Development of an Animal Model of Autoimmune Thyroid Eye Disease

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Development of an Animal Model of Autoimmune Thyroid Eye Disease

M.-C. Many, S. Costagliola, M. Detrait, J.-F. Denef, G. Vassart, and M. Ludgate

In previous studies we have transferred thyroiditis to naïve BALB/c and NOD mice with human thyrotropin (TSH) receptor (TSHR)-primed splenocytes. Because the TSHR has been implicated in the pathogenesis of thyroid eye disease (TED) we have examined the orbits of recipients of TSHR-primed T cells, generated using a TSHR fusion protein or by genetic immunization. In the NOD mice, 25 of 26 animals treated with TSHR-primed T cells developed thyroiditis with considerable follicular destruction, numerous activated and CD8+ T cells, and immunoreactivity for IFN-γ. Thyroxine levels were reduced. Thyroiditis was not induced in controls. None of the NOD animals developed any orbital pathology. Thirty-five BALB/c mice received TSHR-primed spleen cells. Thyroiditis was induced in 60–100% and comprised activated T cells, B cells, and immunoreactivity for IL-4 and IL-10. Autoantibodies to the receptor were induced, including TSH binding inhibiting Igs. A total of 17 of 25 BALB/c orbits displayed changes consisting of accumulation of adipose tissue, edema caused by periodic acid Schiff-positive material, dissociation of the muscle fibers, the presence of TSHR immunoreactivity, and infiltration by lymphocytes and mast cells. No orbital changes or thyroiditis were observed in control BALB/c mice. We have induced orbital pathology having many parallels with human TED, only in BALB/c mice, suggesting that a Th2 autoimmune response to the TSHR may be a prerequisite for the development of TED. The Journal of Immunology, 1999, 162: 4966–4974.

Thyroid eye disease (TED), as its name implies, is an eye disorder occurring in patients with thyroid autoimmunity (reviewed in Ref. 1), especially Graves’ disease (GD). Orbital tissues, particularly the extraocular muscles (EOM), which are grossly enlarged, display edema and lymphocytic infiltration, indicating an autoimmune etiology. Cytokines are elaborated by the infiltrate (2, 3), having pleiotropic effects, including the stimulation of production of glycosaminoglycans by orbital fibroblasts (4), which absorb water causing edema. This seems to be one of the mechanisms responsible for the increase in volume of the EOM and other orbital contents that leads to proptosis. Other features of increasing severity include an accumulation of orbital fat, which also increases orbital volume, chemosis, ulceration of the conjunctiva, and compression of the optic nerve, which may result in blindness. The initiating event and specifically the nature of the autoantigen responsible for the lymphocyte homing to the orbit, are not well characterized. As a consequence, TED remains an autoimmune disease that is poorly managed; therapy is palliative and given only when the disease is well advanced.

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2 M.-C.M. and S.C. contributed equally to this paper.

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4 Abbreviations used in this paper: TED, thyroid eye disease; GD, Graves’ disease; TSH, thyrotropin; TSHR, human TSH receptor; TSAB, thyroid-stimulating Abs; TBI, TSH-binding inhibiting Igs; PAS, periodic acid Schiff; MBP-ECD, extracellular domain of the TSHR produced as a maltose-binding protein fusion, EOM, extraocular muscle; T4, thyroxine; ICC, immunochemistry; B/T, ratio of B cells to T cells.

A number of candidate autoantigens and target tissues have been proposed (reviewed in Ref. 5). Perhaps the most logical is the thyrotropin receptor (TSHR) that is the target of the thyroid-stimulating Abs (TSAB) of GD and the thyroid blocking Abs found in idiopathic myxedema. For this to be the case, the TSHR, or a cross-reacting protein, would have to be expressed in the orbit. A number of approaches have been used to demonstrate TSHR transcripts and/or immunoreactivity, in a variety of orbital tissues in vitro (6). RT-PCR suggests that orbital fat may express TSHR transcripts (7), although results obtained with this methodology are conflicting (reviewed in Ref. 8). Certainly in rodents, the receptor is expressed in fat from most anatomical locations (9) and into adulthood. However, in humans, adipocyte expression of functional TSHR seems to be restricted to the neonate, when TSH and TSAB have been shown to control lipolysis (10). Differentiation and proliferation of human orbital preadipocytes resulting in an increase in adipocytes, have recently been demonstrated in vitro, although the hormones and/or cytokines responsible remain to be determined (11).

