The involvement of IL-12 in murine experimentally induced autoimmune thyroid disease

Paola Zaccone[1], Paddy Hutchings[2], Ferdinando Nicoletti[3], Giuseppe Penna[4], Luciano Adorini[4] and Anne Cooke[2]

[1] Istituto di Microbiologia, Universita di Catania, Catania, Italy
[2] Department of Pathology, University of Cambridge, Cambridge, GB
[3] Institute of Microbiology, University of Milan, Milan, Italy
[4] Roche Milano Ricerche, Milan, Italy

Experimental autoimmune thyroid disease (EAT) can be induced experimentally in mice following immunization with mouse thyroglobulin (mTg) and the adjuvants lipopolysaccharide (LPS) or complete Freund's adjuvant (CFA). EAT can also be transferred to naive recipients by $CD4^+$ T cells from mTg-primed mice. Here we demonstrate a role for IL-12 in the development of EAT by the ability of neutralizing antibody to IL-12 to reduce disease severity and by the lack of significant levels of thyroid infiltration in IL-12p40-deficient mice following immunization with mTg and CFA. A single injection of 300 ng IL-12 at the time of initial immunization with mTg and LPS was able to increase the degree of thyroid infiltration. These data are all consistent with EAT being a Th1-mediated disease. Conversely, however, administration of IL-12 over a prolonged period markedly inhibited the induction of EAT by mTg and CFA and, if given to recipients, inhibited the transfer of EAT by mTg-primed lymph node cells. The development of an autoantibody response to mTg was also inhibited when IL-12 was administered throughout the experimental period, suggesting that sustained exposure to IL-12 can be immunosuppressive.

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1 Introduction

Autoimmune thyroid disease can be experimentally induced in mice by administration of thyroid antigens such as mouse thyroglobulin (mTg) together with CFA or LPS [1, 2]. The severity of the thyroid infiltration is dependent on the strain of mouse and the adjuvant employed [3]. It can also be induced in naive mice following transfer of mTg-specific T cell lines or clones [4, 5] or mTg-primed $CD4^+$ T cells activated *in vitro* with Con A or mTg [6–8]. The composition and characteristics of the thyroid infiltrate may vary depending upon the immunization regime. An autoimmune granulomatous thyroiditis similar to subacute thyroiditis (de Quervain's syndrome) in man has been described following the use of mTg together with CFA [9] and a more lymphocytic infiltrate is generated when LPS is used as the adjuvant or when experimental autoimmune thyroid disease (EAT) is caused by transfer of primed $CD4^+$ T cells [2, 6, 7].

IFN-γ has been shown to exacerbate EAT [10] when given *in vivo* at the time of antigen priming, while its neutralization has been shown to reduce both the incidence and the severity of EAT [11], leading to the assumption that EAT is a Th1-mediated disease. However, other studies show that a more severe form of granulomatous thyroid disease is induced in recipients of mTg-primed spleen cells if IFN-γ is neutralized either during the *in vitro* culture step required for disease transfer or within the recipient [12], therefore suggesting that the situation may not be so straightforward. To clarify the relative roles of Th1 and Th2 cells in EAT, we have studied the involvement of IL-12 in this condition.

IL-12 is a key cytokine in determining whether a Th1 a Th2 response evolves following antigen administration [13]. Administration of IL-12 has been shown to accelerate the onset of insulin-dependent diabetes mellitus (IDDM), a Th1-mediated autoimmune disease [14] in nonobese diabetic (NOD) mice and anti-IL-12 antibody treatment has been shown to inhibit experimental allergic encephalomyelitis (EAE) [15], autoimmune colitis [16]

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Abbreviations: EAT: Experimental autoimmune thyroid disease **mTg:** Mouse thyroglobulin **IDDM:** Insulin-dependent diabetes mellitus **EAE:** Experimental allergic encephalomyelitis

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and experimental autoimmune uveitis [17]. However, the observation that IL-12 can also depress the induction of autoimmunity in several experimental models [18–20] shows that factors such as the adjuvant or the dose and timing of cytokine administration might be important. To determine the involvement of IL-12 in autoimmune thyroid disease, we have administered either IL-12 or a neutralizing antibody to IL-12 around the time of induction of autoimmune thyroiditis. In addition, we have analyzed EAT induction in IL-12-deficient mice. These studies suggest that IL-12 plays a pivotal role in determining the severity of thyroid infiltration.

