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Regulation of cell death and survival in intestinal intraepithelial lymphocytes

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Abstract

Intraepithelial lymphocytes (IEL) of the small murine bowel represent a unique population of mostly CD8+ T lymphocytes that reside within the epithelial cell layer of the intestinal mucosa. The close interaction with epithelial cells appears to be crucial for IEL survival since isolation and *ex vivo* culture induces massive apoptosis in this lymphocyte population. Here, we provide evidence that this form of IEL cell death may be mediated at least in part by endogenously produced glucocorticoids since adrenalectomy or treatment of mice with a glucocorticoid receptor antagonist significantly enhanced *ex vivo* survival of IEL. We further demonstrate that *ex vivo* activation of IEL induces upregulation of anti-apoptotic gene products, compensates for the lack of survival cytokines and rescues from apoptotic cell death. Thus, similar to thymocytes and T cell hybridomas, IEL survival may be regulated by the antagonistic action of TCR activation and glucocorticoids. *Cell Death and Differentiation* (2001) **8,** 706–714.

Keywords: intraepithelial lymphocytes; T cell activation; glucocorticoid; intestinal mucosa; inhibitors of apoptosis

Abbreviations: AICD, activation-induced cell death; CD95, Fas/APO-1; CD95L, CD95 ligand; sCD95-Fc, soluble CD95-human IgG1 fusion protein; cIAP, cellular inhibitor of apoptosis; dex, dexamethasone; HBS, HEPES-buffered saline; IL, interleukin; IEL, intraepithelial lymphocytes; PMA, phorbolmyristate acetate; RT–PCR, reverse transcription polymerase chain reaction; RU-486, mifepristone; TGFβ, transforming growth factor β; TCR, T cell receptor; TNFα, tumor necrosis factor α; TUNEL, TdT-mediated dUTP nick end labeling

Introduction

Intraepithelial lymphocytes are a unique population of lymphocytes whose function in the immune defense and regulation is still poorly understood. IEL of the small murine intestine comprise mostly of CD8+ T cells, and whereas in the peripheral lymphatic organs, only T cells with a CD8$\alpha\beta$ heterodimer are found, a significant portion of IEL expresses CD8$\alpha\alpha$ homodimers. Similarly, T cell receptor (TCR) $\gamma\delta$-expressing lymphocytes are rarely found in the spleen and lymph nodes, but represent an important population in the intestinal mucosa (reviewed in [1–3]). Recent evidence suggests that CD8$\alpha\alpha$ IEL may not originate from thymic precursors, but develop and mature directly in the intestinal mucosa in so-called cryptopatches.[4]

Although IEL have been suggested to play an important role in host defense, in particular in the protection of the intestinal mucosa from infection by parasites and viruses,[5–8] the investigation of IEL functions has been complicated by the low survival rate of isolated *ex vivo* cultured IEL.[9–11] The close association of IEL with epithelial cells of the intestinal mucosa appears to be crucial for IEL survival. Isolation of IEL from their epithelial environment and *ex vivo* culture leads to accelerated apoptotic cell death. This form of death-by-neglect is presumably caused by the lack of epithelial cell-derived survival factors since epithelial cell-derived cytokines, such as interleukin-7 and -15, significantly enhance IEL survival.[9,11]

Although IEL poorly proliferate in response to *ex vivo* stimulation, they exhibit potent *ex vivo* cytotoxicity.[7,12,13] Yet, differential responses of IEL to TCR stimulation are still poorly understood. Here we now show that stimulation of IEL via TCR or by addition of phorbolmyristate acetate (PMA) and ionomycin can compensate for the lack of epithelial cell-derived survival factors and induces *ex vivo* survival. This enhanced survival is most likely caused by induction of anti-apoptotic gene products since IEL activation leads to enhanced expression of cellular inhibitor of apoptosis (cIAP)-1 and -2, and Bcl-x_L. Our data provide further evidence that death-by-neglect of *ex vivo* cultured IEL may be (at least in part) caused by glucocorticoids. IEL isolated form adrenalectomized animals showed enhanced *ex vivo* survival. Similarly, *in vivo* treatment of animals with the glucocorticoid receptor antagonist RU-486 (mifepristone) resulted in reduced *ex vivo* apoptosis whereas *in vivo* treatment with glucocorticoids lead to accelerated cell death. Our data indicate that TCR stimulation and glucocorticoids exhibit antagonistic activity on IEL survival, similarly to that observed in thymocytes and T cell hybridomas.[14,15]

Results

Analysis of *ex vivo* IEL apoptosis

In this study we have analyzed the regulation of cell death and survival of murine intestinal intraepithelial lymphocytes. Figure 1A shows a typical section through the small intestine. IEL are detected between epithelial cells at the basolateral side of the epithelial cell layer. To study *ex vivo*

