The Journal of Immunology

An Essential Role for TNF in Modulating Thresholds for Survival, Activation, and Tolerance of CD8+ T Cells

Ioannis Chatzidakis, Georgia Fousteri, Debbie Tsoukatou, George Kollias and Clio Mamalaki

---

References   This article **cites 66 articles**, 36 of which you can access for free at:
http://www.jimmunol.org/content/178/11/6735.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

average

Subscription   Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions   Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts   Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

An Essential Role for TNF in Modulating Thresholds for Survival, Activation, and Tolerance of CD8$^+$ T Cells[1]

Ioannis Chatzidakis,*[†] Georgia Fousteri,*[†] Debbie Tsoukatou,* George Kollias,[‡] and Clio Mamalaki[2]*

TNF and its receptors p55 and p75 are known to be important in the homeostasis of the peripheral immune system. Previous studies have presented apparently contradictory evidence for an in vivo role of TNF in T cells. In this study, we analyzed TNF-deficient mice crossed with the F5 TCR-transgenic animals. We show that endogenous TNF modulates several aspects of homeostasis of peripheral F5 CD8 T cells. We found that F5/TNF$^{-/-}$ mice had reduced numbers of peripheral F5 T cells, F5/TNF$^{-/-}$ CD8 T cells exhibited reduced survival potential, and furthermore that T cell-derived TNF is required for optimum recovery of naive CD8 T cells in lymphopenic hosts, suggesting its involvement in the survival of peripheral CD8 T cells. Both peptide activation and ensuing Ag-induced apoptosis are quantitatively reduced in TNF$^{-/-}$ CD8 T cells. The latter observations can be related to decreased binding activities of NF-κB and NF-ATp observed in Ag-stimulated F5/TNF$^{-/-}$ T cells. Finally, in a CD8 T cell tolerance model, endogenous TNF was necessary for several parameters of CD8 T cell tolerance induction. Collectively, our results provide evidence that endogenous TNF modulates thresholds in several ligand-driven T cell responses. *The Journal of Immunology,* **2007, 178: 6735–6745.**

T he shaping of the peripheral T cell pool depends on TCR engagement by peptide:MHC complexes. TCR engagement is required for survival of naive T cells, activation by pathogen-derived foreign Ags, and induction of tolerance upon encounter of self-Ags in the periphery. Several different factors have being identified that can influence the outcome of the encounter of TCR with its ligands. It has been shown that survival of CD8 T cells requires continuous ligation by MHC molecules (1–3). In addition, a crucial role of cytokines, such as IL-7 in the survival of naive CD8 T cells has been demonstrated (4). Similarly, under conditions of lymphopenia, where homeostasis of the peripheral pool is re-established by proliferation of naive T cells, both interaction with self-peptide:MHC and cytokine signaling are involved (5–7). In contrast high-avidity recognition of peptide-MHC can lead among others to activation or tolerance depending on the dose and duration of antigenic stimulus as well as the activation status of APCs (8).

During an immune response TNF is expressed early by activated CD8 T cells (9) and there are several studies that suggest a role of TNF on T cell activation and TCR signaling. Thus endogenously secreted (10) or addition of exogenous TNF (11–14) have been shown to enhance T cell activation. Chronic exposure of T cells to

TNF has been shown to down-regulate TCRζ expression and to uncouple distal TCR-signaling pathways (15–17), thus suggesting a direct effect of TNF on TCR signaling.

Although initial studies with TNF knockout mice did not reveal an involvement of TNF in T cell responses (18–20), more recent studies suggest that TNF/TNF receptors (TNFR)[3] pathways act as T cell regulators in vivo (21–26).

In this study, we directly address the in vivo role of endogenous TNF on CD8 T cell function by introducing the F5 TCR-transgenic model (27) in the TNF-deficient background (18). F5 T cells express TNF upon Ag stimulation and this system allows us to study the behavior of F5 T cells that have developed in the absence of endogenous TNF. We demonstrate that CD8 T cells that develop in the absence of TNF have higher thresholds for TCR responses. This results in impaired survival, homeostatic proliferation, and peptide-induced activation of naive peripheral T cells accompanied by reduced NF-κB and NFAT activation; in contrast, we demonstrate that recognition of self-Ags in the absence of endogenous TNF leads to impaired tolerance of autoreactive T cells. Collectively, our results reveal a novel role of TNF in shaping the peripheral lymphoid pool of CD8 T cells, by modulating thresholds of TCR signaling.

Materials and Methods

Mice

Generation of F5 TCR-transgenic (27), H2NP47-transgenic (28), Rag1$^{-/-}$ mice (29), GFP-transgenic mice (30), and TNF$^{-/-}$ (18) mice has been previously described. All mice were backcrossed to the C57BL/10 background. Mice were intercrossed and maintained in the Institute of Molecular Biology and Biotechnology (IMBB) conventional colony. All experiments were conducted according to institutional guidelines.

Cell staining and flow cytometry

Single-cell suspensions of spleen and thymus were prepared and erythrocytes were removed from splenocyte preparations by quick water lysis. A total of

*Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, Vassilika Vouton, Crete, Greece; [†]Department of Biology, University of Crete, Crete, Greece; and [‡]Biomedical Sciences Research Center Alexander Fleming, Vari, Greece

[2] Address correspondence and reprint requests to Dr. Clio Mamalaki, Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, Vassilika Vouton, P.O. Box 1385 GR 711 10 Heraklion, Crete, Greece. E-mail address: mamalaki@imbb.forth.gr

[3] Abbreviations used in this paper: TNFR, TNF receptor; PI, propidium iodide; AICD, activation-induced cell death; SNARF, carboxylic acid acetate succinimidyl ester.

10^6 cells were stained at 4°C for 30 min and analyzed with flow cytometry. Abs used were anti-CD8-FITC, anti-CD8-PE, anti-CD4-PE, anti-CD69-FITC, anti-CD44-PE, anti-CD25-PE, anti-p75-PE, anti-p55, hamster IgG1 λ1-PE isotype control, hamster IgG1 κ isotype control, anti-hamster mAb mixture biotin-conjugated (BD Pharmingen), anti-Vβ11-biotin, (Serotec), followed by streptavidin-Red 670 (Invitrogen Life Technologies). Annexin V binding in combination with propidium iodide (PI) staining was performed according to the manufacturer's instructions (Immunotech). Removal of erythrocytes from blood samples before cytometric analysis was conducted using FACS Lysing Solution (BD Biosciences). Flow cytometry was performed on a Coulter Epics Elite or FACSCalibur (BD Biosciences) cytometer and analyzed with WinMDI software. Whenever indicated, statistical significance was estimated by two-tailed t test.

Proliferation of T cells and cell culture

Cell proliferation was assayed by labeling with CFSE (Molecular Probes) as previously described (31). Briefly, single-cell suspensions of spleens were prepared in HBSS supplemented by 5% FCS 10 mM HEPES, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine. Erythrocytes were removed using Lympholyte-M (Cedarlane Laboratories) and splenocytes (5×10^7/ml) were labeled with 10 μM CFSE in PBS, for 10 min at 37°C. Labeling was stopped with 5 vol of ice-cold RPMI 1640, 10% FCS, for 5 min on ice, followed by three washing cycles in HBSS, 5% FCS. Cells (1×10^6/ml) were stimulated with irradiated (2000 rad) C57BL/10 splenocytes (3×10^6/ml) preloaded with the appropriate concentration of influenza NP68 peptide (NP366–374) as previously described (27). Analysis of CFSE profile was performed by flow cytometry.

For anti-CD3 stimulation, CFSE-labeled splenocytes were incubated with indicated amounts of plate bound anti-CD3 Ab. Binding of anti-CD3 Ab was performed in 1× PBS for 3 h at 37°C followed by two washes with ice-cold 1× PBS.

[^{3}H]Thymidine incorporation was assayed essentially as previously described (27). Single-cell suspensions of splenocytes were stimulated in the same way as CFSE-labeled cells. Normalization on equal numbers of CD8$^+$ cells was performed keeping a constant ratio of C57BL/10 splenocytes:CD8$^+$ T cells. After 40 h of culture, cells were pulsed with [^{3}H]thymidine (Amersham Biosciences; 1 μCi/well) for 6 h.

Adoptive transfers

Erythrocytes were removed by quick water lysis of splenocytes; cells were CFSE- or carboxylic acid, acetate succinimidyl ester; Molecular Probes (SNARF)-labeled, where appropriate, and a volume of 200 μl, containing the indicated numbers of CD8$^+$ T cells, was administered i.v. For SNARF labeling, 5×10^7/ml splenocytes were incubated with 25 μM SNARF1 essentially in the same way as with CFSE.

In vivo cytotoxic assay

In vivo cytotoxic assay was performed similarly as previously described (32). Specifically, splenocytes from C57BL/10 mice were pulsed with 1 μM NP68 peptide for 90 min at 37°C, washed, and labeled with CFSE concentration of 10 μM, as described. Uncoated splenocytes were labeled at CFSE concentration of 1 μM. A total of 5×10^6 splenocytes from each population were injected i.v. in mouse recipients. Specific cytotoxicity was determined by analyzing splenocytes from recipients 19 h after transfer. Labeled cells with differential CFSE intensity could be quantitated by flow cytometry. To obtain specific cytotoxicity values, the ratio between CFSE^{low} (uncoated) and CFSE^{high} (NP68-coated) was calculated.

Nuclear extracts preparation and EMSA

Preparation of nuclear extracts was conducted as described with some minor modifications (33). Single-cell suspensions from spleen and thymus were prepared and $2–6 \times 10^7$ cells were lysed in 10 mM Tris-HCl (pH 8.0), 0.32 M sucrose, 3 mM CaCl$_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, and 0.5% (v/v) Nonidet P-40, for 3 min on ice. Nuclei were washed once with lysis buffer without Nonidet P-40 and frozen and thawed three times in 20% glycerol, 25 mM HEPES (pH 7.9), 115 mM KCl, 2 mM EDTA, 0.1% (v/v) Nonidet P-40, 1 mM DTT, 0.5 mM PMSF, 10 mM NaF supplemented with protease inhibitor mixture tablet (complete mini EDTA-free; Roche).

