Graves' ophthalmopathy: in search of better markers and better treatment

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From in vitro studies using cultures of orbital fibroblasts, it has become clear that cytokines play an important role in the orbital inflammation in Graves’ ophthalmopathy (GO). Orbital fibroblasts seem to be the key target cells of the autoimmune attack, and they are capable to express the Thyrotropin-Receptor (TSH-R). In vivo data on the presence of cytokines in orbital tissues are sparse, and mostly limited to samples obtained from patients with end-stage, inactive GO; the same holds true for presence of the TSH-R. The aim of the present study was to determine whether the cytokine profile and TSH-R expression differ in the active vs. the inactive stage of GO. Orbital fat/connective tissue was obtained from 6 patients with active, untreated GO undergoing emergency orbital decompression, and from 11 patients with inactive GO subjected to rehabilitative decompressive surgery. The mRNA levels of various cytokines and the TSH-R were assessed by real-time polymerase chain reaction (PCR) using the LightCycler. Data are expressed as ratios (unknown mRNA/β-actin mRNA).

Active GO patients had much higher TSH-R expression than inactive patients: 4/0–24 vs. 0/0–9, P=0.01 (median value/range). TSH-R expression was related to the Clinical Activity Score (r = 0.595, P=0.015). Patients with active GO compared to those with inactive GO had higher mRNA levels of the proinflammatory cytokines IL-1β (445/153–877 vs. 0/0–455, P=0.001), IL-6 (1583/968–18825 vs. 559/0–7181, P=0.01), IL-8 (1422/38–7579 vs. 32/0–1081, P=0.046) and IL-10 (145/58–318 vs. 27/0–189, P=0.002). In active GO there also existed a trend towards a predominance of Th1-derived cytokines as evident from higher IL-2 (37/0–158 vs. 0/0–68, P=0.043), IFN-γ (20/0–79 vs. 0/0–16, P=0.12) and IL-12 (2.3/0–14.8 vs. 0/0–1.6, P=0.10) mRNA’s. IL-1RA, IL-2R, IL-3, IL-4, IL-5, IL-13, IL-18, and TNF-α mRNA’s were similar in both groups. These data show that at the mRNA level, TSH-R expression is largely present only during the active stages of GO. The active phase is characterized by the presence of proinflammatory and Th1 derived cytokines, whereas other cytokines, among them Th2 derived cytokines, do not seem to be linked to a specific stage of GO.

Introduction

In recent decades our understanding of the immunopathogenesis of Graves’ ophthalmopathy (GO) has increased substantially. There is growing evidence that the orbital fibroblast is the target cell of the autoimmune attack that comprises an influx of predominantly T lymphocytes, together with some B-cells, plasma cells and mast cells. The finding that extraocular muscle cells are histologically intact and the fact that fibroblasts are involved in other extrathyroidal manifestations of Graves’ disease (pretibial dermopathy and acropachy) supports this view. The autoantigen responsible for this autoimmune attack is still not defined, but there is accumulating evidence that the Thyrotropin Receptor (TSH-R) plays an incompletely understood role in the initiation or perpetuation of the orbital autoimmunity. After the initial demonstration of TSH-R mRNA in orbital tissues, we now know that the TSH-R protein is expressed on the orbital fibroblasts of GO patients and to a lesser extent on orbital fibroblasts of healthy individu-
From recent in vitro studies, it has also become evident that retrobulbar fibroblasts, or preadipocytes, need to differentiate before they express the TSH-R. Furthermore, TSH-R expression can be enhanced in vitro by the addition of certain cytokines like Tumour Necrosis Factor-α (TNF-α) or interferon-γ (IFN-γ). This raises the question whether the TSH-R may be expressed differently during the active vs. the inactive stages of GO. This question has been rarely studied using active specimens. Sampling has largely been done in the inactive stages of disease, mostly during or after extensive immunosuppressive treatment regimens.

