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T cell activation requires a threshold amount of TCR-mediated signals, an amount that is reduced by signals mediated through costimulatory molecules expressed on the T cell surface. Here the role of TNFR2 (p75) as a putative costimulatory receptor for T cell activation was examined. It was found that p75 deficiency in CD8+ T cells increased the requirements for TCR agonist approximately 5-fold. Furthermore, p75−/− T cells display a marked reduction in the proliferative response to TCR agonist. This hypoproliferative response was associated with delayed kinetics of induction of the acute activation markers CD25 and CD69 as well as a marked decrease in the production of IL-2 and IFN-γ. The net result is that very few cells are recruited into the dividing population. Interestingly, CD28 costimulation was only partially effective in rescuing the proliferative defect of p75−/−CD8+ T cells. Thus, p75 provides an important costimulatory signal in addition to that provided by CD28 toward optimal T cell proliferation. The Journal of Immunology, 2001, 167: 6812–6820.

T cells occupy a central role in the initiation and regulation of the adaptive immune response (reviewed in Ref. 1). T cell activation is tightly regulated, involving coordinated integration of multiple signals to ensure Ag-specific clonal expansion and differentiation (1). Although the TCR plays a major role in defining the fine specificity of an immune response, additional mechanisms have evolved to ensure self-tolerance (2). The general requirement of costimulatory signals for the optimal activation of T cells in addition to the Ag-specific signal delivered through the TCR is a major means by which self-tolerance is achieved. The Ag-specific signal is integrated with costimulatory signals, and the convergence of positive signals pushes the T cell toward activation (3). Such mechanisms serve to protect against untoward autoimmune reactions and to focus the immune response on the infected target tissue (2). Clearly, the elucidation of the multiple mechanisms that regulate T cell behavior is crucial toward elucidation of their potential role in disease processes, such as immunodeficiency and autoimmunity.

In the context of infection, a major costimulatory pathway involves the CD28 molecule on the T cell surface (reviewed in Ref. 1). APCs express CD28 ligand (B7) upon activation by foreign pathogen (4). Infectious agents are processed into antigenic peptides, and APCs thus display both Ag and costimulatory ligands to Ag-specific T cells (4). Therefore, the induction of B7 on the surface of the APC alerts Ag-specific T cells of the presence of infectious agents. These signals are integrated within the responding T cell, and, in effect, costimulation serves to lower the threshold amount of signals required through the TCR complex for optimal T cell activation (5). Conversely, in the absence of infection, APCs do not up-regulate costimulatory molecules, and T cells normally remain functionally inactive (1). A corollary of this model is that without costimulatory signals, the signals mediated through the TCR complex are normally insufficient for mounting a functional Ag-specific T cell response.

However, studies using CD28-deficient mice have shown that costimulation through the CD28 pathway is not an absolute requirement for T cell activation (6–10). These studies strongly suggest that the intensity and duration of the antigenic signal mediated through the TCR are also critical factors (5, 8, 10). Furthermore, although CD28 represents a major costimulatory pathway, both in vitro and in vivo studies strongly suggest that there is an additional costimulatory pathway(s) that can lead to a functional T cell response. Indeed, infectious agents can trigger numerous costimulatory molecules and cytokines in addition to B7, including the proinflammatory cytokine TNF-α.

Several studies have implicated TNF-α in playing a costimulatory role in T cell proliferation (11–16). Indeed, APCs not only express CD28 ligand in response to infectious agents, but also express TNF-α when activated. Interestingly, there is a substantial increase in T cell proliferation in response to TCR agonist when TNF-α is added exogenously, which is comparable to that found when IL-2 is added exogenously (11). Importantly, the proliferative response of T cells to stimulation through the TCR complex is essentially abolished when a neutralizing Ab to TNF-α is added (11). In addition, TNF-α is expressed early during T cell activation (17), suggesting that it may serve as a regulatory control point. Taken together, this suggests that TNF-α, provided both exogenously upon APC activation by infectious agents and endogenously upon T cell activation, can act through a costimulatory pathway and may be a critical regulatory point for the progression of the T cell response.

