Erschienen in: European Journal of Immunology ; 39 (2009), 11. - S. 3171-3180
https://dx.doi.org/10.1002/eji.200939554

Soluble TNF-α but not transmembrane TNF-α sensitizes T cells for enhanced activation-induced cell death

Stefan Müller, Silvia Rihs, Johanna M. Dayer Schneider, Bruno E. Paredes, Ingeborg Seibold, Thomas Brunner and Christoph Mueller

Institute of Pathology, Division of Experimental Pathology, University of Bern, Bern, Switzerland

In addition to its proinflammatory effects, TNF-α exhibits immunosuppression. Here, we compared the capacities of transmembrane TNF-α (tmTNF) and soluble TNF-α (sTNF) in regulating expansion of activated T cells by apoptosis. Splenic $CD4^+$ T cells from wtTNF, TNF-α-deficient ($TNF^{-/-}$) and $TNF^{-/-}$ mice expressing a non-cleavable mutant tmTNF showed comparable proliferation rates upon TCR-mediated stimulation. Activation-induced cell death (AICD), however, was significantly attenuated in tmTNF and $TNF^{-/-}$, compared with wtTNF $CD4^+$ T cells. Addition of sTNF during initial priming was sufficient to enhance susceptibility to AICD in tmTNF and $TNF^{-/-}$ $CD4^+$ T cells to levels seen in wtTNF $CD4^+$ T cells, whereas addition of sTNF only during restimulation failed to enhance AICD. sTNF-induced, enhanced susceptibility to AICD was dependent on both TNF receptors. The reduced susceptibility of tmTNF $CD4^+$ T cells for AICD was also evident in an *in vivo* model of adoptively transferred $CD4^+$ T-cell-mediated colonic inflammation. Hence, the presence of sTNF during T-cell priming may represent an important mechanism to sensitize activated T cells for apoptosis, thereby attenuating the extent and duration of T-cell reactivities and subsequent T-cell-mediated, excessive inflammation.

Key words: Activation-induced cell death · $CD4^+$ T cells · Colitis · TNF-α · Transmembrane TNF

Introduction

TNF is a pleiotropic cytokine involved in innate and adaptive immunity. It is regarded as the prototypic proinflammatory cytokine and is crucial in immune defense against many pathogens but also during development of various autoimmune diseases [1].

The type I transmembrane 26 kDa precursor TNF molecule (tmTNF) is preferentially cleaved by the extracellular metalloproteinase TNF-α-converting-enzyme (TACE) to release trimers of the 17 kDa soluble form (sTNF) [2]. The generation of non-cleavable mutants of TNF revealed that tmTNF and sTNF exert distinct biological functions. A main function ascribed to tmTNF is the induction of cell death [3]. Some of the proinflammatory effects ascribed to TNF are also mediated by tmTNF as shown by the tmTNF-induced up-regulation of ICAM-1 and VCAM-1 on endothelial cells *in vitro* [4]. Mice overexpressing a non-cleavable tmTNF mutein are prone to develop arthritis [5] and signs of hepatitis after Concanavalin A (Con A) administration [6]. tmTNF transgenic mice [4] and tmTNF knock-in mice [7] are, in contrast to wtTNF mice, protected from LPS-induced death, thus demonstrating the distinct roles exerted by tmTNF and secreted TNF. Treatment of mice with a synthetic TACE inhibitor also protects mice from endotoxin-mediated death [8], indicating that TACE may represent a target to prevent the fatal effects of excessive sTNF production while maintaining the local TNF-mediated effects required in the host response against intracellular pathogens. The fact that tmTNF mice, but not TNF-deficient mice, are still capable of forming granulomas following infection with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and thus are protected from this mycobacterial infection [9] are in support of such a concept.

Correspondence: Dr. Stefan Müller
e-mail: stefan.mueller@dkf.unibe.ch

However, several studies also revealed anti-inflammatory effects of TNF. For example, Liu *et al*. observed exacerbation of demyelation and inflammation in an EAE model in the absence of TNF [10]. In type I diabetes in the NOD mouse, disease-promoting, as well as disease-attenuating effects by TNF have been observed depending on the time point of TNF administration [11], and in acute airway response to endotoxin, T-cell-derived TNF down-regulated the inflammation [12]. Exposure of T cells to TNF, in particular during chronic inflammatory conditions, has been reported to exert anti-inflammatory effects on T cells, in particular by attenuating TCR-mediated signaling and proliferative expansion [13]. Prolonged exposure to TNF *in vitro* leads to hyporesponsiveness of T cells [14] possibly by uncoupling proximal [15] as well as distal [16] TCR signals, thus indicating an important and direct immunomodulatory function of TNF on activated T cells.

At present, no information is available on the relative capacity of sTNF and tmTNF to mediate these anti-inflammatory activities. This prompted us to directly determine how absence of sTNF affects T-cell proliferation and expansion in the presence, or absence, of the 26 kDa transmembrane form of TNF. Our results clearly demonstrate that sTNF increases susceptibility of T cells to subsequent activation-induced cell death (AICD) *in vitro* and *ex vivo via* a complex, yet unresolved, mechanism involving both TNF receptors. Hence, we propose that the presence of sTNF during priming of T cells represents an important mechanism to confer susceptibility for efficient apoptosis of activated T cells upon TCR engagement, thereby limiting the extent and duration of T-cell reactivities and subsequent T-cell-mediated, excessive inflammatory responses.

Results

wtTNF and tmTNF CD4$^+$ T cells show comparable proliferation *in vitro*

To compare the proliferation rates of wtTNF and tmTNF CD4$^+$ T cells, splenic CD4$^+$ T cells from wtTNF and tmTNF mice were stimulated with different concentrations of anti-CD3ε and pulsed with ^{3}H-thymidine (^{3}H-TdR) on day 3. As shown in Fig. 1A proliferative activities of tmTNF CD4$^+$ T cells and wtTNF CD4$^+$ T cells were comparable, although at higher anti-CD3ε concentrations, ^{3}H-TdR uptake was decreased in wtTNF T cells. To assess the proliferative behavior on a single cell level *in vitro*, unfractionated spleen cells from wtTNF and tmTNF mice were labeled with CFSE and stimulated with anti-CD3ε. The resulting proliferation indices depicted in Fig. 1B clearly show that the proliferative activities of wtTNF and tmTNF CD4$^+$ T cells were almost identical over the 3-day culture period.

tmTNF CD4$^+$ T cells are less susceptible to AICD than wtTNF CD4$^+$ T cells

The size of the T-cell pool is not only controlled by the proliferative activity of the T cells but also by the rate of apoptosis induction in long-term activated T cells by AICD. To assess how the presence, or absence, of secreted TNF affects AICD of T cells, we generated Con A-stimulated splenic T-cell blasts from wtTNF, tmTNF, and TNF$^{-/-}$ mice, which were restimulated on day 5 for 6 h with plate-bound anti-CD3ε. As shown in Fig. 2A, a more pronounced induction of AICD occurred in wtTNF CD4$^+$ T cells, while tmTNF and TNF$^{-/-}$ CD4$^+$ T cells showed a comparable, attenuated induction of AICD. Similarly, an attenuated apoptosis induction was also observed in CD8$^+$ T cells from tmTNF or TNF$^{-/-}$ mice when compared with wtTNF CD8$^+$ T cells (data not shown). These results indicate that the absence of sTNF, rather than the sole presence of tmTNF, led to increased resistance of activated T cells against AICD. To directly address this notion, recombinant mouse TNF (simply referred to as sTNF in the following) was added during the entire culture period (Fig. 2B). While addition of sTNF only slightly increased AICD in activated wtTNF T cells, it substantially increased the frequencies of annexin V-positive CD4$^+$ T-cell blasts from tmTNF mice to levels close to those observed with wtTNF CD4$^+$ T cells. Next, we added sTNF during different intervals, *i.e*. throughout the entire culture period as in Fig. 2B, during priming of T cells only, and during the restimulation period only. These experiments unambiguously revealed that addition of exogenous sTNF during priming of T cells is required and sufficient to confer increased susceptibility to AICD in tmTNF T-cell blasts, while it has no effect when added only during restimulation (Fig. 2C). AICD in CD4$^+$ T cells was previously described to depend on the simultaneous expression of Fas and FasL and subsequent Fas-induced apoptosis [17]. In contrast, TNF/TNFR interaction was suggested to contribute to AICD in CD8$^+$ T cells [18]. Hence, FasL/Fas, TNF/TNFR, or both interactions were selectively inhibited by addition of blocking antibodies to CD4$^+$ T-cell cultures. AICD of

