The Journal of Immunology

This information is current as of April 24, 2017.

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*J Immunol* 2011; 186:4693-4706; Prepublished online 14 March 2011;

doi: 10.4049/jimmunol.1002631

http://www.jimmunol.org/content/186/8/4693

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/03/14/jimmunol.1002631.DC1

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IFN-α Mediates the Development of Autoimmunity both by Direct Tissue Toxicity and through Immune Cell Recruitment Mechanisms

Nagako Akeno,* Eric P. Smith,* Mihaela Stefan,† Amanda K. Huber,† Weijia Zhang,‡ Mehdi Keddache,§ and Yaron Tomer†,*¶

IFN-α is known to play a key role in autoimmunity, but the mechanisms are uncertain. Although the induction of autoimmunity by IFN-α is consistent with primarily immunomodulatory effects, the high frequency of nonautoimmune inflammation suggests other mechanisms. We used thyroiditis as a model to dissect these possibilities. IFN-α treatment of cultured thyrocytes increased expression of thyroid differentiation markers, thyroglobulin, thyroid-stimulating hormone receptor, thyroid peroxidase, and sodium iodide transporter. RNAseq analysis demonstrated that pathways of Ag presentation, pattern recognition receptors, and cytokines/chemokines were also stimulated. These changes were associated with markedly increased nonapoptotic thyroid cell death, suggesting direct toxicity. To corroborate these in vitro findings, we created transgenic mice with thyroid-specific overexpression of IFN-α under control of the thyroglobulin promoter. Transgenic mice developed marked inflammatory thyroid destruction associated with immune cell infiltration of thyroid and surrounding tissues leading to profound hypothyroidism, findings consistent with our in vitro results. In addition, transgenic mice thyroid showed upregulation of pathways similar to those observed in cultured thyrocytes. In particular, expression of granzyme B, CXCL10, a subset of the tripartite motif-containing family, and other genes involved in recruitment of bystander cytotoxic immune responses were increased. Pathways associated with apoptosis and autophagy were not induced. Taken together, our data demonstrate that the induction of tissue inflammation and autoimmunity by IFN-α involves direct tissue toxic effects as well as provocation of destructive bystander immune responses. The Journal of Immunology, 2011, 186: 4693–4706.

Interferon-α, a critical cytokine in the host immune response to viral infections and tumors, has been extensively used as a therapeutic agent for a growing array of diseases, ranging from malignancies, such as hairy cell leukemia (1), to viral infections, notably chronic hepatitis C virus (HCV) infection (2). IFN-α binds to the type I IFN receptor, a transmembrane glycoprotein dimer with cytoplasmic domains. Binding induces a signaling cascade mediated primarily through the JAK-STAT, Crk, insulin receptor substrate, and MAPK pathways (3). Yet, the full scope of the actions of IFN-α, either produced naturally or by therapeutic delivery, remains poorly understood. Emblematic of this understanding gap is the disparate array of notable side effects associated with IFN-α therapy. These include influenza-like symptoms, hematological perturbations, neuropsychiatric signs, and thyroid disease. Individually and/or cumulatively, these side effects can lead to dose reductions in up to 40% of patients and drug discontinuation in up to 14% of patients (4–7).

IFN-α also plays a major role in the development of autoimmunity. Activation of IFN-α pathways has been implicated in a number of autoimmune diseases, most notably systemic lupus erythematosus (SLE) (8). The association of IFN-α therapy with the development of autoimmune thyroiditis has, in particular, supported a role for IFN-α in provoking autoimmune responses (9, 10). However, the mechanisms by which IFN-α induces autoimmunity are still unknown. Conceptually, the induction of autoimmunity is consistent with various immunomodulatory effects of IFN-α, such as activation of cytokines and adhesion molecules, and, of cardinal importance, stimulation of MHC class I Ag expression (11, 12). Moreover, IFN-α shifts the immune response to a Th1 pattern (13–15), resulting in the production of IFN-γ and IL-12, two potent proinflammatory cytokines (15). IFN-α can induce the release of other cytokines, such as IL-6, a cytokine that has been associated with autoimmune thyroiditis (16).

Although the immune effects of IFN-α provide an attractive basis for the induction of autoimmunity by IFN-α, the high frequency of nonautoimmune tissue inflammatory conditions, most notably destructive thyroiditis in many cases of thyroiditis associated with IFN-α therapy (10), suggests that IFN-α can induce pathologic processes also by atypical immune and/or nonimmune mechanisms. We, therefore, hypothesized that IFN-α triggers autoimmunity in genetically susceptible individuals by a combination of direct tissue toxicity and immune modulation. To test this hypothesis, we used IFN-induced thyroiditis (IIT) as a model.

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Received for publication August 2, 2010. Accepted for publication February 4, 2011.

This work was supported in part by Grants DK61659 and DK073681 from the National Institute of Diabetes and Digestive and Kidney Diseases and by a Veterans Administration Merit Award (to Y.T.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AITD, autoimmune thyroid disease; bTg, bovine thyroglobulin; Ct, cycle threshold; FCM, flow cytometric analysis; HCV, hepatitis C virus; IFN, interferon; iNOS, inducible nitric oxide synthetase; mIFN, mouse IFN; NIS, sodium iodide symporter; QPCR, quantitative PCR; SLE, systemic lupus erythematosus; Tg, thyroglobulin; TG, transgenic; TPO, thyroid peroxidase; TRIM, tripartite motif containing; TSH, thyroid-stimulating hormone; WT, wild-type.
IIT is a well-recognized syndrome associated with IFN-α therapy that was first described in 1983 in patients treated with IFN-α for carcinoid tumors (17, 18) and breast cancer (19). Subsequently, numerous studies reported a high incidence of IIT in patients treated with IFN-α, mostly for hepatitis C infection (10, 20). Intriguingly, IIT can manifest either as autoimmune or nonautoimmune thyroiditis, an observation suggesting that IFN-α induces tissue inflammation by immune as well as nonimmune mechanisms (9–11). Our data, combining in vitro and in vivo studies, support the hypothesis that IFN-α has direct tissue toxic effects, most notably the induction of thyroid cell necrosis. Moreover, we show that IFN-α provokes a clear stimulation of an immune-regulated and destructive inflammatory bystander response that most likely triggers tissue-specific autoimmunity in a genetically susceptible host.

