IL-10/Janus kinase/signal transducer and activator of transcription 3 signaling dysregulates Bim expression in autoimmune lymphoproliferative syndrome

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Background: Autoimmune lymphoproliferative syndrome (ALPS) is a human disorder of T cell homeostasis caused by mutations that impair FAS-mediated apoptosis. A defining characteristic of ALPS is the expansion of double negative T cells (DNTC). Relatively little is known about how defective FAS-driven cell death and the Bcl-2 apoptotic pathway intersect in ALPS patients.

Objective: We studied changes in Bcl-2 family member expression in ALPS to determine whether the Bcl-2 pathway might provide a therapeutic target.

Methods: We used flow cytometry to analyze the expression of pro- and anti-apoptotic Bcl-2 family members in T cells from 12 ALPS patients and determined the *in vitro* sensitivity of ALPS DNTC to the pro-apoptotic BH3 mimetic, ABT-737.

Results: The pro-apoptotic molecule, Bim, was significantly elevated in DNTC. Although no general pattern of individual anti-apoptotic Bcl-2 family members emerged, increased expression of Bim was always accompanied by increased expression of at least 1 anti-apoptotic Bcl-2 family member. Strikingly, Bim levels in DNTC correlated significantly with serum IL-10 in ALPS patients, and IL-10 was sufficient to mildly induce Bim in normal and ALPS T cells via a Janus kinase/signal transducer and activator of transcription 3–dependent mechanism. Finally, ABT-737 preferentially killed ALPS DNTC *in vitro*.

Conclusion: Combined, these data show that an IL-10/Janus kinase/signal transducer and activator of transcription 3 pathway drives Bim expression in ALPS DNTC, which renders them sensitive to BH3 mimetics, uncovering a potentially novel therapeutic approach to ALPS. (J Allergy Clin Immunol 2015;135:762-70.)

Key words: *ALPS, Bim, Bcl-2 pathway, IL-10, BH3 mimetic, ABT-737, T cell homeostasis, double negative T cells, apoptosis*

Maintenance of T cell homeostasis is critical for normal functioning of the immune system. Regulated induction of apoptosis is required to maintain T cell homeostasis and is controlled largely by 2 largely independent pathways: the extrinsic or death receptor-driven pathway and the intrinsic or Bcl-2-regulated pathway. Defects in either pathway result in disorders that are characterized by lymphoproliferation (including lymphoma), abnormal responses to infection, and autoimmunity.[1,2] Thus, appropriate regulation of both extrinsic and intrinsic apoptotic pathways is essential for maintaining T cell homeostasis.

Autoimmune lymphoproliferative syndrome (ALPS) is an example of a genetic disorder of lymphocyte apoptosis caused by defects in the FAS-mediated cell death pathway.[3] The majority of ALPS cases are caused by a germline heterozygous mutation in the FAS receptor.[4,5] Somatic mutations in the FAS receptor and mutations in FAS ligand or the apoptosis-inducing effector enzyme, caspase-10, are additional causes of ALPS.[6-9] Mutations in *NRAS*, *KRAS*, and *caspase-8* have also been reported to cause ALPS-like disorders,[10-13] and about one-third of ALPS cases do not have a known genetic cause.[14] ALPS is manifested by chronic non-malignant lymphoproliferation, autoimmunity, and increased risk of lymphoma.[15,16] The signature of the disease is an expanded homogenous population of T cells that are T cell receptor (TCR) $\alpha\beta^+$, $CD4^-CD8^-$, and co-express B220[16-18]; these are called double negative T cells (DNTC).

The role of DNTC in ALPS disease is also controversial. In lymphoproliferative (lpr) mice, which have a homozygous FAS receptor mutation, DNTC can make IL-17, which likely contributes to disease progression.[19] However, in humans, DNTC do not appear to be major producers of IL-17 or IFN-γ, although they express both granzyme B and perforin.[18,20,21] Further, ALPS patients have dramatic elevations in circulating IL-10, and DNTC are the dominant producers of IL-10 when assessed *ex vivo*.[22] Although immunosuppressive therapy of ALPS patients decreases DNTC and serum IL-10 levels,[23] the roles of DNTC and IL-10 remain unclear.