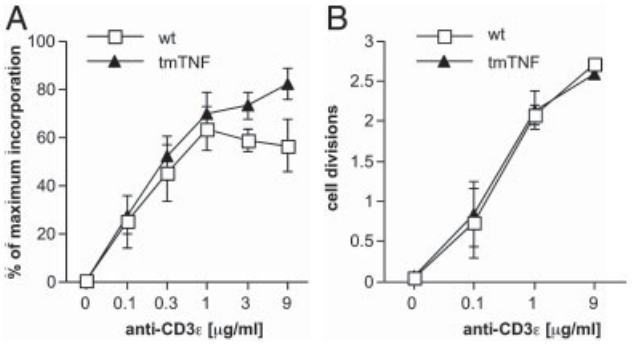

Figure 1. Splenic CD4$^+$ T cells from wtTNF or tmTNF mice have comparable proliferative capacities. (A) Sorted CD4$^+$ splenocytes from wtTNF (squares) or tmTNF mice (triangles) were stimulated with plate-bound anti-CD3ε and soluble anti-CD28, and pulsed with ^{3}H-TdR on day 3. Maximum proliferation rate in each experiment was set to 100%. Mean ± SEM of pooled data from four independent experiments, each with duplicates, are shown. (B) Unfractionated splenocytes from wtTNF (squares) and tmTNF mice (triangles) were labeled with CFSE and stimulated with plate-bound anti-CD3ε. After 3 days the CFSE-profile of the CD4$^+$ population was analyzed by flow cytometry. The average number of T-cell divisions was calculated as described in the *Materials and methods*. Mean ± SEM of pooled data from three independent experiments, each with duplicates, are shown.

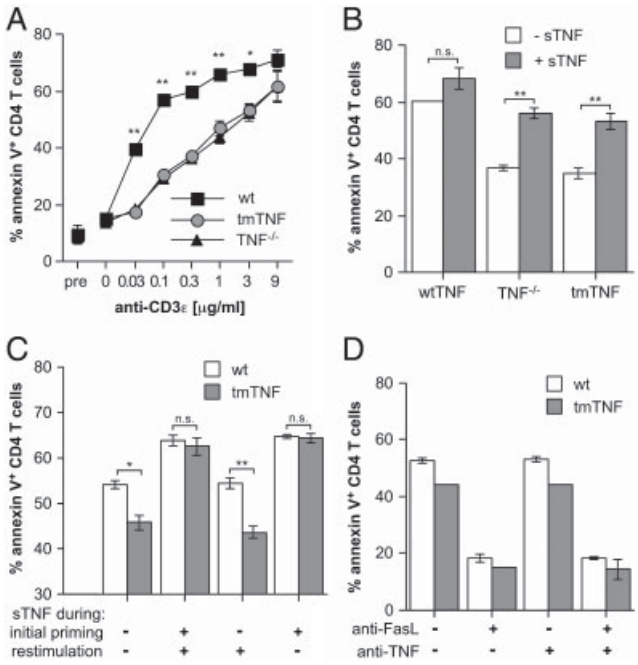

Figure 2. FasL-mediated AICD is reduced in TNF$^{-/-}$ and tmTNF CD4$^+$ T cells but is reverted to levels seen in wtTNF CD4$^+$ T cells by the addition of sTNF during T-cell priming. (A) Frequencies of annexin V-positive CD4$^+$ T-cell blasts from spleens of wtTNF (squares), TNF$^{-/-}$ (circles), and tmTNF mice (triangles) were determined before (pre) and after restimulation with plate-bound anti-CD3ε. (B) Splenic T-cell blasts were restimulated with 0.3 μg/mL plate-bound anti-CD3ε in the absence (open columns) or presence (shaded columns) of 10 ng/mL sTNF. (C) sTNF was either added during the entire experiment; during generation of T-cell blasts only (priming); or during restimulation only, to cultures with wtTNF (open columns) or tmTNF (shaded columns) T cells. (D) Influence of blocking anti-FasL and/or anti-TNF on AICD during restimulation with 0.3 μg/mL plate-bound anti-CD3ε. Data in (A–C) show mean ± SD ($n = 2$) and are representative of three independent experiments. Data in (D) represent mean ± SEM ($n = 3$) and are representative of two independent experiments. Two-tailed p-values were calculated for differences between wt and tmTNF (A and C) or between priming in the presence or absence of sTNF (B) with the unpaired Student's t-test across all experiments; $^*p \leq 0.05$, $^{**}p \leq 0.01$.

CD4$^+$ T cells was completely abrogated when FasL/Fas interactions were blocked, while neutralization of TNF did not affect AICD either in wtTNF or in tmTNF CD4$^+$ T-cell blasts (Fig. 2D).

Activated tmTNF and wtTNF CD4$^+$ T-cell blasts show equal expression activities of FasL and Fas

To assess the possible mechanism(s) that may lead to a reduced AICD of activated tmTNF CD4$^+$ T cells, we analyzed the most proximal event of the death receptor-mediated apoptosis pathway, *i.e.* the extent of FasL/Fas interaction. However, no evidence for a differential surface expression of Fas and FasL on wtTNF, and tmTNF CD4$^+$ T-cell blasts upon anti-CD3ε restimulation was found (Fig. 3A). To measure the functional activity of FasL, CD4$^+$ T-cell blasts (effectors) were co-cultured with ^{3}H-TdR-labeled, Fas-sensitive Jurkat cells (targets) on plate-bound anti-CD3ε to assess the extent of target cell DNA fragmentation. wtTNF and tmTNF CD4$^+$ T-cell blasts were equally potent in inducing Fas-dependent killing of Jurkat cells (Fig. 3B). Addition of sTNF during activation and culture of T-cell blasts did not affect the extent of target cell DNA fragmentation. However, DNA fragmentation was completely abolished when Fas-Fc fusion protein is added during co-culture, confirming that DNA fragmentation in Jurkat cells was entirely FasL-dependent (Fig. 3B). In an attempt to measure potential differences in the extent of Fas-receptor-mediated apoptosis induction between activated wtTNF and tmTNF CD4$^+$ T cells, T-cell blasts were incubated for 8 h in the presence of FasL vesicles produced by the murine FasL transfected neuroblastoma cell line Neuro2A-FasL [19]. Addition of these FasL vesicles equally increased by about 20% the frequencies of apoptotic wtTNF and tmTNF T-cell blasts. Addition of sTNF throughout the experiment did not increase the extent of FasL vesicles-mediated apoptosis. Hence, sTNF did not affect the sensitivity of the Fas-receptor-mediated cell death-signaling pathway in activated, non-TCR-restimulated T cells (Fig. 3C).