Binding reactions were conducted using 2–5 μg of nuclear protein (normalized for equal numbers of CD8$^+$ cells) and 4×10^4 cpm ($3–5 \times 10^5$ cpm/ng) of ^{32}P-labeled double-stranded oligonucleotides. NF-κB binding was assayed in 10 mM HEPES (pH 7.9), 50 mM KCl, 2 mM MgCl$_2$, 4 mM spermidine, 100 ng/ml BSA, 20 nM zinc acetate, 0.05% Nonidet P-40, and 400 μg/ml poly dI-dC, for 40 min on ice. Protein-DNA complexes were separated from free probe on 5% nondenaturing acrylamide gels. For su-

pershifts, 0.5 μg of anti-p50 polyclonal Ab (Santa Cruz Biotechnology) were incubated with extracts on ice for 30 min before the addition of probe. For AP-2, the binding buffer contained 100 μg/ml poly dI-dC NF-AT binding of thymus nuclear extracts was performed for 30 min at room temperature followed by additional incubation for 30 min on ice in 67 μg/ml poly dI-dC. For supershifts, 0.5 μg of anti-NF-ATp mAb (Santa Cruz Biotechnology) were added to reactions before incubation on ice. Sp1 binding of thymus nuclear extracts was performed for 30 min at room temperature in 50 μg/ml poly dI-dC. The oligonucleotides used were: a sequence from the Igκ enhancer (5'-GATCCAGAGGGGACTTTCCGAGAGGCCAT-3') containing an NF-κB-binding site or double-stranded oligonucleotide (5'-GGATCGAACTGACCGCCCGCGGC-3') containing an AP-2-binding site, or a sequence from the IL-4 promoter (5'-TGGTGTAATAAAATTT TCCAATGT-3') containing an NF-AT binding site or double-stranded oligonucleotide (5'-TCGATTCGATCGGGGCGGGGCGAGC-3') containing an Sp1-binding site.

Results

Optimum homeostasis of naive CD8$^+$ T cells requires endogenously expressed TNF

The first indication about the role of endogenous TNF in the survival and maintenance of peripheral pool of CTLs came from the observation that introduction of F5-transgenic mice into the TNF-deficient background led to a decrease in the number of F5 T cells in the periphery of F5/TNF$^{-/-}$ mice. Flow cytometric analysis of spleen cells from F5/TNF$^{-/-}$ mice showed ~50% reduction in both the percentage and absolute number of peripheral CD8$^+$Vβ11$^+$ (F5 TCR-β chain) transgenic T cells compared with F5 mice (Fig. 1, *a* and *b*). This reduction, although less pronounced, was also observed in the Rag1$^{-/-}$ background where all T cells express the F5 TCR (data not shown). Similar reduction in percentages of F5/TNF$^{-/-}$ CD8$^+$ T cells was also observed in the mesenteric lymph nodes (data not shown). It is unlikely that the reduction observed in the number of peripheral F5 T cells is due to minor histocompatibility differences between parental genetic backgrounds, because CD8 T cells from F5/TNF$^{-/-}$ mice are phenotypically naive, i.e., CD44^{low}, CD25^{low}, CD69$^-$. The cellularity and distribution of subpopulations in F5/TNF$^{-/-}$ thymi were apparently normal (data not shown) indicating that the shrunken F5 peripheral pool in F5/TNF$^{-/-}$ mice was not due to altered thymic selection but could be attributed to defective survival of peripheral F5/TNF$^{-/-}$ CD8$^+$ T cells. To directly address this possibility, we labeled splenocytes from F5/Rag1$^{-/-}$ and F5/Rag1$^{-/-}$/TNF$^{-/-}$ mice with two different intracellular dyes (SNARF and CFSE, respectively) and adoptively transferred them as a mixture in C57BL/10 mice. F5 T cell numbers from both donors declined considerably over a 1-wk period. However, while equal numbers of F5/Rag1$^{-/-}$ and F5/Rag1$^{-/-}$/TNF$^{-/-}$ donor T cells were transferred at the time of injection, 1 wk later F5/TNF$^{-/-}$ T cells comprised only about ten percent of donor T cells (Fig. 1*c*) which denotes a compromised potential for survival when compared with their TNF$^{+/+}$ counterparts. To exclude that the observed differential ability of survival was an artifact due to the different dye used for each cohort of T cells, we performed the same experiment now using CFSE for F5/Rag1$^{-/-}$ and SNARF for F5/Rag1$^{-/-}$/ TNF$^{-/-}$ donor T cell labeling, obtaining identical results with the previous experiment (Fig. 1*d*). To rule out the slight possibility that rapid division of labeled cells made them undetectable, equal number of T cells from F5/GFP/Rag1$^{-/-}$ and F5/GFP/Rag1$^{-/-}$/ TNF$^{-/-}$ mice (that express GFP in all their T cells) were labeled with SNARF and transferred separately to C57BL/10 hosts. Fig. 1*e* shows that decline of F5/GFP/TNF$^{-/-}$ T cells is more rapid than their TNF$^{+/+}$ counterparts. Furthermore, no divisions were detected on days 3, 5, 6, and 7 (data not shown) by CFSE or SNARF dilution in any of the above experiments. It appears, therefore, that F5 T cells that have developed in the absence of endogenous TNF
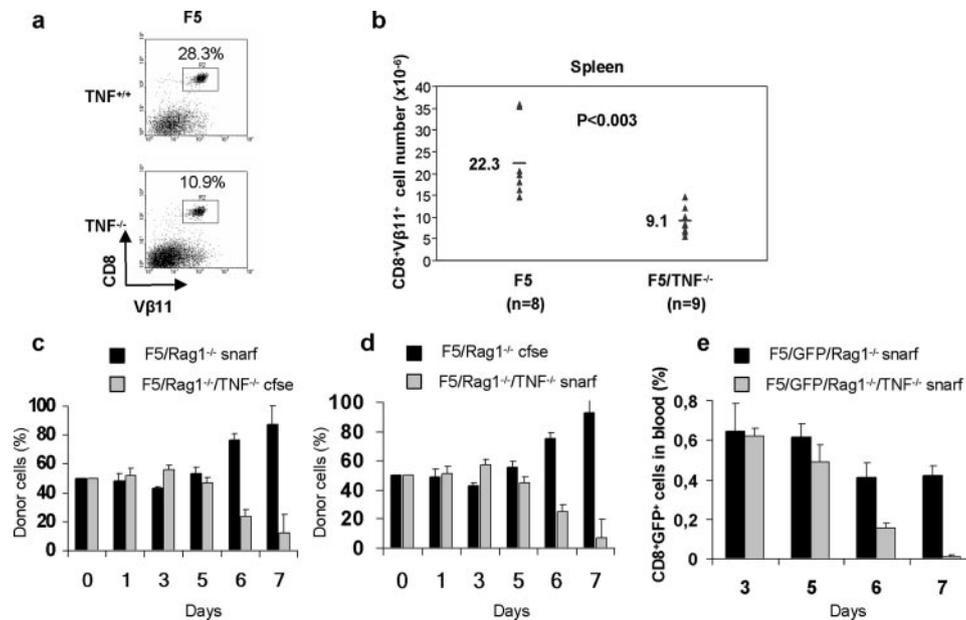
FIGURE 1. Reduced numbers and lifespan of F5/TNF$^{-/-}$ T cells. *a* and *b*, Six- to 12-wk-old mice were sacrificed and spleen cell suspensions were analyzed by flow cytometry. Absolute CD8$^+$Vβ11$^+$ spleen cell numbers were calculated in F5/TNF$^{+/+}$ and F5/TNF$^{-/-}$ mice. ▲, Individual mice and bars indicate mean values. Graph represents a collection of three independent experiments. *c*, Splenocytes from F5/Rag1$^{-/-}$/TNF$^{+/+}$ and F5/Rag1$^{-/-}$/TNF$^{-/-}$ mice containing 8 × 10^6 CD8$^+$ cells were labeled with the intracellular dyes SNARF and CFSE, respectively, mixed, and subsequently transferred to C57BL/10 mice. *d*, Results from a similar experiment, but now splenocytes from F5/Rag1$^{-/-}$/TNF$^{+/+}$ mice were CFSE labeled and splenocytes from F5/Rag1$^{-/-}$/TNF$^{-/-}$ mice were SNARF labeled. For both cases, recipients were analyzed for CD8$^+$Vβ11$^+$ percentage in peripheral blood on days 1, 3, 5, 6, and 7. Results are shown as percentage of the indicated genotype of F5 cells among donor F5 cells. *e*, Splenocytes from F5/GFP/Rag1$^{-/-}$/TNF$^{+/+}$ or F5/GFP/Rag1$^{-/-}$/TNF$^{-/-}$ mice containing 8 × 10^6 CD8$^+$ cells were labeled with SNARF and transferred to C57BL/10 mice separately. Hosts were analyzed on days 3, 5, 6, and 7 for the presence of CD8$^+$GFP$^+$ cells in blood.

have defective peripheral survival capabilities and this cannot be rescued by TNF in the host spleen, because C57BL/10 acceptor mice are TNF$^{+/+}$.
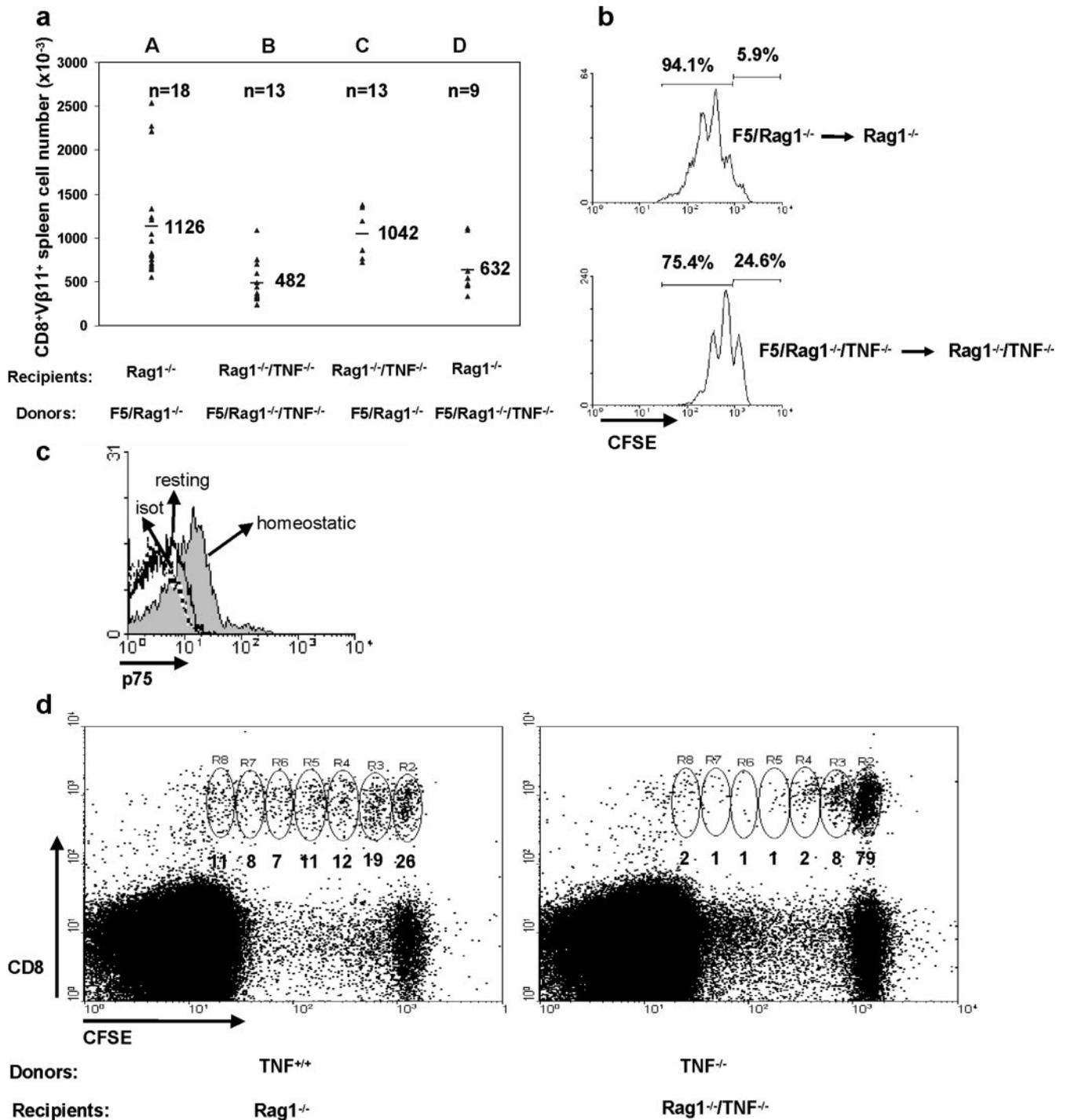
Homeostatic expansion of CD8$^+$ T cells has been shown to be a mechanism of restoring the pool of peripheral T cells under lymphopenic conditions (34–36). This phenomenon often referred to as homeostatic proliferation or lymphopenia induced proliferation occurs through an IL-7 and class I MHC-dependent mechanism (6).

