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Cutting Edge: Distinct TCR- and CD28-Derived Signals Regulate CD95L, Bcl-xL, and the Survival of Primary T Cells

Andreas Kerstan and Thomas Hünig

TCR-driven clonal expansion of T cells is limited by activation-induced cell death through CD95/CD95L interactions. This cell-contact dependent mechanism is attenuated by costimulation through CD28. Here, we show that primary rat lymph node T cells activated by “superagonistic” CD28-specific mAb, which do not require TCR-engagement for full T cell activation, do not up-regulate CD95L. CD28 superagonist activated T cells are highly resistant to artificial CD95 cross-linking, and display a marked up-regulation of the survival factor Bcl-xL. Consistently, NF-κB factors, known to promote Bcl-xL transcription, are strongly activated by superagonistic CD28 mAb stimulation. In contrast, a weaker induction of NFAT, which positively regulates the CD95L gene, in CD28 activated cells as compared with TCR- or TCR/CD28-stimulated cells was observed. Thus, by recruiting the mitogenic activity of CD28 in the absence of TCR engagement, the anti-apoptotic signals provided by costimulation are revealed without interfering proapoptotic effects induced by TCR stimulation. The Journal of Immunology, 2004, 172: 1341–1345.

Activation-induced cell death (AICD) is an important mechanism in the termination of immune responses. Experimentally, this is visualized by restimulation of activated T cells through the TCR which results in apoptosis mediated primarily by the interaction of the death receptor CD95 with its ligand (CD95L) expressed after T cell activation (1–4). In most systems studied, induction of CD95L in response to TCR stimulation is not sufficient to induce AICD in resting CD95 expressing T cells (5–7). Rather, “fratricide” via CD95 only proceeds once susceptibility to CD95-mediated apoptosis has been acquired during the course of T cell activation. Recently, however, Kishimoto and Sprent (8) have demonstrated that strong TCR ligation can induce AICD also in naive murine CD4 T cells, provided that cells expressing costimulatory ligands are rigorously removed.

Costimulation via CD28 does not only support T cell proliferation but also T cell survival (9). CD28-mediated signaling counteracts AICD intracellularly by increasing the expression of anti-apoptotic molecules, most prominently of Bcl-xL (9) which interferes with the depolarization of mitochondria and thus the type II death pathway which acts as an amplification loop in lymphocytes (10). Furthermore, CD28 signals suppress the TCR-induced up-regulation of the CD95L required for CD95 mediated fratricide (11).

Because normally, CD28 stimulation per se does not evoke a cellular response in primary resting T cells, the anti-apoptotic effects of CD28-derived signals are generally studied by comparing costimulation with stimulation through the TCR alone (12). A key problem with this approach is the different degree of activation achieved by stimulation only via the TCR (partial activation without proliferation), and via the TCR and CD28 (full activation), making it difficult to distinguish between anti-apoptotic effects secondary to proliferation vs the differential regulation of pro- and anti-apoptotic factors by the two activating signals. Furthermore, this approach does not allow the analysis of CD28-derived anti-apoptotic signals in the absence of concomitant TCR signals which may complicate the picture.

We have, therefore, followed up these studies by using a system in which the effect of fully activating CD28 signals on AICD can be studied without interfering TCR signals. We used mitogenic or “superagonistic” CD28 mAb which induce proliferation of all primary resting T cells without TCR engagement both in vivo and in vitro (13). T cell activation by superagonistic anti-CD28 mAb occurs without an increase in ZAP-70, TCR-ζ, or LAT phosphorylation (14), and hence without stimulation of the TCR complex.

In the present communication, we have compared AICD of freshly isolated rat T cells stimulated with anti-TCR, anti-TCR plus conventional anti-CD28 (costimulation), or with superagonistic mAb to CD28. We demonstrate that TCR-only stimulated cells undergo CD95-dependent “fratricide” which is attenuated by costimulation at the levels of 1) CD95L expression and 2) sensitivity to CD95 cross-linking. Importantly, T cells...
fully activated by CD28 superagonists are protected from fratricide at both levels, suggesting that in costimulation, TCR-derived signals do not contribute to but rather counteract the anti-apoptotic effect of CD28 ligation.

