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Critical Role of TNF Receptor Type-2 (p75) as a Costimulator for IL-2 Induction and T Cell Survival: A Functional Link to CD28

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CD28 provides important signals that lower the threshold of T cell activation, augment the production of IL-2, and promote T cell survival. The recent identification of a second family of costimulatory molecules within the TNFR family has reshaped the “two-signal” model of T cell activation. In this study the role of p75 as a T cell costimulatory molecule in controlling cell fate during TCR/CD28-mediated stimulation was examined. We found that p75-deficient T cells possess a profound defect in IL-2 production in response to TCR/CD28-mediated stimulation. Examination of key signaling intermediates revealed that TCR proximal events such as global tyrosine phosphorylation and ZAP70 phosphorylation, as well as downstream MAPK cascades are unperturbed in p75-deficient T cells. In contrast, p75 is nonredundantly coupled to sustained AKT activity and NF-kB activation in response to TCR/CD28-mediated stimulation. Moreover, p75-deficient T cells possess a defect in survival during the early phase of T cell activation that is correlated with a striking defect in Bcl-xL expression. These data indicate discrete effects of p75 on the intracellular signaling milieu during T cell activation, and reveal the synergistic requirement of TCR, CD28, and p75 toward optimal IL-2 induction and T cell survival. We propose that p75 acts as one of the earliest of the identified costimulatory members of the TNFR family, and is functionally linked to CD28 for initiating and determining T cell fate during activation. The Journal of Immunology, 2004, 173: 4500–4509.

T cells play a key role in immunity as actors and facilitators in the Ag-specific eradication of potential threats. They are tightly regulated by multiple mechanisms in a spatial- and temporal-dependent manner. The TCR confers specificity of response toward specific Ag, whereas numerous costimulatory molecules affect the response outcome (1, 2). In biochemical terms, although TCR-mediated signal transduction can activate a multitude of signaling cascades, a number of costimulatory molecules have been shown to provide a molecular push toward robust T cell responses, as well as fine-tuning of T cell differentiation toward specific effector mechanisms. Current models of T cell costimulation suggest several costimulatory molecules are required for the development of effective T cell-mediated immunity, reflecting the requirement of several signals in simultaneous action during the course of the T cell response, and the requirement of different signals at different times and stages of T cell differentiation (2).

The extensive literature identifies two general families of costimulatory molecules: CD28-related and the members of the TNFR family (2–4). Their respective characterization indicates overlapping and distinct activity on T cell proliferation, cytokine production, survival, and differentiation. The integration of signals stemming from the TCR and accessory cell surface proteins is thereby an important mode of molecular regulation for T cell activation. An important integration point for TCR-mediated and costimulatory signals toward a robust T cell response is the tight regulation of the critical T cell growth factor IL-2 (5, 6). Early characterization of TCR- and CD28-mediated signals illustrated the two-signal concept for functional T cell activation. Indeed, CD28 was established as a key costimulatory molecule for induction of IL-2 based on its ability to substantially augment expression in T cells stimulated via TCR (7). Transcriptional regulation of IL-2 is achieved by cooperative binding of different transcription factors (including NFAT, AP-1, and NF-kB) (8), which are downstream targets of both the TCR and CD28 signaling pathways (5, 9). Their integration at the IL-2 promoter has been mapped to the CD28 response element, which is similar in sequence to the NF-kB consensus-binding site (10). Thus, coordinated signals provided by external stimuli elicit intracellular signaling pathways that converge qualitatively and quantitatively toward changes in gene expression profile.

CD28 is the best characterized of the growing numbers of identified costimulatory molecules, and has been shown to synergize with the TCR to lower the threshold of T cell activation (11, 12), enhance initial clonal expansion (13), regulate IL-2 production (7, 14), and augment the expression of antiapoptotic members of the Bcl-2 family (15). Initial biochemical studies of CD28 function demonstrated its role in the transcription and stability of IL-2 mRNA (16). Subsequent studies of CD28 signaling showed synergy with those mediated via the TCR complex toward the activation of downstream effectors (17). In particular, optimal activation of the transcription factors NFAT, AP-1, and NF-kB is dependent on “amplification signals” provided by CD28-mediated recruitment and subsequent activation of Vav (18) and PI3K (17). More recently, protein kinase B (AKT) was shown to provide the CD28-mediated costimulatory signal for up-regulation of IL-2,
suggesting that it also uses discrete signaling pathways (19). However, the effects mediated by CD28 were found to be insufficient to sustain long-term T cell survival, and is thus functionally positioned early during T cell activation (within 24 h of Ag encounter) (20).

A number of novel costimulatory molecules have been identified from the TNFR family, including 41BB, CD134 (OX40), and CD27 (2). The discrete costimulatory roles played by these so-identified members of the TNFR family correspond with their expression pattern during the T cell response: peak expression occurs between days 2 and 4, and remains at significant levels thereafter (2). Analysis of CD27-deficient T cells revealed that CD27 provides specific support of Ag-specific expansion, as well as profound effects on T cell memory reflected by delayed response kinetics and reduction of CD8+ T cell numbers during secondary challenge (21). Stimulation of 41BB induces enhanced proliferative response when combined with anti-CD3 and anti-CD28 stimulation (22), sustains established CD4+ and CD8+ T cell responses as well as enhances cell division and effector function (23, 24). Engagement of OX40 promotes effector and memory T cell function, and can also enhance clonal expansion and cytokine production. Characterization of OX40-deficient T cells showed relatively unimpaired IL-2 production, cell division, and clonal expansion but revealed an important role toward sustaining the expression of antiapoptotic molecules Bcl-xL and Bel-2 thereby promoting T cell survival (25). Thus, the roles of 41BB and OX40 for T cell differentiation and function appear to sustain the response (2), whereas CD28 appears to be important for the initial stages of activation and IL-2 induction.