TNF-α binds to two distinct receptors on the cell surface, TNFR-1 (p55) and TNFR-2 (p75) (reviewed in Ref. 18). Studies using agonist Abs have demonstrated that the two receptors signal distinct TNF activities (12). While p55 was shown to be responsible for signaling cytotoxicity and the induction of several genes, p75 was shown to be capable of signaling the proliferation of primary thymocytes and a cytotoxic T cell line (13). It was further determined that agonist Abs toward p75 resulted in the enhanced T cell proliferative response to stimulation observed when adding...
TNF-α exogenously, whereas specific activation of p55 had no effect (15). In addition, it was found that p75 is the predominant TNFR on activated T cells (17). However, these studies do not address whether the p75 signal is important, or if it is actually a redundant pathway.

Recently, mice deficient in p55 (19, 20) and p75 (21) TNFRs were generated to clarify their respective roles in the immune system. These studies confirmed the importance of p55 for the cytotoxicity associated with TNF-α. Interestingly, p55+/− T cells do not exhibit a defect in proliferative response to mitogens such as Con A or agonist Ab specific for the TCR complex (22). This is consistent with the observations that agonists specific for p55 did not affect the proliferative response to TCR agonists (15). Functional analysis of p75−/− T cells did not reveal any changes in responsiveness, including response to the mitogen Con A (21). However, the same group that generated p75−/− mice did not examine the T cell response to specific stimulation through the TCR complex, which is the normal physiological route of T cell activation. Furthermore, no study to date has examined the importance of p75 for T cell proliferation using TCR-specific stimulation. Therefore, a potentially critical costimulatory pathway has been neglected, and the elucidation of the mechanisms that lead to a functional T cell response is incomplete.

This question was addressed in our laboratory by examining the ex vivo response of p75−/− T cells to specific stimulation through the TCR complex. It was determined that p75−/− T cells display a marked reduction in this proliferative response, and that p75 deficiency in CD8+ T cells increased the requirements for TCR agonists (15). Functional analyses revealed that p75−/−CD8+ T cells produce significantly less IL-2 and IFN-γ in response to TCR agonist and display delayed kinetics in the acquisition of the activation phenotype. We propose a model in light of these data that p75 contributes in a nonredundant manner toward the activation and recruitment of transcription factors that are associated with T cell activation.

Materials and Methods

Mice

Breeders for C57BL/6 (B6, H-2b) and B6-p75−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6 mice deficient in the p75 TNFR-α receptor have been previously described (21). The p75−/− mice were genotyped using a PCR strategy. Mice 4–7 wk of age were used for all experiments.

Abs and flow cytometry

The following Abs were used: FITC-conjugated mAbs to mouse CD8 (53.67), PE-conjugated CD4 (GK1.5), and biotin-conjugated CD25 (PC61), CD44 (Pep-1, and CD69 (H1.2F3; all from BD PharMingen (San Diego, CA), supplied by Cedarlane Laboratories, Hornby, Canada). Cell staining and flow cytometric analysis 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 and 2% FCS). The CellQuest software program (BD Biosciences, Mountain View, CA) was used for data acquisition and analysis.

Cells

Lymph nodes were harvested, and single-cell suspensions were prepared from each of the mouse lines. For studies of CD4+CD8+ (CD8-) and CD4+CD8− (CD4) T cell subsets, each of the respective populations was purified from whole lymph node cell suspensions using miniMACS microbeads (Miltenyi Biotec, Auburn, CA) and either mouse CD8+ mAb or CD4+ mAb (CD4). The respective T cell subsets were positively selected using a MACS MS1 separation column and miniMACS magnet according to the manufacturer’s protocol (Miltenyi Biotec), achieving >95% purity. Cells were cultured at 37°C in 5% CO2 in IMEM (Life Technologies, Burlington, Canada) supplemented with 10% (v/v) FBS (Life Technologies), 5 × 10−5 μM 2-ME, and antibiotics (1-medium).