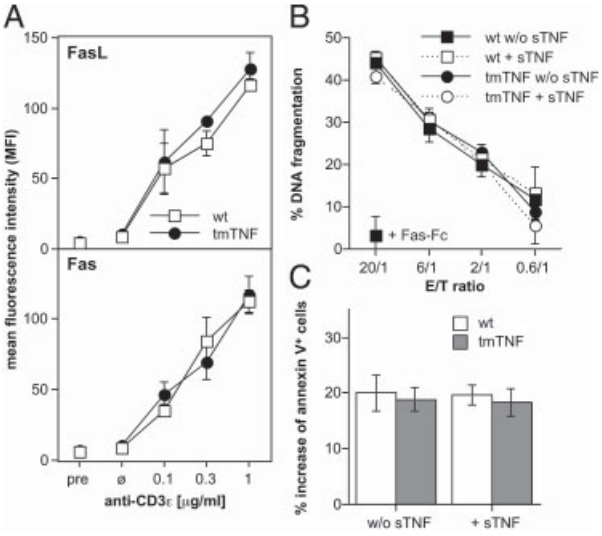

Figure 3. Cell surface expression of FasL and Fas, and FasL-mediated cytotoxicity are identical in wtTNF and tmTNF CD4$^+$ T cells. (A) T-cell blasts from wtTNF and tmTNF mice were restimulated with increasing concentrations of anti-CD3ε and stained for CD4, FasL, and Fas surface expression. Mean ± SD of data pooled from two independent experiments are shown, each with duplicates. (B) T-cell blasts from wtTNF and tmTNF mice, generated in the absence, or presence, of 10 ng/ml sTNF were co-cultured with ^{3}H-TdR-labeled, Fas-sensitive Jurkat cells at different *E/T* ratios in the presence, or absence, of 10 ng/mL sTNF. Fas-Fc fusion protein was added at 10 μg/mL to selected wells of the highest *E/T* ratio. After 12 h, extent of cell death (DNA fragmentation) was calculated as described in the *Materials and methods*. Mean ± SD of data pooled from two independent experiments are shown, each with triplicates. (C) FasL vesicles derived from the Neuro2A-FasL cell line were added for 8 h to wtTNF (open columns) or tmTNF T-cell blasts (shaded columns) generated in the absence, or presence, of 10 ng/mL sTNF. Apoptotic cells were detected by annexin V staining. Mean ± SEM of data pooled from four independent experiments, each with duplicates, are shown.

TNFR1 and TNFR2 are both required for sTNF mediated, enhanced susceptibility to AICD of CD4$^+$ T cells

Soluble and tmTNF have been previously reported to differentially bind and signal through TNFR1 and TNFR2. Hence, we determined next whether the two TNF receptors differ in their capacity to prime T cells by exposure to sTNF for enhanced AICD. To this end, TNFR1$^{-/-}$, TNFR2$^{-/-}$, and TNFR1$^{-/-}$2$^{-/-}$ mice were backcrossed on a tmTNF knock in (tmTNFki) background. The activation of CD4$^+$ T cells of tmTNF mice in the presence of sTNF increased the percentage of T cells undergoing AICD upon subsequent anti-CD3ε-mediated restimulation by about one-third. Addition of sTNF to TNFR1,2$^{-/-}$ tmTNFki T cells, however, had no effect on the number of T cells undergoing AICD following subsequent activation. Intriguingly, deficiency of either TNFR1 or TNFR2 on T cells almost completely abolished the sTNF-mediated increase in AICD (Fig. 4) and binding of sTNF to either TNFR2 or TNFR1 alone during the priming of CD4$^+$ T cells was insufficient to confer an increased AICD induction during subsequent CD3ε-mediated restimulation of the T cells.

Accelerated accumulation of tmTNF CD4$^+$ T cells in tmTNF RAG2$^{-/-}$ recipient mice

To assess how the differential rate of AICD induction in wtTNF and tmTNF CD4$^+$ T cells affects T-cell expansion during inflammatory conditions *in vivo*, we followed the accumulation of transferred T cells in the CD4$^+$CD45RB^{hi} T-cell transfer model of colitis [20, 21]. To this end, colitogenic wtTNF and tmTNF CD4$^+$ T-cell subsets were adoptively transferred into wtTNF and tmTNF RAG2$^{-/-}$ recipients, respectively. Mice were sacrificed on day 8 or 15 post transfer and colonic tissue was sampled for histopathological analysis. H&E-stained longitudinal and cross-sections of the proximal colon of experimental mice (Fig. 5A) revealed moderate signs of colitis on day 8 post adoptive transfer in both groups of recipient mice with a more pronounced mononuclear cell infiltration of the colonic lamina propria in tmTNF RAG2$^{-/-}$ recipients of tmTNF CD4$^+$ T cells compared with wtTNF RAG2$^{-/-}$ recipients of wtTNF CD4$^+$ T cells (Fig. 5A, inset photographs). Histopathological signs of colitis were more pronounced in both groups of mice sacrificed on day 15 post CD4$^+$ T-cell transfer (Fig. 5A). To directly quantitate the expansion of the transferred T cells, isolated CD4$^+$ T cells from the spleen, MLN, colonic epithelium, and colonic lamina propria of recipient mice were enumerated (Fig. 5B). CD4$^+$ T-cell numbers were markedly elevated in recipient mice of tmTNF CD4$^+$CD45RB^{hi} T cells in all lymphoid compartments analyzed. In MLN differences were more pronounced at the earlier time point (day 8) when, apart from an increased mononuclear cell infiltration seen in tmTNF Rag2$^{-/-}$ recipient mice, the colon still appeared histologically normal (Fig. 5A and B). In the colonic lamina propria differences in the number of CD4$^+$ T cells were most pronounced at a more advanced stage of the disease (day 15) when clear signs of severe inflammation were observed (Fig. 5A and B). At this time tmTNF RAG2$^{-/-}$ recipients revealed a slightly higher colitis score of 11.5 ± 1.2, compared with 9.2 ± 1.1 (mean $\pm$ SEM) of wtTNF RAG2$^{-/-}$ recipients of wtTNF CD4$^+$ T cells ($p = 0.17$, Fig. 5C).

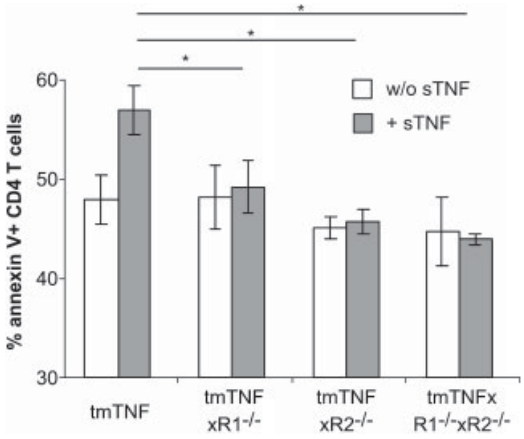

Figure 4. Sensitization of T cells for enhanced AICD by sTNF requires the presence of both TNF receptors, TNFR1 and TNFR2. T-cell blasts from tmTNF, tmTNFxTNFR1$^{-/-}$, tmTNF $\times$ TNFR2$^{-/-}$, and tmTNF $\times$ TNFR1$^{-/-}$2$^{-/-}$ mice were generated in the presence (+sTNF), or absence (w/o sTNF), of 10 ng/mL sTNF. T-cell blasts were restimulated for 4 h with 1 μg/mL plate-bound anti-CD3ε and frequencies of apoptotic cells determined by annexin V staining. Mean $\pm$ SEM of pooled data from six (tmTNF, tmTNF $\times$ TNFR1$^{-/-}$, tmTNF $\times$ TNFR2$^{-/-}$) or mean $\pm$ SD of pooled data from two (tmTNF $\times$ TNFR1$^{-/-}$2$^{-/-}$) individual experiments are shown, each with duplicates. Two-tailed *p*-values were determined across all experiments for the comparison of the relative changes in AICD upon priming in the presence of sTNF between the different transgenic donor mice, using the unpaired Student's *t*-test; *$p \leq 0.05$.

