Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

The BH3-mimetic ABT-737 inhibits allogeneic immune responses

Pietro E. Cippà,[1,2] Anna K. Kraus,[1,2] Ilka Edenhofer,[1,3] Stephan Segerer,[2,3] Jin Chen,[1,2] Martin Hausmann,[4] Yang Liu,[1,2] Annick Guimezanes,[5] Philip D. Bardwell,[6] Rudolf P. Wüthrich[1,2] and Thomas Fehr[1,2]

1 Institute of Physiology, University of Zürich, Switzerland
2 Division of Nephrology, University Hospital Zürich, Switzerland
3 Institute of Anatomy, University of Zürich, Switzerland
4 Division of Gastroenterology and Hepatology, University Hospital Zürich, Switzerland
5 Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, Marseille, France
6 Abbott Bioresearch Center, Worcester, MA, USA

Keywords
ABT-737, allograft, apoptosis, Bcl-2, immunosuppression, transplantation.

Correspondence
Prof. Dr. med. Thomas Fehr, Division of Nephrology, University Hospital, Rämistrasse 100, CH-8091 Zürich, Switzerland. Tel.: +41 44 255 33 84; fax: +41 44 255 45 93; e-mail: thomas.fehr@access.uzh.ch

Conflicts of Interest
P.D.B. is an employee of Abbott, which developed and provided ABT-737. However, no financial sponsoring was received for this study and no conflict of interest exists for the other authors.

Received: 18 November 2010
Revision requested: 23 December 2010
Accepted: 21 April 2011

doi:10.1111/j.1432-2277.2011.01272.x

Summary

Apoptosis controls the adaptive immune system through regulation of central and peripheral lymphocyte deletion. Therefore, substances that selectively interact with the intrinsic apoptosis pathway in lymphocytes offer unexplored opportunities to pharmacologically modulate the immune response. Here, we present evidence that the BH3-mimetic ABT-737 suppresses allogeneic immune responses. *In vitro*, ABT-737 prevented allogeneic T-cell activation, proliferation, and cytotoxicity by apoptosis induction, but without impairing the physiological functions of remaining viable T cells. *In vivo*, ABT-737 was highly selective for lymphoid cells and inhibited allogeneic T- and B-cell responses after skin transplantation. The immunosuppressive effect of ABT-737 was markedly increased in combination with low-dose cyclosporine A, as shown by the induction of long-term skin graft survival without significant inflammatory infiltrates in 50% of the recipients in an MHC class I single antigen mismatched model. Thus, pharmacological targeting of Bcl-2 proteins represents a novel immunosuppressive approach to prevent rejection of solid organ allografts.

Introduction

The identification of alternative pharmacological targets to suppress allo-specific immune responses is a fundamental step in the development of new drugs aiming at the optimization of long-term outcome after solid organ transplantation [1]. It has been shown that the modulation of apoptosis in lymphocytes is responsible for central and peripheral repertoire selection [2,3] and controls the deletion of alloreactive lymphocytes in the induction of peripheral transplantation tolerance [4]. Therefore, targeting apoptosis pathways in lymphocytes represents a potential novel strategy for immunosuppression.

Two distinct but interconnected pathways regulate apoptosis in mammalian cells: the extrinsic pathway is mediated by death receptors at the cell surface (e.g. by Fas- or TNF-receptor), whereas the intrinsic (or mitochondrial) pathway is under the control of pro- and anti-apoptotic members of the Bcl-2 family [5]. Selective small-molecule Bcl-2 antagonists have rationally been developed for the treatment of tumors [6]. The Bcl-2 inhibitor ABT-737 and its orally bioavailable counterpart

© 2011 The Authors

ABT-263 bind the anti-apoptotic proteins Bcl-2, Bcl-XL, and Bcl-w (but not A1 and Mcl-1) and activate the intrinsic apoptosis cascade in human and murine tumor cells [7]. While research on Bcl-2 inhibitors focused on cancer biology, this novel class of drugs offers the opportunity for selective interaction with a fundamental pathway in mammalian cells and could find various clinical applications outside of oncological therapy. Interestingly, it has been shown that ABT-737 has a favorable effect on various autoimmune diseases in mice and inhibits B- and T-cell immune responses [8,9]. Moreover, Carrington *et al.* recently described a beneficial impact of ABT-737 on glucose levels after islet transplantation from (C57Bl/6 × SJL)F_1 to spontaneous nonimmune diabetic RIP-H-2K^b mice [8]. However, BH3-mimetics have never been tested in stringent models of allograft rejection.

Here, we present evidence that the BH3-mimetic ABT-737 suppresses allogeneic T-cell responses *in vitro* because of a clone size reduction in the alloreactive T-cell population by apoptosis induction. *In vivo*, ABT-737 selectively induced apoptosis in lymphatic tissues and potently inhibited allogeneic T- and B-cell responses after skin transplantation in mice synergistically with cyclosporine A (CsA).

Materials and methods

Mice

C57Bl/6 (B6, H-2^b), CBA (H-2^k), B6.C-H2Kbm1 (bm1, H-2^{bm1}), Balb/c (H-2^d), and BM3.3 (H-2^k) mice were housed in specific pathogen-free conditions at the University of Zürich. Bm1 mice (seven nucleotides mutation in the H-2K^b locus) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) [10]. BM3.3 mice express on all CD8 T cells a transgenic T-cell receptor (TCR) selective for a naturally processed octapeptide bound to the allogeneic MHC class I molecule H-2K^b [11,12]. All animal experiments were performed according to protocols approved by the legal authority (Veterinary Office of the Canton of Zürich).

In vivo experiments

Full thickness tail skin (about 0.5–1.0 cm^2) from donor mice was grafted. Recipient mice were treated from day 5 before grafting until allograft rejection. Skin grafts were considered rejected when <10% of the graft remained viable. In some experiments, recipient mice were sacrificed at day 8 after transplantation and the transplanted tissue was harvested for histological evaluation. ABT-737 was provided by Abbott Bioresearch (Worcester, MA, USA) and was injected intra-peritoneally (i.p.) at 50 mg/kg/day. Vehicle consisted of polyethylene glycol, Tween 80, dextrose solution and DMSO. CsA was purchased from Sigma-Aldrich (Buchs, Switzerland) and injected subcutaneously (s.c.) at 10 mg/kg/day.

