Evidence for a Role for Notch Signaling in the Cytokine-Dependent Survival of Activated T Cells

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Notch signaling plays a critical role in several cell-fate decisions during the development and life span of multicellular organisms (1–3). The Notch pathway is mediated by isoforms of the Notch receptor and ligands, which include Jagged and Delta in vertebrates. Signaling is initiated by ligands that liberate the Notch1 intracellular (NIC) domain (NICD) from the membrane, which then translocates to the nucleus (1). In the nucleus, NICD binds the C-promoter-binding factor-1/recombinant signal-binding protein-J-κ complex converting it from a repressor to an activator of transcription (4–6). We have recently described an antiapoptotic role for the Notch pathway in activated T cells expressing mutant Fas ligand are sensitive to apoptosis. Activated T cells isolated on the basis of Notch expression (Notch+) are enriched for Bcl-xL expression and demonstrate reduced susceptibility to apoptosis triggered by neglect or oxidative stress. This article must therefore be hereby marked toward either one of these outcomes in activated T cells.

A complex network of signaling cascades and molecules is required for the regulated survival of T cells at all developmental stages (10, 11). T cells activated by cognate Ag undergo several rounds of cell division and differentiate into functional subsets that perform specialized functions. Following clearance of Ag from the organism, the bulk of Ag-specific activated T cells is deleted. Two pathways regulate activated T cell apoptosis. The stimulation of T cells in response to self-Ags is negatively regulated via a death pathway activated by receptors of the TNFR family. This is referred to as activation-induced cell death (AICD) and in CD4+ T cells is brought about by Fas-mediated activation of the caspase family of proteases (12). Unlike AICD, which removes autoreactive cells, the deletion of T cells activated in response to infection is believed to be triggered by a reduction of sustaining cytokines from the microenvironment. This process is referred to as activated T cell neglect-induced death (A-NID) and reactive oxygen species (ROS) are the principal mediators of this death pathway (13, 14). Members of the Bcl-2 family proteins, which comprise both pro- and antiapoptotic proteins, regulate this event (15, 16).

The cytokine IL-2 functions not only as a growth factor, promoting T cell proliferation and survival, but also primes T cells activated by self-Ags for AICD (17). Differential signaling of receptor components as well as the presence of concomitant stimuli regulate these opposing outcomes. Ectopic expression of NICD blocks AICD in T cell lines (7, 18) presumably by blocking signals downstream of FasR ligation (7). Although persistent signaling via Notch inhibits reactivation responses of T cells (19), in the early stages of T cell activation, Notch promotes IL-2 responsiveness and proliferation by regulating cytokine receptor expression (20, 21). Because IL-2 can drive both the survival and death of Ag-stimulated T cells, here we ask if Notch biases IL-2 signaling toward one of these outcomes in activated T cells.
Materials and Methods
Isolation of T cell subsets and T cell activation

C57BL/6 and C57BL/6J^{Peroxlac} were maintained at the Small Animal Facility at National Centre for Biological Sciences (NCBS). All experiments involving animals were performed with the prior approval of the Institutional Animal Ethics Committees at NCBS. CD3^+^, CD4^+^, or CD8^+^ T cells were isolated from mouse spleens using MagCellect kits (R&D Systems). Activated T cell blasts (TCB) were generated from naïve T cells isolated from spleens of 6- to 8-wk-old C57BL/6 mice. T cells were stimulated with beads coated with Abs to CD3-CD28 (Dynal) and in some experiments with plate-bound anti-CD3 (1 µg/ml, clone 2C11; R&D Systems) and soluble anti-CD28 (5 µg/ml; BD Biosciences). After 48 h, TCB were washed and used in assays or continued in culture with the cytokine IL-2 (R&D Systems). For peptide-stimulated T cells, splenocytes from 8-wk-old p14 TCR-transgenic mice were cultured with 200 ng/ml peptide for 12 h. Cells were washed to remove unbound peptide and cultured in Click’s medium containing 0.5 µM IL-2.

Chemicals and reagents

Hoechst 33342, Mm(II)tetrakis(4-benzoic acid) porphyrin chloride (MnT-BAP), the γ-secretase inhibitors t-685,458 (GSI-X), WPE-III-31C (GSI-XVII), and DAPT (GSI-IX) were obtained from Calbiochem (EMD Bio-sciences). Dihydroethidium (DHE) was obtained from Molecular Probes (Invitrogen Life Technologies) and the recombinant rat Notch1/Fc chimera and recombinant mouse Fas/Fc chimera were obtained from R&D Systems.