In vivo activated wtTNF CD4$^+$ T cells are more susceptible to *ex vivo* AICD than tmTNF CD4$^+$ T cells

We subsequently assessed whether *in vivo* primed CD4$^+$ T cells from tmTNF donor mice are also more resistant to *ex vivo* AICD than wtTNF CD4$^+$ T cells. wtTNF RAG2$^{-/-}$ recipients of wtTNF CD4$^+$CD45RB^{hi} spleen cells and tmTNF RAG2$^{-/-}$ mice transplanted with tmTNF CD4$^+$CD45RB^{hi} T cells were sacrificed on day 15 post adoptive transfer for isolation of MLN cells and colonic lamina propria lymphocytes (cLPL). Isolated cells were restimulated for 8 h with plate-bound anti-CD3ε and frequencies of apoptotic CD4$^+$ T cells determined. As shown in Fig. 6A, tmTNF CD4$^+$ T cells from MLN and colonic lamina propria are less susceptible to *ex vivo*-induced AICD than wtTNF CD4$^+$ T cells. In line with the results obtained *in vitro*, addition of exogenous sTNF to the primed CD4$^+$ T cells during *ex vivo* TCR restimulation alone did not increase the frequencies of annexin V-positive CD4$^+$ T cells from wtTNF or tmTNF origin (Fig. 6B).

Discussion

The role of TNF as the prototypic proinflammatory cytokine and its excessive production in autoimmune diseases [1, 22] makes it

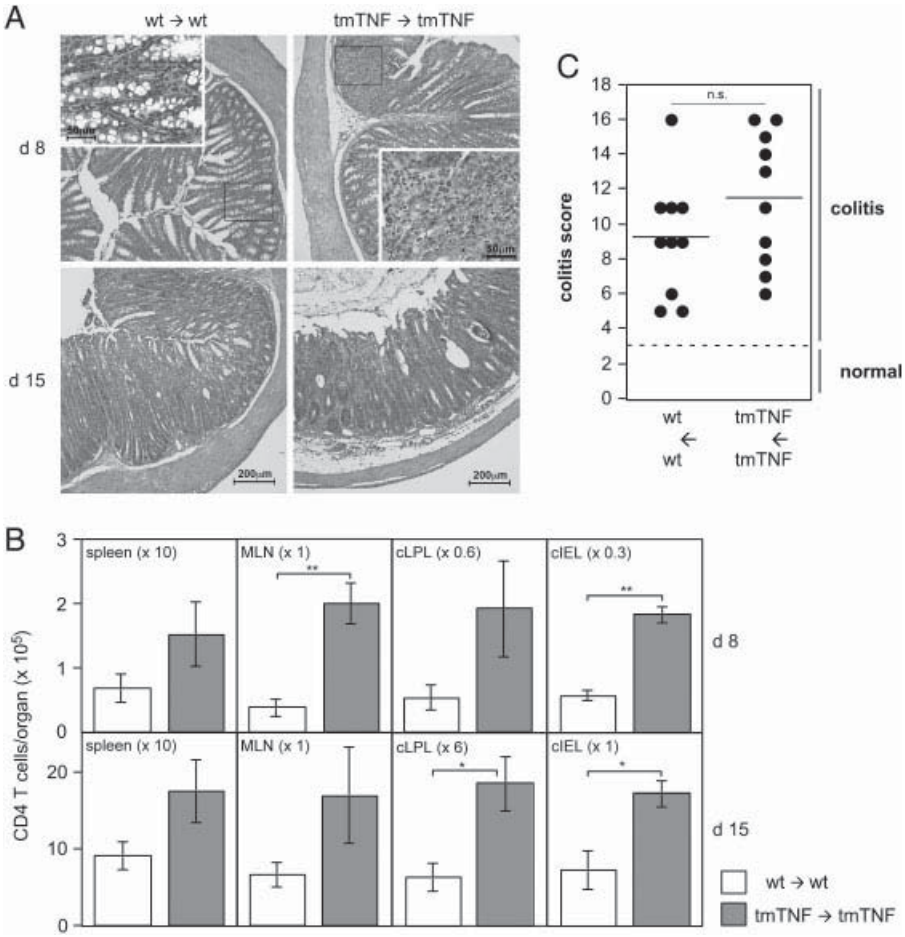

Figure 5. Adoptively transferred tmTNF $CD4^+$ T cells accumulate more rapidly in tmTNF $RAG2^{-/-}$ recipient mice than wtTNF $CD4^+$ T cells during colitis induction in wtTNF $RAG2^{-/-}$ mice. (A) Histopathology of the proximal colon of wtTNF $RAG2^{-/-}$ recipients of wtTNF $CD4^+CD45RB^{hi}$ T cells (wt→wt) and of tmTNF $RAG2^{-/-}$ recipients of tmTNF $CD4^+CD45RB^{hi}$ T cells (tmTNF→tmTNF) on day 8, and day 15, post cell transfer. Histology is representative of three, and ten recipient animals analyzed in each group on days 8 and 15, respectively. (B) Absolute numbers of $CD4^+$ T cells recovered from spleen, MLN colonic lamina propria, and colonic epithelium on days 8 and 15 post transfer of wtTNF $CD4^+$ T cells into wtTNF $RAG2^{-/-}$ recipients (open bars) and of tmTNF $CD4^+$ T cells into wtTNF $RAG2^{-/-}$ recipients (shaded bars). Mean ± SEM of pooled data from three individual mice *per* group are shown. Two-tailed *p*-values were calculated with the unpaired Student's *t*-test; $*p \leq 0.05$, $**p \leq 0.01$. (C) Colitis disease score was determined on day 15 post transfer on H&E-stained histology sections. ($n = 10$ for both groups); n.s. = not significant (two-tailed Student's *t*-test for difference of the means).

an obvious target in the treatment of chronic inflammatory disorders. The often-dramatic results seen in patients with fistulizing Crohn's disease, and patients with rheumatoid arthritis treated with mAb against TNF (*e.g.* infliximab, adalimumab), and/or TNF-binding soluble TNFR2 fusion protein (etanercept) support such a concept. In the present study, however, we clearly demonstrate an anti-inflammatory role of TNF *in vitro* and *in vivo* by promoting sensitization of T cells for subsequent AICD. This sensitization requires the presence of the soluble form of TNF already during the early priming phase of the T cells, and which involves concerted signaling *via* both TNF receptors. These observations nicely fit with our earlier finding that the exclusive presence of TNF as a 26 kDa non-cleavable mutant accelerates colonic inflammation in a T-cell transfer model of colitis [23]. Lack of sensitization of primed T cells for AICD in the absence of sTNF may even be potentiated by tmTNF mediated delay of AICD [24]. However, in our experiments, no additional tmTNF-mediated anti-apoptotic effect was found as tmTNF and $TNF^{-/-}$ $CD4^+$ T cells were equally resistant to AICD (Fig. 2A).

