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A Role for IL-15 in Driving the Onset of Spontaneous Autoimmune Thyroiditis?1

Pete Kaiser,2* Lisa Rothwell,* Dusan Vašíček,3† and Karel Hala‡

The obese strain (OS) of chickens, which suffers from spontaneous autoimmune thyroiditis, is an excellent animal model for Hashimoto’s thyroiditis and provides a unique opportunity to investigate the mechanisms underlying and driving the onset of the disease. Following recent advances in cloning chicken cytokines, we can now begin to investigate the role of cytokines in driving the lymphoid infiltration of the thyroid seen in these birds from day 7 posthatch. Using real-time quantitative RT-PCR, we characterized the expression of IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-15, and IL-18 in thyroids from OS birds and control CB line birds, both in the embryo just before hatch (embryonic day 20) and at 3 and 5 days posthatch. All of these cytokines were up-regulated compared with levels in thyroids from CB birds, at least at some time points, with some evidence for coordination of regulation, e.g., for the proinflammatory cytokines IL-1β and IL-8. Only IL-15 was up-regulated at all time points. IL-15 was also shown to be up-regulated in spleens of OS birds at embryonic day 20 and 5 days posthatch, suggesting that IL-15 is constitutively up-regulated in this line of birds. This could explain the general immune system hyperreactivity exhibited by OS chickens and may be a factor driving the lymphoid infiltration of the thyroid.

Recent progress in the cloning of avian cytokines has led to the development of reagents with which to measure cytokine production in response to infection in the chicken. This should allow a greater insight into the mechanisms controlling the responses of the chicken to disease, both infectious and autoimmune, at both a cellular and molecular level. The avian orthologues of the Th1 cytokines IFN-γ, IL-2, and IL-18 recently have been cloned (10–12), as have the proinflammatory cytokines IL-1β (13) and IL-6 (14); IL-15 (accession no. AF152927), which is closely related to IL-2; and the chemokine IL-8 (15, 16). The genomic sequences and gene structure for IFN-γ (17), IL-2 (18), IL-18 (P. Kaiser, unpublished data), IL-1β (P. Kaiser, unpublished data), IL-15 (P. Kaiser, unpublished data), and IL-8 (19) have been fully determined. A partial genomic sequence for IL-6 has also been isolated recently (P. Kaiser, unpublished data). Gene structure information makes possible the design of probes and primers to specifically quantify cytokine mRNA levels using real-time quantitative RT-PCR.

We aimed to determine the levels of mRNA of these cytokines in the thyroids of 20-day-old embryos, 3- and 5-day-old OS, and unaffected CB birds to try to determine which cytokines, if any, might be driving the initial lymphocyte infiltration of the thyroid from 7 days of age in OS birds.

Materials and Methods

Experimental animals

OS leukemia-free chickens (5), bred as a closed flock homozygous for the MHC haplotype B13, were maintained under standardized conventional conditions (20) in the Central Laboratory Animal Facilities of the Medical Faculty, University of Innsbruck. Chickens of the unrelated leukemia-free inbred CB line (MHC haplotype B12) were used as a control line.

The designation of MHC haplotypes conforms to the nomenclature adopted at the International Workshop of the Chicken MHC (21). For details on the lines used, see the review by Hala and Plachy (22). To eliminate breeding errors, the MHC status of all animals was serologically determined.

Real-time quantitative RT-PCR

Cytokine mRNA levels in thyroids and spleens from OS and CB birds were quantified using a method based on that of Kaiser et al. (23).