2 Results

2.1 Exogenous IL-12 enhances EAT induced by mTg and LPS

In our hands, the use of CFA as an adjuvant for induction of EAT in CBA/J mice characteristically results in severe thyroid lesions whereas that induced by mTg and LPS or transfer of mTg-primed cells is less severe (histological score of 4.4 ± 0.45 using CFA compared to 0.8 ± 0.2 for LPS for four separate experiments). The difference in severity of the infiltrate between the two inductive regimes could either signify a different pathogenic process or a more magnified response elicited by CFA. Given the severity of the infiltrate induced employing CFA as the adjuvant it is unlikely that any enhancing effect of IL-12 could be detected in this system. Initially, we therefore examined the ability of 300 ng IL-12 to modulate EAT induced by mTg and LPS. It was clear that if IL-12 administration was given around the time of the immunization with mTg and LPS, significant enhancement of EAT occurred ($p < 0.0001$) (Fig. 1 a). It is known that IL-12 can replace the priming LPS injection in the elicitation of the Schwartzmann reaction through its ability to cause IFN-γ production [21, 22] and indeed some mice given 300 ng IL-12 as well as mTg and LPS died. This was particularly marked when IL-12 was given with both LPS injections. To minimize this mortality, we examined whether one dose of IL-12 administered before the first injection of mTg and LPS would still enhance EAT. Enhanced thyroid infiltration was seen 4 weeks after immunization whether one or two injections of IL-12 were given ($p < 0.0001$) (Fig. 1 a). This enhanced infiltration was still evident at 8 weeks after immunization ($p < 0.0001$) (Fig. 1 b), suggesting that the differences in level of infiltration were not simply due to a change in the kinetics of the autoimmune response. The autoantibody response to mTg was also increased at 4 weeks after initial priming and analysis of the isotype profile of the induced autoantibodies suggested that this increased autoantibody response was due predominantly to an

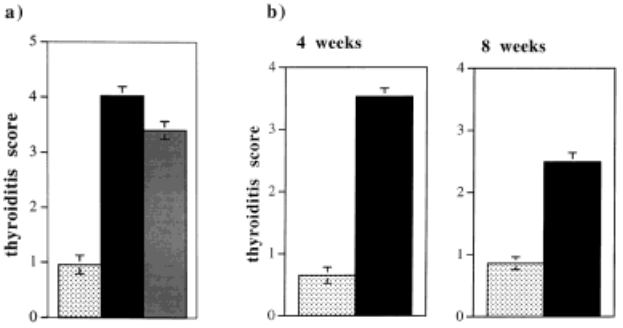

Figure 1. IL-12 increases the severity of thyroid lesions induced by administration of mTg and LPS. (a) Thirty-six CBA/J mice immunized with mTg and LPS received 300 ng IL-12 with both immunizations or with the first immunization. Controls received PBS instead of IL-12. Initially each group consisted of 12 mice but a mortality of 50 % in the group given two injections of IL-12 and 40 % in the group given one IL-12 injection reduced numbers in these groups to six and seven mice, respectively. Control, ⊠ ; IL-12, one injection, ▩ ; IL-12, two injections, ▨ . (b) Thirty CBA/J mice were immunized with mTg and LPS. Twenty mice received 300 ng IL-12 with the first immunization but due to a 25 % mortality 15 mice remained. Thyroids were taken at 4 and 8 weeks from eight and seven mice, respectively, in the IL-12-treated group and five mice in the control groups. The histological score of thyroid lesions induced by mTg and LPS and 4 and 8 weeks in the presence or absence of IL-12 is shown together with the SEM. Control, ⊠ ; IL-12 treated, ■.

enhanced IgG2a and IgG2b response to the autoantigen ($p < 0.0001$) (Fig. 2). An elevated autoantibody response was still evident at 8 weeks after priming in IL-12-treated mice compared to non-cytokine-treated control mice and once more there was a greater enhancement in the IgG2a component of the autoantibody response than that seen for IgG1 (Fig. 2 b).

2.2 Endogenous IL-12 is involved in EAT induced by mTg and CFA

There are some data to suggest that the infiltration seen when mTg is given with LPS differs from that with CFA [2, 6, 7]. mTg and CFA elicits a severe thyroid infiltration in CBA/J mice which has granulomatous characteristics. As this thyroiditis is severe we decided that the best way of determining whether IL-12 played a role in disease induction with this autoantigen/adjuvant combination was to assess whether neutralization of IL-12 by a mAb influenced the incidence or severity of EAT. Therefore, 100 μg of a neutralizing antibody to IL-12 was administered on alternate days for 3 weeks to CBA/J mice immunized with mTg and CFA. Control mice received an irrelevant IgG. From Fig. 3 a it can be seen that neutrali-