The initial characterization of $TNF^{-/-}$ mice not only confirmed the potent proinflammatory activities of TNF but, unexpectedly, also provided compelling evidence for an anti-inflammatory role of this cytokine [10]. The complexity of TNF-mediated pro-, and anti-inflammatory effects in the induction of deleterious autoimmune responses has been demonstrated in a mouse model of multiple sclerosis: while TNF was found to be essential in promoting acute EAE in susceptible mouse strains, it subsequently limited the expansion of myelin oligodendrocyte glycoprotein-reactive T cells, and thus, the chronic phase of the disease. In particular, the expansion of antigen-experienced T cells was prolonged in $TNF^{-/-}$ mice and, in contrast to wtTNF mice, EAE was readily induced by a second administration of

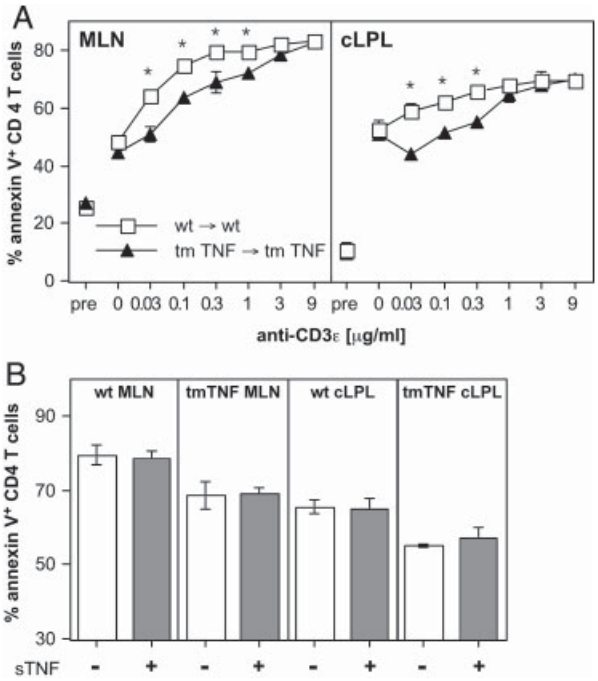

Figure 6. AICD is reduced in tmTNF $CD4^+$ T cells isolated from MLN and colonic lamina propria of colitic mice when compared with wtTNF $CD4^+$ T cells. wtTNF $RAG2^{-/-}$ recipients of wtTNF $CD4^+CD45RB^{hi}$ T cells (squares) and tmTNF $RAG2^{-/-}$ recipients of tmTNF $CD4^+$ $CD45RB^{hi}$ cells (triangles) were sacrificed on day 15 post transfer and $CD4^+$ T cells from MLN and colonic lamina propria were restimulated for 8 h with (A) increasing concentrations, or (B) a constant concentration of 0.3 μg/mL anti-CD3ε in the absence or presence of 10 ng/mL sTNF. Annexin V-positive $CD4^+$ T cells were determined before (pre) and after restimulation. Data show mean ± SEM ($n = 3$) and are representative of four (A) or two (B) independent experiments. Two-tailed p-values were calculated in (A) for differences between the isolated and restimulated wtTNF and tmTNF $CD4^+$ T cells from the MLN or colonic lamina propria using the unpaired Student's t-test. $^*p \leq 0.05$.

myelin oligodendrocyte glycoprotein peptide [25]. In a spontaneous mouse model of insulin-dependent diabetes mellitus, the NOD mouse, TNF led to increased [26, 27] or attenuated [28–30] islet infiltration, depending on timing and duration of exposure of the immune system to TNF [11]. Indications for an immunoregulatory role for TNF have been also reported in mouse models of infectious diseases, such as in schistosome [31] and *M. tuberculosis*/BCG infections [32], where TNF was found to be required to limit immunopathologies in the liver and lung, respectively. In humans, anti-inflammatory effects of TNF may be responsible for the unexpected failure of treating patients with multiple sclerosis with a soluble TNFR1 fusion protein (lenercept) [33]. The molecular mechanisms of these anti-inflammatory effects of TNF are not entirely understood. Given the substantial proliferative capacities of activated T cells [34] the differences in the extent of AICD induction observed in the present study will greatly affect the size of the pool of reactive T cells generated during an immune response. Hence, the sTNF-mediated enhanced AICD of highly activated T cells might represent an important mechanism of immunosuppression, particularly, since not only $CD4^+$ T cells but also splenic $CD8^+$ T cells can be primed by sTNF (but not tmTNF) for enhanced FasL-Fas-mediated AICD (data not shown).

The molecular mechanisms of how sTNF sensitizes primary T cells for accelerated AICD are presently unknown. The presence of sTNF during the priming phase did not affect surface expression levels of Fas and FasL (Fig. 3) and also the kinetics of mRNA expression of anti-apoptotic genes such as $FLIP_L$, Bcl-X_L, or cIAP-2 during priming and reactivation, assessed by quantitative RT-PCR, were not significantly different between tmTNF and wtTNF T cells (data not shown). For $FLIP_L$ these results were also confirmed by Western blotting (data not shown). The proapoptotic protein Bim, which has been implicated in the deletion of activated T cells *in vivo* [35], did also not show differential expression patterns on a Western blot, between wtTNF and tmTNF $CD4^+$ T-cell blasts (data not shown). However, our data confirm previous results that show a cooperation between the Fas and the TNF system in autoreactive T-cell control [36]. Furthermore, the lack of sTNF-mediated sensitization of tmTNF $CD4^+$ T cells for FasL-mediated apoptosis in the absence of anti-CD3ε-restimulation (Fig. 3C) suggests that TCR distal events yet to uncover may be involved in this sensitization process. We failed to consistently detect changes in the expression profile of pro-, *versus* anti-apoptotic molecules in these primary T cells when primed in the presence, *versus* absence, of sTNF. Despite these failed attempts, however, we still favor the hypothesis that TNF-mediated changes in the ratio of pro-, *versus* anti-apoptotic signals in a given primed T cell will affect its fate during subsequent restimulation. Such a differential susceptibility to AICD, however, may not be further influenced by TNF during restimulation of the T cells and is solely executed *via* FasL/Fas interactions since TNF signaling during TCR restimulation had no effect on AICD (Fig. 2D).

For the observed sTNF-mediated enhancement of AICD induction in T cells, both TNFR1 and TNFR2 were required as T cells from both $TNFR1^{-/-}$ and $TNFR2^{-/-}$ tmTNF mice showed no increase in AICD when primed in the presence of sTNF. Such a co-operative effect of TNFR1 and TNFR2 in the induction of cytotoxicity has been initially ascribed to a ligand passing effect of TNFR2, which may concentrate TNF on the cell surface for subsequent signaling *via* TNFR1 [37]. A few years later, the synergistic activities of both TNFR types in TNFR1-mediated cytotoxicity were ascribed to TNFR2-mediated negative regulation of TRAF2 functions [38]. In agreement with these findings, AICD and contraction of activated, TNFR2-deficient CD4 T cells is impaired, leading to the preferential expansion of TNFR2-deficient CD4 T cells upon co-transfer with wtCD4 T cells into lymphopenic mice. As a consequence, transferred TNFR2-deficient CD4 T cells induce an accelerated onset of colitis in $RAG2^{-/-}$ mice [39].