Several lines of evidence suggest that the prototypic member of the TNFR family, namely TNFR-2 (p75), plays an important costimulatory role for T cells. First, it was found that adding exogenous TNF-α to T cell cultures stimulated via TCR cross-linking substantially augmented the proliferative response to a degree comparable to T cell cultures supplemented with exogenous IL-2 (26). Furthermore, anti-TCR-induced proliferation in T cells is essentially abolished by a neutralizing Ab to TNF-α (26). Second, agonistic Abs identified that of the two receptors for TNF-α, p75 signals the enhancement in the proliferative response (27). In correlation with these findings, it was found that p75 is the dominant TNFR during T cell activation (28). Moreover, the known p75 ligands are expressed by activated T cells and APCs: TNF-α is one of the earliest cytokines expressed during activation of lymphocytes and APCs (28), and lymphoxygenin-α is expressed on activated T and B cells (29). Biochemical characterization of the cytoplasmic domain of the p75 TNFR revealed a TNFR-associated factor-binding domain and was shown to recruit TNFR-associated factor 2 (30), an adaptor molecule through which costimulatory members of the TNFR family are thought to mediate their function.

More recently, we have shown that p75 decreases the threshold of T cell activation using p75-deficient mice (31). Further cellular characterization of p75-deficient CD8+ T cells revealed a costimulatory role for p75 toward a number of aspects of T cell activation, including IFN-γ and IL-2 production, as well as the proliferative response that was only partially rescued by CD28 coligation (31). These data strongly suggested an important relationship between TCR, CD28, and p75 toward optimal T cell responses. In this study we show that T cells deficient in p75 possess a marked defect in IL-2 production in response to TCR/CD28-mediated stimulation, demonstrating for the first time the costimulatory role of p75 in relation to TCR and CD28-mediated signals toward IL-2 induction. Biochemical characterization showed that p75 provides distinct qualitative signals via AKT and NF-κB, whereas TCR-proximal signaling events and MAPK family cascades were largely intact. Moreover, we found that p75-deficient CD8+ T cells are more susceptible to apoptosis during the early phase of T cell activation in response to TCR/CD28-mediated stimulation. This decreased survival of p75-deficient CD8+ T cells correlated with a defect in the expression of the antiapoptotic molecule Bel-2/BI.

These results support a model of costimulation that implicates p75 in a critical role during TCR/CD28-mediated stimulation toward IL-2 induction, clonal expansion, and survival and that these functional aspects are correlated with a distinct role in the intracellular signaling milieu during the early phase of the T cell response.

Materials and Methods

Mice

Breeders for C57BL/6 (B6, H-2b) and B6 p75-deficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6 mice deficient in the p75 have been previously described (32). p75-deficient mice were genotyped using a PCR strategy. Mice 4–7 wk of age were used for all experiments.

Cells

Lymph nodes were harvested and single cell suspensions prepared from each of the mouse lines. The CD4+ CD8+ T cell subset was purified from whole lymph node cell suspensions using miniMACS microbeads (Miltenyi Biotec, Auburn, CA). CD8+ T cells were positively selected using a MACS MS+ Separation column and MiniMACS magnet, as per manufacturer’s protocol (Miltenyi Biotec), achieving >95% purity. Cells were cultured at 37°C and 5% CO2 in Iscove’s DMEM (Invitrogen Life Technologies, Burlington, Ontario, Canada) supplemented with 10% (v/v) FBS (Invitrogen Life Technologies), 5 × 10−5 μM 2-ME, and antibiotics (I-media).

Abs and flow cytometry

PE-conjugated hamster anti-mouse p75 mAb (TR75-89) was used to study p75 expression (BD Pharmingen, Mississauga, Ontario, Canada). Cell staining and flow cytometry were performed according to standard procedures. Briefly, cells were incubated with the relevant Abs for at least 15 min at 4°C, and subsequently washed twice with FACS medium (PBS plus 2% FCS). The CellQuest software program (BD Biosciences, Mountain View, CA) was used for data acquisition and analysis. For TNF-α neutralization studies, two Abs were used: rat anti-mouse TNF-α used (MP6-XT3; BD Pharmingen) and rat anti-mouse TNF-α (V1q; Cedarlane Laboratories, Hornby, Ontario, Canada).

7-Aminoactinomycin D (7-AAD) assay

CD8+ T cells (1 × 106) were stimulated with 10 μg/ml immobilized anti-CD3ε (2C11) and 10 μg/ml anti-CD28 (35.71) in a flat-bottom 24-well plate for various periods of time. Cells were harvested and stained with 7-AAD (10 μg/ml in FACS buffer), fixed with 4% paraformaldehyde, and subsequently analyzed by FACS (as previously described).