Thus, although the nature of the autoantigen is still speculative, we know that the lymphocytic infiltrate can profoundly affect the orbital fibroblasts. These in vitro studies have demonstrated that cytokines (presumably produced by the infiltrating lymphocytes) when added to fibroblast cultures, induce their proliferation and glycosaminoglycan production. These effects can be blocked by, for example IL-1 Receptor Antagonist (IL-1RA), irradiation or corticosteroids. Furthermore, the expression of several adhesion molecules on the fibroblast cell surface is upregulated under the influence of cytokines, which also induce the expression of regulatory proteins such as Heat shock protein 72 or HLA-DR.

These in vitro findings have raised the prospect of more specific immunomodulatory (e.g. anti-cytokine) therapies, especially because the results of specific therapies, for example anti-TNF-α, show promise in other autoimmune diseases such as Crohn’s disease and rheumatoid arthritis. However, a major impediment is that the choice for an immune therapy in GO would be based on in vitro studies and not on data derived from in vivo observations. At present it is largely unknown whether the cytokines used in the culture experiments are in fact present in the orbital tissues of GO patients, and whether different cytokines are present in the active and/or the inactive stages of this disease. The scarce in vivo data that are available have been obtained in extensively treated and probably inactive or in poorly defined patients. Cytokines act in a network, and are produced by many different celltypes. They exert their effects mostly in a paracrine or even autocrine fashion. If we opt to treat GO patients with specific anticytokine therapies we need to have more knowledge of the cytokine environment in the active stage of GO. We therefore decided to perform a study aimed at comparing the cytokine milieu in patients with active GO vs. in patients with inactive, endstage eye disease. We used real-time PCR to quantitatively study the amounts of mRNA coding for a variety of cytokines and cytokine receptors, as well as the TSH-R, in orbital fat/connective tissue biopsies taken during decompressive surgery.

Patients and methods

Patients We studied orbital tissue from 17 patients with GO. Six patients had untreated very active GO and underwent an emergency orbital decompression operation because of optic neuropathy (n=5) or fixation of the globes (n=1). The activity of the eye disease was assessed by the Clinical Activity Score (CAS). A score of ≥5 (maximal score 10) including a worsening in the previous 2 months indicates active GO. Eleven patients underwent elective orbital decompression purely for rehabilitative reasons in the inactive stage of the disease. Inactive eye disease was defined as no changes in eye status in the previous six months. From these eleven patients four had not received immunosuppressive therapy at any time and seven had been treated in the past with corticosteroids, orbital irradiation or both (median 11 months/ range 6–42, prior to surgery). All patients gave informed consent. The local committee on medical ethics approved this study.
Table 1: Characteristics of patients with active or inactive Graves' ophthalmopathy.

<table>
<thead>
<tr>
<th></th>
<th>Active GO n=6</th>
<th>Inactive GO n=11</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex F/M</td>
<td>6/0</td>
<td>9/2</td>
<td>0.295</td>
</tr>
<tr>
<td>Age</td>
<td>50 ±12</td>
<td>45 ±13</td>
<td>0.271</td>
</tr>
<tr>
<td>Smoking no/yes</td>
<td>0/6</td>
<td>2/8#</td>
<td>0.270</td>
</tr>
<tr>
<td>Duration GO (months)</td>
<td>5.5 (1–26)</td>
<td>36 (11–216)</td>
<td>0.003</td>
</tr>
<tr>
<td>Duration Thyroid disease (months)</td>
<td>6 (2–204)</td>
<td>60 (16–276)</td>
<td>0.08</td>
</tr>
<tr>
<td>TSH (mU/l)</td>
<td>1.14 (&lt;0.01–8.1)</td>
<td>0.62 (0.02–6.9)</td>
<td>0.6</td>
</tr>
<tr>
<td>TT3 (nmol/l)</td>
<td>2.2 (1.65–2.85)</td>
<td>2.2 (1.45–3.35)</td>
<td>0.6</td>
</tr>
<tr>
<td>TT4 (nmol/l)</td>
<td>108 (82–150)</td>
<td>135 (65–175)</td>
<td>0.2</td>
</tr>
<tr>
<td>TBII (U/l)</td>
<td>78 (27–366)</td>
<td>2.5 (2.5–12)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Immunosuppressive treatment in the past no/yes</td>
<td>6/0</td>
<td>4/7†</td>
<td>0.026</td>
</tr>
<tr>
<td>Clinical Activity Score</td>
<td>7.3 ±1.9</td>
<td>2.3 ±1.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total Eye Score</td>
<td>28 ±7.3</td>
<td>12 ±7.7</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*analysis by t-test for values expressed as mean ± SD, or by Mann-Whitney U-test for values expressed as median with range
# Smoking habits are unknown for one patient
†corticosteroids n=2, radiotherapy n=4 or both n=1