At present, one of the main concerns of a treatment with TNF-neutralizing agents is the potentially enhanced risk of opportunistic infections, particularly with *M. tuberculosis* [40]. As a possible way to circumvent this problem, the use of specific TACE inhibitors has been proposed. Indeed, in the absence of sTNF,

tmTNF is sufficient to induce the formation of granulomas and to protect mice from BCG infection [9] while possibly being inferior to sTNF in its capacity to induce inflammation in joints and central nervous system [7]. However, our data presented in this report provide compelling evidence that absence of sTNF may lead to adverse effects: absence of sTNF-mediated sensitization for AICD may result in excessive activation and accumulation of reactive T cells *in vivo*. As a consequence, the risk for mounting autoimmune reactions that may even lead to T-cell-driven autoimmune disorders may be enhanced [41]. The reported effect of TNF on the susceptibility of T cells to Fas-mediated cell death at immunopriviliged sites such as the eye [42] further illustrates the importance of such a sTNF-mediated control of AICD induction for maintaining local tissue homeostasis.

In conclusion, the present study demonstrates that sTNF but not tmTNF exerts immunoregulatory function by priming T cells for accelerated AICD. Such an sTNF-mediated effect on AICD may limit the extent of T-cell activation and expansion during inflammatory responses to eventually reduce – as part of a feedback mechanism – the risk of excessive systemic TNF levels that may lead to life-threatening conditions such as septic shock or cachexia. Our results obtained *in vivo* further indicate that the differential effect of sTNF and tmTNF on induction of T-cell apoptosis needs to be carefully evaluated when blocking of TNF shedding by TACE inhibitors is to be considered as a therapeutic strategy to control inflammatory disorders.

Materials and methods

Mice

C57BL/6J (B6) mice were originally purchased from Harlan (Horst, The Netherlands). RAG2$^{-/-}$ and TNF$^{-/-}$ mice, backcrossed to a B6 background, were originally purchased from C.D.T.A. – CNRS (Orléans, France). tmTNF tg $\times$ TNF$^{-/-}$ $\times$ lymphotoxin-$\alpha^{-/-}$ mice [4] were crossed at least six times with TNF$^{-/-}$ mice to obtain tmTNF tg LT$\alpha^{+/+}$ mice on a B6 background. RAG2$^{-/-}$ $\times$ TNF$^{-/-}$ mice and RAG2$^{-/-}$ $\times$ tmTNF tg mice were obtained by F2 breeding of the parental strains. tmTNFki mice on a B6 background [7] were kindly provided by Dr. Jon Sedgwick, DNAX, Palo Alto, USA. RAG2$^{-/-}$ $\times$ tmTNFki mice were generated by F2 breeding of the parental strains. Comparative analysis at the beginning of the study demonstrated that experiments with tmTNF tg and tmTNFki mice yield identical results and, therefore, the term "tmTNF" is subsequently used for both tmTNF tg, and tmTNFki mice. TNFR1 knock out (TNFR1$^{-/-}$), TNFR2$^{-/-}$, and TNFR1 and 2 double knock out (TNFR1$^{-/-}$2$^{-/-}$) mice were generously provided by Dr. Horst Blüthmann (F. Hoffmann-LaRoche, Switzerland) and were crossed with tmTNFki mice. tmTNFki $\times$ TNFR1$^{-/-}$, tmTNFki $\times$ TNFR2$^{-/-}$, and TmTNFki $\times$ TNFR1,2$^{-/-}$ mice were generated by F2 breeding of the parental strains. All animal experiments were approved by the local authorities of the Canton of Bern.

Anti-mouse antibodies and cytometry reagents

Anti-CD4-PE (GK1.5), anti-CD45-PE/Cy5 (30-F11), anti-CD45RB-FITC (16A), anti-FasL (MFL-3), anti-Fas (Jo2), anti-CD28 (37.51), and annexin V-FITC were purchased from BD Pharmingen (San Diego, CA, USA). Anti-CD3ε (145-2C11), anti-TCRαβ (H57–597), anti-CD8α (53–6.7), anti-CD45/B220 (RA3 6B2), and anti-CD11b/Mac-1 (M1/70) were purified from hybridoma supernatants by protein G. FITC conjugation of H57-597 and biotinylation of 53–6.7, RA3 6B2, and M1/70 was performed according to standard protocols. Biotinylated goat anti-hamster IgG and streptavidin-PE were purchased from Caltag Laboratories (Burlingame, CA, USA). Streptavidin-coupled paramagnetic microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Polyclonal anti-mouse TNF (IP400) was purchased from Genzyme (Boston, MA, USA).

Cell isolations and CD4$^+$ T-cell enumeration

Splenocytes and MLN cells were mechanically released and counted before staining for CD45 and CD4. Colonic intraepithelial lymphocytes and cLPL were isolated as described previously [43, 44]. For *ex vivo* AICD experiments, cLPL were enriched by discontinuous Percoll gradient centrifugation (Pharmacia, Uppsala, Sweden), while for *in vivo* accumulation analyses, crude isolates were assessed.

Proliferation of CD4$^+$ T cells

^{3}H-TdR incorporation: 96-well U-bottom plates were coated with serially diluted anti-CD3ε in 50 mM Tris, pH 9.0, overnight at 4°C. A total of 1.5×10^5 sorted cells were placed in 100 μL IMDM (GIBCO/Life Technologies, Gaithersburg, MA, USA)+10% FBS (GIBCO/Life Technologies) in the presence of 1 μg/mL anti-CD28 and cultured for 3 days at 37°C, 5% CO_2. An aliquot of 0.5 μCi/well ^{3}H-TdR (Pharmacia) was added for 6 h and incorporation measured on a TopCount™ microplate β-counter (Perkin Elmer, Schwerzenbach, Switzerland).

CFSE profile: whole splenocytes were isolated from wtTNF and tmTNF mice, resuspended at 5×10^7/mL PBS+5% FBS. CFSE (Molecular Probes, Eugene, OR, USA) was added at a final concentration of 3.5 μM and unincorporated fluorochrome washed away after 1 h. After a 3-day culture period in anti-CD3ε-coated 96-well plates, the CFSE profile of the CD4$^+$ population was analyzed by flow cytometry. The average number of cell divisions of the whole CD4$^+$ population was calculated from MFI in FL1 (FITC) according to the following equation: $x = \ln(p/y)/\ln(2)$; p = MFI of non-divided cells, y = MFI of whole CD4$^+$ population.

Induction of AICD

In vitro

Splenocytes were cultured at 37°C, 5% CO_2 for 48 h in IMDM+10% FBS+2 μg/mL Con A (Sigma, Buchs, Switzerland) at 3×10^6 cells/

mL. Medium was subsequently replaced by IMDM+10% FBS+ 100 units/mLrecombinant human IL-2 (Proleukin™, Chiron, Emeryville, MA, USA) for further 72 h. Dead cells were removed by discontinuous density gradient centrifugation (Lympholyte M®, Cedarlane, Burlington, NC, USA). Viable cells were cultured for 6 h at 1.5×10^5 cells/well in anti-CD3ε-coated 96-well U-bottom plates and subsequently stained with anti-CD4-PE and annexin V-FITC. Frequencies of apoptotic $CD4^+$ T cells were determined by flow cytometry. Murine recombinant TNF (sTNF, R&D Systems, Minneapolis, MN, USA) was added to the cultures at 10 ng/mL in all experiments at the indicated time points.

Ex vivo

MLN cells and cLPL were cultured for 8 h at 1.5×10^5 cells/well in anti-CD3ε-coated 96-well U-bottom plates. Annexin V-positive $CD4^+$ T cells were determined as described in the previous section (*in vitro* introduction of ACID).

