Autoimmune thyroid disease induced by thyroglobulin and lipopolysaccharide is inhibited by soluble TNF receptor type I

Paola Zaccone[1], Zoltán Fehérvári[1], Lucy Blanchard[1], Ferdinando Nicoletti[2], Carl K. Edwards III[3] and Anne Cooke[1]

[1] Department of Pathology, Immunology Division, Cambridge University, Cambridge, GB
[2] Department of Biomedical Sciences, Section of General Pathology, University of Catania, Catania, Italy
[3] Amgen Inc., Thousand Oaks, USA

Experimental autoimmune thyroiditis (EAT) is inducible in mice by immunization with thyroglobulin and adjuvant. Previous studies have shown that EAT is an autoimmune Th1-mediated disease but its characteristics differ with the adjuvant. Granulomatous lesions with marked follicular disruption develop following administration of thyroglobulin (Tg) and complete Freund's adjuvant (CFA) whereas when lipopolysaccharide (LPS) is used as the adjuvant only focal infiltrates of mononuclear cells are observed. The pro-inflammatory cytokine, TNF-α, is associated with Th1 autoimmune-mediated conditions. Cytokine antagonists have been used as potential therapeutic agents in several experimental autoimmune models. Soluble cytokine receptors belong to this category and may naturally be shed from cell membranes to inhibit cytokine activity. We show that the administration of the soluble TNF receptor type I (sTNFR I) in the induction of EAT has very different effects on the two models of induced autoimmune thyroiditis. sTNFR I treatment inhibits the induction of EAT only when mouse Tg is given with LPS not with CFA, suggesting an important difference in the pathogenic processes.

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1 Introduction

Experimental autoimmune thyroiditis (EAT) is inducible in susceptible strains of mice by immunization with mouse thyroglobulin (mTg) and either lipopolysaccharide (LPS) or complete Freund's adjuvant (CFA) as adjuvant. However, there are differences between the lesions which develop when using the two adjuvants. When CFA is used as the adjuvant with mTg the lymphoid lesions which develop are granulomatous and the lymphoid follicular architecture is severely disrupted. On the other hand when LPS is used as the adjuvant together with mTg only focal infiltrates of mononuclear cells are observed [1]. While the differences observed may be simply due to the degree of disease severity induced by the two adjuvants it could also be accounted for by a different underlying mechanism of pathogenesis. Previous studies have inferred that EAT induced by both adjuvants is a "Th1-like disease". For example, administration of IL-10 has a protective effect on EAT induced by mTg/LPS [2], and progression to full-blown EAT requires an intact IFN-γ system [3], finally neutralization of IFN-γ diminishes the severity of thyroid lesions induced by mTg and CFA [4].

TNF-α is another pro-inflammatory cytokine associated with Th1 autoimmune mediated conditions such as SLE-like syndrome, autoimmune diabetes in nonobese diabetic (NOD) mice, collagen-induced arthritis (CIA) or experimental allergic encephalomyelitis (EAE) [5]. TNF-α may play a direct pathogenic role or indirectly influence disease pathogenesis through effects on macrophage activation and/or B cell differentiation and antibody production. Individual cytokine antagonists or combinations of them have been used in experimental autoimmune models as potential therapeutic agents. Soluble cytokine receptors belong to this category and may naturally be shed from cell membranes to inhibit cytokine activity, but their release is normally insufficient to entirely neutralize the cytokine's effect. Two different soluble forms of the TNF receptor (sTNFR) have been identified, sTNFR I

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Abbreviations: sTNFR I: Soluble TNF receptor type I **EAT:** Experimental autoimmune thyroiditis **mTg:** Mouse thyroglobulin **NOD:** Nonobese diabetic

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(p55) and sTNFR II (p75) [6]. Both receptors bind with equal affinity to TNF-α and lymphotoxin [7]. We show here that the administration of sTNFR I in the induction of EAT produces different effects depending on the adjuvant used to immunize the mice. We show that the sTNFR I treatment inhibited induction of EAT only when mTg is given with LPS but not with CFA, suggesting an important difference in the role of TNF-α in the two models of EAT.