We have found TSHR transcripts, by Northern blot, in adipose tissue from a patient with TED (12). Subsequently we have obtained immunocytochemical staining of orbital biopsies from TED patients, associated with elongated cells adjacent to clusters of fat cells and in the cytoplasm of adipocytes, using two different mAbs to the TSHR (our unpublished work). Together these in situ patient data support the hypothesis that the TSHR is expressed in the orbital adipose compartment, at least in TED, and thus may be a target autoantigen and link with the thyroid gland.

In a number of previous studies, we have established an animal model by immunizing with TSHR fusion protein (13), transferring TSHR-primed T cells (14), and immunizing with a cDNA for the TSHR (15) in an expression vector. The type of response induced varies with the genetic background (16) such that thyroiditis, TSH-binding inhibiting Igs (TBI), and elevated circulating thyroxine (T4) are obtained in BALB/c (H2d) mice, whilst a destructive thyroiditis and reduced circulating T4 are obtained in NOD.
(H2g) animals. Analysis of the phenotype of the lymphocytic infiltrate of the two strains indicates that both contain activated T cells expressing the receptor for IL-2 but the BALB/c contain B cells and IL-10- and IL-4-producing cells, suggesting a Th2 response, whereas the NOD display hallmarks of a Th1 response (reviewed in Ref. 17) with destruction of the gland.

In view of our human in situ studies demonstrating the TSHR in ocular tissues, we have examined the orbits of BALB/c and NOD mice and subjected them to transfer of TSHR-primed T cells generated using the various induction protocols. Data from three separate experiments show induced orbital pathology, which has many parallels with human TED, exclusively in BALB/c mice.

Materials and Methods

Induction protocols

Generation of in vivo primed T cells: transfer to naive recipients. Groups of four 6-wk-old female BALB/c and NOD mice were hyperimmunized i.p. with the extracellular domain of the human TSHR produced as a maltose-binding protein fusion (MBP-ECD) (18) in an adjuvant of alum plus attenuated Bordetella pertussis toxin as described previously in detail (13). Six weeks later, the animals were killed and their spleens and thyroids removed, the latter to ensure that thyroiditis had been induced. Untreated mice of the same age and sex were also killed to provide controls. Spleen cells from MBP-ECD-treated and control animals were mechanically disrupted and cultured in RPMI 1640 medium supplemented with 10% FCS, β-mercaptoethanol, and 20 µg/ml of MBP-ECD, as described previously (14).

Following this period of in vitro priming, the splenocytes were extensively washed and a portion of them were passed through a column (Isocell mouse CD4 isolation kit, Pierce, Rockford, IL) to generate a CD4+-enriched population, as described previously (14).

Groups of 6-wk-old female BALB/c and NOD mice were immunized in the tail vein with a total volume of 100–200 µl of PBS containing ~10^6 unfractionated splenocytes from primed or control syngeneic animals or 10^6 CD4+-enriched T cells from primed syngeneic mice, as described previously (14). In two separate experiments, animals were killed at 4 and 8 or 12 wk after transfer.

Genetic immunization to generate primed T cells: transfer to naive recipients. Two 6-wk-old BALB/c mice were injected in the anterior tibialis muscle with the cDNA for the full-length human TSHR in the pcDNAIII expression plasmid, in PBS following pretreatment with cardiotoxin, as described previously (14). Immunizations with 100 µg of MBP-ECD, as described previously (14).

Serum total free T4 levels were measured by Gammacoat RIA, in duplicate and at two different dilutions. Results are expressed as µg/dl.