Total RNA was prepared from pooled thyroids and spleens of five birds of each strain (OS and CB) at embryonic day 20 (E20), 3 days of age.
Table I. Real-time quantitative RT-PCR probes and primers

<table>
<thead>
<tr>
<th>RNA Target</th>
<th>Probe/Primer Sequence</th>
<th>Exon Boundary</th>
<th>Acc. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>28S</td>
<td>F 5'- (VIC) -AGGACGGCTAGCCAGGATTCCACCA- (TAMRA) -3'</td>
<td>3/4</td>
<td>X59733</td>
</tr>
<tr>
<td></td>
<td>R 5'- GACGACGGATCTGGCAGTCGTC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F 5'- (FAM) -TGGCCAGCCTGCAGGAC- (TAMRA) -3'</td>
<td>3/4</td>
<td>Y07922</td>
</tr>
<tr>
<td></td>
<td>R 5'- GTTCTTCTGCTGACAGGAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>F 5'- (FAM) -CCACTCAGTGTGGTGGTAGG- (TAMRA) -3'</td>
<td>5/6</td>
<td>AJ245728</td>
</tr>
<tr>
<td></td>
<td>R 5'- TGGAGTTGCTGCGGATGAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>F 5'- (FAM) -ACTGAGCGAGGTGGACCC- (TAMRA) -3'</td>
<td>2/3</td>
<td>AJ224516</td>
</tr>
<tr>
<td></td>
<td>R 5'- TGGAGAATATGCAAGAACACAGTAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>F 5'- (FAM) -AGGAGAAGCTGCTGAGGCTTC- (TAMRA) -3'</td>
<td>3/4</td>
<td>AJ250838</td>
</tr>
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<td></td>
<td>R 5'- GCTGCGGCGGCTGCA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>F 5'- (FAM) -TCTTTATCCAGTCCTTCTGACCA- (TAMRA) -3'</td>
<td>1/2</td>
<td>AJ009800</td>
</tr>
<tr>
<td></td>
<td>R 5'- GCCCTCCTCCTGGTTCTGA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15</td>
<td>F 5'- (FAM) -AGATCGTAAATTTCTCAGAAAAAC (TAMRA) -3'</td>
<td>4/5</td>
<td>AJ416937</td>
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<tr>
<td></td>
<td>R 5'- TTTCTGCTGCTGACGTAACAT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>F 5'- (FAM) -CCGCGCCCTCAGGAGGAT- (TAMRA) -3'</td>
<td>4/5</td>
<td>AJ276026</td>
</tr>
<tr>
<td></td>
<td>R 5'- ACCCTGACGGCTGAAATC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Acc. No., genomic DNA sequence; F, forward; R, reverse; FAM, 5-carboxyfluorescein.

Thyroid expression in the thyroid before the clinical onset

Serially diluted RNA for the different reactions, as shown in Table II. There was a linear relationship between the amount of input RNA and the \( C_i \) values for the various reactions as shown in Table II. Regression analyses of the \( C_i \) values generated by the log10 dilution series gave \( R^2 \) values for all reactions in excess of 0.98 (see Table II for details). The increase in cycles per log10 decrease in input RNA for each specific reaction, as calculated from the slope of the respective regression line, is given in Table II.

To control for variation in sampling and RNA preparation, the \( C_i \) values for cytokine-specific product for each sample were standardized using the \( C_i \) value of 28S rRNA product for the same sample. The \( C_i \) values for 28S rRNA did not alter significantly from sample to sample; the average 28S rRNA \( C_i \) values for all samples ranged from 9.12 to 10.46. Cytokine-specific \( C_i \) values varied from sample to sample and from cytokine to cytokine. The \( C_i \) values for 28S rRNA thus appeared to be independent of cytokine production and disease. Therefore, they were taken to be representative of the level of RNA extracted from all samples. To normalize RNA levels between samples within an experiment, the mean \( C_i \) value for 28S rRNA-specific product was calculated by pooling values from all samples in that experiment. Tube-to-tube variations in 28S rRNA \( C_i \) values about the experimental mean

<table>
<thead>
<tr>
<th>Table II. Standard curve data from real-time quantitative RT-PCR on total RNA extracted from stimulated splenocytes</th>
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<tbody>
<tr>
<td>( \Delta Rn )</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>28S</td>
</tr>
<tr>
<td>IFN-γ</td>
</tr>
<tr>
<td>IL-1β</td>
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<td>IL-2</td>
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<td>IL-6</td>
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<td>IL-8</td>
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<tr>
<td>IL-15</td>
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<tr>
<td>IL-18</td>
</tr>
</tbody>
</table>

* \( \Delta Rn \), Change in the reporter dye; \( C_i \), the cycle at which the change in the reporter dye levels detected passes the \( \Delta Rn \); \( R^2 \), coefficient of regression.