2 Results

2.1 Effect of continuous sTNFR I treatment on EAT

When CFA is used with mTg to prime for EAT there is a more aggressive disease than when LPS is used as the adjuvant [1]. To explore the involvement of TNF-α in disease pathogenesis, mice immunized with mTg and LPS or mTg and CFA, were given sTNFR I three times a week. It can be seen in Fig. 1 that while administration of sTNFR I markedly reduced all thyroglobulin-specific immunoglobulin isotypes in mTg/LPS-immunized mice, neutralization of this cytokine had no effect on autoantibodies induced by mTg/CFA. This effect on autoantibody titers was mirrored in the levels of induced thyroiditis where neutralization of TNF-α decreased the severity of thyroid lesions in mTg/LPS-immunized mice but did not influence the extent of thyroid infiltration seen following mTg/CFA. Furthermore, this effect of TNF-α neutralization was seen even when sTNFR I administration was delayed until the second priming dose of mTg and adjuvant. At the end of the experiment spleens (at day 28 from mTg/LPS immunized mice) and lymph node (at day 21 from mTg/CFA immunized mice) cells from control and sTNFR-treated mice were analyzed phenotypically and functionally. There was no difference in numbers or surface level of expression of CD4, CD8, B220, or CD62 ligand (CD62L) nor was there any difference in the proliferative response and in the cytokine secretion to mTg, LPS or Con A (data not shown).

2.2 sTNFR I protects mice from mTg and LPS-induced EAT even when administered only at the time of immunization

To determine whether TNF-α was important during immunization with LPS, the soluble receptor was only given for 3 days around the time of immunization itself and not throughout the experiment. From Fig. 2 it can be seen that this more restricted protocol was just as effective at reducing pathology as sustained administration of sTNFR I. On day 28 all subclasses of IgG antibodies in

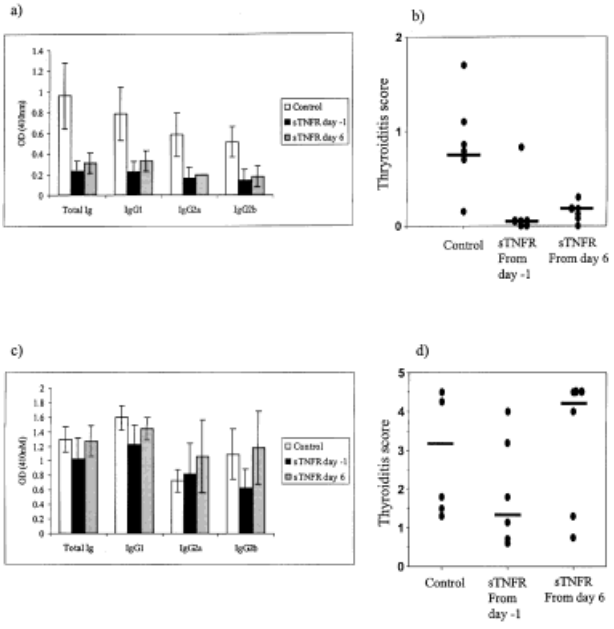

Fig. 1. sTNFR I decreases severity of thyroid lesions and anti-thyroglobulin antibodies only when thyroiditis is induced by administration of mTg and LPS. Eighteen CBA/J mice were immunized with mTg/CFA or mTg/LPS (Sect. 4). Some of these (6 mice) received 5 mg/kg sTNFR 3 days/week over a period of 21 (mTg/CFA) or 28 (mTg/LPS) days starting the day before the first immunization. A second group (5 mice) received the same dose of sTNFR starting the day before the second immunization. Control group (7 mice) received PBS 3 days/week for 28 days. (a) Serum (diluted 1/800) antibody response to mTg using LPS as adjuvant. Controls were significantly higher than both of sTNFR-treated groups for all the isotypes tested ($p<0.004$). (b) Histological score of thyroid lesions induced by mTg/LPS. Controls were significantly higher than sTNFR from day –1 ($p<0.025$) and sTNFR from day 6 ($p<0.025$). (c) Serum (diluted 1/8,000) response to mTg using CFA as adjuvant. (d) Histological score of thyroid lesions induced by mTg/CFA. None of the readouts for the mTg/CFA showed significant differences between any of the groups.

the serum were decreased in mice after sTNFR I treatment. Interestingly, serum taken on day 10 after the first immunization showed that the IgM level in the sTNFR I-treated group was comparable with the control group. In contrast, the IgG antibody levels were diminished in the sTNFR I-treated group (Fig. 3a). This result suggests that sTNFR I treatment prevents isotype switching from IgM to IgG, confirming the role of TNF-α in B cell maturation. To analyze whether this effect on IgG was due to sTNFR I treatment preventing germinal center formation and B cell development, spleens were taken on day 10 and sections examined for germinal centers by staining with peanut agglutinin (PNA) and antibody to B220. No significant differences were found between sTNFR-treated