Mixed lymphocyte reaction (MLR) and cell-mediated lympholysis (CML) assay

Mixed lymphocyte reaction were performed with BM3.3 splenocytes stimulated by irradiated CD8 T-cell depleted splenocytes from B6 (allogeneic) and CBA or BM3.3 (syngeneic) mice. Splenocytes were sorted by magnetic cell separation according to the protocols of Miltenyi Biotec (Bergisch Gladbach, Germany) to allow a selective analysis of alloantigen-specific CD8 T cells. ABT-737 or DMSO-containing vehicle were added to the MLR at the beginning of the culture. For the CML assay B6 splenocytes were cultured with irradiated Balb/c (allogeneic) and B6 (syngeneic) lymphocytes during 5 days (stimulation phase). Thereafter, ^{51}Cr-labeled concanavalin-A-stimulated Balb/c lymphocytes were added to the serially diluted culture for 4 h (killing phase) and alloantigen-specific cytotoxicity was assessed by measurement of ^{51}Cr release in the supernatant. For a compensation of the alloreactive clone size reduction in certain experiments the same number of viable ABT-737-treated responder cells was used for the killing phase as measured in vehicle control cultures. Alloantigen-specific lysis was calculated as: Specific lysis (%) = (experimental release − spontaneous release)/(total release − spontaneous release) × 100.

Fluorescence activated cell sorting (FACS) and detection of allospecific antibodies

FACS was performed with a BD-FACSCanto (Becton Dickinson, Allschwil, Switzerland). Anti-mouse CD3-FITC, CD4-PE, CD8-APC, CD8-PE, CD45R/B220 PE-Cy5, anti-IgG-FITC, anti-IgM-PE, annexin V-FITC, and propidium iodide (PI) were purchased from eBioscience (Frankfurt, Germany), anti-mouse CD25-PE/Cy7 and interferon-γ (IFN-γ)-APC from Biolegend (Uithoorn, the Netherlands), carboxyfluorescein succinimidyl ester (CFSE) from Promega (Dübendorf, Switzerland). Allospecific antibodies were detected using FACS: after Fc-receptor blockade, allogeneic splenocytes were incubated with recipients' serum and subsequently stained with a secondary anti-mouse anti-IgG and anti-IgM antibody. Mean fluorescence intensity (MFI) was determined in FACS gating on B220 negative cells.

ELISA, Western blotting, and blood analyses

Interferon-γ ELISA was performed using a kit purchased from Biolegend and used according to the manufacturer's

© 2011 The Authors

protocol. Western blot analysis was performed using a rabbit anti-caspase-3 antibody (Cell Signaling Technology, Danvers, MA, USA). A peroxidase-conjugated donkey anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK) was used as secondary reagent. Haematological analyses were performed in the laboratory of the Division of Hematology at the University Hospital Zürich with an ADVIA 2120 flow cytometer (Siemens, Eschborn, Germany).

Histology, immunohistochemistry, TUNEL and analysis of isolated intestinal epithelial cells

Histology and immunohistochemistry were performed as previously described [13]. Monoclonal anti-CD3 antibody was purchased from AbD Serotec (Düsseldorf, Germany), anti-CD8a from OriGene (Rockville, MD, USA). For detection of apoptotic cells using immunohistochemistry the monoclonal antibody F7-26 (Chemicon International, Inc. Temecula, CA, USA) was used as previously described [14]. F7-26 binds to single-stranded DNA after thermal denaturation. A peroxidase-conjugated monoclonal rat anti-mouse IgM antibody (Zymed, San Francisco, CA, USA) was used as secondary reagent. Apoptosis in intestinal mucosa associated lymphatic tissue was quantified by TUNEL technology with the *In Situ* Cell Death Detection Kit (Roche, Basel, Switzerland) as described by the manufacturer. Intestinal epithelial cells were isolated and analyzed using FACS as previously described [15].

Statistics

Student *t*-test, Mann–Whitney test, ANOVA test or Fisher's exact test were used to compare values between groups as appropriate; skin graft survival was compared using log-rank test. GRAPH PAD PRISM Software Version 5.0 was used for calculations.

Results

Bcl-2 inhibition suppresses allogeneic T-cell responses *in vitro*

The Bcl-2 inhibitor ABT-737 was first tested *in vitro* in an MLR model. To analyze the immunosuppressive effect of ABT-737 on a homogeneous population of alloreactive T cells, we used the transgenic mouse model BM3.3. The BM3.3 mouse expresses on all CD8 T cells a transgenic TCR specific for a naturally processed octapeptide bound to the allogeneic MHC class I molecule H-2K^b [11,12]. After 3 days of a classical MLR culture with BM3.3 responders and irradiated B6 stimulators, we registered a potent, concentration-dependent inhibition of transgenic CD8 T-cell activation (data not shown), proliferation (CFSE dilution, Fig. 1a) and IFN-γ synthesis (ELISA of culture supernatant and intracellular IFN-γ staining in transgenic CD8 T cells, Fig. 1b) in cultures exposed to ABT-737. Analogous results were observed at the cytotoxicity level in a CML assay using the BM3.3 transgenic model (data not shown) and in the nontransgenic fully MHC-mismatched combination B6 to Balb/c (Fig. 1c).