**Materials and Methods**

*Abs and reagents*

mAb to rat αβTCR (R73, IgG1), conventional (JJ319, IgG1) and mitogenic (JJ316, IgG1) mAb to rat CD28 have been previously described (13). Polyclonal Ab to Bcl-xL (M-125), β-actin (C-11), USF-2 (C-20), and NF-κB e-Rel (N), p65 (A) and p50 (NLS) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-bcl-2 (10C4) was from Biocarta (Hamburg, Germany), mouse anti-NFATc1 (7A6) from Alexis (Grüneberg, Germany). Rabbit polyclonal Ab to NFATc2 were kindly provided by A. Rao (University of Boston, MA). Sheep anti-mouse IgG (ShMilg) was from Boehringer Mannheim (Mannheim, Germany), and goat anti-mouse IgG-peroxidase as well as goat anti-rabbit IgG-peroxidase were from Dianova (Hamburg, Germany).

**Stimulation of rat T cells**

Freshly isolated lymph node T cells from 6- to 8-wk-old LEW rats kept under pathogen-free conditions were obtained by nylon wool passage. In all experiments performed purity of T cells was >95% and cells were stimulated at a cell density of 7.5 × 10^4 cells/ml (unless otherwise indicated) in supplemented X-VIVO medium. Experiments shown in Figs. 1 and 2 were performed in 96-well plates, whereas 9 cm plastic dishes (Figs. 3A and 4) and 6-well plates (Fig. 3B) were used for large scale preparations.

For TCR and TCR/CD28 costimulation, cells were incubated on plastic dishes (Greiner, Frickenhausen, Germany) precoated with ShMilg as described (14) followed by 2 μg/ml anti-TCR mAb (R73), without or with 0.5 μg/ml soluble conventional anti-CD28 mAb (JJ319). For stimulation with anti-CD28 Abs alone, cells were cultured on ShMilg-coated plates in the presence of 5 μg/ml soluble superagonistic (JJ316) or conventional (JJ319) anti-CD28 mAb. Proliferation was determined by pulsing triplicate cultures with [3H]thymidine (0.5 μCi/ml; Amersham, Braunschweig, Germany) 6 h before harvesting.

**Induction, prevention, and detection of apoptosis**

To determine CD95-dependent apoptosis, T cells were cultured in the presence of a neutralizing CD95L mAb (MFL4; BD PharMingen, San Diego, CA). Sensitivity to CD95-mediated apoptosis was assessed by the addition of a soluble CD95L-Flag-tag fusion protein (Alexis) which was cross-linked via an anti-Flag mAb (Enhancer; Alexis) for the last 6 h of culture. Cells were harvested and annexin V binding buffer was added (0.01M HEPES, pH 7.4, 0.14M NaCl, 2.5 mM CaCl₂) containing FITC-labeled annexin V and 7-aminoactinomycin D (7-AAD). After 15 min at 4°C in the dark, samples were diluted in buffer and immediately analyzed in a FACS Calibur flow cytometer (BD Biosciences, Mountain View, CA). Results shown in Fig. 1 were obtained with 7-AAD only.

**IL-2 ELISA**

IL-2 was detected using the OptEIA rat IL-2 set from BD PharMingen according to the manufacturer’s instructions.

**Western blot**

Cells were washed twice in PBS and resuspended in lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, pH 7.9, 50 mM β-glycerolphosphate, 1% Triton X-100, 10% glycerol, 50 mM NaF, 0.04% NaN₃, 1 mM DTT, 1 mM Na₃VO₄, 2 μM leupeptin, 1 mM pefabloc). Cells were kept on ice for 15 min and centrifuged at 12,000 × g for 20 min. Lysates from 10⁶ cells per lane were separated by SDS-10%-PAGE, transferred to nitrocellulose membranes (Hybond; Amersham), and subjected to immunoblotting using the indicated Abs. Preparation and analysis of nuclear extracts were conducted as previously described (14).

**RNase protection assay**

Total RNA was extracted using TRRozl reagent (Life Technologies, Gaithersburg, MD) and processed using BD PharMingen’s RPA system (rat APO-1) according to the manufacturer’s instructions. Membranes were sealed in saran wrap and image data collected with a PhosphoImager (Fuji Photo, Düsseldorf, Germany).