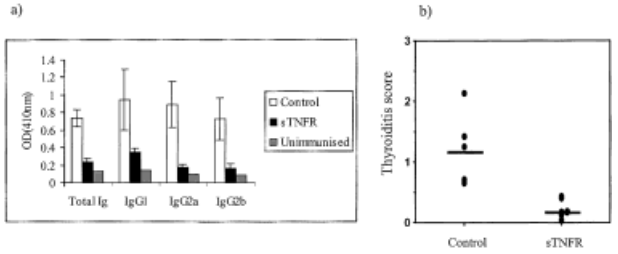

Fig. 2. sTNFR decreases thyroiditis when given for a short period of 3 days with mTg and LPS. CBA/J mice (12) were immunized with mTg/LPS. One group (6 mice) received 5 mg/kg sTNFR over a period of 3 days coinciding with the first and second immunizations of mTg/LPS. Control group (6 mice) were treated with PBS alone. (a) Serum antibody response to mTg (sera diluted 1/800). Controls were significantly higher than both of sTNFR-treated groups for all the isotypes tested ($p<0.002$). (b) Histological score of thyroid lesions induced by mTg/LPS. Controls showed a significantly higher ($p<0.025$) thyroiditis score than the sTNFR group.

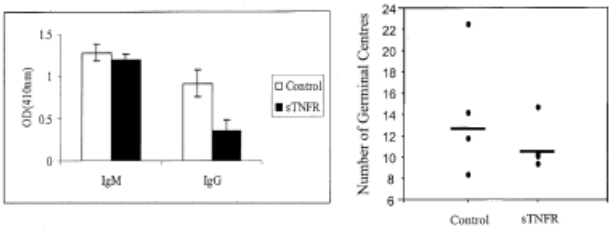

Fig. 3. sTNFR treatment prevents isotype switching from IgM to IgG without affecting germinal cener formation. Five CBA/J mice were immunized with mTg/LPS and treated with sTNFR as described in Fig. 2. The five control mice received PBS alone as treatment (white bars). Mice were killed 10 days after the first immunization. (a) IgG (1/100) and IgM (1/800) serum antibody response to mTg. IgG production by the controls was significantly higher ($p<0.0001$) than the sTNFR-treated group whereas IgM was not significant. (b) Number of splenic germinal centers seen 10 days after first immunization. The number of germinal centers was not significantly different between the two groups. Four individual spleens examined at three different levels for both of the treatment groups.

and control groups (Fig. 3b). From Fig. 3b it can be seen that germinal centers are similar in number and Fig. 4 shows that there are no apparent qualitative differences between the germinal centers of the two groups.

2.3 sTNFR I also protects NOD mice from EAT induced by mTg/LPS

In normal mouse strains the thyroid lesions induced following administration of mTg and CFA resolves within 6 weeks. However, studies in NOD mice have shown that this autoimmune-prone mouse strain fails to resolve the induced thyroid lesion [8]. We compared the production of TNF-α by activated peritoneal macrophages in CBA/J and NOD mice (Fig. 5) and found much higher levels produced by NOD mice ($p<0.01$). To determine whether neutralization of TNF-α might improve disease outcome in autoimmune prone mouse strains such as NOD we examined whether sTNFR I treatment might also prevent EAT induction in NOD mice. Fig. 6 shows that as in the CBA/J mouse, sTNFR I treatment is capable of reducing both autoantibody and severity of thyroid lesions in NOD mice. To investigate the mechanism by which sTNFR I modulates thyroid autoimmunity, splenocytes from mTg/LPS-immunized mice were taken at day 10 after the first immunization and compared with those from sTNFR I-treated but comparably immunized mice. We measured both proliferation and cytokine response to the mitogens Con A and LPS *in vitro*. The data presented in Fig. 7 show a reduced proliferative response and IFN-γ production following sTNFR I treatment (Fig. 7a and b). There was also an increased IL-10 response by the LPS-stimulated splenocytes of sTNFR I-treated, mTg/LPS-immunized mice. The increased production of IL-10 might contribute to a diminished *in vivo* autoimmune response to thyroid antigens in NOD mice treated with sTNFR I. If lymph node cells, from mTg/CFA-immunized mice, are taken 10 days after the first immunization and cultured *in vitro* with mTg (50 μg/ml) or Con A (2 μg/ml), no differences are found between sTNFR-treated and untreated mice (data not shown).

