A neoepitope generated by an FLT3 internal tandem duplication (FLT3-ITD) is recognized by leukemia-reactive autologous CD8+ T cells

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The FLT3 receptor tyrosine kinase is expressed in more than 90% of acute myelogenous leukemias (AMLs), up to 30% of which carry an internal tandem duplication (ITD) within the FLT3 gene. Although varying duplication sites exist, most FLT3-ITDs affect a single protein domain. We analyzed the FLT3-ITD of an AML patient for encoding HLA class I–restricted immunogenic peptides. One of the tested peptides (YVDFREYEYY) induced in vitro autologous T-cell responses restricted by HLA-A*0101 that were also detectable ex vivo. These peptide-reactive T cells recognized targets transfected with the patient’s FLT3-ITD, but not wild-type FLT3, and recognized the patient’s AML cells.

Our results demonstrate that AML leukemic blasts can in principle process and present immunogenic FLT3-ITD neoepitopes. Therefore, FLT3-ITD represents a potential candidate target antigen for the immunotherapy of AML. (Blood. 2007; 109:2985-2988)

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Synthesis products were analyzed by high-performance liquid chromatography (HPLC) (Varian star, Darmstadt, Germany) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (GSG-future, Bruchsal, Germany). Peptides of less than 80% purity were purified by HPLC.

**In vitro stimulation of JC-FLT3-ITD–specific T cells**

In vitro stimulation (IVS) was performed as published with slight modifications. JC-PBMCs were seeded in 96-well plates (5 × 10⁴ per well) in AIM-V supplemented with 5% human serum, IL-2 (20 U/mL), IL-7 (10 ng/mL), IL-4 (10 ng/mL), and synthetic JC-FLT3-ITD peptides (5 μg/mL) and restimulated on day 7. One of the IVS-responder populations was cloned by limiting dilution. Feeders were allogeneic AK-EBV-B lymphocytes (irradiated, 2 × 10⁶ per well). Stimulators were EF-DCs (irradiated, 1 × 10⁶ per well) transfected with JC-FLT3-ITD mRNA (1 μg per 0.2 × 10⁶ DCs) using TransMessenger Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Restimulations were performed in 7-day intervals.

**ELISPOT assays**

IFN-γ and granzyme B (GrB) enzyme-linked immunospot (ELISPOT) assays were performed as described. Spots were evaluated with computer-assisted video image analysis. Briefly, image acquisition was performed with an AxioCam MRc camera attached to a Zeiss Axio Imager M1 microscope via a 0.63× adapter. Spots were counted with a 3.15× magnification at a resolution of 1388×1040 pixels. The software was KS ELISPOT version 4.8. Targets were K562/HLA cells electroporated with mRNA at 300 μF and 250 V or loaded with synthetic peptides (10 μg/mL).

**Results and discussion**

The FLT3-ITD identified in AML cells (JC-FLT3-ITD) encoded a duplication of amino acids 591 to 599 (Figure 1). The same duplication event has already been found in another AML patient, which is in line with the rather limited location of FLT3-ITD occurrence. Five peptides with high combined prediction scores were selected (Figure 1) and used for IVS with JC-PBMCs collected in CR. T-cell responses against peptide YVDFREYEY (YVDA1), predicted to bind to HLA-A*0101, could be repeatedly generated. The peptide was only recognized in association with HLA-A*0101 but not with any other HLA-A or -B allele of patient J.C. (not shown). YVDA1-reactive IVS responders recognized K562/A1 transfected with full-length JC-FLT3-ITD mRNA but not K562/A1 transfected with wtFLT3 mRNA (Figure 2A). This indicated that the peptide YVDA1 was endogenously processed and proved the existence of JC-FLT3-ITD–specific T cells.

IVS-responder populations containing YVDA1-reactive T cells also recognized JC-AML cells (Figure 2A). We cloned IVS JC#1 using HLA-I–matched EF-DCs transfected with JC-FLT3-ITD as stimulators. Ten of 19 T-cell clones recognized YVDA1 and JC-AML equally well (Figure 2B), which demonstrated that JC-FLT3-ITD was expressed and processed by JC-AML cells leading to the presentation of YVDA1 on their cell surface. When YVDA1 was titrated, half-maximal recognition was observed at 20 nM (Figure S1, available on the Blood website; see the Supplemental Figure link at the top of the online article), which is in the range of moderate avidity T cells.

YVDA1-specific T cells were detectable ex vivo at similar frequencies in the patient’s peripheral blood lymphocytes (Figure 2C) and in bone marrow lymphocytes (not shown) both drawn at diagnosis. This indicated an in vivo expansion of these T cells. In addition, ex vivo CD8⁺ T cells secreted GrB in response to YVDA1, which demonstrated their cytotoxic ability. It is widely accepted that progressive malignant disease in the presence of antitumor T cells is not an oxymoron considering the complexity of resistance mechanisms already discovered.