Results

Thyroid histology

Of the BALB/c and NOD mice treated with the MBP-ECD protein or the cDNA of the human TSHR to generate primed spleen cells, 75% and 100%, respectively, developed thyroiditis. Briefly this comprised lymphocytic infiltration so that >15% of the surface was occupied by interstitium in the BALB/c and lymphocytic infiltration accompanied by follicular destruction in the NOD. Spleens from these animals were dissociated for use in the transfer experiments. Control spleens from untreated age/sex matched animals, shown histologically to be free from thyroiditis, were also used.

In earlier experiments we have demonstrated thyroiditis in naive syngeneic recipients of TSHR protein-primed splenocytes, 2 wk after transfer. In the present study, reporting three separate experiments, we observed that the lymphocytic infiltration persisted until at least 12 wk and retained the characteristics we have already observed (see below).

Thyroiditis was induced in all but 1 of the 26 NOD mice receiving TSHR-primed T cells, irrespective of the experiment, the sampling time or the T cell population used.

In the first experiment, 60% of the BALB/c recipients of MBP-ECD-primed T cells displayed thyroiditis at 4 wk; 75% at 8 wk. However, this rate increased to 100% in the second experiment, the same percentage persisting until 12 wk. Thyroiditis was induced in all BALB/c recipients of cDNA-primed T cells. In both strains, recipients of nonprimed T cells were free from thyroiditis, as were untreated age/sex matched controls. The results of the thyroid examination of all of the mice in the study are summarized in Table I.

All five BALB/c mice receiving spleen cells from animals immunized with the cDNA for the human TSHR, displayed lymphocytic infiltration comprised of activated T cells and B cells. There
were no signs of gland destruction, but there were signs of thickening of the epithelial layer and follicular budding. Similar features were observed in the thyroids of BALB/c recipients of MBP-ECD-primed T cells, particularly those examined at 12 wk, as shown in Fig. 1A. In contrast, the infiltration in the thyroids of NOD recipients of TSHR-primed spleen cells replaced large areas of the gland and resulted in considerable follicular destruction, as shown in Fig. 1B.

Activated T cells were present both in BALB/c and NOD thyroids (Fig. 2, A and D) and significant numbers of B cells only in the former (Fig. 2, B and E).

We have performed staining for IFN-γ, IL-4, and IL-10, the first of these cytokines being the hallmark of a Th1 and the last two of a Th2 immune response. IL-4 and IL-10 immunoreactivity was abundant in the BALB/c mice but not in the NOD, which displayed instead IFN-γ staining, as shown in Fig. 2, C and F. No staining was obtained in the absence of first and/or second Ab or when substituting an isotype control (data not shown).

The ratio of B:T cells and of IL-4:IFN-γ in the thyroid lymphocytic infiltrate were calculated in the BALB/c and NOD animals in which orbital pathology had been evaluated. Thyroiditis was induced in all 15 NOD mice and in no instance did the B:T cell ratio exceed 0.45 or the IL-4:IFN-γ ratio exceed 0.13, indicating the Th1 nature of the disease, as shown in Table II. In contrast, in the BALB/c mice in which thyroiditis had been induced, the B:T cell ratio was >1 increasing to almost 2 and the IL-4:IFN-γ ratio was >1.8 increasing to 3.5, suggestive of type Th2 disease, as shown in Table II.

In addition, the surface occupied by interstitium was calculated in the BALB/c thyroids and compared with the orbits (see below).

In both strains of mice an evolution in the thyroiditis was observed, so that the degree of infiltration increased with the length of time following transfer and, in the NOD mice, the extent of follicular destruction.

**Histology of the orbit**

Orbits of all of the NOD, irrespective of the sampling time and all of the BALB/c mice treated with nonprimed splenocytes displayed normal histology with intact muscle fibers, no signs of edema or fat accumulation and no immune infiltrate. Examples are shown in Fig. 3, A and B.

