npg
Cell Death and Differentiation (2004) 11, 416–423
© 2004 Nature Publishing Group All rights reserved 1350-9047/04 $25.00
www.nature.com/cdd

Reactive oxygen species regulate quiescent T-cell apoptosis via the BH3-only proapoptotic protein BIM

H Sade[1] and A Sarin*,[1]

[1] National Centre for Biological Sciences, UAS-GKVK Campus, Bangalore, India

* Corresponding author: A Sarin, National Centre for Biological Sciences, UAS-GKVK Campus, New Bellary Road, Bangalore 560065, Karnataka, India. Tel: +91 80 363 6420; Fax: +91 80 363 6462; E-mail: sarina@ncbs.res.in

Received 12.6.03; accepted 16.9.03; published online 9.1.04
Edited by G Nunez

Abstract

The survival of quiescent T cells in the peripheral immune system is dependent on signals transmitted from the extracellular environment. The requirement for survival factors is also manifested *in vitro*, providing a robust system to examine molecular mechanisms underlying T-cell death. We show that peripheral T cells cultured in the absence of survival factors accumulate reactive oxygen species (ROS), upregulate BIM (Bcl-2-interacting mediator of death) and inducible nitric oxide synthase (iNOS) expression, culminating in Fas-independent neglect-induced death (NID). We have examined ROS, iNOS and cytokine modulation of T-cell NID. Antioxidants inhibit BIM induction, caspase activation and apoptosis but do not promote cell cycle entry. iNOS-deficient T cells are protected from apoptosis, implicating iNOS in the regulation of NID via suppression of Bcl-x_L expression and consequent inhibition of BIM activity. Finally, we show that the prosurvival cytokine IL-7 elevates Bcl-x_L expression and transcriptionally regulates iNOS but not BIM expression in T cells.

Cell Death and Differentiation (2004) **11,** 416–423. doi:10.1038/sj.cdd.4401347
Published online 9 January 2004

Keywords: T cells; apoptosis; BIM; ROS; NOS

Abbreviations: IL-7, interleukin-7; NID, neglect-induced death; NOS, nitric oxide synthase; ROS, reactive oxygen species

Introduction

Cell death decisions in metazoans are effected through conserved signaling pathways.[1,2] In mammalian cells, apoptosis is initiated via transmembrane receptor-mediated apoptotic signaling[3,4] or proapoptotic molecules that converge on the mitochondrion.[5,6] Transmembrane receptors of the tumor necrosis factor receptor (TNFR) superfamily trigger apoptosis principally via the activation of procaspase 8/10.[3,4] Caspase-induced apoptosis is regulated by endogenous antagonists such as FLICE-like inhibitor protein (FLIP) that prevent processing and antiapoptotic proteins of the inhibitors of apoptosis protein (IAP) family that block function.[7] Antiapoptotic members of the Bcl-2 family principally block the release of apoptogenic intermediates from the mitochondrial intermembrane space that occurs as an initial step during mitochondrially regulated apoptosis.[8]

T cells at all developmental stages require extrinsic signals for survival.[9,10] In the thymus, negative selection ensures the deletion of autoreactive thymocytes and allows maturation of $CD4^+$ or $CD8^+$ T cells.[9] Interactions with the thymic stroma ensure immature T-cell survival and thereby influence the peripheral T- cell repertoire. Thymic maturation into $CD4^+$ or $CD8^+$ subsets of T cells is accompanied by the emigration of these cells to peripheral lymphoid organs. The requirement for stromal interactions continues to be an essential survival signal for peripheral T cells, which require continuous signals from the major histocompatibility complex (MHC) for survival.[11–15] MHC signals are linked to the expression of antiapoptotic proteins such as Bcl-2[16] and are distinct from those that trigger proliferation. Naïve T-cell survival is also linked to the expression of the lung-Kruppel like factor.[17] Susceptibility to diverse apoptotic stimuli is seen in all developmental stages of T cells, but regulation of apoptotic signaling is maturation-stage specific.[18–21] Similarly, the requirement for extrinsic signals is also common to all developmental stages, but the underlying mechanisms of survival signaling in T cells are likely to be distinct in different maturational stages.

Bcl-2 family proteins are pivotal intermediates in apoptotic signaling in mammalian cells, particularly in the maintenance of lymphocyte homeostasis.[6,22] The proapoptotic proteins Bax and Bak interact with the BH3-only protein BIM (Bcl-2-interacting mediator of death) to initiate death in T cells. Thus, mutations in BIM or Bax/Bak have profound effects on T-cell numbers and homeostasis.[22,23] BIM is sequestered in the cytosol by its association with the dynein light chain (LC-8),[24] and is regulated by transcriptional or post-translational mechanisms that function to disrupt the cytosolic sequestration of the protein. In mature, activated T cells, BIM is post-transcriptionally regulated during apoptotic signaling,[25] whereas in immature T cells the regulation is transcriptional.[26,27] However, BIM cannot trigger apoptosis in mice lacking both Bax and Bak.[28] A role for BIM in regulating T-cell homeostasis is established, but its regulation in different T-cell maturational stages is incompletely understood. We observed that BIM protein is increased in quiescent T cells undergoing neglect-induced death (NID), and in this study we have examined the mechanism underlying this event.

During apoptotic signaling, perturbations of the outer mitochondrial membrane result in the release of apoptogenic intermediates such as cytochrome *c*, which catalyzes the activation of caspase-9 and thereby a proteolytic apoptotic cascade.[29] However, all mitochondrially regulated apoptotic signaling does not converge on the activation of caspases. Diverse apoptotic stimuli may activate either caspase-independent death pathways[30,31] or cascades with minimal involvement of caspases.[32–35] In antigen-stimulated,

activated T cells, activation-induced death (AID) is regulated by reactive oxygen species (ROS) and caspase activity principally restricted to mediating nuclear damage in this pathway.[33] We and others have reported a limited involvement of caspases in quiescent T-cell apoptosis.[34,35] In this study, we show that quiescent T cells cultured *in vitro* in the absence of growth factors undergo Fas-independent NID. We provide evidence that ROS are key mediators of NID and regulate BIM induction, caspase activation and apoptosis in quiescent T cells. Our data implicate inducible-nitric oxide synthase (iNOS) as a regulator of T-cell death independent of BIM. Finally, we show that the cytokine IL-7, which promotes T-cell survival, regulates the expression of iNOS, but not the induction of BIM in T cells.