To investigate the role of TNF in homeostatic expansion of F5 T cells, we compared recovery of adoptively transferred F5 T cells in Rag1$^{-/-}$ lymphopenic hosts in conditions of TNF sufficiency or deficiency; we found that, 26 days following transfer, <50% of F5 T cells are recovered when both donor cells and recipient mice are TNF-deficient compared with when they are TNF-sufficient (Fig. 2*a*, columns A and B). Moreover, by transferring F5 T cells to TNF$^{-/-}$/Rag1$^{-/-}$ hosts and F5/TNF$^{-/-}$ T cells to Rag1$^{-/-}$ hosts (Fig. 2*a*, columns C and D), we found out that it is the genotype of donor T cells that mainly determines F5 T cell recovery, indicating that T cell-derived TNF plays a positive role in homeostatic expansion of F5 T cells.

To test directly whether the recovery of fewer F5/TNF$^{-/-}$ T cells was related to impaired homeostatic proliferation rather than increased cell death, we determined the proliferation profile of F5 T cells after adoptive transfer to lymphopenic recipient mice. Fig. 2*b* shows that when both donor and recipient mice were of wild-type TNF, at least three divisions were detectable by day 7 with only 6% of F5 T cells remaining undivided; in contrast in the absence of TNF from donors and recipients, proliferation of donor F5/TNF$^{-/-}$ T cells was delayed by approximately one division with 25% of them still being undivided (Fig. 2*b*). Thus, reduced recoveries of donor F5/TNF$^{-/-}$ T cells are, at least partly, due to their impaired homeostatic proliferation potential.

To provide an insight about the relative contribution of each TNFR to homeostatic proliferation of CD8 T cells, we analyzed surface expression of p55 and p75 on homeostatically expanding CD8 T cells from F5/Rag1$^{-/-}$ donor mice. As shown in Fig. 2*c*, p75 is expressed in homeostatically proliferating F5 CD8 T cells on day 2, whereas we were unable to detect p55 expression (data not shown). Similar expression patterns are obtained on day 5 as well (data not shown). Interestingly, p75 expression is more pronounced compared with resting naive CD8 T cells, which for the first time demonstrates that p75 is up-regulated by homeostatic proliferation. Considering the crucial role of the IL-7-IL-7R pathway on homeostatic proliferation of T cells (4–6), we compared surface expression of IL7-Rα on lymphopenia-induced cycling F5/Rag1$^{-/-}$ and F5/Rag1$^{-/-}$/TNF$^{-/-}$ CD8 T cells. No direct role of TNF on IL-7Rα was revealed because no difference was observed on days 2 and 5 following transfer (data not shown).

To rule out the possibility that the observed differences in homeostatic proliferation were restricted to the F5 specificity, we transferred polyclonal C57BL/10 (TNF$^{+/+}$) and TNF$^{-/-}$ CFSE-labeled T cells in Rag1$^{-/-}$ and Rag1$^{-/-}$/TNF$^{-/-}$ recipient mice, respectively, and compared their proliferation profile 3 days after transfer. As depicted in Fig. 2*d*, donor TNF$^{+/+}$ CD8$^+$ T cells have undergone considerably more divisions than TNF$^{-/-}$ ones, which is in good agreement with the transfer experiments performed with the F5 specificity, indicating that most if not all of CD8$^+$ T cell clones require TNF for maximal homeostatic proliferation. The ratio of CD8$^+$CFSE$^+$:CD8$^-$CFSE$^+$ of TNF$^{-/-}$ cells was calculated and found similar before and after transfer, thus indicating that accumulation of undivided CD8$^+$ cells (Fig. 2*d*, *right panel*) is due to their impaired proliferative capacity rather than death of divided cells.

FIGURE 2. Impaired CD8 T cell homeostatic expansion in the absence of TNF. *a*, Splenocytes from F5/Rag1$^{-/-}$/TNF$^{+/+}$ and F5/Rag1$^{-/-}$/TNF$^{-/-}$ mice, containing 3×10^6 CD8$^+$ cells, were transferred to TNF$^{+/+}$ and TNF$^{-/-}$ lymphopenic hosts as indicated. Hosts were sacrificed on day 26 and CD8$^+$Vβ11$^+$ spleen cells were calculated. ▲, Individual mice and bars indicate mean values. *b*, Flow cytometry analysis of CFSE-labeled CD8$^+$Vβ11$^+$ cells on day 7 following transfer. *c*, On day 2 following transfer, homeostatically expanded CD8$^+$cells from hosts and resting naive CD8$^+$ cells from F5/Rag1$^{-/-}$ mice were analyzed for surface p75 expression (dotted line: isotypic control, solid line: F5/Rag1$^{-/-}$ CD8$^+$ cells, shaded line: CD8$^+$ cells from lymphopenic hosts). *d*, Splenocytes from C57BL/10 (TNF$^{+/+}$) and TNF$^{-/-}$ mice containing 6×10^6 CD8$^+$ cells were transferred to Rag1$^{-/-}$ and Rag1$^{-/-}$/TNF$^{-/-}$ hosts, respectively. Hosts were sacrificed on day 3 and CFSE profile of CD8$^+$ cells was determined by flow cytometry. Each number indicates the percentage of CD8$^+$ cells belonging to the above division.

Because numbers of T cells in adult TNF$^{-/-}$ and wild-type mice are not different, we reasoned that a role of TNF in the optimum maintenance of homeostatic equilibrium would be apparent early in life, when seeding of peripheral lymphoid organs is not complete and a lymphopenic state occurs naturally (37, 38). To test this hypothesis, we compared percentages and absolute numbers of CD4 and CD8 cells in spleens of TNF$^{-/-}$ and wild-type mice at day 17 of age; at this age, the number of T cells is half the number of the one found in adult mice (Table I and Ref. 39). Table I shows a significant reduction in both percentage and number of CD8 and CD4 T cells in TNF$^{-/-}$ mice, thus providing direct evidence in the role of TNF in shaping of naive peripheral T cell repertoire.

Table I. *Less accumulation of peripheral T cells in young TNF$^{-/-}$ mice[a]*

|	% CD4	CD4$^+$ ($\times 10^6$)	% CD8	CD8$^+$ ($\times 10^6$)
C57BL/10 17 day ($n = 11$)	12.0 ± 1.37	8.22 ± 2.26[b]	5.95 ± 0.85	3.73 ± 1.05[c]
TNF$^{-/-}$ 17 day ($n = 15$)	8.50 ± 1.48	4.25 ± 0.74[b]	4.25 ± 0.10	2.13 ± 0.48[c]
C57BL/10 adult ($n = 5$)	25.4 ± 3.61	39.9 ± 7.49	14.3 ± 1.84	20.1 ± 5.60
TNF$^{-/-}$ adult ($n = 4$)	24.8 ± 4.34	36.0 ± 6.60	13.11 ± 1.56	18.00 ± 2.32

[a] Seventeen-day-old C57BL/10 or TNF$^{-/-}$ mice were sacrificed and absolute numbers of CD4$^+$ and CD8$^+$ splenocytes were determined by flow cytometry. Results represent a collection of three independent experiments. For comparison, average numbers from C57BL/10 and TNF$^{-/-}$ adult mice are shown (all p values between adult C57BL/10 and TNF$^{-/-}$ adults were >0.38).
[b] Value of $p < 3 \times 10^{-5}$.
[c] Value of $p < 2 \times 10^{-4}$.