Cytokine ELISA

A total of 2 × 106 CD8+ T cells were cultured in a flat-bottom 24-well plate coated with 10 μg/ml 2C11 for 20 h. The amount of IL-2 and IFN-γ in the supernatant was determined by ELISA. The capture and detection Abs used for IL-2 were JS66-1A12 and JS66-5H4, respectively (obtained from BD Pharmingen). The capture and detection Abs used for IFN-γ were R4-6A2 and XMG1.2, respectively (BD Pharmingen). Briefly, plates were coated with the capture Ab (4 μg/ml in carbonate buffer) and blocked with 1% BSA/0.1% azide in PBS. Wells were washed with PBS-Tween 20 and samples were then added in three dilutions, with each plate containing one well of cytokines. The wells were washed and the detection Ab (1 μg/ml in 1% BSA/0.1% azide in PBS) was added. The wells were then washed and streptavidin-alkaline phosphatase (BD Pharmingen) was added (1/2000 in 1% BSA/0.1% azide in PBS). After washing the wells, substrate (104; Sigma-Aldrich, Milwaukee, WI) was added and plates were subsequently analyzed with an ELISA plate reader at 405 nm.

Abbreviations used in this paper: 7-AAD, 7-aminoactinomycin D; CQ-PCR, competitive and quantitative PCR; WT, wild type; HPRT, hypoxanthine phosphoribosyltransferase; GSK, glycogen synthase kinase.
Cytokine competitive and quantitative RT-PCR

Competitive and quantitative PCR (CQ-PCR) was used to determine the steady-state levels of IL-2 and TNF mRNA. A total of 2 × 10⁶ T cells were cultured in 1.0 ml of media in flat-bottom 24-well plates coated with 5 μg/ml 2C11 for 9 h. Cells were then harvested and total RNA was prepared according to the manufacturer’s recommendations using the RNasey Mini kit (Qiagen, Valencia, CA). CDNAs were generated from the total RNA preparation as previously described (33). CQ-PCR was then performed as previously described (33). Briefly, the amount of cDNA was normalized between p75-deficient and wild-type (WT) T cells using the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT). The linearized pGQRS plasmid was used as the competitor (gift from R. Locksley, University of California, San Francisco, CA). The sequences for the 5’ and 3’ oligonucleotide primers used for IL-2 were, respectively: 5’-GCA GCT CTA CAG CGG AAG-3’ and 3’-GAG TCA AAT CCA GAA CAT GCC GCA-3’. The sequences for the 5’ and 3’ oligonucleotide primers used for TNF-α were, respectively: 5’-GTT CTA TGG CCC AGA CCC TCA CAC-3’ and 3’-TCC CAG GTA TAT GGG TTC ATA CCA AG-3’. The sequences for the 5’ and 3’ oligonucleotide primers used for HPRT were, respectively: 5’-CGA GGT AGG CTG GCC TAT AGG CT-3’ and 3’-GAG GTG AGG CTG GCC TAT AGG CT-3’. The PCR products were subjected to electrophoresis on a 2% agarose gel and visualized with ethidium bromide. Densitometry was performed using the Alphalager software.

Western blot

A total of 2 × 10⁶ CD8⁺ T cells were cultured in a flat-bottom 24-well plate coated with 10 μg/ml anti-CD3 (2C11) alone and in combination with 10 μg/ml soluble anti-CD28 (37.51) for various periods of time. Whole cell lysates were prepared as previously described. Briefly, cells were harvested, washed with PBS, and treated with lyssis buffer (1% Triton X-100, TNE (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA), 1 mM sodium orthovanadate, 1 mM sodium molybdate, and complete protease inhibitor mix; Roche Diagnostic, Laval, Quebec, Canada) and 10% glycerol for 20 min on ice. Total protein was quantitated using DC Protein Assay (Bio-Rad, Hercules, CA), equivalent amounts were resolved by SDS-PAGE, and then transferred to Immobilon-P membrane (Millipore, Bedford, MA).

The following Abs (all from Cell Signaling Technology, Pickering, Ontario, Canada) were used: rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204) Ab, rabbit anti-p44/42 MAPK Ab, rabbit anti-phospho-p38 MAPK Ab, rabbit anti-phospho-stress-activated protein kinase/JNK (Thr183/Tyr185) Ab, rabbit anti-phospho-stress-activated protein kinase JNK Ab, rabbit anti-phospho-ZAP70 (Ty319) Ab, rabbit anti-phospho-AKT (Ser473) Ab, rabbit anti-AKT Ab, rabbit anti-phospho-IκBα (Ser32) Ab, rabbit anti-IκBα Ab. Tyrosine phosphorylation was assayed using 4G10, a mouse IgG2b mAb (Santa Cruz Biotechnology, Santa Cruz, CA).

AKT activation was measured using the AKT kinase assay kit, which involves immunoprecipitating AKT from cell extracts, incubation with cold ATP and glycogen synthase kinase (GSK)-3αβ fusion protein as substrate, and detecting for phosphorylated GSK-3αβ by Western blot as per manufacturer’s protocol (Cell Signaling Technology).