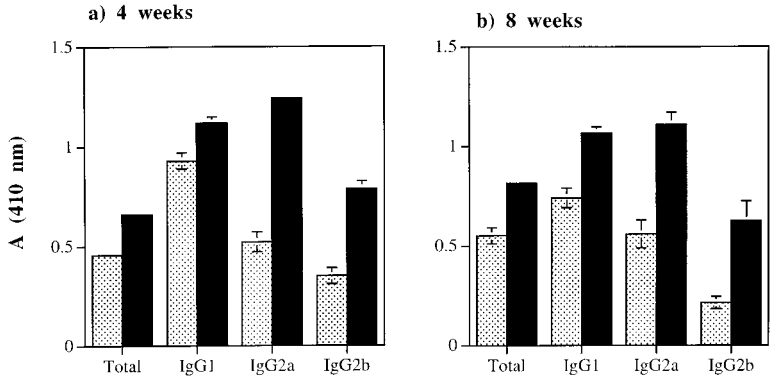

Figure 2. IL-12 increases the levels of autoantibody induced by administration of mTg and LPS. Thirty CBA/J mice were immunized with mTg and LPS and 20 mice received 300 ng IL-12 with the first immunization. Due to a 25 % mortality in the IL-12 treated group the data shown are from 15 mice in this group and ten mice in the PBS-treated one. Serum antibody response to mTg and 4 weeks (a) and 8 weeks (b) after immunization was assessed by ELISA and the absorbance values obtained for sera diluted 1/800 are shown. Control, ⊠ ; IL-12 treated, ■.

zation of IL-12 significantly inhibited the development of EAT ($p < 0.0001$). The levels of serum autoantibodies to mTg were also slightly reduced (Fig. 3 b) with no pronounced shift in isotype profile. When lymph node cells were taken from the mice when they were killed, and cultured *in vitro* with mTg it was observed that cells from mice treated with anti-IL-12 secreted less TNF-α (29 pg/ml at 72 h compared to the control value of 94 pg/ml) and IFN-γ (46 U/ml at 72 h compared to the control value of 1808 U/ml) and also produced less NO (7 mM/ml compared to the control value of 21 mM/ml).

2.3 Targeted disruption of the IL-12 gene inhibits the induction of EAT

Another way of assessing the need for IL-12 in the induction of EAT with mTg and CFA is to determine whether EAT can be induced in mice in which the gene encoding IL-12 is disrupted through targeted mutagenesis. We therefore determined whether EAT could be induced in IL-12-deficient mice. Although C57BL/6 mice are low responders to the induction of EAT with mTg and CFA, this was to our advantage since changes in the extent of infiltration would be more readily detected in a more moderate response. Significant levels of thyroid infiltration were induced in control C57BL/6 mice whereas mice on the C57BL/6 background, but bearing the disrupted IL-12 gene, developed little thyroiditis following immunization with mTg and CFA (Fig. 4 a). Thus, the genetic absence of IL-12 profoundly impairs the development of induced EAT. However, no statistically significant effect of this absence of IL-12 was seen on antibody responses as both control and IL-12-deficient mice made equivalent autoantibody responses (Fig. 4 b).

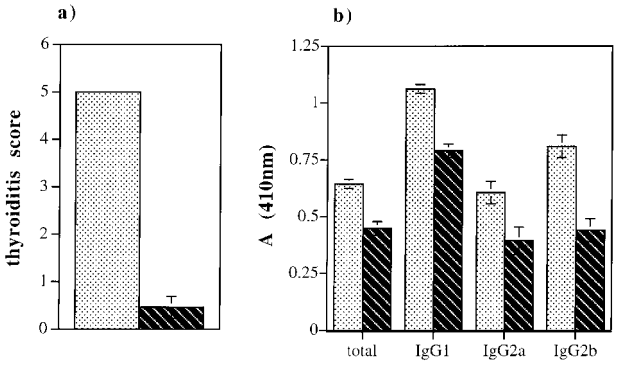

Figure 3. Neutralization of IL-12 reduces the severity of EAT and autoantibodies induced by mTg and CFA. Fifteen CBA/J mice were immunized with mTg and CFA. Eight mice were given 100 μg antibody to IL-12 and seven mice were given an irrelevant isotype-matched control antibody on alternate days throughout the experiment. After 3 weeks mice were killed and thyroids and serum were taken for histology and autoantibody determination, respectively. (a) The histological score of thyroid lesions (± SEM) induced by mTg and CFA in the presence or absence of neutralizing antibodies to IL-12. (b) The isotype profile induced by mTg and CFA in the presence or absence of neutralizing antibodies to IL-12. Data are presented as mean ± SEM for the following serum dilutions, total Ig: 1/3600; IgG1: 1/2400; IgG2a and IgG2b: 1/800. Irrelevant antibody, ⊠ ; anti-IL-12 antibody, ▨ .