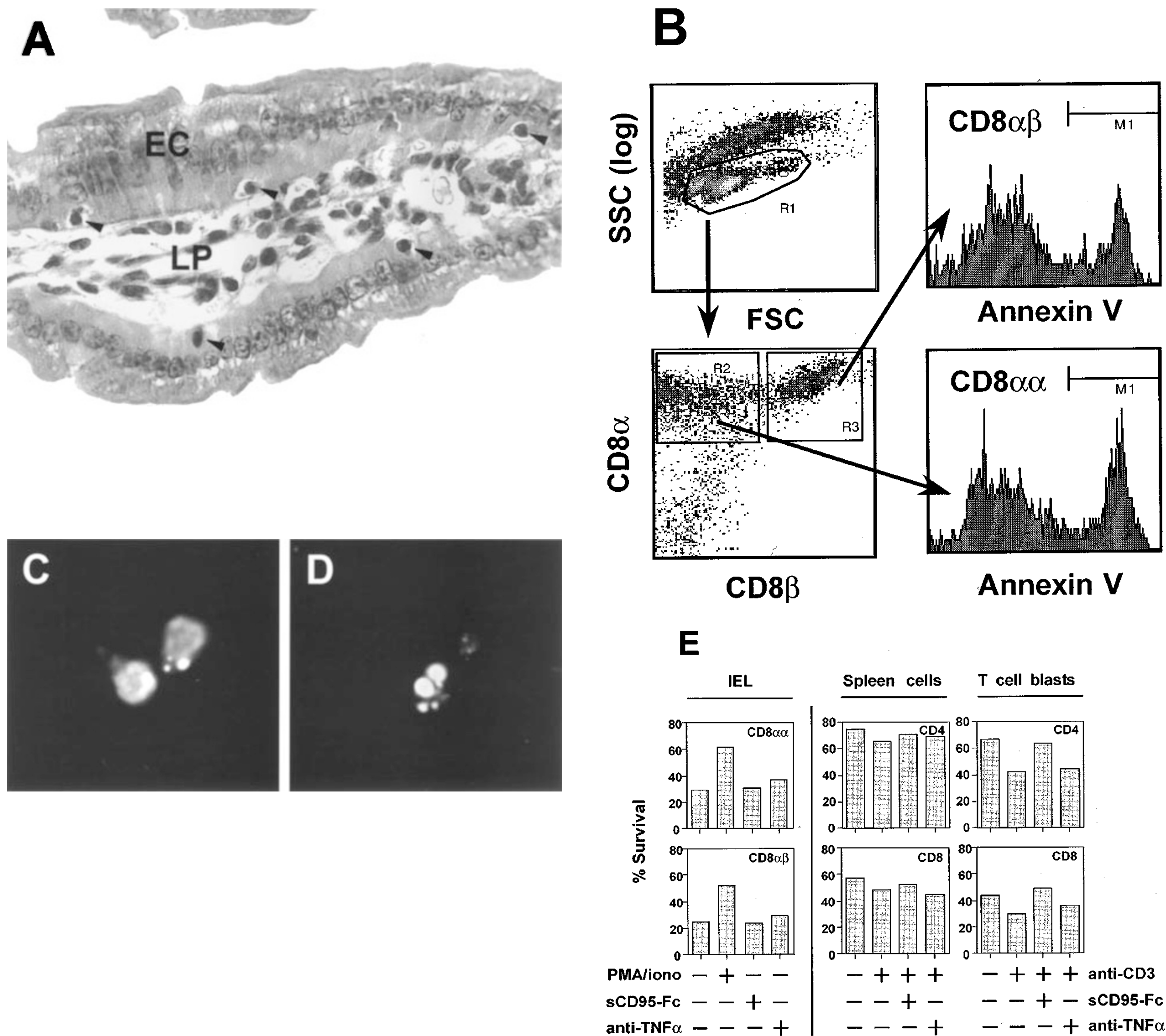

Figure 1 *Ex vivo* apoptosis of IEL is not caused by CD95L or TNFα. (**A**) Histological section through the mucosa of the small intestine. IEL are indicated by arrows, EC, epithelial cells, LP, lamina propria. (**B**) Assessment of IEL apoptosis. IEL preparations were stained with anti-CD8α-Cy-chrome (FL-3), CD8β-PE (FL-2) and Annexin V-FITC (FL-1) as described in Materials and Methods, and analyzed by flow cytometry. Electronic gates were set around lymphocytes to discriminate epithelial cells. Annexin V binding was then assessed in the CD8αα$^+$ and CD8αβ$^+$ IEL populations. (**C, D**) Assessment of apoptotic morphology in freshly isolated IEL **(C)**, and IEL cultured for 5 h *ex vivo* **(D)**. Cells were stained with ethidium bromide and acridine orange and analyzed by fluorescence microscopy. (**E**) Analysis of *ex vivo* IEL apoptosis. Freshly isolated IEL or spleen cells, or T cell blasts were cultured with PMA (20 ng/ml) and ionomycin 200 ng/ml) (IEL) or plate-bound anti-CD3 (2 ug/ml) (spleen cells, T cell blasts). Death receptor-induced apoptosis was blocked by addition of soluble CD95-Fc or neutralizing anti-TNFα. Survival of the different T cell subsets after overnight culture was assessed by Annexin V staining

survival, IEL were isolated from the epithelial cell layer of the small bowel and enriched by density centrifugation. Either freshly isolated or upon *ex vivo* culture, apoptotic cell death was assessed by simultaneous staining for cell surface CD8α and CD8β, and detection of phosphatidylserine exposure by Annexin V (Figure 1B).[16] This methodological approach allowed comparative and quantitative analysis of early apoptosis in the two major IEL subsets, i.e. CD8αα$^+$ and CD8αβ$^+$ IEL. Figure 1B also shows that IEL undergoing apoptotic cell death show a characteristic reduction in cell size (FSC) and increase in light scatter (SSC). This apoptotic morphology was further confirmed by TUNEL (TdT-mediated dUTP nick end labeling) (data not shown) and fluorescence microscopy using ethidium bromide/acridine orange staining. Figure 1D shows a typical example of an apoptotic IEL with a fragmented nucleus.