EMSA

A total of 4 × 10⁶ CD8⁺ T cells were cultured in a flat-bottom 24-well plate coated with anti-CD3 (2C11) mAb (10 μg/ml) in the presence or absence of soluble anti-CD28 (37.51) mAb (10 μg/ml) for 8 h. Nuclear extracts were prepared as previously described (34). Briefly, cells were harvested and washed with PBS. Cell pellets were resuspended in buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 15 min. Samples were centrifuged, resuspended in buffer C (20 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 20 min. Cellular debris was removed by centrifugation. Protein concentration of nuclear extracts was quantified using DC Protein Assay (Bio-Rad).

Oligonucleotide probe used for NF-κB was end-labeled with [γ-³²P]GTP. The 5’ and 3’ sequences used for generating three-nucleotide overhangs of the oligonucleotide probe were respectively: 5’-ATG TGA GGG GAC TTT CCC-3’ and 3’-ACT CCC GAC AAA GGG TCC-5’. The fill-in reaction was mediated using the large fragment (Klenow) of DNA polymerase I (Invitrogen Life Technologies).

Nuclear extracts (4 μg) were incubated with radioactively labeled probe for 20 min at room temperature, and then resolved by PAGE (5% acrylamide). Gel shifts were analyzed using Molecular Dynamics PhosphorImager SI system (Amersham Biosciences, Sunnyvale, CA) and ImageQuant (version 5.2) software (Amersham Biosciences).

Results

p75 TNFR2 expression during T cell activation

Patterns of cell surface expression of receptors can provide insight to their usage and function. The kinetics of expression of costimulatory members of the TNFR family appear to correlate with their temporally dependent functions during T cell activation (2). Early work on the two TNFRs reported that p75 is the dominant TNFR on activated T cells (28), indicating that the pleiotropic effects of TNF-α can be attributed to the differential expression of the TNFRs. However, the kinetics of p75 expression is unknown, and its examination may reveal insight to the temporal aspects of its function during T cell activation. We therefore examined p75 expression during TCR/CD28-mediated stimulation of naïve T cells to assess its potential temporal usage during T cell activation. Immediately ex vivo CD8 T cells displayed constitutive expression of p75, and increased in its expression after 48 h of TCR/CD28-mediated stimulation (Fig. 1). As expected, p75-deficient T cells did not display any expression, confirming that there was no leaky

**FIGURE 1.** CD8⁺ T cells displayed constitutive expression of p75 and increased expression after TCR/CD28-mediated stimulation. A, p75 expression on naive and activated T cells. A total of 1 × 10⁶ CD8⁺ T cells were stimulated with 10 μg/ml plate-bound anti-CD3 in combination with soluble anti-CD28 (10 μg/ml) for various times, harvested and subjected to analysis by flow cytometry (see Materials and Methods). p75-deficient T cells (dotted line), which involves immunoprecipitating AKT from cell extracts, incubation with cold ATP and glycogen synthase kinase (GSK)-3αβ fusion protein as sub-
expression over the course of culture examined. The constitutive expression of p75 in naive T cells suggests that it plays a very early role in T cell activation, and continues to exert its effect as its expression is induced during the early phase of the response.

**IL-2 induction during T cell activation is dependent on both CD28 and p75**

The induction of IL-2 gene during T cell activation is a highly regulated process requiring both TCR-mediated and costimulatory signals. These signals provided at the initiation of the T cell response can control commitment to cytokine production and proliferation. CD28 is well characterized in its ability to costimulate robust IL-2 production. We previously reported the defect in the proliferative response to TCR/CD28-mediated stimulation by p75-deficient CD8+ T cells (31). Moreover, IL-2 induction was defective in p75-deficient CD8+ T cells stimulated via TCR-mediated stimulation. We sought to determine whether p75 is critical for optimal IL-2 induction in T cells stimulated via both TCR and CD28. The amount of IL-2 that was secreted in response to TCR cross-linking alone and in combination with CD28 coligation was measured by ELISA performed on culture supernatants. As shown in Fig. 1B, p75-deficient CD8+ T cells stimulated with anti-CD3 displayed a marked reduction in the amount of IL-2 detected in the culture supernatant compared with WT CD8+ T cells. CD28 coligation led to enhancement of IL-2 production in WT CD8+ T cells, which is consistent with its known function in costimulating IL-2 induction. Strikingly, CD28 coligation did not rescue the defect in IL-2 production of p75-deficient CD8+ T cells, demonstrating the importance of p75 as a costimulator of IL-2 during T cell activation. This suggests that p75 and CD28 may act synergistically toward costimulating optimal IL-2 induction.

A key point of IL-2 regulation is at the transcriptional level (9). To study whether the observed reduction in IL-2 production by p75-deficient CD8+ T cells stemmed from a reduction in the steady-state levels of cytokine transcript, IL-2 mRNA was measured by QPCR. This technique permits quantitative analysis of relative transcript levels as a function of competitor dose. When stimulated with anti-CD3 alone, p75-deficient CD8+ T cells displayed a reduction in steady-state levels of IL-2 transcript compared with WT CD8+ T cells (Fig. 2). As expected, CD28 coligation caused a substantial (10-fold) increase in steady-state levels of IL-2 transcript in WT CD8+ T cells. In striking contrast, CD28 coligation only caused a modest increase for p75-deficient CD8+ T cells, which displayed a 10-fold reduction in IL-2 transcript compared with WT (Fig. 2). This result reveals for the first time the importance of p75 in the optimal induction of IL-2 at the level of cytokine transcript in response to TCR/CD28-mediated stimulation. The defect in steady-state levels of IL-2 transcript suggests that p75 and CD28 may converge on common intracellular signaling effectors to achieve fine control over the activation of transcription factors that assemble at the IL-2 promoter for its optimal induction.