Mechanism of immunosuppression by Bcl-2 inhibition *in vitro*

Previous studies have shown that ABT-737 initiates the intrinsic apoptosis cascade by mitochondrial release of cytochrome c [6]. However, apart from their role as apoptosis regulators, Bcl-2 proteins seem to be involved in additional cellular functions in lymphocytes [16,17]. Here, we show that the immunosuppressive effect of ABT-737 results from clone size reduction in the alloreactive T-cell population by apoptosis induction. However, the fraction of alloreactive CD8 T cells that remained viable after exposure to a concentration of ABT-737 not inducing a complete T-cell deletion was not impaired in its physiological effector functions. For this purpose, we analyzed the fate of alloreactive lymphocytes in culture after treatment with ABT-737. Within hours ABT-737 induced apoptosis in resting lymphocytes as shown by phosphatidylserine exposure (annexin V positivity) in PI negative cells using FACS analysis (Fig. 2a) and by caspase 3 activation in Western blot (Fig. 2b). Similar results were obtained for wild type and for transgenic BM3.3 lymphocytes. The specific examination of transgenic alloreactive CD8 T cells in the BM3.3 to B6 model revealed that ABT-737 markedly increased the number of apoptotic cells in this population after 3 days of MLR in a concentration dependent manner (data not shown). However, the small fraction of alloreactive CD8 T cells that survived exposure to ABT-737 (PI negative population) was not altered in its activation status (CD25 expression, Fig. 2c) and proliferation capacity (CFSE dilution, Fig. 2d, ABT-737 plots) even at high ABT-737 concentrations. Moreover, the cytotoxicity of surviving lymphocytes was not reduced compared with control, as shown in experiments, in which after activation under the effect of ABT-737 the number of viable cells was adjusted before starting the killing phase (Fig. 2e). The relevance of such observations was confirmed with an *ex vivo* experiment. After five daily injections of ABT-737 (50 mg/kg/day) BM3.3 mice presented marked lymphopenia in blood compared with control animals (Fig. 2f). The mice were sacrificed and their splenocytes used for MLR with irradiated B6 lymphocytes stimulators. *Ex vivo* CD8 T-cell activation (data not shown) and proliferation

© 2011 The Authors

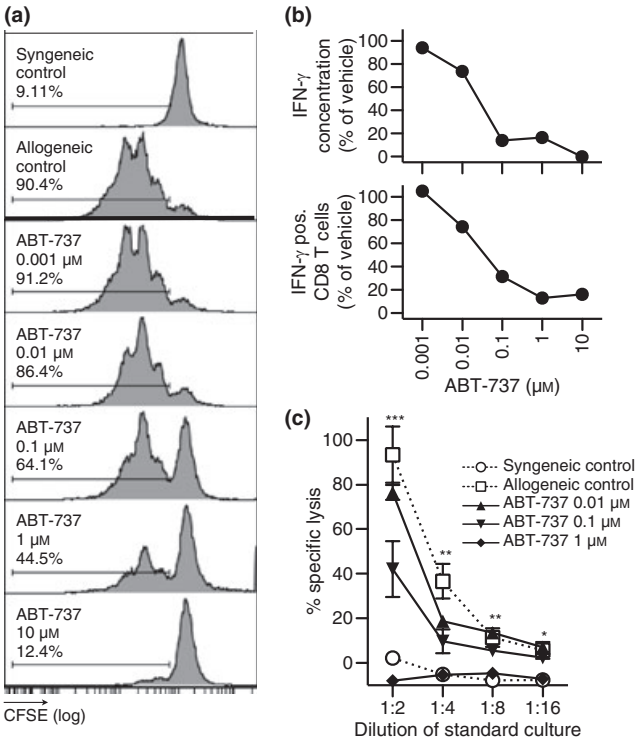


Figure 1 ABT-737 suppresses allogeneic T-cell responses *in vitro*. (a) Mixed lymphocyte reaction (MLR) with transgenic BM3.3 splenocytes stimulated with CD8-depleted irradiated B6 splenocytes. After 3 days of MLR, ABT-737 suppressed CD8 T-cell proliferation in a concentration-dependent manner as shown by carboxyfluorescein succinimidyl ester (CFSE)-dilution in FACS. Representative results of one of three independent experiments are shown. (b) ABT-737 inhibited IFN-γ synthesis, as shown by reduction of IFN-γ concentration in the culture supernatant measured by ELISA (three independent experiments). The effect on allo-reactive BM3.3 CD8 T cells was confirmed determining the number of IFN-γ positive transgenic CD8 T cells in an additional independent experiment. Percent of values measured in cultures exposed to vehicle are given. (c) B6 lymphocytes were stimulated with Balb/c splenocytes in the presence of ABT-737 or DMSO-containing vehicle during 5 days. Allo-antigen specific cytotoxicity was tested in a ^{51}Cr release assay against concavalin A-stimulated Balb/c lymphocytes. ABT-737 exposure during the stimulation phase suppressed alloantigen-specific cytotoxicity in a concentration-dependent manner. Representative results of one of three independent experiments are shown (one-way ANOVA comparing the four allogeneic stimulated groups, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

(Fig. 2f) were not reduced by the previous exposure to ABT-737. Therefore, the immunosuppressive effect of ABT-737 appears to be explained by clone size reduction in the alloreactive lymphocyte population by apoptosis, sparing the physiological functions of remaining viable CD8 T cells. This contrasted with the mechanism of action of established small-molecule immunosuppressive drugs: in the same experimental setting CsA, rapamycin, and mycophenolic acid directly suppressed proliferation, whereas ABT-737 reduced the alloreactive clone size (Fig. 2d).

ABT-737 is highly selective for lymphatic tissues

The intrinsic apoptosis pathway is fundamental and universal in mammalian cells. Therefore, systemic toxicity might limit the clinical application of Bcl-2 inhibition as an immunosuppressive principle. It has been shown that the relevance of different apoptosis regulatory mechanisms varies among tissues, eventually determining the selectivity of different Bcl-2 inhibitors [18] and – as a consequence – the toxicity of the treatment. To assess the tissue selectivity of ABT-737, we analyzed by immunohistochemistry the number of apoptotic cells in different organs after injection of ABT-737 (50 mg/kg) or DMSO-containing vehicle. Six hours after injection, ABT-737-treated mice presented a 10-fold increase in the number of apoptotic cells in spleen and a twofold increase in the thymus compared with vehicle-treated controls (Fig. 3a and b). Parenchymal organs such as kidney, liver, or heart were minimally affected by the treatment. To exclude a role of a possible variability in ABT-737 exposure among different organs, the selectivity of ABT-737 was further assessed by a specific analysis of epithelial and lymphatic cells from one and same organ: the intestine. We found that after five daily injections of ABT-737 (50 mg/kg/day),