Spontaneous *ex vivo* apoptosis of IEL is not caused by CD95 ligand or TNFα

Previous reports have shown that *ex vivo* cultured intestinal IEL are very susceptible to apoptotic cell death.[9–11] Similarly, we have found that both major IEL subsets, CD8αβ$^+$ and CD8αα$^+$ IEL, exhibited significant apoptotic cell death already

after 5 h *ex vivo* culture and very few cells survived overnight culture (Figure 1E, and data not shown). In different experiments, we always observed high rates of spontaneous *ex vivo* apoptosis of IEL (average of 11 independent experiments: $60\pm16\%$ Annexin V^+ cells after 6–8 h *ex vivo* culture). Experiments with highly purified IEL (sorted by flow cytometry) indicate that dying contaminating epithelial cells within the IEL preparation are not the underlying cause of IEL death since similar or even elevated rates of apoptosis were observed in purified *vs* enriched IEL, respectively ($73.2\pm7.4\%$ (n=6) *vs* $64.2\pm15.9\%$ (n=12), mean$\pm$S.D.). Members of the TNF/TNF receptor family have been implicated in T lymphocyte apoptosis (reviewed in[17–20]). Restimulation of previously activated T cell blasts causes CD95/CD95L-dependent activation-induced cell death (AICD) which is blocked by soluble CD95-Fc fusion protein (Figure 1E),[21–24] but not by neutralizing anti-TNFα (Figure 1E).[25] As reported previously, freshly isolated splenic T lymphocytes were found to be relatively resistant to spontaneous and activation-induced apoptosis (Figure 1E).[26] Our data, however, indicate that CD95/CD95 ligand or TNFα/TNF receptors interactions are most likely not responsible for the spontaneous *ex vivo* demise of IEL since antagonistic soluble CD95-Fc fusion protein or neutralizing anti-TNFα could not reverse the high rate of IEL apoptosis (Figure 1E). And, whereas restimulation of T cell blasts caused CD95/CD95L-dependent AICD, stimulation of IEL rather induced a significant reduction in *ex vivo* apoptosis (Figure 1E).

A role for glucocorticoids in *ex vivo* death-by-neglect

The underlying reason for the high rate of *ex vivo* IEL apoptosis is currently unclear. It has been suggested that epithelial cell-derived cytokines, such as IL-7 and IL-15, may regulate IEL survival *in vivo* and the lack of these survival factors may contribute to IEL apoptosis *in vitro*.[11,27–29] IEL have been reported previously to be exposed to and be affected by glucocorticoids *in vivo* and *in vitro*.[30–32] Glucocorticoids are potent inducers of apoptosis in thymocytes and mature T cells (reviewed in[33]). Interestingly, although glucocorticoids can induce apoptosis, they can also block AICD in T cells, as well as TCR stimulation can inhibit glucocorticoid-induced cell death.[15,34,35] We therefore investigated whether *in vivo* generated steroids may be the trigger of this form of death-by-neglect. To test this hypothesis, we first injected mice with dexamethasone (a synthetic glucocorticoid) and analyzed IEL survival upon *ex vivo* culture, with or without activation. Figure 2A shows that IEL isolated from dexamethasone-treated animals exhibited a reduced survival rate *ex vivo*, which however, was partially reverted upon *ex vivo* stimulation. Spleen cells, analyzed in parallel for comparison, were found to follow a similar pattern, yet with reduced sensitivity to dexamethasone (Figure 2A).

We then further assessed whether inhibition of glucocorticoid action would reduce IEL *ex vivo* apoptosis. The adrenal glands are a major source of glucocorticoids and adrenalectomy should significantly reduce *in vivo* glucocorticoid generation. Both major subsets of IEL, CD8$\alpha\alpha$ and CD8$\alpha\beta$, isolated from adrenalectomized animals showed enhanced *ex vivo* survival, compared to IEL from sham-operated mice (Figure 2B). This observation was made in three independent experiments.

Since the adrenal glands may not represent the only significant source of glucocorticoids,[14] we further aimed at blocking endogenous glucocorticoid activity by *in vivo* administration of RU-486, a glucocorticoid receptor antagonist.[36] Therefore, mice were injected with 1 or 10 mg RU-486, control diluent or 1 mg dexamethasone as positive control. IEL were isolated 4 h later and apoptosis in freshly isolated or *ex vivo* cultured cells was analyzed. While freshly isolated IEL from control animals and RU-486-treated animals showed only minimal Annexin V binding, cells from dexamethasone-treated animals displayed already significant phosphatidylserine flip (data not shown). *Ex vivo* culture of control IEL for 6 h induced high rates of apoptosis, as described above, which were further exceeded in cells isolated from dexamethasone-treated animals (Figure 2C). Interestingly, however, inhibition of glucocorticoid activity by RU-486 led to a significant enhancement of *ex vivo* IEL survival in both major subsets (Figure 2C). This effect of RU-486 was dose-dependent since injection of 10 mg further enhanced *ex vivo* IEL survival (data not shown). Thus, we conclude that *ex vivo* death-by-neglect is, at least in part, caused by endogenously produced glucocorticoids.

Activation induces resistance to death-by-neglect

The experiment described in Figure 1C suggested that IEL activation results in enhanced *ex vivo* survival, rather than induction of AICD. This may be due to the reported antagonistic action of TCR and glucocorticoids.[15,34,35] In order to investigate the role of activation signals on IEL apoptosis, we stimulated IEL with increasing concentrations of plate-bound anti-CD3 antibody and analyzed IEL apoptosis after 6 h. Whereas stimulation of primed peripheral T cell blasts caused CD95/CD95L-dependent AICD (Figure 1C),[22] activation of IEL led to a significant reduction in *ex vivo* apoptosis. Rescue from this form of death-by-neglect was dependent on the strength of stimulus since plate-bound anti-CD3 dose-dependently enhanced IEL survival (Figure 3A).

