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*J Immunol* 2012; 189:3043-3053; Prepublished online 10 August 2012; doi: 10.4049/jimmunol.1200311

http://www.jimmunol.org/content/189/6/3043

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Genetically Driven Target Tissue Overexpression of CD40: A Novel Mechanism in Autoimmune Disease

Amanda K. Huber,* Fred D. Finkelman,† Cheuk Wun Li,* Erlinda Concepcion,* Eric Smith,‡ Eric Jacobson,‡ Rauf Latif,§ Mehdi Keddache,¶ Weijia Zhang,* and Yaron Tomer*‡§

The CD40 gene, an important immune regulatory gene, is also expressed and functional on nonmyeloid-derived cells, many of which are targets for tissue-specific autoimmune diseases, including β cells in type 1 diabetes, intestinal epithelial cells in Crohn’s disease, and thyroid follicular cells in Graves’ disease (GD). Whether target tissue CD40 expression plays a role in autoimmune disease etiology has yet to be determined. In this study, we show that target tissue overexpression of CD40 plays a key role in the etiology of autoimmunity. Using a murine model of GD, we demonstrated that thyroidal CD40 overexpression augmented the production of thyroid-specific Abs, resulting in more severe experimental autoimmune GD (EAGD), whereas deletion of thyroidal CD40 suppressed disease. Using transcriptome and immune–pathway analyses, we showed that in both EAGD mouse thyroids and human primary thyrocytes, CD40 mediates this effect by activating downstream cytokines and chemokines, most notably IL-6. To translate these findings into therapy, we blocked IL-6 during EAGD induction in the setting of thyroidal CD40 overexpression and showed decreased levels of thyroid stimulating hormone receptor-stimulating Abs and frequency of disease. We conclude that target tissue overexpression of CD40 plays a key role in the etiology of organ-specific autoimmune disease. The Journal of Immunology, 2012, 189: 3043–3053.

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d40, a member of the TNFR superfamily, is genetically associated with multiple autoimmune diseases including Graves’ disease (GD) (1), rheumatoid arthritis (RA) (2), multiple sclerosis (MS) (3), asthma (4), Crohn’s disease (5), and systemic lupus erythematosus (6). In GD, the CC genotype of a C/T single nucleotide polymorphism (SNP) in CD40 at the −1 position of the Kozak sequence is strongly associated with disease, increasing the risk for GD by 30–80% (7). Functionally, it has been shown that the CC genotype induces a 15–32% increase in CD40 protein expression (8). Moreover, the association is significantly stronger in a subset of GD patients having high titers of thyroid-specific Abs (i.e., anti-thyroid stimulating hormone receptor [TSHR], anti-thyroglobulin [Tg], and/or anti-thyroid peroxidase [TPO]) (8–10).

Although the CD40 gene is a general autoimmunity gene, it is unique among autoimmunity genes because it is expressed and functional in many nonimmunie tissues, where it has been shown to contribute to nonspecific inflammatory responses (11–16). Interestingly, many of the tissues that express CD40 are themselves targets for various tissue-specific autoimmune conditions (17–21), including thyroid follicular cells, the target of the autoimmune thyroid disease GD (15, 22). However, whether thyroid-specific CD40 expression plays a role in GD etiology has yet to be determined. The aim of this study was to test the hypothesis that thyroid-specific expression of CD40 is critical to the development of autoimmunity using experimental autoimmune GD (EAGD) as a model.

Materials and Methods

Generation of TG-CD40 mice

Studies were approved by the University of Cincinnati and Mount Sinai School of Medicine institutional animal care and use committees. Mouse CD40 cDNA, obtained from Dr. D. Wagner (University of Colorado, Denver CO), was cloned into a pSG5 plasmid using BamHI and PacI sites, inserting CD40 downstream of the β-globin intron. The Stul/SallI fragment from pSG5/CD40, containing the β-globin intron and CD40, was then cloned into the EcoRI/SallI site of the pSKbTg plasmid downstream of the bovine Tg (bTg) promoter (obtained from Dr. J. Fagin, Memorial Sloan-Kettering Cancer Center, New York, NY) (see Ref. 23). This pSKbTg-CD40 construct was cut using Xbal and XhoI, and this fragment was microinjected into fertilized C57BL/6 mouse eggs, which were implanted into pseudopregnant female mice. The pups were confirmed by PCR and Southern blotting to have integrated the transgene. Litters were continued from the founders by crossing them with wild-type (WT) C57BL/6 mice.