3 Discussion

EAT is commonly used as a model of human (Hashimoto's) autoimmune thyroiditis. This form of hypothyroidism is characterized histopathologically by a focal mononuclear cell infiltrate in the thyroid and serologically by circulating antibodies specific for thyroid peroxidase and thyroglobulin. In susceptible mouse strains, both CFA and LPS serve as potent adjuvants for inducing EAT with mTg. Most studies refer to EAT as a model of Th1 autoimmunity regardless of the adjuvant used to induce the disease [2, 4]. Some studies refer generally to "adjuvants" without specifying whether CFA or LPS was used to induce EAT [3]. In our laboratory both adjuvants were used to induce EAT and the differences investigated. The level of serum anti-thyroglobulin autoantibodies (Fig. 1a, serum dilution 1:800; Fig. 1c, serum dilution 1:8,000) and the size of thyroid are significantly greater in mice immunized with CFA than in those immunized with LPS (Fig. 1b and d). Following CFA immunization the follicular architecture is destroyed and the thyroid infiltrate con-

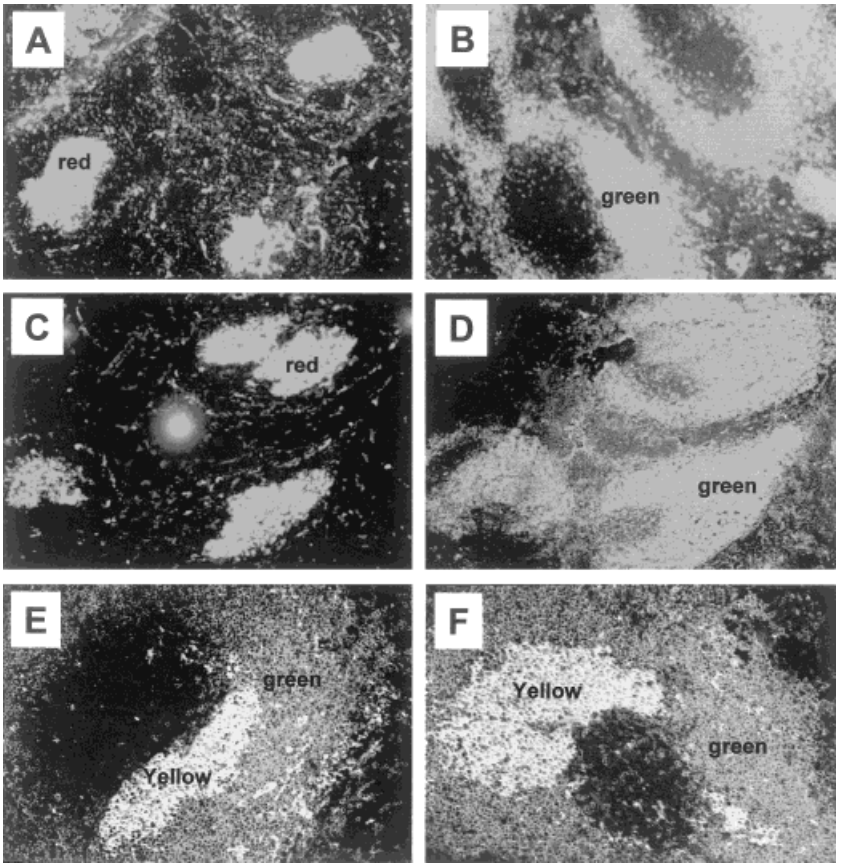

Fig. 4. sTNFR I treatment does not prevent germinal center formation in the spleen. Spleen sections were stained with PNA to examine the number of germinal centers and their architecture. Sections were also co-stained with B220 to examine the frequency and distribution of B cells. (A) PNA (red) staining of spleen sections from control mice; (B) B220 (green) staining of spleen sections from control mice; (C) PNA (red) staining of spleen sections from sTNFR I-treated mice; (D) B220 (green) staining of spleen sections from sTNFR I-treated mice (×100 magnification); (E) Overlay of PNA (yellow) and B220 (green) staining of spleen sections from control mice; (F) Overlay of PNA (yellow) and B220 (green) staining of spleen sections from sTNFR I-treated mice. (×200 magnification).