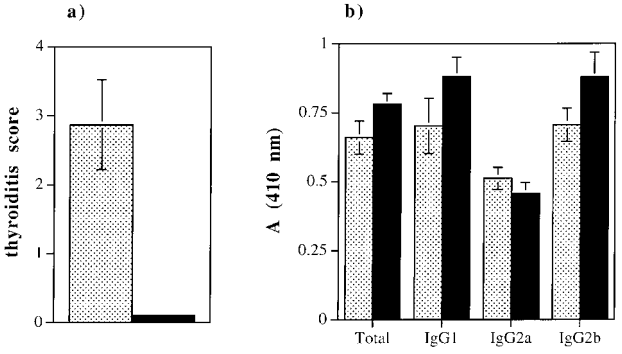

Figure 4. The effect of targeted disruption of the IL-12 p40 gene on induction of EAT. Thyroid lesions were induced by mTg and CFA in eight normal C57BL/6 mice and eight C57BL/6 mice with a targeted disruption of the IL-12p40 gene. (a) The histological score for these lesions at 3 weeks (± SEM) and (b) the autoantibody response at 3 weeks (± SEM) for sera diluted 1/6400 are shown. Control, ⊠; IL-12p40-deficient, ■.

2.4 IL-12 enhanced the pathogenicity of primed T cells in EAT transfer

Draining lymph node cells from mice primed with mTg and CFA are able to transfer mild EAT to naive recipients following a 3-day *in vitro* culture with mTg [8, 23]. To determine whether IL-12 could have any effect on primed T cells, lymph node cells from donor mice primed with mTg and CFA were cultured for 3 days in the presence or absence of 10 μg/ml IL-12. Analysis of the culture supernatants showed higher levels of IFN-γ (461 U/ml at 72 h compared to the control value of 161 U/ml at this time point) and IL-10 secretion (1028 pg/ml at 72 h compared to a control value of 89 pg/ml) in those cultures containing IL-12. It was additionally found that lymph node cells cultured with IL-12 were able to transfer more severe thyroid disease ($p < 0.001$) (Fig. 5 a) and there was an increase in the antibody response to mTg in the recipients of these cells (Fig. 5 b).

Inclusion of the neutralizing anti-IL-12 antibody in this 3-day *in vitro* culture step did not, on the other hand, have any effect on the ability of the primed lymph node cells to transfer EAT (data not shown).

2.5 The effect of exogenous IL-12 on EAT induced by mTg and CFA

Although, as stated previously, our experimental protocol using mTg and CFA frequently induces such severe EAT in CBA/J mice that we could not expect IL-12 to further enhance the extent of thyroid infiltration, we thought that effects on autoantibody levels might still be detected. As the Schwartzman reaction was not a problem when using CFA as adjuvant, we administered 300 ng IL-12 five times per week throughout the experimental period. Unexpectedly, this resulted in very little thyroid infiltration and only low levels of autoantibody of all isotypes (Figs. 6 b and 7 b). Thyroid infiltration in this model achieves a peak incidence at 21 days before gradually resolving and therefore we also analyzed the extent of infiltration at an earlier time point (14 days) in case the kinetics of infiltration had been altered. Compa-

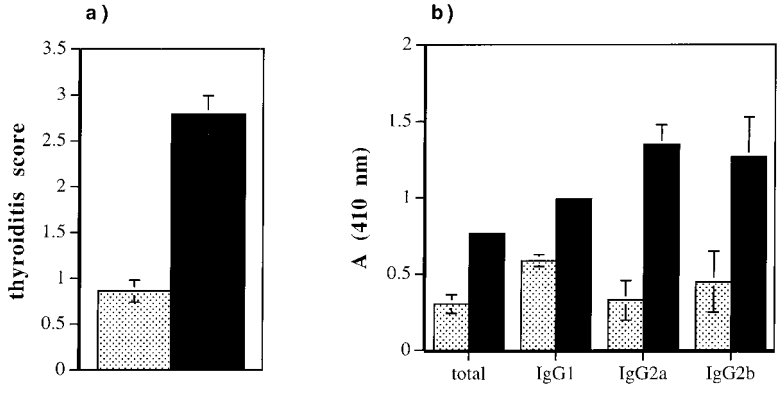

Figure 5. The effect of *in vitro* IL-12 on the ability of mTg-primed lymph node cells to transfer EAT and influence autoantibody responses. CBA/J mice were immunized with mTg and CFA on day 0 and day 7. Lymph node cells were taken on day 15 and cultured in the presence or absence of IL-12 before transfer to recipients. Six mice received lymph node cells cultured with IL-12 and four mice received lymph node cell cultured without IL-12. (a) The histological scores of the thyroid lesions induced at 3 weeks (± SEM) and (b) the serum autoantibody response (± SEM) at 3 weeks for sera diluted 1/800 are shown. Control, ⊠; IL-12 treated, ■.