Results

BIM is induced during quiescent T-cell apoptosis

$CD3^+$ T cells isolated from adult mouse spleens and cultured *in vitro* in the absence of trophic factors undergo NID. The apoptotic response of T cells during NID assessed either by DNA damage (Figure 1a) or cell lysis (Figure 1b) is blocked by the cytokine interleukin-7 (IL-7), which prolongs T-cell survival over a period of days (Figure 1a and b). The BH3-only protein BIM is implicated in the death of T cells *in vitro*.[23] Therefore, we assessed the expression of this protein in T cells that had been cultured *in vitro* in the absence of trophic factors. The expression of two BIM isoforms, BIM-extra-long (BIM-EL) and BIM-long (BIM-L), is upregulated in T cells cultured in the absence of IL-7 (Figure 1c, NID). We observed some variations in the extent of BIM-EL induction in different animals and the data presented are representative of these patterns of induction. BIM induction is apparent by 4 h, when there is no detectable apoptotic damage and the expression increases progressively thereafter (Figure 1d). Of the three prototypic isoforms of BIM, BIM-EL and BIM-L are always detected, whereas BIM-small (BIM-S) is rarely seen. A transient increase in Bax expression was also seen in some experiments as shown in the experiment in Figure 1d. However, the modulation of Bax expression was not consistent in all experiments. There is no increase in the expression of Bcl-x_L (Figure 1d) and nor did the expression of other proteins, such as p38MAPK (used to establish parity of loading (LC)), change significantly in culture. From these results we concluded that BIM expression was upregulated in quiescent T cells, similar to immature T cells,[26,27] also indicating that its regulation in quiescent T cells may be distinct from the post-transcriptional regulation of BIM in antigen-stimulated, cycling, T cells.[25]

Antioxidants block T-cell death

Caspase activity is largely confined to mediating nuclear damage in T cells undergoing NID.[34,35] ROS regulate caspase activation and apoptotic damage in antigen-stimulated T cells, cultured *in vitro* in the absence of growth factors.[33,36] Therefore, we asked if ROS signaling regulates cell death in quiescent T cells. Both nuclear damage (Figure 2a) and cell lysis (Figure 2b) are inhibited by the

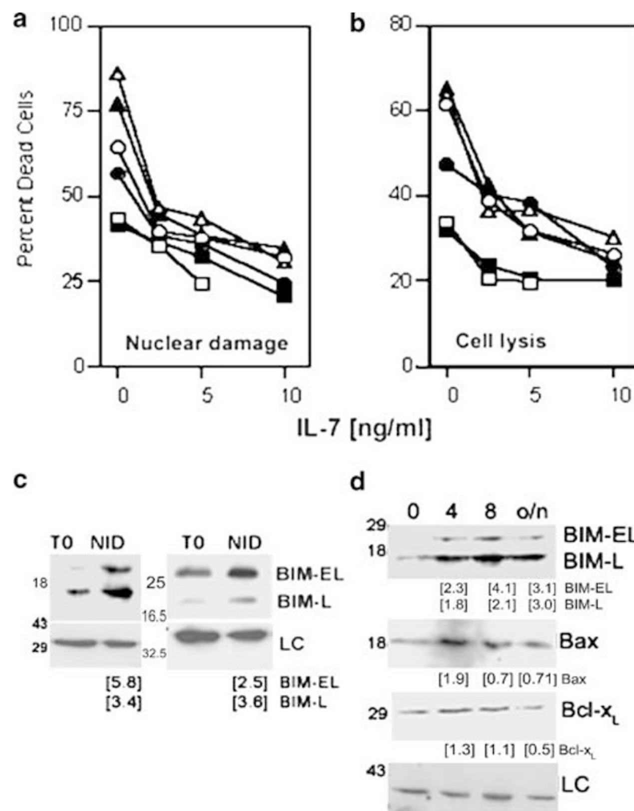

Figure 1 T cells upregulate BIM and undergo NID *in vitro*. (**a**, **b**) T cells were cultured *in vitro* in the presence of indicated concentrations of IL-7. Cells were analyzed for apoptotic nuclear morphology (**a**) or loss of membrane integrity (**b**) after 18 (squares), 36 (circles) or 72 (triangles) h. Open and closed symbols indicate two separate sets of T-cell preparations. (**c**) Expression of BIM isoforms in total cell lysates of freshly isolated T cells (T0) or cells cultured without IL-7 for 12 h (NID) by Western blot analysis. Parity of loading was established with p38MAPK in all experiments unless stated otherwise (LC). (**d**) Total cell lysates of T cells that are freshly isolated or cultured *in vitro* for indicated times were analyzed by sequential strip and reprobes of membranes for the expression of BIM, Bcl-x_L and Bax by Western blot analysis. Values in parentheses below the blots represent the fold change relative to the control/T0

nonthiol, superoxide dismutase (SOD) mimic MnTMPyP (Mn(III)terakis(1-methyl-4-pyridyl) porphyrin pentachloride) and the antioxidant PDTC (pyrrolidine dithiocarbamate) (Figure 2a and b). The induction of apoptosis is independent of Fas-death receptor signaling and as shown in Figure 2c, T cells from mice with a deficiency in Fas signaling (B6.*lpr*) are as susceptible to apoptosis as T cells from wild-type mice (C57Bl/6). Apoptosis in both wild-type and Fas mutant mice is inhibited by another SOD mimic MnTBAP (Mn (III) tetrakis (5, 10, 15, 20-benzoic acid porphyrin) (MnTBAP) (Figure 2c). MnTMPyP inhibited the mitochondrial efflux of cytochrome *c* in cells undergoing NID (Figure 2d). We failed to detect the processing of caspase-9 in these cells (Figure 2d), but the processing of caspase-7 is blocked in cells treated with MnTMPyP (Figure 2e). These data indicate that ROS-dependent signaling is an early event and precedes caspase activation and mitochondrial events in the apoptotic pathway. MnTMPyP also inhibits the processing of caspase-3 and the cleavage of PARP to its 85 kDa form in dying cells.