Southern blotting

Genomic DNA from mouse tails was digested sequentially with SpeI and SalI. Digested DNA product was run on a 1% agarose gel. DNA was then transferred onto Hybond XL nylon membrane (GE Healthcare, Piscataway, NJ) and probed for CD40 using the SpeI- and SalI-digested pSKbTg plasmid fragment (containing the bTg promoter and CD40) that was radiolabeled. The expected size of the band was 3.8 kb.
PCR genotyping

Briefly, DNA was amplified using the following primer pairs: pSbKbTg plasmid-specific primers: forward primer, 5′-GGTGGACCCCTGAT-TGTTCTT-3′, and reverse primer, 5′-AGGGGCGGTTGGTATGCTC-3′; and the following primers for control gene TSH-B (to check for presence of genomic DNA): forward primer, 5′-TCTCTCAAGATGCTCTATAG-3′, and reverse primer, 5′-GTAATCTCAGCTGAAAGT-3′. PCR was performed in 20-μl reaction mixtures containing 0.3 μl genomic DNA, 2 μl of each primer (5 μM stock), 2 μl PCR buffer containing 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l MgCl2, 0.5 mmol/l genomic DNA, 2 μl of each primer (5 μM stock), 2 μl PCR buffer containing 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l MgCl2, 0.5 μl 2′-deoxyuracil, 5′-triphosphates (200 μmol/l dATP, dGTP, dTTP, and dCTP), and 1 μl REDTaq DNA polymerase (Sigma-Aldrich, St. Louis, MO). Reaction mixtures were heated to 95°C for 5 min and then cycled 35 times as follows: 30 s at 95°C, 30 s at 55°C, and 50 s at 72°C and finally one cycle for 50 s at 72°C before being cooled to 4°C. Products were run on a 1% agarose gel. A band of 336 bp (pSbKbTg plasmid-specific product) indicated that the transgene was expressed, and a band of 386 bp (TSH-B product) confirmed that DNA was present in the reaction.

F1 hybrid mice

TG-CD4O mice were generated on a C57BL/6 background. F1 hybrids (F1H) were used in all experiments (except the chimeric experiments). Therapeutically, the donor mouse of origin was a TG-CD40 C57BL/6 crossed with a WT BALB/c mate (CXB). These have been shown to be susceptible to EAGD with ~50% of immunized mice developing disease (24, 25).

Immunohistochemistry

Thyroid tissue was removed from both WT and CD40 transgenic (TG) mice and placed in OCT compound in plastic mold; these were immediately frozen with dry ice. Sections were cut 4 μm thick, placed on a slide, and fixed for 10 min in ice-cold acetone. Slides were blocked for 10 min with avidin block, for 10 min with biotin block, for 30 min with biotin A block, and for 10 min with biotin B block (all from Invitrogen, Carlsbad, CA). Anti-CD40 clone 3/23, BD Biosciences, San Jose, CA; primary Ab was added at 1:250 dilution in 2% mouse serum and incubated for 60 min. Rabbit anti-rat IgG secondary Ab, mouse adsorbed (Vector Laboratories, Burlingame, CA), was then added at a dilution of 1:250 for 10 min. Slides were incubated for 15 min in HRP–streptavidin (Invitrogen), followed by diaminobenzidine (DakoCytomation, Glostrup, Denmark) for 3 min, counterstained with hematoxylin (DakoCytomation) for 1 min, and then mounted. All rinses were with PBS/Tween. Sections were visualized using an Olympus BX51 microscope, digital image captured using a Diagnostic Instruments digital camera, Model 74 Slider, and image saved digitally using Spot Advanced Diagnostic Instruments software Windows (version 4.6).

