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

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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 metalloprotease 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.
However, several studies also revealed anti-inflammatory effects of TNF. For example, Liu et al. observed exacerbation of demyelination and inflammation in an EAE model in the absence of TNF [10]. In type 1 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 3H-thymidine (3H-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, 3H-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-CD3c. 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/C0. As shown in Fig. 2A, a more pronounced induction of AICD occurred in wtTNF CD4+ T cells, while tmTNF and TNF/C0 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/C0 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
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 ³H-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-signalizing pathway in activated, non-TCR-restimulated T cells (Fig. 3C).

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 0.5 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 < 0.05, **p < 0.01.

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 ³H-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 plates. DNA fragmentation in Jurkat cells was entirely FasL-dependent (Fig. 3B). Addition of sTNF during activation and culture of T-cell blasts did not affect the extent of target cell DNA fragmentation. (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⁺ CD45RBhi 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⁺ CD45RBhi 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±SEM) of wtTNF RAG2⁻/⁻ recipients of wtTNF CD4⁺ T cells (p = 0.17, Fig. 5C).

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⁺ CD45RBhi spleen cells and tmTNF RAG2⁻/⁻ mice transplanted with tmTNF CD4⁺ CD45RBhi 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...
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
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, Bel-Xo, or c-FLAP in the absence of sTNF (but not tmTNF) for enhanced FasL-Fas-mediated AICD (data not shown). For FLIP, these results were also confirmed by Western blotting (data not shown). The pro-apoptotic protein Bim, which has been implicated in the deletion of activated T cells in vivo [35], did also not show differential expression patterns. 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<0.05.

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+/CD45RBhi T cells (squares) and tmTNF RAG2-/- recipients of tmTNF CD4+/CD45RBhi cells (triangles) were sacrificed on day 15 post transfer and CD4+ T cells from MLN and colonic lamina propria were restimulated for 8h 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+S.E.M. (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<0.05.
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 immunoprophylactic 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 not be tmTNF exerts immunoregulatory function by priming T cells for maintaining local tissue homeostasis.

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 × TNF−/− × lymphoxygen-α−/− mice [4] were crossed at least six times with TNF−/− mice to obtain tmTNF tg LTα+/+ mice on a B6 background. RAG2−/− × TNF−/− × mice and RAG2−/− × 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−/− × 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−/− × TNFR2−/−) mice were generously provided by Dr. Horst Blüthmann (F. Hoffmann-LaRoche, Switzerland) and were crossed with tmTNFki mice. tmTNFki × TNFR1−/−, tmTNFki × TNFR2−/−, and tmTNFki × 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-CD3e (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 S3–6.7, 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

3H-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 × 105 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% CO2. An aliquot of 0.5 µCi/well 3H-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 × 107/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% CO2 for 48 h in IMDM+10% FBS+2 µg/mL Con A (Sigma, Buchs, Switzerland) at 3 × 106 cells/
mL. Medium was subsequently replaced by IMDM+10% FBS+100 units/mL recombinant human IL-2 (ProliferaTm, 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 x 10^5 cells/well in anti-CD3e-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 x 10^5 cells/well in anti-CD3e-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 x 10^5 cells/mL in IMDM+5% FBS and serially diluted in 96-well U-bottom plates coated with 1 µg/mL anti-CD3e. As targets, Fas-sensitive Jurkat target cells were labeled with 5 µCi/mL ^3H-TdR for 3 h and 10^5 ^3H-TdR-labeled Jurkat target cells were added per well. The plates were incubated for 12 h at 37°C, 5% CO2. 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 t/cpm C0)) x 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.

**Adaptive CD4+CD45RBhi T-cell transfer-mediated colitis**
T- and B-cell-deficient RAG2−/− and tmTNF−/− x RAG2−/− mice were reconstituted i.p. with 2 x 10^5 sorted CD4+CD45RBhi 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).

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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  ·  3H-TdR: 3H-thymidine

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