**Results and Discussion**

**Omission of the TCR signal in CD28 driven proliferation increases viability of primary T cells**

To evaluate the impact of TCR and CD28 signals during T cell activation on the development of cell-contact dependent apoptosis (fratricide), we initially compared the viability of freshly isolated rat lymph node T cells after TCR-stimulation, costimulation, and activation with superagonistic CD28-specific mAb. Cultures were set up at various cell densities, and apoptosis was assessed after 24 h by 7-AAD staining and flow cytometry (Fig. 1A). When T cells were cultured at high but not at low cell densities, TCR ligation induced a >2-fold increase in 7-AAD-permeable cells relative to cells in medium alone.

Costimulation by TCR/CD28 moderately reduced cell density-dependent apoptosis compared with TCR stimulation alone. In marked contrast, stimulation of T cells through superagonistic CD28 mAb without TCR engagement completely failed to cause cell death at all cell densities tested. In fact, T cells thus activated contained fewer dead cells than cells left in medium alone; results obtained with conventional CD28 mAb, which is highly effective in costimulating TCR-triggered responses but unable to induce T cell proliferation by itself, were indistinguishable from medium controls.

One explanation for the enhanced survival of superagonistic CD28 activated compared with costimulated T cells could be a difference in their rates of proliferation. As shown in Fig. 1B

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**FIGURE 1.** A. Apoptosis of activated primary T cells. Purified peripheral rat T cells were stimulated at various cell-densities ranging from 0.1 to 2 million cells/ml (see inset) with the indicated stimulating mAb for 24 h. Cells were harvested and percentage of apoptotic cells was determined by 7-AAD staining and FACS analysis. B. Proliferative responses. T cells (7.5 × 10⁴/ml) were stimulated with the indicated mAb for 24 h and pulsed with [3H]thymidine for the last 6 h of culture. Mean cpm of triplicate wells ± SEM are shown. C. IL-2 secretion. Cells were stimulated as in B. At 24 h, supernatants were assayed for IL-2 by ELISA.
and in confirmation of our earlier results (13), the proliferative response of TCR/CD28 costimulated and superagonistic CD28 mAb-activated T cells was indistinguishable as assessed by [3H]thymidine incorporation, and high levels of IL-2 were produced under both conditions (Fig. 1C). Furthermore, although DNA synthesis had been fully initiated in costimulated and CD28 superagonist-activated cultures, cell division had not yet occurred at the time point when cell death was evaluated (24 h; data not shown), excluding a contribution of newly generated cells to the viable population. These findings indicate that T cells fully activated through superagonistic anti-CD28 mAb, i.e., without a TCR signal, are less prone to cell-contact-dependent cell death than TCR/CD28 costimulated cells.

Omission of the TCR signal in CD28 driven proliferation reduces both the ability to induce and the susceptibility to undergo apoptosis via CD95

Because TCR- and TCR/CD28-stimulation induced apoptosis only at high cell densities (see Fig. 1A), and cell-contact-dependent AICD in T cells is attributed primarily to CD95/CD95L interactions (1–4), we investigated the contribution of this receptor-ligand pair to the loss of viable cells. Purified T cells were stimulated for 24 h in the absence or presence of a blocking CD95L-specific mAb, thereby preventing CD95L/CD95 interactions during culture. In parallel, the sensitivity of cultured T cells to CD95-mediated cell death was determined by artificially cross-linking CD95 with a soluble recombinant ligand for the last 6 h of culture.

As shown in Fig. 2, neither CD95L blockade nor CD95 cross-linking significantly modified the frequency of apoptotic cells in unstimulated cultures, confirming earlier data by others (5). In contrast, continuous CD95L blockade completely prevented cell death induced by TCR stimulation. Moreover, TCR-stimulated cells were highly sensitive to apoptosis-induction by CD95 ligation.

When TCR/CD28 costimulated cells were assayed (Fig. 2), inclusion of the blocking CD95L-specific mAb greatly improved the viability of the recovered cells, indicating that also under these conditions of activation, the cell density dependent death of activated T cells is mediated by CD95/CD95L interactions. In addition, artificial CD95 triggering efficiently induced apoptosis in these proliferating cells, showing their sensitivity to CD95-mediated cell death.