Proliferation assays and cell surface marker expression

Proliferation assays were performed by incubating 1 × 105 cells with varying concentrations (0–10 μg/ml) of plate-bound anti-CD3ε (2C11). Cells were cultured in triplicate in a volume of 0.2 ml in flat-bottom 96-well plates, and 1 μCi [3H]thymidine was added for the last 10 h of a 72-h culture period. In some cultures exogenous IL-2 was added at a concentration of 20 U/ml. To assay cell surface marker expression, 1 × 106 cells were incubated in flat-bottom 96-well plates coated with 10 μg/ml 2C11 for various periods of time. The activation markers CD25 and CD69 were analyzed by FACS (described above). In other cultures, 10 μg/ml soluble anti-CD28 (37.51) mAb was included.

CFSE fluorescence assay

Purified CD8+ T cells (1 × 105) were labeled with 1 μM CFSE and incubated with 10 μg/ml plate-bound 2C11 in a flat-bottom 24-well plate for various periods of time. Cells were harvested and stained with the indicated Abs or 7-amino-actinomycin D (7-AAD)3 and subsequently analyzed by FACS (described above).

7-AAD assay

CD8+ T cells (1 × 105) were incubated with 10 μg/ml plate-bound 2C11 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 medium), fixed with 4% paraformaldehyde, and subsequently analyzed by FACS (described above).

Cytokine ELISA

CD8+ T cells (2 × 105) were cultured in 1.0 ml I-medium in a flat-bottom 24-well plate coated with 10 μg/ml 2C11 for 20 h. The amounts of IL-2 and IFN-γ in the supernatant were determined by ELISA. The capture and detection Abs used for IL-2 were JES6-1A12 and JES6-5H4, 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 added in three dilutions, with each plate containing wells for standard. 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 (no. 104, Sigma, St. Louis, MO) was added, and plates were subsequently analyzed with an ELISA plate reader at 405 nm.

Intracellular cytokine assay

CD8+ T cells (1 × 105) were incubated with 10 μg/ml plate-bound 2C11 for 36 h, with the last 6 h in the presence of Golgi Stop (BD PharMingen), and subsequently stained intracellularly for cytokine expression as described previously (23). Briefly, cells were harvested and stained with anti-CD8-PE and anti-CD4-TRICOLOR. Cells were fixed with 2% paraformaldehyde and permeabilized with 0.3% (v/v) saponin in FACS medium. Anti-cytokine Abs were then added; for IL-2, FITC-anti-IL-2 (JES6-5H4) was used, and for IFN-γ, PE-anti-mouse IFN-γ (XMG1.2) was used. Cells were washed with permeabilization buffer and analyzed by FACS.

Cytokine competitive and quantitative RT-PCR (CQ-PCR)

CQ-PCR was used to determine the intracellular levels of IL-2 and TNF mRNA. T cells (2 × 105) were cultured in 1.0 ml I-medium 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 RNaseasy Mini Kit (Qiagen, Valencia, CA). cDNAs were generated from the total RNA preparation as previously described (24). CQ-PCR was performed as previously described (24). Briefly, the amount of cDNA was normalized between p75−/− and wild-type T cells using the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT). The linearized pPQRS plasmid was used as the competitor (gift from R. Locksley). The sequences for the 5′ and 3′ oligonucleotide primers used for IL-2 were, respectively, 5′-CCACTTCAAGCTCTACAGCG-3′ and 5′-CAGGAGAACTGGTATGGGTA-3′.

3 Abbreviations used in this paper: 7-AAD, 7-amino-actinomycin D; AIDC, activation-induced cell death; CQ-PCR, competitive and quantitative PCR; FSC, forward light scatter; HPRT, hypoxanthine phosphoribosyl transferase.
GAAG-3’ and 5’-GATCTCAATCCAGACAGCTGCA-3’. The sequences for the 5’ and 3’ oligonucleotide primers used for TNF-α were, respectively, 5’-GTTCATCGCCAGGACCTCACA-C-3’ and 5’-TCCAGGGCATATGGTGTTCTACACAG-3’. The sequences for the 5’ and 3’ oligonucleotide primers used for HPRT were, respectively, 5’-GT TGATACAGGCACTTTTGTG-3’ and 5’-GAGGTAGGGTGCTGCCCTAGAGC-3’. The PCR products were subjected to electrophoresis on a 2% agarose gel and visualized with ethidium bromide. Densitometry was performed using Alphalmager software (Alpha Innotech, San Leandro, CA).