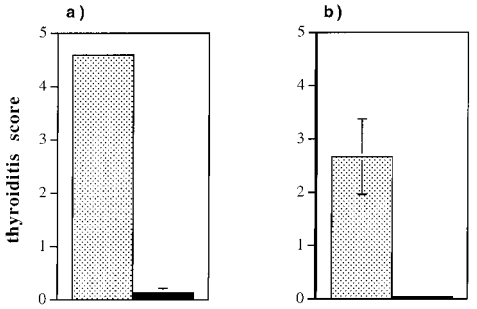

Figure 6. The effect of exogenous IL-12 on the severity of thyroid lesions induced by mTg and CFA. Twenty-seven CBA/J mice were immunized with mTg and CFA in the presence or absence of 300 ng IL-12. The IL-12 was administered five times a week throughout the experiment. Mice were killed at 2 weeks (13 mice) or 3 weeks (14 mice) after the initial immunization and the severity of thyroid lesions in each group assessed histologically. The histological score of the induced thyroid lesions at (a) 2 weeks, for six IL-12-treated and seven control mice and at (b) 3 weeks, for eight IL-12-treated and the six control mice is shown. Control, ⊠ ; IL-12 treated, ■ .

rable results to those obtained at 21 days were obtained at 14 days; IL-12 inhibited both thyroid infiltration and total autoantibody responses (Figs. 6 a and 7 a). This effect of IL-12 on thyroiditis was also seen when 300 ng IL-12 was only given with the first injection of antigen (average score of IL-12 group 0.99 ± 0.4 compared to control group average score 2.67 ± 0.08). The autoantibody response was, however, not significantly affected by a single administration of IL-12 although there was a trend towards an enhanced IgG2a response and a reduced IgG1 response. Thus, IL-12 given at the time of initial exposure to antigen and the adjuvant CFA inhibited the induction of thyroid infiltration and repeated exposure to IL-12 inhibited both the induction of EAT and the development of Tg autoantibodies. This differs from the results seen when LPS was used as the adjuvant where a single dose of IL-12 enhanced both thyroid lesions and autoantibody responses.

As stated previously, EAT can be transferred to naive recipients by lymph node cells from a thyroglobulin primed donor mouse following a 3-day culture of the primed lymph node cells with thyroglobulin. We had already seen that IL-12 added to the culture enhanced the transfer of EAT (Fig. 5 a), therefore the effect of IL-12 on the ability of the donor mouse to generate T cells capable of transferring disease was assessed. Lymph node cells taken from mice which were primed with mTg and CFA and given 300 ng IL-12 five times per week were cultured *in vitro* with mTg in the absence of IL-12 and their ability to transfer EAT assessed in comparison to control cells from mTg/CFA-primed mice which did not receive IL-12. Cells from mice treated with IL-12 had a markedly reduced ability to transfer EAT (the average score in this group was 0.03 ± 0.06 with three out of four mice failing to develop any thyroid infiltrate) compared to the control group of recipients where all mice developed EAT (average score 1.92 ± 0.71). This inability of lymph node cells from IL-12-treated donor mice to transfer EAT was mirrored in the reduced proliferative response of these lymph node cells to mTg *in vitro* (Fig. 8) and the reduced autoantibody response in recipients (data not shown).

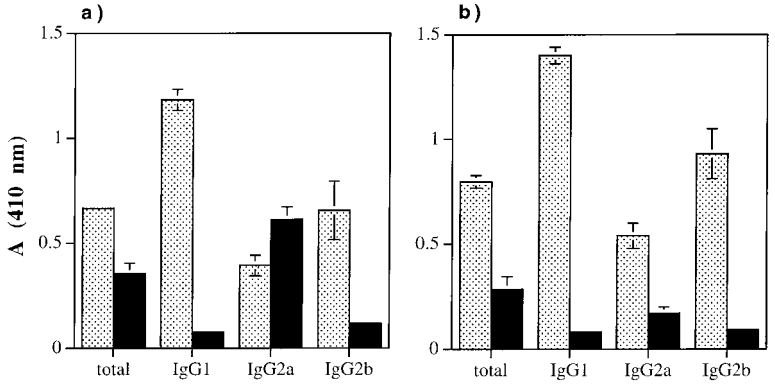

Figure 7. The effect of exogenous IL-12 on the levels of autoantibody induced by mTg and CFA. CBA/J mice were immunized with mTg and CFA with or without 300 ng IL-12 which was administered throughout the experiment. Thirteen mice were killed at 2 weeks and 14 at 3 weeks after the initial immunization and sera were taken for autoantibody measurement. The mTg autoantibody levels at (a) 2 weeks and (b) 3 weeks were assessed by ELISA. Data are presented as absorbance values (± SEM) with sera diluted for total Ig (a) 1/400 and (b) 1/400; IgG1 (a) 1/3200 and (b) 1/1600; IgG2a (a) 1/200 and (b) 1/800; IgG2b (a) 1/800 and (b) 1/800. Control, ⊠ ; IL-12 treated, ■.