Finally, and in line with their survival even at high cell densities (Fig. 1A), T cells activated by superagonistic CD28-specific mAb showed no intrinsic CD95L-mediated apoptosis. Similarly to costimulated cultures, however, they exhibited some susceptibility to artificial CD95 cross-linking. Consistently, this sensitivity to CD95-mediated induction of apoptosis was lower than in costimulated cultures.

Taken together, T cells activated via the TCR only proliferate poorly, kill each other efficiently via CD95/CD95L ligand interactions, and are highly sensitive to artificial CD95 cross-linking. TCR/CD28 costimulated cells and T cells activated via CD28 superagonists proliferate at the same high rate, but the omission of the TCR signal results in a reduction in CD95L-mediated fratricide and an increased apoptosis resistance to artificial CD95 ligation.

Differential regulation of pro- and anti-apoptotic molecules by TCR and CD28 stimulation

Several studies have shown that the CD95L is up-regulated after TCR stimulation (1–3), and that costimulation via CD28 can prevent this effect (11). Therefore, we compared the levels of CD95L mRNA over time in T cells activated by TCR-stimulation alone, by TCR/CD28 costimulation, and by CD28 superagonist, relative to the expression of a household gene, L32. As shown in Fig. 3A, TCR stimulation resulted in a robust induction of CD95L mRNA which persisted at high levels throughout the culture period. Additional stimulation of CD28 partially prevented this up-regulation. Importantly, even lower levels of CD95L mRNA were found in T cells proliferating in response to superagonistic CD28 signals without TCR stimulation.

The low levels of CD95L mRNA in T cells stimulated for 24 h by costimulation or with superagonistic CD28 mAb provide an explanation for the lower levels of contact dependent cell death compared with that in TCR-stimulated cells (Figs. 1 and 2). In addition, however, there was also a gradient in sensitivity to CD95-mediated apoptosis ranging from very high in TCR-only stimulated, via intermediate in costimulated, to low in CD28 superagonist activated T cells (Fig. 2). These differences were not due to differences in CD95 expression, which was actually highest in T cells activated by the most “protective” protocol, i.e., stimulation with superagonistic anti-CD28 mAb (Fig. 3A).

Accordingly, the expression of the anti-apoptotic factor Bcl-xL, known to interfere with CD95-mediated cell death in lymphocytes, was also investigated. As previously described by others (9), Bcl-xL was poorly expressed in unstimulated or TCR-only activated T cells, but up-regulated by costimulation via CD28 at both the mRNA and protein levels (Fig. 3). Furthermore, Bcl-xL induction was even more pronounced in T cells activated by CD28 superagonists. In contrast, Bcl-2, an anti-apoptotic protein related to Bcl-xL, was constitutively expressed in unstimulated or TCR-stimulated T cells activated by CD28 superagonists. In contrast, Bcl-2, an anti-apoptotic protein related to Bcl-xL, was constitutively expressed and not further up-regulated by stimulation, in agreement with observations by others (9, 15).

In summary, these results suggest that the superior resistance to CD95-triggered apoptosis in T cells activated by superagonistic CD28 mAb stimulation as compared with TCR/CD28 costimulation may be due to a more effective up-regulation of anti-apoptotic molecules, in particular of Bcl-xL, in the absence of a TCR signal.
Differential regulation of NFAT and NF-κB by TCR- and CD28-derived signals

It was recently shown that NFAT factors positively regulate the CD95L promoter (16–18). Therefore, we next investigated whether diminished CD95L mRNA expression observed after CD28 costimulation (Fig. 3A) correlated with reduced activation of NFAT factors in our experimental system. To this end, we compared nuclear levels of NFATc1 (NFATc) and c2 (NFATp), in T cells activated by TCR-stimulation alone, by TCR/CD28 costimulation and by CD28 superagonists over a time course of 48 h. As expected, TCR stimulation led to a strong induction and accumulation of the nuclear NFATc1 isoform, NFATc1/A (19), which persisted up to 48 h of stimulation. Additional stimulation via CD28 (costimulation) also resulted in a strong initial induction of NFATc1 which was, however, followed by a decline between 24 and 48 h of culture. Strikingly, nuclear NFATc1 expression was much lower in T cells activated by CD28 superagonists throughout the culture period, and was barely detectable after 2 days (Fig. 4).