EAGD induction

Mice were induced with EAGD according to the Nagayama model (26), with the modifications by Rapoport (27, 28). Adenoviral vector containing the β-subunit of the TSH receptor (AdTSHR-289) or the control LacZ (AdLacZ) cDNA, acquired from Dr. B. Rapoport (Cedars Sinai Medical Center, Los Angeles, CA), was propagated by Viracast (North Liberty, IA) to give stock solutions of 1.2 × 1010 particles/ml. Mice were injected i.m. with 5.0 × 107 particles in 50 μl 10% glycerol (in PBS) in the thigh muscle. A series of three i.m. injections were given at 3-wk intervals (Fig. 1A). Blood was drawn for T4 and TSHR Ab (TRAb) levels 1 wk after the second injection and at the time of sacrifice, 3 wk after the third injection. Criteria used to classify a mouse as having experimental autoimmune GD were the presence of higher than normal (mean ± 2 SD [SD] of the pooled control AdLacZ mice levels) TRAb and T4 levels. There was no significant increase in TRAb or T4 levels in WT and TG AdLacZ-immunized mice.

Anti–IL-6 treatment of EAGD

To test the effects of anti–IL-6 treatment on EAGD, we used a modified Nagayama model. Mice were immunized at days 0 and 21. Before the boost at day 21, mice were bled for a baseline TRAb and T4 measurement and then treated with 1 mg rat IgG1 anti-mouse IL-6 Ab (Ip7) or isotype control (GL113) (both purified by ammonium sulfate fractionation and cation exchange chromatography from ascites grown in pristane-primed athymic nude mice). Because the half-life of the Ab is ∼5–7 d, mice were retreated with Ab 5 d later, at day 26. In addition, mice were bled at days 26, 31, and at sacrifice on day 35 (Fig. 4A). Blood was used to determine TRAb and T4 levels.

Creation of chimeric mice

BALB/c and BALB/cBy mice used for chimera experiments were purchased from Taconic Farms (Hudson, NY), whereas the CD40 knockout (KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Four- to 8-wk-old BALB/cBy and CD40KO mice were given two doses of 475 rad of irradiation 3 h apart. Within 1 h after the second round of irradiation, mice were reconstituted with 5–6 × 106 cells of donor bone marrow (BM) recovered from the femurs and tibulas of 5- to 12-wk-old donor (BALB/c) mice. One donor mouse engrafts two to four recipient mice. Engraftment was confirmed by flow cytometry of mouse lymphocytes, checking for the expression of nonclassical MHC molecule Qa2 in the BALB/cBy mice and CD40 in the KO mice. Once engraftment was confirmed, mice were immunized to induce EAGD.

Quantitative PCR

RNA was extracted from mouse thyroids using the MELT total RNA isolation kit (Applied Biosystems/Ambron, Austin, TX). cDNA was synthesized by reverse transcription using the Superscript III First-Strand Synthesis kit (Invitrogen). Gene expression of CD40, IL-6, and TNF-α was assessed using TaqMan gene expression assays from Applied Biosystems (Foster City, CA).

Primary thyroid cell cultures

Studies were approved by University of Cincinnati and Mount Sinai School of Medicine institutional review boards. Normal human thyroids (healthy lobe in patients undergoing surgery for thyroid tumors) were obtained from University Hospital (University of Cincinnati Cancer Center Human Tissue Procurement Facility, Cincinnati, OH) and the Department of Pathology, Mount Sinai Medical Center (New York, NY). Tissue was minced and digested in a 50-ml Falcon tube with type II collagenase (Worthington Biochem, Lakewood, NJ) at a concentration of 5 mg/ml (units per vial between 18,000 and 20,000) for 3 h at 37°C. After 3 h, 10 ml medium (DMEM, 4.0 mM l-glutamine and sodium pyruvate, 10% FBS, and 1% antibiotic–antimycotic solution [all from HyClone/Thermo Fisher, Wal- tham, MA]) was added to the tube, and the product was strained through a 70-μm cell strainer (BD Falcon, San Jose, CA) into another 50-ml Falcon tube. The tube was centrifuged at 1000 rpm for 5 min. The supernatant was carefully removed from the pellet, and the pellet was washed with another 10 ml medium. Again, the tube was spun at 1000 rpm for 5 min. The medium was removed from the pellet. The pellet was suspended in 10 ml medium, and the cells were plated in a 25-cm2 flask (Corning) and placed in an incubator at 37°C with 5% CO2.