Materials and Methods

Materials and reagents

DMEM and penicillin-streptomycin were purchased from Fisher Scientific (Pittsburgh, PA). MTT, a tetrazole, Coon’s modification of Ham’s F12 media, thyroid-stimulating hormone (TSH), insulin, and apotransferrin, and hydrocortisone were purchased from Sigma-Aldrich (St. Louis, MO). TRIzol solution and reagent were purchased from Invitrogen (Carlsbad, CA). Stratagene quantitative PCR (QPCR) cDNA synthesis kit and Brilliant SYBR Green QPCR reagents were purchased from Stratagene (La Jolla, CA). Mouse anti-human TSH receptor (TSHR) Ab and FITC-conjugated rabbit anti-mouse IgG were purchased from Serotec (Raleigh, NC). FITC-conjugated mouse anti-rat MHC class I mAb and mouse anti-human β-actin mAb were purchased from Abcam (Cambridge, MA). FITC-conjugated nonspecific mouse IgG1 control was purchased from BD Biosciences (San Jose, CA). PE-conjugated goat anti-mouse IgG Ab, normal mouse IgG1, and flow cytometric analysis (FCM) wash buffer were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Purified hamster anti-mouse CD3ε, biotin rat anti-mouse CD45R/B220, and purified rat anti-mouse F4/80-like receptor mAbs were purchased from BD Biosciences.

Generation of IFN-α transgenic mice

Creation of the transgenic (TG) mice and all mouse studies were reviewed and approved by the University of Cincinnati and Mount Sinai Institutional Animal Care and Use Committees. The mouse IFN-α (mIFN-α) cDNA, provided by T. Michiels (University of Louvain, Brussels, Belgium), was digested with BamH I and XhoI into a 0.6-kb mIFN-α construct, digested with EcoRV and SalI sites. The final product (designated pBSK-bTg; provided by Dr. J. Fagin, Memorial Sloan Kettering Cancer Center) at the EcoRV and SalI sites. The plasmid containing the bTg–mIFNa construct was digested with BamH I and XhoI into a 0.6-kb mIFN-α construct and ligated into a pBluescriptSK+ vector containing the bovine Tg (bTg) promoter (5′-AGGGGCC-9G-3′; forward primer) and mIFN-α cDNA, 5′-CTCC-3′; reverse primer). Control reference was TSHR gene; forward primer (5′-GTGTT-GGGGCCCTTGATTGTTCTT-3′) and reverse primer (5′-AGGGGCC-CGGTTTGGACTC-3′). Reference control was TSHR gene; forward primer (5′-GTAACTACTCACTGCAAAATG-3′) and reverse primer (5′-TCC-TCAAAATGCTGATTAG-3′). In order to verify the integrity of the transgene, Southern blotting of XbaI/SalI–digested genomic DNA (size 3.4 kb) from tail biopsies was performed using standard techniques (Fig. 3C).

Cell culture

Human thyroid primary cells were prepared from fresh, noncancerous thyroid tissue adjacent to thyroid tumors that were removed at surgery. Tissues were obtained from the University of Cincinnati tissue bank. The use of deidentified anonymous human thyroid was approved by the University of Cincinnati institutional review board. Tissue was minced and incubated in 200 U/ml collagenase solution for 1 h at 37°C. Cells were harvested and cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were passaged at 1:2 dilution and cultured until confluent (P1). P1 or P2 cells were used for experiments. Cells were confirmed to be thyroid cells by Western blot analysis for thyroglobulin (Tg) (data not shown).

The well-differentiated, nontransformed rat thyroid cell line, PCCL3, was provided by J. Fagin (Memorial Sloan-Kettering Cancer Center) and propagated in H4 complete medium, which consisted of Coon’s modification of Ham’s F12 media containing 5% FBS, glutamine (286 µg/ml), apotransferrin (5 µg/ml), hydrocortisone (10 nmol/l), insulin (10 µg/ml), TSH (10 mIU/ml), penicillin, and streptomycin. Cells were cultured at 37°C in 5% CO2; medium was replaced every 48 h. Cells were counted with a Z1 Coulter Counter Cell and Particle Counter (Beckman Coulter, Fullerton, CA).

Real-time QPCR

Total cellular RNA was extracted from rat tissues and cells using TRIzol reagent (1). For cell culture and RNAseq confirmation QPCR experiments, 5 µg RNA was used to synthesize cDNA by Stratagene Stratascript QPCR cDNA synthesis kit. mRNA levels were measured by real-time QPCR using a SmartCycler System (Cepheid, Sunnyvale, CA) using the primers shown in Supplemental Table 3 and SYBR Green for detection of amplicon. For QPCR determination of mRNA expression levels of different genes, cDNA was obtained from 1 µg RNA using the Superscript III first-strand cDNA synthesis kit (Invitrogen), and QPCR was performed using an ABI 7300 machine and TaqMan probe system (Applied Biosystems, Foster City, CA). The IFN-α gene does not contain introns (22) and, therefore, intron-spanning primers could not be provided by Applied Biosystems. Accordingly, after RNA extraction and before cDNA synthesis, the RNA preparation was treated with DNase using the reagent kit, Turbo DNA-free (Ambion, Austin, TX), according to instructions, then reverse transcribed. The primers used to measure mIFN-α levels were obtained from the inventoried set available from ABI (mIFN-α; assay ID: Mm00833961_s1). The reference gene was mouse β-actin (mouse ACTB, VIC-MGB, ID: 4362341F;0708008). Other RNAs assayed using inventoried Applied Biosystems primer/probe mixes included the following: mouse OAS1a (ID: 00836412.m1); mouse granzyme B (Mm00423874.m1); mouse tripartite motif containing (TRIM)21 (Mm00447364.m1); mouse CXCL10 (Mm0999972.m1); rat CXCL10 (Rn00594648.m1); rat CXCL10 (Rn00021731); human OAS1 (Hs00973635.A1); human TRIM21 (Hs001726.m1); and human granzyme B (Hs00188051.m1). Reactions were performed in triplicate. For time- and dose–course experiments, normalized expression was determined by applying the cycle threshold (Ct) values for target and reference genes to the Q–Gene program (23) (Figs. 8, 9). For the QPCR experiments performed to confirm the RNAseq results (Supplemental Table 2), we calculated the −ΔΔCt, as follows: Ct values of tested genes were first normalized to the Ct values of β-actin to obtain a ΔCt (i.e., Ct of tested gene − Ct of β-actin). The ΔCt value of the wild-type (WT) mice was then subtracted from the ΔCt value of the TG mice for the same gene. The obtained value of ΔΔCt is equal to the difference of the logarithm base 2 of the ratio of mRNA levels in the TG mice to mRNA levels in the WT mice, and therefore, the −ΔΔCt value is shown in Supplemental Table 2 for consistency with the RNAseq results.

