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STAT3 gain-of-function mutations associated with autoimmune lymphoproliferative syndrome like disease deregulate lymphocyte apoptosis and can be targeted by BH3 mimetic compounds

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ABSTRACT

Autoimmune lymphoproliferative syndrome (ALPS) is typically caused by mutations in genes of the extrinsic FAS mediated apoptotic pathway, but for about 30% of ALPS-like patients the genetic diagnosis is lacking. We analyzed 30 children with ALPS-like disease of unknown cause and identified two dominant gain-of-function mutations of the Signal Transducer And Activator Of Transcription 3 (STAT3, p.R278H, p.M394T) leading to increased transcriptional activity. Hyperactivity of STAT3, a known repressor of FAS, was associated with decreased FAS-mediated apoptosis, mimicking ALPS caused by FAS mutations. Expression of BCL2 family proteins, further targets of STAT3 and regulators of the intrinsic apoptotic pathway, was disturbed. Cells with hyperactive STAT3 were consequently more resistant to intrinsic apoptotic stimuli and STAT3 inhibition alleviated this effect. Importantly, STAT3-mutant cells were more sensitive to death induced by the BCL2-inhibitor ABT-737 indicating a dependence on anti-apoptotic BCL2 proteins and potential novel therapeutic options.

1. Introduction

The Canale-Smith or autoimmune lymphoproliferative syndrome (ALPS) is a genetic disorder of disturbed apoptosis [1–3]. This physiological form of cell death is of fundamental importance for the regulation of cell numbers in the immune system [4]. In human cells apoptosis is conveyed *via* two major pathways. In the extrinsic pathway, cell surface death receptors (FAS, TRAIL-R1/2 and TNF-R1) are activated upon binding of their cognate death ligands (FAS-L, TRAIL, TNFα) [5]. Cell death is instantly initiated by intracellular recruitment of adapter proteins and inactive procaspase-8 and -10 into a death-inducing signaling complex that mediates the activation of the caspases. A caspase cascade is triggered by cleavage mediated activation of downstream executioner caspases. Cellular proteins are proteolyzed leading to the death of so-called type I cells (*e.g.* activated T cells). In most cells, however, the amount of death receptor activated caspases is insufficient to induce cell death (type II cells). In these cells, the signal needs to be amplified by the intrinsic mitochondria mediated pathway through cleavage of the BCL-2 protein BID by caspase-8 or -10. This leads to the formation of active truncated BID (tBID) that translocates to the mitochondria and activates the pro-apoptotic BCL-2 family members BAX and BAK1 to mediate the release of cytochrome *c* from the mitochondria into the cytosol. This triggers the assembly of the apoptosome and subsequent activation of effector caspases and cell death execution. The intrinsic apoptotic pathway can also be initiated independent of the extrinsic pathway by multiple signals, including cytokines and cellular stress. Members of the BCL2 family regulate each other through complex bimolecular interaction networks and function together as a rheostat, controlling the sensitivity of cells to apoptotic stimuli [6].

All members of the BCL2 family contain one to four conserved sequence motifs called the BCL2 homology (BH1–4) domains. Among

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the pro-apoptotic members of the BCL2 family, there are the so-called BH3-only proteins (including BID and BIM) that contain only the single BH3 domain [7]. The BH3 domain comprises a 9-amino acid amphipathic α-helix that binds to a hydrophobic pocket of BCL2-like anti-apoptotic proteins (including BCL2 and BCL-XL). In this manner, pro-apoptotic BH3 only proteins are kept inactive. A class of small molecule drugs (including ABT-737 [8]) mimics BH3-domain proteins and aims at disrupting this complex, thereby sensitizing cells to apoptosis.

In 70% of patients the genetic causes of ALPS are germline or somatic mutations in the genes of the extrinsic apoptotic pathway encoding the Fas receptor (*FAS*), its ligand (*FASLG*) or caspase-10 (*CASP10*) [3]. Defective death receptor signaling results in the accumulation of lymphocytes and failure to remove auto-reactive lymphocytes. As a consequence ALPS patients characteristically show enlargement of lymph nodes, spleen and liver and increased numbers of peripheral, otherwise rare terminally differentiated, activated, double-negative T lymphocytes that do not express the surface markers CD4 and CD8, but have a functional alpha/beta T-cell receptor (so called α/β-DNT cells). Although lymphoproliferation is initially non-malignant, ALPS patients have an increased risk of developing B and T cell malignancies (in particular lymphomas) early in life caused by inappropriately surviving lymphocytes with oncogenic potential [9,10]. Autoimmune disease in ALPS patients is mainly caused by B lymphocytes and directed towards blood cells. Increased numbers of B cells producing autoreactive antibodies cause various autoimmune problems: clinically significant hemolytic anemia, thrombocytopenia with bleeding tendency, autoimmune neutropenia and risk of bacterial infection. Other organs such as skin, liver, kidney, endocrine or nervous system may be affected by autoimmunity [11].