Results

Proliferative response to TCR cross-linking is reduced in lymph node T cells from p75−/− mice

To determine whether p75 plays a role in the proliferative response of T cells to TCR cross-linking, lymph node cells from B6 and B6-p75−/− mice were stimulated with immobilized anti-CD3ε mAb (2C11) to induce proliferation. It was found that the proliferative response of p75−/− lymph node T cells was significantly reduced (Fig. 1A). We next investigated whether exogenous IL-2 could rescue the hypoproliferative response displayed by p75−/− T cells. As shown in Fig. 1B, the addition of exogenous IL-2 was able to rescue the proliferative response of p75−/− T cells to TCR cross-linking. This suggests that p75−/− T cells are able to respond to IL-2, and that the reduction in the proliferative response to TCR cross-linking may be due at least in part to a lack of IL-2 production. The hypoproliferative response of p75−/− T cells is not due to defects in TCR expression, since these cells express similar levels as wild-type cells (data not shown). The p75−/− and wild-type T cells used in these studies were similar in cell surface expression of activation/memory markers (i.e., CD25, CD69, and CD44; data not shown). Furthermore, a similar proliferative defect in response to TCR cross-linking was observed in p75−/− T cells even after depletion of CD44-positive cells (data not shown). Thus, naïve T cells lacking p75 expression are hypoproliferative in response to TCR cross-linking. This proliferative defect was not observed for p55−/− T cells (data not shown), demonstrating that the biological effect of TNF on T cell proliferation is restricted to p75.

p75 lowers the activation threshold for proliferation by CD4+ and CD8+ T cells

To examine whether p75 modulates the threshold of T cell activation for proliferation, the proliferative response of purified p75−/−/CD8+ T cells to varying doses of 2C11 was determined. As shown in Fig. 2A, CD8+ T cells deficient in p75 required approximately 5-fold greater 2C11 stimulation for an equivalent response by wild-type CD8+ T cells. This result suggests that p75 decreases the threshold of activation for proliferation by lowering the requirement for signals derived from the TCR. Similarly, the proliferative response of CD4+ T cells deficient in p75 was also affected, indicating that p75 plays an important role for both T cell subsets (Fig. 2B). To determine whether exogenous IL-2 could rescue the hypoproliferative response of p75−/− T cells, the proliferation assays were performed in cultures supplied with exogenous IL-2. As shown in Fig. 2, A and B, exogenous IL-2 markedly enhanced the proliferative potential for both cell types. At lower doses of 2C11 exogenous IL-2 was able to rescue the proliferative response of p75−/− CD4+ and CD8+ T cells. Since IL-2 is a critical growth factor for T cell proliferation, this observation is consistent with the hypothesis that the induction of IL-2 in response to TCR cross-linking is limited for T cells deficient in p75.

We noted in Fig. 2A that at higher doses of 2C11 and in the presence of exogenous IL-2, p75−/−/CD8+ T cells displayed a greater proliferative response than wild-type CD8+ T cells. Wild-type CD8+ T cells undergo activation-induced cell death (AICD) as a means of limiting the extent of expansion in an autoregulatory manner (25). We have previously shown that p75−/−/CD8+ T cells are highly resistant to Fas-mediated AICD (26). To determine whether this increase in proliferative response could be due to the resistance of p75−/−/CD8+ T cells to AICD, CD8+ T cells were stimulated with 2C11 in the presence of exogenous IL-2 and subsequently stained with 7-AAD as a means of determining the percentages of viable cells. As shown in Fig. 3A, cultures of p75−/−/CD8+ T cells contained more live cells (74% 7-AAD negative) than wild-type cultures (19% 7-AAD negative). The p75−/− population also contains fewer early apoptotic cells (1%) compared with the wild-type population (19%). This observation is consistent with the hypothesis that the increase in the proliferative response of p75−/−/CD8+ T cells compared with wild-type cells when activated with high concentrations of 2C11 and exogenous...
cells were harvested at 72 h of stimulation with plate-bound 2C11, additional support to the hypothesis that activated p75 compared with wild-type cultures (Fig. 3). The percentages refer to 7-AAD-negative cells (live cells). B, CD4 T cells deficient in p75 are not resistant to AICD. Culture conditions are similar to those in A, except CD4 T cells were used, and the cells were harvested at 78 h. The results were reproduced in three independent experiments.