As an overall measurement of disease severity we used the Total Eye Score (TES), calculated as the sum of multiplying each NO SPECSS class present by the grade in that class (for that purpose we substituted 1, 2, and 3 respectively, for grades a, b, and c).\(^2\) We also noted the duration of both the eye disease and the thyroid disease in months since the first signs and symptoms.

At the time of surgery, all patients were clinically euthyroid on antithyroid drugs (11 patients), L-T\(_4\) (4 patients), or without any drugs (2 patients).

RT-PCR analysis of cytokine and TSH-R gene expression Surgical specimens of orbital fat/connective tissue were obtained and immediately snap-frozen in liquid nitrogen until use. RNA was extracted using TriPure Isolation Reagent (Roche Molecular Biochemicals, Mannheim, Germany), and cDNA synthesis was performed with the first-strand cDNA synthesis kit for RT-PCR (AMV) (Roche Molecular Biochemicals). Published primer pairs were used to amplify β-actin, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-13, IFN-γ, TNF-α, and soluble IL-1 Receptor Antagonist (sIL-IRA).\(^2\) We designed primer pairs for IL-18 (forward: 5' GGT TGA ATC TAA ATT ATC AGT C 3', reverse: 5' GAA GAT TCA AAT TGC ATC TTA T 3'), and for the TSH-R (forward: 5' AGCCACTGCTGTGCTTTTAAAG 3', reverse: 5' CCAAAACCAATGATCTCATCC 3') and checked the specificity of the product with Southern blotting. All primers spanned intron/exon boundaries to exclude amplification of contaminating genomic DNA.

Real-time PCR was performed for the quantitative estimation of the above mentioned cytokines and the TSH-R. Standards for the
different mRNA's were prepared from RNA of phytohaemoagglutinin-stimulated peripheral blood mononuclear cells, T-cells or human thyroid tissue. For each mRNA assayed, a sequence-specific standard curve was generated using 10-fold serial dilutions of this target standard PCR product and the same primers used to amplify the cDNA. For each gene the standard protocol was optimized; however, the same amount of cDNA was always added. Twenty-µl PCR reactions were set up with final concentrations of 5mM MgCl$_2$, 2 µl SYBR green mastermix (Roche Molecular Biochemicals), 5 µl 1:10 diluted cDNA, 0.3 µM of both forward and reverse primers.

The reactions were then cycled in the LightCycler (Roche Molecular Biochemicals) with the following parameters: denaturation for one cycle at 95 °C for 10 s, 45 cycles (temperature transition of 20 °C/s) of 95 °C 0s, 50°C 10 s, 72 °C 15 s and fluorescence reading taken at 72 °C, melting curve analysis with continuous fluorescence reading. If necessary, optimization of the protocol was achieved by changing MgCl$_2$ concentrations and/or reading fluorescence at a higher temperature and/or using LightCycler-FastStart DNA master SYBR Green I (Roche Molecular Biochemicals). The LightCycler software generated a standard curve (measurements taken during the exponential phase of the amplification) that enabled the amount of each gene in each test sample to be determined.

For each particular mRNA, all patients' samples were run in one amplification together with a negative control and a standard curve. Because not all biopsies were of equal size, we corrected for input differences by expressing each gene product as the ratio to its corresponding β-actin value. β-actin did not differ significantly between active and inactive biopsies.

Total T$_4$ and T$_3$ were determined with an in-house radioimmunoassay (RIA). TSH was measured in a chemiluminescent enzyme immunoassay (Immulite Third Generation TSH kit, DPC, Los Angeles, CA). TBI was measured by TRAK assay (BRAHMS Diagnostica, Berlin, Germany).