In the first MBP-ECD transfer experiment (at 8 wk), one BALB/c mouse receiving unfractionated T cells and two BALB/c mice receiving the CD4⁺-enriched population exhibited considerable orbital changes, described below, which were also present in

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**Table I. Summary of thyroiditis and orbital pathology results**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Examined Weeks</th>
<th>Transfer Cells</th>
<th>Thyroiditis</th>
<th>Orbital Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>4</td>
<td>Whole ECD</td>
<td>3/5</td>
<td>ND</td>
</tr>
<tr>
<td>BALB/c</td>
<td>4</td>
<td>CD4⁺ ECD</td>
<td>3/5</td>
<td>ND</td>
</tr>
<tr>
<td>BALB/c</td>
<td>4</td>
<td>Whole cDNA TSHR</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>BALB/c</td>
<td>8</td>
<td>Whole ECD</td>
<td>8/10</td>
<td>5/10</td>
</tr>
<tr>
<td>BALB/c</td>
<td>8</td>
<td>CD4⁺ ECD</td>
<td>4/5</td>
<td>2/5</td>
</tr>
<tr>
<td>BALB/c</td>
<td>8</td>
<td>Control whole</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>BALB/c</td>
<td>12</td>
<td>Whole ECD</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>BALB/c</td>
<td></td>
<td>Untreated</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>NOD</td>
<td>4</td>
<td>Whole ECD</td>
<td>4/5</td>
<td>ND</td>
</tr>
<tr>
<td>NOD</td>
<td>4</td>
<td>CD4⁺ ECD</td>
<td>6/6</td>
<td>ND</td>
</tr>
<tr>
<td>NOD</td>
<td>4</td>
<td>Control whole</td>
<td>0/3</td>
<td>ND</td>
</tr>
<tr>
<td>NOD</td>
<td>8</td>
<td>Whole ECD</td>
<td>7/7</td>
<td>0/7</td>
</tr>
<tr>
<td>NOD</td>
<td>8</td>
<td>CD4⁺ ECD</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>NOD</td>
<td>8</td>
<td>Control whole</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
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<td>Whole ECD</td>
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<td>0/3</td>
</tr>
<tr>
<td>NOD</td>
<td></td>
<td>Untreated</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

* Weeks after transfer of T cell population.
* Whole ECD, T cells primed with extracellular domain of the TSHR fusion protein and not fractionated; CD4⁺ ECD, T cells primed with the extracellular domain of the TSHR fusion protein and fractionated to yield CD4⁺ enriched; control whole, T cells not primed, not fractionated; whole cDNA TSHR, T cells primed by genetic immunization, not fractionated; untreated, mice receiving no T cells.

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**FIGURE 1.** Semithin (0.5 μm) sections of (A) BALB/c and (B) NOD thyroids from recipients of TSHR-primed T cells, 12 wk after transfer. In the BALB/c the immune infiltrate does not result in follicular destruction, in contrast to the NOD, in which the extensive immune infiltrate surrounds destroyed follicles. Magnification, ×320.
four of the five BALB/c mice at 8 wk in the second experiment and in all five BALB/c mice at 12 wk. The orbital changes were mirrored in all five BALB/c mice, examined at 4 wk after receiving the splenocytes primed via immunization with TSHR cDNA, as shown in Fig. 3, C and D. There were histologic signs of vasodilation and infiltration by immune cells, notably mast cells that were present in all orbits displaying modification.

The EOM were disrupted by edema that was shown to be PAS-positive, as shown in Fig. 4a. Large deposits of adipose tissue were also present, particularly noticeable in the toluidine blue-stained section in Fig. 4b. In the same section, the edematous material was lightly stained suggesting the absence of charge. Activated T cells, macrophages, and B cells were also present in the most severely affected orbits, as shown in Fig. 4, c and d.

Staining with a mAb for the TSHR, which produces dense immunostaining on the basolateral surface of mouse thyroid (Fig. 5A), displayed considerable immunoreactivity to the receptor in the BALB/c mice with orbital pathology but no staining was detectable in control BALB/c orbits (Fig. 5B) or in the absence of first and/or second Ab or substituting an isotype control (data not shown). The presence of edema resulted in an increase in the background staining of affected orbits when compared with controls from recipients of nonprimed T cells. Aside from this, the strongest immunostaining was associated with elongated fibroblast-like cells that were often in close proximity to fat tissue. The fat tissue also displayed staining, although less noticeable (Fig. 5C).