FIGURE 3. A, CD8 T cells deficient in p75 are resistant to AICD. Cells were harvested at 72 h of stimulation with plate-bound 2C11 exogenous IL-2, stained with 7-AAD, and analyzed using FACS (see Materials and Methods). The percentages refer to 7-AAD-negative cells (live cells). B, CD4 T cells deficient in p75 are not resistant to AICD. Culture conditions are similar to those in A, except CD4 T cells were used, and the cells were harvested at 78 h. The results were reproduced in three independent experiments.

FIGURE 4. p75 CD8 T cells are more susceptible to apoptosis when stimulated with anti-CD3 without exogenous IL-2. CD8 T cells (1 × 10^6) were stimulated with 10 μg/ml plate-bound anti-CD3 alone or in the presence of exogenous IL-2 and harvested after 48 and 72 h. A, The total number of viable cells in each culture was determined using a hemocytometer. Stained B6 wild-type CD8 T cells; □, unstimulated controls; ■, stimulated p75 CD8 T cells. B, Cultures were also stained with 7-AAD and analyzed using FACS (see Materials and Methods). The data shown are representative of three independent experiments performed.

Cells that have undergone division. The hypoproliferative response of p75 T cells was further investigated by examining their susceptibility to apoptosis when stimulated with TCR agonist alone. The number of viable cells recovered after 72 h of anti-CD3 stimulation was much lower for p75 T cells (27). We used this technique to determine the number of cellular divisions by p75 T cells (26). Staining of the p75 T cells de

FIGURE 5. p75 CD8 T cells display marked reduction in the proportion that have undergone division in response to TCR cross-linking. CD8 T cells (5 × 10^6) were labeled with 1 μM CFSE and stimulated with 10 μg/ml plate-bound anti-CD3 alone or in the presence of exogenous IL-2 for 60 h (see Materials and Methods). The shaded gray curve indicates the stimulated cells; the black line shows CFSE-labeled unstimulated cells (negative control). The results were reproduced in three independent experiments.

CFSE

Cell Number

B6.wt

B6.p75

B6.wt + IL-2

B6.p75 + IL-2

CFSE

p75 CD8 T cells display a marked reduction in the proportion that divide in response to TCR cross-linking CFSE is a dye that binds irreversibly to cellular proteins and permits analyses of the parameters and kinetics of cell division events (27). We used this technique to determine the number of cellular divisions by p75 and wild-type CD8 T cells that have been activated by TCR cross-linking. As shown in Fig. 5, there was a marked reduction in the proportion of p75 CD8 T cells that have divided after 60 h of 2C11 stimulation. Wild-type CD8 T cells underwent considerable division, as nearly all cells had decreased CFSE fluorescence relative to unstimulated cells that remained CFSE bright. This indicates that the marked reduction in the proliferative response of p75 CD8 T cells in the absence of exogenous IL-2 is due to a marked reduction in the proportion of cells that have undergone division.
CFSE-labeled cells were also stimulated in the presence of exogenous IL-2 to study whether it could rescue the hypoproliferative response observed for p75–/–CD8+/H11001 T cells. As expected, cultures that were stimulated in the presence of exogenous IL-2 had undergone a greater number of divisions than those in the absence of exogenous IL-2, as reflected in the relative decrease in CFSE fluorescence. Consistent with the results from the [3H]thymidine incorporation assay (Fig. 2), p75–/–CD8+/H11001 T cells stimulated in the presence of IL-2 were able to divide to at least the same extent as wild-type cells. In addition, essentially all the p75–/–CD8+/H11001 T cells that were stimulated in the presence of exogenous IL-2 had undergone division. Together, these results demonstrate that p75–/–CD8+/H11001 T cells are able to use IL-2 when supplied exogenously, and that the induction of this critical growth factor in response to TCR cross-linking may be limited in p75-deficient T cells.