Activation induces expression of anti-apoptotic gene products

The results described above suggest that stimulation induces the expression of anti-apoptotic gene products, preventing the rapid *ex vivo* demise of IEL. Members of the Bcl-2 family have been previously shown to regulate lymphocyte survival, in particular death-by-neglect (reviewed in[37,38]). We therefore analyzed the expression of Bcl-2 and Bcl-x_L by RT–PCR in freshly isolated IEL, with and without TCR stimulation, and compared expression levels to those found in spleen cells. Bcl-2 was expressed only at low levels and no significant gene induction was observed after stimulation (data not shown). In contrast, Bcl-x_L was not detected in unstimulated IEL, but was efficiently induced upon IEL activation. In spleen cells, Bcl-x_L was already expressed in unstimulated cells and only a

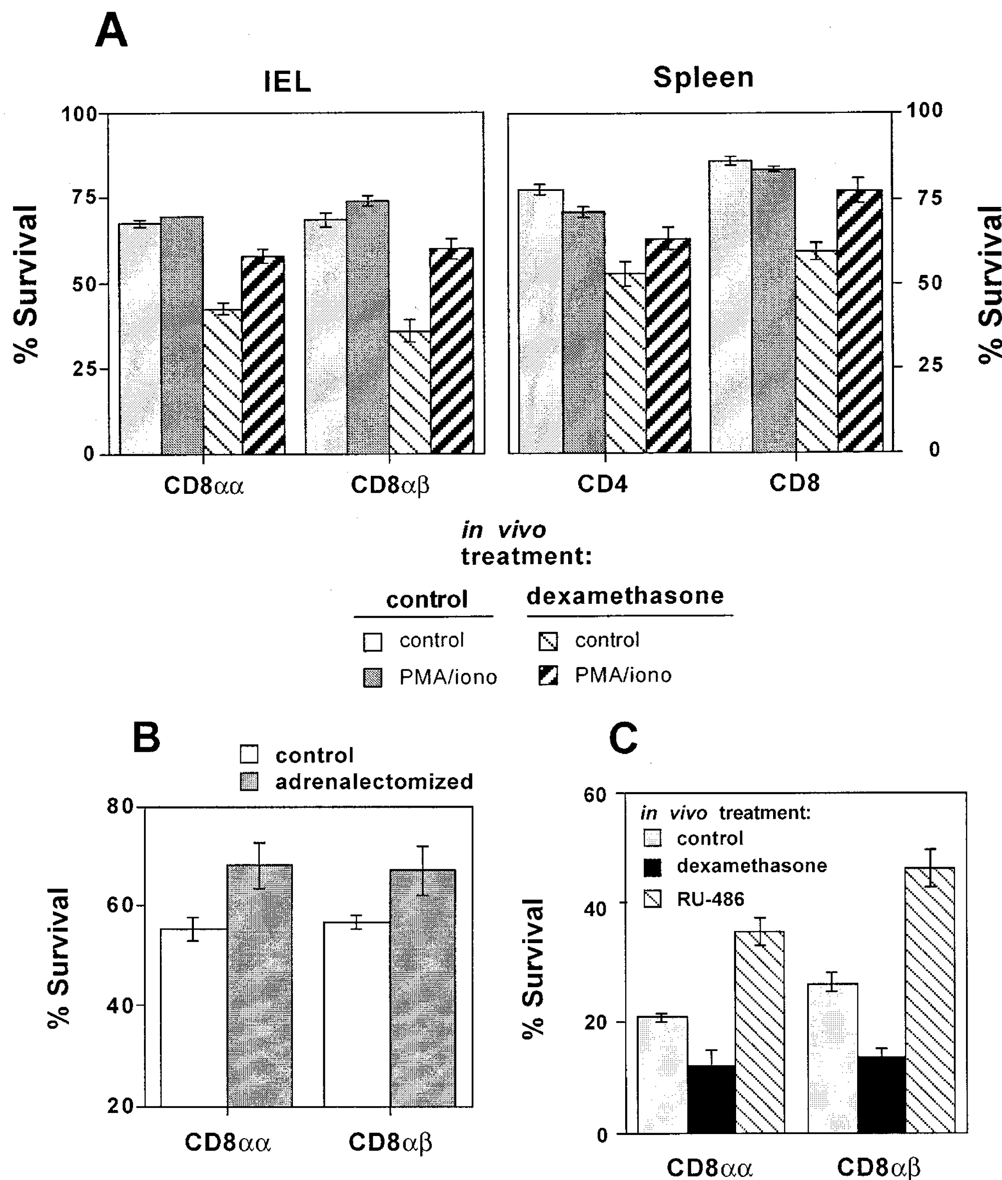

Figure 2 Role for glucocorticoids in IEL apoptosis. (**A**) Mice were injected with control diluent or 1 mg dexamethasone for 4 h. IEL and spleen cells were isolated and cultured in the presence or absence of PMA (20 ng/ml) and ionomycin (200 ng/ml) for further 5 h. Survival was assessed by Annexin V staining as described above. **(B)** IEL were isolated from adrenalectomized animals or sham-operated animals (control) and survival after 6 h culture in the different IEL subsets was assessed. (**C**) Inhibition of glucocorticoid receptor activity induces protection from *ex vivo* apoptosis. Mice were injected with control diluent, 1 mg dexamethasone or 1 mg RU-486 for 4 h. IEL were isolated and survival was assessed after 5 h *ex vivo* culture

minimal gene induction was observed upon TCR stimulation.