© 2011 The Authors

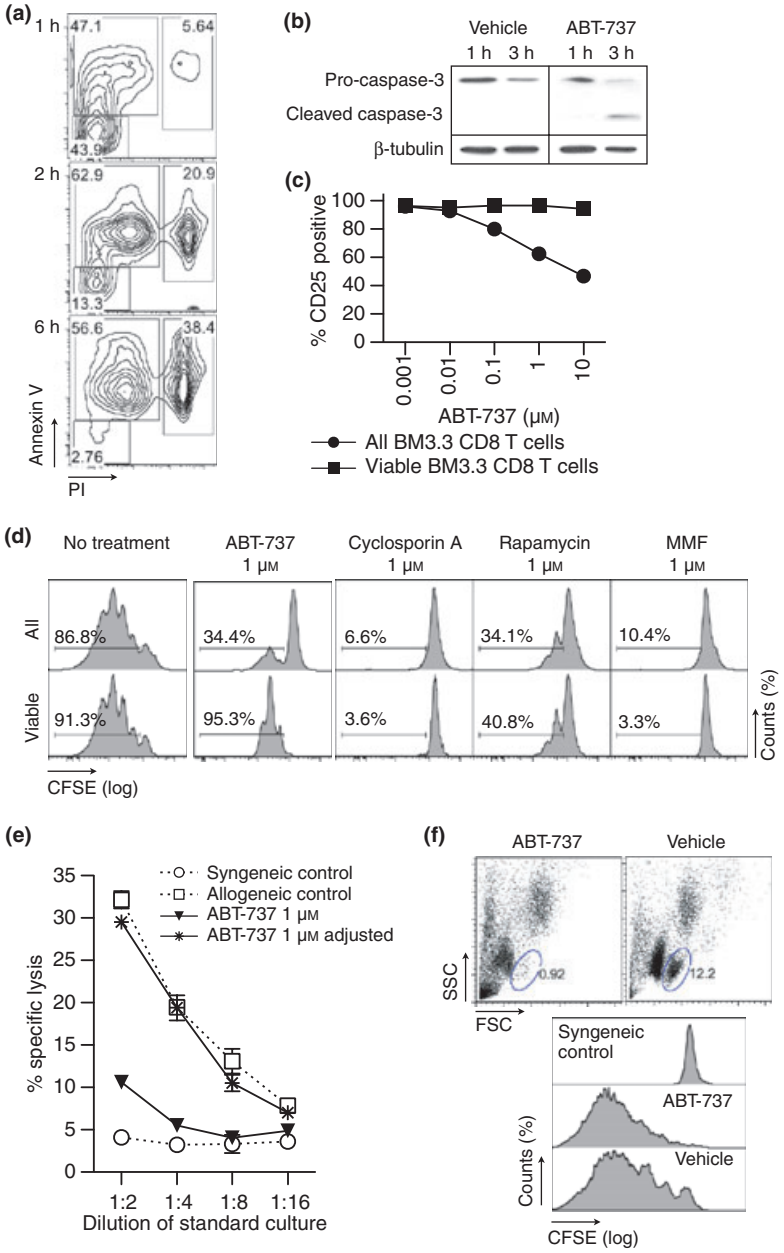


Figure 2 The immunosuppressive mechanism of ABT-737. (a and b) ABT-737 (5 μM) induced apoptosis in B6 splenocytes within hours as shown by exposure of phosphatidylserine in propidium iodide negative cells (FACS) (a) and caspase 3 activation (Western blot) (b). (c and d) In mixed lymphocyte reaction (MLR) with BM3.3 splenocytes stimulated by irradiated CD8-depleted B6 splenocytes ABT-737 inhibited CD8 T-cell activation (c) and proliferation (d) in a concentration-dependent manner if all responder BM3.3 cells were considered. By contrast, activation and proliferation were not impaired by ABT-737 in a selective analysis of viable (PI negative) responder BM3.3 cells. Similar results were obtained from several experiments with different MLR conditions. Representative data obtained after 3 days of MLR are shown and compared with the effect of cyclosporine A, rapamycin and mycophenolate mofetil (MMF) in the same experimental setting for CD8 T-cell proliferation (d). (e) Modified cell-mediated lympholysis (CML) assay to compensate the reduced number of viable responder cells after the stimulation phase under the effect of ABT-737. The inhibitory effect of ABT-737 on cytotoxicity was completely compensated if the same number of viable treated cells was added to the ^{51}Cr-labeled target cells during the killing phase. Data from a CML in the fully MHC-mismatched combination B6 to Balb/c are shown (no statistical difference between allogeneic control and ABT-737 1 μM adjusted in all dilutions of standard culture). (f) BM3.3 mice were treated with ABT-737 or vehicle during 5 days. Treatment efficacy was demonstrated by lymphocyte depletion in blood (FACS). Splenocytes from ABT-737 and vehicle-treated mice did not differ in their proliferation capacity after 4 days of MLR against B6 lymphocytes, as shown by carboxyfluorescein succinimidyl ester (CFSE) dilution.