The degree of proliferation of T cells in lymphopenic hosts has been shown to be influenced by the TCR specificity and to be related to the affinity of the TCR for self- peptides:MHC. A possible explanation for reduced numbers in young TNF$^{-/-}$ mice can be that their T cells have reduced functional avidity for peripheral selecting ligands and as a consequence reduced rate of
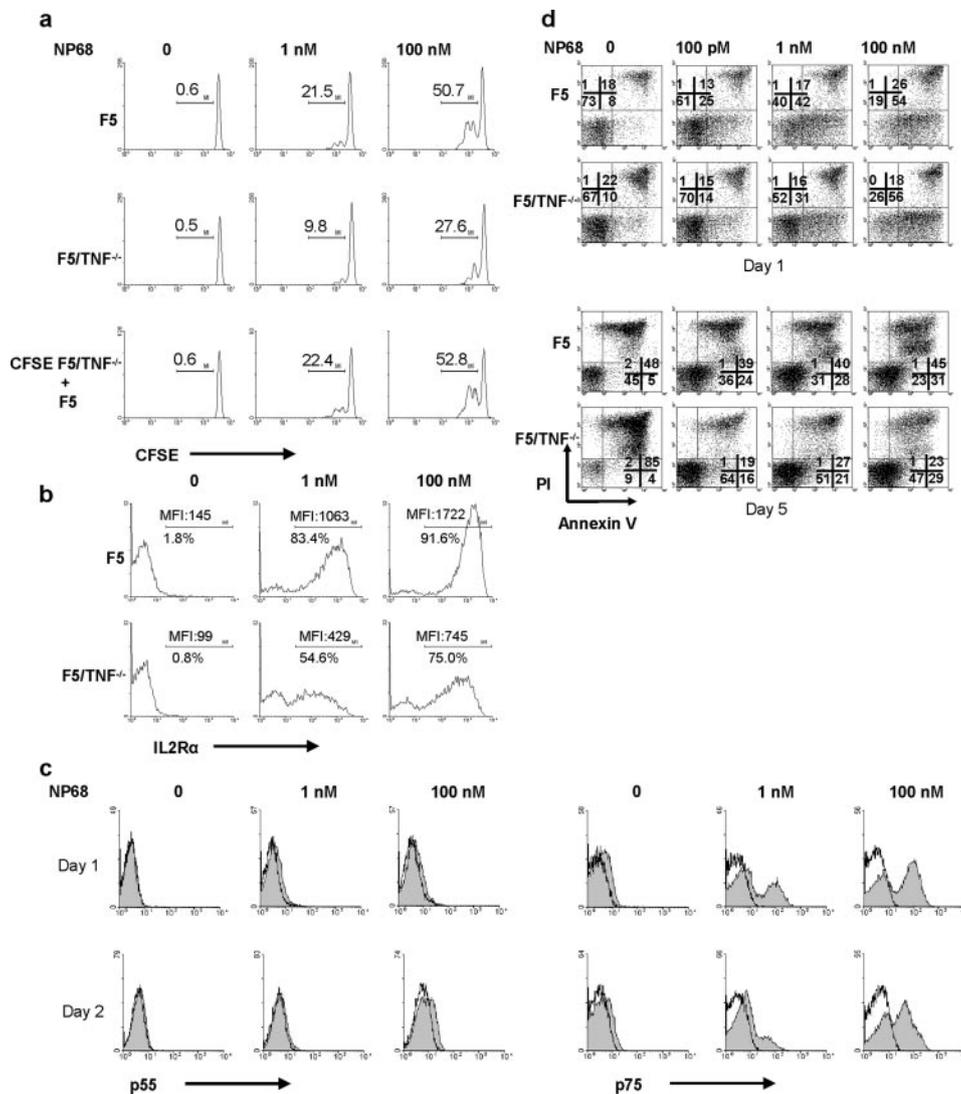
FIGURE 3. Defective activation and AICD of F5/TNF$^{-/-}$ T cells in vitro. Splenocytes from the below-mentioned mice were cocultured with irradiated C57BL/10 spleen cells loaded with various concentrations of NP68 peptide and cultures were analyzed on day 2. *a*, CFSE profile of CD8$^+$ T cells from F5/Rag1$^{-/-}$ or F5/Rag1$^{-/-}$/TNF$^{-/-}$ mice (*top* and *middle panels*, respectively). CFSE profile of labeled F5/Rag1$^{-/-}$/TNF$^{-/-}$ CD8$^+$ T cells cocultured with unlabeled F5/Rag1$^{-/-}$ CD8$^+$ T cells in a ratio 1:4, respectively (*lower panel*). Percentages denote the fraction of CD8$^+$ cells that have undergone at least one division. *b*, Splenocytes from F5/Rag1$^{-/-}$ or F5/Rag1$^{-/-}$/TNF$^{-/-}$ mice were analyzed for expression of stimulation-related surface molecule IL-2Rα. All histograms were gated on CD8$^+$ lymphocytes. Mean fluorescence intensity (MFI) of cells positive for the examined surface molecule. Similar results were obtained in at least two independent experiments. *c*, Surface p55 and p75 expression on NP68-stimulated F5/Rag1$^{-/-}$ CD8$^+$ T cells after 1 and 2 days in culture (solid line: isotypic control, shaded line: a-p55 or a-p75). *d*, Spleen cells from F5/Rag1$^{-/-}$ or F5/Rag1$^{-/-}$/TNF$^{-/-}$ were cultured in the presence of various concentrations of soluble NP68 peptide. On days 1 and 5, total CD8$^+$ cells were analyzed for annexin V binding and PI staining. For each graph, live, nonapoptotic CD8$^+$ cells are depicted in the *low left area*; early apoptotic CD8$^+$ cells are depicted in the *bottom right*; and secondary necrotic are depicted in the *upper right*.
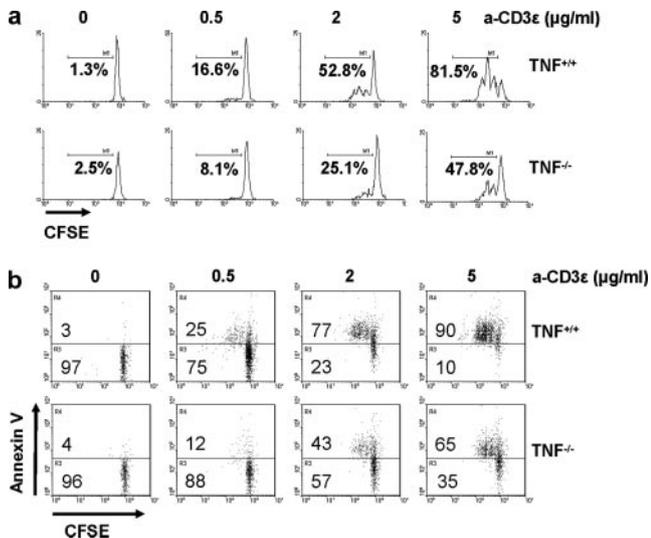
FIGURE 4. TNF is indispensable for optimal proliferation of CD8$^+$ T cells in vitro. Splenocytes from TNF$^{+/+}$ and TNF$^{-/-}$ mice were cultured on plates precoated with varying concentrations of anti-CD3 mAb and cultures were analyzed by flow cytometry on day 2. *a*, CFSE profile of CD8$^+$ T cells from indicated mice. Percentages denote the fraction of CD8$^+$ T cells that have divided at least once. *b*, Cultures were analyzed for annexin V binding. Percentages in the *upper region* indicate early apoptotic cells among live CD8$^+$ T cells. Dot plots were gated on live CD8$^+$ cells as judged by forward and side scatter.

proliferation. In contrast, the observed phenotype can be attributed to impaired peripheral survival of early thymic emigrants with low avidity for self-ligands resulting in reduced numbers of peripheral

T cells. Later in life, there is no apparent difference in cell number probably because of accumulation and/or expansion of T cells with high avidity.

### Altered response of TNF$^{-/-}$ CD8$^+$ T cells after TCR stimulation

To examine high-affinity interactions of F5 TCR with agonist peptide:MHC in the absence of endogenous TNF, CFSE-labeled F5/Rag1$^{-/-}$, and F5/Rag1$^{-/-}$/TNF$^{-/-}$ T cells were in vitro stimulated with increasing concentrations of the NP peptide. As shown in Fig. 3*a*, F5/TNF$^{-/-}$ T cells proliferated poorly in response to Ag when compared with their wild-type counterparts. Similar impaired dose response was observed in [^{3}H]thymidine incorporation experiments (data not shown). The observed defective proliferation can be rescued by paracrine production of TNF, as demonstrated by experiments where CFSE-labeled F5/TNF$^{-/-}$ T cells are activated in the presence of excess unlabeled F5/TNF$^{+/+}$ CD8 T cells (Fig. 3*a*, *lower panel*). F5/TNF$^{+/+}$ CD8 T cells produce high amounts of TNF from as early as 6 h to at least 24 h after antigenic stimulation, as judged by intracellular staining (data not shown).

In response to Ag, up-regulation of the activation marker CD25 (IL-2Rα chain) was far less pronounced in F5/TNF$^{-/-}$ T cells (Fig. 3*b*); less surface expression was also observed for the activation markers CD44 and CD69 (data not shown). Addition of exogenous IL-2 in the culture of stimulated F5/TNF$^{-/-}$ T cells did not restore activation at the same level as in TNF wild-type cells (data not shown) indicating that impaired proliferation was not due solely to defective IL-2 production.