Transient transfection of cell lines

Transient transfection of cell lines was performed using the Lipofectamine 2000 kit, according to the manufacturer’s instructions.

Flow cytometry

PCCL3 rat thyroid cells were seeded into 6-cm dishes. Cells were harvested in PBS, washed with PBS, and resuspended in FCM wash buffer supplemented with 0.02% sodium azide. For detection of MHC class I, cells (5 × 106) were incubated for 30 min at 4°C with 10 µl FITC-conjugated mouse anti-rat MHC class I mAb or FITC-conjugated nonspecific IgG1 control and washed twice. For TSHR, 5 × 105 cells were incubated with 1 µg mouse anti-human TSHR Ab (or control IgG1) for 30 min at 4°C and washed twice with FCM wash buffer, followed by incubation with PE-conjugated goat anti-mouse IgG (1:200). Ab binding was quantified by flow cytometry (mean fluorescence intensity), using a COULTER EPICS XL-MCL flow cytometer (Beckman Coulter).

MTT assay

PCCL3 thyroid cells or human thyroid primary cells were plated into 96-well plates at 3 × 103 or 1 × 104 cells/well and treated with various concentrations of IFN-α for 48 h. Cell viability was measured by MTT assay, as previously described (24).
Apoptosis assay

PCCCL3 rat thyroid cells or human thyroid primary cells were cultured in 6- cm dishes and treated with IFN-α for 48 h. Cells were harvested and washed in cold PBS. Necrotic and apoptosis cells were measured by a Vybrant apoptosis assay kit 3 (Invitrogen).

Thyroid transcriptome analysis in IFN-α TG thyroids and in primary human thyroid cells exposed to IFN-α

High throughput next generation sequencing for mRNA quantitation. Human thyroid primary cells were grown, as described above, and exposed to 5000 U/ml IFN-α for 12 and 24 h. Total RNA was purified from cells using TRIzol, DNase treated, and electrophoresed to confirm integrity. For the analysis of mouse thyroid tissues, we used a cohort of TG animals, high expressing IFN-α line 91 in a ~75% C57BL/625%CBA/J background, which were given T4 supplementation until 6 mo of age, and were then taken off T4 replacement for ~2 mo. The animals were sacrificed at 8 mo of age. The atrophied, but dissectible thyroids were removed and pooled for RNA extraction (total of 10 animals). Control thyroids from littermate controls of normal size were also pooled for analysis (n = 8). Because of constraints related to the small size of the TG thyroids and the cost of the RNaseq, we performed RNaseq of pooled samples. Therefore, to increase the power of our analyses, the cutoff for significance was adjusted to be robust and the threshold number of sequences required for significance was increased (see below). The RNA from the thyroids was extracted using the RNeasy mini kit (Qiagen, Valencia, CA), DNase treated (RNase-free DNase) (Qiagen), and analyzed for integrity by agarose gel electrophoresis. RNaseq is a method of transcriptome analysis that consists of sequencing a cDNA library by high throughput next-generation sequencing. The number of reads aligning to a specific gene sequence is proportional to the abundance of that gene in the sample from which the cDNA library was prepared. We used the mRNA-Seq sample preparation kit from Illumina (San Diego, CA), according to the manufacturer’s recommendation. Briefly, mRNA was extracted from 2 μg total RNA using oligo(dT) magnetic beads and fragmented at high temperature using divalent cations. A cDNA library compatible with the Illumina next-generation sequencing technology was then prepared from the fragmented mRNA by reverse transcription, second-strand synthesis, and ligation of specific adapters. The amount of dDNA in each library was accurately quantified by spectrophotometric analysis using the Qbit system from Invitrogen and diluted to a 10 nM concentration. Next-generation sequencing was performed on an Illumina Genome Analyzer Ix, according to the manufacturer’s recommendations, using the Single-Read Cluster Generation Kit v2 and the SBS Sequencing Kit v3. Image analysis and base calling were conducted in real time by the SDI software. The reads were statistically analyzed against public reference sequence databases of human (ucsc hg18) or mouse (mm9) genome, RefSeq exons, splicing junctions, and contamination databases, including ribosome and mitochondria sequences using Burrows-Wheeler Aligner, and the alignment files in SAM format were generated. After filtering reads mapped to contamination databases, the reads that were uniquely aligned to each exon and splicing-junction sites were extracted and then counted. The read count for each RefSeq transcript was calculated by combining the counts for exons and splicing junctions of corresponding transcript and also normalized to relative abundance in fragments per kilobase of exon model per million to compare transcription level among samples.

To compare the expression levels of transcripts across samples, the read counts of transcripts in each sample were normalized by leveling the total read counts in each sample to the maximum of the read counts in all samples. The read count data were then formatted into microarray-like data that could be analyzed using a variety of microarray statistical analysis tools. The differentially expressed transcripts were identified using a M-based random sampling method implemented in DESeq package in BioConductor (http://bioconductor.org/packages/2.5/bioc/html/DEGseq.html). The transcripts were further filtered at >2-fold change and a minimum read count of 50 in either condition.

Ingenuity pathway analysis. The differentially expressed transcripts were subjected to pathway analysis by Ingenuity Pathway Analysis (IPA) system, version 8.6 (http://www.ingenuity.com/). The IPA program identifies biological networks and/or pathways representing interactions between the differentially expressed genes in the tested samples and/or with other genes in the database. The fold changes of these genes were converted to log2 Ratios and then imported into the IPA along with gene symbols. Fisher’s exact test was used to calculate a p value for the probability that a pathway was significantly enriched in input genes compared with the genome, and the pathways/networks were ranked by the p values. The significance level and the percentages of upregulated or downregulated genes of each pathway were summarized in scatter bar charts (Fig. 6). The graphical presentation of gene–gene interactions and deregulated genes for enriched pathways were visualized in Canonical Pathway Explorer (Fig. 7).