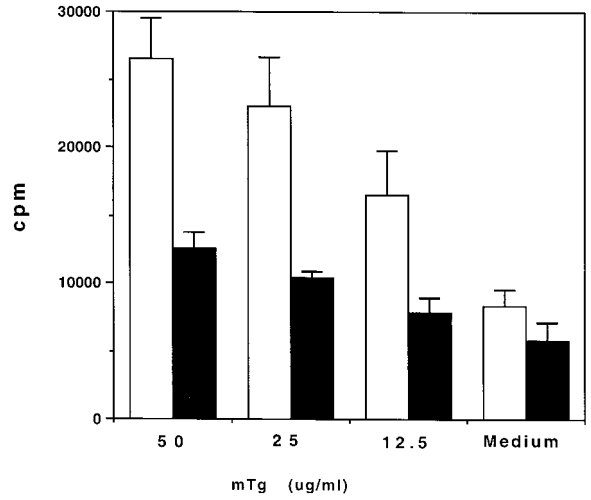

Figure 8. The effect of IL-12 *in vivo* on the proliferative response of mTg-primed lymph node cells to mTg *in vitro*. CBA/J mice were immunized with mTg and CFA on day 0 and day 7 with or without 300 ng IL-12 which was administered five times a week throughout the experiment. Popliteal lymph node cells were taken on day 15 and their proliferative response to mTg after 72 h in culture was assessed and is shown together with the SEM. Control, □; IL-12 treated, ■.

2.6 The effect of IL-12 or anti-IL-12 mAb on the ability of thyroiditogenic T cells to transfer EAT

Data presented in previous sections suggested that IL-12 *in vitro* could enhance the ability of primed T cells to transfer EAT (Fig. 5 a) and neutralization of IL-12 could prevent EAT induction (Fig. 3 a). We therefore questioned whether alteration in IL-12 levels in the recipient had any effect on the ability of primed T cells to transfer EAT. Recipients of thyroiditogenic T cells were therefore given either 300 ng IL-12 or PBS five times a week for 2 weeks starting at the time of transfer and the thyroid histology and autoantibody titers were assessed 14 days later. The data clearly showed that IL-12 treatment of recipients could inhibit the ability of thyroiditogenic T cells to transfer EAT (recipient thyroiditis score, 0.85 ± 0.16, control recipients 3.08 ± 0.17, $p < 0.0001$) and furthermore reduced the amount of autoantibody synthesized irrespective of isotype. Neutralization of IL-12 with anti-IL-12 antibody in the recipient of mTg-primed lymph node cells had no effect on either the transfer of EAT or autoantibody production (data not shown).

3 Discussion

Thyroid autoimmunity can be induced in rodents following immunization with thyroid antigens and adjuvant and the autoreactive response monitored by the degree of thyroid infiltration and the development of autoantibodies [1, 2, 23]. The characteristics and degree of the thyroid infiltrate vary with the use of different adjuvants and different strains of mice [3, 23]. Immunization of high responder strains of mice with mTg and CFA results in the development of granulomatous lesions in the thyroid and a pathology resembling subacute thyroiditis (de Quervain's) in humans [9]. In our experiments the thyroids of CBA/J mice immunized with mTg and CFA were extensively infiltrated with mononuclear cells, polymorphonuclear cells, macrophages and giant cells with few thyroid follicles remaining. This histological picture concurs with that originally described in 1983 by Imahori and Vladutiu [9]. Experimental autoimmune thyroiditis is also induced by immunization of mice with mTg and LPS [2, 23]. In our hands immunization of CBA/J mice with mTg and LPS results in less severe thyroid lesions than those seen when this mouse strain is immunized with mTg and CFA. The composition of the inflammatory infiltrates also differs and with LPS as an adjuvant they largely consist of mononuclear cells. Our data suggest in both these experimental systems that IL-12 plays a key role in the development of EAT. When mTg and CFA are used to elicit EAT, anti-IL-12 antibody treatment profoundly inhibits the development of disease. Furthermore, targeted disruption of the IL-12 gene renders the mice resistant to the induction of EAT by mTg and CFA. The lack of an effect of genetic absence of IL-12 on the autoantibody response may be attributable to redundancy within the cytokine network. IL-12 and IL-18, for example, share many biological functions [24] and it may be that, in the genetic absence of IL-12, IL-18 takes over some of the roles normally performed by this cytokine.