Next, we monitored p55 and p75 surface expression levels on F5 T cells on days 1 and 2 after antigenic activation. As shown in Fig.
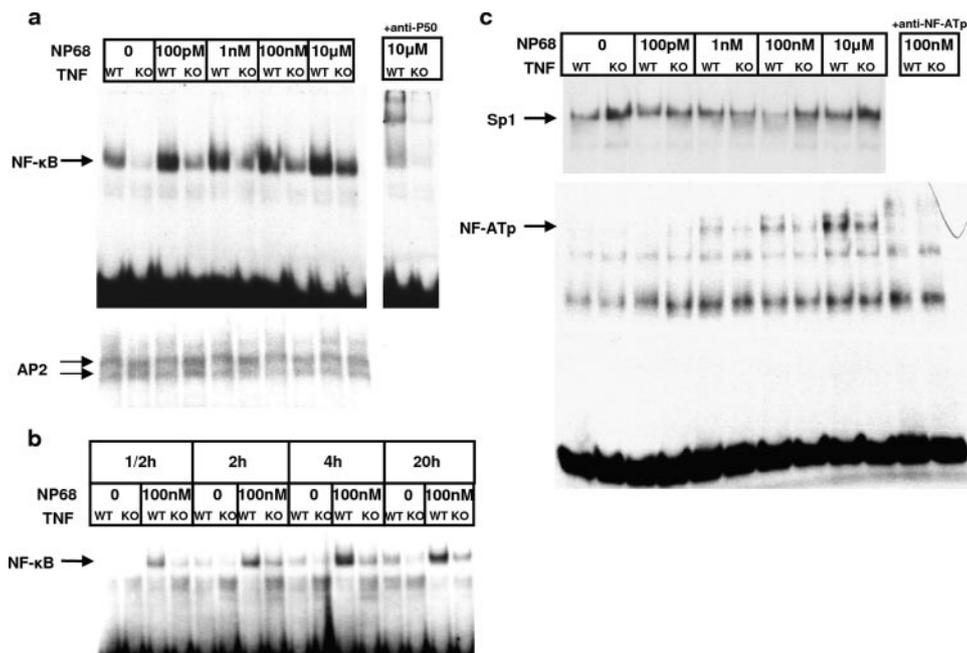
FIGURE 5. Decreased NF-κB and NF-AT DNA-binding activity in F5/TNF$^{-/-}$ T cells. A total of 3×10^7 splenocytes were harvested from F5/Rag1$^{-/-}$ (wild type (WT)) or F5/Rag1$^{-/-}$/TNF$^{-/-}$ (knockout (KO)) mice and stimulated with the indicated concentrations of NP68 peptide. Nuclear extracts were prepared and assayed for NF-κB-binding activity with EMSA. *a*, Peptide dose response of WT or KO F5 T cells after 4 h stimulation. For the supershift, a polyclonal anti-p50 Ab was used. AP-2 binding to an appropriate oligonucleotide was used to verify similar quantity/quality among samples. One representative experiment of three performed is shown. *b*, Time course of NP68 stimulation of F5 WT and KO T cells. *c*, Thymocytes from F5/Rag1$^{-/-}$ (WT) or F5/Rag1$^{-/-}$/TNF$^{-/-}$ (KO) mice were stimulated with the indicated concentrations of NP68 peptide for 30 min, and nuclear extracts were assayed for NF-AT-binding activity with EMSA. NF-ATp (NF-ATc2/NF-AT1) was found to be one of the components of the inducible complex as confirmed by supershift with anti-NF-ATp-specific Ab. Sp1 binding to an appropriate oligonucleotide was used to verify similar quantity/quality among samples. One representative experiment of three performed is shown.
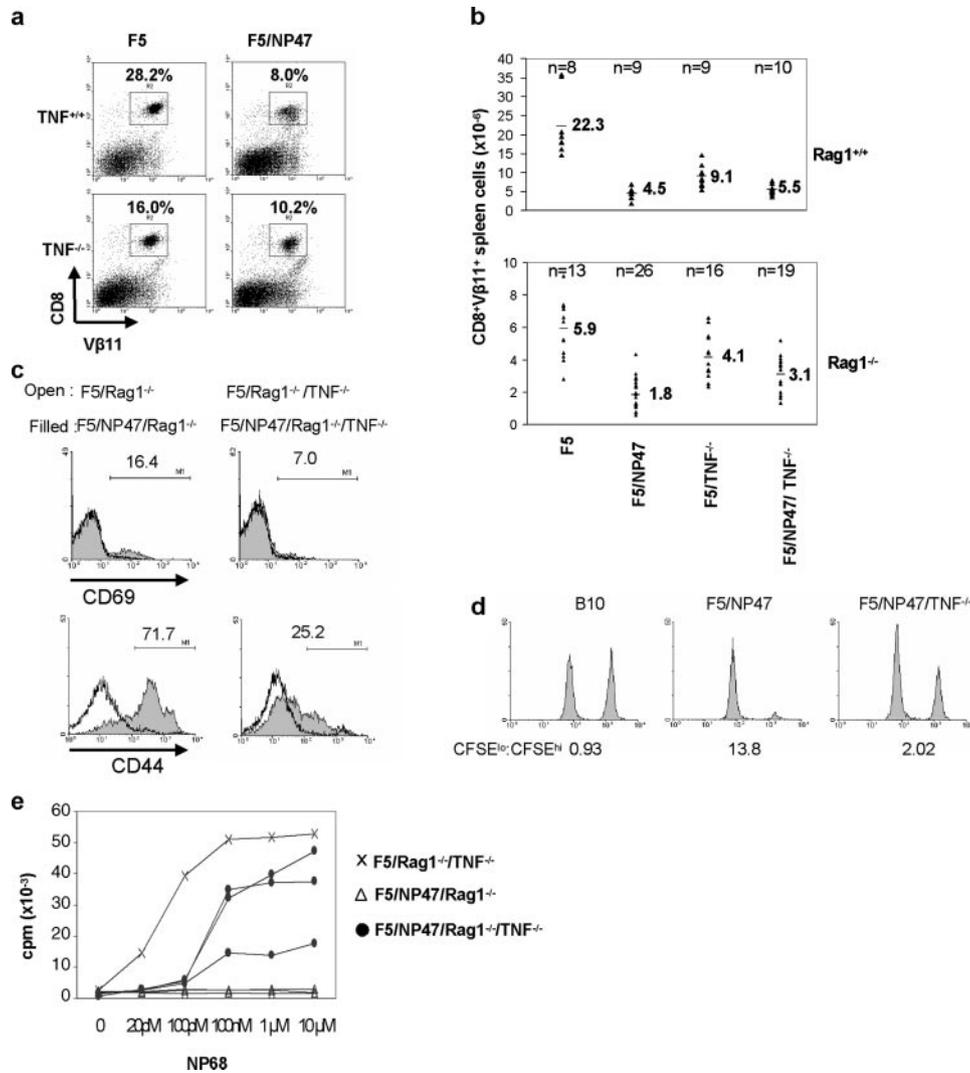
FIGURE 6. TNF deficiency affects critical parameters of F5 T cell tolerance. *a* and *b*, Six- to 12-wk-old mice were sacrificed and spleen cell suspensions were analyzed by flow cytometry for CD8 and Vβ11 staining. Absolute CD8$^+$Vβ11$^+$ spleen cell numbers were calculated in F5/TNF$^{-/-}$ and F5/NP47/TNF$^{-/-}$, as well as F5 and F5/NP47 control mice, in both Rag1$^{+/+}$ and Rag1$^{-/-}$ backgrounds. ▲, Individual mice and bars indicate mean values. Each graph represents a collection of at least three independent experiments. *c*, Expression of activation markers CD44 and CD69 on CD8$^+$ gated cells is shown. Percentages refer to CD44$^+$ or CD69$^+$ cells in the CD8$^+$ population. *d*, In vivo cytotoxicity assay. A total of 5×10^6 splenocytes from C57BL/10 mice labeled with low concentration of CFSE (CFSE^{low}) were mixed with equal numbers of high concentration CFSE (CFSE^{high}) splenocytes loaded with NP68 peptide, and i.v. injected to recipient mice as indicated. Nineteen hours later, recipients were sacrificed and CFSE profile on splenocytes was determined by flow cytometry. NP68-specific killing is expressed as the ratio of CFSE^{low} to CFSE^{high} cells (CFSE^{low}:CFSE^{high}). *e*, Proliferation assay on spleens cells from F5/NP47/Rag1$^{-/-}$, F5/NP47/Rag1$^{-/-}$/TNF$^{-/-}$, and F5/Rag1$^{-/-}$/TNF$^{-/-}$ control mice activated with different concentrations of peptide. Samples were normalized for equal numbers of CD8$^+$ cells. Each curve represents an individual mouse. This experiment was performed at least three times with similar results.

3*c*, only a moderate expression of p55 is observed on F5 CD8 T cells and only after 2 days of activation. In contrast, we observed high expression levels of surface p75 in low and high Ag dosages and at both time points tested.

To study the role of TNF in the process of activation-induced cell death (AICD), we followed apoptotic death of in vitro stimulated of F5/Rag1$^{-/-}$ and F5/Rag1$^{-/-}$/TNF$^{-/-}$ T cells in the presence of increasing concentrations of NP peptide by annexin V staining. Fig. 3*d* shows results from one such experiment on days 1 and 5 after stimulation. We observed that within both F5 and F5/TNF$^{-/-}$ populations the proportion of early apoptotic cells increases in a dose-dependent manner with a concomitant reduction in the proportion of live cells. However, at all concentrations of NP peptide tested, the percentage of viable cells was higher in the F5/TNF$^{-/-}$ T cells with a parallel decrease of apoptotic cells. Similar results were obtained on days 2–4 after stimulation (data not shown). It is of interest that in the absence

of peptide the opposite effect was observed: cell death was more pronounced in unstimulated F5/TNF$^{-/-}$ T cells after 5 days in culture (Fig. 3*d*). The last observation correlates well with our in vivo findings that survival signals are impaired in naive F5/TNF$^{-/-}$ T cells.

To determine whether TNF ablation affects TCR-mediated stimulation of most of CD8$^+$ T cell clones and not only F5, we stimulated T cells from C57BL/10 (TNF$^{+/+}$) and TNF$^{-/-}$ mice with increasing concentrations of plate bound anti-CD3 Ab. As observed with the F5 specificity, TNF$^{-/-}$ CD8$^+$ T cells exhibited a declined proliferative response to anti-CD3 Ab by a factor 2–3 (Fig. 4*a*) comparing with TNF$^{+/+}$ CD8$^+$ T cells. Additionally, early apoptotic CD8$^+$ T cells (annexin V$^+$) among live cells were fewer in cultures from TNF$^{-/-}$ than from TNF$^{+/+}$ mice (Fig. 4*b*). Notably, in our system apoptosis is in analogy with cell divisions and that makes reasonable the observation that fewer divisions in TNF$^{-/-}$ CD8$^+$ T cells result to less apoptosis; thus, it is

FIGURE 7. In vivo Ag-induced expansion, clonal deletion, and "anergy" of transferred F5 T cells are impaired in the absence of TNF. *a*, Splenocytes from F5/Rag1$^{-/-}$ or F5/Rag1$^{-/-}$/TNF$^{-/-}$ donor mice containing 6×10^6 CD8$^+$ cells were adoptively transferred to NP47/Rag1$^{-/-}$ or NP47/Rag1$^{-/-}$/TNF$^{-/-}$ recipient mice, respectively. On days 1, 2, 3, 7, 14, and 26, recipients were analyzed for CD8$^+$Vβ11$^+$ percentage in peripheral blood. Results are expressed as fold change in percent of CD8$^+$Vβ11$^+$ cells = (% − % first day)/% first day. Six mice from each type, from one of two such experiments, are shown. Bars, Mean values. *b*, On day 26, mice were sacrificed and splenocytes from both kinds of recipients containing equal numbers of CD8$^+$ cells were assayed for proliferative capacity in response to NP68 in vitro. Each point represents the mean value from five animals and bars represent SD. Equal number of CD8$^+$ cells from an F5/Rag1$^{-/-}$ spleen was used as a control.
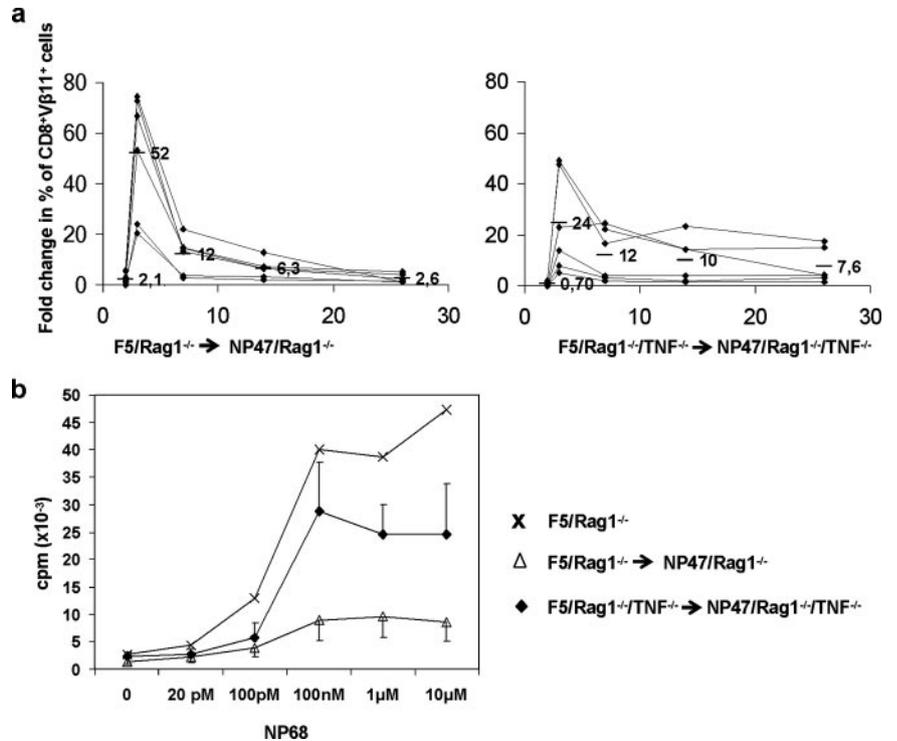
not clear whether the observed AICD phenotype in TNF$^{-/-}$ CD8$^+$ T cells is due solely to impaired activation or whether lack of TNFR-mediated T cell death also contributes to that phenotype.