© 2011 The Authors

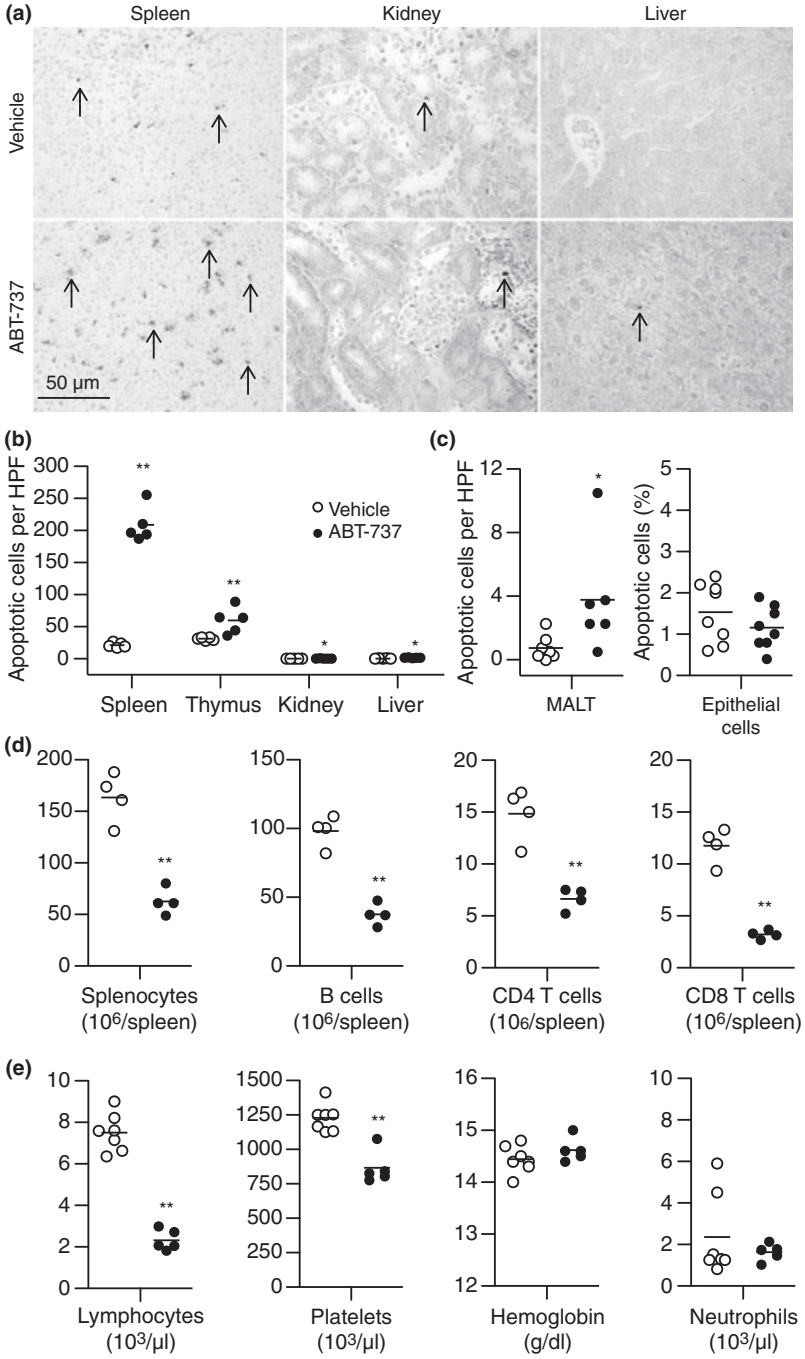


Figure 3 The pro-apoptotic effect of ABT-737 is selective for lymphocytes. (a and b) B6 mice were sacrificed 6 h after injection of ABT-737 (50 mg/kg) or vehicle. Apoptosis detection by immunohistochemistry for single-stranded DNA revealed a 10-fold increase in the number of apoptotic cells in the spleen and a twofold increase in the thymus. Kidney and liver tissue presented a low number of apoptotic cells and were minimally affected by the treatment [mean number of F7-26 positive cells per 10 high power fields (HPF), $n = 5$ mice/group; $^{*}P < 0.05$, $^{**}P < 0.01$]. (c) After five daily injections of ABT-737 (50 mg/kg/day), the intestine of B6 mice was harvested. Treatment with ABT-737 significantly increased the number of apoptotic cells in the mucosa associated lymphatic tissue (MALT) as shown by TUNEL staining (mean number of apoptotic cells per HPF), but had no impact on the frequency of apoptotic cells in isolated intestinal epithelial cells as measured in FACS ($n = 6$–9 mice/group, $^{*}P < 0.05$). (d) Analysis of splenocyte subpopulations using FACS after five daily injections revealed that B and T cells were similarly affected by ABT-737 (number of cells/spleen, $n = 4$ mice/group; $^{**}P < 0.01$). (e) After 18 daily injections of ABT-737 (50 mg/kg/day), B6 mice presented a significant lymphopenia and a moderate thrombocytopenia compared with control animals. Erythrocytes and neutrophils were not affected by the treatment ($n = 5$–7 mice/group).

© 2011 The Authors

B6 mice presented a significant increase in the number of apoptotic cells in the mucosa associated lymphatic tissue (Peyer's patches), but not in the epithelium (TUNEL staining). FACS analysis of isolated intestinal epithelial cells from colonic mucosa confirmed that ABT-737 did not increase the fraction of apoptotic cells in this tissue (Fig. 3c).

Spleen FACS analyses after five daily injections revealed that ABT-737 exposure resulted in a 60–65% reduction in the number of total splenocytes and that different lymphocyte subpopulations were similarly affected by the treatment (Fig. 3d).

In addition, haematological analyses were performed. After 18 daily injections of ABT-737 (50 mg/kg/day), B6 mice showed a significant reduction of lymphocyte and platelet counts compared with vehicle-treated controls, but erythrocytes and neutrophils were not affected (Fig. 3e).

ABT-737 inhibits skin graft rejection synergistically with low-dose cyclosporine A