Statistical analysis To analyse differences in continuous variables we used either t-tests or, in case of an abnormal distribution or zero values, the Mann-Whitney U (exact)-test. To analyse differences in categorical data the Fisher's Exact test was used. Nonparametric correlations were calculated with two-tailed Spearman's rho correlation coefficients. T helper (Th)1, Th2 and inflammatory cytokines score were calculated as described previously.

In short, mRNA measurements were divided in three categories based on the 25th and 75th percentile of the ordered data of all patients. Category 1 contains the data lower than the 25th percentile, category 2 contains the data between the 25th and 75th percentile and category 3 contains the data above the 75th percentile. Cytokines mRNA's in category 1 were assigned a value of 0, in category 2 a value of 2 and in category 3 a value of 4. The values of IL-2, IL-12p40, IL-18 and IFNγ in each sample were than summed to arrive to a total Th1 score. The values of IL-4, IL-5 and IL-13 were summed to arrive to a total Th2 score. Although IL-6 is also a Th2 cytokine, its primary action is proinflammatory. Therefore all cytokines with a predominantly proinflammatory action were used for the total inflammation score (IL-1β, IL-6, IL-8, TNF-α and IL-1RA). The role of IL-10 can be proinflammatory, immunosuppressive and immunoregulatory, therefore we did not include IL-10 in a cytokine score.

Results

Table 1 shows the patient characteristics. The active patients had more severe eye disease and a shorter duration of GO than the inactive patients.
IL-1β and TSH-R mRNA were significantly more often present in orbital tissue of active GO patients than in inactive patients (Table 2). The different mRNA/β-actin ratios indicated that patients with active, severe, untreated GO had not only significantly higher levels of TSH-R and the pro-inflammatory cytokine IL-1β, but also higher levels of the proinflammatory cytokines IL-6, IL-8 and IL-10 (Figure 1 and Table 2). The Th1 cytokine IL-2 was also significantly higher in patients with active GO. Other Th1 cytokines such as IFN-γ and IL-12p40 tended to be higher in patients with active GO, although this did not reach statistical significance. All other measured cytokines were comparable in both groups. IL-4 and IL-2 Receptor (IL-2R) could not be found in these orbital tissues. The cytokine profile was not likely to be influenced by prior immunosuppressive therapy, because we did not find differences in mRNA expression between patients who were never treated for their eye disease (n=4) versus those who were (n=7). In addition, immunosuppressive therapy was given 11 months (median) before biopsy.

The inflammation score was significantly higher in orbital tissue of active vs. inactive GO patients (11[8–16] median[range] vs. 6[0–16], P=0.016). The Th1 score (10[4–16] vs. 4[0–8], P=0.10) and Th2 score (0[0–4] vs. 0[0–8], P=0.656) were similar in both active and inactive patients.

Table 2: TSH receptor and cytokine mRNA expression in orbital fat/connective tissue samples of patients with active vs. inactive GO.

<table>
<thead>
<tr>
<th>% Biopsies positive for each mRNA</th>
<th>mRNA/β-actin ratio*</th>
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<tbody>
<tr>
<td>Active n=6</td>
<td>Inactive n=11</td>
</tr>
<tr>
<td>TSH-R</td>
<td>83</td>
</tr>
<tr>
<td>IL-1β</td>
<td>100</td>
</tr>
<tr>
<td>IL-2</td>
<td>83</td>
</tr>
<tr>
<td>IL-2R</td>
<td>0</td>
</tr>
<tr>
<td>IL-3</td>
<td>0</td>
</tr>
<tr>
<td>IL-4</td>
<td>0</td>
</tr>
<tr>
<td>IL-5</td>
<td>17</td>
</tr>
<tr>
<td>IL-6</td>
<td>100</td>
</tr>
<tr>
<td>IL-8</td>
<td>100</td>
</tr>
<tr>
<td>IL-10</td>
<td>100</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>67</td>
</tr>
<tr>
<td>IL-13</td>
<td>17</td>
</tr>
<tr>
<td>IL-18</td>
<td>100</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>50</td>
</tr>
<tr>
<td>TNF-α</td>
<td>33</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>67</td>
</tr>
</tbody>
</table>

median values with range; * Fisher exact test; **Mann-Whitney U (exact)-test
The amount of TSH-R mRNA did correlate with the Clinical Activity Score ($r=0.595$, $P=0.015$) and with IL-1β mRNA ($r=0.0653$, $P=0.005$; Figure 2). The amount of TSH-R mRNA was also related to the inflammation score ($r=0.696$, $P=0.002$) and the Th1 score ($r=0.480$, $P=0.051$), but not to the Th2 score ($r=-0.046$, $P=0.860$). The amount of IL-2 mRNA was correlated with a shorter duration of eye disease ($r=0.606$, $P=0.01$).