Reduced NF-κB and NF-ATp activation in TNF-deficient F5 cells

Next, we wanted to examine the biochemical mechanisms underlying the observed effects of TNF in T cell responses. NF-κB plays a positive role in T cell development and function (40, 41), as well as a protective role in TNF-induced apoptosis (42, 43). NF-κB is mostly retained in the cytoplasm of unstimulated T cells and translocates into the nucleus upon a variety of stimuli including TNF and TCR stimulation (41, 44).

To assess whether the absence of TNF influences NF-κB activation following Ag-specific stimulation, we used EMSA to compare NF-κB binding activity in nuclear extracts from equal number of T cells from F5/Rag1$^{-/-}$ and F5/Rag1$^{-/-}$/TNF$^{-/-}$ splenocytes that had been exposed to peptide in vitro. We show that NF-κB-binding activity is consistently lower in activated F5/Rag1$^{-/-}$/TNF$^{-/-}$ T cells at all concentrations of NP peptide (Fig. 5*a*) and time points tested (Fig. 5*b*). Binding of constitutively active transcription factor AP-2 is not affected in TNF$^{-/-}$ nuclear extracts, thus excluding protein degradation due to cell death.

NF-AT is another family of inducible transcription factors, which plays a central role in mitogen-driven gene transcription in T cells, while there is no evidence for its activation by TNF. NF-ATp/NF-AT1/NF-ATc2 is very rapidly dephosphorylated and translocates from the cytoplasm into the nucleus after TCR or calcium ionophore stimulation (45). The effect of TNF-deficiency on NF-ATp activation following Ag-induced TCR signaling was assessed by EMSA experiments on thymocytes from F5/Rag1$^{-/-}$/TNF$^{-/-}$ mice following peptide activation (Fig. 5*c*); thymocytes rather than splenocytes were used, because NF-ATp-binding activity was more discernible in these cells. We observed that at all concentrations of peptide tested, specific binding activity of nuclear NF-ATp was consistently less in F5/Rag1$^{-/-}$/TNF$^{-/-}$ compared with control F5/Rag1$^{-/-}$ thymocytes. Protein integrity was

demonstrated by binding of ubiquitous transcription factor Sp1 (Fig. 5*c*). Our results suggest that endogenous TNF has the potential to modulate TCR-triggered signaling pathways.

Endogenous TNF alters in vivo tolerance induction of F5 T cells

The fact that our results reveal defects in several F5/TNF$^{-/-}$ T cell responses led us to investigate the possible involvement of TNF in tolerance induction of peripheral cytotoxic F5 T cells. We analyzed doubly transgenic mice that express both the F5 TCR and its cognate Ag NP in the TNF$^{-/-}$ background; we used an NP-transgenic line (NP47) (28) where tolerance of F5 T cells is achieved in the periphery by interaction with NP-expressing bone marrow-derived cells (32). In doubly transgenic F5/NP47/Rag1$^{-/-}$ mice, the Ag NP is not expressed in a functional way in the thymus and encounter of Ag in the periphery leads to clonal deletion of autospecific T cells with the remaining T cells being functionally impaired as manifested by their complete inability to proliferate upon challenge with Ag in vitro. However, peripheral T cells from these mice express activation markers and retained some killer function in vivo (32). Fig. 6*a* shows that introduction of this doubly transgenic mouse model in the TNF-deficient background leads to significantly less reduction of autospecific F5 T cells. Thus, there was an 80% reduction of F5 T cells when F5 and F5/NP47 spleens were compared, while only 40% reduction was observed if comparison was between F5/TNF$^{-/-}$ and F5/NP47/TNF$^{-/-}$ spleens (Fig. 6*b*). Similarly, in the Rag1$^{-/-}$ background, we observed a 70% F5 T cell reduction in F5/NP47/Rag1$^{-/-}$ spleens due to the expression of endogenous Ag but only 24% when the TNF mutation is introduced (Fig. 6*b*). This can be due to either impaired clonal deletion or/and increased in vivo proliferation or F5/NP47/TNF$^{-/-}$ T cell. In addition, surface expression of activation markers CD44 and CD69 was considerably less pronounced in remaining peripheral F5 T cells in F5/NP47/Rag1$^{-/-}$/TNF$^{-/-}$ mice (Fig. 6*c*). This suggests that impaired response to self-Ag in the TNF-deficient background results in less in vivo priming and as a consequence, in less deletion of autospecific T cells.

As shown in Fig. 6d, defective in vivo priming of F5 T cells by self-Ag in the TNF-deficient background resulted in reduced capacity to kill target cells in an in vivo cytotoxic assay. However, Fig. 6e shows that following further in vitro stimulation with NP peptide, T cells from F5/NP47/Rag1$^{-/-}$/TNF$^{-/-}$ spleens underwent considerable cycling compared with anergic cells from F5/NP47/Rag1$^{-/-}$ mice that fail to proliferate.

Naive F5/TNF$^{-/-}$ T cells chronically exposed to Ag in vivo establish a compromised "anergic" phenotype through differential Ag-driven responses

Transfer of naive F5 T cells to NP47 hosts has been shown to promote an early expansion phase, followed by period of massive contraction. Additionally, Ag-experienced F5 T cells that survived this kind of AICD, exhibit a homogenous "anergic" phenotype (32).

To test whether acquisition of this "anergic" phenotype is affected by the absence of endogenous TNF, we transferred naive F5 and F5/TNF$^{-/-}$ T cells to NP47 and NP47/TNF$^{-/-}$ hosts, respectively. In accordance to Stamou et al. (32), we observed accumulation of F5 T cells in NP47 recipients early after transfer followed by a sharp reduction already prominent in day 7 after transfer. However, expansion of F5/TNF$^{-/-}$ T cells in NP47/TNF$^{-/-}$ hosts was less pronounced (Fig. 7a). This observation is in agreement with the decreased activation potential of F5/TNF$^{-/-}$ T cells when challenged with Ag in vitro. Moreover, the contraction phase of F5/TNF$^{-/-}$ T cells was much smoother than that of F5, an observation that can be well-correlated with the lower degree of activation induced apoptosis we observed in vitro.

Twenty-six days after adoptive transfer, hosts were sacrificed and F5 T cells recovered from spleens were examined for proliferative capacity after re-exposure to Ag in vitro. F5 T cells recovered from NP47 hosts were not able to proliferate in response to Ag in vitro when compared with naive F5 T cells. In contrast, recovered F5/TNF$^{-/-}$ T cells from NP47/TNF$^{-/-}$ hosts still retained much of their proliferative potential (Fig. 7b). In that sense, establishment of the anergic phenotype after antigenic encounter in vivo was less efficient for transferred F5/TNF$^{-/-}$ T cells.

Discussion

In this study, we demonstrate that endogenous TNF modulates thresholds of ligand-driven responses of CTLs, thus revealing a novel role for this cytokine in peripheral lymphoid selection, homeostasis, and function. Specifically, we provide evidence that TNF is required for optimum survival of peripheral T cells, maximal antigenic responses (both proliferation and Ag-induced apoptosis) as well as tolerance induction. Although we detect the effect of TNF deficiency only in peripheral T cells, we cannot exclude the possibility that a defect due to the absence of TNF at early stages of their development is partly responsible for the observed phenotype, because TNF is known to be expressed in the thymus (46).

An important aspect of T cell-mediated immunity is the formation of T cell repertoire and the maintenance of homeostatic equilibrium of mature T cells. It is now well-established that survival of naive peripheral T cells requires both IL-7/IL-7R signaling (4) and continuous TCR interaction with MHC molecules. Similar, but not identical, mechanisms appear to regulate lymphopenia-induced proliferation of T cells when found in a T cell-deficient environment. In this study, we provide evidence that endogenous TNF modulates thresholds of CD8 T cells for both lymphopenia-induced proliferation and long-term survival. Thus, the decreased frequency and absolute number of F5 T cells found in the periphery of F5/TNF$^{-/-}$ mice (Fig. 1, *a* and *b*) in combination with the requirement of TNF for maximum accumulation of F5 T cells transferred into hosts (Fig. 2, *a* and *b*) suggest that endogenous

TNF is involved in mechanisms shaping peripheral maintenance of naive T cells. The fact that TNF in host mice cannot restore optimal survival and lymphopenia-induced proliferation of TNF-deficient T cells suggests a T cell-intrinsic defect for cells that have developed in the absence of endogenous TNF. Furthermore, our results showing significant up-regulation of p75, but not p55, on F5 T cells undergoing homeostatic proliferation (Fig. 2c), suggest a novel role for this receptor in T cell homeostasis under lymphopenia.

It has been recently shown that the strength of the TCR signaling determines the relative capacity of naive T cells to compete for factors that support T cell survival and homeostatic proliferation (7, 47–49). Our data, showing modulation of thresholds of T cell survival by TNF, predict that although TNF$^{-/-}$ and wild-type mice show no apparent differences in their T cell compartment (18, 19), differences in their T cell repertoire should exist with TNF giving a survival advantage to T cell clones with lower affinity for self-ligands. This prediction is further supported by the decrease in the rate of filling up the peripheral T cell pool in young TNF$^{-/-}$ mice suggested by the lower CD4$^+$ and CD8$^+$ T cell numbers (Table I). Homeostatic proliferation has been shown to be a mechanism for the shaping of peripheral T cell pool in neonates (50) and we are currently investigating the relative contribution of impaired homeostatic proliferation and survival of T cells in the shrunk peripheral pool of young TNF-deficient mice.

Our data demonstrate that endogenous TNF is important for optimal responses of CD8 T cells to cognate Ag peptide. We observed a quantitative defect in Ag responses in vitro of TNF-deficient CTLs, a higher concentration of Ag being required to elicit the same responses as TNF wild-type cells (Figs. 3 and 4). This is in agreement with previous reports showing that TNF is essential for efficient primary CD4$^+$ T cell responses, only under suboptimal conditions of stimulation (22).

The fact that in our experiments C57BL/10 TNF-sufficient cells are used for Ag presentation indicates that impaired Ag stimulation cannot be solely due to the known modulation of MHC class I or other molecules expression on APCs by TNF. Our experiments demonstrate that both Ag-induced activation and apoptosis are reduced in TNF-deficient CD8 T cells suggesting that less T cell apoptotic death in TNF-deficient mice can be attributed to less TCR signaling in addition to the possible contribution of TNFR signaling in AICD (51). The latter could be mediated by expression of p55 on stimulated F5 T cells (Fig. 3c) in agreement to previous studies using p55$^{-/-}$ mice, p55 was found to contribute in activation-induced T cell deletion following strong peptide stimulation (52). However, a role of p75 in promoting cell death cannot be excluded (53). Both in vitro and in vivo encounter of Ag by TNF-deficient T cells leads to weaker responses, demonstrated by less up-regulation of surface molecules (Figs. 3b and 6c) known to be involved in both T cell activation and apoptosis. In agreement with previous experiments in human T lymphocytes (10), we showed that endogenous TNF regulates the surface expression of the high-affinity IL-2R in activated murine T cells. IL-2/IL-2R signals are known to facilitate Ag-induced T cell expansion but also play an important role in terminating this response by clonal deletion in a feedback mechanism (51). Regulation of surface expression of the high-affinity IL-2R by TNF in activated murine T cells could account at least partly for the dual role of TNF in activation and death of F5 T cells. Reduced proliferative response and delayed kinetics of CD25 induction were also reported in p75$^{-/-}$ mice (23). In addition, a costimulatory function of p75 for Ag-driven T cell responses has been reported in vitro (54) and in vivo (55) but during viral infection overlapping roles for the two receptors in regulating CD8 T cells responses were reported (56).