Thyroid function tests

TSH assay was performed by S. Refetoff and R. Weiss (Divison of Endocrinology, University of Chicago), as previously described (25). Whereas normal range for adult mice is strain and gender specific for most strains, TSH <10 μU/ml is normal. The intra-assay and interassay coefficients of variations at the higher range of the assay are 10 and 24%, respectively. Total T4 levels were measured from blood spotted on filter paper using the neonatal T4 kit (Diagnostic Products, Los Angeles, CA), according to the manufacturer’s instructions. Normal range for total T4 was 2–8 μg/dl.

Maintaining the lines on T4 supplementation

Following documentation of profound hypothyroidism in the high-expressing IFN-α TG line, the animals’ chow was changed to Modified Pico-Lab Rodent 20/250 ppm thyroid powder (TestDiet, Greenfield, IN).

ELISA for measurement of serum IFN-α levels

The serum levels of mIFN-α were determined by using a mIFN-α ELISA kit from PBL Biomedical Laboratories (Piscataway, NJ), according to the manufacturer’s instructions.

Thyroid histology and immunocytochemistry

After sacrifice, the thyroids were dissected in two of the following ways: 1) most thyroid specimens were dissected with the overlying strap muscles removed; 2) nonthyroid hormone-treated thyroid specimens from the two high-expressing IFN-α lines were dissected with inner layer of overlying muscles unperturbed due to inflammatory changes in the surrounding tissue. Gross thyroid images were using a Leica Z24 stereoscopic microscope at variable magnification; digital images were captured using a Nikon Coolpix 950 camera. For routine H&E staining, the trachea were cut above and below the area of the thyroid and the tissue was placed as a block into 10% formalin for at least 48 h for tissue fixation, followed by storage in 70% ethanol. After embedding in paraffin, standard 5-μm sections were obtained for staining. For immunocytochemistry, dissected tissues were embedded in OCT, sectioned, and placed onto slides. Prior to staining, slides were refixed in cold PBS containing 0.2% glutaraldehyde for 10 min. After staining, slides were washed in PBS, dehydrated through an ethanol series, and then coverslipped. Immunocytochemistry was performed by the Comparative Pathology Core at University of Cincinnati College of Medicine, Department of Pathology. Positive control for each of the three primary immune cell classes, CD3e (T cells), CD45R/B220 (B cells), and F4/80-like receptor (macrophages), were spleen sections processed identically and from the same group of animals used for immunocytochemistry. Serial dilutions of the primary Ab were performed with the spleen sections to optimize specific signal versus background, followed by simultaneous of spleen and thyroid tissue section on the same slides under identical conditions. Final concentrations of primary Ab were as follows: CD3e: 1:200, CD45R/B220: 1:5000, F4/80-like receptor, 1:200. Sections were visualized using an Olympus BX51 microscope; digital image captured using a Diagnostc Instruments digital camera, Model 74 Slider; and image saved digitally using Spot Advanced Diagnostic Instruments software Windows version 4.6.

Results

IFN-α expression in different tissues and effects of IFN-α on thyroid-specific gene expression

To ascertain whether IFN-α can exert biological effects on thyroid cells, we first confirmed that IFN-α was expressed in the thyroid. Eleven human tissues were analyzed by QPCR for IFN-α gene expression. Liver tissues showed the highest expression level of IFN-α (26). Interestingly, thyroid tissues showed the second highest levels of expression of IFN-α among the tissues examined (Fig. 1A). We next examined the effects of IFN-α on the expression of the following thyroid-specific genes: TSHR, Tg, thyroid peroxidase (TPO), and sodium iodide symporter (NIS). We used the MHC class I gene, known to be upregulated by IFN-α, as a positive control. PCCCL3 cells (a differentiated rat thyroid cell line) were incubated with rat IFN-α. Both dose-dependent responses and time-dependent responses were assessed. Time-
course experiments performed with a constant IFN-α concentration of 5000 U/ml showed a peak expression of TSHR mRNA at 24 h that persisted through 48 h (Fig. 1B). The increase in TSHR was also apparent at the protein level, as determined by FACS analysis (Fig. 1C). Similarly, the expression levels of Tg, TPO, and NIS mRNA were significantly increased at 24 h by IFN-α; Tg expression persisted even at the 48-h time point, but the expression levels of TPO and NIS decreased to baseline by 48 h (Fig. 1D–F). To analyze the mechanism of increased expression of thyroid-specific genes, promoter activities were determined by a luciferase assay for Tg, TPO, and NIS, respectively. pGL4.10 was used as a negative control. Only the Tg promoter showed a significant increase in activity induced by IFN-α (p < 0.05), suggesting that TPO and NIS are upregulated by IFN-α through other mechanisms. Finally, as expected, there was a dose-dependent and time-dependent increase in the expression of MHC class I mRNA when PCCL3 cells were incubated with rat IFN-α (Fig. 1G, 1H), as well as at the protein level (Fig. 1I).

**Effects of IFN-α on thyroid cell survival**

To test our hypothesis that IFN-α is directly toxic to thyroid cells, we investigated the effect of IFN-α on thyrocyte viability in cultured thyroid cells. A significant IFN-α dose-dependent decrease in cell viability was seen in both PCCL3 rat and human thyroid cells in primary cultures, with maximum cell death occurring at concentrations of 5000 U/ml IFN-α (Fig. 2A, 2B). To determine whether IFN-α–induced thyroid cell death was caused by apoptosis or necrosis, thyrocytes were treated with 5000 U/ml IFN-α for up to 48 h and analyzed by the Vybrant apoptosis assay kit. Both PCCL3 and human thyroid cells (in primary culture) showed significant increase in necrotic cell numbers at 24 and 48 h of IFN-α treatment, but apoptotic cell numbers were unchanged (Fig. 2C, 2D). mRNA levels of caspase-3 (the major mediator of apoptosis) were also measured by QPCR. Whereas a trend toward increased caspase-3 levels was seen during IFN-α treatment of PCCL 3 cells, this change was not statistically significant (Fig. 2E). A third mechanism of cell death, autophagy (27, 28), involving inhibition of the mammalian target of rapamycin signaling pathway (29), was tested by measuring S6K phosphorylation. There was no
change of phospho-S6K levels on Western blot analysis after treatment with IFN-α (Fig. 2F), suggesting no role for autophagy in the observed cell death.