Analysis of protein expression by flow cytometry

A total of 0.5 × 10^6 cells were fixed with 1% paraformaldehyde for 30 min, washed twice in permeabilization buffer (PBS with 0.3% saponin, 1% FCS, and 0.1% sodium azide), and stained with the appropriate isotype control or Ab of interest. Abs were diluted in permeabilization buffer and all incubations were of 40-min duration on ice. Cells were resuspended in saponin-free buffer for analysis by flow cytometry (CellQuest software; BD Biosciences). For the analysis of cell surface expression of Notch1, Ab (clone A6) was freshly conjugated to Zenon Alexa Fluor 488 and nonpermeabilized cells stained as per the manufacturer’s instructions (Invitrogen Life Technologies-Molecular Probes). Notch expression was analyzed by flow cytometry.

Isolation of Notch^+^ T cells

A total of 1–2 × 10^7 T cells stimulated for 24 h with anti-CD3-CD28 were washed and resuspended in 1 ml of MagCellct Binding Buffer (R&D Systems) containing 6 µg of anti-Notch1 Ab (clone A6). Cells were incubated for 20 min at 4°C, washed to remove unbound Ab and resuspended in 1 ml of MagCellct Buffer containing an appropriate dilution of goat-anti-mouse Ig-Ferrifluor (R&D Systems) and incubated at 4°C according to the manufacturer’s instructions. Unbound cells isolated after three to four rounds of selection are the Notch-low (Notch^-^) subset and cells bound to beads after two rounds of selection used as the Notch^+^ subset. All subsets were normally divided into two groups for analysis; these include: cells continued in culture for functional assays and a second group used to generate lysates for Western blot analysis.

Manipulations of Notch processing in activated T cells

Stock solutions of GSI-X, GSI-XVII, and GSI-IX in DMSO were obtained commercially (Calbiochem). Activated T cells were cultured in cytokine in the presence of the GSI or the equivalent volume of DMSO. Clone A6 was added in soluble form and purified mouse Ig was used as the control. In experiments with the Notch/Fc, the reagent was coated on wells following the manufacturer’s instructions. The Fas/Fc-chimera from the same company was used as the control for Notch/Fc.

Retroviral infections

Retroviruses pseudotyped with vesicular stomatitis virus G envelope were produced by transient transfection of 293T cells with the pMIG retrovector carrying the gene of interest, and packaging plasmids encoding pCMV-VS5-G and pUVMVC3-gag-pol (Aldevron) using Fugene 6 (Roche Molecular Biochemicals). Media was changed at 24 h and viral supernatant was collected 48 h posttransfection. pMIG-NIC-GFP comprises aa 1759 – 2556 of the human NICD subcloned into the pMIG vector backbone and was collected 48 h posttransfection. pMIG-NIC-GFP comprises aa 1759 – 2556 of the human NICD subcloned into the pMIG vector backbone and was collected 48 h posttransfection. Retroviruses pseudotyped with vesicular stomatitis virus G envelope were produced by transient transfection of 293T cells with the pMIG retrovector carrying the gene of interest, and packaging plasmids encoding pCMV-VS5-G and pUVMVC3-gag-pol (Aldevron) using Fugene 6 (Roche Molecular Biochemicals). Media was changed at 24 h and viral supernatant was collected 48 h posttransfection. pMIG-NIC-GFP comprises aa 1759 – 2556 of the human NICD subcloned into the pMIG vector backbone and was collected 48 h posttransfection. Retroviruses pseudotyped with vesicular stomatitis virus G envelope were produced by transient transfection of 293T cells with the pMIG retrovector carrying the gene of interest, and packaging plasmids encoding pCMV-VS5-G and pUVMVC3-gag-pol (Aldevron) using Fugene 6 (Roche Molecular Biochemicals). Media was changed at 24 h and viral supernatant was collected 48 h posttransfection. pMIG-NIC-GFP comprises aa 1759 – 2556 of the human NICD subcloned into the pMIG vector backbone and was collected 48 h posttransfection. Retroviruses pseudotyped with vesicular stomatitis virus G envelope were produced by transient transfection of 293T cells with the pMIG retrovector carrying the gene of interest, and packaging plasmids encoding pCMV-VS5-G and pUVMVC3-gag-pol (Aldevron) using Fugene 6 (Roche Molecular Biochemicals). Media was changed at 24 h and viral supernatant was collected 48 h posttransfection. pMIG-NIC-GFP comprises aa 1759 – 2556 of the human NICD subcloned into the pMIG vector backbone and was collected 48 h posttransfection. In some experiments with plate-bound anti-CD3 (1 µg/ml, clone 2C11; R&D Systems) and soluble anti-CD28 (5 µg/ml; BD Biosciences). After 48 h, TCB were washed and used in assays or continued in culture with the cytokine IL-2 (R&D Systems). For peptide-stimulated T cells, splenocytes from 8-wk-old p14 TCR-transgenic mice were cultured with 200 ng/ml peptide for 12 h. Cells were washed to remove unbound peptide and cultured in Click’s medium containing 0.5 µM IL-2.