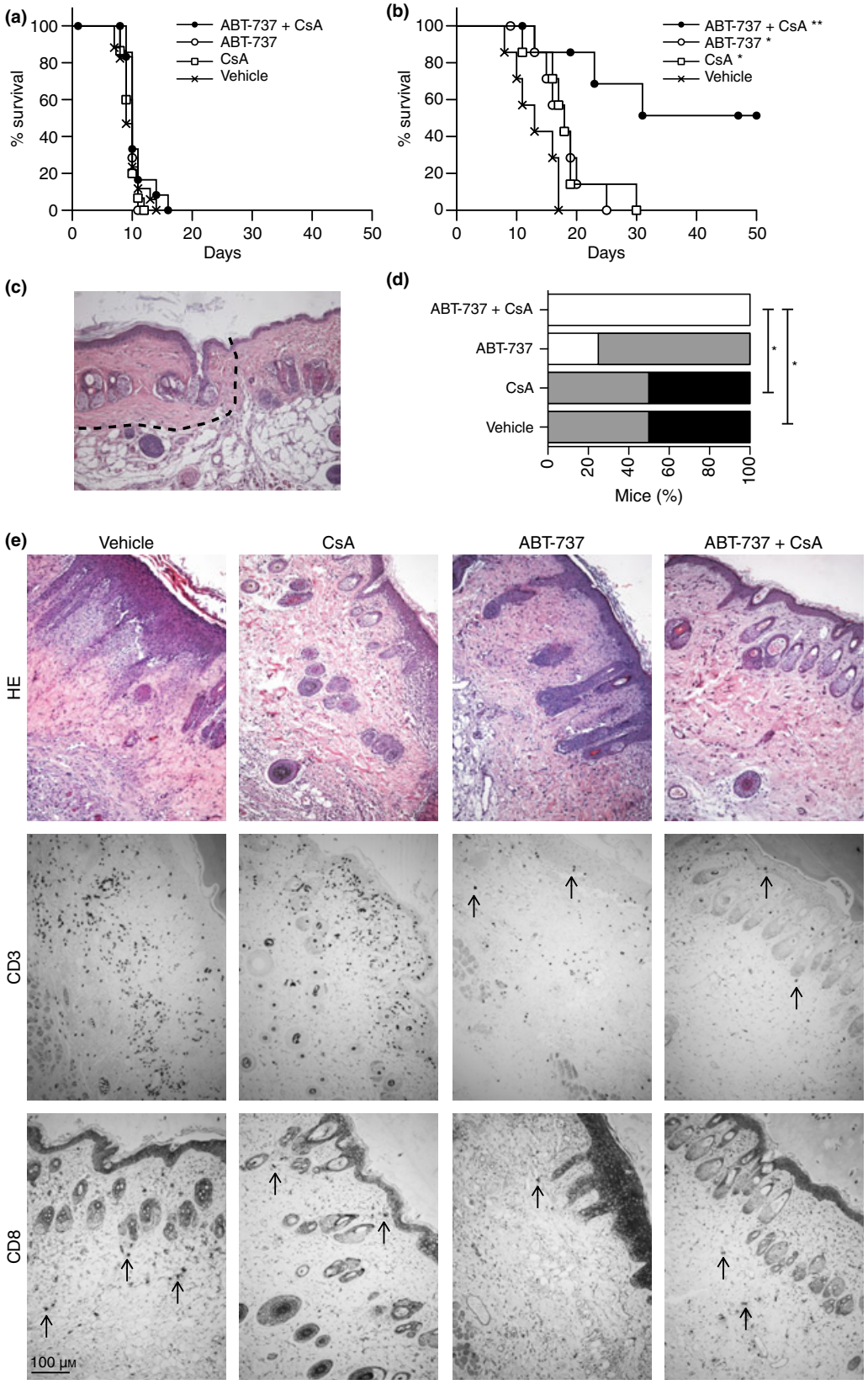
The immunosuppressive effect of ABT-737 on T-cell mediated rejection was assessed *in vivo* in a murine skin graft model. In the fully MHC-mismatched combination Balb/c to B6 neither ABT-737 (50 mg/kg/day) nor low-dose CsA (10 mg/kg/day) or a combination of ABT-737 and low-dose CsA were sufficient to significantly prolong skin graft survival (Fig. 4a). However, in the MHC class I single antigen mismatched combination bm1 to B6, daily injections with ABT-737 from day 5 before transplantation until rejection significantly prolonged skin graft survival compared with vehicle control (median graft survival vehicle versus ABT-737: 13 vs. 18 days, $P = 0.03$, Fig. 4b). Notably, ABT-737 and CsA displayed a similar, significant but rather modest effect as single agents in this model, but their immunosuppressive potency was markedly increased in combination: a long-term graft survival was achieved in 50% of recipients treated with ABT-737 (50 mg/kg/day, i.p.) and CsA (10 mg/kg/day, s.c.) from day 5 before transplantation (median graft survival CsA versus ABT-737 + CsA: 18 days versus undefined, $P = 0.008$; vehicle versus ABT-737 + CsA, $P = 0.001$, Fig. 4b). Fifty days after transplantation recipients that had not rejected their grafts were sacrificed. The histological analysis revealed an almost complete absence of inflammatory infiltrates (Fig. 4c), supporting the hypothesis of a suppression of the allospecific immune response. Moreover, the immunosuppressive effect of ABT-737 and the synergistic effect in combination with CsA were confirmed in an additional experiment, in which skin grafts were analyzed histologically 8 days after transplantation. At that time point, a prominent reduction of CD3 positive T cells was seen in mice treated either with ABT-737 or the combination of ABT-737 and CsA (vehicle versus ABT-737 + CsA, $P = 0.03$, Fig. 4d). CD8 positive T cells were low in number but also demonstrated a trend toward a reduced infiltration (Fig. 4e). Notably, syngeneic skin grafts were not affected by ABT-737 treatment with or without CsA (follow up of more than 150 days, data not shown), indicating that ABT-737 did not negatively interfere with the healing process after surgery.

ABT-737 inhibits allospecific humoral responses

The serum level of allospecific antibodies was measured 15 days after transplantation of Balb/c skin grafts onto B6 recipients. Although not sufficient to prolong skin graft survival in this setting (Fig. 4a), ABT-737 significantly inhibited the production of allospecific antibodies as measured by indirect FACS (vehicle versus ABT-737, IgG, $P = 0.002$, Fig. 5). Interestingly, also the B-cell response was further inhibited in the group treated with a combination of ABT-737 (50 mg/kg/day) and low-dose CsA (10 mg/kg/day). Similar results were obtained for IgG and

Figure 4 ABT-737 inhibits allospecific T-cell responses *in vivo*. (a) In the fully MHC-mismatched combination Balb/c to B6 neither ABT-737 (50 mg/kg/day), nor CsA (10 mg/kg/day) or a combination of both prolonged skin graft survival (data pooled from two identical experiments, $n = 7$–12 mice/group). (b and c) In the single MHC-mismatched model bm1 to B6 ABT-737 inhibited skin graft rejection synergistically with CsA (Log-rank test in comparison to the vehicle group, $^*P < 0.05$, $^{**}P < 0.01$, $n = 7$–8 mice/group). As a result, long-term graft survival was observed in 50% of the recipients in the combination group. Biopsies of the non-rejected skin grafts did not present any histological sign for rejection at day 50 after transplantation, as shown by the comparison of transplanted (left to the dashed line) and native skin (right to the dashed line) in one representative example. (d and e) Histological analysis of bm1 skin grafts at day 8 after transplantation onto B6 recipients confirmed a marked inhibition of T-cell mediated rejection by ABT-737 in synergism with CsA. Representative examples of the morphological evaluation by routine histology (hematoxylin and eosin staining, HE) and immunohistochemistry for CD3 and CD8 from mice treated with vehicle, CsA (10 mg/kg/day), ABT-737 (50 mg/kg/day) or a combination of both drugs are shown. Please note the prominent thickening of the epidermis and cellular infiltration in the vehicle-treated mice (HE, upper left). A marked reduction of the CD3 positive infiltrating cells (arrows in second row, third, and fourth column) could be demonstrated. The number of CD8 positive cells was low when compared with the CD3 positive cells (arrows in the lower panels). Semi-quantitative analyses for CD3 are presented in (d) according to a 3 degree score (white: no infiltration, gray: infiltration in less than 50% of the graft, black: infiltration in more than 50% of the graft; Fisher's exact test comparing no infiltration versus any infiltration, $^*P < 0.05$; $n = 3$–4 mice/group).