The anti-apoptotic activity of many members of the Bcl-2 family may be limited to apoptosis stimuli involving signaling via the mitochondria (reviewed in[39]). In contrast, cellular inhibitors of apoptosis (cIAP) act on the level of caspase activation, central signaling and execution proteases of most forms of apoptotic cell death (reviewed in[40]). We have therefore analyzed the expression of cIAP-1 and cIAP-2 in unstimulated and stimulated IEL. Both, cIAP-1 and -2 were hardly detectable in unstimulated IEL, however TCR triggering led to a strong induction of mRNA expression. Levels of both anti-apoptotic genes were already elevated in unstimulated spleen cells and were found unaltered upon activation (Figure 3D).

IEL stimulation prevents the degradation of apoptosis inhibitors

De novo gene induction of anti-apoptotic molecules may not represent the only mechanism how *ex vivo* IEL apoptosis is prevented by cell stimulation. Yang *et al*. have recently reported on the glucocortiocid-induced proteosome-mediated

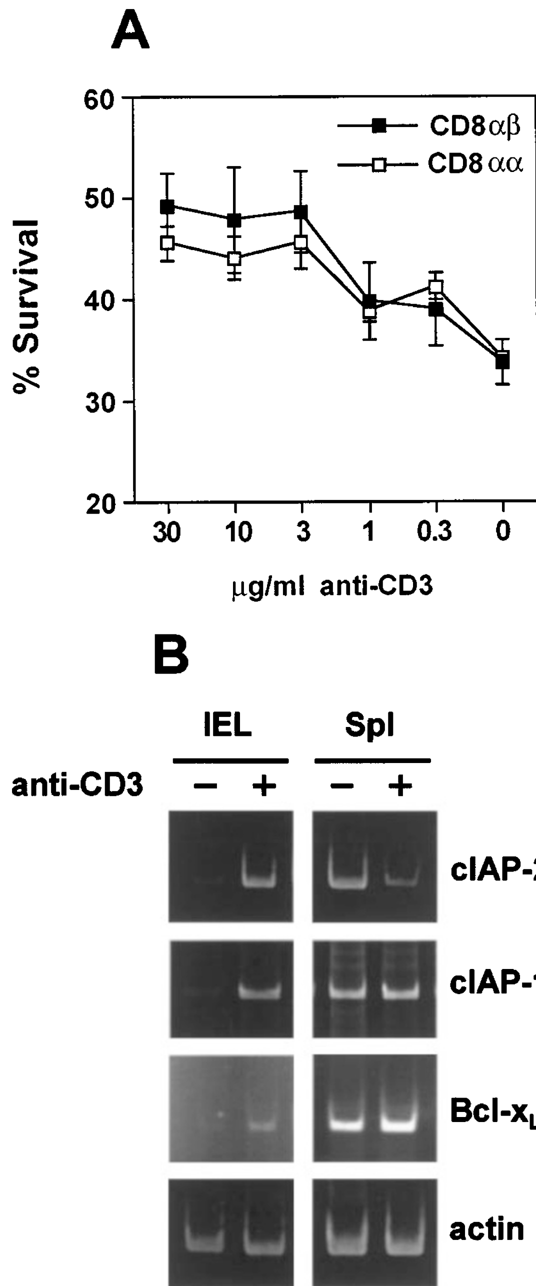

Figure 3 IEL stimulation induces survival. (**A**) IEL were stimulated with increasing concentrations of plate-bound anti-CD3 for 6 h and survival in CD8$\alpha\alpha^+$ or CD8$\alpha\beta^+$ IEL was assessed by Annexin V staining. (**B**) Activation of IEL induces anti-apoptotic genes. IEL and spleen cells were stimulated with plate-bound anti-CD3 for 4 h and mRNA expression of cIAP-1, cIAP-2, Bcl-x_L and actin was assessed by RT–PCR