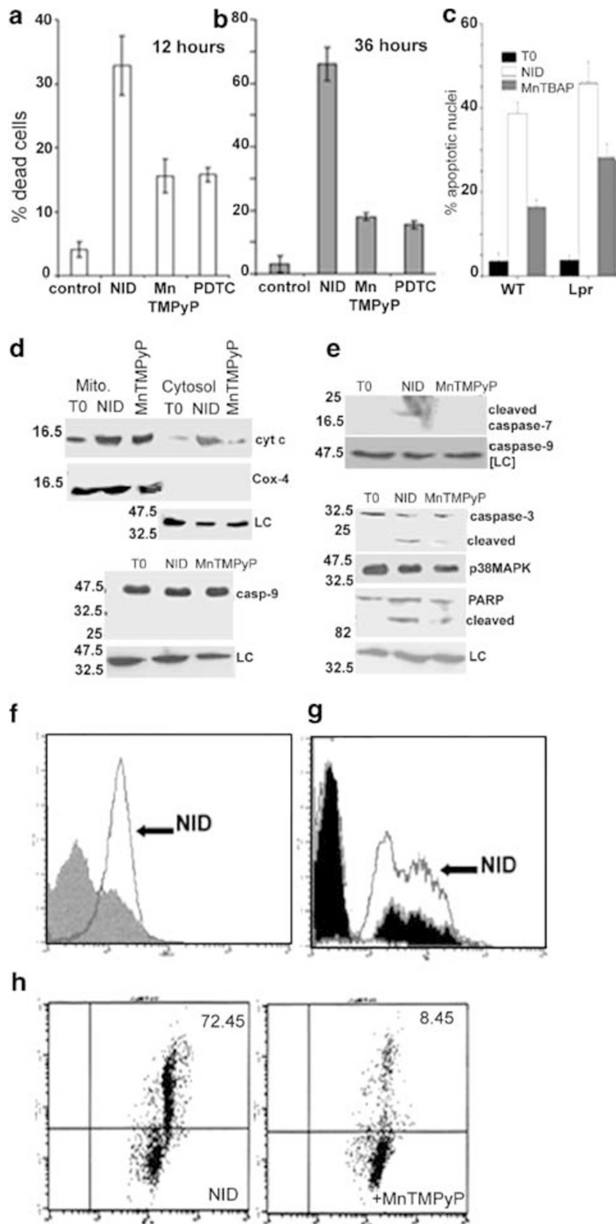

Figure 2 Effect of antioxidants on T cells cultured *in vitro* and undergoing NID. (**a**, **b**) T cells were cultured as such (NID) or with 100 μM MnTMPyP or 100 μM PDTC and analyzed for apoptotic nuclear damage (**a**) and lysis (**b**) after 12 and 36 h, respectively. Control refers to freshly isolated T cells. (**c**) T cells from C57BL/6 (WT) and B6.*lpr* (lpr) mice were cultured as such (NID, open bars) or with 50 μM MnTBAP (gray bars). After 12 h, cells were harvested and assessed for DNA damage. Freshly isolated T cells are indicated as T0 (black bars). (**d**) Subcellular fractions of freshly prepared T cells (T0, lanes 1 and 4), cells cultured as such (NID, lanes 2 and 5) or with MnTMPyP (lanes 3 and 6) for 4 h were probed for cytochrome *c*. The blots were reprobed for Cox-4 to ascertain the purity of the fractions. Total cell lysates from the same conditions as described above were probed for the processing of caspase-9. (**e**) Lysates of freshly prepared T cells (lane 1, T0) or cells cultured *in vitro* (lane 2, NID) or with 100 μM MnTMPyP (lane 3) for 12–15 h were assessed by Western blot analysis for the expression of cleaved caspase-7, caspase-3 and PARP. (**f**, **g**) Flowcytometric analysis of T cells stained with DihydroRhodamine-123 (**f**) or DHE (**g**) as described in Materials and Methods. Open histograms indicate T cells undergoing NID and filled histograms are freshly isolated T cells. (**h**) T cells cultured with (panel 2) or without (panel 1) 100 μM MnTMPyP were stained with DHE and analyzed by flowcytometry. The upper right quadrant indicate cells that fluoresce brightly with the dye

Consistent with the inhibition of apoptosis in T cells by the antioxidants, we detected intracellular accumulation of reactive intermediates using the reagents DHR 123 or dihydroethidium (DHE) (open histograms in Figure 2f and g). In freshly prepared T cells, a small subset is positive for ROS (filled histograms in Figure 2f and g). However, after 6–8 h in culture, almost all cells show increased fluorescence of DHR123 (open histograms in Figure 2f) or DHE (Figure 2g). Expectedly, MnTMPyP also blocked the generation of ROS in these cells (Figure 2h); numbers in the upper right quadrant indicate the percentage of ROS-high subsets. Taken together, these data favored the hypothesis that ROS accumulation may be causally linked to the induction of apoptosis in T cells.

Antioxidants block the induction of BIM

To assess if ROS generation influenced apoptotic outcome via the modulation of BIM, we tested if PDTC or MnTMPyP regulated the expression of BIM. Indeed, as shown in Figure 3a, both inhibitors significantly reduced the induction of BIM, suggesting that ROS target the BIM expression in T cells. The increase in Bax expression is not modulated by PDTC (Figure 3a) or MnTMPyP (data not shown). Neither the cytokine IL-7 (Figure 3b, lane 3) nor the broad-spectrum caspase inhibitor BD-fmk (Figure 3b, lane 4) blocked the induction of BIM. We have recently shown that IL-7 induces the expression of Bcl-x_L in T cells[34] (Figure 3b). Elevated levels of Bcl-x_L, especially if sustained over a period of time, would antagonize the proapoptotic effects of BIM and promote survival even in the continued presence of BIM. Caspase-mediated apoptotic damage is principally confined to the nucleus in quiescent T cells,[34,35] and the lack of effect of BD-fmk is consistent with delayed activation of caspases in this apoptotic cascade.