sists not only of mononuclear cells but additionally polymorphonuclear cells typifying granulomatous lesions. Histologically, CFA induced EAT resembles more a human form of subacute thyroiditis, known as De Quervain's thyroiditis, which is often associated with a generalized infectious process such as tuberculosis and syphilis. When LPS is used with mTg to induce EAT the thyroid is not enlarged, the follicular structure is preserved, and the infiltration is focal and consists almost entirely of mononuclear cells. If CFA is used the acute infiltration in the thyroid peaks at day 21 and tends to resolve spontaneously within 6 weeks after the first immunization with mTg. In contrast LPS/mTg causes a peak infiltration at 28 days after the first immunization which does not resolve for 2 months or longer. Different outcomes of sTNFR I treatment in the two models of induced thyroiditis might be due to differences in the underlying pathological process or differences in the kinetics of the response.

From the present study it is clear that sTNFR I treatment is able to diminish both thyroid infiltration and thyroglobulin autoantibodies only if mice are immunized with thyroglobulin and LPS (Fig. 1a, b, c and d). This effect of sTNFR I was seen in both CBA/J and NOD mice. These two strains were chosen because one, the CBA/J mouse is a good responder in terms of induced thyroid autoimmunity, while the other, the NOD mouse, spontaneously develops autoimmune pathologies. Although the NOD mouse is primarily known for its development of diabetes type I, it additionally develops Sjogren's syndrome and thyroiditis (albeit at a low incidence). The incidence of autoimmune thyroid disease in NOD mice can, however, be increased by elevated intake of dietary iodine [9, 10].

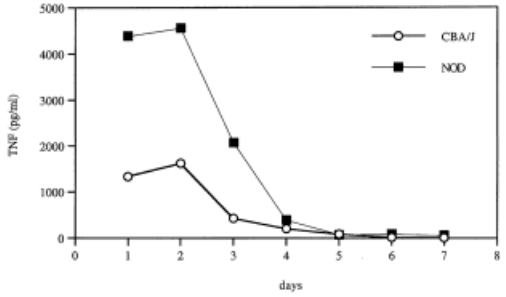

Fig. 5. Peritoneal exudate cells (PEC) from NOD thioglycolate-treated mice produce elevated levels of TNF-α compared to those from CBA/J mice. PEC were obtained from CBA/J and NOD mice. Cells were incubated in 96-well flat-bottom tissue culture plates and stimulated with 5 μg/ml LPS over a period of 7 days. Supernatants were sampled every 24 h for concentration of TNF-α. Unstimulated cells did not produce detectable amounts of TNF-α (not shown). NOD PEC produced significantly higher levels of TNF-α than the CBA controls ($p<0.01$).

This strain is additionally highly sensitive to the induction of autoimmune thyroid disease by thyroglobulin and adjuvant or by TSHR (thyroid-stimulating hormone receptor) and adjuvant [11]. The low level of expression of the inhibitory FcγRIIb by NOD mice may further contribute to the severity of the developed thyroid pathology. Autoimmune thyroid disease induced experimentally with CFA does not resolve in NOD mice as rapidly as it does in CBA/J mice, leading to the suggestion that there may be some defect in immune regulation in NOD mice [8].

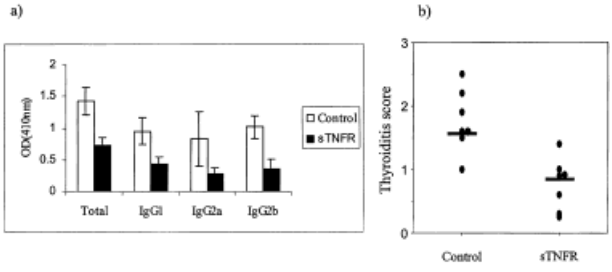

Fig. 6. sTNFR I treatment decreases EAT development in NOD as well as CBA/J mice. Seven NOD mice were immunized with mTg/LPS and treated with sTNFR as described in Fig. 2. The seven control NOD mice were also immunized with mTg/LPS and received PBS alone as treatment. (a) Serum antibody response to mTg (sera diluted 1/400). Controls were significantly higher than both of sTNFR-treated groups for all the isotypes tested ($p<0.006$). (b) Histological score of thyroid lesions induced by mTg/LPS. Controls showed a significantly higher ($p<0.001$) thyroiditis score than the sTNFR I groups.