Recently, Scholl and colleagues showed the potency of FLT3-ITD–encoded peptides and related mimotopes to bind to HLA-I molecules. However, whether these peptides were processed and recognized by T cells was not explored. Analogous to JC-FLT3-ITD, fusion proteins occurring in leukemia subsets have been found to induce T-cell responses (e.g., ETV6/AML1 and TEL/AML1). As newly synthesized FLT3-ITD is retained in the endoplasmic reticulum because of inefficient folding and chaperoning, it appears likely that FLT3-ITD molecules are preferentially degraded after synthesis and introduced into the HLA-I–presenting pathway. This would favor T-cell recognition in case peptides are immunogenic.

It has been shown that FLT3-ITDs are present in leukemia stem cells. Targeting of leukemogenic molecules with T cells might improve the chances to eliminate also the quiescent fraction of leukemia stem cells as compared with small inhibitory molecules.

To fully recognize the potential of FLT3-ITD as an immunotherapeutic target, it will be necessary to study in a next step a cohort of FLT3-ITD–positive patients for anti–FLT3-ITD T-cell responses and to include CD8⁺ as well as CD4⁺ T-cell responses via all individual HLA-I and -II alleles.

**Acknowledgments**

C.G. was a fellow of the research training group 1043, “antigen-specific immunotherapy,” (project A3) and a PhD candidate at the Johannes Gutenberg-Universität in Mainz; T.W. was supported by the collaborative research initiative SFB 432 (project A1); and both projects were funded by the Deutsche Forschungsgemeinschaft. This work was further supported by a MAIFOR grant provided by the Medical Faculty of the Johannes Gutenberg-Universität (E.H. and T.F.); SFB 490 (project E6) (S.T.); and the Deutsche Krebshilfe (grant 70-2427-Hul) (C.H.). The authors thank S. Stevanovic.
They acknowledge the contributions by members of the Tumor Vaccination Center, particularly technical assistance by B. Schuch, M. Brkic, and K. Bechtold and FACS analysis by A. Konur. The authors also thank S. Debo and D. Eberts for technical assistance and M. Fatho and V. Lennerz for helpful discussions.

Authorship

Contribution: C.G. performed the experiments and drafted the manuscript as part of her PhD thesis; F.H. performed FLT3-ITD sequencing and cloning; S.T. and M.P.R. performed peptide predictions; F.K.S. performed PCR-based FLT3-ITD screening and prepared clinical samples; C.M.B. generated stable K562/HLA transfectants; C.H. contributed to the design of the project; T.F. contributed to the design of the project and to the writing of the manuscript; and T.W., C.G.’s thesis supervisor, designed the project and finalized the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Figure 2. AML-reactive CD8+ T cells of patient J.C. specifically recognized the HLA-A*0101–restricted peptide YVDREYEYY (YVD/A1) encoded by JC-FLT3-ITD. (A) Specificity of IVS-responder populations. JC-PBMCs collected in CR after induction chemotherapy were stimulated with peptide YVD/A1 in independent IVS. IVS responders were tested on day 13 in a 20-hour IFN-γ ELISPOT assay for recognition of unloaded, YVD/A1-loaded, wtFLT3 mRNA-transfected, or JC-FLT3-ITD mRNA-transfected K562/A1 cells (1 × 10⁵ per well) as well as for recognition of autologous AML cells (1 × 10⁵ per well). The results obtained with IVS JC#1, JC#2, and JC#4 are shown as representative examples. (B) Specificity of T-cell clones derived from IVS JC#1. JC#1 responders were cloned on day 14 by limiting dilution using HLA-I–compatible EF-DCs transfected with JC-FLT3-ITD mRNA as stimulators. T-cell clones were tested 20 days later against unloaded or YVD/A1-loaded K562/A1 cells (1 × 10⁵ per well) as well as against autologous AML cells (1 × 10⁵ per well) in a 20-hour IFN-γ ELISPOT assay. Three representative clones, JC#1.5, JC#1.6, and JC#1.12, are shown. (C) Reactivity against YVD/A1 detectable in ex vivo CD8+ T cells. CD8+ T cells were positively selected from JC-AML cells with immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and tested in a 30-hour IFN-γ and in a 30-hour GrB ELISPOT assay against unloaded or peptide-loaded untransfected K562 cells (1 × 10⁵ per well) as well as against unloaded or peptide-loaded K562/A1 cells (1 × 10⁵ per well). Peptides were YVD/A1- and known HLA-A1–binding peptides from HCMV pp65, tyrosinase, and influenza A basic polymerase 1 (pp65 364 to 373, SEHPTFTSQY; tyrosinase 146 to 156, SSDYVIPIGTY; PB1 591 to 599, VSDGGPNLY, respectively). The pp65 peptide served as a positive control; tyrosinase and influenza A peptides were negative controls. Data are means of duplicates. Notably, YVD/A1 recognition required the presence of HLA-A1 on K562 cells, because peptide-loaded but untransfected K562 cells did not induce spot formation in any test.

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