Recently, BIM has been identified as a target of the forkhead (FoxO) family of transcription factors.[37,38] Transcriptional activity of FoxO proteins is repressed by phosphorylation mediated by phosphatidylinositol-3 kinase (PI3K)-dependent Akt/PKB signaling,[39] which prevents nuclear localization of the transcription factor. We observed a reduction of the phosphorylated FoxO (Thr^{24}) species at a time (60 min, Figure 3c, lane 3) that preceded the increase in BIM protein expression in T cells (data not shown).The loss of the phosphorylated form was complete by 120 min. Furthermore, MnTMPyP appeared to prevent the hypophosphorylation of FoxO (Figure 3c, lanes 2 and 4). However, the total levels of FoxO protein are also increased in MnTMPyP-treated cells. Thus, the apparent increase in the phosphorylated species may be due to the increase in FoxO in MnTMPyP-treated cells.

To assess if blocking ROS-mediated damage restored functional competence in these cells, we tested for T-cell receptor-dependent proliferation in cells rescued from death by the antioxidants. T cells that had been cultured for 48 h in the presence or absence of the antioxidants were transferred to plates coated with anti-CD3, a condition that crosslinks the receptor and stimulates proliferation in uncompromised T cells (Figure 3d, T0). The proliferative response of T cells that have been previously cultured for 48 h in the absence of

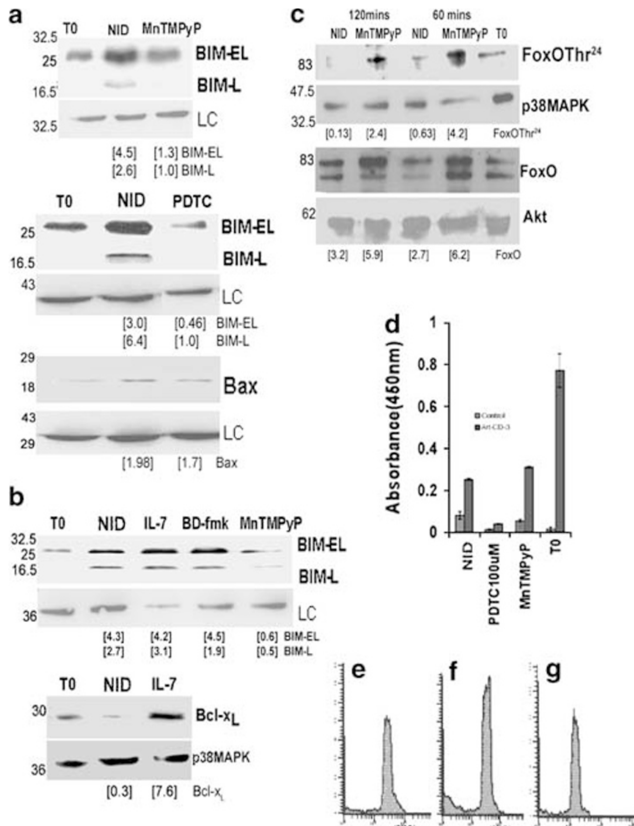

Figure 3 Antioxidants block BIM induction but do not promote entry into cell cycle. (**a**) Whole-cell lysates of freshly isolated T cells (T0) or T cells cultured as such (lane 2) or with 100 μM MnTMPyP (lane 3) or 100 μM PDTC (lane 3, lower panel) for 12 h were analyzed by Western blot for the expression of BIM and Bax. (**b**) Lysates were prepared from the following conditions: freshly isolated T cells (lane 1, T0), cells cultured as such (lane 2, NID) or with 10 ng/ml IL-7 (lane 3) or 25 μM BD-fmk (lane 4) or 100 μM MnTMPyP (lane 5) for 12 h. Expression of BIM and Bcl-x_L was determined by Western blot analysis. (**c**) Freshly isolated T cells (T0, lane5) or cells cultured with (lanes 2 and 4) or without (lanes 1 and 3) 100 μM MnTMPyP were probed for the expression of phosphorylated-Thr^{24} of FoxO or FoxO at 60 min (lanes 3 and 4) or 120 min (lanes 1 and 2) by Western blot analysis. In (**a**–**c**), values in parentheses below the blots indicate the fold change relative to the control/T0. (**d**) Freshly isolated T cells (T0) or T cells cultured for 48 h as such (NID) or with 100 μM PDTC or 100 μM MnTMPyP were washed and continued in culture (gray bars) or cultured on plastic-bound anti-CD-3 (black bars). After 12 h, cell proliferation was determined using XTT as described in Materials and Methods. (**e**–**g**) Freshly isolated T cells (**e**) or T cells cultured with (**g**) and without (**f**) MnTMPyP were analyzed by flowcytometry for DNA content as described in Materials and Methods