(Thyroids only), and 5 days of age using Trizol reagent (Life Technologies, Gaithersburg, MD) following the manufacturer’s instructions. Purified RNA was stored at -70°C.

For both cytokine and 28S rRNA-specific amplification, primers and probes were designed using the Primer Express software program (PE Applied Biosystems, Foster City, CA). Details of the probes and primers are given in Table I. All cytokine probes were designed, from the sequence of the relevant genes, to lie across intron/exon boundaries. Cytokine probes were labeled with the fluorescent reporter dye 5-carboxyfluorescein at the 5' end and with the quencher N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end. The 28S probe was labeled with the fluorescent reporter dye VIC (PE Applied Biosystems) at the 5' end and with TAMRA at the 3' end.

RT-PCR was performed using the TaqMan EZ RT-PCR kit (PE Applied Biosystems). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) with the following cycle profile: one cycle of 50°C for 2 min, 96°C for 5 min, 60°C for 30 min, and 95°C for 5 min, and 40 cycles of 94°C for 20 s, 59°C for 1 min.

Quantification was based on the increased fluorescence detected by the ABI PRISM 7700 Sequence Detection System due to hydrolysis of the target-specific probes by the 5' nuclease activity of the rTth DNA polymerase during PCR amplification. The passive reference dye 6-carboxy-x-rhodamine, which is not involved in amplification, was used to correct for rhodamine, which is not involved in amplification. The passive reference dye 6-carboxy-x-rhodamine, which is not involved in amplification. The 28S rRNA was used as a reference for normalization of the reporter signal. Results are expressed in terms of the threshold cycle value (\( C_t \)), the cycle at which the change in the reporter dye passes a significance threshold. In this work, the threshold values of the change in the reporter dye are as shown in Table II for all reactions described.

To generate standard curves for the cytokine and 28S rRNA-specific reactions, total RNA, extracted from appropriately stimulated lymphoid cells, was serially diluted in sterile RNase-free water, and dilutions were made from \( 10^{-1} \) to \( 10^{-5} \). Each RT-PCR experiment contained three no-template controls, test samples, and a log10 dilution series. Each experiment was performed in triplicate, with replicates performed on different days. Regression analysis of the mean values of six replicate RT-PCR for the log10 diluted RNA was used to generate standard curves.

Results

Cytokine expression in the thyroid before the clinical onset of SAT

Replicate measurements on different days were highly repeatable, with a coefficient of variation for six replicate RT-PCRs of log10.
were calculated. The slope of the 28S rRNA log_{10} dilution series regression line was used to calculate differences in input total RNA. Using the slopes of the respective cytokine log_{10} dilution series regression lines, the difference in input total RNA, as represented by the 28S rRNA, was then used to adjust cytokine-specific C_{t} values. Fig. 1 shows the effect of standardizing cytokine-specific C_{t} values to correct for tube-to-tube variation in RNA levels. Standardization does not dramatically alter the distribution of the results as a whole.

IL-18 mRNA expression is up-regulated in OS E20 thyroids compared with CB E20 thyroids (Fig. 2). At 3 and 5 days posthatch, there is no difference in IL-18 mRNA expression between OS and CB thyroids. IFN-γ mRNA is down-regulated in OS E20 thyroids compared with CB E20 thyroids, it is of equal expression at 3 days posthatch, and it is up-regulated by 5 days posthatch (Fig. 2).

Up-regulation of the expression of the proinflammatory cytokines in OS thyroids compared with CB thyroids is biphasic (E20 and 5 days posthatch) for IL-1β and IL-8, and at 5 days posthatch only for IL-6 (Fig. 3). At the other time points, there are no differences in mRNA expression between OS and CB thyroids for these cytokines, with the exception being IL-8 at 3 days posthatch, which is down-regulated in thyroids from OS birds.