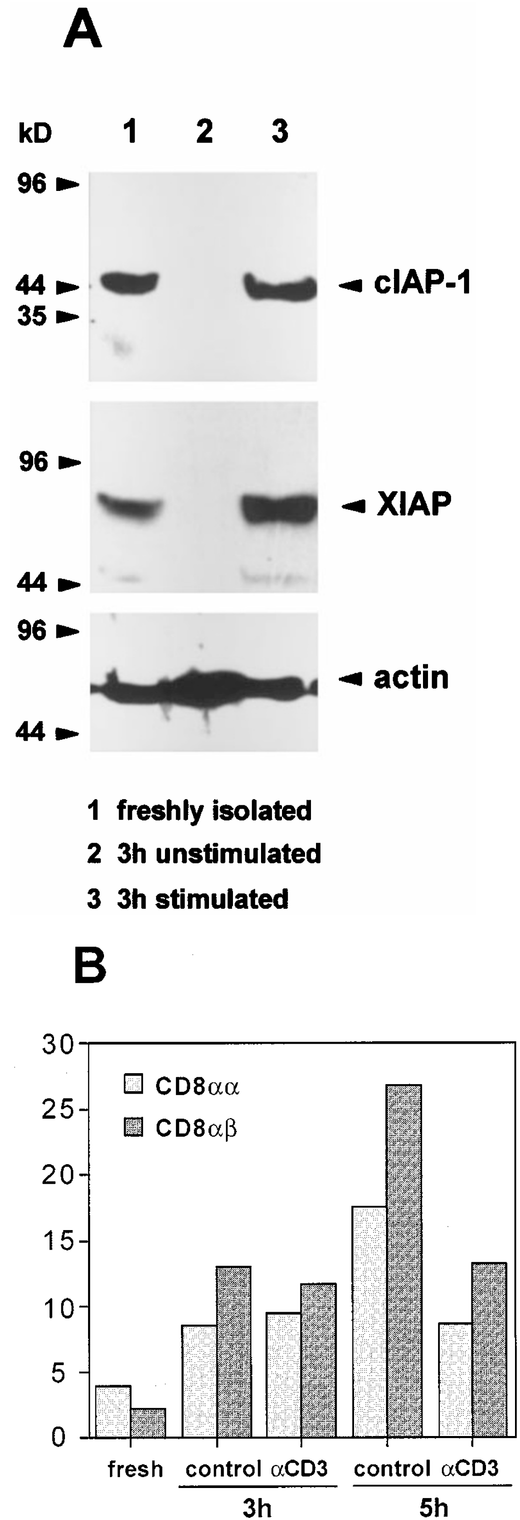

Figure 4 Stimulation prevents degradation of inhibitors of apoptosis protein. (**A**) Protein levels of cIAP-1, XIAP, and actin in freshly isolated IEL, or cells culture with or without anti-CD3 stimulation for 3 h, were analyzed by Western blotting. Molecular weights of protein standards are indicated. (**B**) Annexin V binding on CD8$\alpha\alpha^+$ and CD8$\alpha\beta^+$ IEL of the same experiment were analyzed in parallel

degradation of IAPs in thymocytes[41]. We therefore analyzed whether IAPs may be degraded during IEL apoptosis and whether stimulation antagonizes this process. cIAP-1 and XIAP (X-linked inhibitor of apoptosis)[42] levels in freshly isolated IEL, or cells that were left untreated or stimulated for 3 h, were analyzed by Western blot. Surprisingly, significant protein levels of cIAP-1 and XIAP were found already in freshly isolated IEL (Figure 4A), although only

minimal mRNA levels of cIAP-1 and -2 were detected in these cells (Figure 3D). *Ex vivo* culture for 3 h was sufficient to reduce cIAP-1 and XIAP protein to undetectable levels, suggesting glucocorticoid-induced degradation of these inhibitors of apoptosis.[41] However, significant levels of cIAP-1 and XIAP were maintained if IEL were stimulated with plate-bound anti-CD3. Interestingly, while increased IEL apoptosis was already detected after 3 h *ex vivo* culture, no difference was observed between unstimulated and stimulated IEL (Figure 4B). However, after 5 h *ex vivo* culture high rates of cell death were observed in unstimulated cells, whereas apoptosis in anti-CD3 stimulated IEL was stabilized (Figure 4B). Thus, degradation of cIAP-1 and XIAP precedes detection of phosphatidylserine exposure. We further conclude that both, activation-induced gene induction as well as activation-induced prevention of degradation of inhibitors of apoptosis may contribute to *ex vivo* IEL survival.

Discussion

Although T lymphocytes of the intestinal epithelial cell layer represent an important number of all immune cells, their role in host defense and/or immune regulation is still largely unknown. Recent evidence, however, suggest that IEL-mediated cytotoxicity is protective during viral infections.[7,8] In addition, certain subsets of IEL may represent regulatory T cells, maintaining immune homeostasis of the intestinal mucosa and oral tolerance (reviewed in[43]). Studies on the role of IEL in host defense and immune regulation have been complicated by the rapid demise of IEL upon *ex vivo* culture.[9–11] The intimate contact of IEL with their neighboring epithelial cells appears to deliver important survival signals and dissociation of IEL from the epithelial cell layer rapidly induces apoptosis. Epithelial cells of the intestinal mucosa are a rich source of a wide variety of cytokines. For example, epithelial cells produce IL-7 and -15, two important cytokines in IEL development and survival.[11,28,29] Whereas IL-7 has been described as an essential growth factor for extrathymic maturating IEL progenitors,[28] IL-15 has mitogenic and survival-inducing activity in mature IEL.[9,11] Similarly, targeted disruption of the common γ-chain of the IL-7 and IL-15 receptor results in reduced development of TCR$\gamma\delta^+$ CD8$\alpha\alpha^+$ IEL.[44]

While epithelial-derived cytokines can rescue IEL from *ex vivo* apoptosis,[9,45] the trigger of this death-by-neglect has not been investigated so far. Our data presented here provide strong evidence that, at least in part, endogenously produced glucocorticoids may be involved in this form of apoptotic cell death. *In vivo* administration of dexamethasone accelerated the *ex vivo* demise of IEL and, similarly to spontaneous apoptotic cell death, was antagonized upon IEL activation. Furthermore, either inhibition of glucocorticoid synthesis by adrenalectomy or inhibition of glucocorticoid receptor activation by RU-486 resulted in a marked increase in IEL *ex vivo* survival. Although the cellular source and the kinetic of synthesis of the endogenous glucocorticoids involved in IEL apoptosis induction are unknown, we may speculate that glucocorticoids must prime IEL for apoptosis already *in vivo*. *In vivo* administration of RU-486 significantly reduced *ex vivo* apoptosis, whereas RU-486 added after the IEL isolation process had only minimal effect on IEL survival (data not shown). Recent studies have shown that stress or burn can lead to generation of high levels of endogenous glucocorticoids, which may trigger *in vivo* apoptosis of lymphocytes and IEL.[30,46] Thus, euthanizing mice with CO_2 could provoke sufficient stress to release glucocorticoids from the adrenal glands. On the other hand, it is possible that low levels of glucocorticoids are constitutively present in the intestinal mucosa (possibly produced by other sources than the adrenal glands?), as a negative regulatory element of the ‘physiological inflammation’ in the intestine. While adrenalectomy significantly reduced IEL apoptosis, a stronger effect was observed upon *in vivo* administration of RU-486. Like IEL, thymocytes are extremely sensitive to glucocorticoid-induced apoptosis. Recently, Vacchio and colleagues reported that thymic epithelial cells are an important alternative source of glucocorticoids and therefore may affect thymocyte survival.[14] It is thus possible that other sources of glucocorticoids than adrenal glands, possibly even cells within the intestinal mucosa, contribute to apoptosis induction in IEL.