CD28 costimulation partially rescues the hypoproliferative response of p75–/–CD8+/H11001 T cells

To examine whether CD28 costimulation could rescue the hypoproliferative response by p75–/–CD8+/H11001 T cells, the cells were stimulated with anti-CD28 in addition to anti-CD3. As expected, CD28 costimulation increased the proliferative potential of anti-CD3-stimulated wild-type CD8+/H11001 T cells (Fig. 6). This is in agreement with the well-documented decrease in the threshold of activation that CD28 costimulation mediates (5). CD28 costimulation caused a partial increase in proliferative potential by p75–/–CD8+/H11001 T cells, but was only restored to the level of wild-type T cells stimulated with anti-CD3 alone. This indicates that p75 plays an important and nonredundant costimulatory role in T cell activation that is distinct from CD28 costimulation.

p75–/–CD8+/H11001 T cells display a reduction in activation phenotype in response to TCR cross-linking

Acute activation markers such as CD25 and CD69 are expressed at the cell surface during T cell activation and serve as a measure of the extent of activation. Given that T cells deficient in p75 proliferated poorly in response to TCR cross-linking, it was of interest to determine whether these cells also display a defect in acquiring an activated phenotype. The cell surface expression of CD25 in response to TCR cross-linking was important to study, since it is the critical component of the high affinity IL-2R, which is the functional receptor for IL-2 in the physiological context. As shown in Fig. 7, the cell surface expression of CD25 was similar between the two cell types at the earliest time point tested (3 h). However, at 8 h...
of 2C11 stimulation the percentage of cells that were CD25+ was reduced for p75−/− CD8+ T cells compared with the percentage of wild-type cells (50 vs 84%, respectively). Since CD25 expression is essential for optimal IL-2 signaling, the marked reduction in the proportion of cells that are CD25+ for p75−/− CD8+ T cells may be an important factor that contributes to the marked reduction in the proportion of cells that divide in response to TCR cross-linking.

CD69 is an early activation marker of T cells, and its expression gives a measure of the extent of T cell activation. The pattern of induction of CD69 for p75−/− CD8+ T cells stimulated with 2C11 closely parallels that of CD25 (Fig. 7). With greater duration of TCR-mediated stimulation, there is greater recruitment of p75−/− CD8+ T cells into the CD69high population, demonstrating the increased requirement in the amount of TCR-mediated signals to generate a particular response for the p75−/− T cell population compared with wild-type cells.

The forward light scatter (FSC) pattern was also examined in this kinetic study to assess blastogenesis in response to TCR cross-linking. As both the kinetics of induction of CD25 and CD69 indicate that p75−/− CD8+ T cells are activated with delayed kinetics (due to increased requirements for TCR-mediated stimulation in the absence of p75), it would be expected that FSC should also display the same pattern. Indeed, as shown in Fig. 7, the percentage of cells that have increased in size in response to TCR cross-linking is markedly reduced at the 20 h point, and there is greater recruitment of p75−/− CD8+ T cells into the FSChigh population with greater duration of TCR-mediated stimulation.

p75−/− T cells display a reduction in the production of IL-2 in response to TCR cross-linking

Based on the observation that exogenous IL-2 was able to rescue the marked reduction of the proliferative response by p75−/− T cells, the amount of IL-2 produced in response to TCR cross-linking was examined to assess whether this was one of the limiting factors.

The amount of IL-2 that was secreted in response to TCR cross-linking was measured by ELISAs performed on the culture supernatant. As shown in Fig. 8A, there was a marked reduction in the amount of IL-2 in the culture supernatant of p75−/− CD8+ T cells compared with wild-type cells at 20 h of 2C11 stimulation. This suggests that the amount of IL-2 secreted is limiting for the proliferative response of p75−/− CD8+ T cells. Furthermore, when the culture supernatant of p75−/− CD8+ T cells was assayed for IL-2 at 40 h of 2C11 stimulation, the amount of IL-2 was equivalent to the amount produced by wild-type cells at 20 h. This indicates that the production of IL-2 occurs in a delayed manner for p75−/− CD8+ T cells, as a greater duration of TCR-mediated stimulated allows for increased accumulation of TCR-mediated signals. This is consistent with the threshold model of T cell activation, in this case for IL-2 production, such that p75 lowers the requirement for TCR-mediated stimulation.