Functional FasL assay

Functional cell surface expressed FasL was detected as described previously [17], Briefly, T-cell blasts (effector cells) were resuspended at 4×10^6 cells/mL in IMDM+5% FBS and serially diluted in 96-well U-bottom plates coated with 1 μg/mL anti-CD3ε. As targets, Fas-sensitive Jurkat target cells were labeled with 5 μCi/mL ^{3}H-TdR for 3 h and 10^4 ^{3}H-TdR-labeled Jurkat target cells were added *per* well. The plates were incubated for 12 h at 37°C, 5% CO_2. Fas-Fc fusion protein was generated as described previously [17] and used to confirm FasL specificity of the killing. The extent of cell death (% DNA fragmentation) was calculated as (1 - [cpm *e*/cpm *t*]) × 100; *e* = wells with effector and target cells, *t* = target cells only.

Assessment of functional Fas expression

Functional Fas expression was assessed by incubating T-cell blasts for 8 h in the presence of FasL vesicles freshly produced by the murine FasL transfected neuroblastoma cell line Neuro2A-FasL [19] (kindly provided by Dr. A. Fontana, University Hospital of Zurich, Switzerland) and subsequent staining with annexin V.

Adoptive $CD4^+CD45RB^{hi}$ T-cell transfer-mediated colitis

T- and B-cell-deficient $RAG2^{-/-}$ and $tmTNF^{-/-} \times RAG2^{-/-}$ mice were reconstituted i.p. with 2×10^5 sorted $CD4^+CD45RB^{hi}$ splenocytes from wtTNF or tmTNF mice as described previously [45]. $CD4^+$ T cells that express high levels of CD45RB are generally considered naïve T cells and induce progressive colonic inflammation upon adoptive transfer in lymphopenic mice [20]. After 8 or 15 days post transfer, mice were euthanized and tissue samples removed for histological assessment of colonic inflammation, cell isolations, and functional assays.

Histopathology

H&E-counterstained longitudinal and cross-sections of paraffin-embedded colonic tissue were scored for the grade of colitis independently by two persons in a blinded fashion. To the final score (0–16) the following aspects contributed with 0 = normal, 1 = mild/few, 2 = moderate/notable, and 3 = severe/excessive, unless stated otherwise: (i) thickness of mucosa; (ii) mononuclear infiltration; (iii) loss of goblet cells; (iv) presence of crypt abscesses; (v) epithelial defects (0 = absent, 1 = erosions); (vi) abundance and extent of dilated blood and lymph vessels (edemas in combination with mononuclear infiltrates).

Acknowledgements: The authors would like to thank Jon Sedgwick, Horst Blüthmann, and Adriano Fontana for generously providing mouse strains and reagents, Claudio Vallan and Bernadette Wider for cell sorting, Sabine Jakob for preparation of the Fas-Fc fusion protein and technical assistance, and Nadia Corazza for helpful discussions. This work was supported by Grants 31-53961.98 and 31-65307.01 from the Swiss National Science Foundation and a Senior Research Award from the Crohn's and Colitis Foundation of America to C.M.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Kollias, G., Douni, E., Kassiotis, G. and Kontoyiannis, D.,** On the role of tumor necrosis factor and receptors in models of multiorgan failure, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease. *Immunol. Rev.* 1999. **169**: 175–194.

2 **Moss, M. L., Jin, S. L., Milla, M. E., Burkhart, W., Carter, H. L., Chen, W. J., Clay, W. C. *et al.*,** Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* 1997. **385**: 733–736.

3 **Perez, C., Albert, I., DeFay, K., Zachariades, N., Gooding, L. and Kriegler, M.,** A nonsecretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. *Cell* 1990. **63**: 251–258.

4 **Mueller, C., Corazza, N., Trachsel-Loseth, S., Eugster, H. P., Buhler-Jungo, M., Brunner, T. and Imboden, M. A.,** Noncleavable transmembrane mouse tumor necrosis factor-alpha (TNFalpha) mediates effects distinct from those of wild-type TNFalpha *in vitro* and *in vivo*. *J. Biol. Chem.* 1999. **274**: 38112–38118.

5 **Alexopoulou, L., Pasparakis, M. and Kollias, G.,** A murine transmembrane tumor necrosis factor (TNF) transgene induces arthritis by cooperative p55/p75 TNF receptor signaling. *Eur. J. Immunol.* 1997. **27**: 2588–2592.

6 **Kusters, S., Tiegs, G., Alexopoulou, L., Pasparakis, M., Douni, E., Kunstle, G., Bluethmann, H. *et al.*,** *In vivo* evidence for a functional role of both tumor necrosis factor (TNF) receptors and transmembrane TNF in experimental hepatitis. *Eur. J. Immunol.* 1997. **27**: 2870–2875.

7 **Ruuls, S. R., Hoek, R. M., Ngo, V. N., McNeil, T., Lucian, L. A., Janatpour, M. J., Korner, H. *et al.*,** Membrane-bound TNF supports secondary

lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. *Immunity* 2001. **15**: 533–543.

8 **Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerretti, D. P., Alderson, M., Kerwar, S. S., Torrance, D. S. *et al.*,** Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature* 1994. **370**: 218–220.

9 **Olleros, M. L., Guler, R., Corazza, N., Vesin, D., Eugster, H. P., Marchal, G., Chavarot, P. *et al.*,** Transmembrane TNF induces an efficient cell-mediated immunity and resistance to Mycobacterium bovis bacillus Calmette-Guerin infection in the absence of secreted TNF and lymphotoxin-alpha. *J. Immunol.* 2002. **168**: 3394–3401.

10 **Liu, J., Marino, M. W., Wong, G., Grail, D., Dunn, A., Bettadapura, J., Slavin, A. J. *et al.*,** TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. *Nat. Med.* 1998. **4**: 78–83.

11 **Yang, X. D., Tisch, R., Singer, S. M., Cao, Z. A., Liblau, R. S., Schreiber, R. D. and McDevitt, H. O.,** Effect of tumor necrosis factor alpha on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. *J. Exp. Med.* 1994. **180**: 995–1004.

12 **Togbe, D., Grivennikov, S. I., Noulin, N., Couillin, I., Maillet, I., Jacobs, M., Maret, M. *et al.*,** T cell-derived TNF down-regulates acute airway response to endotoxin. *Eur. J. Immunol.* 2007. **37**: 768–779.

13 **Cope, A. P.,** Studies of T-cell activation in chronic inflammation. *Arthritis Res.* 2002. **4**: S197–S211.

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

15 **Isomäki, P., Panesar, M., Annenkov, A., Clark, J., Foxwell, B., Chernajovsky, Y. and Cope, A.,** Prolonged exposure of T cells to TNF down-regulates TCR zeta and expression of the TCR/CD3 complex at the cell surface. *J. Immunol.* 2001. **166**: 5495–5507.

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

17 **Brunner, T., Mogil, R. J., LaFace, D., Yoo, N. J., Mahboubi, A., Echeverri, F., Martin, S. J. *et al.*,** Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature* 1995. **373**: 441–444.

18 **Zheng, L., Fisher, G., Miller, R. E., Peschon, J., Lynch, D. H. and Lenardo, M. J.,** Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature* 1995. **377**: 348–351.

19 **Rensing-Ehl, A., Frei, K., Flury, R., Matiba, B., Mariani, S. M., Weller, M., Aebischer, P. *et al.*,** Local Fas/APO-1 (CD95) ligand-mediated tumor cell killing *in vivo*. *Eur. J. Immunol.* 1995. **25**: 2253–2258.

20 **Powrie, F., Leach, M. W., Mauze, S., Caddle, L. B. and Coffman, R. L.,** Phenotypically distinct subsets of CD4+T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int. Immunol.* 1993. **5**: 1461–1471.