Cytokine analysis

Primary thyroid cell cultures were washed with 10 ml PBS after 24 h, and then, 10 ml medium (DMEM, 4.0 mM l-glutamine and sodium pyruvate, 10% FBS, 1% antibiotic–antimycotic solution [all from HyClone/Thermo Fisher]) was added to the flask. This was allowed to incubate at 37°C with 5% CO2 overnight. The next day, primary thyroid cells were washed with PBS and plated evenly in a 12-well plate. Cells were incubated 24 h to allow for adherence to the wells. Again, the cells were washed, and at this time, the cells were treated for 0–3 d with 1 μg anti–CD40–stimulating Ab (G28.5). At the end of 3 d, the medium was removed from the cells and used to analyze the presence of cytokines IL-6, IL-8, TNF-α, IL-12, IL-1, and IFN-γ. Cytokines were assayed using a Millipore multiplex bead based array (Millipore, Billerica, MA) as described by the manufacturer. Assays were read on a bio-plex reader (Bio-Rad, Hercules, CA), which detects individual beads by flow cytometry. Standards were run in parallel to samples to determine cytokine concentration.

T4 assay

T4 was measured using a radioimmunoassay kit for neonatal T4 (Coat-a-Count/Siemens). Blood samples from mice were collected as a blood drop on filter paper. Assays were performed according to the manufacturer’s protocol and analyzed using a gamma counter. Calibrators run simultaneously with mouse serum samples were used to create a standard curve that was used to determine sample concentrations.

TRAb measurements

A commercial radioimmunoassay kit (Kronus, Star, ID) was used to measure TRAb levels in mice. Serum was collected by allowing whole mouse blood to clot and then centrifuging to separate. An assay was performed according to the manufacturer’s protocol and analyzed using a gamma counter. Calibrators run simultaneously with mouse serum samples...
were used to create a standard curve that was used to determine sample concentrations.

**Thyroid transcriptome analysis in CD40 transgenic thyroids and in CD40-stimulated primary human thyroid cells.** High-throughput next-generation sequencing for mRNA quantitation. Human thyroid primary cells were grown and treated for 1–3 d with 1 μg stimulating anti-CD40 Ab (G28.5) as described above. Total RNA was purified from cells of thyroids using TRIzol and was then DNase treated. For the analysis of mouse thyroid tissues, we collected thyroids at the time of sacrifice from WT non-EAGD, WT EAGD, transgenic non-EAGD, and transgenic EAGD mice either 5 or 9 wk after the initiation of TSHR immunization. Because of constraints related to cost and amount of RNA needed for RNAseq, extracted thyroid RNA was pooled (WT non-EAGD, n = 3; WT EAGD, n = 4; TG non-EAGD, n = 6; and TG EAGD, n = 6).

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 per 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 adaptors. The amount of dsDNA in each library was accurately quantified by spectrofluorometric analysis using the Qbit system from Invitrogen and diluted to a 10 nM concentration. Next-generation sequencing was performed on an Illumina Genome Analyzer IIX according to the manufacturer’s recommendations using the Illumina Single-Read Cluster Generation Kit version 2 and the SBS Sequencing Kit version 3. Image analysis and base calling was conducted in real time by the SDS 2.5/RTA1.5 software. The reads with good quality were aligned to 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 alignment files in SAM format were generated. After filtering reads that mapped to contamination databases, the reads that were uniquely aligned to each exon and splicing–junction sites were extracted and counted. The count for each refseq transcript was calculated by combining the counts for exons and splicing junctions of corresponding transcript normalized to relative abundance in fragments per kilobase of exon model per million to compare transcription levels 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 number 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. Differentially expressed transcripts were identified using M-A based random sampling method implemented in DEGseq 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 the 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 log2Ratio and then imported into the IPA tool 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.