Chemicals and reagents

Abs were from the following sources: Akt, NICH (M20 or C-20), Hes-1 (sc-25392), Bcl-xL, and p38MAPK (Santa Cruz Biotechnology); Akt-phospho serine 473 and phosphorylated substrates of Akt (Cell Signaling Technology); actin, Bax (clone 2C8), Notch-1 (clone A6), and tubulin (Neo-Markers); Jagged1, p64^kDa, BIM, PE-conjugated Notch-1-ICD (clone mNIA), and FITC-conjugated Bcl-2 as well as appropriate isotype controls for the latter two reagents (BD Biosciences); Notch1 cytoplasmic domain (rabbit polyclonal) and P3K (Upstate Biotechnology); Notch-1 C-terminal domain clone C17.9C6 (DSSH); CD25, CD127, CD69, and isotype controls all conjugated to PE and CD28 (eBioscience); anti-Hes-1 (Chemicon International).

For immunoprecipitations, 5–8 × 10^6 cells were lysed using buffer from Pierce Biotechnology supplemented with protease and phosphatase inhibitors. Cell lysates were incubated with relevant Abs (1 µg of Ab/10^6 cells) for 1 h. Immune complexes were precipitated for 2 h at 4°C using SephaLOSE-G plus beads (Pierce Biotechnology). The beads bound to immunoprecipitate were washed, before analysis of the immunoprecipitated proteins. In the Western blot analysis, Abs from Upstate Biotechnology and BD Biosciences were used at concentrations recommended by the manufacturers; clones A6 (NeoMarkers), C-20 and M20 (Santa Cruz Biotechnology) were used at 2 µg/ml, cleaved Notch1 (Cell Signaling Technology) at 1/300, and membranes were incubated for 12–18 h with the Abs.

Results

Notch expression is regulated by TCR-dependent signals

Activation of naïve T cells triggers the induction and processing of Notch1 as indicated by the appearance of proteins recognized by Abs to the Notch C terminus (NCT)/NICD in Western blot analysis. These include reactivity to M-20, C-20, C17.9C6 (Fig. 1A), as well as to an Ab recognizing a specific cleavage product of Notch1 (NCT-Val 1744) (Fig. 1A). Although naïve T cells demonstrate poor reactivity to the aforementioned Abs, they express full-length Notch1 (Fig. 1B). A comparable induction of the NICD also occurs in CD3^+^ T cells activated in vivo following i.v. or i.p. challenge with allogenic spleen cells (Fig. 1C). As reported by other groups, the Notch1 target protein Hes1 is induced when naïve T cells are stimulated to generate activated TCB (Fig. 1D). No changes are apparent in the expression of the ligand Jagged1, which is detected in both naïve and activated T cells (Fig. 1E).

Interfering with Notch in activated T cells triggers ROS-dependent apoptosis

Cytokines such as IL-2 promote the survival of activated T cells. To assess Notch function in T cell survival, reagents that block Notch processing were assessed for the effect on activated T cells. Notch processing is initiated by ligand binding to the Notch ectodomain, which triggers two successive cleavages that release the intracellular domain from its association with the ectodomain (22). The first cleavage is mediated by an extracellular metallo-protease, which in turn facilitates the cleavage and release of the intracellular domain by γ-secretases. Two γ-secretase inhibitors,
FIGURE 1. T cell activation triggers Notch1 expression. A, Detection of NICD by Abs to the NICD in freshly isolated naive (N) T cells (lane 1) or the same preparation activated with plate-bound Ab to CD3 and CD28 for 48 h (lane 2) to generate TCB. The arrow indicates the correct band. B, Expression of the 230-kDa full-length receptor in naive T cells and TCB C. Levels of NICD in CD3⁺ T cells isolated from mice injected i.v. or i.p. with allogenic spleen cells 48 h before harvest and analysis. CD3⁺ T cells from saline injected mice are similar to naive (N) T cells in patterns of Notch1 expression and data from a saline injected (i.v.) group is presented. D, Levels of Hes-1 protein in naive and TCB detected using rabbit polyclonal Abs from two sources, Chemicon International (upper panel) and Santa Cruz Biotechnology (lower panel). The arrow indicates the specific band. E, Expression of the ligand Jagged1 in naive and activated T cells. LC indicates the loading control.