**TCR-proximal signaling events function independently of p75**

The biochemical events following TCR engagement are well characterized, involving changes in the intracellular compartment of T cells that ultimately lead to alteration of gene expression profile (35). TCR-mediated signal transduction involves phosphorylation of key tyrosine residues in the TCR complex by Src-family protein tyrosine kinases that lead to recruitment and activation of adaptor molecules such as ZAP70 at the T cell surface (36). Tyrosine kinases then couple the TCR complex to the Ras- and Rho-family GTPases signaling cascades, leading to the multiple biochemical pathways including ERK1/2 MAPK, and regulation of inositol phospholipid metabolism, which controls intracellular calcium and activity of diverse serine/threonine kinases including protein kinase C and PI3K-controlled serine kinases (18).

Given the important contribution made by p75 toward IL-2 induction during TCR/CD28-mediated stimulation, we sought to investigate the signaling events through which p75 exerts its effect in relation to those mediated through the TCR and CD28. We first examined whether p75 affects TCR-proximal signaling events in response to stimulation with anti-CD3 alone, or in combination with either anti-CD28 or exogenous TNF-α (10 ng/ml), by assaying for global tyrosine phosphorylation. Notable TCR-proximal substrates indirectly observed by examination of the tyrosine phosphorylation profile include ZAP70 (70 kDa) and its immediate downstream target linker of activated T cells (36–38 kDa), both of which are recruited and activated at the TCR complex. As shown in Fig. 3A, the tyrosine phosphorylation profiles of p75-deficient CD8+ T cells appeared largely similar to WT. In addition, anti-CD28 and exogenous TNF-α treatment of p75-deficient CD8+ T cells did not affect global tyrosine phosphorylation. These data suggest that p75 is not required for tyrosine phosphorylation of substrates that are immediately coupled to the TCR complex, and that TCR-mediated stimulation is sufficient for these early signaling events. Consistent with this finding, ZAP70 phosphorylation assayed directly by Western blot showed that this TCR-proximal signaling event is not coupled to p75 (Fig. 3B). Hence, TCR-proximal signaling events of CD8+ T cells are functional and independent of p75 at early time points of stimulation.
MAPK family cascades are not coupled to p75 during T cell activation

TCR engagement is directly linked to the MAPK pathway, which includes ERK, JNK, and p38 (37). Signals are delivered from the TCR complex to the ERK pathway via homology 2 domain-containing leukocyte protein of 76 kDa and Grb2 (38), whereas JNK and p38 pathways appear to be coupled to both CD28 and TCR (39, 40). MAPK pathways are notable for their role in activating transcription factors such as Elk1, c-Fos, and c-Jun (components of the heterodimeric transcription factor AP-1) (35, 41), which is known to be important for the regulation of IL-2 gene expression (40). We sought to determine the relative roles of TCR, CD28, and p75 toward these MAPK pathways at various time points of stimulation using Western blot analysis. We were particularly interested in examining the intracellular signaling milieu around the 10-h time point because we observed that steady-state levels of IL-2 transcript were markedly reduced in p75-deficient CD8+ T cells at this time point. As shown in Fig. 4, anti-CD3 treatment was sufficient to phosphorylate ERK1/2, JNK, and p38 in both p75-deficient and WT CD8+ T cells, which persisted up to 10 h of stimulation. Moreover, CD28 coligation did not lead to an increase in phosphorylation of these MAPKs. These data suggest that the MAPK pathways are sufficiently activated by TCR-mediated stimulation and do not require p75.

p75 in AKT phosphorylation

TCR signaling is coupled to PI3K, which then leads to AKT recruitment and activation (42). A previous report demonstrated that AKT acts as a mediator for CD28 costimulation of IL-2 induction (19), indicating that both TCR and CD28 signal toward AKT phosphorylation (42). The finding that CD28 coligation failed to rescue the defect in IL-2 induction suggested that p75 may be functionally linked to CD28 in regulating this critical growth factor. We therefore examined the possibility that AKT phosphorylation may be a downstream target of convergence between p75 and CD28. It was found that AKT underwent robust phosphorylation in response to anti-CD3 stimulation in both WT and p75-deficient CD8+ T cells at early time points (Fig. 5). This phosphorylation was sustained over the 10 h of anti-CD3 stimulation examined for WT CD8+ T cells, but was dramatically decreased for p75-deficient CD8+ T cells. Interestingly, CD28 coligation failed to rescue this defect in AKT phosphorylation. To directly measure the activation of AKT, we used a nonradioactive AKT assay that measures the phosphorylation of the substrate, a GSK-3αβ fusion protein (see Materials and Methods). As shown in Fig. 5B, WT and p75-deficient CD8+ T cells displayed similar immediate activation of AKT after 10 min of stimulation as indicated by similar levels of phosphorylated GSK-3αβ. Strikingly, AKT activity was dramatically less in p75-deficient T cells compared with WT after 10 h of stimulation, and CD28 coligation did not rescue this defect. These data demonstrate the importance of p75 toward sustaining the activation of AKT during T cell activation, and indicate a novel functional link between the CD28 and p75 costimulatory pathways.