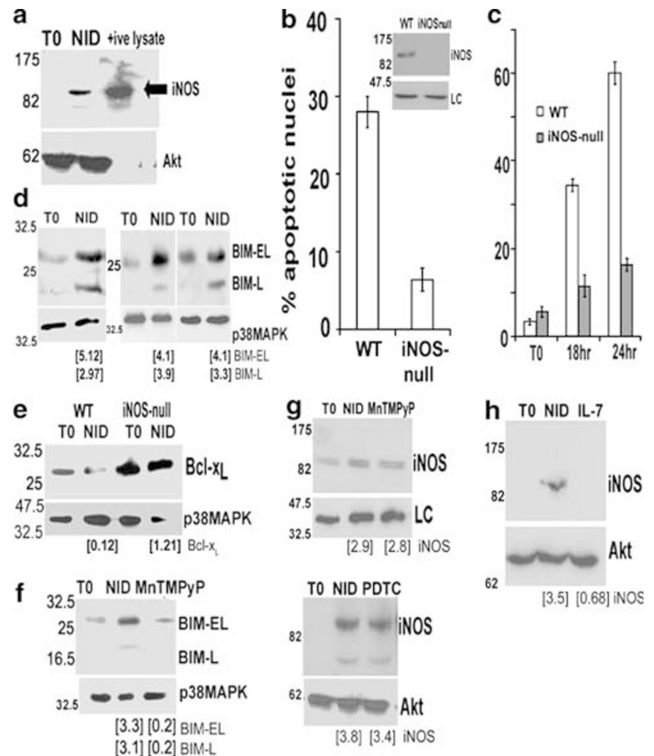

Figure 4 T cells from iNOS-null mice are protected from NID. (**a**) Lysates of freshly isolated T cells (lane 1, T0) and cells cultured as such (lane 2, NID) were analyzed for the expression of iNOS protein. The positive control was provided from the manufacturer and is a lysate of the RAW 264.7 cell line. (**b**) Apoptotic nuclear damage assessed in T cells from WT or iNOS-null mice was cultured for 12 h. Values have been normalized for apoptotic damage in freshly isolated T cells and the data derived from six independent animals for each set are shown. Inset: expression of iNOS protein in T cells derived from wild-type (WT) or iNOS-null mice cultured *in vitro* for 12 h. (**c**) Apoptotic nuclear damage in freshly isolated T cells (T0) or T cells from wild-type (open bars) or iNOS-null mice (gray bars) cultured *in vitro* for 18 and 24 h. (**d**–**h**) Expression of various indicated proteins determined by Western blot analysis in freshly isolated T cells (T0), cells cultured in the absence of survival factors (NID) or cells cultured with MnTMPyP or PDTC. (**d**, **f**) T cells from iNOS-null mice. (**g**–**h**) T cells from wild-type mice. (**e**) T cells from one animal each of an age-matched wild-type control (WT) or an iNOS-null mouse (iNOS-null). Values in parentheses below the blots indicate fold change in protein expression relative to control/T0 for each blot

trophic factors is severely impaired (Figure 3d, NID). Despite the fact that both PDTC and MnTMPyP block death, neither reagent could promote entry into cell cycle in T cells. We analyzed the DNA content of T cells that have been cultured *in vitro* in the presence (Figure 3g) or absence of MnTMPyP (Figure 3f) and find that there is no progression into cell cycle compared to freshly isolated T cells (Figure 3e).

Quiescent T cells from iNOS-null mice do not require trophic support for survival *in vitro*

T cells undergoing NID upregulate iNOS protein (Figure 4a), suggesting a role for iNOS in T-cell homeostasis. We wished to test this observation in a genetic model and obtained T cells from mice with a targeted disruption of the iNOS gene. T cells from mutant mice were protected from death when cultured in the absence of IL-7 (Figure 4b). To assess if there was a secondary, latent pathway that triggered apoptosis with slower kinetics, iNOS-null T cells were continued in culture for an extended period. However, we did not observe a significant increase in apoptotic cells at 18 or 24 h (Figure 4c) or at later time points (data not shown). Expectedly, iNOS protein is not detected in cells derived from mutant mice (Figure 4b, inset). T cells from mutant mice express BIM (Figure 4d, lanes 1, 3 and 5), which indicated that BIM turnover in T cells was not under the control of iNOS. The induction of BIM in mutant cells cultured in the absence of IL-7 (Figure 4d) was comparable to that observed in wild-type mice in earlier experiments. It should be noted that the expression of endogenous Bcl-x_L is substantially higher in T cells of mutant mice (Figure 4e, compare lanes 1 and 3) and does not

go down in culture (Figure 4e), which would ensure protection from death in these T cells. Furthermore, MnTMPyP negatively regulates induction of BIM in iNOS-null T cells (Figure 4f), indicating that the mechanism of regulation of this proapoptotic protein is conserved and most likely mediated by reactive derivatives of oxygen in T cells. MnTMPyP or PDTC (Figure 4g) do not regulate expression of iNOS protein. Since both IL-7-treated and T cells from iNOS-null mice had high levels of Bcl-x_L, we tested the possibility that IL-7 regulates iNOS induction in T cells. As shown in Figure 4h, the induction of iNOS is inhibited in cells cultured *in vitro* in the presence of the cytokine. Thus, it appears that prosurvival cytokines such as IL-7 may increase Bcl-x_L expression by transcriptional suppression of iNOS, which in turn negatively regulates Bcl-x_L expression in T cells.

Discussion

We report that BIM and iNOS proteins are regulated in quiescent, peripheral T cells undergoing NID and show that autocrine ROS modulate BIM expression and quiescent T-cell death. From the analysis of T cells from iNOS-deficient mice, we rule out a role for iNOS in the regulation of BIM. Finally, we show that prosurvival cytokines such as IL-7 regulate the expression of iNOS and possibly thereby the expression of Bcl-x_L and survival in T cells (Figure 5).

BIM converges on the mitochondrial pathway of apoptosis through activation of the BH-3 multidomain proteins Bax or Bak. Quiescent T-cell death in BIM knockout or the Bax–Bak double knockouts is impaired,[22,23] indicating a redundancy in the function of these proteins in ensuring T-cell deletion. Bax and Bak are constitutively expressed in all cells and BH3-only proteins, such as BIM, are believed to function to regulate the apoptotic response in a cell- and stimulus-specific manner.[40] However, BIM is not sufficient to trigger death in the absence of Bax and Bak.[28] The transient increase in Bax expression observed in some experiments was not regulated by the antioxidants that block the increased expression of BIM. We show that NID is independent of Fas signaling and identify a role for ROS in the regulation of BIM expression in quiescent T cells. We do not have an insight into the mechanism of ROS generation in T cells undergoing NID. It is apparent from the data from other laboratories on the BIM-deficient mouse that BIM likely triggers an early and key event in the apoptotic process in T cells. We would suggest that ROS lead to BIM activation, but do not rule out the possibility that ROS (possibly of mitochondrial origin) are the mediators of nuclear damage and cell lysis in T cells. Given the complexity and multi-compartment nature of ROS signaling, we cannot specifically identify the different ROS that are generated in these cells. We establish a role for ROS principally through the use of the metalloporphyrins, which are synthetic antioxidant compounds demonstrated to dismutate superoxide with high efficacy.[41] These compounds also mimic catalase and scavenge peroxynitrite.[42] In cells, the dismutation of superoxide leads to the production of toxic products such as hydrogen peroxide, which is expected to be neutralized by the catalase activity of the compounds used in this study. Both PDTC and MnTMPyP modify NADPH/NADP oxidoreductase function, which is also a key component of the plasma membrane oxidoreductase system.[43,44] Thus, our study implicates cytoplasmic compartments in the generation of ROS that regulate BIM expression during NID in quiescent T cells. In addition, this study extends earlier studies in antigen-stimulated T cells[33] to identify a role for ROS as key regulators of T-cell NID.