21 **Morrissey, P. J., Charrier, K., Braddy, S., Liggitt, D. and Watson, J. D.,** CD4+ T cells that express high levels of CD45RB induce wasting disease when transferred into congenic severe combined immunodeficient mice. Disease development is prevented by cotransfer of purified CD4+T cells. *J. Exp. Med.* 1993. **178**: 237–244.

22 **Owens, T., Wekerle, H. and Antel, J.,** Genetic models for CNS inflammation. *Nat. Med.* 2001. **7**: 161–166.

23 **Corazza, N., Brunner, T., Buri, C., Rihs, S., Imboden, M. A., Seibold, I. and Mueller, C.,** Transmembrane tumor necrosis factor is a potent inducer of colitis even in the absence of its secreted form. *Gastroenterology* 2004. **127**: 816–825.

24 **Zhang, H. G., Liu, C., Su, K., Yu, S., Zhang, L., Zhang, S., Wang, J. *et al.*,** A membrane form of TNF-alpha presented by exosomes delays T cell activation-induced cell death. *J. Immunol.* 2006. **176**: 7385–7393.

25 **Kassiotis, G. and Kollias, G.,** 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.* 2001. **193**: 427–434.

26 **Hunger, R. E., Carnaud, C., Garcia, I., Vassalli, P. and Mueller, C.,** Prevention of autoimmune diabetes mellitus in NOD mice by transgenic expression of soluble tumor necrosis factor receptor p55. *Eur. J. Immunol.* 1997. **27**: 255–261.

27 **Green, E. A., Eynon, E. E. and Flavell, R. A.,** Local expression of TNFalpha in neonatal NOD mice promotes diabetes by enhancing presentation of islet antigens. *Immunity* 1998. **9**: 733–743.

28 **Satoh, J., Seino, H., Abo, T., Tanaka, S., Shintani, S., Ohta, S., Tamura, K. *et al.*,** Recombinant human tumor necrosis factor alpha suppresses autoimmune diabetes in nonobese diabetic mice. *J. Clin. Invest.* 1989. **84**: 1345–1348.

29 **Jacob, C. O., Aiso, S., Michie, S. A., McDevitt, H. O. and Acha Orbea, H.,** Prevention of diabetes in nonobese diabetic mice by tumor necrosis factor (TNF): similarities between TNF-alpha and interleukin 1. *Proc. Natl. Acad. Sci. USA* 1990. **87**: 968–972.

30 **Grewal, I. S., Grewal, K. D., Wong, F. S., Picarella, D. E., Janeway C. A., Jr. and Flavell, R. A.,** Local expression of transgene encoded TNF alpha in islets prevents autoimmune diabetes in nonobese diabetic (NOD) mice by preventing the development of auto-reactive islet-specific T cells. *J. Exp. Med.* 1996. **184**: 1963–1974.

31 **Davies, S. J., Lim, K. C., Blank, R. B., Kim, J. H., Lucas, K. D., Hernandez, D. C., Sedgwick, J. D. and McKerrow, J. H.,** Involvement of TNF in limiting liver pathology and promoting parasite survival during schistosome infection. *Int. J. Parasitol.* 2004. **34**: 27–36.

32 **Zganiacz, A., Santosuosso, M., Wang, J., Yang, T., Chen, L., Anzulovic, M., Alexander, S. *et al.*,** TNF-alpha is a critical negative regulator of type 1 immune activation during intracellular bacterial infection. *J. Clin. Invest.* 2004. **113**: 401–413.

33 **The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group.** TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. *Neurology* 1999. **53**: 457–465.

34 **Butz, E. A. and Bevan, M. J.,** Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* 1998. **8**: 167–175.

35 **Hildeman, D. A., Zhu, Y., Mitchell, T. C., Bouillet, P., Strasser, A., Kappler, J. and Marrack, P.,** Activated T cell death *in vivo* mediated by proapoptotic bcl-2 family member bim. *Immunity* 2002. **16**: 759–767.

36 **Zhou, T., Edwards C. K., III, Yang, P., Wang, Z., Bluethmann, H. and Mountz, J. D.,** Greatly accelerated lymphadenopathy and autoimmune disease in lpr mice lacking tumor necrosis factor receptor I. *J. Immunol.* 1996. **156**: 2661–2665.

37 **Tartaglia, L. A., Pennica, D. and Goeddel, D. V.,** Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. *J. Biol. Chem.* 1993. **268**: 18542–18548.

38 **Weiss, T., Grell, M., Siemienski, K., Muhlenbeck, F., Durkop, H., Pfizenmaier, K., Scheurich, P. and Wajant, H.,** TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J. Immunol.* 1998. **161**: 3136–3142.

39 **Dayer Schneider, J., Seibold, I., Saxer-Sekulic, N., Paredes, B. E., Saurer, L. and Mueller, C.,** Lack of TNFR2 expression by CD4(+) T cells exacerbates experimental colitis. *Eur. J. Immunol.* 2009. **39**: 1743–1753.

40 **Keane, J., Gershon, S., Wise, R. P., Mirabile-Levens, E., Kasznica, J., Schwieterman, W. D., Siegel, J. N. and Braun, M. M.,** Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N. Engl. J. Med.* 2001. **345**: 1098–1104.

41 **Ohashi, P. S.,** T-cell signalling and autoimmunity: molecular mechanisms of disease. *Nat. Rev. Immunol.* 2002. **2**: 427–438.

42 **Elzey, B. D., Griffith, T. S., Herndon, J. M., Barreiro, R., Tschopp, J. and Ferguson, T. A.,** Regulation of Fas ligand-induced apoptosis by TNF. *J. Immunol.* 2001. **167**: 3049–3056.

43 **Müller, S., Bühler-Jungo, M. and Mueller, C.,** Intestinal intraepithelial lymphocytes exert potent protective cytotoxic activity during an acute virus infection. *J. Immunol.* 2000. **164**: 1986–1994.

44 **Müller, S., Lory, J., Corazza, N., Griffiths, G. M., Z'graggen, K., Mazzucchelli, L., Kappeler, A. and Mueller, C.,** Activated CD4+and CD8+ cytotoxic cells are present in increased numbers in the intestinal mucosa from patients with active inflammatory bowel disease. *Am. J. Pathol.* 1998. **152**: 261–268.

45 **Corazza, N., Eichenberger, S., Eugster, H. P. and Mueller, C.,** Nonlymphocyte-derived tumor necrosis factor is required for induction of colitis in recombination activating gene (RAG)2($^{-/-}$) mice upon transfer of CD4 (+)CD45RB(hi) T cells. *J. Exp. Med.* 1999. **190**: 1479–1492.

Abbreviations: **AICD:** activation-induced cell death · **BCG:** bacillus Calmette-Guérin · **B6:** C57BL/6J · **cLPL:** colonic lamina propria lymphocytes · **sTNF:** soluble TNF-α · **TACE:** TNF-α-converting-enzyme · **tmTNF:** transmembrane TNF-α · **tmTNFki:** tmTNF knock in · **^{3}H-TdR:** ^{3}H-thymidine

Full correspondence: Dr. Stefan Müller, Department of Clinical Research, Division of Gastroenterology, University of Bern, CH-3010 Bern, Switzerland
Fax: +41-31-632-3297
e-mail: stefan.mueller@dkf.unibe.ch

Additional correspondence: Dr. Christoph Mueller, Institute of Pathology, P. O. Box 62, University of Bern, CH-3010 Bern, Switzerland
e-mail: christoph.mueller@pathology.unibe.ch