IL-2 mRNA expression is up-regulated in OS thyroids compared with CB thyroids at 3 days posthatch only (Fig. 4). By contrast, IL-15 mRNA expression is up-regulated in OS thyroids at all time points in this study (Fig. 4).
been shown to induce IFN-γ and to activate neutrophils (32). To date, chicken IL-18 has only been shown to be up-regulated in the thyroids of patients suffering from the disease (26, 33–36). This report shows that these cytokines are expressed in the thyroids of OS chickens before the onset of the lymphoid infiltration. Interestingly, for two of these cytokines (IL-1β and IL-8) the up-regulation of their mRNA is biphasic, occurring both in the embryo (E20) and just before the onset of the lymphoid infiltration associated with the disease (day 5 posthatch). IL-6, in contrast, is only up-regulated 5 days posthatch.

IL-2 has been implicated in the pathogenesis of both Hashimoto’s thyroiditis (33, 37) and SAT (7, 38). However, IL-2 mRNA is only up-regulated in the thyroids of OS birds at 3 days posthatch and therefore seems an unlikely candidate to be driving the lymphoid infiltration of the thyroid seen from day 7 posthatch. By contrast, the closely related cytokine IL-15 is up-regulated at all points in this study. In mammals, IL-15 shares many of the biological properties of IL-2. As well as being up-regulated in the thyroid of OS birds, IL-15 is also constitutively up-regulated in the spleens of OS birds at E20 and day 5 posthatch. Therefore, IL-15 represents a good candidate for the general immune system hyperreactivity in OS birds (7) and may play a role in driving the lymphoid infiltration of the thyroid (IL-15 can be chemotactic for lymphocytes (39, 40)). There is one report of IL-15 mRNA being up-regulated in the thyroids of Hashimoto’s thyroiditis patients (41).

At present, a lack of IL-15–specific reagents prevents us from investigating the role of this cytokine in SAT further. One obvious candidate for the overexpression of IL-15 in OS chickens would be a promoter polymorphism. However, although we have determined the gene structure for chicken IL-15, as yet we have no information on the promoter of chicken IL-15. Although bioactive recombinant chicken IL-15 has recently been expressed (42), as yet there are no mAbs described for chicken IL-15. However, mAbs specific for the α-chain of the IL-15R recently have been described (43), two of which inhibit the proliferative effect of IL-15 on T cells. This raises the possibility of blocking experiments in ovo and in vivo to determine whether IL-15 has a direct role in driving SAT, although these are far from simple in avian species because murine Abs do not fix chicken complement.

One potential drawback of real-time quantitative PCR is that, for cytokines, mRNA does not necessarily equate to bioactive protein. For example, both IL-1β and IL-18 in mammals are produced initially as propeptides, which are then cleaved into an active form by the action of caspase-1 (44–47). The same mechanism is thought to apply for the avian orthologues of these two cytokines (12, 13). IL-15 production in mammals, in contrast, is predominantly controlled posttranslationally, and mRNA levels may be greater than protein levels (48–51). For other cytokines, mRNA levels correlate extremely well with bioactive protein. For example, in the chicken, IFN-γ and IL-6 mRNA levels (as measured by real-time quantitative PCR) and protein levels (as measured by bioassays) are in close agreement (23). However, we only have reliable bioassays for a limited number of chicken cytokines (type I IFN, IFN-γ, IL-1β, IL-2, IL-6, and IL-18) and mAbs to even fewer (type I IFN, IFN-γ, and IL-2). Overall, real-time quantitative PCR is currently the only method that allows us to quantify a wide variety of avian cytokines within a particular disease model.

The question remains as to which cells in the thyroid of OS birds are expressing the cytokines we have identified in this study. To
this end, we intend to develop in situ hybridization for chicken cytokines in the thyroid, which in combination with immunohistochemistry should enable us to determine the phenotype of the cells expressing various cytokine mRNA before the onset of the lymphoid infiltration.

Acknowledgments

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References