The fact that F5 T cells express both TNFRs after in vitro stimulation (Fig. 3c) in parallel with high TNF production (data not shown) is consistent with a role for both receptors in mediating TNF effect on T cell responses.

The strength of TCR signaling and duration of Ag encounter are critical parameters for the fate of naive CD8 T cells toward death, anergy, or retainment of naive phenotype (8). It is, therefore, conceivable that factors influencing TCR signaling would affect developmental fates of CD8 T cells after Ag engagement. We report here that TNF mutation decreases naive F5 T cell responses to Ag in vitro. Consequently, responses of F5 T cells to self-Ag are affected too. F5 NP47/TNF$^{-/-}$ T cells were less "Ag-experienced" than their TNF$^{+/+}$ counterparts, as judged by the high proportion of CD44$^-$ and CD69$^-$ cells among them (Fig. 6c). Therefore, they exhibit a more naive-like phenotype, which is in accordance with their ability to proliferate after re-exposure to Ag in vitro (Fig. 6e). Because our analysis involves only live T cells, we cannot exclude the possibility that a small proportion of autospecific T cells dies fast after activation, thus escaping detection.

Similar defects in "anergy" are found in F5/TNF$^{-/-}$ CD8 T cells recovered from NP47/TNF$^{-/-}$ hosts that can proliferate substantially after NP68-stimulation in vitro (Fig. 7b). Moreover, F5/NP47 CD8 T cells have acquired a cytolytic activity against NP-loaded transferred cells presumably through priming by endogenous Ag. In contrast, F5/NP47/TNF$^{-/-}$ CD8 T cells cannot kill a significant fraction of target cells (Fig. 6d) and this can be attributed to insufficient in vivo T cell priming in the absence of TNF. This lytic activity seems contradictory to a tolerant phenotype, but in a normal host a low frequency of autoreactive clones incapable to expand would be insufficient to provoke tissue damage. A similar kind of T cell tolerance profile has been described in a different mouse model (57), rendering proliferation impotency a more critical parameter for maintaining tolerance. Collectively, the role of TNF/TNFRs pathways on CD8 T cell tolerance relies on its costimulatory effect to TCR signaling. Because TCR-derived signals not only determine the efficacy of T cell immunity but also tolerance induction (8) and T cell viability (58), it is anticipated that costimulatory molecules can have an influence on these parameters of T cell biology. Indeed, CD28 costimulation is essential for optimal peripheral tolerance of CD8 T in different models (59, 60). In our model of T cell tolerance, F5 T cells with a naive phenotype are exported from thymus and interact with NP-expressing bone marrow-derived cells (32), where they integrate a TCR signal capable to drive them through a differentiation program to the specific tolerant phenotype. In the case of TNF deficiency, TCR signaling is not strong enough to establish tolerance to the same level and a large fraction of F5/NP47/TNF$^{-/-}$ (Fig. 6)—or F5/TNF$^{-/-}$ recovered from NP47/TNF$^{-/-}$ hosts (Fig. 7)—CD8 T cells share similar characteristics with naive CD8 T cells. This would result in potentially more autoaggressive cells and enhanced autoimmune responses; this is in agreement with results showing that in TNF-deficient mice, enhanced myelin-specific T cell reactivity is observed, leading to exacerbated experimental autoimmune encephalomyelitis (61). Thus, endogenous TNF plays an important role in many aspects of T cell tolerance such as clonal deletion, proliferative potential and cytotoxicity.

We demonstrate that during T cell responses endogenous TNF is crucial for the activation and recruitment of the transcription factor NF-κB (Fig. 5, a and b) which is important in development and responses of T cells (40, 41). Persistent exposure to exogenously added TNF has been shown to uncouple proximal TCR signaling as well as to selectively target TCR distal pathways of CD4 T cells (15, 17, 62). Additionally, studies with p75$^{-/-}$ T cells revealed that p75 signaling is critical for NF-κB activation after TCR stim-

ulation (54). Interestingly, partial inhibition of NF-κB has been associated with decreased peripheral CD8 T cell numbers and proliferative responses (63, 64). Impaired NF-κB activity could, therefore, well account for decreased survival and activation potential of naive TNF$^{-/-}$ T cells in our experimental system.

Our data, for the first time, show that lack of the endogenous TNF can affect NF-ATp activation during TCR ligation (Fig. 5c), indicating that TNF can cross-talk with novel pathways, different from the ones already described in TNF/TNFRs signaling. It is well-documented that NF-ATp positively regulates the expression of the IL-2Rα chain (65), which might explain its decreased expression in F5/TNF$^{-/-}$ CD8$^+$ T cells. Moreover, NF-ATp signaling has a positive role on establishing a T cell tolerant phenotype, as manifested in NF-ATp$^{-/-}$ T cells receiving an anergizing stimulus (66).

Although our results clearly demonstrate that T cells, which developed in the absence of TNF, have an intrinsic defect in several ligand-driven responses, questions still remain to be answered concerning the relative contribution of soluble and membrane-bound TNF as well as distinct roles of the two receptors that mediate TNF signaling.

Collectively, our data point toward a model in which endogenous TNF is a key regulator in the developmental decisions of peripheral T cells by modulating TCR signaling thresholds. Thus, mutation of TNF leads to switches from survival to death and from clonal deletion and anergy, to responsiveness of autospecific T cells. Further elucidation of the cellular and molecular mechanisms governing the role of TNF in early and late T cell responses will be important for designing intervention in autoimmune disease treatment.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

References

1. Tanchot, C., F. A. Lemonnier, B. Perarnau, A. A. Freitas, and B. Rocha. 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science* 276: 2057–2062.
2. Brown, I. E., M. Mashayekhi, M. Markiewicz, M. L. Alegre, and T. F. Gajewski. 2005. Peripheral survival of naive CD8$^+$ T cells. *Apoptosis* 10: 5–11.
3. Jameson, S. C. 2005. T cell homeostasis: keeping useful T cells alive and live T cells useful. *Semin. Immunol.* 17: 231–237.
4. Schluns, K. S., W. C. Kieper, S. C. Jameson, and L. Lefrancois. 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat. Immunol.* 1: 426–432.
5. Tan, J. T., E. Dudl, E. LeRoy, R. Murray, J. Sprent, K. I. Weinberg, and C. D. Surh. 2001. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proc. Natl. Acad. Sci. USA* 98: 8732–8737.
6. Seddon, B., and R. Zamoyska. 2002. TCR and IL-7 receptor signals can operate independently or synergize to promote lymphopenia-induced expansion of naive T cells. *J. Immunol.* 169: 3752–3759.
7. Kieper, W. C., J. T. Burghardt, and C. D. Surh. 2004. A role for TCR affinity in regulating naive T cell homeostasis. *J. Immunol.* 172: 40–44.
8. Redmond, W. L., and L. A. Sherman. 2005. Peripheral tolerance of CD8 T lymphocytes. *Immunity* 22: 275–284.
9. Brehm, M. A., K. A. Daniels, and R. M. Welsh. 2005. Rapid production of TNF-α following TCR engagement of naive CD8 T cells. *J. Immunol.* 175: 5043–5049.
10. Pimentel-Muinos, F. X., M. A. Munoz-Fernandez, and M. Fresno. 1994. Control of T lymphocyte activation and IL-2 receptor expression by endogenously secreted lymphokines. *J. Immunol.* 152: 5714–5722.
11. Shalaby, M. R., T. Espevik, G. C. Rice, A. J. Ammann, I. S. Figari, G. E. Ranges, and M. A. Palladino. 1988. The involvement of human tumor necrosis factors-α and -β in the mixed lymphocyte reaction. *J. Immunol.* 141: 499–503.
12. Yokota, S., T. D. Geppert, and P. E. Lipsky. 1988. Enhancement of antigen- and mitogen-induced human T lymphocyte proliferation by tumor necrosis factor-α. *J. Immunol.* 140: 531–536.
13. Banerjee, D., H. C. Liou, and R. Sen. 2005. c-Rel-dependent priming of naive T cells by inflammatory cytokines. *Immunity* 23: 445–458.
14. Sepulveda, H., A. Cerwenka, T. Morgan, and R. W. Dutton. 1999. CD28, IL-2-independent costimulatory pathways for CD8 T lymphocyte activation. *J. Immunol.* 163: 1133–1142.
15. Isomaki, P., M. Panesar, A. Annenkov, J. M. Clark, B. M. Foxwell, Y. Chernajovsky, and A. P. Cope. 2001. Prolonged exposure of T cells to TNF

down-regulates TCRζ and expression of the TCR/CD3 complex at the cell surface. *J. Immunol.* 166: 5495–5507.

16. Clark, J. M., A. E. Annenkov, M. Panesar, P. Isomaki, Y. Chernajovsky, and A. P. Cope. 2004. T cell receptor ζ reconstitution fails to restore responses of T cells rendered hyporesponsive by tumor necrosis factor α. *Proc. Natl. Acad. Sci. USA* 101: 1696–1701.

17. Church, L. D., J. E. Goodall, D. A. Rider, P. A. Bacon, and S. P. Young. 2005. Persistent TNF-α exposure impairs store operated calcium influx in CD4+ T lymphocytes. *FEBS Lett.* 579: 1539–1544.

18. Pasparakis, M., L. Alexopoulou, V. Episkopou, and G. Kollias. 1996. Immune and inflammatory responses in TNF α-deficient mice: a critical requirement for TNF α in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J. Exp. Med.* 184: 1397–1411.

19. Marino, M. W., A. Dunn, D. Grail, M. Inglese, Y. Noguchi, E. Richards, A. Jungbluth, H. Wada, M. Moore, B. Williamson, et al. 1997. Characterization of tumor necrosis factor-deficient mice. *Proc. Natl. Acad. Sci. USA* 94: 8093–8098.

20. Korner, H., D. S. Riminton, D. H. Strickland, F. A. Lemckert, J. D. Pollard, and J. D. Sedgwick. 1997. Critical points of tumor necrosis factor action in central nervous system autoimmune inflammation defined by gene targeting. *J. Exp. Med.* 186: 1585–1590.

21. Hill, G. R., T. Teshima, V. I. Rebel, O. I. Krijanovski, K. R. Cooke, Y. S. Brinson, and J. L. Ferrara. 2000. The p55 TNF-α receptor plays a critical role in T cell alloreactivity. *J. Immunol.* 164: 656–663.