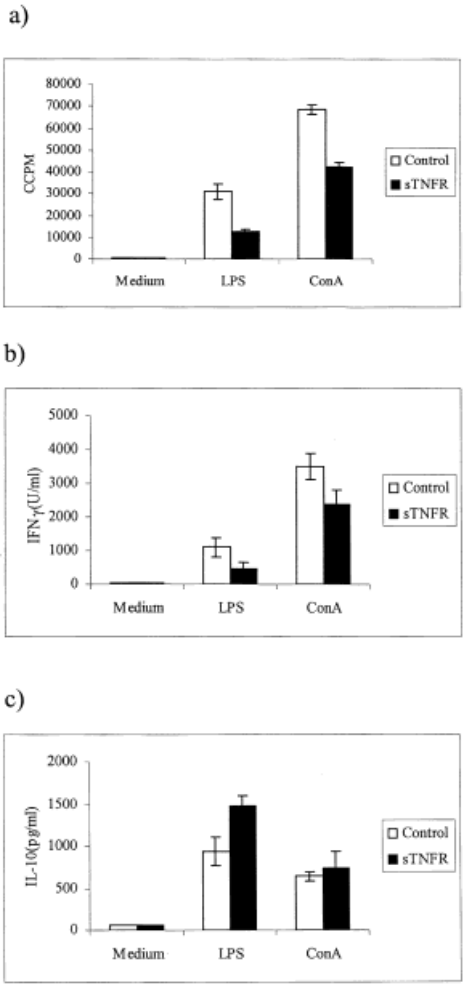

Fig. 7. sTNFR I inhibits both mitogen-induced proliferation and IFN-γ production but increases LPS-induced IL-10 *in vitro*. Six NOD mice were immunized with mTg/LPS and treated with sTNFR as described in Fig. 2. The six control NOD mice were immunized with mTg/LPS and received PBS alone as treatment. At 10 days after the first immunization mice were killed and splenocytes prepared for functional analysis. (a) Proliferation in response to LPS and ConA. Controls were significantly higher ($p<0.0001$) than the sTNFR-treated group. (b) IFN-γ production in response to LPS and ConA. Controls were significantly higher ($p<0.001$) than the sTNFR treated group. (c) IL-10 production in response to LPS and ConA. Controls produced significantly higher ($p<0.0001$) amounts of IL-10 than the sTNFR-treated group when stimulated by LPS.

LPS-induced EAT is referred to as a Th1-like autoimmune disease since IL-12 administration given at the same time as the first and the second immunization has been shown to increase the level of thyroid infiltration seen in CBA/J mice following mTg and LPS [1]. Comparisons of levels of cytokine production by macrophages following LPS stimulation revealed that NOD macro-

phages produce significantly more TNF-α (Fig. 5) and IL-12 (data not shown) than CBA/J macrophages. TNF-α is a major product of macrophages stimulated by LPS and, together with IL-12 and IFN-γ, it drives a pathogenic Th1-like response. TNF-α additionally stimulates monocytes to produce other inflammatory mediators like IL-1 and nitric oxide that also serves to amplify the effects of the adjuvant during antigen presentation. The ability of TNF-α to up-regulate MHC class II and adhesion molecule expression on endothelial cells also contributes to inflammation. The pre-disposition of NOD mice to develop a greater pro-inflammatory response to LPS may therefore lead to the increased levels of the thyroid pathology (Fig. 2 and 6) seen in this strain compared to CBA/J mice. The ability of sTNFR I to modulate EAT in NOD mice suggests a pivotal role for TNF-α and lymphotoxin in thyroid pathology and furthermore demonstrates the efficacy of their neutralization as a therapeutic agent in individuals genetically pre-disposed to develop autoimmunity. Systemic ablation of TNF-α causes an enhanced production of IL-10 and diminished secretion of IFN-γ by LPS-stimulated splenocytes (Fig. 7b). The increased IL-10 and lowered levels of IFN-γ (Fig. 7c) would favor reduced Th1 responsiveness. A reduction in Th1 responsiveness would also contribute to the reduced B cell IgG antibody response that is seen following sTNFR I treatment of mTg/LPS-immunized mice. In this study it is not possible to definitely determine whether the sTNFR I-mediated inhibition of EAT and the cytokine/serum Ig effects are due to TNF and/or lymphotoxin blockade. Distinguishing between these scenarios could be performed by use of appropriate blocking antibodies or knockout mice.