The signal transducer and activator of transcription 3 (STAT3) is a member of the STAT protein family that controls apoptosis, proliferation and cell growth by regulation of gene expression in response to extracellular cytokines, IFNγ or growth hormones [12]. Somatic gain-of-function (GOF) mutations of *STAT3* cause lymphoproliferative neoplasms, aplastic anemia, and myelodysplastic syndrome [13–15], whereas germline *STAT3* gain-of-function mutations have been reported in patients suffering from recurrent infections, multi-organ autoimmunity, hypogammaglobulinemia and lymphoproliferation indicating a potential overlap of phenotype with ALPS disease [16–18]. The underlying molecular basis for lymphoproliferation in *STAT3* GOF caused disease is not yet understood. Due to this lack of knowledge and the lack of clinically approved STAT3 inhibitors, targeted treatment is not yet available.

2. Material and methods

2.1. Patients, relatives and healthy controls

Patients, relatives and healthy controls were enrolled in this study after obtaining written informed consent. All experiments were approved by the Ethical Review Boards of the Hadassah Hebrew University, the Israeli Ministry of Health and the University Hospital Düsseldorf. Whole-exome sequencing of DNA derived from peripheral blood was performed on a HiSeq2000 (Illumina, San Diego, CA).

2.2. Sanger sequencing of germline and somatic mutations in classical ALPS genes

To exclude germline or somatic variations in classical ALPS associated genes as genetic cause of the disease we amplified exons including exon/intron borders of *FAS*, *FASLG* and *CASP10* by PCR and carried out Sanger sequencing as described earlier [19].

2.3. Whole-exome sequencing and bioinformatic analysis

To identify the disease causing mutations next generation sequencing was carried out after targeted enrichment of whole exonic regions from sheared genomic DNA for the patient and family members using the SeqCap EZ Exome Library 2.0 kit (Roche/Nimblegen, Madison, WI). 100 bp single-read sequencing was performed on a HiSeq2000 (Illumina, San Diego, CA) essentially as described [20]. Sequencing data was aligned to the human genome assembly hg19 (GRCh37) using BWA. Sequencing data was converted using Samtools. Variation calls were obtained employing GATK, HapMap, OmniArray and dbSNP134 datasets (The Broad Institute, Cambridge, MA). Single nucleotide variations were annotated using the Variant Effect Predictor, based on the Ensemble database (v70). Variations were imported into a proprietary MySQL database driven workbench (termed Single Nucleotide Polymorphism Database, SNuPy).

2.4. Validation of STAT3 sequence variation

Validation of the nucleotide variations in the *STAT3* gene were performed by PCR/Sanger sequencing using genomic DNA from the patients and family members. The following primers were used: STAT3-R278H (forward: 5′ CCTTGTTCTTATTGTAGTGGTCTCC 3′, reverse: 5′ AAAGAGAAGATGGGCTCACG 3′), STAT3-M394T (according to [17]) (forward: 5′ CTTCATCCTCCGGCTACTTG 3′, reverse: 5′ GAGCTCCTCCCACATACCAA 3′). DNA fragments were amplified by PCR employing the Phusion High Fidelity PCR Master Mix (NEB, Ipswich, MA), 0.5 μM each primer and 20 ng of template genomic DNA. Cycling conditions: 30 s at 98 °C followed by 30 cycles of 7 s at 98 °C, 23 s at 60 °C, 30 s at 72 °C and a final extension of 10 min at 72 °C. Sanger sequencing was carried out by a core facility (BMFZ, University Duesseldorf, Germany). The nucleotide variations were visualized using sequencher software (Gene Codes, Ann Arbor, MI).

2.5. Isolation and cultivation of mononuclear cells

Peripheral blood was obtained from the patients, relatives and healthy individuals. Mononuclear cells were isolated using density gradient centrifugation and cultured in medium consisting of RPMI1640 (Life Technologies, Darmstadt, Germany) and Panserin 401 (PAN-Biotech, Aidenbach, Germany) mixed 1:1, supplemented with 10% fetal calf serum (FCS) and 100 μg gentamycin (Life Technologies) and 30 U/ml IL2 (Miltenyi, Bergisch Gladbach, Germany). For the first 4 days, cells were activated by addition of 7 μg/ml phytohemagglutinine (PHA, Life Technologies).

2.6. Transformation of primary B lymphocytes

Cell lines of R278H mutant STAT3 B cells and healthy controls were generated by transformation with Epstein-Barr virus (EBV) (ATCC, Wesel, Germany) as described previously [21] and cultured in RPMI1640 supplemented with 20% FCS, 2 mM L-glutamine, 1% penicillin/streptomycin (Life Technologies).

2.7. Immunoblotting

Cells treated with IL6 (25 ng/ml, Miltenyi) for 24 h or left untreated were lysed in buffer containing 1% NP-40, 50 mM Tris, pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 2 mM dithiothreitol, protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). Proteins were separated on 8–15% polyacrylamide gels, transferred to polyvinylidene fluoride membranes and detected by chemiluminescence (GE Healthcare, Freiburg, Germany). The following primary antibodies were used: β-actin (Sigma-Aldrich, St. Louis, MO), STAT3 (R&D Systems, Wiesbaden, Germany), phospho-STAT3 (Tyr705, Cell Signaling, Frankfurt am Main, Germany), BCL2 (Santa Cruz Biotechnology, Santa Cruz, CA), BCL-XL (Cell Signaling), tubulin-HRP (Cell Signaling).