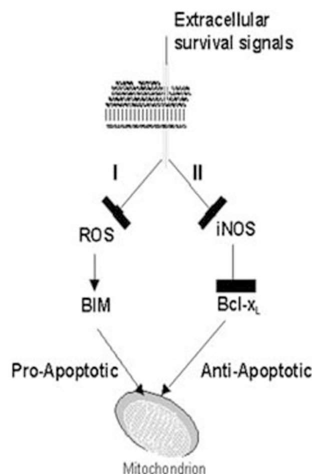

Figure 5 Extrinsic signals can promote survival by regulating pathway I or II. Blocking induction of ROS (pathway I) will result in reduced transcription of BIM and thereby favor survival. Blocking iNOS induction or function (pathway II) will result in increased expression of Bcl-x_L, antagonize BIM and protect cells from mitochondrial damage and apoptosis. Extrinsic signals may also converge on the mitochondrion via other intermediates not depicted in the model[51]

In the immune system, cytokines regulate lymphocyte homeostasis including cell division, differentiation and survival, by activating multiple signal transduction pathways. Cytokines such as IL-7 that signal via the γc chain have been implicated in T-cell survival[45–47] Signals through IL-7 receptor result in the docking of transcription factors that regulate apoptotic gene expression and can also activate prosurvival pathways regulated by PI3K signaling.[34,48] Although BIM is regulated via PI3K modulation of FoxO transcription factors, we suggest that in T cells there may be alternative mechanisms of regulating BIM transcription, as constitutive activation of the PI3K pathway achieved by IL-7[34,35] does not down-regulate BIM (Figure 3b). Our data indicate that iNOS is not essential for induction of BIM, but the expression of Bcl-x_L is elevated in mutant T cells (Figure 4), suggesting that Bcl-x_L is negatively regulated by iNOS. The possibility that iNOS may regulate the subcellular localization of BIM has not been tested in this study. The observation that IL-7, which regulates Bcl-x_L expression, blocked iNOS upregulation in dying T cells, suggests one mechanism by which cytokines may function to regulate survival without regulating the expression of BIM in T cells. iNOS transcription in mesangial cells is regulated via STAT-3 activation[49] and IL-7 has been reported to activate STAT-3,[50] suggesting a mechanism by which IL-7 may regulate iNOS expression. That the cytokine IL-7 blocks death in the continued presence of BIM suggests that quiescent T-cell survival can result from the suppression of BIM induction (pathway I, Figure 5) or the activity of antiapoptotic proteins such as Bcl-x_L that antagonize BIM function (pathway II, Figure 5). Cell survival may also be

regulated via pathways that regulate other intermediates that influence mitochondrial integrity.[51] As discussed before, these experiments do not discriminate between the various compartments that contribute to the generation of ROS in cells undergoing NID. Thus, BIM might well trigger an increase of mitochondrial ROS that would function as effectors of apoptotic damage in cells undergoing NID.

What are the implications of these observations on the regulation of peripheral T-cell survival *in vivo*? Since T-cell viability can be sustained despite increased amounts of BIM (as in iNOS-deficient T cells, Figure 4), it appears that the immune system activates several mechanisms to ensure T-cell survival. These mechanisms have likely developed to function as checkpoints that prevent uncontrolled deletion of quiescent, circulating T cells. This would ensure that the peripheral T-cell repertoire is not compromised by the loss of T-cell subsets required to initiate the immune response against pathogens. Apart from cytokines such as IL-7, circulating T cells are dependent on signals from the MHC to stay alive.[11–15] The molecular pathway of death regulated by this signaling is relatively unexplored. We now suggest that ROS are pivotal intermediates in the death pathway that is regulated by MHC-dependent signals. Further experiments will be required to reveal if signals via CD4 and CD8 coreceptors on T cells indeed counteract ROS generation and the subsequent induction of BIM in quiescent T cells.

Materials and Methods

Cells and reagents

C57Bl/6-iNOS null, C57Bl/6.lpr and age- and strain-matched control mice were obtained from Jackson Labs (Bar Harbor, ME, USA) and accessed at the Small Animal Facility at NII, New Delhi. For experiments not involving mutant mice, C57Bl/6 mice were maintained at the Small Animal Facility at NCBS, Bangalore. All animal experiments were performed with the approval of the respective Institutional Animal Ethics Committees at NII and NCBS. For all experiments, $CD3^+$ T cells were isolated from 4–6-week mouse spleens, using a previously described protocol.[34] All chemicals were obtained from Calbiochem (San Diego, CA, USA) unless specified otherwise. Boc-D-fmk was obtained from Enzyme Systems Products (Dublin, CA, USA). DHR123 and DHE were obtained from Molecular Probes (Eugene, OR, USA). Recombinant murine IL-7 and antibody to cytochrome *c* and Bcl-x_L were obtained from R&D Systems (Minneapolis, USA). Antibodies to BIM and Cox-4 were from BD Pharmingen (San Diego, USA), to Bax, phosphorylated forkhead, forkhead, caspase-3, cleaved caspase-7 and caspase-9 were from Cell Signaling Technology (MA, USA), to iNOS/NOS-II from Upstate Biotechnology (Lake Placid, NY, USA) and to PARP, p38MAPK and Akt from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

NID, assays of apoptotic damage and proliferation

T cells isolated from mouse spleens were cultured at 2×10^6/ml for 12–18 h in routine assays of NID. Apoptotic damage was assessed relative to freshly isolated T cells (T0). Wherever used, IL-7 (10 ng/ml), MnTMPyP (100 μM), MnTBAP (50 μM) or PDTC (100 μM) were added at the initiation of culture. Data have been derived from a minimum of three experiments unless stated otherwise. Apoptotic nuclear morphology using the dye Hoechst 33342 and cell lysis using propidium iodide uptake were assessed as described.[52] All flowcytometric analyses were performed on a Becton Dickinson FACS® (Becton Dickinson, Mount View, CA, USA) using CELLQuest software. Proliferation was assessed using an XTT-based cell proliferation kit as per the manufacturer's instructions (Biological Industries Co., Beit Haemek, Israel). Assays were terminated after 4 h incubation with the dye.