**Discussion**

In this study we found some striking differences between active and inactive GO patients. First, we observed that 83% (five out of six) of the orbital connective tissue samples from active GO patients had detectable levels of TSH-R mRNA compared with only 18% (two out of 11) of the biopsies from inactive patients. This differential expression of TSH-R between the active and inactive stages suggests that the TSH-R is not present continuously in the human orbit and that its presence is induced during the autoimmune response at the active stage of GO. TSH-R mRNA was present, however, in two biopsies from inactive patients, one of them had been treated with corticosteroids and the other one received no prior therapy. From the inactive patients these two had the highest Clinical Activity Score (4 and 5 respectively) as opposed to 1.75 ± 1 in the remaining nine inactive patients.

The differential expression of TSH-R may explain why some investigators could not find TSH-R mRNA in their studies using biopsies possibly obtained from patients with inactive eye disease. The TSH-R (mRNA and protein) has been found, *in vitro*, in orbital fibroblasts of patients with GO as well as in healthy controls, although the immunoreactivity appeared less abundant in the controls. Others found TSH-R expression in GO patients but not in healthy con-

![Figure 1](image-url): Individual and median (indicated by a line) values of IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, IFN-γ, IL-18 and the TSH-R to β-actin mRNA ratio's in 6 patients with active and 11 patients with inactive GO.
These differences might be explained by the sensitivity of the techniques used, such as RT-PCR, *in situ* hybridization and immunohistochemistry, or by differences in expression regulation. Animal model studies suggest that an autoimmune response against the TSH-R causes both Graves thyroid disease and Graves' ophthalmopathy. Our finding that TSH-R mRNA is predominantly found in active GO as compared to inactive GO would agree with a recent finding that orbital adipocytes only express the TSH-R during certain stages of their differentiation. When and if the autoimmune attack has begun it may well be self-perpetuating. However, it is also possible that the TSH-R is present in very small amounts in normal human orbital connective tissue and that it is further upregulated during the autoimmune attack. This might be supported by a recent finding that the TSH-R gene expression and/or TSH-dependent cAMP production in orbital fibroblasts can be altered under different conditions *in vitro*. On the contrary, it is also possible that the expression of the TSH-R is secondary to autoimmunity induced by another antigen, although its presence can still be important in the perpetuation of the inflammatory process. This might involve humoral autoimmunity against the TSH-R and could explain the strong association we previously found between TSH-R antibody titers and the activity of the eye disease. Therefore we hypothesize that the orbital fibroblast has the ability to express the TSH-R, and is able to upregulate the expression during an autoimmune attack. This might be under the influence of cytokines produced by T lymphocytes. After the TSH-R expression on the orbital fibroblast is upregulated, this might induce further lymphocyte recruitment or cause binding of TSH-R antibodies. This might lead to activation of other fibroblasts.