© 2011 The Authors



© 2011 The Authors

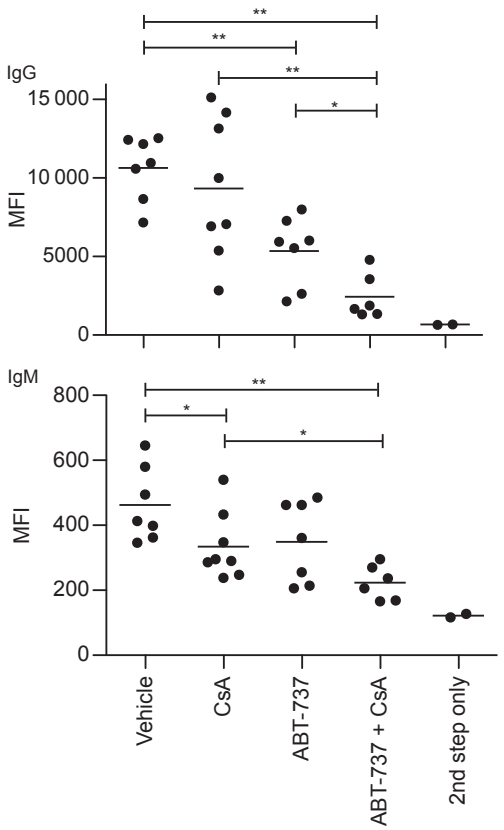


Figure 5 ABT-737 inhibits allospecific B-cell responses *in vivo*. Serum levels of allospecific IgG and IgM antibodies were measured 15 days after skin transplantation in the Balb/c to B6 combination. ABT-737 (50 mg/kg/day) synergistically with CsA (10 mg/kg/day) inhibited allospecific humoral responses (MFI, mean fluorescence intensity; $*P < 0.05$, $**P < 0.01$, $n = 6$–8 mice/group). Representative results of one of two experiments are shown.

IgM allospecific antibodies (vehicle versus ABT-737 + CsA, IgG $P = 0.001$, IgM $P = 0.001$; CsA versus ABT-737 + CsA, IgG $P = 0.003$, IgM $P = 0.028$). Thus, ABT-737 potently inhibited allospecific T- and B-cell responses *in vivo* and displayed a marked synergistic effect with the calcineurin inhibitor CsA.

Discussion

BH3-mimetics represent a new class of drugs to inhibit detrimental immune responses such as in autoimmunity [9], allergy [19] and chronic inflammation. Here, we report that ABT-737 potently suppressed allospecific T- and B-cell responses. The immunosuppressive effect of ABT-737 was markedly increased in combination with low-dose CsA.

In our *in vitro* and *ex vivo* models, immunosuppression by ABT-737 was a sole manifestation of clone size reduction in the alloreactive T-cell population induced by apoptosis. Although T-cell depletion represents a well established immunosuppressive approach [1], the mechanism of action described here differs from strategies in current clinical protocols. In contrast to depleting antibodies such as antithymocyte globulin or cytotoxic drugs such as cyclophosphamide, Bcl-2-antagonists interact with pathways physiologically regulating peripheral T-cell deletion and offer opportunities to better understand and to modulate these fundamental mechanisms as a novel immunosuppressive approach.

The efficacy of this concept was confirmed *in vivo* in a skin transplantation model. As a single agent ABT-737 significantly prolonged skin graft survival, but was not sufficient to fully prevent rejection in a MHC class I mismatched model. As ABT-737 did not completely deplete lymphocytes *in vivo* (Fig. 3d), we speculate that a subpopulation of alloreactive lymphocytes survived the pro-apoptotic effect of ABT-737 and was responsible for graft rejection. By contrast, in combination with low dose CsA ABT-737 completely prevented rejection in 50% of the recipients, as shown in the histological analysis of the grafts at day 50 after transplantation (Fig. 4b and c). Because of the high immunogenicity of the skin, it is not surprising that a pharmacological combination was required to completely exploit the immunosuppressive potential of ABT-737. Of particular interest, the addition of a Bcl-2 inhibitor increased the immunosuppressive potency of CsA at a dosage that by its own was not sufficient to prevent graft rejection. We hypothesize that in the setting of a reduced clone size by ABT-737, a lower concentration of CsA was sufficient to control the pool of alloreactive T cells. This hypothesis is supported by the fact that the combination therapy was not sufficient to inhibit the larger pool of allo-reactive cells in the fully MHC-mismatched combination. However, additional interactions between the calcineurin and the intrinsic apoptosis pathways have been previously described and might play a role in this setting [17].

Bcl-2 inhibitors suppress immune responses through a novel pharmacological target and open new options for pharmacological combinations. Because of the favorable synergistism with CsA, ABT-737 might find a clinical application as part of a low dose calcineurin inhibitor (CNI) based regime and help reducing CNI long-term toxicity. Moreover, based on its well-established anti-neoplastic properties, we expect that ABT-737 would reduce the incidence of tumors in transplant recipients. This aspect would differentiate Bcl-2 inhibitors from most current immunosuppressive drugs, and might have a major impact on survival in this high-risk situation for cancer development.