**cAMP measurement using CRE luciferase.** A total of 30,000 cells/well of stable HEK-TSHR cells, which express the TSHR on the surface, were seeded into a 96-well plate in media (DMEM, 4.0 mM L-glutamine and sodium pyruvate, 10% FBS, and 1% antibiotic antimitotic solution (all from Hyclone/Thermo Fisher Waltham, MA]) and incubated at 37°C with 5% CO2. The following day, the cells were transfected using 0.3 μg PG14.29[luc2P/CRE/Hygro] (a gift from Dr. F. Fan, Promega, Madison, WI) per well using Xfectamine (BD Clontech, Mountain View, CA) as per the manufacturer’s protocol. After 4 h at 37°C, the transfection medium was washed and replaced by 200 μL complete DMEM and incubated for 48 h prior to testing the samples. Mouse IgG was purified from pooled serum samples of WT isotype-treated controls, WT, anti-IL-6-treated mice, TG isotype-treated controls, and TG anti-IL-6-treated mice, using a melon gel IgG spin purification kit (Thermo Scientific, Rockford, IL) as per the manufacturer’s protocol. IgG samples were quantified and diluted in media (DMEM, 4.0 mM L-glutamine and sodium pyruvate, 10% FBS, and 1% antibiotic anti-mycotic solution (all from Hyclone/Thermo Fisher Waltham, MA]) to a concentration of 10 μg/mL. HEK-TSHR cells, described above, were treated with 70 μL purified mouse IgG and allowed to incubate at 37°C with 5% CO2. After 5 h, 70 μL Bright Glo luciferase substrate (Promega) was added to each well. To lyse the cells, the plate was shaken for 2 min and immediately read using the FLUOstar Omega (BMG Labtech, Cary, NC).

**Mouse Ig isotyping.** Serum was collected by allowing whole mouse blood to clot and then centrifuged to separate. Mouse Ig isotypes were determined using a Milipore multiplex bead based array (Millipore Billerica, MA) as described by the manufacturer. Assays were read on a bio-plex reader (Bio-Rad Hercules, CA), which detects individual beads by flow cytometry. Standards were run in parallel to samples to determine the concentration of each Ig isotype per sample.

**Statistical analysis and power calculations.** The severity of EAGD was analyzed using a one-tailed Student t test, because our hypothesis was that TG mice would have more severe disease (i.e., higher T4 and TRAb levels). Frequency of EAGD was compared between TG and WT mice using the nonparametric χ2 test. A p value < 0.05 was considered statistically significant for both tests.

**Results**

**Generation of CD40 transgenic mice.** To test the effects of thyroidal CD40 expression on the development of EAGD, we overexpressed CD40 in the thyroid of mice, using CD40 cDNA placed under the control of the bTg promoter, as described in Materials and Methods. This promoter has been shown to specifically target transgene expression to the thyroid in mice (23). The final plasmid, designated bTg-CD40 (Supplemental Fig. 1A), was confirmed by digestion and sequencing.

The bTg-CD40 construct was microinjected into fertilized C57BL/6 mouse eggs, which were implanted into pseudo-pregnant female mice. Founders and positive offspring were screened by PCR, using primers targeting the rabbit intron of the construct, which is expressed only in the transgene insert (Supplemental Fig. 1B). Expression was confirmed by Southern blotting (Supplemental Fig. 1C), using a probe specific to the entire insert. High levels of CD40 expression in the thyroid was confirmed both by quantitative RT-PCR (Supplemental Fig. 1D), as well as by immunohistochemistry (Supplemental Fig. 1E) of thyroid sections from mice. From here on, mice overexpressing CD40 in the thyroid are referred to as TG mice (not to be confused with the abbreviation for thyroglobulin, Tg). TG mice did not show any phenotype when followed for up to a year.

**Thyroidal CD40 overexpression and EAGD.** Transgenic mice overexpressing CD40 in the thyroid (TG) were generated in a C57BL/6 mouse background. This background is resistant to EAGD; however, crossing these mice with BALB/c for one generation (F1H or CxB) makes these F1H mice susceptible to EAGD, with 50–65% of mice developing disease (24, 27). All EAGD experiments performed in this study used F1H transgenic CD40 (TG) and WT F1H littermates. EAGD was induced as described previously (Fig. 1A) (26–28).

**Total autoantibody levels.** Because CD40 has been implicated in the development of thyroid-specific Abs in Graves’ patients (9, 10), we first analyzed the effects of CD40 overexpression on the development of TSHR binding Abs (TRAb) in all immunized mice. There was no difference in the incidence of TRAb in TG and WT mice immunized with adenosinergic vector containing the A-subunit of the TSH receptor (AdTSHR-289; see Materials and Methods)
of WT and TG mice that developed EAGD (WT, n = 27; TG, n = 37). TG mice had higher levels of TRAb compared with WT EAGD mice, which correlated with a more severe disease in TG mice (p = 0.004) (Fig. 1C). In addition, there was a significant increase in thyroid hormone (T4) levels in TG mice with EAGD compared with WT littermates with EAGD (p = 0.005) (Fig. 1D), indicating that TG mice produced more stimulating Abs than WT mice. These data demonstrated that thyroidal CD40 expression not only augmented TRAb levels but also that these Abs were pathogenic in nature, resulting in increased thyroid hormone release (i.e., more severe disease). These data are consistent with our previous findings that CD40 is associated with pathogenic thyroid-specific Abs (10).