Molecules of the TNF superfamily and their receptors play a crucial role in germinal center formation, B cell differentiation and antibody production. Moreover, it has been shown that mice lacking either lymphotoxin-α (LT-α) or TNFR I fail to develop germinal centers in peripheral lymphoid organs [12]. In our study sTNFR treatment did not appear to significantly affect germinal center formation in the spleen (Fig. 3b and 4). The reduction in IgG but not IgM autoantibody production does not therefore appear to be due to a failure of germinal center formation *per se,* but may be due to an inability to isotype switch. It cannot, however, be ruled out that sTNFR I administration acts simply to reduce the numbers of IgG-producing plasma cells.

sTNFR type I and type II are found in the plasma of patients with SLE, progressive systemic sclerosis and rheumatoid arthritis. The sTNFR triggers an “off” signal during an inflammatory process, however, physiological levels of sTNFR found in autoimmune individuals are normally not sufficient to ameliorate the disease but still represent a possible therapy for autoimmune disease. The efficacy of blockade of TNF-α in RA (rheumatoid arthritis) in alleviating disease symptoms supports its key role in the pathology of such diseases. Our study shows that sTNFR I inhibited both the cell-mediated and humoral arm of autoimmune thyroid disease and suggests its possible utility as a therapeutic agent in treatment of some forms of autoimmune thyroid disease of humans. Furthermore, this study highlights the fact that different adjuvants, while initiating a Th1 response, may elicit a different cytokine cascade. Neutralization of TNF or lymphotoxin in our studies reduced IL-10 production and diminished the generation of pro-inflammatory cytokines when LPS was used as an adjuvant. This effect was not seen using CFA. Therefore, care should be taken in the use of different adjuvants to induce experimental animal models of human autoimmune disease and the interpretation of the subsequent results.

4 Materials and methods

4.1 Mice

EAT susceptible 6–8-week-old CBA/J mice (H-2^k) of both sexes were obtained from Harlan U.K. Ltd Bicester Oxon. NOD (H-2^{g7}) mice were obtained from breeding colonies established in the Pathology Department, University of Cambridge facilities. During the experiments all the mice were maintained in the same standard conditions at the Department of Pathology with free access to food and water.

4.2 Induction of EAT using adjuvant

mTg was prepared by extraction from the pooled homogenized thyroids of normal outbred mice as previously described [13]. Mice were given two immunizations with 50 μg mTg emulsified in CFA (H37Ra Difco Laboratories, Detroit, MI). The first, on day 0, was given s.c. at the base of the tail and the second, on day 7, was divided between the two hind foot pads. The mice were bled on day 21 and killed for histological examination of the thyroids. Alternatively, mice were given 50 μg mTg followed 3 h later by 20 μg LPS (*Salmonella enteritides,* Sigma Chemical Co., St. Louis, MO) both injected i.v. This was repeated on day 7, and the mice were bled and the thyroids taken on day 28.

4.3 sTNFR I treatment

PEGylated recombinant human sTNFR I (p55), produced and characterized as described elsewhere [14], was diluted in sterile PBS and injected i.p. at 5 mg/kg body weight. Control mice were injected with PEG diluted in sterile PBS using the same regimen. The sTNFR I was administered 3 days a week for 3 weeks in the CFA/mTg-induced EAT and for 4

weeks in the LPS-induced EAT, starting at the same time as the first immunization. The sTNFR I was also injected at the same dose and with the same regime described above but starting concurrently to the second immunization. The sTNFR I was also administered for two2 periods of 3 days starting the day before the first and the second immunization. Control mice were injected i.p. with the same amount of sterile PBS.

4.4 Proliferation assay

Spleens were taken on day 10 or 28 from CBA/J and NOD mice treated with sTNFR I or with PBS and immunized with mTg and LPS on day 0 and 7. Single-cell suspensions were prepared and adjusted to 5×10^5/well in 96-well flat-bottom plates (Falcon, Cedex, France) in 200 μl RPMI-1640 supplemented with 5% FCS (Gibco), 2 mM L-Glutamine (Gibco), 100 mg/ml streptomycin, 100 U/ml penicillin (Gibco). mTg (100 μg/ml), LPS (5 μg/ml) (Sigma), and Con A (2 μg/ml) (Sigma) were added *in vitro* as shown and after 72 h in culture, with [^{3}H]thymidine (37 kBq/well) present for the last 8 h of culture, the wells were harvested onto glass-fiber filters and counted on a β-plate counter (LKB Wallac, Pharmacia, Finland). Results are expressed as the ccpm (mean corrected counts per minute ± standard deviation) of triplicate wells.