22. Kassiotis, G., K. Kranidioti, and G. Kollias. 2001. Defective CD4 T cell priming and resistance to experimental autoimmune encephalomyelitis in TNF-deficient mice due to innate immune hypo-responsiveness. *J. Neuroimmunol.* 119: 239–247.

23. Kim, E. Y., and H. S. Teh. 2001. TNF type 2 receptor (p75) lowers the threshold of T cell activation. *J. Immunol.* 167: 6812–6820.

24. Schmaltz, C., O. Alpdogan, S. J. Muriglan, B. J. Kappel, J. A. Rotolo, E. T. Ricchetti, A. S. Greenberg, L. M. Willis, G. F. Murphy, J. M. Crawford, and M. R. van den Brink. 2003. Donor T cell-derived TNF is required for graft-versus-host disease and graft-versus-tumor activity after bone marrow transplantation. *Blood* 101: 2440–2445.

25. Poehlein, C. H., H. M. Hu, J. Yamada, I. Assmann, W. G. Alvord, W. J. Urba, and B. A. Fox. 2003. TNF plays an essential role in tumor regression after adoptive transfer of perforin/IFN-γ double knockout effector T cells. *J. Immunol.* 170: 2004–2013.

26. Grivennikov, S. I., A. V. Tumanov, D. J. Liepinsh, A. A. Kruglov, B. I. Marakusha, A. N. Shakhov, T. Murakami, L. N. Drutskaya, I. Forster, B. E. Clausen, et al. 2005. Distinct and nonredundant in vivo functions of TNF produced by T cells and macrophages/neutrophils: protective and deleterious effects. *Immunity* 22: 93–104.

27. Mamalaki, C., T. Norton, Y. Tanaka, A. R. Townsend, P. Chandler, E. Simpson, and D. Kioussis. 1992. Thymic depletion and peripheral activation of class I major histocompatibility complex-restricted T cells by soluble peptide in T-cell receptor transgenic mice. *Proc. Natl. Acad. Sci. USA* 89: 11342–11346.

28. Mamalaki, C., M. Murdjeva, M. Tolaini, T. Norton, P. Chandler, A. Townsend, E. Simpson, and D. Kioussis. 1996. Tolerance in TCR/cognate antigen double-transgenic mice mediated by incomplete thymic deletion and peripheral receptor downregulation. *Dev. Immunol.* 4: 299–315.

29. Spanopoulou, E., C. A. Roman, L. M. Corcoran, M. S. Schlissel, D. P. Silver, D. Nemazee, M. C. Nussenzweig, S. A. Shinton, R. R. Hardy, and D. Baltimore. 1994. Functional immunoglobulin transgenes guide ordered B-cell differentiation in Rag-1-deficient mice. *Genes Dev.* 8: 1030–1042.

30. de Boer, J., A. Williams, G. Skavdis, N. Harker, M. Coles, M. Tolaini, T. Norton, K. Williams, K. Roderick, A. J. Potocnik, and D. Kioussis. 2003. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. *Eur. J. Immunol.* 33: 314–325.

31. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* 171: 131–137.

32. Stamou, P., J. de Jersey, D. Carmignac, C. Mamalaki, D. Kioussis, and B. Stockinger. 2003. Chronic exposure to low levels of antigen in the periphery causes reversible functional impairment correlating with changes in CD5 levels in monoclonal CD8 T cells. *J. Immunol.* 171: 1278–1284.

33. Hatzis, P., A. S. Al-Madhoon, M. Jullig, T. G. Petrakis, S. Eriksson, and I. Talianidis. 1998. The intracellular localization of deoxycytidine kinase. *J. Biol. Chem.* 273: 30239–30243.

34. Rocha, B., N. Dautigny, and P. Pereira. 1989. Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. *Eur. J. Immunol.* 19: 905–911.

35. Ernst, B., D. S. Lee, J. M. Chang, J. Sprent, and C. D. Surh. 1999. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* 11: 173–181.

36. Goldrath, A. W., and M. J. Bevan. 1999. Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. *Immunity* 11: 183–190.

37. Garcia, A. M., S. A. Fadel, S. Cao, and M. Sarzotti. 2000. T cell immunity in neonates. *Immunol. Res.* 22: 177–190.

38. Min, B., R. McHugh, G. D. Sempowski, C. Mackall, G. Foucras, and W. E. Paul. 2003. Neonates support lymphopenia-induced proliferation. *Immunity* 18: 131–140.

39. Haaijman, J. J., H. S. Micklem, J. A. Ledbetter, J. L. Dangl, L. A. Herzenberg, and L. A. Herzenberg. 1981. T cell ontogeny. Organ location of maturing pop-

40. Kuo, C. T., and J. M. Leiden. 1999. Transcriptional regulation of T lymphocyte development and function. *Annu. Rev. Immunol.* 17: 149–187.

41. Siebenlist, U., K. Brown, and E. Claudio. 2005. Control of lymphocyte development by nuclear factor-κB. *Nat. Rev. Immunol.* 5: 435–445.

42. Beg, A. A., and D. Baltimore. 1996. An essential role for NF-κB in preventing TNF-α-induced cell death. *Science* 274: 782–784.

43. Van Antwerp, D. J., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma. 1996. Suppression of TNF-α-induced apoptosis by NF-κB. *Science* 274: 787–789.

44. Bonizzi, G., and M. Karin. 2004. The two NF-κB activation pathways and their role in innate and adaptive immunity. *Trends Immunol.* 25: 280–288.

45. Macian, F. 2005. NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.* 5: 472–484.

46. Deman, J., M. T. Martin, P. Delvenne, C. Humblet, J. Boniver, and M. P. Defresne. 1992. Analysis by in situ hybridization of cells expressing mRNA for tumor-necrosis factor in the developing thymus of mice. *Dev. Immunol.* 2: 103–109.

47. Freitas, A. A., F. Agenes, and G. C. Coutinho. 1996. Cellular competition modulates survival and selection of CD8+ T cells. *Eur. J. Immunol.* 26: 2640–2649.

48. Ge, Q., V. P. Rao, B. K. Cho, H. N. Eisen, and J. Chen. 2001. Dependence of lymphopenia-induced T cell proliferation on the abundance of peptide/MHC epitopes and strength of their interaction with T cell receptors. *Proc. Natl. Acad. Sci. USA* 98: 1728–1733.

49. Kassiotis, G., R. Zamoyska, and B. Stockinger. 2003. Involvement of avidity for major histocompatibility complex in homeostasis of naive and memory T cells. *J. Exp. Med.* 197: 1007–1016.

50. Le Campion, A., C. Bourgeois, F. Lambolez, B. Martin, S. Leaument, N. Dautigny, C. Tanchot, C. Penit, and B. Lucas. 2002. Naive T cells proliferate strongly in neonatal mice in response to self-peptide/self-MHC complexes. *Proc. Natl. Acad. Sci. USA* 99: 4538–4543.

51. Lenardo, M., K. M. Chan, F. Hornung, H. McFarland, R. Siegel, J. Wang, and L. Zheng. 1999. Mature T lymphocyte apoptosis–immune regulation in a dynamic and unpredictable antigenic environment. *Annu. Rev. Immunol.* 17: 221–253.

52. Speiser, D. E., E. Sebzda, T. Ohteki, M. F. Bachmann, K. Pfeffer, T. W. Mak, and P. S. Ohashi. 1996. Tumor necrosis factor receptor p55 mediates deletion of peripheral cytotoxic T lymphocytes in vivo. *Eur. J. Immunol.* 26: 3055–3060.

53. Gaur, U., and B. B. Aggarwal. 2003. Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. *Biochem. Pharmacol.* 66: 1403–1408.

54. Kim, E. Y., and H. S. Teh. 2004. Critical role of TNF receptor type-2 (p75) as a costimulator for IL-2 induction and T cell survival: a functional link to CD28. *J. Immunol.* 173: 4500–4509.

55. Kim, E. Y., J. J. Priatel, S. J. Teh, and H. S. Teh. 2006. TNF receptor type 2 (p75) functions as a costimulator for antigen-driven T cell responses in vivo. *J. Immunol.* 176: 1026–1035.

56. Suresh, M., A. Singh, and C. Fischer. 2005. Role of tumor necrosis factor receptors in regulating CD8 T-cell responses during acute lymphocytic choriomeningitis virus infection. *J. Virol.* 79: 202–213.

57. Teague, R. M., B. D. Sather, J. A. Sacks, M. Z. Huang, M. L. Dossett, J. Morimoto, X. Tan, S. E. Sutton, M. P. Cooke, C. Ohlen, and P. D. Greenberg. 2006. Interleukin-15 rescues tolerant CD8+ T cells for use in adoptive immunotherapy of established tumors. *Nat. Med.* 12: 335–341.

58. Marrack, P., and J. Kappler. 2004. Control of T cell viability. *Annu. Rev. Immunol.* 22: 765–787.

59. Vacchio, M. S., and R. J. Hodes. 2003. CD28 costimulation is required for in vivo induction of peripheral tolerance in CD8 T cells. *J. Exp. Med.* 197: 19–26.

60. Yu, X. Z., M. H. Albert, P. J. Martin, and C. Anasetti. 2004. CD28 ligation induces transplantation tolerance by IFN-γ-dependent depletion of T cells that recognize alloantigens. *J. Clin. Invest.* 113: 1624–1630.

61. Kassiotis, G., and G. Kollias. 2001. Uncoupling the proinflammatory from the immunosuppressive properties of tumor necrosis factor (TNF) at the p55 TNF receptor level: implications for pathogenesis and therapy of autoimmune demyelination. *J. Exp. Med.* 193: 427–434.

62. Cope, A. P., R. S. Liblau, X. D. Yang, M. Congia, C. Laudanna, R. D. Schreiber, L. Probert, G. Kollias, and H. O. McDevitt. 1997. Chronic tumor necrosis factor alters T cell responses by attenuating T cell receptor signaling. *J. Exp. Med.* 185: 1573–1584.

63. Attar, R. M., H. Macdonald-Bravo, C. Raventos-Suarez, S. K. Durham, and R. Bravo. 1998. Expression of constitutively active IκB β in T cells of transgenic mice: persistent NF-κB activity is required for T-cell immune responses. *Mol. Cell. Biol.* 18: 477–487.

64. Ferreira, V., N. Sidenius, N. Tarantino, P. Hubert, L. Chatenoud, F. Blasi, and M. Korner. 1999. In vivo inhibition of NF-κB in T-lineage cells leads to a dramatic decrease in cell proliferation and cytokine production and to increased cell apoptosis in response to mitogenic stimuli, but not to abnormal thymopoiesis. *J. Immunol.* 162: 6442–6450.

65. Schuh, K., T. Twardzik, B. Kneitz, J. Heyer, A. Schimpl, and E. Serfling. 1998. The interleukin 2 receptor α chain/CD25 promoter is a target for nuclear factor of activated T cells. *J. Exp. Med.* 188: 1369–1373.

66. Macian, F., F. Garcia-Cozar, S. H. Im, H. F. Horton, M. C. Byrne, and A. Rao. 2002. Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* 109: 719–731.