### Frequency of EAGD

We analyzed whether the increase in serum levels of TRAb resulted in an increased frequency of disease in TG mice. Interestingly, there was no significant difference in the frequency of disease between TG mice (52.8%) and WT littermates (62.96%; p = 0.32) (Table I), suggesting that tissue-specific expression of CD40 is not required to trigger clinical disease. However, in view of the higher TRAb levels in immunized TG mice, we tested whether severity of disease differed between TG and WT mice.

### Severity of disease

TG mice that developed EAGD had significantly higher titers of TRAb in their serum compared with WT mice with EAGD (p = 0.004) (Fig. 1C). In addition, there was a significant increase in thyroid hormone (T4) levels in TG mice with EAGD compared with WT littermates with EAGD (p = 0.005) (Fig. 1D), indicating that TG mice produced more stimulating Abs than WT mice. These data demonstrated that thyroidal CD40 expression not only augmented TRAb levels but also that these Abs were pathogenic in nature, resulting in increased thyroid hormone release (i.e., more severe disease). These data are consistent with our previous findings that CD40 is associated with pathogenic thyroid-specific Abs (10).

### EAGD in mice lacking thyroidal CD40

So far, we have shown that thyroidal CD40 plays a role in autoantibody production and severity of GD. However, our experiments did not test whether thyroidal CD40 expression is necessary for the development of GD in mice. To investigate whether thyroidal CD40 expression is necessary for disease development, we knocked out thyroidal CD40 expression using a chimeric approach. We lethally irradiated CD40KO mice and reconstituted them with WT BALB/c BM to generate mice with normal expression of CD40 in marrow-derived cells, and no CD40 expression in the thyroid, as well as in other non–marrow-derived tissues. It should be noted that our chimeric mice did not have a deletion of CD40 only in the thyroid because CD40 was not expressed in all non–BM-derived cells. However, deleting CD40 in all non–BM-derived cells was actually advantageous to our model. CD40 is expressed in endothelial cells and the thyroid is highly vascular. Therefore, KO of CD40 only in thyroid follicular cells might have still allowed the endothelial CD40 to have a significant effect on triggering EAGD. By deleting CD40 in all non–BM-derived tissues, we ensured that CD40 expression in endothelial and other non–follicular cells within the thyroid did not mask this effect (note that from here on we refer to the chimeric mice with deletion of CD40 in all non-BM cells as chimeric-KO mice). Because the CD40KO mice were on a BALB/cBy background, the controls for these experiments were WT BALB/cBy mice reconstituted with BALB/c BM. Experiments performed by us have shown that BALB/c and BALB/cBy mice are both susceptible to EAGD with a similar frequency of disease (Supplemental Fig. 2). Reconstitution of control and KO mice was confirmed by flow cytometry of mouse lymphocytes. For WT mice, the presence of a nonclassical MHC molecule Qa2, found in

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**Table I. Number of TSHR Abs-positive mice and frequency of EAGD after immunization with adenovirus–TSHR**

<table>
<thead>
<tr>
<th>Group</th>
<th>Ab-Positive Mice (%)</th>
<th>Frequency of EAGD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (n = 27)</td>
<td>27 (100%)</td>
<td>17/27 (62.96)</td>
</tr>
<tr>
<td>TG (n = 37)</td>
<td>36 (97.3%)</td>
<td>19/37 (52.8)</td>
</tr>
<tr>
<td>WT Reconstituted (n = 51)</td>
<td>48 (94.1%)</td>
<td>32/51 (62.7)</td>
</tr>
<tr>
<td>KO Reconstituted (n = 40)</td>
<td>21 (52.5)*</td>
<td>13/40 (32.5)**</td>
</tr>
</tbody>
</table>

*p = 1.6 × 10⁻³, **p = 4.0 × 10⁻³.*
BALB/c mice and not BALB/cBy mice, was used (Fig. 2A, top row), and in KO mice, the presence of CD40 was used, as markers of reconstituted cells (Fig. 2A, bottom row).