To study whether the observed reduction in IL-2 production by p75−/− CD8+ T cells arose from a reduction in the transcript, IL-2 mRNA was measured by CQ-PCR. As shown in Fig. 8B, p75−/− CD8+ T cells displayed a 2- to 3-fold reduction in the amount of IL-2 transcript in response to TCR cross-linking compared with wild type. This result suggests that p75 provides a costimulatory signal that lowers the threshold of activation for IL-2 gene induction.

TNF-α is another important cytokine that is expressed during T cell activation (23). Previous studies have shown that TNF-α can enhance the proliferative response of T cells to TCR cross-linking (11). To determine whether p75 is required for TNF-α expression upon TCR-mediated stimulation, the level of TNF-α mRNA was measured using CQ-PCR. It was found that p75−/− CD8+ T cells displayed a modest decrease in the level of TNF-α transcript compared with wild-type cells (Fig. 9). Interestingly, this decrease was not as marked as that for IL-2. This is probably due to differential requirements in the assembly and constitution of the transcription factor complex for the induction of the two cytokine genes (28). Indeed, previous reports have shown that TNF-α is expressed earlier than IL-2 (23), suggesting that the latter cytokine has more stringent requirements in the transcription factor complex toward
its induction. Furthermore, TNF-α was found to be the first cytokine produced by T cells upon activation (23), suggesting that it may be an important early checkpoint for the progression of the T cell response.

**p75<sup>-/-</sup> CD8<sup>+</sup> T cells display a reduction in the expression of IFN-γ in response to TCR cross-linking**

A third cytokine that is transcriptionally regulated and expressed in particularly large amounts by activated CD8<sup>+</sup> T cells is IFN-γ. The total amount of secreted cytokine was measured using ELISA. As shown in Fig. 10A, p75<sup>-/-</sup> CD8<sup>+</sup> T cells produced markedly less IFN-γ than wild-type cells. To examine whether this difference was due to a reduction in the number of cells expressing IFN-γ or to a reduction in the amount of IFN-γ produced per cell, an intracellular cytokine immunostaining assay was used. As shown in Fig. 10B, there was both a marked reduction in the number of cells expressing IFN-γ (25% for wild-type vs 9% for p75<sup>-/-</sup> CD8<sup>+</sup> T cells) as well as a marked decrease in the amount produced per cell, as measured by mean fluorescence intensity. Thus, deficiency of p75 in T cells results in the diminished recruitment of cells in the IFN-γ-expressing population in response to TCR-mediated stimulation as well as a marked reduction in the intracellular levels of cytokine produced per cell.

**Discussion**

T cell activation is a complex process and involves not only the engagement of the TCR with its antigenic ligand, but also a myriad of adhesion molecules and costimulatory ligands that are found on APCs activated by pathogenic stimuli. Engagement of B7-1 and B7-2 on APCs by CD28 molecules on T cells provides a critical signal for cell cycle progression, IL-2 production, and clonal expansion (5). However, it is becoming clear that CD28 is part of a larger family of related counter-receptors that play an essential role in regulating the T cell response. These additional molecules play an important role, together with CD28, in regulating the acquisition of effector function and/or induction of tolerance in T cells (14). In this study we examined the importance of the p75 TNF-α receptor (TNFR-2) for T cell activation and more broadly assessed the role that TNF-α plays as an environmental cue toward eliciting an efficient T cell response.

Previous reports have shown that TNF-α can act through p75 to enhance the proliferative response of T cells to TCR agonists (11, 15, 16). However, the mechanism by which p75 elicits this enhancement has not been determined. Moreover, it is not clear whether p75 performs an essential or redundant function in T cells. We addressed these questions by investigating T cells from p75<sup>-/-</sup> mice. It was determined that p75 performs an important costimulatory function that effectively lowers the threshold of activation and thus lowers the requirement for TCR agonist to produce a given proliferative response. This conclusion is partly based on the observation that a much higher concentration of anti-CD3 is required by p75<sup>-/-</sup> T cells to achieve the same level of proliferation as wild-type T cells. This observation also provides the basis for a novel means of modulating the T cell response.