In line with other reports (20), TCR-induced activation of nuclear NFATc2 (DeP-NFATc2) was very short lived. No differences were observed between the three modes of T cell activation used. Corresponding results to those presented here by Western blotting were also obtained using EMSAs (data not shown).

This suggests that in contrast to NFATc2, the induction and nuclear accumulation of NFATc1/A is TCR-dependent and negatively regulated by costimulation via CD28. How CD28 signals act on the proposed autoregulatory loop of NFATc1/A induction (21) is presently unknown. In any event, the levels of NFATc1 activation in the three groups correlate with the levels of CD95L mRNA (Fig. 3) and the degree of apoptosis mediated by endogenous CD95L (Fig. 2), and could thus contribute to the opposing effects of TCR and CD28 signals on contact-dependent cell death (Fig. 1A). Of note, the failure of the CD28 superagonist to efficiently induce nuclear accumulation of NFATc1 while promoting optimal IL-2 synthesis (Fig. 1C) is consistent with the unimpaired capacity of NFATc1-deficient mice to produce this cytokine (22).

Several NF-/NF-κB response elements have recently been identified in the human and murine Bcl-xL promoters (23–25). Accordingly, we examined whether enhanced expression of Bcl-xL observed after TCR/CD28 costimulation or superagonistic CD28 mAb stimulation (Fig. 3) correlated with the extent of NF-/NF-κB activation. Western blotting of nuclear extracts for c-Rel, p65 and p50 demonstrated comparable and sustained activation of these components after TCR/CD28 costimulation and superagonistic CD28 mAb stimulation (Fig. 3) correlated with the extent of NF-κB activation. Western blotting of nuclear extracts for c-Rel, p65 and p50 demonstrated comparable and sustained activation of these components after TCR/CD28 costimulation and superagonistic CD28 mAb stimulation (Fig. 3) correlated with the extent of NF-κB activation. Western blotting of nuclear extracts for c-Rel, p65 and p50 demonstrated comparable and sustained activation of these components after TCR/CD28 costimulation and superagonistic CD28 mAb stimulation (Fig. 3).

In contrast, stimulation with conventional CD28 mAb did not induce NF-/NF-κB, and TCR stimulation induced only a low level of nuclear NF-/NF-κB. These results confirm and extend our earlier results obtained in short-term assays by EMSA and Western blot regarding the NF-/NF-κB components p50 and p65 (14, 26).

Taken together, these data correlate the effects of TCR and CD28 signals on CD95L and Bcl-xL expression with the activation of two key transcription factors positively regulating these genes, i.e., NFAT and NF-κB, respectively. We therefore speculate that these transcription factors play a pivotal role in the apoptosis-promoting effects of isolated TCR signals, and the protective effects of costimulation by CD28.
In conclusion, we have compared cell-contact mediated and artificially induced apoptosis via the death receptor CD95 in primary T cells activated through the TCR alone, by TCR/CD28 costimulation, or with superagonistic CD28-specific mAb, and observed opposing roles of these two key cell surface receptors in clonal contraction via AICD. Our results show that in the absence of TCR ligation, CD28-mediated T cell activation does not induce CD95L-mediated fratricide, and indeed only minimal transcription of the CD95L gene. In contrast, the anti-apoptotic factor Bcl-xL is more efficiently induced. These effects confirm previous results obtained with conventionally costimulated cells and extend them with regard to the contribution of TCR signals. Thus, NF-κB factors are activated in a CD28-dependent fashion in conventional systems of costimulation (27), and are known to promote Bcl-xL expression (23–25); here we show that both effects occur in the absence of TCR stimulation and thus can be mapped to the signaling cascade emanating from CD28. In contrast, NFAT factors promote CD95L transcription, and both a reduction of CD95L transcripts and nuclear NFAT were observed when T cells were activated via CD28 superagonists.

In physiological T cell activation, the relative contribution of TCR-derived and costimulatory signals varies depending on TCR affinity and the availability of cognate MHC/peptide complexes on the one, and of costimulatory ligands on the other hand. Although T cell stimulation by CD28 superagonists does not address the proximal TCR signaling machinery (14), it is dependent on “tonic” TCR signals (K. Dennehy, T. Hünig, unpublished observations) and thus may mimic one end of this spectrum, i.e., optimal costimulation in the presence of a minimal contribution of the TCR which is required to enable CD28 function.

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