Glucocorticoids have been suggested to affect the transcriptional activity of NF-κB.[47–51] Therefore, expression NF-κB-dependent survival factors[52–54] may be antagonized by glucorticoids. Inhibition of NF-κB, by overexpression of IκB or inhibition by lactacystin, has been shown to sensitize cells to a variety of apoptosis-inducing stimuli.[55–57] Similarly, recent evidence indicates that dexamethasone induces a proteosome-mediated degradation of cIAP-1 and XIAP in thymocytes, and subsequently apoptotic cell death.[41] Our findings that ‘spontanous’ *ex vivo* apoptosis and dexamethasone-induced apoptosis of IEL is reduced upon TCR activation would be consistent with this model of IEL survival. TCR activation leads to strong induction of NF-κB activity and NF-κB-dependent survival genes, such as cIAP-1 and -2 (our own data).[53] While in the absence of survival-promoting cytokines, endogenous glucocorticoids may induce apoptosis, IEL activation via TCR signals appears to antagonize glucocorticoid-induced death by inducing and maintaining expression of anti-apoptotic gene products. Glucocorticoids not only induce apoptosis, but may also regulate immune responses through inhibition of inflammatory cytokine production (reviewed in[58,59]) and cytotoxic effector functions.[34,35] Thus, the controlled antagonism between glucocorticoid action and T cell activation may be important for the regulation of immune homeostasis in the intestinal mucosa.

Material and Methods

Reagents and solutions

Anti-CD8α-Cy-chrome, anti-CD8β-PE, and anti-CD4-PE were purchased from PharMingen (La Jolla, CA, USA); anti-CD3ε (145-2C11) anti-TCR$\alpha\beta$ (H57) and anti-TCR$\gamma\delta$ (GL-3) were purified from hybridoma supernatant by protein A columns and conjugated to FITC; neutralizing rabbit anti-mouse TNFα (IP-400) was from Genzyme (Cambridge, MA,

USA) (anti-TNFα-neutralizing activity was tested in a L929 bioassay)[60]; Annexin V-FITC was obtained from Bender Biosystems (Vienna, Austria). The generation of soluble CD95-immunoglobulin fusion protein (sCD95-Fc) has been reported previously.[22,61]

Culture medium consisted of RPMI 1640, 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin, 50 uM β-mercaptoethanol, 20 mM HEPES pH 7.4. HEPES-buffered salt solution (HBS) consisted of 10 mM HEPES pH 7.2, 25 mM $NaHCO_3$, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 137 mM NaCl, 5.6 mM D-glucose. Percoll and Ficoll were purchased from Pharmacia (Uppsala, Sweden). Isotonic Percoll solution was made by adding 1/10 of the volume of 10 times concentrated HBS. Recombinant interleukin (IL)-2 (proleukin) was from Chiron (Suresnes, France). Concanavalin A (Con A), phorbolmyristate acetate (PMA), dexamethasone were from Sigma (St. Louis, MO, USA), ionomycin from Calbiochem (La Jolla, CA, USA).

Isolation of IEL from small intestine

Male Balb/c mice were bred and maintained in the animal facility of the Faculty of Medicine, University of Bern, Switzerland. Age-matched male adrenalectomized or sham-operated Balb/c mice were obtained from Harlan (The Nederlands). Mice were euthanized with CO_2 and IEL from the small bowel of 7–10 week old mice were isolated as described previously.[12,62] Briefly, epithelial cells and IEL were dissociated in HBS, 1 mM DTT and separated on a 40%/70% Percoll gradient. The interphase containing enriched IEL (usually between 40–85% $CD8^+$ cells) was washed, resuspended in culture medium and directly used for experiments. Purified cells usually consisted of 36.6$\pm$12.6% $CD8\alpha\beta^+$, 39.7$\pm$3.8% $CD8\alpha\alpha^+$, 60.2$\pm$19.2% $TCR\alpha\beta^+$, 23.8$\pm$7.5% $TCR\gamma\delta^+$ lymphocytes (mean$\pm$S.D. of eight isolations) and were approximately 95% viable, as monitored by Annexin V binding.[16] In some experiments, IEL were further sorted on a FACSvantage (Becton Dickinson, Mountain View, CA, USA) based on the lymphocyte gate in the forward-side scatter.

Spleen cells were isolated by meshing up the spleen between frosted microscopy glass slides and subsequent hypotonic lysis to remove erythrocytes.[12] After resuspension in complete medium, cells were either directly used for experiments (spleen cells) or stimulated for 2 days with 1 ug/ml concanavalin A (Sigma) and subsequent culture with 100 U/ml recombinant IL-2 for 5 days to generate T cell blasts. Before being used in an experiment, T cell blasts were purified by Ficoll density centrifugation to remove dead cells.