As in the case of the induced thyroiditis, there was an evolution in the severity of the orbital pathology with mast cells and edema occurring first, followed by the appearance of lymphocytes. A summary of the orbital histology results is given in Table I.

The incidence of orbital pathology showed no correlation with circulating T4 or TBII levels (see below) when the mice were killed, but it was always accompanied by thyroiditis.

The severity of the thyroiditis and orbital pathology was quantified as the surface occupied by the interstitium. In the absence of thyroiditis or orbital pathology, the interstitium occupied <15% and 10%, respectively, of the surface of each tissue and the thyroid B:T ratio was <1. In cases of mild thyroiditis, the interstitium...
occupied 15–20% and the B:T ratio was 1–1.3, again there was no orbital pathology.

In the most severe thyroiditis, the interstitium occupied 25–30% and the B:T cell ratio was 1.6–1.9, in the corresponding orbits, the orbital pathology occupied 15–20% and the B:T ratio was 1–1.3, again there was no orbital pathology.

Thus orbital pathology depended on the most severe thyroiditis that itself displayed the most skewed Th2 response as defined by the B:T cell ratio (>1.6) and IL-4:IFN-γ ratio (>2.5).

### Circulating Abs to the TSHR

In our previous study in which we examined BALB/c and NOD mice 16 days following transfer of primed cells, circulating Abs to the MBP-ECD Ag used to generate the primed cells were not induced, as assessed by ELISA. In the present study, circulating Abs were present in both strains of mice and persisted until 12 wk. Control animals had OD values up to 0.2, whilst recipients of TSHR-primed T cells had values from 0.4 to 1.2 (individual data not shown).

### Biological activities of TSHR Abs

The amount of TSH bound was never <80% that of the mean of the controls (defined as 100%) when testing individual control sera. The results from the the MBP-ECD transfer protocol showed that, at 8 wk, 10 of 15 BALB/c mice and 6 of the 12 NOD mice, and, at 12 wk, 4 of 5 BALB/c mice and 2 of 3 NOD mice sera had values of <80% for TSH binding, with a minimum of 45% for the BALB/c and 55% for the NOD (individual data not shown).

### Circulating thyroid hormone levels

In previous experiments, in which disease has been induced directly with Ag or with transfer of primed T cells, we have observed considerable heterogeneity in thyroid hormone levels, even in animals before treatment. However, the BALB/c mice appeared to be more adversely affected by the transfer of syngeneic T cells because even the recipients of nonprimed spleen cells had reduced T4 levels: six mice had T4 levels of 0.5–0.8 µg/dl, and the remaining four mice had 0.8–1.3 µg/dl.

As expected from the destruction of the thyroid in the NOD mice, 10 of 15 recipients of TSHR-primed T cells tested had reduced T4 levels: six mice had 0.4 vs 3.7 ± 0.2 at 4 wk and 2.8 ± 0.4 at 8 wk, respectively (mean ± SD). Thus when evaluating BALB/c recipients of receptor-primed T cells, by comparison with untreated animals they have slightly reduced circulating free T4 but using the recipients of nonprimed T cells as a baseline, they were elevated in 16 of 20 mice tested (individual data not shown).

### Discussion

These experiments are an extension of our previous study (14) in which we were able to transfer thyroiditis, as assessed at 2 wk, to naive syngeneic recipients (BALB/c and NOD mice) of TSHR fusion protein-primed splenocytes. In the present study, we have demonstrated that the thyroiditis persists for at least 12 wk and increases in severity with time. Abs to the immunizing Ag are induced by 4 wk after transfer and may have bioactivity in being TBI. The thyroiditis induced in the NOD mice displays follicular destruction, a B:T cell ratio of <0.45 and IL-4:IFN-γ ratio of <0.13, indicating a Th1 autoimmune response. In contrast, the