We investigated the nature of the hypoproliferative response of p75<sup>-/-</sup> T cells. Since T cell proliferation is particularly dependent on signals mediated through the high affinity IL-2R, we assessed the kinetics of expression of CD25, a component of the high affinity IL-2R, and the amount of IL-2 produced in response to TCR cross-linking. It was found that p75<sup>-/-</sup> CD8<sup>+</sup> T cells possess delayed kinetics in the recruitment of cells to the CD25<sup>high</sup> population. This observation can be interpreted according to a threshold
model for T cell activation. In the absence of p75, greater amounts of TCR-mediated signals are required to elicit a particular response. With longer incubation times with TCR agonist, there is a net accumulation and increase in the amount of TCR-mediated signals. Thus, over time there is an increase in the proportion of cells that are CD25^+/. For wild-type CD8^+ T cells, this activation threshold is achieved with much shorter incubation times with anti-CD3 Abs compared with p75^-/-/CD8^+ T cells. This demonstrates the costimulatory importance of p75, in that much less stimulation is required to reach the activation threshold for the induction of CD25 in the entire wild-type CD8^+ population.

Interestingly, the response of p75^-/-/CD8^+ T cells displays a bimodal distribution, giving rise to two apparent populations: those that are CD25^+ and the others that are CD25^-/. The observation that a proportion of p75^-/-/CD8^+ T cells were CD25^+ at the 8 h point may be due to a stochastic phenomenon in which the amount of TCR-mediated signals received permits a particular frequency of CD25^+ cells, similar to the stochastic pattern of effector responses described by Keslo et al. (29). An alternative explanation is that there exists a subpopulation of p75^-/-/CD8^+ T cells that readily up-regulate CD25 and another subpopulation that requires a greater amount of TCR-mediated signals. It is of interest to determine whether subpopulations of CD8^+ T cells have an intrinsically lower threshold of activation.

The threshold model for a particular T cell response can be applied to the induction of cytokines. As Itoh and Germain (28) pointed out, there exists a critical signaling threshold for elicitation of a particular cytokine response, and signals that rise above this point lead to an increase in the overall amount of cytokine produced by a cell population (28). Three cytokines that are expressed during T cell activation are TNF-α, IL-2, and IFN-γ. Consistent with the hierarchical organization of TCR signaling thresholds proposed by Itoh and Germain (28), we observed that the induction of TNF-α was similar between p75^-/- and wild-type CD8^+ T cells, whereas there was a marked decrease in the induction of IL-2 and IFN-γ for p75^-/- T cells. This suggests that the TCR signaling threshold for TNF-α gene expression is lower than those for the other two cytokines studied. This is also consistent with previous studies on the kinetics of cytokine induction, which demonstrate that TNF-α is expressed earlier than IL-2 and IFN-γ (23).
The observation that exogenous IL-2 rescued the hypoproliferative response of p75+/−/CD8+ T cells suggested that its expression might be limiting. Indeed, as determined at the level of secreted cytokine as well as the IL-2 transcript, there is a marked reduction in the level of IL-2 gene induction in the absence of p75. This is consistent with the threshold model of activation, in that greater amounts of TCR-mediated signals are required to reach the threshold for p75+/−/CD8+ T cells. Furthermore, since IL-2 is a critical growth factor and important for the proliferative response, it can account at least in part for the reduction in proliferation observed for p75+/−/ T cells.

The expression of IFN-γ followed the same pattern as IL-2, consistent with the threshold model of activation. There was a significant reduction in the recruitment of p75+/−/CD8+ T cells into the IFN-γ-expressing population as well as a reduction in the intracellular levels per cell. This is interpreted to mean that for wild-type CD8+ T cells, the amount of signals generated at the time point tested was sufficient to reach and rise beyond the threshold of activation. This is reflected in both the significant recruitment of wild-type cells into the IFN-γ-expressing population as well as a marked increase in the intracellular levels per cell relative to p75+/−/CD8+ T cells.

What is the mechanism by which p75 provides costimulation for T cell activation? A model that can account for the costimulatory role of p75 in T cell proliferation is shown in Fig. 11. This model suggests that p75 provides an important signal that recruits and uses distinct signaling pathways, convergence with the pathways activated through the TCR complex. However, curiously through the TCR complex.

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