Lymph node T cells from immunized mice are able to transfer EAT to recipients but only if they are activated *in vitro* prior to transfer. Inclusion of IL-12 in the *in vitro* culture step markedly increased the severity of the thyroiditis induced by these transferred T cells and an analysis of the culture supernatants revealed that IL-12 increased the production of IFN-γ. These observations would be consistent with EAT induced by this protocol being a Th1-mediated disease. It is also clear from the data in our report that anti-IL-12 or disruption of the IL-12 gene inhibits the induction of thyroid lesions by thyroglobulin and CFA. Cells from mice depleted of IL-12 secreted less TNF-α and IFN-γ which would be consistent with a diminished Th1 response. However, no pronounced shift in isotype profiles was observed in mice treated with

anti-IL-12 which might argue against immune deviation being the underpinning mechanism.

Furthermore, when disease was induced with mTg and LPS administration of IL-12 around the time of immunization resulted in the development of more extensive thyroid infiltration with a relative increase in the contribution of IgG2a and IgG2b isotypes in the autoantibody response to mTg. This preferential increase in thyroglobulin-specific IgG2a and IgG2b autoantibodies would also be consistent with a deviation to a Th1 response. All the data discussed above therefore support the view that IL-12 plays an important role in the induction of Th1-mediated autoimmune diseases, as documented in several experimental models [14–17].

In contrast to its enhancing effect on EAT induced by mTg and LPS, IL-12 appeared to play an immunosuppressive role when EAT was induced using mTg together with CFA. Both EAT and autoantibody responses were inhibited when the cytokine was given throughout the experimental period. It was striking that such exposure to IL-12 was not only able to prevent the induction of disease but also to inhibit the ability of thyroglobulin-primed cells to transfer EAT when recipients were given the cytokine throughout the experimental period. Inhibition of autoimmune responses by IL-12 has been noted under specific experimental conditions [18–20]. In particular, studies on collagen induced arthritis have suggested that the reduced autoimmune response following high dose IL-12 treatment may reflect the action of IL-10 [18]. IL-12 has been shown to induce IL-10 production [13], thus leading to the suggestion that this may represent a negative feedback loop which plays a physiological role in dampening down tissue pathology. We have no evidence that IL-10 is playing a down-regulatory role in our experiments but it is interesting to note that IL-10 at high doses has been shown to inhibit induction of EAT [25]. Recent studies suggest that immune suppression mediated by IL-12 may be due to induction of IFN-γ which in turn induces nitric oxide (NO) synthase within macrophages [26]. Inhibitors of NO synthase are able to prevent IL-12-mediated inhibition of T cell responses. It will be possible to establish whether either of these mechanism(s) is responsible for the inhibition seen in our experimental systems by neutralizing IL-10 or inhibiting NO production.

Our data highlight the key role that IL-12 plays in the induction of EAT. Absence of IL-12 through targeted mutagenesis results in a failure to induce thyroid lesions but permits autoantibody formation, and neutralization of IL-12 also inhibited the generation of thyroid lesions. Consistent with these findings is the observation that exogenous administration of IL-12 can increase the severity of EAT induced by thyroglobulin and LPS. However, it would appear that the dose or timing of administration of exogenous IL-12 or the adjuvant employed may be critical for the outcome, as this cytokine was also shown to be capable of potently inhibiting not only the induction of EAT but also the ability of thyroiditogenic T cells to mediate pathology. Further analysis of the basis for exacerbation or inhibition of EAT by IL-12 might provide and insight into strategies for therapeutic intervention in autoimmune disease.

4 Materials and methods

4.1 Mice

EAT-susceptible 6–8-week-old CBA/J mice (H-2^k) of both sexes and 6–8-week-old female C57BL/6 mice were obtained from Harlan U.K. Ltd Bicester Oxon. IL-12p40-deficient C57BL/6 mice [27] were kindly provided by J. Magram (Hoffmann-La Roche, Inc., Nutley, NJ). The mice were maintained in our own facilities at the Department of Pathology with free accesss to food and water.

4.2 Induction of EAT using adjuvant

mTg was prepared by extraction from pooled homogenized thyroids from normal outbred mice as previously described [23, 28]. 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 enteritidis*, 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 Induction of EAT by transfer of primed cells after *in vitro* stimulation

This model offers three separate opportunities to intervene in the system by treating either the donors, the recipients or by adding reagents *in vitro*. Donor mice were immunized with mTg/CFA on day 0 and 7 as described above and the popliteal lymph nodes were taken on day 15. Cells from these nodes were cultured for 3 days in petri dishes (Nalge Nunc International, Denmark) at 5×10^6/ml in RPMI 1640 (Gibco, Paisley, Scotland) with 5 % FCS and other standard supplements [8] together with 40 μg/ml mTg. This culture period enabled the activation and expansion of the effector T cells which were washed and then 2×10^7 viable cells were injected i.v. into normal syngeneic recipients.