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**Table II. B:T and IL-4:IFN-γ ratios in thyroids of BALB/c and nonobese diabetics recipients of TSHR primed T cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BALB/c</th>
<th>Nonobese Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thyroiditis</td>
<td>IL-4:IFN-γ</td>
</tr>
<tr>
<td>Examed 8 wk post receipt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>whole MBP-ECD primed T cells</td>
<td>0.8</td>
<td>1.32</td>
</tr>
<tr>
<td>+</td>
<td>1.2</td>
<td>1.98</td>
</tr>
<tr>
<td>+</td>
<td>1.3</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>1.3</td>
<td>2.12</td>
</tr>
<tr>
<td>+</td>
<td>1.7</td>
<td>2.99</td>
</tr>
<tr>
<td>+</td>
<td>1.8</td>
<td>3.18</td>
</tr>
<tr>
<td>+</td>
<td>1.8</td>
<td>3.09</td>
</tr>
<tr>
<td>+</td>
<td>1.9</td>
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</tr>
<tr>
<td>+</td>
<td>1.9</td>
<td>3.41</td>
</tr>
<tr>
<td>Examed 8 wk post receipt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBP-ECD CD4⁺⁺ T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>0.8</td>
<td>1.33</td>
</tr>
<tr>
<td>+</td>
<td>1.2</td>
<td>1.93</td>
</tr>
<tr>
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<tr>
<td>+</td>
<td>1.9</td>
<td>2.61</td>
</tr>
<tr>
<td>Examed 12 wk post receipt</td>
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<td></td>
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<tr>
<td>MBP-ECD whole T cells</td>
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<td></td>
</tr>
<tr>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
<td>+</td>
<td>1.9</td>
<td>3.5</td>
</tr>
</tbody>
</table>

**Notes:**

- Treatments are as follows: 8 wk whole T, mice examined 8 wk after transfer of T cells primed with the extracellular domain of the TSHR fusion protein and not fractionated; 8 wk CD4⁺⁺, mice examined 8 wk after transfer of T cells primed with the extracellular domain of the TSHR fusion protein and fractionated to yield CD4⁺⁺ enriched; 12 wk whole T, mice examined 12 wk after transfer of T cells primed with the extracellular domain of the TSHR fusion protein and not fractionated.
- Abs to CD4⁺⁺ Ag in the BALB/c recipients, without thyroiditis, were present in both strains of mice and persisted until 12 wk.
- Abs to CD4⁺⁺ Ag in the BALB/c recipients, with thyroiditis, were present in both strains of mice and persisted until 12 wk.
- Abs to CD4⁺⁺ Ag in the NOD recipients, without thyroiditis, were present in both strains of mice and persisted until 12 wk.
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BALB/c thyroiditis displayed no follicular destruction, a B:T cell ratio >1 and IL-4:IFN-γ ratio >2, indicating a Th2 autoimmune response. Furthermore, the Th1 and Th2, preferences of the NOD and BALB/c mice were conserved throughout the 12-wk experiment. Genetic immunization is equally effective at generating the TSHR-primed spleen cells for transfer as hyperimmunization with fusion protein.

In addition to confirming and extending information regarding our transfer protocol, we have obtained orbital pathology, having many of the characteristics displayed by patients with TED (e.g., edema, accumulation of adipose tissue, formation of PAS-positive material, vasodilation, TSHR immunoreactivity, and infiltration by mast cells, T cells, and B cells); in 17 of 25 BALB/c mice treated with TSHR-primed spleen cells, produced either by an MBP-ECD fusion protein or the cDNA for the human TSHR. None of the NOD mice developed these ocular changes. Furthermore, in separate experiments, New Zealand black mice treated with the cDNA for the TSHR but not the

FIGURE 3. Semithin sections (0.5 μm) of orbital muscular tissue from ×130 (A) and ×320 (B) BALB/c recipients of nonprimed T cells. The histology is normal with intact muscle fibers: ×130 (C) and ×320 (D) BALB/c recipient of TSHR-primed T cells 12 wk after transfer. Organization of muscle bundles has been lost with individual muscles being dissociated by edema.

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luteinizing hormone receptor developed thyroiditis but showed no signs of ocular change (data not shown).