GSI-X (L685,458) or GSI-XVII (WPE-III-31C), and a recombinant rat Notch1-Fc chimera and an Ab to the extracellular domain (clone A6) (19, 23) that would intercept ligand-receptor interactions, were tested. Differences in efficacy notwithstanding, the GSIs and rNotch/Fc triggered an increase in cellular ROS (measured by increased fluorescence of the oxidized form of the dye DHE) as compared with cells treated with IL-2 (Fig. 2A). Consistent with the increase in DHE oxidation, all reagents also compromised the viability of activated CD4⁺ or CD8⁺ T cells (Fig. 2, B–D). In both subsets, treatment with the GSI or the Ab resulted in a loss of processed Notch1 (Fig. 2E), which preceded the induction of apoptotic damage. This was accompanied by a minimal change if any in expression of total Notch1 (Fig. 2E). We also report a concomitant loss of expression of the Notch target Hes1 in activated T cells following treatment with the GSI (Fig. 2F). We tested whether blocking ROS induction ameliorates apoptosis triggered by the disruption of the Notch pathway. MnTBAP, a broad-spectrum antioxidant with catalase and superoxide dismutase activity which blocks mature T cell apoptosis (13, 14, 24), inhibited GSI-X-induced apoptosis or A-NID (Fig. 2G). The loss of processed Notch1 in cells in the condition of A-NID was prevented in cells treated with MnTBAP (Fig. 2H). There was only a small increase noted in cellular levels of Notch protein (Fig. 2H). Similarly, Hes1 is not completely lost in cells treated with MnTBAP (Fig. 2H). Thus, the molecular features of apoptotic damage triggered by reagents that disrupt Notch signaling in activated T cells phenocopies those of T cells undergoing A-NID.

Apoptosis triggered by inhibiting Notch activity is not Fas dependent

ROS are key intermediates in both AICD and A-NID, performing distinct functions in both death pathways. Because ROS are also required for the induction of Fas ligand in AICD (25), we tested whether apoptosis triggered in cells treated with the GSIs culminates in Fas-mediated apoptosis. Two approaches were taken to address this question. First, a murine Fas-Fc chimera that blocks Fas ligand-Fas mediated apoptosis in TCB (data not shown) was tested and was observed to have a marginal effect on GSI-X-induced apoptotic damage (Fig. 3A). Another inhibitor GSI-IX was also tested in these experiments and gave similar results (Fig. 3A). Second, activated T cells were generated from gld/gld mice, which have a point mutation in Fas ligand and the effect of the GSIs tested on activated T cells derived from these mice. Both GSI-X and GSI-XVII triggered an accumulation of ROS as measured by DHE fluorescence (Fig. 3B), and a substantial loss in T cell viability (Fig. 3C). These effects were comparable to those observed in wild-type mice (Fig. 2). These data indicate that Fas receptor signaling plays a minimal role, if at all, in the apoptotic pathway triggered by the disruption of Notch in activated T cells.

Disrupting Notch processing reduces cellular levels of Bcl-xL

In an earlier study, we have shown that ectopically expressed NICD increases cellular levels of Bcl-xL in T cell lines (7). Induction of Bcl-xL also results from signals transmitted via IL-2 in T cells. We tested whether inhibiting Notch processing disrupts Bcl-xL expression in activated CD4⁺ and CD8⁺ T cells. Treatment with GSI-X or GSI-XVII resulted in a loss of Bcl-xL protein (Fig. 4A). Levels of the antiapoptotic protein Bcl2 were also reduced but to a smaller extent (Fig. 4A). Similarly, a loss of Bcl-xL was also observed in cells treated with clone A6 (Fig. 4B).

Notch triggers a PI3K and Akt/PKB-dependent survival pathway in T cell lines (7) and in immature T cells (26). IL-2 activates Akt/PKB, which regulates the expression and function of several proteins implicated in T cell survival (27). We have shown that Notch1 is present in a complex with PI3K and the Src-family non-receptor tyrosine kinase p56Lck in activated T cells and T cell lines (7). We now show that the NICD-Pi3K-p56Lck complex in activated T cells is immunoprecipitated by the T cell costimulatory molecule CD28, which is reported to contribute to the activation of PI3K in T cells (28) (Fig. 4C). Further, CD28 is present in a complex with C-20 (Fig. 4D). Notch1 immunoprecipitated by clone C-20 is recognized by C17.9C6 (Fig. 4D). Activation of Akt/PKB requires phosphorylation of serine 473 and GSI-X-treated CD3⁺ and CD8⁺ cells present a significant reduction in the phosphorylation of the serine 473 residue in Akt/PKB indicating reduced kinase activity (Fig. 4E). This was also confirmed by a loss of phosphorylated substrates of Akt/PKB in GSI-X treated cells, compared with cells cultured in IL-2 alone (Fig. 4F).