Assays for intracellular ROS

Staining for DHR123 was performed according to the manufacturer's instructions (Molecular Probes). For DHE, cells were stained with 2 μM DHE in complete medium for 40 min at 37°C. Cells were washed once and analyzed immediately by flowcytometry. Incubations with the dyes were performed in the absence of inhibitors.

Analysis of DNA content

Cells (2×10^6) were pelleted by centrifugation. Pellets were loosened by tapping and gently resuspended in chilled DNA-fragmentation buffer (0.1% Triton X-100, 0.1% sodium citrate and 25 μg/ml propidium iodide). Samples were stored protected from light, at 4°C for 18 h before analysis by flowcytometry.

Subcellular fractionation

A digitonin-based buffer was used to separate the membrane (mitochondrial) fraction from the soluble (cytoplasmic) fraction of cell lysates. Briefly, cell pellets were resuspended in a digitonin-based buffer (40 μg/ml digitonin in 120 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 20 mM Hepes-Tris, pH 7.2 supplemented with protease inhibitors) and samples were held on ice for 5 min. Unlysed cells and debris were removed by centrifugation at 800 *g* and samples were then centrifuged at 14 000 *g* for 15 min. The resulting pellet was enriched in mitochondria and the soluble fraction was representative of the cytoplasmic fraction. The distribution of cytochrome *c* oxidase (Cox4) was used to determine that cytoplasmic cytochrome *c* did not result from mitochondria damaged in the course of the protocol.

Western blot analysis

Cell lysates were resolved on SDS gels and protocols recommended by the manufacturer were used for Western blot analysis. Blots were developed by chemiluminiscence using Super Signal (Pierce, USA) as described before.[34] p38MAPK was used to establish parity of loading in 10–12% SDS gels and Akt was used as the loading control (LC) for 8–10% SDS gels. All blots were sequentially stripped and reprobed in different combinations of antibodies to confirm the data. Densitometry analysis was performed using Image gauge software v 3.0. Fold increase or decrease in proteins was calculated relative to the control (T0) for each blot.

Acknowledgements

We are very grateful to Satyajit Rath and Vineeta Bal (National Institute of Immunology, New Delhi) for generous access to mutant strains of mice used in this study. We acknowledge Veronica Rodrigues, Gaiti Hasan, MK Mathew and Satyajit Rath for discussion and comments on the manuscript. The work was supported by an International Senior Research Fellowship in Biomedical Science Wellcome Trust, UK and the Department of Science and Technology, India.