Previous work has shown that IL-2 and related cytokines can activate AKT (43). Given that p75-deficient CD8+ T cells possess a defect in IL-2 induction, we sought to determine whether the defect in AKT phosphorylation was indirectly due to limited IL-2 production by p75-deficient CD8+ T cells. We addressed this question using a blocking Ab for IL-2R during stimulation with anti-CD3 and CD28 coligation. The dose of anti-IL2Rβ used was...
sufficient to completely abrogate T cell proliferation (data not shown). It was found that anti-IL-2Rβ treatment did not substantially affect AKT phosphorylation in WT CD8+ T cells after 10 h of stimulation, demonstrating that the IL-2 is not necessary for AKT phosphorylation during the early phase (up to 10 h) of the response and thereby suggests that this period represents an IL-2-independent phase of T cell activation. This strongly suggests that the defect in AKT activation observed for p75-deficient CD8+ T cells is due to lack of a direct signal toward AKT via p75.

**p75 is critical for NF-κB activation**

NF-κB is one of a crucial set of transcription factors that coordinately binds to the IL-2 promoter (44). It is downstream of both TCR and CD28 signaling via protein kinase Cθ and AKT (45). In its latent form, it is found in a complex with IκBα in the cytoplasm (46). Activation signals target this complex by phosphorylating IκBα, leading to its degradation mediated by ubiquitin. The liberated NF-κB then translocates across the nuclear membrane and subsequently acts upon on gene targets. Hence, NF-κB activation can be measured directly by its trans-localization into the nucleus, and indirectly by the phosphorylation of IκBα. Previous work has shown that AKT phosphorylation observed in p75-deficient CD8+ T cells, as well as the important role that NF-κB in IL-2 gene expression, we sought to determine whether p75 couples to IκBα and subsequent degradation is defective in p75-deficient CD8+ T cells. The steady-state level of IκBα phosphorylation in p75-deficient CD8+ T cells displayed similar level of IκBα phosphorylation relative to unstimulated control. These data suggest that TCR-mediated stimulation is sufficient for immediate phosphorylation of IκBα, and that p75 provides an important contribution to sustain this response during T cell activation.

To directly measure the activation of NF-κB, its presence in the nucleus of T cells upon TCR- and TCR/CD28-mediated stimulation was determined using EMSA. Consistent with the IκBα phosphorylation data, the steady-state levels of NF-κB in the nucleus of WT CD8+ T cells at 10 h of stimulation, indicating its degradation subsequent to phosphorylation. In contrast, p75-deficient CD8+ T cells displayed similar level of IκBα protein relative to unstimulated control. These data suggest that TCR-mediated stimulation is sufficient for immediate phosphorylation of IκBα, and that p75 provides an important contribution to sustain this response during T cell activation.

**p75 promotes T cell survival and is required for Bcl-xL expression during T cell activation**

T cell survival is an essential means by which the strength of the immune response is regulated. T cell activation can lead to a number of cell fate outcomes, depending on the context of signals provided by its local environment. Costimulation mediated by
CD28 controls initial clonal expansion and provides early signals for expression of the Bcl-2 family member Bcl-xL (15). The costimulatory TNFR family member OX40 was shown to be important for maintaining high levels of Bcl-xL later during activation (days 4–8), indicating the temporally linked functions of CD28 and OX40 toward T cell survival (48). Given that p75-deficient T cells possess defect in the proliferative response to TCR/CD28-mediated stimulation, we next addressed whether this defect is due to an inability to survive during T cell activation. We analyzed cell death using 7-AAD staining as a function of CFSE by flow cytometry. The CFSE fluorescence revealed that anti-CD3 and anti-CD28 cotreatment led to recruitment of p75-deficient CD8+ T cells to the dividing population after 65 h of culture (Fig. 7A), although the total clone size as measured by the number of viable cells recovered was substantially reduced in p75-deficient CD8+ T cell cultures (Fig. 7B). The latter result suggested that p75-deficient T cells are defective for survival during T cell activation. Examination of 7-AAD fluorescence revealed that the percentage of live cells is reduced in p75-deficient T cell cultures compared with WT (42 and 71%, respectively), and that proportionately more cells that were recruited into the dividing population stained as apoptotic. This suggests that p75 is important for the survival of T cells during the proliferative response, and indicates that dividing cells possess an increased propensity to undergo apoptosis in the absence of survival signals provided by p75.

The observation that CD28 coligation failed to rescue the survival defect of p75-deficient T cells suggested that the expression of antiapoptotic molecules attributable to CD28 signaling is also defective. We therefore examined TCR/CD28-mediated up-regulation of Bcl-xL using Western blot analysis. Consistent with previous reports (15, 49), CD28 coligation led to an increase in Bcl-xL expression over TCR-mediated stimulation alone. Strikingly, the expression of this antiapoptotic molecule was completely abolished in p75-deficient CD8+ T cells, and CD28 coligation failed to rescue this defect. This strongly suggests that p75 acts early toward survival via expression of Bcl-xL during the very early phase of T cell activation.

