herein, unprimed healthy individuals (either adult buffy coat or umbilical cord blood donations) were analyzed for reactivity against immunogenic FLT3-ITDs identified in leukemia patients **VE, IN, QQ, FL** and **JC** and restricted in the autologous setting by HLA-B*27:05, -A*32:01, -A*11:01, -A*01:01 and -A*01:01, respectively (**Table 5.3.1.1**).

By means of limiting dilution-like microculture stimulation allogeneic $CD8^+$ T-cell responses against FLT3-ITDs could be detected in eight of nineteen healthy buffy coat donors (**Table 5.3.1.3** and **Figure 5.3.1.4**). ITD-reactive T cells could be propagated beyond the initial detection in four of these eight individuals involving monocytes as antigen-presenting cells. The frequencies of FLT3-ITD-reactive T cells in the peripheral blood of those donors was estimated to be in the range of approximately 3 to 12 per 10^{-7} of $CD8^+$ T cells (**Table 5.3.2.1**). These frequencies were equivalent to a log-3 to log-4 reduction when compared to frequencies observed in the peripheral blood of AML patients (**Figure 5.2.2.1**; Graf *et al.,* unpublished). This supported the view that the anti-ITD responses found in patients with FLT3-ITD-positive AML resulted from the interaction of their T-cell system with leukemia cells bearing length mutations of FLT3-ITD. None of the T-cell microcultures recognized wt-FLT3 which indicated an exquisite leukemia reactivity of the T cells. Although the estimated frequencies of FLT3-ITD-reactive T cells are rather low but the $CD8^+$ T cells against individual FLT3-ITDs can be detected in different donors (**Figure 5.3.1.4, Figure 5.3.1.5** and **Figure 5.3.1.6**).

Restricting HLA class I alleles of buffy coat-derived allogeneic T cells against FLT3-ITDs were identified in two donors. In donor #7926, **VE_**FLT3-ITD recognition was restricted by HLA-C*07:02 (**Figure 5.3.2.7**), while recognition of the same FLT3-ITD had been restricted by HLA-B*27:05 in the original patient-derived autologous model system (**Figure 5.3.1.1**). But all efforts to establish a stable clonal anti-FLT3-ITD T-cell line from allogeneic sources by stimulation with RNA-transfected APCs and non-specific expansion with OKT3 failed. In donor #1853, **IN_**FLT3-ITD recognition was restricted by HLA-B*35:01 (**Figure 5.3.2.5** and **5.3.2.6**), which also had

been observed in patient **IN** (**Table 5.3.1.1**). By performing C-terminal fragmentation of the *FLT3-ITD* cDNA of patient **IN,** it was possible to identify a 10-mer peptide **CPSDNEYFYV** recognized by the allogeneic T cells in association with HLA-B*35:01 (**Figures 5.3.4.4** and **5.3.4.5**).

It is reported that naïve CD45RA-positive $CD8^+$ T cells represent a prime source for leukemia-reactive CTL in unprimed healthy individuals (Distler *et al.,* 2008; Quintarelli *et al.,* 2008; Bleakley *et al.,* 2010; Albrecht *et al.,* 2011), and that such CTLs target polymorphic mHags and non-polymorphic LAAs (Quintarelli *et al.,* 2008; Bleakley *et al.,* 2010; Wölfel *et al.,* 2008). Interestingly, the adoptive transfer of naïve T cells was shown to mediate a superior antitumor immunity as compared to central memory T cells (Hinrichs *et al.,* 2009). Recently, Distler and colleagues have also suggested that depletion of naïve T cells will impair the generation of donor-derived $CD8^+$ CTLs recognizing leukemia cells in the context of shared HLA molecules (Distler *et al.,* 2011). Umbilical cord blood mononuclear cells (UCBMC) collected from the umbilical cord and placenta of newborns represent another source of hematopoietic stem cells in allo-HSCT. It is known that UCB-T cells predominantly exhibit a naïve phenotype and their TCR repertoire is fully diversified (Garderet *et al.,* 1998; Merindol *et al.,* 2010; Merindol *et al.,* 2011). Naïve T cells can be phenotypically distinguished from effector and memory cells by the expression of distinct cell surface markers, such as CD45RA, CD62L, and CCR7 (Sallusto *et al.,* 1999). CD62L and the chemokine receptor CCR7 are both involved in homing of naïve and central memory $CD8^+$ T cells to secondary lymphoid organs (Weninger *et al.,* 2002). The superior antitumor immunity mediated by $CD62L^+$ $CCR7^+$ $CD8^+$ T cells in murine adoptive immunotherapy studies can be attributed to their increased proliferative and migratory abilities, compared to $CD62L^-$ $CCR7^-$ effector memory $CD8^+$ T cells (Gattinoni *et al.,* 2005). Therefore, these major differentiation markers (i.e. $CD45RA^+$, $CD62L^+$, $CD27^+$ and $CCR7^+$) were used herein for flow cytometric analysis of UCB-derived $CD8^+$ T cells. As shown in five UCB donations (**Table 5.4.1.2**) between 85-99% of UCB-$CD8^+$ T cells expressed a naïve phenotype.