Assessment of apoptosis in distinct T cell subsets

Apoptosis was assessed by the detection of phosphatidylserine on the cell surface of dying cells.[16] Enriched or sorted IEL were resuspended at 5×10^4 or 1×10^5 cell per 200 ul and cultured in flat-bottom 96 well plates. If cells were treated with anti-CD3, plates were previously coated overnight with appropriate concentrations of anti-CD3 in 50 mM Tris pH 9.0 at 4°C and washed three times with PBS. Other stimuli or reagents were directly added to the cells in appropriate dilutions at 1/20 of the culture volume. Final DMSO or ethanol concentrations were always $\leqslant$0.1%. After either 5–6 h or overnight incubation, cells were harvested, washed once with cold PBS, 1% calf serum and then stained for 20 min with anti-CD8α Cy-chrome and anti-CD8β PE at 4°C. Cells were subsequently washed with Annexin V binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) and then stained with Annexin V-FITC (0.5 ug/ml) for 5 min. After two washes with Annexin V binding buffer, cells were fixed in 1% paraformaldehyde in Annexin V binding buffer and analyzed on a FACScan flow cytometer using Cellquest analysis software (Becton Dickinson, Mountain View, CA, USA). Cells in the lymphocyte gate were analyzed for their expression of CD8α and CD8β, and $CD8\alpha\alpha^+$ or $CD8\alpha\beta^+$ populations were examined for cell death by means of their Annexin V staining.

For comparison, freshly isolated spleen cells or day 7 T cell blasts were treated in parallel. Apoptosis was analyzed as described above, besides that anti-CD4PE and anti-CD8α Cy-chrome were used to detect Annexin V binding on $CD4^+$ and $CD8^+$ T cells, respectively.

To further confirm the apoptotic phenotype of IEL, either freshly isolated cells or *ex vivo* cultured cells were harvested and stained with ethidium bromide (100 ug/ml) and acridine orange (100 ug/ml) in PBS, and immediately analyzed by fluorescence microscopy.[63] In some experiments, DNA fragmentation in apoptotic IEL was detected by TUNEL using a commercially available kit (Roche Diagnostics (Rotkreuz, Switzerland).

RT–PCR for Bcl-2, Bcl-x_L, cIAP-1 and cIAP-2

In some experiments, IEL or spleen cells were left either untreated or stimulated for 5 h with plate-bound anti-CD3. After two washes in PBS, cells were lysed in Tri-reagent RNA isolation medium (Sigma) and total RNA was isolated as recommended by the manufacturer. cDNA was synthesized from 2 ug total RNA using a commercial cDNA kit (Promega, Madison, WI, USA) using oligo-dT primers and manufacturer's suggested conditions. PCR for murine Bcl-2, Bcl-x_L, cIAP-1, cIAP-2 and actin as a control was performed as described previously.[53,64–66] Following primers were used: mBcl-2 sense: 5′-AGCCCTGTGCCACCATGTGTC-3′; mBcl-2 antisense: 5′-GGCAGGTTTGTCGACCTCACT-3′; mBcl-x_L sense: 5′-TAGTCCAGC-CAGGGCACGT-3′; mBcl-x_L antisense: 5′-GGCTGATATCATACTG-CAT-3′; actin sense: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; actin antisense: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′; mcIAP-1 sense 5′-GCCATTGTCTTTTCTGTCAC-3′, mcIAP-1 antisense 5′-CTGCGTCTGCATTCTCATC-3′; mcIAP-2 sense 5′-ACCTAGTGTTC-CTGTTCAGC-3′, mcIAP-2 antisense 5′-CCTTCTCCTCTTCTCTTCT-CTC-3′. PCR was performed in a PTC-100 thermocycler (MJ Research Inc., Watertown, MA, USA). Bcl-2, cIAP-1, cIAP-2 and actin cDNAs were amplified for 32 cycles at 1 min 94°C, 1 min 55°C, 1 min 72°C, followed by 10 min extension at 72°C; Bcl-x_L cDNA was amplified for 32 cycles at 1 min 94°C, 1 min 50°C, 1 min 72°C, followed by 10 min extension at 72°C. PCR products were resolved on a 4% acrylamide-1$\times$TBE gel and visualized by ethidium bromide staining.

Detection of cIAP-1 and XIAP by Western blot

IEL were cultured for times indicated, harvested and washed in PBS. Cell pellets were then lyzed in 1$\times$reducing SDS–PAGE sample buffer (50 mM Tris pH 6.8, 1 mM DTT, 2% SDS, 10% glycerol, 0.1% bromophenol blue). Equal amounts of protein were separated on a 12% gel and transferred to PVDF membrane. Membranes were then blocked in 5% non-fat dry milk and incubated with anti-cIAP-1, anti-XIAP (R&D Systems, Minneapolis, MN, USA) (kindly provided by S Martin) or, to assess equal loading, anti-actin (Amersham). Antibody binding was detected with appropriate horse-radish peroxidase-conjugated secondary antibodies and ECL (Amersham).

In vivo treatment of IEL with glucocorticoid and RU-486

Balb/c mice were either injected i.p. with carrier only (olive oil, Sigma), 1 mg dexamethasone (Sigma), or 1 or 10 mg of the glucocorticoid receptor antagonist mifepristone (RU-486; kindly provided by Dr. R Sitruk-Ware, Laboratoires Exelgyn, Paris, France).[36] After 3 h, mice were sacrificed, and spleen cells and IEL were isolated as described above. Viability was assessed by Annexin V staining either directly after isolation or after 6 h culture with agents and stimuli indicated. The animal studies have been performed after approval by the Animal Study Review Board of the State of Bern, Switzerland.

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