IL-2 and IL-7 receptor expression following disruption of Notch

The induction of apoptosis and modulations described in the preceding sections were observed in the continued presence of IL-2, prompting an assessment of the IL-2R α-chain (CD25) expression following interference with Notch signaling. Perhaps owing to differences in the activation state of the T cells and the continued presence of exogenous cytokine, the reduction of CD25 expression observed in activated T cells is not as complete as reported in T cells during primary encounter with Ag (20). However, surface expression of CD25 was consistently reduced in activated T cells.
cultured in IL-2 and concomitantly treated with either GSI-X or GSI-XVII (Fig. 5, A and B).

Although small, the changes in CD25 expression could still account for the observed attenuation of IL-2-mediated survival by the GSI. Therefore, we tested the effect of the GSI in the context of activated T cell survival mediated by the cytokine IL-7. IL-2 and IL-7 share a common c/H9253 chain in their receptor complexes (29) and A-NID is also inhibited by IL-7. The GSIs attenuate the IL-7-mediated survival of activated T cells (Fig. 5C), but cells continue to express substantial amounts of CD127, the a-chain of the IL-7R (Fig. 5, D and E). Thus, although GSI down-modulate CD25 expression even after relatively short exposures, the possibility that Notch signaling converges on other pathways downstream of receptor signaling is likely in these conditions.

Notch expression is modulated following TCR signaling

The experiments thus far indicate that Notch signaling impinges on several aspects of IL-2-mediated signaling and survival in peripheral T cells. However, IL-2 does not up-regulate Notch1 expression in the absence of concomitant signaling via the TCR. Thus,
the levels of NICD were comparable in freshly isolated CD3\(^+\) T cells (Fig. 6A, day 0) and cells cultured for 24 (data not shown) and 48 h in IL-2 (Fig. 6A). NICD is indeed up-regulated if T cells of either the CD4\(^+\) or CD8\(^+\) subset are activated using CD3 and CD28 (Fig. 6B and C). Cell surface Notch1 is undetectable on naive T cells (data not shown) but the expression is increased following stimulation with CD3-CD28 and is comparable in CD4\(^+\) and CD8\(^+\) T cells across multiple experiments (Fig. 6D). The expression of Notch on T cells following their activation has not been characterized and since activated T cells are destined to undergo apoptosis, we hypothesized that the pathway will not be sustained in activated T cells for extended durations. In agreement with this, flow cytometric analysis of cell surface Notch on activated T cells revealed heterogeneity emerging in the pattern of Notch expression following the discontinuation of Ag receptor stimulation, with only a subset of activated cells expressing higher levels of the receptor following culture in IL-2 (Fig. 6D).

To confirm the modulation of Notch expression observed in polyclonally stimulated T cells, we extended the analysis to peptide-stimulated CD8\(^+\) transgenic T cells from P14-transgenic mice, which recognize the lymphocytic choriomeningitis virus Db-gp33 epitope. Closely paralleling the pattern of polyclonally stimulated T cells (data not shown), cell surface Notch1 is induced in CD8\(^+\) T cells stimulated by peptide using established protocols (14). Following stimulation with antigenic peptide, there is gradual increase in the number of cells positive for cell surface Notch1 (Fig. 6E). Subsequent to this increase, Notch1 expression is down-regulated by 72 h and is detected only on a comparatively small subset (Fig. 6E). In summary, Notch1 expression is modulated following Ag receptor signaling and is not retained on the surface of all activated T cells.