It was analyzed whether human UCB-derived $CD8^+$ T cells required a ‘‘third signal’’ for a productive response *in vitro*. As shown in **Table 5.4.1.4**, naïve $CD8^+$ T cells attained a maximum differentiation towards the effector memory phenotype ($CD8^+$ T_{EM}; $CD45RA^+$ $CD45RO^+$ $CCR7^+$) with a cytokine cocktail containing IL-12, IL-7 and IL-15. This is in consistency with McCarron and Reen, (2010) who has demonstrated that IL-12 is necessary for the differentiation of activated human neonatal $CD8^+$ T cells into effector T cells. Previously, it has been reported that IL-12-priming during antigenic stimulation dramatically increased the population of effector and memory $CD8^+$ T cells mainly by preventing activation-induced cell death (AICD) (Chang *et al.,* 2004), suggesting that the development of effector and memory cells could be regulated by

early IL-12 signalling, which is mediated by down-regulation of CD43 expression (Lee and Chang, 2010).

Antigen-specific T cells are contained in umbilical cord blood and can be primed and expanded as shown for T cells against CMV, adenovirus, Epstein Barr virus, influenza virus and HIV-1 (Sun *et al.,* 1999; Marchant *et al.,* 2003; Park *et al.,* 2006; Rastogi *et al.,* 2007; Hanley *et al.,* 2009). Direct evidence that umbilical cord blood might be used as a source for generating anti-tumor CTLs is rather limited. Recently, Merindol *et al.,* (2010) showed that Melan-A/A2-specific $CD8^+$ T cells can be detected in UCB. Germenis and Karanikas. (2010) reported on the generation of CTLs against an HLA-A2-restricted MAGE-A3 peptide from UCBMCs. In addition, it has been demonstrated that cord blood contains T cells specific for maternal mHag HA-1 and may contribute to *in vivo* GvL activity after transplantation (Mommaas *et al.,* 2005). Recently, Krishnadas and colleagues (2011) described the presence of WT1-specific $CD8^+$ T cells in umbilical cord blood.

Using quasi-limiting dilution conditions, as described in **Method sections** (**4.8** and **4.9.2**) and as schematically summarized in **Figure 5.4.2.1**, $CD8^+$ T-cell responses against distinct FLT3-ITDs were generated from UCB lymphocytes. Anti-FLT3-ITD responses were observed in five of seven UCB donors stimulated against the five different FLT3-ITDs using either $CD8^-$ UCBMCs (**Figure 5.4.2.2a** and **b**) or FastDCs as antigen-presenting cells (**Figure 5.4.2.4**). The frequencies of ITD-reactive T cells were estimated to be in the range of 4 to 18 per 10^6 $CD8^+$ T cells, which is approximately 15-fold higher than the frequencies observed in healthy individuals (see **Result section 5.3**). These data indicate that UCB is superior to buffy coats as a source of FLT3-ITD-reactive T cells. The UCB-precursor frequencies are considerably lower than the frequency reported for Melan-A/A2-specific UCB-$CD8^+$ T cells, i.e. 400 CTLs per 10^6 $CD8^+$ T cells (Merindol *et al.,* 2010), but higher than the precursor frequency reported for MAGE-A3/A2 peptide-specific UCB-$CD8^+$ T cells, i.e. 2 to 3 CTLs per 10^6 $CD8^+$ T cells *(*Germenis and Karanikas*,* 2010).

HLA I- alleles restricting FLT3-ITD-derived peptides could be identified in four of five UCB donations with anti-FLT3-ITD reactivity (**Table 5.4.3.1**). Evidently, in single UCB donations T cells against different FLT3-ITDs were detectable and the recognition of single FLT3-ITDs by lymphocytes from the same UCB donation was restricted by different HLA I alleles **(Figure 5.4.3.2).** At this juncture, as with buffy coat donors, the restricting HLA I molecules were different from those observed in the original patient-derived systems. In donor MZUCB #2, FLT3-ITD-specific $CD8^+$ T cell responses were observed against two distinct ITDs. Their HLA-restriction alleles were HLA-A*02:01 for **FL**_FLT3-ITD and HLA-A*03:01 for **VE**_FLT3-ITD (**Figure 5.4.3.3**). The **FL**_FLT3-ITD-reactive T cells recognized the 10-mer peptide