**Generation of TG mice overexpressing IFN-α in the thyroid**

In order to examine the direct and indirect effects of IFN-α on thyrocytes in vivo in a more direct physiologic manner, we generated TG mice overexpressing IFN-α in the thyroid. To generate the TG mice, we used a construct in which the IFN-α gene was placed under the control of the bTg promoter (Fig. 3A). To confirm that immunoreactive IFN-α could be produced by the construct, PCCL3 thyroid cells were transfected with the bTg–mIFN-α construct and mIFN-α protein levels were measured by ELISA. Significant amounts of mIFN-α protein were secreted from PCCL3 cells after transfection with the bTg–mIFN-α construct (Fig. 3B). TG mice, generated as described in Materials and Methods, were screened by PCR (Fig. 3C). PCR screening of the offspring identified seven founders. Six of the seven founders (animal lines 91, 99, 22, 100, 30, and 2) appeared to have intact insertion of the bTg–mIFN-α transgene, as determined by Southern blot of F1 offspring, and were investigated further. Fig. 3D shows a representative Southern analysis of two lines, 91 and 100. In order to confirm the tissue-specific and relative line expression of IFN-α, we performed QPCR analysis of IFN-α mRNA extracted from representative tissues. Fig. 3E shows high levels of thyroidal expression of IFN-α in an intermediate copy number line 100. Note the very low levels of expression in the other tissues tested.

**Further TG line characterization**

Lines 91, 99, and 22, which demonstrated greater copy number by Southern analysis, exhibited reduced fertility and increased morbidity and mortality within the litters. Litter size ranged from ∼3 to 8 in numbers with variable running in the TG pups. Pups that survived to weaning, but were runted initially, proved to be invariably TG positive, yet displayed catch-up growth on T4 supplemented chow. Fig. 4A shows the pronounced running with reduced body hair of the TG-positive animals in a representative litter from line 91 at day 14 of life. To assess for systemic toxicity of IFN-α, we assayed serum from TG mice for IFN-α by ELISA, but did not find detectable IFN-α levels. However, T4 and TSH levels demonstrated severe primary hypothyroidism in the three high copy number lines (91, 22, and 99). For example, Fig. 4B shows representative hormone levels in TG+ and TG− mice from line 91, a high-expressing line. T4 levels were low in TG+ mice at the earliest time tested (∼4 mo) and were dramatically lower than in non-TG littermate controls (Fig. 4B). Fig. 4C and 4D show markedly low T4 levels coupled with high TSH levels in line 91 compared with an intermediate copy number line 100.

At the time of neck dissection of the high-expressing lines, we observed surrounding inflammatory changes coupled with small thyroid size. Fertility, morbidity, and mortality were substantially improved with initiation of T4-supplemented chow that normalized T4 and TSH levels (data not shown). Histologically, thyroids from untreated hypothyroid animals at ∼3–4 mo of age showed profound inflammatory necrosis of the thyroid (Fig. 4F). Fig. 4E and 4H show a normal appearing thyroid, histologically and by gross inspection taken from euthyroid intermediate copy number line 100; Fig. 4F and 4I show the histology and gross appearance of the thyroid from high-expressing line 91 at ∼4 mo of age without thyroid hormone replacement demonstrating the marked inflammatory necrosis of the gland. Interestingly, T4 replacement partially reversed the severe thyroid inflammation and cell death (Fig. 4G, 4J) without decreasing thyroidal levels of IFN-α (Fig. 4K), suggesting that T4 replacement may have a thyroid-protective effect in the setting of thyroid autoimmunity, as has been suggested in pregnant autoimmune thyroid disease (AITD) patients (30). The partial preservation of thyroid tissue with thyroid hormone intervention in the higher-expressing lines (91, 22, and 99) allowed for dissection of sufficient thyroid gland tissue for RNA isolation. Fig. 4K shows the relative expression of IFN-α mRNA by QPCR in five of the six lines. Lines 91 and 22, requiring thyroid hormone treatment for survival and fertility,
demonstrated markedly high IFN-α expression in a range similar to the two intermediate-expressing lines 100 and 30 that were not on T4 replacement therapy at the time of sacrifice at \(\sim4\) mo of age.

Interestingly, attempts to backcross high-expressing lines, initially in a predominantly C57BL/6 background, to a CBA/J background, a strain known to be susceptible to autoimmune thyroiditis (31), were unsuccessful. Even on thyroid hormone replacement, litters were infrequent and small in numbers, and most of the pups were runted and did not survive. However, intermediate-expressing lines (lines 100 and 30) and a low-expressing line (line 2) were successfully backcrossed up to six generations to CBA/J without obvious fertility or viability challenges. Taken together, these data support the hypothesis that local IFN-α production in a susceptible individual can induce thyroiditis and thyroid dysfunction.

**Immunohistochemical analyses of TG thyroids**

To study the contribution of immune cell infiltration to the thyroidal destructive processes, representative thyroids from normal-, intermediate-, and high-expressing IFN-α lines, ages 3–4 mo old, were processed for H&E histochemistry as well as immunocytochemistry for specific immune cell markers. Fig. 5B depicts normal-appearing histology from a WT thyroid for comparison. There were inflammatory infiltrates in tissues surrounding the thyroid glands of high-expressing lines (Fig. 5C), suggesting a strong thyroidal chemokine response. Examination of thyroids of high expressors showed no detectable intrathyroidal staining for macrophages (F4/80-like receptor) or B cells (CD45R/B220). There was, however, consistent, but infrequent and scattered presence of cells staining positively for the T cell marker, CD3e (Fig. 5D). Hypothesizing that at the time of sacrifice most infiltrating mononuclear cells have disappeared as the thyroid was almost completely necrotic, we analyzed thyroids of 1-day-old pups from high-expressing lines 22 and 91 and their WT littermates. Fig. 5D reveals marked inflammatory infiltration of the thyroid and surrounding tissues from a TG line 91 animal; corresponding tissue section from a WT pup is shown in Fig. 5A.
Effects of IFN-α on thyroid transcriptome