**Notch\(^+\) cells are resistant to apoptotic stressors**

Receptivity to Notch signaling is determined by the expression of the receptor on the cell surface. Because T cells express Notch ligands, the Notch\(^+\) subset offers a system to study Notch signaling in a more physiological context wherein conventional activation of Notch is possible. Therefore, we isolated and analyzed the Notch\(^+\) subset for survival capabilities. Using an Ab to extracellular Notch (clone A6), T cells expressing cell surface Notch (Notch\(^+\)) were isolated 24–36 h poststimulation. The expression of cell surface Notch1 in Notch-low (Notch\(^-\)) cells is clearly lower than unfraccionated (UF) or Notch\(^+\) cells (Fig. 7A). However, both subsets express comparable levels of activation Ags such as CD25, CD69, and Sca1 (data not shown). In comparison to Notch\(^-\) cells, the Notch\(^+\) subset demonstrates improved survival in an assay of A-NID (Fig. 7B), and apoptosis induced by oxidative stress i.e., exogenously added hydrogen peroxide (Fig. 7C). Apoptosis triggered by a range of concentrations of the synthetic corticosteroid dexamethasone is also substantially lower in the Notch\(^+\) subset (Fig. 7D). Notch\(^+\) cells are not generally resistant to cell death, as susceptibility to apoptosis triggered by the genotoxic drug etoposide or staurosporine is unchanged (data not shown). To assess reactivation responses of cells isolated by this protocol, the following analysis was undertaken. Following a 7-day rest phase in low dose IL-2, restimulation via the TCR resulted in an induction (relative to the unstimulated cells) of CD25 and CD69 in the Notch\(^+\) and the UF/Notch\(^+\) subsets as compared with cells cultured in IL-2 alone (Fig. 7E). Furthermore, consistent with their improved survival and expression of Notch1, NICD is detected in Notch\(^+\) cells even when cultured for 48 h in the absence of cytokine (Fig. 7F). Expectedly, Notch\(^-\) cells present a dramatic loss of this species when cultured in the absence of IL-2 (Fig. 7F). Analysis of Bcl-2 family proteins showed that expression of Bcl-2 is not substantially different between subsets (Fig. 7G). However, Notch\(^+\) cells express substantially high levels of Bcl-x\(_L\) (Fig. 7H). Unexpectedly, the expression of the proapoptotic protein BIM is reduced in this subset. The expression of Bax is comparable in both groups (Fig. 7H). Similarly, Notch\(^-\)-activated subsets of p14 T cells are also enriched for the expression of Bcl-x\(_L\) (Fig. 7I).
Overexpression of activated Notch1 in T cells

The experiments thus far are consistent with a protective role for Notch in A-NID in T cells. To test this more directly and analyze the specificity of the reagents used, we constitutively expressed activated human NIC using a retroviral expression system in activated T cells. Activated T cells infected with pMIG-NIC-RES-GFP or pMIG-RES-GFP were tested for susceptibility to apoptosis induced by the GSI. Apoptotic damage triggered by GSI-X (at 20 and 10 μM) and GSI-XVII (at 15 and 10 μM) was significantly lower ($p < 0.005$) in cells expressing NIC as compared with cells infected with GFP alone (Fig. 8A). Subsequently, T cells infected with pMIG-NIC-RES-GFP or pMIG-RES-GFP were assessed for the induction of apoptotic nuclear damage in conditions of A-NID. Induction of neglect-induced death was lower in activated T cells expressing the NICD cultured in the absence of IL-2 as compared with cells transfected with GFP (Fig. 8B). When the difference in induction of apoptotic damage relative to the IL-2-treated group is considered, cells expressing NIC are significantly more protected ($p < 0.005$) from apoptosis triggered by IL-2 withdrawal than cells expressing GFP alone.

**Discussion**

Cytokine deprivation-induced apoptosis is characterized by irreversible changes in cellular metabolism triggered by reduced nutrient uptake and a decreased rate of cellular glycolysis. This process is negatively regulated by Bcl-2 family proteins, which promote mitochondrial integrity and by protein kinases that regulate the uptake and assimilation of nutrients (30, 31). In this study, we describe a role for the Notch pathway in the cytokine-dependent survival of activated T cells. A Notch1/Fc chimera or an Ab to Notch1 that interferes with Notch-ligand interactions and chemical inhibitors of β-secretase that disrupt Notch processing, implicate Notch signaling in T cell survival. The antiapoptotic function suggested by these experiments was substantiated by the ectopic expression of an active form of Notch1 that protected activated T cells from A-NID. Furthermore, the consequences of Notch signaling in activated T cells are manifested in elevated levels of the antiapoptotic protein Bcl-xL and enhanced Akt/PKB activity.

Cell-cell interactions regulating proliferation, survival, fate specification, and differentiation are critical at many stages in development and the Notch pathway is implicated in these events in flies, worms, and mammals (1–3). Notch1 regulates commitment to the T cell lineage (32) and the survival of pre-T cells following β-selection (26). Mature T cells express Notch receptors and their ligands, which renders them susceptible to regulation via this pathway. Thus, the role of the Notch pathway in mature T cell function is being investigated by several groups. Both inhibitory and activating functions have been attributed to Notch in the context of peripheral T cell proliferation (reviewed in Ref. 33). Notch signaling is implicated in the acquisition of effector function by mature T cells (34–38) and interactions between Notch and other transcription factors have been demonstrated to regulate the TH1 or TH2 polarization of mature T cells (34, 37). In this context, it is noteworthy that the conditional deletion of Notch1 in mature T cells indicated that there is redundancy in Notch-receptor signaling during T cell differentiation (39). Furthermore, in peripheral T cells, Notch receptor engagement concomitant with Ag-receptor stimulation is tolerogenic to T cells (19, 36). Consequently, the expectation from these studies that the pathway is stringently regulated in activated T cells, is validated by the observed down-regulation of cell surface Notch1 that occurs in T cells following their activation (Fig. 6). Another member of the family, Notch-3, is implicated in the generation of regulatory T cells (40). More recently, regulatory T cells have been reported to exert immunosuppressive effects by the activation of the Notch pathway in target cells (41). Thus, the Notch pathway influences diverse functions in the T cell lineage.