Although DNTC can arise in animals with defects in either the extrinsic or intrinsic cell death pathway, DNTC origin appears pathway-specific. The presence in most lpr mice of $CD8^{lo}$ cells that express TCR αβ, which are likely DNTC precursors,[24-26] and the absence of DNTC in β2-microglobulin-deficient lpr mice suggest that DNTC arise from overstimulated $CD8^+$ T cells. Consistent with this, 1 study found an extensive overlap of particular CDR3 sequences between DNTC and $CD8^+$ T cells in ALPS patients, sequences that were not represented in $CD4^+$ T cells.[27] Chronic stimulation regimens often induce the death receptor

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Abbreviations used
ALPS: Autoimmune lymphoproliferative syndrome
CDR: Complementarity determining region
DNTC: Double negative T cells
IL-10R: IL-10 receptor
Jak: Janus kinase
lpr: Lymphoproliferative
MFI: Mean fluorescence intensity
NF-κB: Nuclear factor-κB
STAT: Signal transducer and activator of transcription
TCR: T cell receptor

pathway,[28] suggesting that chronic stimulation of $CD8^+$ T cells may facilitate their elimination via FAS driven death, while in the absence of FAS signaling, CD8 is lost, and $B220^+$ DNTC accumulate. In contrast, mice that lack expression of Bim or overexpress Bcl-2 also accumulate DNTC, but these cells lack B220 expression.[29,19] However, why the intrinsic cell death pathway is not sufficient to control DNTC in ALPS patients remains unclear.

Indeed, while the apoptotic defect in ALPS results from defective FAS signaling, the role of the intrinsic apoptotic pathway in ALPS pathophysiology is not known. There appear to be overlapping roles for the extrinsic and intrinsic apoptotic pathways in murine T cell homeostasis.[30] In ALPS patients, steady-state Bim levels were elevated in some patients, and repeated TCR stimulation of $CD8^+$ T cells from these patients slightly increased Bim levels.[31] However, baseline levels of Bcl-2 family members have not yet been examined extensively in T cell subsets from ALPS patients. Here, we report a dysregulated Bcl-2 pathway in ALPS, most notably that DNTC from ALPS patients have significantly increased levels of Bim. We also show that IL-10 signaling through STAT3 contributes to this increased Bim expression. Lastly, we investigated whether this dysregulated expression of Bim could facilitate DNTC targeting by a drug that suppresses the activity of anti-apoptotic Bcl-2 family members.[32-34] ABT-737 binds to and suppresses the anti-apoptotic effects of Bcl-2, Bcl-xL, and Bcl-w (but not Mcl-1 or A1[34]) and prevents their ability to neutralize pro-apoptotic Bcl-2 family members.[32] Further, we have previously shown that treatment of normal mice with ABT-737 promotes the Bim-dependent loss of T cells.[35] Our results with this approach suggest that it could be useful for targeting these cells in patients with ALPS or other disorders of immune dysregulation.

METHODS

Human subjects

Patients were evaluated at Cincinnati Children's Hospital Medical Center. Blood samples were taken after informed consent was obtained according to an Institutional Review Board research protocol. Control samples were obtained from healthy volunteers associated with the Cincinnati Children's Hospital Diagnostic Immunology Laboratory. Samples were held at room temperature before analysis.

Clinical data, genetic mutations, DNT cell percentage, and cytokine level data were obtained from medical records. PBMC from patients and controls were separated from whole blood by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Pittsburgh, Pa). Cells were then washed, pelleted, and stained for flow cytometry, cultured or frozen in FBS with 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, Mo).