To elucidate potential mechanisms by which IFN-α leads to destructive thyroiditis in the TG mice, we performed a transcriptome analysis comparing the entire thyroid transcriptome in the TG mice and the WT mice. A cohort of TG mice and a selection of non-TG WT littermates from the high-expressing IFN-α line 91 without thyroid hormone replacement for ~2 mo were sacrificed at ~8 mo of age, and the thyroids were dissected. The small size of the TG thyroids necessitated their pooling (see Materials and Methods) prior to RNA extraction. RNA was isolated, and the transcriptome was analyzed by RNAseq (see Materials and Methods), a method that allows for greater sensitivity, specificity, and dynamic range than hybridization-based expression microarrays, while providing the potential for characterizing the expression of all transcripts in a given RNA sample. Bioinformatic pathway analysis of the RNAseq data was performed by the IPA program (Ingenuity Systems, Redwood City, CA), and the data are available using Gene Expression Omnibus accession GSE25115 at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE25115. As expected and corroborating the marked induction of IFN-α protein in the TG thyroids, the classical IFN-α signaling pathways were upregulated in the IFN-α TG mice ($p = 7.08 \times 10^{-8}$; Fig. 6A). In addition to the IFN-α pathway, four other pathways were also significantly upregulated, as follows: Ag presentation ($p = 5.13 \times 10^{-6}$), complement system ($p = 5.25 \times 10^{-5}$), granzyme B ($p = 3.89 \times 10^{-5}$), and pattern recognition receptor ($p = 2.04 \times 10^{-4}$). In addition, the IL-6 pathway was upregulated, but to a smaller degree, with significant upregulation of the IL-6R (2.6-fold increase), albeit the expression of the IL-6 gene itself was not increased. Two pathways, arachidonic acid metabolism ($p = 1.95 \times 10^{-3}$) and fatty acid metabolism ($p = 1.00 \times 10^{-2}$), were downregulated. Supplemental Table 1 lists individual genes upregulated or downregulated with the pathways identified by Ingenuity; Supplemental Table 2 shows the results of a subset of genes selected to confirm the validity of the RNAseq results by standard QPCR. In all the genes selected for confirmation, QPCR mRNA levels matched the RNAseq data (Supplemental Table 2). Fig. 7 depicts a representative detailed outline of one of the most significantly upregulated canonical pathways, Ag presentation.

To determine whether the transcriptome results in the TG mice reflected acute or chronic changes induced by IFN-α, we determined whether pathways that were affected in the TG mice might also be regulated in human thyroid follicular cells exposed to IFN-α for 12 and 24 h. Human thyroid follicular cells in...
primary cultures were exposed to high levels of IFN-α (5000 U/ml) for 0, 12, or 24 h, followed by RNA isolation (see Materials and Methods). The samples for the three time points were pooled at each time point and performed in duplicates (0, 12, 24 h). Not unexpectedly, this intervention, reflecting an acute stimulatory effect of IFN-α, provoked a marked increase in classical IFN-α signaling pathways as seen after the chronic IFN-α exposure in the TG thyroids (p = 8.13 × 10^{-8}; Fig. 6B). In addition, the IPA showed six other pathways that were significantly altered by IFN-α (Fig. 6B). Three pathways were upregulated and overlapped with the pathways increased in the TG thyroids, as follows: 1) Ag presentation (p = 6.61 × 10^{-9}); 2) complement system (p = 1.10 × 10^{-3}); and 3) pattern recognition receptor pathways (p = 2.29 × 10^{-3}). Two pathways were uniquely increased in the human thyroid cells exposed to IFN-α: apoptosis (p = 2.19 × 10^{-3}) and retinoic acid receptor (p = 6.17 × 10^{-4}). Only one pathway, the cell cycle pathway, was downregulated (p = 5.01 × 10^{-5}), most likely reflecting the decreased cell viability. Thus, the Ag presentation, complement, and pattern recognition receptor pathways were acutely activated, whereas other inflammatory pathways, notably granzyme B, most likely reflected chronic exposure of the tissue to IFN-α.

**Further assessment of IFN-α-regulated genes**

We selected four key inflammation-related genes shown to be induced chronically in both TG mouse thyroids and acutely in human thyroid cells exposed to IFN-α. We tested these genes by QPCR in TG mice and human thyroid cells in primary cultures. These genes were OAS1, a gene that is increased as a part of the early antiviral response induced by IFN-α; TRIM21, a nuclear protein that binds to and regulates the function of IgG, and is involved with several autoimmune diseases (32–34); granzyme B, a serine protease that is a cytotoxic T cell marker (35–37) (Fig. 8); and CXCL10, a major chemokine for the recruitment of mononuclear cells (38–42) (Fig. 9). We performed time-course and dose-response experiments (Figs. 8, 9); the TG mice thyroid RNA, for this comparison, was obtained from the same RNA preparations used for surveying IFN-α expression (Fig. 4K). As in the RNAseq analysis, all of these four genes showed significant upregulation in our QPCR analysis (Figs. 8, 9). CXCL10 was significantly induced upon acute exposure of human thyroid cells in culture and was strikingly induced with chronic exposure of thyrocytes to IFN-α in the high-expressing TG lines. This suggested that CXCL10 has both acute and chronic effects on thyroidal inflammation. In contrast and as expected, granzyme B, thought to be primarily expressed by immune cells, was increased only in the TG thyroids, reflecting the cytotoxic T cell damage to thyrocytes. The increased granzyme B levels, seen at the 6-h time point in thyroid cell cultures, most likely represent some contamination of the culture with thyroid-resident lymphocytes, and were not observed later than 6 h (most likely due to death of contaminating lymphocytes). Moreover, no increase in granzyme B levels in thyrocytes was observed in our dose–response experiments (Fig. 8). TRIM21, with a less marked difference among the TG lines, showed a significant increase in the cultured thyrocytes, suggesting that its effects are only acute. TRIM21 appeared to be representative of a number of this class of genes found to be induced in the RNAseq analysis. For example, in the TG mice thyroid RNA, TRIM14 and TRIM5 were increased 3.4- and 2.94-fold, respectively. In the human thyrocytes, TRIM14 was increased 2.96-fold (24 h) and TRIM5 was increased 3.36-fold (24 h).