In the immune response to infection, Ag-receptor engagement triggers multiple rounds of cell division, resulting in a dramatic increase in effector T cells. Ag clearance is accompanied by the deletion of a majority of activated cells and the emergence of an Ag-specific, memory pool capable of extended survival in the absence of Ag (42). The requirement for memory T cells to transit through an activated effector phase before differentiation is unresolved (43, 44) and may not be equivalent for CD4 and CD8 T cell subsets. Nonetheless, surviving the contraction phase of the immune response is necessary for the generation of memory and the Notch pathway may be critical for the transition through this survival checkpoint. Emerging data indicates that ROS-mediated postactivation apoptosis is a key event defining this checkpoint that cells must overcome before differentiation into memory (13, 14, 45). Thus, inhibitors of ROS block activated T cell death (13) and cause an increase in circulating pools of memory cells in vivo (14). In our experiments, both the GSIs and the Notch/Fc chimera trigger accumulation of ROS and consequent loss of viability, suggesting that Notch can negatively regulate cellular ROS, a key intermediate in the deletion of Ag-reactive T cells. An earlier study has reported that cells treated with MnTBAP accumulate Notch
transcripts (13). We find that the inhibition of ROS by a broad-spectrum antioxidant stabilizes the expression of NICD although we do not observe a substantial difference in the expression of Notch protein in these cells. Although regulation of Notch expression by ROS may have a transcriptional component, the accumulation of ROS following disruption of Notch signaling may not require new gene expression. These data suggest interactions between cellular ROS and the Notch pathway and possible feedback circuits regulating outcomes, although the mechanism(s) are yet to be elucidated. In earlier studies, mutations in Notch have been linked to mitochondrial dysfunction in humans and Notch has also been reported to regulate expression of mitochondrial enzymes in

FIGURE 6. Distribution of Notch in activated T cells in culture. A, Flow cytometric analysis of freshly isolated T cells (left panel) and cells cultured for 48 h with 20 U/ml IL-2 (right panel), permeabilized, and stained with a PE-conjugated Ab (clone mNIA) to the NICD (black histogram). B and C, NICD expression (black histogram) in CD3+ T cells (B) or CD4+ or CD8+ T cells (C) cultured for 48 h with CD3-CD28. In A and B, gray histograms and in C the open histograms represent staining with an isotype-matched Ab. D, Cell surface expression of Notch-1 (clone A6) in CD4+ or CD8+ T cells stimulated with CD3-CD28 for 48 h. The filled histogram represents staining with A6. Data shown are representative of two to three experiments. E, Cell surface expression of Notch-1 (clone A6) in naive T cells stimulated with CD3-CD28 for 48 h, followed by culture with IL-2 for an additional 2 days. Upper panel, staining with an isotype-matched control. NECD indicates Notch extracellular domain. F, Splenocytes from p14-TCR-transgenic mice were stimulated (200 ng/ml peptide) in vitro for 12 h and continued in culture without peptide. Cells were stained for Thy 1.2 and gated cells analyzed for cell surface Notch1 by flow cytometry. Panels on the left are isotype controls. The results in all panels represent three to four independent experiments.
Furthermore, a more recent study has shown that hypoxia modulates NIC turnover and Notch activity (48). Notch processing is closely allied to TCR stimulation and is not observed in naive T cells cultured with IL-2 or IL-7 (data not shown). Thus, the Notch pathway may offer a means of regulating effector T cell survival without compromising the deletion of autoreactive or inappropriately activated T cells.

Enhanced cellular levels of the antiapoptotic protein Bcl-xL also marks the subset that stabilizes cell surface Notch expression. Conversely, perturbation of Notch signaling by the GSI in activated T cells results in a substantial reduction in cellular levels of Bcl-xL, which cannot be overcome by IL-2. Because IL-2 sustains Bcl-xL expression and promotes T cell survival, it may be argued that the observed effects of Notch are a reflection of its regulation of IL-2R expression. Although a small but consistent reduction in levels of IL-2R expression is indeed observed, the GSIs do not modulate the expression of the IL-7R although they regulate both IL-2- and IL-7-mediated survival. Without excluding the possibility that the regulation of IL-2R expression is a component of Notch-dependent survival, we expect that Notch signaling in activated T cells likely intersects with multiple signaling cascades in T cells.