**Discussion**

The two-signal model for T cell activation has been substantially modified because it was first proposed more than 30 years ago (50). The “second signal” that was initially attributed to CD28 has now grown in complexity based on observations that other cell surface molecules can modulate specific aspects of the T cell response. The identification of other costimulatory molecules are important in light of the ability of CD28-deficient T cells to undergo a robust response depending on the strength of signal mediated through the TCR. We demonstrate the critical role of p75 in relation to TCR/CD28-mediated stimulation in the induction of IL-2. The striking finding that IL-2 production in p75-deficient T cells cannot be rescued by CD28, strongly suggests that both receptors are important for costimulating IL-2 production and thereby advances the current costimulatory model of IL-2 induction during T cell activation, for which CD28 has been understood to be the dominant costimulatory receptor. Our results reveal a hitherto unknown functional link between p75 and TCR/CD28 in up-regulating the state-steady levels of IL-2 transcript, and that this relationship is correlated with specific defects in the activation of AKT and NF-κB in p75-deficient CD8+ T cells. We found that p75 is expressed earliest among the identified costimulatory members of the TNFR family, with constitutive expression in naive CD8 T cells, set to costimulate T cell activation. Moreover, our data show that p75 is important for T cell survival during TCR/CD28-mediated activation, with a correlated defect in the expression of the antiapoptotic molecule Bcl-xL.

**Biochemical mechanism of p75 and CD28 on IL-2 induction**

Many studies have examined the biochemical nature of CD28 costimulation in relation to TCR signaling. The body of evidence strongly suggests that CD28 uses both qualitative and quantitative signals. Michel et al. (17) showed that CD28 costimulation potentiates TCR signaling by amplifying phospholipase C-γ1 activation and intracellular calcium response, demonstrating quantitative signaling and synergy between the TCR and CD28 signaling pathways. Kane et al. (19) showed that CD28 signal transduction involving AKT is needed for activating CD28 response element in the IL-2 promoter, demonstrating qualitative signals that are uniquely mediated by CD28 toward IL-2 induction. In examining the biochemical nature of the costimulatory role of p75 in IL-2 induction, we observed that TCR-proximal events such as tyrosine phosphorylation and ZAP70 phosphorylation were not coupled to the p75 pathway. Moreover, MAPK family cascades (ERK, JNK,
and p38) were also found to be largely unaffected by p75 deficiency in CD8+ T cells, demonstrating that TCR-mediated signaling is adequate to activate the MAPK pathways. These findings strongly suggested that p75 might provide a distinct and qualitative signal for its costimulatory properties in T cells. Indeed, we showed specific defect of the AKT and NF-κB pathways in p75-deficient CD8+ T cells, demonstrating that a costimulatory member of the TNFR family couples specifically with AKT/NF-κB, and that these impairments are not rescued by CD28 coligation. Further, blockade of IL-2 signaling (using anti-IL-2Rβ) up to 10 h in culture did not abrogate the phosphorylation of AKT for WT T cells, indicating that defective AKT activation observed for p75-deficient T cells is not due to indirect effects associated with defective IL-2 production. The data suggest that the p75 pathway is not coupled to TCR-proximal signaling events or MAPK family cascades, but rather acts through a distinct signal to activate the AKT/NF-κB pathway. The observation that p75 is required for sustaining AKT phosphorylation at the 10 h time point suggests that TNF-α is produced during the first 10 h of activation and then signals in an autocrine fashion through p75. If this were the case, then we should be able to block AKT activation in WT cells by adding a neutralizing anti-TNF-α Ab during the first 10 h of activation. We used two neutralizing anti-TNF-α mAbs from commercial sources (Materials and Methods) but neither Ab was effective in inhibiting anti-TCR induced proliferation in WT cells. The lack of availability of an effective neutralizing anti-TNF-α Ab prevented us from testing this hypothesis.

The finding that CD28 costimulation only led to a marginal increase in the steady-state levels of IL-2 transcript and secreted cytokine in p75-deficient T cells (though leading to a substantial increase in WT cells) suggests a novel relationship between these two costimulatory molecules, and an interesting functional link between the CD28-related and TNFR families. The marginal increase demonstrates that CD28 is still functional in p75-deficient T cells, and suggests that p75 provides an important contribution relative to CD28 for IL-2 production during T cell activation. The relative signaling from these two costimulatory molecules appear to converge at the level of AKT/NF-κB as a critical integration point toward optimal IL-2 production. The cytoplasmic tail of CD28 is known to contain a binding domain for PI3K, which is immediately upstream of AKT phosphorylation. Further work is required to examine the biochemical relationship immediately proximal to these cell surface receptors.

Position of p75 within the model of costimulation (relative to CD28 and members of the TNFR family)

On a temporal basis, CD28 controls the early outcomes of T cell activation by costimulating IL-2 induction and antiapoptotic members of the Bcl-2 family, reflecting its constitutive expression on naive T cells. CD28 is therefore positioned among the earliest costimulatory molecules in a temporal model of T cell activation, affecting threshold of signaling required for progression of T cell activation upon TCR engagement such as initial clonal expansion, survival, and IL-2 induction. Engagement of CD28 then leads to efficient expression of TNFR family members (such as OX40, 41BB, and CD27), in a temporal sequence of costimulatory modulation of the T cell response. Indeed, the growing number of costimulatory members identified in the TNFR family suggests that there are specific and simultaneous signals that function in a temporal manner toward modulating T cell response outcomes (2). Our data provide the first case of a costimulatory member of the TNFR family acting in the earliest phase of T cell activation, positioned temporally with CD28 in a nonredundant manner toward the induction of IL-2 and promoting T cell survival. Moreover, this is the first TNFR member identified to have a functional link to CD28 toward IL-2 induction, in contrast to the temporal link that exists between CD28 and other costimulatory members of the TNFR family.