**Discussion**

The mechanisms by which IFN-α induces autoimmunity are still not fully understood. In particular, the roles of immune modulation versus target tissue effects are still controversial. In this study, using thyroiditis as a model, we examined the role of endogenous induction of IFN-α in an attempt to dissect the direct tissue toxic effects of IFN-α versus its immune-regulatory actions. Because IFNs are induced in all cells in response to viral infection, it appears that the notion of selectively increasing IFNs independent of the viral stimulus has not been frequently considered in the past (43) as a strategy to understand IFN’s pathologic mechanisms. As such, our findings have novel implications to other tissues that are exposed to high local levels of IFN-α, either during infections, or as an apparent side effect of large doses of IFN-α administered for chemotherapy, or when induced by the DNA damage that is associated with autoimmune diseases, such as SLE. It is likely that high local levels of IFN-α in certain tissues can contribute to the development of an autoimmune response to that tissue by a bystander mechanism, as was suggested in type 1 diabetes (44).

Our results demonstrate that TG mice overexpressing IFN-α in the thyroid develop severe inflammatory thyroiditis, leading to profound hypothyroidism requiring T4 supplementation for survival. These results are consistent with previous studies showing that TG mice overexpressing IFN-α in the pancreatic β cells develop severe insulin-dependent diabetes (45). However, this study did not analyze the mechanisms by which IFN-α induced tissue-specific autoimmunity. Interestingly, Akwa et al. (43)
demonstrated that TG mice overexpressing IFN-α in the CNS developed a progressive inflammatory encephalopathy associated with a predominantly lymphocytic infiltration and induction of IFN-α–responsive genes such as MHC class I and OAS1. We found a similar inflammatory response in the thyroid accompanied by inflammatory changes in tissues adjacent to the thyroid, and, more globally, demonstrated that IFN-α activated the Ag presentation, complement, and pattern recognition receptor pathways. These effects would be predicted to induce inflammatory destruction of the thyroid coupled with direct toxicity potentially through granzyme B–mediated actions.

The primary actions of IFN-α in cultured thyrocytes appeared to be an early increase in the expression of thyroid-specific proteins, a significantly increased activity of several pathways involved in innate and adaptive immune responses, and, ultimately, markedly increased nonapoptotic thyroid cell death. Consistent with these in vitro findings, TG mice overexpressing IFN-α in the thyroid displayed striking destructive changes in the thyroid that were distinctly exacerbated in an autoimmune-susceptible background. Moreover, the same innate and adaptive immune response pathways were upregulated in the TG mice. The high local levels of IFN-α in the thyroid, importantly without evidence for systemic elevation of IFN-α, appear to lead also to nonimmune tissue destruction, the severity of which is modulated by background autoimmune susceptibility. Our data are consistent with recent studies demonstrating that cytokines such as IFN-γ can directly inhibit thyrocyte function, and suggest a new mechanism for induction of hypothyroidism in the setting intrathyroidal cytokine secretion (46, 47). Indeed, lymphocytic infiltration of the thyroid alone may not be sufficient to cause hypothyroidism (48). Even though the local levels of IFN-α in the TG mice are significantly higher than the serum levels in HCV patients treated with IFN-α, it is likely that the thyroidal levels of IFN-α in patients are higher than serum levels due to local effect of the HCV virus itself (48).

Our in vitro studies demonstrated a consistent IFN-α dose-dependent increase in thyroid-specific proteins, TPO, NIS, TSHR, and Tg. This induction of thyroid–Ag expression by IFN-α provides an attractive potential mechanism accounting for the development of autoimmune thyroiditis (49) in the setting of thyroidal inflammation [e.g., caused by the HCV virus itself (50)]. Specifically, peptides resulting from the degradation of these upregulated self proteins could be presented to T cells. In support of this possibility, a significant increase in Ag presentation pathways leading to presentation of thyroid-specific peptides and activation of cytotoxic T cells has been observed in other model systems (51). Thus, the IFN-α–induced upregulation of thyroid-specific Ags and of Ag presentation pathways, coupled with IFN-α–induced thyroid cell necrosis observed in our tissue culture and in vivo experiments, could result in the generation and presentation of pathogenic peptides derived from thyroid-specific Ags (see Fig. 10). Of note, a previous study by Caraccio et al. (52) showed decreased thyroid–Ag expression by IFN-α. However, in their investigation, the experimental conditions were different and thyroid–Ag gene expression was tested at a later time point than in our study, suggesting that the decreased expression observed most likely reflected the late thyrocyte necrotic cell death associated with IFN-α exposure.
A number of cytokines were increased in the TG mice thyroids and the cultured human thyrocytes. CXCL10 levels were in particular markedly increased consistent with data showing that blood levels of this cytokine are not only in high circulating concentrations in autoimmune thyroiditis (42), but also correlate with a greater probability of thyroid dysfunction in IFN-α–treated HCV patients (39). More recently, Antonelli et al. (53), similar to our studies, have shown that IFN-α markedly induces CXCL10 in cultured human thyrocytes. CXCL10 is associated with a number of conditions characterized by the recruitment of cytotoxic lymphocytes such as cutaneous lupus erythematosis (54) and regression of melanoma (55). Another relevant cytokine pathway that was increased in the TG mice was the IL-6 pathway, a pathway that has been shown to play a role in autoimmune thyroiditis (16, 56).

Of particular interest was the finding of a marked induction by IFN-α of the granzyme B pathway in the TG thyroids. Granzyme B is a cytotoxic T cell marker, and is a part of a well-established pathway used by killer lymphocytes to destroy target cells (57). Typically, granzyme B is thought to be injected into target cells from recruited cytotoxic T cells via perforin channels formed on the cell membrane. Apoptosis rapidly ensues (58). Moreover, recent data suggest that granzyme B can degrade target cell proteins; as a result, pathogenic peptides released during the degradation can augment the autoimmune response (59). This mechanism of autoimmune tissue destruction has been confirmed to operate in several well-characterized autoimmune diseases in which pathogenic peptides are generated, including β cell destruction in type 1 diabetes (60), flares of SLE (61), and multiple sclerosis (60). Our results suggest that in vivo exposure of the thyroid to increased IFN-α, as a result of infection or IFN therapy, may induce locally high levels of granzyme B derived from cytotoxic T cells. The high levels of granzyme B may generate pathogenic thyroid peptides that can trigger and/or augment a destructive autoimmune response.