Orbital pathology was present only in those BALB/c mice with severe thyroiditis, i.e., with a B:T cell ratio >1.6, and an IL-4:IFN-γ ratio >2.5 and >24% of the surface occupied by interstitium. Severity of thyroiditis increased in both mouse strains with time. Does this indicate a temporal requirement, with development of thyroiditis preceding the ocular changes, as occurs in many patients with TED (1)?

At first glance, priming with TSHR cDNA seems to be the most efficient method of transferring induced ophthalmopathy, because all five animals in this treatment protocol displayed ocular changes. However, in the first MBP-ECD transfer experiment, there was considerable heterogeneity with two of the animals producing no response in terms of thyroiditis or TBII, suggesting that, despite the presence of thyroiditis in the donor animals, the transfer population was less effective than in the second experiment in which 100% had thyroiditis and 90% had ocular changes.

The eye signs did not correlate with the levels of TBII or circulating T4 measured only at a single time point (i.e., at death), so it is unwise to draw firm conclusions. However, it was noteworthy that the BALB/c mice were adversely affected by transfer of T cells, even nonprimed, because all animals had reduced T4 levels when compared with untreated mice although recipients of TSHR-primed T cells had elevated T4 compared with the nonprimed recipients. Furthermore, reduced T4 concurrent with autoimmune response to the TSHR are not sufficient to induce eye disease, as evidenced by the NOD model. Future studies should address this issue in detail, by following T4 and circulating TSH levels from the time of transfer through to death.
The model poses several questions: The eye signs we have induced seem to depend on a Th2 response to the TSHR, yet ICC revealed TSHR immunoreactivity only in the affected orbits and not in control. 1) Where is the TSHR or receptor cross-reactive protein in the orbit that is the target of the priming immune recognition event? The ICC detection method is robust but may lack the sensitivity to reveal low-level TSHR immunoreactivity, particularly that associated with a cell type that may not be abundant in the orbit, such as a preadipocyte. The pattern of immunostaining we obtained, elongated fibroblast-like cells, is consistent with this notion and will be the subject of further investigation.

Studies from other laboratories have shown that some guinea pig adipose tissue can bind TSH with high affinity (21) and in most anatomical locations expresses TSHR transcripts (9). 2) If the same holds true for mice and human, why does an autoimmune response to the TSHR result in organ-specific GD and TED rather than systemic disease involving all adipose compartments? Experiments are underway to address this issue, at least in the mouse models, by examining other fat depots for the presence of lymphocytic infiltration.

Mast cells were present in all of the affected orbits suggesting that they were early arrivals in the disease process. 3) What is the mechanism driving mast cell homing to the orbit and is it Ag specific? In the same context, the material accumulating between the muscle fibers was shown to be PAS positive, because of the presence of sugar moieties. 4) Where does it originate? If it were shown to be one of the glycosaminoglycans, then orbital fibroblasts could be the source, as in well established human TED. If not, it could be the result of vasodilation, which was observed in many orbits, or the degranulation of the mast cells themselves.