The findings in our study are particularly important given the recent advances in understanding of the signaling networks that are linked to various costimulatory molecules (2). These have largely been differentiated in their respective roles from a temporal standpoint toward common signaling targets. The constitutive expression of p75 on T cells and subsequent up-regulation early during T cell activation, together with the observation that IL-2 induction is defective in p75-deficient T cells, which cannot be rescued by CD28 coligation, strongly argues that p75 is a critical costimulatory receptor for early T cell activation events during which T cell fate is determined.

Integral role of AKT as a common signaling mediator of costimulatory molecules

AKT activity is functionally associated with TCR/CD28-mediated induction of IL-2, expression of antiapoptotic molecules of the Bcl-2 family, and T cell survival (20, 21). Indeed, the important role of AKT in T cell survival was shown by Song et al. (20) using ectopic expression of active and dominant negative variants of AKT. A link between costimulatory members of the TNFR family and AKT was previously postulated (2). More recently, it was shown that OX40, a member of the TNFR family, functions to maintain AKT activity over time: OX40-deficient CD4+ T cells displayed normal levels of phosphorylated AKT over the first 24 h of Ag-specific stimulation, but did not maintain large amounts of phosphorylated AKT from days 2 to 4 (20). Sustained AKT signaling mediated via OX40 was correlated with cell survival over time, and thereby shown to have an integral role in regulating T cell longevity. Our observation that AKT phosphorylation is defective in p75-deficient T cells during the first 24 h of stimulation suggests that the functional link between costimulatory molecules is temporally regulated through AKT as a common signaling mediator. Moreover, the early role of p75 on the AKT/NF-κB pathway during T cell activation suggests that it is an important regulatory point for initiation and progression of the T cell response. CD28, OX40 and now p75 are therefore identified as costimulatory molecules that are linked with the AKT pathway to exert discrete effects during the T cell response. Costimulation toward crucial activation events (i.e., IL-2 induction, clonal expansion, and survival) may therefore be achieved on a temporal basis through discrete action of costimulatory molecules via AKT.

Regulation of T cell survival by p75 during T cell activation

T cell fate outcome is dependent on the signals that are provided during antigenic challenge, which regulate pathways involved in cell survival and death. CD28-mediated costimulation was thought to be the primary signal for commitment of responding T cells to clonal expansion, IL-2 production, and survival, depending on the strength of TCR-mediated signals (11). Inadequate level of signaling (i.e., subthreshold) via CD28 and TCR-mediated signals leads to either anergy or death (1), whereas a sufficient level of signaling through these pathways leads to induction of costimulatory members of the TNFR family, which subsequently functions to sustain the T cell response by promoting survival (2). We found in our study that p75 functions at early time points during TCR/CD28-mediated activation toward T cell fate outcome early during activation by controlling survival. The data suggest that the progression of the T cell response beyond day 1 is largely dependent on p75. Indeed, p75-deficient CD8+ T cells
show an increased propensity toward apoptosis during TCR/CD28-mediated stimulation, leading to reduced clone size. This suggests that the balance between life and death in controlling T cell fate outcomes is determined by early signals provided by TCR, CD28, and p75.

Interestingly, the expression of Bcl-xL was found to be up-regulated as early as 10 h of TCR/CD28-mediated stimulation, which was completely abrogated in p75-deficient CD8+ T cells. A previous study by Noel et al. (49) showed that anti-CD3 treatment led to Bcl-xL expression, and that CD28 coligation led to even greater levels of Bcl-xL after 24 h of stimulation. The same study also showed that CD28-deficient T cells and CTLA4-Ig treatment displayed a substantial decrease in Bcl-xL expression after 24 h of stimulation. Our study examined Bcl-xL expression after 9 h of stimulation, showing that anti-CD3 treatment in WT CD8+ T cells led to expression of Bcl-xL and that CD28 coligation was additive toward greater expression. Although CD28 coligation led to an increase in expression, levels of Bcl-xL was still significant after 9 h of anti-CD3 treatment alone, conditions that would be similar to examining CD28-deleted T cells at the same early time point.

Overall, the data show that p75 acts as the earliest of the identified costimulatory members of the TNFR family, functionally linked to CD28 during the early phase of the T cell response. Deficiency of p75 in CD8+ T cells led to specific biochemical defects in AKT, NF-kB, and Bcl-xL as early as 10 h of TCR/CD28-mediated stimulation. The importance of p75 during TCR/CD28-mediated stimulation reveals an important framework of cell surface molecules from distinct receptor families toward crucial activation events such as IL-2 induction, clonal expansion, and survival. It also points to the importance of the temporal and environmental context in dictating T cell fate outcomes during the earliest stages of activation.

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References