4.4 Proliferation assay

Popliteal lymph nodes were taken on day 16 from CBA/J mice treated with 300 ng IL-12 or with PBS containing 100 μg/ml mouse serum albumin five times a week starting on day-1 and immunized with mTg on day 0 and 7. The cells were pooled from groups of five mice and single-cell suspensions were prepared and adjusted to 3×10^5/well in 96-well flat-bottom plates (Falcon, Becton Dickinson Labware Europe, 38241 Meylan, Cedex France) in 200 μl RPMI 1640 supplemented as described above. mTg was added *in vitro* as shown and after 72 h in culture, with [^{3}H] thymidine (0.5 μCi/well) present for the last 8 h, the wells were harvested onto glass-fiber filters and counted on a β-plate counter (LKB Wallac 1205 Betaplate Liquid Scintillation counter, Pharmacia, Finland). Results are expressed as the cpm (mean ± SEM) of triplicate wells.

4.5 IL-12 treatment

Recombinant mouse IL-12, produced as previously described [14], was diluted in PBS containing 100 μg/ml mouse serum albumin (Sigma Chemical Co., St. Louis, MO) and injected i.p. at 300 ng/mouse. For EAT induced with mTg/CFA, the IL-12 was administered 5 days a week for 3 weeks commencing on the day before priming with antigen. When inducing EAT with mTg/LPS, the IL-12 was administered in a single dose after the mTg but before the LPS on day 0 (and in some experiments also on day 7). In each case, control mice received identical volumes of the vehicle alone. In experiments involving the transfer of primed cells, IL-12 was administered 5 days a week for 2 weeks to either the donors of the recipients or added at 0.5 ng/ml to the *in vitro* stimulation with mTg. An equivalent volume of PBS was added to control cultures.

4.6 Anti-mouse IL-12 mAb treatment

The rat anti-mouse IL-12 mAb (10F6) is a neutralizing IgG2b antibody which reacts with the IL-12 p40 subunit [29]. Mice which had been immunized with mTg/CFA were given 100 μg anti-IL-12 diluted in PBS i.p. on alternate days for 3 weeks starting on the day of immunization. In transfer experiments, anti-IL-12 was administered to either donors or recipients on alternate days for 2 weeks commencing on the day of immunization or transfer, respectively. The antibody was added to the *in vitro* cultures at a concentration of 10 μg/ml. In all experiments, an irrelevant rat IgG2b antibody was used as a control.

4.7 Detection of cytokines in culture supernatants

Supernatants were collected from effector cell cultures at 48 and 72 h and tested for IFN-γ and IL-4 using an ELISA. Capture antibodies DB1 (for IFN-γ), 11B11 (for IL-4) 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 and for IL-4 was 0.3 ng/ml. A standard kit (Endogen Inc. Cambridge MA) was used for the detection of TNF-α. The sensitivity of the assay was < 10 pg/ml.

4.8 Detection of anti-Tg autoantibodies

These were assayed using standard ELISA methods described in detail elsewhere [30]. Briefly, plates were coated overnight with mTg diluted to 10 μg/ml in a coating buffer, pH 9.6, and left at 4 °C. 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 polyvalent anti-mouse IgG at 1/500 (Sigma Chemicals, Poole, GB) or anti-mouse IgG1, IgG2a, or IgG2b at 1/1000 (Southern Biotechnology Associates Inc., Birmimgham, AL) and the plates incubated for a further hour and then washed. The substrate, 1 mg/ml p-nitrophenyl phosphate (Sigma Chemicals), was added at 100 μl/well and the reaction stopped with 25 μl/well 4 M NaOH after which the absorbance was read at 410 nm.

In all assays a dilution curve was carried out for experimental and control serum samples. For clarity of data presentation, specific serum dilutions were selected which are given in the figure legends.

4.9 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 [23]. 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 descernible, 5 = no follicles detectable. The severity of the infiltration was assessed blind and the final score for each mouse is the total divided by the number of observations. Therefore the average thyroiditis for each group is the mean ± SEM of all the mice in the group.

4.10 Statistical analysis

Data were analyzed by ANOVA.

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Correspondence: Anne Cooke, Immunology Division, Department of Pathology, University of Cambridge, Cambridge CB21QP, GB
Fax: +44-1223 33 3716
e-mail: ac@mole.bio.cam.ac.uk