In the TG mice and cultured human thyrocytes exposed to IFN-α, we observed cell death without evidence for apoptosis or autophagy and, therefore, by exclusion, it is likely to be by necrosis. Cell death can occur by at least three primary mechanisms, as follows: apoptosis, autophagy, and necrosis (62). Apoptosis is a programmed cell death triggered by specific receptors and
signaling pathways (63), whereas autophagy is a process of sequestration of cytoplasmic constituents including organelles that leads to their eventual degradation in lysosomes and cell death (27). Death by necrosis does not have a uniform definition and is often identified as the cause of cell death by excluding apoptosis and autophagy. Although the dogma has been that necrosis is a nonprogrammed uncontrolled relatively end-stage form of cell death, there is growing evidence for the regulation of cell necrosis by discrete signaling pathways (64). In the TG mice, the thyrocyte necrosis might have been induced by cytotoxic T cells; however, our findings in thyrocytes in culture suggested that IFN-α also induced thyroid cell death by direct toxicity.

The downregulation of the arachidonic acid and fatty acid metabolism pathways is not as clearly linked to thyroid inflammation. This was observed dramatically, but only in the TG thyroid tissue. Whether these changes are epiphenomena or directly involved in the thyroid pathology is not clear, but could represent decreased synthesis of membranes in the cells affected by the inflammatory damage.

Interestingly, thyroid tissue destruction increased when the high-expressing TG lines were backcrossed from a predominantly C57BL/6 background to an autoimmunity-susceptible CBA/J background. This thyroid destruction, possibly coupled with other harmful inflammatory changes in surrounding tissues, prevented the establishment of high-expressing lines on the CBA/J-susceptible background and suggested a strong genetic susceptibility to the thyroid toxic effects of IFN-α. This notion is supported by the observations that the presence of thyroid Abs, considered a preclinical marker of genetic susceptibility to AITD (65), prior to the initiation of IFN-α therapy, is a significant risk factor for the development of AITD during IFN-α treatment (20). Moreover, we have shown that injecting IFN-α to NOD H2h4 mice, a strain genetically susceptible to spontaneous autoimmune thyroiditis, caused a higher frequency of autoimmune thyroiditis, supporting the notion that IFN-α triggers thyroiditis in genetically susceptible individuals (66). Finally, one study by our group showed an association between several immune-regulatory genes and IIT (12).

Our results (Supplemental Table 1) demonstrated a broad induction of the TRIM family of proteins. There are >64 members of the TRIM family in mice (67), and, although primarily described in the context of the antiviral responses of IFNs (68), their functions have been increasingly linked to an extensive range of
biological actions, including innate immunity. A subset consisting of five members of the TRIM family was found to be increased by RNAseq in the TG thyroids. Of particular interest was TRIM21 [also known as Ro52 (69)], a nuclear protein that binds to and regulates the function of IgG, and is a target autoantigen in several autoimmune diseases, most notably Sjogren’s syndrome and SLE. Moreover, genetic polymorphisms of TRIM21/Ro52 have been associated with the onset of Sjogren’s (70) and SLE (71). More recently, TRIM/Ro52/ mice were shown to develop evidence for SLE that could be reversed by disrupting the IL-23/IL-17 pathway, thought to mediate some of the inflammatory changes in SLE (72). Our studies showed a dose- and sustained time-dependent increase of TRM21 in response to IFN-α in cultured human thyrocytes and a greater expression in the higher IFN-α–expressing TG lines (Fig. 8).

Taken together, our data suggest that IFN-α induces tissue-specific autoimmunity by direct tissue toxic effects as well as by immune recruitment bystander mechanisms. The most notable

FIGURE 10. A proposed model for the induction of thyroiditis by IFN-α through direct actions on thyroid cells. The effects of IFN-α involve at least two primary processes, as follows. 1) The first pathway involves the induction and release of cytokines/chemokines that mediate the recruitment of immune cells that induce autoimmune cell death. This bystander mechanism can lead to the activation of resident T cells within the thyroid, initiating an autoimmune response. The induction of granzyme B in the thyroids from TG mice strongly suggested the activation of this well-described pathway of immune-mediated cell death, which can trigger thyroiditis. This process could be augmented in genetically susceptible individuals. Indeed, backcrossing the TG animals to the CBA/J thyroiditis-susceptible strain worsened the phenotype, suggesting genetic augmentation of this mechanism. 2) The second pathway involves a direct toxic action of IFN-α on thyroid cells. The observation of thyroid cell death in vitro and in vivo in the high-expressing lines strongly suggested direct thyroid toxicity independent of the recruitment of the peripheral immune system. The thyroid-specific effects we observed, upregulation of thyroid Ags, Ag presentation pathways, and innate immune response pathways, coupled with induction of thyroid cell death by IFN-α, could lead to a T cell response to the thyroid and development of thyroid autoimmunity.
components of IFN actions. Thyroid may help distinguish the beneficial versus destructive pathologic autoimmune phenomena demonstrates, as suggested by Akwa et al. (43), that IFN-α is a “two-edged sword” that provides protection from viral illness and yet can induce tissue injury. Further study of our model of overexpression of IFN-α in the thyroid may help distinguish the beneficial versus destructive components of IFN actions.

Acknowledgments

We thank Dr. Robert Franco and Mary Palacask (University of Cincinnati) for assisting with the PACS analyses, Rita Angel (University of Cincinnati) for performing the FACS analyses, and Drs. Roy Weiss and Samuel Reffetto (University of Chicago) for performing the TSH assays.

Disclosures

The authors have no financial conflicts of interest.

References


### SUPPLEMENTARY TABLES:

**Table I:** Pathway genes disregulated in thyroid glands of transgenic mice over-expressing interferon alpha in the thyroid

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**Fatty acid metabolism**

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**Other interferon-related genes (TRIM genes)**

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**Table II:** Confirmation by QPCR of genes shown to be upregulated in TG thyroids by RNAseq expression analysis.

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<td>Gzmb</td>
<td>granzyme B</td>
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<td>Il18bp</td>
<td>interleukin 18 binding protein</td>
<td>3.141</td>
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<td>Il6ra</td>
<td>interleukin 6 receptor, alpha</td>
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Table III: Primers used for SYBR Green Q-PCR experiments.

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