### Table III. Correlation between severity of thyroiditis and orbital pathology in BALB/c mice

<table>
<thead>
<tr>
<th>Treatment a</th>
<th>Thyroiditis b</th>
<th>% Interstitium c</th>
<th>B:T Cell Ratio d</th>
<th>Orbital Pathology e</th>
<th>% Interstitium f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examined 8 wk post</td>
<td>-</td>
<td>12</td>
<td>0.8</td>
<td>-</td>
<td>7.6</td>
</tr>
<tr>
<td>receipt MBP-ECD primed whole T cells</td>
<td>+</td>
<td>20</td>
<td>1.2</td>
<td>-</td>
<td>8.8</td>
</tr>
<tr>
<td>+</td>
<td>20</td>
<td>1.3</td>
<td>-</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>19</td>
<td>1.3</td>
<td>-</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>24</td>
<td>1.7</td>
<td>+</td>
<td>14.2</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>25</td>
<td>1.8</td>
<td>+</td>
<td>14.4</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>28</td>
<td>1.8</td>
<td>+</td>
<td>16.6</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>29</td>
<td>1.9</td>
<td>+</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>28</td>
<td>1.9</td>
<td>+</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>Examined 8 wk post</td>
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<td>15</td>
<td>0.8</td>
<td>-</td>
<td>8.7</td>
</tr>
<tr>
<td>receipt MBP-ECD primed CD4+ cells</td>
<td>+</td>
<td>19</td>
<td>1.2</td>
<td>-</td>
<td>9.5</td>
</tr>
<tr>
<td>+</td>
<td>20</td>
<td>1.2</td>
<td>-</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>28</td>
<td>1.8</td>
<td>+</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>30</td>
<td>1.9</td>
<td>+</td>
<td>17.7</td>
<td></td>
</tr>
<tr>
<td>Examined 12 wk post</td>
<td>-</td>
<td>26</td>
<td>1.6</td>
<td>+</td>
<td>17.2</td>
</tr>
<tr>
<td>receipt MBP-ECD primed whole T cells</td>
<td>+</td>
<td>26</td>
<td>1.7</td>
<td>+</td>
<td>16.6</td>
</tr>
<tr>
<td>+</td>
<td>28</td>
<td>1.7</td>
<td>+</td>
<td>18.6</td>
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<tr>
<td>+</td>
<td>28</td>
<td>1.8</td>
<td>+</td>
<td>16.4</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>30</td>
<td>1.9</td>
<td>+</td>
<td>18.7</td>
<td></td>
</tr>
</tbody>
</table>

a Treatments are as follows: 8 wk whole T, mice examined 8 wk after transfer of T cells primed with the extracellular domain of the TSHR fusion protein and not fractionated; 8 wk CD4+, mice examined 8 wk after transfer of T cells primed with the extracellular domain of the TSHR fusion protein and fractionated to yield CD4+ enriched; 12 wk whole T, mice examined 12 wk after transfer of T cells primed with the extracellular domain of the TSHR fusion protein and not fractionated.

b +, presence of thyroiditis; -, absence of thyroiditis.

c The percent interstitium is estimated on toluidine blue-stained sections by point counting.

d The numbers of intrathyroidal CD3+ T cells and B cells in BALB/c mice were evaluated at a magnification of ×250 in 10 microscopic fields chosen at random and given as the B:T cell ratio.

e +, presence of orbital pathology; -, absence of orbital pathology.
The induced ophthalmopathy was always accompanied by thyroiditis and indeed animals with orbital pathology showed the most severe thyroiditis with the highest proportion of B cells in the infiltrate. In humans, it has been possible to amplify fragments of Ig variable chain, especially the lambda light chain, from orbital mRNA, indicating the presence of B cells (22) and possibly even TSAB, because the majority of these are of the IgG1 lambda subtype. Further evidence for the presence of B cells secreting TSAB in TED orbits is provided by studies of TED orbital tissue xenografts into SCID mice. Several weeks after transplantation, circulating TSAB were found in seven of nine mice receiving TED orbital tissue (23). However, even though TED in humans is often associated with high levels of TSAB, a proportion of patients have no TSAB and are euthyroid.

Because biopsy material from patients with TED is generally available only when the disease is well advanced, several studies have shown that cytotoxic mechanisms (24) or a combination of cell mediated and humoral responses are in play (25). The results in our model suggest that in the initial stages inflammatory and nondestructive Th2 responses hold sway. There is some evidence that the same is true in human TED. Analysis of the cytokines present in orbital biopsies, by RT-PCR, demonstrates a profile more consistent with a Th2 response, with transcripts for IL-4 but not IFN- 

...tion and also of dissecting the immunopathogenic mechanisms that result in this sight-threatening disorder. In particular it should be investigated whether it is possible to induce TED in NOD mice, by skewing the immune response toward Th2 by including in the immunization protocol the cDNA for IL-4 and also by producing disease specific T cell clones.

In conclusion, we have extended our observations of the disease induced by transferring TSHR-primed T cells to BALB/c and NOD mice. We have induced orbital pathology, having many parallels with human TED, in BALB/c but not in NOD mice, treated with TSHR-specific T cells.

Acknowledgments

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References