We extend earlier data to show that in activated T cells the Notch1-PI3K-p56lck complex includes CD28, which is a key molecule for T cell survival and the development of effectors (28) and

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**FIGURE 7.** Characterization of Notch$^+$ T cells. A, UF, Notch$^+$, and Notch$^-$ activated T cells were stained (filled histogram) for cell surface expression of Notch-1 (clone A6). B, Apoptotic damage in Notch$^+$ and Notch$^-$ activated T cells cultured for 18 h in the presence (●) or absence (□) of IL-2. C, Apoptotic damage in Notch$^+$ and Notch$^-$ activated T cells cultured for 18 h with 50 μM H$_2$O$_2$ (●). D, Apoptotic damage in Notch$^+$ and Notch$^-$ activated T cells cultured for 10 h with dexamethasone. E, Notch$^+$ and Notch$^-$ cells were cultured for 5 days in low levels of IL-2. Cells were continued as such or restimulated for 8 h with beads coated with Abs to CD3-CD28 and stained for CD25 and CD69. The histograms (open: isotype control; filled: CD69; open gray: CD25) represent expression profiles of stimulated cells. The MFI for cells cultured in IL-2 alone or IL-2 and CD3-CD28 are shown in the graph. A representative experiment of two separate analysis is shown. F, Cell lysates of Notch$^+$ or Notch$^-$ cells cultured for 48 h without IL-2 and levels of NICD (C17.9C6) assessed by Western blot analysis. G, Notch$^+$ and Notch$^-$ T cells were permeabilized and stained for Bcl-2 expression by flow cytometry. The open histogram indicates the isotype control. H, Cell lysates derived from Notch$^+$ and Notch$^-$ activated T cells were analyzed for the levels of indicated proteins by Western blot analysis. I, Cell lysates of Notch$^+$ and Notch$^-$ cells isolated 24 h after peptide stimulation of p14-transgenic T cells analyzed for the expression of Bcl-2 and Bcl-xL. The Student t test was used as the test of significance (*, p < 0.05).

**FIGURE 8.** Ectopic expression of NIC protects activated T cells from GSI-induced apoptosis and A-NID. A, Activated T cells expressing pMIG-GFP or pMIG-NIC-GFP were cultured with IL-2 in the presence of the indicated concentrations of GSI-X and GSI-XVII. After 18 h, apoptotic nuclear damage was assessed as described in Materials and Methods. B, Activated T cells expressing pMIG-GFP or pMIG-NIC-GFP were cultured in the presence or absence of IL-2. After 18 h, apoptotic nuclear damage was assessed in the different groups as described in Materials and Methods. The data presented are derived from five independent infections. Differences between various groups have been analyzed using the Student t test (**, p < 0.05).
confirm that the Notch pathway converges on the activation of Akt/PKB (Fig. 4). Although we report on Akt/PKB signaling, it should be noted that the possibility of Notch interacting with other signaling pathways that regulate cell survival (30, 49, 50) is yet to be examined. In agreement with our earlier study and the data presented here, Zúñiga-Pflücker and colleague (26) have reported a positive interaction between Notch and the Akt/PKB pathway. The activation of Akt/PKB favors the argument that Notch signaling can modulate nutrient uptake to maintain bioenergetic homeostasis (29, 30). Indeed, Notch-ligand interactions have been shown to maintain glucose transport and metabolism in double-negative thymocytes (26). Whether these outcomes reflect a transcriptional function of Notch or interactions with membrane-localized receptor complexes (27–29) is, as yet, unclear.

One mechanism by which this or similar interactions may be effected is via associations with receptors for cytokines. In activated T cells, disruption of Notch signaling compromises both IL-2- and IL-7-mediated survival. The receptors for these cytokines, along with some others, contain a \( \gamma_c \) chain in their receptor complexes, which is implicated in the activation of JAK-STAT, PI3K, and Ras (MAPK) pathways (29). Despite considerable overlap in signaling pathways, cytokines trigger distinct outcomes because of the differential regulation of receptor expression and because the \( \gamma_c \) functions in association with other (IL-7R, IL-2R, or IL-2R\( \beta \) ) receptor chains (29, 49). In the absence of clear evidence of a physical association with cytokine receptor components, the possibility that Notch signaling independently converges on common intermediates cannot be excluded. A recent report of Notch localization in the immunological synapse in CD4\( ^+ \) T cells (51) is consistent with Notch functioning as a component of the program that regulates activation and survival of effector T cells. Thus, we propose that in addition to the functions described for Notch in promoting Ag-dependent T cell proliferation and differentiation, Notch augments immune function by modulating cytokine-dependent survival of Ag-stimulated T cells.

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**Disclosures**

The authors have no financial conflict of interest.

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