YESDNEYFYV, designated as **YES/A2**, that was predicted from the putative **FL_**FLT3-ITD amino acid sequence with different algorithms (**Figure 5.4.4.2**). The same procedure, however, did not lead to the identification of the peptide recognized by HLA-A*03:01-restricted T cells against **VE_**FLT3-ITD (**Figure 5.4.4.3**). The reasons for this are manifold and involve e.g. insufficient peptide prediction algorithms as well as posttranslational protein or peptide modification (Skipper *et al.*, 1996) or even splicing of non-contiguous peptide segments (Vigneron *et al.*, 2004; Warren *et al.*, 2006). The antigenic capability of the **FL_**FLT3-ITD-derived peptide **YES/A2** was validated by peptide stimulation of HLA-A*02:01-positive UCB lymphocytes (schematically explained in **Figure 5.4.4.4**). T cells stimulated with **YES/A2** were found to recognize 293Tcells transfected with HLA-A*02:01 and loaded with **YES/A2** as well as 293T cells co-transfected with HLA-A*02:01 and either full-length **FL**_*FLT3-ITD* cDNA or the **FL**_*FLT3-ITD*$_{Exon\ 13\text{-}16}$ cDNA fragment (**Figure 5.4.4.5**), indicating that **YES/A2** is naturally processed.

A recent study identified an ITD insertion site within the beta1-sheet (aa 610-615) of the tyrosine-kinase domain-1 (TKD1) as a particularly unfavourable prognostic factor associated with resistance to chemotherapy and inferior outcome in AML (Kayser *et al.,* 2009). The AML cells of patient **IN** carry an immunogenic FLT3-ITD located within TKD1 (**Table 5.3.1.1**; Graf *et al.*, unpublished). This indicates that T-cell responses can also be seen in FLT3-ITDs affecting TKD1, which provide a rational basis for immunotherapeutic approaches even in this subgroup with enhanced poor prognosis. The elimination of drug-resistant and/or quiescent leukemia-initiating cells in residual disease is a major issue of all attempts to improve survival and to cure patients with AML (Graham *et al.,* 2002). Although small molecules inhibiting the FLT3-receptor appear promising for the treatment of FLT3-ITD$^+$ AML (Giles *et al.,* 2003; Zhang *et al.,* 2008; Metzelder *et al.,* 2009), kinase inhibitors cannot reliably eliminate minimal residual disease according to past experience (Levis and Small, 2005). But, as shown recently, sorafenib monotherapy may synergize with GvL effects after allo-HSCT to induce durable remissions in FLT3-ITD-positive AML (Metzelder *et al.,* 2012). Especially, since leukemia-initiating cells were shown to express FLT3-ITD (Van Driessche *et al.,* 2005), the additional generation and maintenance of an effective FLT3-ITD-specific immune response would improve chances to eliminate FLT3-ITD$^+$ leukemia stem cells. However, the strong variation of ITD duplications requires to design an individual and patient-specific immunotherapy. As another limitation of FLT3-ITD-specific immunotherapy, a small, but consistent, portion of AMLs initially harboring FLT3-ITDs lacks these alterations at relapse (Nakano *et al.*, 1999; Kottaridis *et al*., 2002; Shih *et al*., 2002; McCormick *et al.*, 2010). Obviously, in these cases relapse is driven by leukemia cells that do not harbor this mutation. Furthermore, in some relapse AML samples new FLT3-ITDs are observed, whereas the one identified at initial diagnosis cannot be detected anymore, suggesting that distinct FLT3-ITDs are present in different AML subpopulations (Kottaridis *et al*.,

2002; Shih *et al*., 2002). For this minority of patients suffering from FLT3-ITD$^+$ AML additional therapeutic options would have to be developed.

The presented work aimed at the generation of FLT3-ITD-specific T-cell populations from different sources of T cells, which were either primed or naïve towards mutated FLT3. HLA class I-restricted, FLT3-ITD-reactive T cells were successfully expanded not only from peripheral blood of patients with FLT3-ITD-positive AML, but also from healthy buffy coat and from umbilical cord blood lymphocytes using *in vitro*-stimulation techniques. These data strengthen the idea to target FLT3-ITDs with T cell-based immunotherapy. Thus, it appears worth to find out,

-if and how anti-FLT3-ITD T-cell responses are influenced by chemotherapy and treatment with kinase inhibitors,

-if their presence or spontaneous enhancement is associated with improved prognosis,

-if FLT3-ITD$^+$ AML cells develop signs of immune-selection and immune-resistance in the course of the disease, and, finally,

-whether the enhancement and maintenance of T-cell responses against the individual FLT3-ITD, e.g. by vaccination or adoptive T-cell transfer, can eliminate minimal residual disease after successful conventional therapy.

7. References

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8. Appendix

Declaration

''I hereby declare that this thesis represents my original work, without any unauthorized assistance and only with the support that I have indicated in the thesis. All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such. Moreover, I declare that this is a true copy of my thesis, including any final revisions, and that this thesis has not been submitted for a higher degree to any other University or Institution. In carrying out this research, I complied with the rules of standard scientific practice as formulated in the statutes of the Johannes Gutenberg-University Mainz to insure standard scientific practice.''

Vijay Kumar Singh
Place: Mainz,
Date: 18/ 04/ 2013

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Curriculum vitae