Flow cytometry

Fresh or thawed human PBMCs were incubated with human IgG (Sigma-Aldrich) to block Fc receptors, then stained with combinations of the following cell-surface antibodies: anti-CD3, -*CCR7* (BD Biosciences, San Jose, Calif), -CD4, -CD45RO (BioLegend, San Diego, Calif), -CD8, -CD45RA and -B220 (eBioscience, San Diego, Calif). Intracellular staining was performed with 1 of the following antibodies: anti-Bim, -Bcl-xL (Cell Signaling Technology, Beverly, Mass), -Bcl-2 (Caltag Laboratories, Burlingame, Calif), -Mcl-1 (Rockland, Gilbertsville, Pa), -A1 (AbCam, Cambridge, Mass) or anti IL-10RA (EMD Millipore, Billerica, Mass). For detection of Phospho-STAT3, human PBMCs were incubated for 2 hours at 37°C, stained for cell surface CD3, then stimulated with 50 ng/mL of recombinant human IL-10 (PeproTech, Rocky Hill, NJ) for 20 minutes. The cells were then washed, fixed, permeabilized, and stained with anti-phospho-STAT3 (Tyr705) (Cell Signaling Technology, Beverly, Mass). For evaluation of cell viability, cells were stained with 7-aminoactinomycin D stain or the Blue Live/Dead Fixable Dead Cell Stain kit (Life Technologies, Grand Island, NY). A minimum of 2.5×10^5 events was acquired on a BD LSR II or BD FACSCanto flow cytometer and analyzed by FACSDiva (BD Biosciences, San Jose, Calif). In order to convert values from mean fluorescence intensity (MFI) to molecules of equivalent soluble fluorochrome, we used Cyto-Cal Multifluor plus Violet Fluorescence Intensity Calibrator (Thermo Scientific, Pittsburgh, Pa). The beads were run on the cytometer at the same time as we acquired samples, using the same photomultiplier tube voltage and compensation settings. The data were analyzed using the Cali curve data analysis program, according to the manufacturer's instructions.

Cell culture

PBMCs from ALPS patients or normal controls were cultured in RPMI 1640 media (supplemented with 2 mM L-glutamine, 10 mM HEPES, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% FBS) at 37°C in 5% CO_2.

ABT-737, a generous gift of Abbott Laboratories (Chicago, Ill), was dissolved in dimethyl sulfoxide and diluted in a solution composed of 35% polyethylene glycol, 5% Tween-80 (Fisher Biotechnologies, Pittsburgh, Pa), and 65% dextrose in water. Human cells were cultured in the presence of the indicated ABT-737 concentration or vehicle overnight. Cells were washed and stained, and cell viability was determined by flow cytometry as above. In order to account for the rate of spontaneous apoptosis *in vitro*, the percentage of cell survival was calculated according to the following formula: percentage of cell survival = 100 − {[(% live cells in vehicle − % live cells treated)/% live cells in vehicle] × 100%}. For exogenous IL-10 culture experiments: human PBMCs from normal controls were incubated in the presence or absence of 50 ng/mL recombinant human IL-10 for 12 hours before or after T cell activation. Cells were activated for 3 days with 2 μg/mL plate-bound anti-CD3 (OKT3 mAb) and 2 μg/mL anti-CD28 (eBioscience, San Diego, Calif), ±25 IU/mL recombinant human IL-2. ALPS PBMCs were incubated in the presence or absence of 50 ng/mL recombinant human IL-10 for 12 hours in the presence of IL-2. Cells were then harvested, washed, and stained for surface markers and intracellular Bim. In some experiments, cells were incubated with 7.5 μM Stattic V, a STAT3 inhibitor (Santa Cruz Biotechnology, Santa Cruz, Calif) or 250 nM Ruxolitinib, a Jak1 inhibitor (LC Laboratories, Woburn, Mass) for 45 minutes prior to adding IL-10.

Statistical analysis

Statistical significance was estimated using a 2-sample paired Student *t* test or Wilcoxon matched pairs signed rank test. For estimation of significance for Bim elevation, a 1-sample *t* test was used to compare mean values to a value of 1. Associations between cytokine level and Bim were assessed by separate linear regression of each cytokine to Bim level. All statistical analyses were performed using Prism 6.0 (GraphPad Software Inc, La Jolla, Calif), and differences were considered significant at $P \leq .05$.

RESULTS

Bim is elevated in ALPS T cells

While ALPS is a disorder of the extrinsic apoptotic pathway, little is known about the role of the intrinsic pathway of apoptosis