4.5 Preparation of peritoneal exudate cells

To maximize macrophage recovery CBA/J and NOD mice were pre-treated with thioglycollate (Difco, East Molesely, GB) by injection with 2 ml of a 3% solution into the peritoneal cavity. After 5 days macrophages were eluted using sterile ice-cold PBS containing streptomycin (100 mg/ml) and penicillin (100 U/ml). Erythrocytes were lysed and PEC washed and re-suspended in complete medium (RPMI 1640 plus supplements). Cells were stimulated by the addition of 5 μg/ml LPS and plated out in 96-well flat-bottom plates at 2×10^5 cells/well. Plates were then incubated at 37°C in 5% CO_2 and supernatants removed for analysis of cytokines at 24-h intervals over a 7-day period.

4.6 Detection of cytokines in culture supernatants

Supernatants were collected from effector cell cultures at 48 h and tested for IFN-γ, IL-12, IL-10, and TNF-α using ELISA. Capture and secondary antibodies were all obtained from PharMingen (PharMingen, San Diego, CA) and the assays performed according to the manufacturer's instructions. The sensitivity of detection for IFN-γ was 12.5 U/ml, for IL-10, 62.5 pg/ml, for IL-4, 8 pg/ml, for IL-12, 15 pg/ml and for TNF-α was 31 pg/ml.

4.7 Detection of anti-thyroglobulin autoantibodies

These were assayed using standard ELISA methods. Briefly, plates were coated overnight with mTg diluted to 10 μg/ml in a coating buffer pH 9.6 and left at 4^0C. After blocking with PBS/Tween/BSA, the sera were appropriately diluted and incubated on the plates for 1–2 h at room temperature. Developing antibodies, conjugated to alkaline phosphatase, were added after washing; these were goat anti-mouse polyvalent immunoglobulins at 1/500 (Sigma Chemicals, Poole, Dorset), goat anti-mouse IgM (μ-chain specific) (Sigma Chemicals) or anti-mouse IgG1, 2a or 2b at 1/1,000 (Southern Biotechnology Associates Inc., Birmingham, AL) and the plates incubated for a further hour and then washed. The substrate, p-nitrophenyl phosphate (Sigma Chemicals) 1 mg/ml, was added at 100 μl/well and the reaction stopped with 25 μl/well 4 M NaOH after which the OD was read at 410 nm. In all assays a dilution curve was carried out for experimental and control serum samples. For clarity of presentation, specific serum dilutions were selected which are given in the figure legends.

4.8 Evaluation of thyroid infiltration

Thyroids were fixed in 10% phosphate buffered formalin and serial sections at six levels were stained with hematoxylin-eosin. The criteria used for scoring were those previously employed in our laboratory [13]. For each mouse there are 12 possible scores since there are two lobes to the gland and six levels for each. Grade 0 = no infiltration, 1 = any definite infiltration up to 20%, 2 = between 20 and 50%, 3 = between 50 and 75%, 4 = gland totally infiltrated but follicles still discernible, 5 = no follicles detectable. The severity of the infiltration was assessed blind and the final score is the arithmetic mean of the two lobes in each of the six sections for each mouse.

4.9 Immunofluorescent staining

Frozen spleen sections were rehydrated in PBS then blocked with 20% normal mouse serum for 30 min. Sections were washed three times in PBS and incubated at room temperature for 45 min with 25 μl of biotin-labeled PNA (Lectin from *Arachis hypogea*, Sigma) at a concentration of 20 μg/ml. Sections were washed three times in PBS and incubated as previously described with 25 μl of TRITC-conjugated streptavidin (Seralab, Leicestershire, GB) at a concentration of 40 μg/ml. Sections were washed again three times in PBS and co-stained with 25 μl of anti-mouse B220 FITC-conjugated antibody (PharMingen) at a concentration of 10 μg/ml. Finally, sections were washed three times in PBS and observed under a fluorescence microscope for evaluation of germinal center size and number. Four different spleens were examined for each treatment group. Each spleen had 9 possible scores since they were examined in triplicate at three different levels.

4.10 Statistical analysis

Serum data were analyzed by means of a one-way ANOVA and histology by the Wilcoxon signed rank test. Median values for histological scores are represented by bars in the appropriate figures. Cytokine data were examined by one-way ANOVA and a paired *t*-test.

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Correspondence: Anne Cooke, Department of Pathology, Immunology Division, Cambridge University, Cambridge, CB2 1QP, GB
Fax: +44-1223-333-914
e-mail: ac@mole.bio.cam.ac.uk