References

1. Aravind L, Dixit VM and Koonin EG (1999) The domains of death: evolution of the apoptosis machinery. Trends Biochem. Sci. 24: 47–53
2. Tittel JN and Steller H (2000) A comparison of programmed cell death between species. Genome Biol. 1: 0003.1–0003.6
3. Ashkenazi A and Dixit VM (1998) Death receptors: signaling and modulation. Science 281: 1305–1308
4. Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV and Boldin MP (1999) Tumor necrosis factor receptor and Fas signaling mechanisms. Annu. Rev. Immunol. 17: 331–367
5. Green DR and Reed JC (1998) Mitochondria and apoptosis. Science 28: 1309–1312
6. Vander Heiden MG and Thompson CB (1999) Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? Nat. Cell Biol. 1: E209–216
7. Deveraux QL and Reed JC (1999) IAP family proteins – suppressers of apoptosis. Genes Dev. 13: 239–252
8. Gottlieb E, Vander Heiden MG and Thompson CB (2000) Bcl-x(L) prevents the initial decrease in mitochondrial membrane potential and subsequent reactive oxygen species production during tumor necrosis factor alpha-induced apoptosis. Mol. Cell. Biol. 20: 5680–5689
9. Goldrath AW and Bevan MJ (1999) Selecting and maintaining a diverse T-cell repertoire. Nature 402: 255–262
10. Freitas AA and Rocha B (1999) Peripheral T cell survival. Curr. Opin. Immunol. 11: 152–156
11. Brocker T (1997) Survival of mature CD4T lymphocytes is dependent on major histocompatibility complex class II-expressing dendritic cells. J. Exp. Med. 186: 1223–1232
12. Kirberg J, Berns A and von Boehmer H (1997) Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. J. Exp. Med. 186: 1269–1275
13. Tanchot C, Lemonnier FA, Perarnau B, Freitas AA and Rocha B (1997) Differential requirements for survival and proliferation of CD8 naive or memory T cells. Science 276: 2057–2062
14. Murali-Krishna K, Lau LL, Sambhara S, Lemonnier F, Altman J and Ahmed R (1999) Persistence of memory CD8T Cells in MHC class I-deficient mice. Science 286: 1377–1381
15. Takeda S, Rodewald HR, Arakawa H, Bluethmann H and Shimizu T (1996) MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span. Immunity 5: 217–228
16. Veis DJ, Sorenson CM, Shutter JR and Korsmeyer SJ (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75: 229–240
17. Kuo CT, Veselits ML and Leiden JM (1997) LKLF: A transcriptional regulator of single-positive T cell quiescence and survival. Science 277: 1986–1990
18. Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M, Soengas MS, Elia A, de la Pompa JL, Kagi D, Khoo W, Potter J, Yoshida R, Kaufman SA, Lowe SW, Penninger JM and Mak TW (1998) Differential requirement for caspase 9 in apoptotic pathways *in vivo*. Cell 94: 339–352
19. Boehme SA and Lenardo MJ (1996) TCR-mediated death of mature T lymphocytes occurs in the absence of p53. J. Immunol. 156: 4075–4078
20. Kuida K, Haydar TF, Kuan CY, Gu Y, Taya C, Karasuyama H, Su MS, Rakic P and Flavell RA (1998) Reduced apoptosis and cytochrome *c*-mediated caspase activation in mice lacking caspase 9. Cell 94: 325–337
21. Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM and Mak TW (1998) Apaf1 is required for mitochondrial pathways of apoptosis and brain development. Cell 94: 739–750
22. Rathmell JC, Lindsten T, Wei-Xing Z, Cinalli RM and Thompson CB (2002) Deficiency in bak and bax perturbs thymic selection and lymphoid homeostasis. Nat. Immunol. 3: 932–939
23. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F, Adams JM and Strasser A (1999) Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. Science 286: 1735–1738
24. Puthalakath H, Huang DC, O'Reilly LA, King SM and Strasser A (1999) The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol. Cell 3: 287–296
25. Hildeman DA, Zhu Y, Mitchell TC, Bouillet P, Strasser A, Kappler J and Marrack PC (2002) Activated T cell death *in vivo* mediated by proapoptotic bcl-2 family member bim. Immunity 16: 759–767
26. Bouillet P, Purton JF, Godfrey DI, Zhang LC, Coultas L, Puthalakath H, Pellegrini M, Cory S, Adams JM and Strasser A (2002) BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. Nature 415: 922–926
27. Wang Z, Malone MH, He H, McColl KS and Distelhorst CW (2003) Microarray analysis uncovers the induction of the pro-apoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. J. Biol. Chem. 278: 23861–23867
28. Zong W-X, Lindstein T, Ross AJ, MacGregor GR and Thompson CB (2001) BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev. 15: 1481–1486
29. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR and Martin SJ (1999) Ordering the cytochrome *c*-initiated cascade: hierarchical activation of caspases-2, -3, -6, -7, -8 and -10 in a caspase-9 dependent manner. J. Cell Biol. 144: 285–291
30. Deas O, Dumont C, MacFarlane M, Rouleau M, Hebib C, Harper F, Hirsch F, Charpentier B, Cohen GM and Senik A (1998) Caspase-independent cell death induced by anti-CD2 or staurosporine in activated human peripheral T lymphocytes. J. Immunol. 161: 3375–3383
31. Susin SA, Lorenzo H, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM and Kroemer G (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397: 441–446
32. Sarin A, Williams MS, Alexander-Miller MA, Berzofsky JA, Zacharchuk CA and Henkart PA (1997) Target cell lysis by CTL granule exocytosis is independent of ICE-Ced-3 family proteases. Immunity 6: 209–215
33. Hildeman DA, Mitchell TC, Teague TK, Henson P, Day BJ, Kappler J and Marrack PC (1998) Reactive oxygen species regulate activation-induced T cell apoptosis. Immunity 10: 735–744
34. Sade H and Sarin A (2003) Interleukin-7 blocks caspase-independent apoptosis in mature T cells via phosphatidylinositol-3 kinase (PI3-K)-mediated survival signalling. Eur. J. Immunol. 33: 913–919
35. Rathmell JC, Farkash EA, Gao W and Thompson CB (2001) IL-7 enhances the survival and maintains the size of naïve T cells. J. Immunol. 167: 6869–6876
36. Devadas S, Zaritskaya L, Rhee SG, Oberley L and Williams MS (2002) Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and fas ligand expression. J. Exp. Med. 195: 59–70
37. Dijkers PA, Medema RH, Lammers J-W.J, Koenderman L and Coffer PJ (2000) Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol. 1201–1204
38. Stahl M, Dijkers PF, Kops GJ, Lens SM, Coffer PJ, Burgering BM and Medema RH (2002) The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. J. Immunol. 168: 5024–5031
39. Brunet A, Datta SR and Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr. Opin. Neurobiol. 11: 297–305
40. Puthalakath H and Strasser A (2002) Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ. 9: 505–512
41. Pasternack RF, Banth A, Pasternack JM and Johnson CS (1981) Catalysis of the disproportionation of superoxide by metalloporphyrins III. J. Inorg. Biochem. 15: 261–267
42. Zingarelli B, Day BJ, Crapo JD, Salzman AL and Szabo C (1997) The potential role of peroxynitrite in the vascular contractile and cellular energetic failure in endotoxic shock. Br. J. Pharmacol. 120: 259–267
43. Faulkner KM, Liochev SI and Fridovich I (1994) Stable Mn(III) porphyrins mimic superoxide dismutase *in vitro* and substitute for it *in vivo*. J. Biol. Chem. 269: 23471–23476
44. Wright MV and Kuhn TB (2002) CNS neurons express two distinct plasma membrane electron transport systems implicated in neuronal viability. J. Neurochem. 83: 655–664

45. Nakajima H, Shores EW, Noguchi M and Leonard WJ (1997) The common cytokine receptor gamma chain plays an essential role in regulating lymphoid homeostasis. J. Exp. Med. 185: 189–195
46. Maraskovsky E, Teepe M, Morrissey PJ, Braddy S, Miller RE, Lynch DH and Peschon JJ (1996) Impaired survival and proliferation in IL-7 receptor-deficient peripheral T cells. J. Immunol. 157: 5315–5323
47. Tan JT, Dudl E, LeRoy E, Murray R, Sprent J, Weinberg KI and Surh CD (2001) IL-7 is critical for homeostatic proliferation and survival of naive T cells. Proc. Natl. Acad. Sci. USA 98: 8732–8737
48. Lin J-X, Migone TS, Tsang M, Friedmann M, Weatherbee JA, Zhou L, Yamauchi A, Bloom ET, Mietz J, John S and Leonard WJ (1995) The role of shared receptor motifs and common stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13 and IL-15. Immunity 2: 331–339
49. Yu Z, Zhang W and Kone BC (2002) Signal transducers and activators of transcription 3 (STAT3) inhibits transcription of the inducible nitric oxide synthase gene by interacting with nuclear factor κB. Biochem. J. 367: 97–105
50. Pallard C, Stegmann AP, van Kleffens T, Smart F, Venkitaraman A and Spits H (1999) Distinct roles of the phosphatidylinositol 3-kinase and STAT5 pathways in IL-7-mediated development of human thymocyte precursors. Immunity 10: 525–535
51. Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB and Hay N (2001) Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. Genes Dev. 15: 1406–1418
52. Varghese J, Chattopadhyay S and Sarin A (2001) Inhibition of p38 kinase reveals a tumour necrosis factor alpha (TNF)-mediated, caspase-dependent, apoptotic death pathway in a human myelomonocyte cell line. J. Immunol. 166: 6570–6577