Second, we observed a difference in the cytokine profile between patients with active and inactive eye disease. In our six active patients we found significantly more proinflammatory cytokines such as IL-1β, IL-6 and IL-8 than in patients with inactive eye disease. Also IL-10 levels were increased in patients with active GO. It is difficult to interpret the higher IL-10 levels as this cytokine is known to exert many actions including Th1, Th2 and immunoregulatory. There was a trend to Th1 cytokines such as IL-2 and IFN-γ. Th2 cytokines were similar and thus do not seem to play a major role in active eye disease. Although the presence of cytokine mRNA has been studied before qualitatively, our quantitative approach shows a marked difference between active and inactive GO. Other cytokines such as IL-13, IL-18, IL-1RA and TNF-α mRNA's had similar levels in both active and inactive patients. To our knowledge this is the first study in which mRNA's for many different cytokines are quantitatively determined in orbital connective tissue of well-defined GO patients. A potential drawback of our study is that our methods do not allow us to identify the source of the
cytokines measured. They may be produced by lymphocytes, macrophages, fibroblasts and/or adipocytes. However, our aim was to gain insight into the overall cytokine milieu present in the orbits of patients with active eye disease vs. those with inactive disease. The final goal being to get *in vivo* data that will help in choosing specific immunomodulatory therapies. The *in vitro* studies published so far have used fibroblasts as the target cell for several cytokines. This approach is valuable, but does not take into account that a) these fibroblasts themselves are probably also capable of producing cytokines and b) that cytokines are known to act in an extensive network. We therefore feel that our *in vivo* approach has its own advantages, although our mRNA results need to be supported at the protein level.

Our results are in agreement with a study using a different approach. Aniszewski *et al.* analysed T cell clones and found particularly Th1-type T cell clones in early GO as compared to late GO. Also Pappa *et al.* found, with the same approach, more T cells in early as compared to late GO.

Some of the cytokines we found, such as IL-2, IL-10 and IL-5 have been found by McLachlan *et al.* in a very small group of well-defined GO patients. However, they were not able to detect any IFN-γ mRNA, which might be because their patients had inactive GO. We found IFN-γ mRNA in only two out of 11 (18%) inactive patients as opposed to three out of six (50%) active subjects, and with much higher levels in the active then in the inactive patients (20/0–79 vs. 0/0–16). In a large study, Hiromatsu *et al.* found mRNA of various cytokines such as IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6 and IL-10, in orbital connective tissue of extensively treated patients. As this group of patients had been treated with radiotherapy and corticosteroids it is reasonable to assume that they were in the inactive stage of their eye disease. Our results in the inactive patients are in accordance with theirs, with the exception of IL-4.

Orbital fibroblasts can act as both target and effector cells in the autoimmune process. Cytokines can, at least *in vitro*, stimulate the orbital fibroblast to proliferate and, for example, IFN-γ, IL-1 and transforming growth factor-β stimulate glycosaminoglycan production. They can also stimulate the fibroblast to increased HLA-DR expression and inhibit IL-1RA expression. Therefore, it might be possible that cytokines also have other effects on their target cells, the orbital fibroblasts, such as inducing TSH-R expression leading to a possible binding site for TBII antibodies, thus leading to secondary pathways and perpetuating the autoimmune response.

In delineating possibilities for specific immunomodulatory therapies, e.g. anti-cytokine therapies, in the future we need to know the sequence of events and the molecules involved in the autoimmune process. This study demonstrates in the orbital fat/connective tissue of active GO patients a cytokine environment of predominantly pro-inflammatory cytokines and a trend towards Th1 cytokines, but not Th2 cytokines. Drugs, such as anti-TNF-α, currently used in rheumatoid arthritis and Crohn’s disease do not seem to be a good choice in view of our findings. Drugs that counteract a Th1 or inflammatory cytokine may have more potential. In this respect blocking IL-2 or IFN-γ might be considered, thus trying to antagonize Th1 effects. Another approach might be to administer IL-1RA, which by competition for the IL-1 receptor blocks the IL-1 effect and thereby a major route in the inflammatory pathway. Recently Cunnane *et al.* showed a beneficial effect of IL-1RA treatment on the inflamed synovial membrane in rheumatoid arthritis patients, with a reduction of mononuclear cell infiltration. The long-term effects of these new drug therapies are not yet known, but tolerability seems to
be reasonable and side-effects acceptable. In the future we might consider treating patients with one of these specific anticytokine drugs. We conclude that in orbital fat/connective tissue of active, untreated Graves’ ophthalmopathy patients there is significantly more TSH receptor mRNA than in inactive patients, which suggests a role for the TSH-R in perpetuating the orbital autoimmune process. The cytokine environment in the orbital fat tissues of these active GO patients consists mainly of proinflammatory cytokines and a trend to Th1 but not Th2 cytokines. Thus there seems to be an initial predominantly cellular autoimmune attack, which might be extended by a humoral autoimmune response.

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