© 2011 The Authors

Apoptosis is a fundamental cellular mechanism and tissue selectivity of Bcl-2 inhibitors is crucial to limit systemic toxicity. Although a detailed analysis of the mechanisms determining tissue selectivity of ABT-737 was beyond the objectives of this study, our experiments showed that ABT-737 is selective for lymphatic cells and tissues. These results, which are supported by data from clinical trials using the ABT-737 orally bioavailable counterpart ABT-263 [20], suggest a favorable toxicity profile for ABT-737. Further analyses are required for a better understanding of the regulation of Bcl-2 proteins in inflamed tissues and to better assess the selectivity of ABT-737 after surgery. In this context, the normal survival and wound healing of syngeneic grafts in ABT-737-treated mice represent a promising results, thereby distinguishing ABT-737 from anti-proliferative drugs such as mTOR inhibitors.

Current available therapeutical options to control humoral rejection are limited. ABT-737 as a single agent and particularly in combination with CsA suppressed the allo-antibody response in a fully MHC-mismatched skin graft model. Although our mechanistic studies primarily focused on T-cell responses, we observed that B and T cells were similarly depleted by ABT-737 (Fig. 3d). Therefore, we suggest B-cell depletion as the principal explanation for this effect of ABT-737, but a concomitant T-cell help inhibition is likely to be involved and may explain the synergistic effect with CsA. Further studies are required to better assess the effect of ABT-737 on plasma cells.

Thus, our data strongly support further evaluation of Bcl-2 inhibitors as novel class of immunosuppressive drugs with potential broad clinical application in the field of allo-transplantation.

Authorship

PEC and TF: designed and performed the experiments, and wrote the paper. AKK, IE, JC, MH, and YL: performed the experiments. SS: analyzed the data. AG and PDB: contributed mice and important reagents. RPW: designed the experiments.

Funding

The project was supported by the Swiss National Science Foundation (grant no. 323530-133893 to P.E.C., 310000-121979 to T.F.; 32003B-129710 to S.S.), the Olga Mayenfisch Stiftung and the Zürich Center for Integrative Human Physiology (ZIHP).

Acknowledgements

We thank A.-M. Schmitt-Verhulst for providing the BM3.3 mouse strain, K. Leucht and M. Krebs for intestinal cell isolation and K. Bruni and her team for the blood analyses.

References

1. Halloran PF. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 2004; **351**: 2715.
2. Bouillet P, Purton JF, Godfrey DI, *et al.* BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* 2002; **415**: 922.
3. Rathmell JC, Thompson CB. Pathways of apoptosis in lymphocyte development, homeostasis, and disease. *Cell* 2002; **109**(Suppl.): S97.
4. Wells AD, Li XC, Li Y, *et al.* Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med* 1999; **5**: 1303.
5. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. *N Engl J Med* 2009; **361**: 1570.
6. Oltersdorf T, Elmore SW, Shoemaker AR, *et al.* An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* 2005; **435**: 677.
7. Hann CL, Daniel VC, Sugar EA, *et al.* Therapeutic efficacy of ABT-737, a selective inhibitor of BCL-2, in small cell lung cancer. *Cancer Res* 2008; **68**: 2321.
8. Carrington EM, Vikstrom IB, Light A, *et al.* BH3 mimetics antagonizing restricted prosurvival Bcl-2 proteins represent another class of selective immune modulatory drugs. *Proc Natl Acad Sci USA* 2010; **107**: 10967.
9. Bardwell PD, Gu J, McCarthy D, *et al.* The Bcl-2 family antagonist ABT-737 significantly inhibits multiple animal models of autoimmunity. *J Immunol* 2009; **182**: 7482.
10. Schulze DH, Pease LR, Geier SS, *et al.* Comparison of the cloned H-2Kbm1 variant gene with the H-2Kb gene shows a cluster of seven nucleotide differences. *Proc Natl Acad Sci USA* 1983; **80**: 2007.
11. Auphan N, Curnow J, Guimezanes A, *et al.* The degree of CD8 dependence of cytolytic T cell precursors is determined by the nature of the T cell receptor (TCR) and influences negative selection in TCR-transgenic mice. *Eur J Immunol* 1994; **24**: 1572.
12. Guimezanes A, Barrett-Wilt GA, Gulden-Thompson P, *et al.* Identification of endogenous peptides recognized by *in vivo* or *in vitro* generated alloreactive cytotoxic T lymphocytes: distinct characteristics correlated with CD8 dependence. *Eur J Immunol* 2001; **31**: 421.
13. Segerer S, Hudkins KL, Taneda S, *et al.* Oral interferon-alpha treatment of mice with cryoglobulinemic glomerulonephritis. *Am J Kidney Dis* 2002; **39**: 876.
14. Segerer S, Eitner F, Cui Y, Hudkins KL, Alpers CE. Cellular injury associated with renal thrombotic microangiopathy in human immunodeficiency virus-infected macaques. *J Am Soc Nephrol* 2002; **13**: 370.
15. Grossmann J, Walther K, Artinger M, *et al.* Progress on isolation and short-term ex-vivo culture of highly purified

© 2011 The Authors

non-apoptotic human intestinal epithelial cells (IEC). *Eur J Cell Biol* 2003; **82**: 262.
16. Ludwinski MW, Sun J, Hilliard B, *et al.* Critical roles of Bim in T cell activation and T cell-mediated autoimmune inflammation in mice. *J Clin Invest* 2009; **119**: 1706.
17. Shibasaki F, Kondo E, Akagi T, McKeon F. Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. *Nature* 1997; **386**: 728.
18. Vogler M, Dinsdale D, Dyer MJ, Cohen GM. Bcl-2 inhibitors: small molecules with a big impact on cancer therapy. *Cell Death Differ* 2009; **16**: 360.
19. Karlberg M, Ekoff M, Huang DC, Mustonen P, Harvima IT, Nilsson G. The BH3-mimetic ABT-737 induces mast cell apoptosis *in vitro* and *in vivo*: potential for therapeutics. *J Immunol* 2010; **185**: 2555.
20. Wilson WH, Tulpule A, Levine AM, *et al.* A phase 1/2a study evaluating the safety, pharmacokinetics, and efficacy of ABT-263 in subjects with refractory or relapsed lymphoid malignancies. *Blood* 2007; **110**: Abstract 1371.

© 2011 The Authors