Autoantibody levels. EAGD was induced in 40 chimeric CD40KO (chimeric-KO) and 51 chimeric control mice (WT BALB/cBy mice reconstituted with BALB/c BM) by i.m. injection of AdTSHR-289. All mice were analyzed for autoantibody and T4 production. Although nearly all control mice immunized with AdTSHR-289 developed TRAb (94.1%), only 52.5% of chimeric-KO mice developed TRAb (p = 0.179). TRAb levels of WT and KO EAGD mice (WT, n = 32; KO, n = 13). TRAb levels were lower in KO compared with WT mice (586.813 and 625.056, respectively), but the difference was not significant (p = 0.398) (Fig. 2C). There was also no difference in the level of T4 in chimeric-KO and WT mice that developed EAGD. Error bars on all graphs represent SE. Dotted line represents the T4 level upper limit of normal.

Frequency of GD. There was a significant decrease in the incidence of EAGD in thyroid chimeric-KO mice compared with control chimeric mice (13 of 40, 32.5% in chimeric KO, versus 32 of 51, 62.7% in WT mice; p = 0.004) (Table I). This demonstrates that expression of CD40 by non–BM-derived cells (including thyrocytes) enhances but is not absolutely required for the development of EAGD.

Severity of disease. Disease severity was determined by TRAb and T4 levels in the serum. In mice that developed EAGD, mean levels of TRAb were lower in chimeric-KO mice (438.3 U/l) compared with control chimeric mice (625.056 U/l). However, this difference was not statistically significant (p = 0.179) (Fig. 2C). There was also no difference in the level of T4 in chimeric-KO and WT mice (Fig. 2D), demonstrating that even though the...
chimeric-KO mice had a significant decrease in disease frequency, those mice that developed disease had similar disease severity to the WT mice.

**Effect of thyroidal CD40 expression on thyroid transcriptome**

To elucidate potential mechanisms by which thyroidal CD40 overexpression causes a more severe disease in EAGD mice, we used a discovery approach and performed a transcriptome analysis comparing the entire thyroid transcriptome in TG and WT mice with and without EAGD at acute stage of disease (5 wk after initiation) and chronic stage of disease (9 wk after initiation). EAGD and non-EAGD cohorts from TG and WT mice were sacrificed, and their thyroids were collected. RNA was isolated, and the transcriptome was analyzed by RNAseq (see Materials and Methods). IPA was performed to examine pathways associated with EAGD in general and those specific to the more severe EAGD observed when there is thyroidal CD40 overexpression (Table II). Bioinformatic pathway analysis of the RNAseq data was performed by the IPA program (Ingenuity Systems, Redwood City, CA), and the data are available for viewing using Gene Expression Omnibus accession number GSE39081 at the Web site http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39081.

In WT mice with EAGD at acute stage of disease, upregulated pathways included those associated with nonspecific inflammation including those found in the oxidative stress response pathway ($p = 5.62 \times 10^{-5}$), complement pathway ($p = 2.04 \times 10^{-3}$), and IL-10 signaling pathway ($p = 6.6 \times 10^{-5}$). This differed in WT chronic EAGD, where upregulated pathways were associated with the adaptive immune response. These included dendritic cell maturation ($p = 1.51 \times 10^{-5}$), IL-8 signaling ($p = 0.004$), CD28 signaling in Th cells ($p = 0.002$), and Ag presentation pathways ($p = 0.005$). These findings are consistent with a shift from nonspecific inflammation to adaptive immune response activation as the disease progresses. Next, to examine the difference in disease development between TG and WT mice, we compared acute EAGD in TG mice (where CD40 was overexpressed on thyroid follicular cells) to acute EAGD in WT mice. Two interesting pathways were significantly upregulated, IGF-1 and IL-6 signaling pathways (Table II). In chronic EAGD, TG versus WT, two immune pathways were upregulated, CXCR4 signaling ($p = 0.002$) and IL-1 signaling ($p = 0.03$), in TG mice compared with WT mice (Table II).

Interestingly, both in acute and chronic EAGD in TG and WT mice, the parathyroid hormone/vitamin D receptor pathways were upregulated. Although vitamin D has been shown to play many roles in the immune system and disease (reviewed in Refs. 29 and 30), we believe this could be an artifact from our mouse thyroid samples being slightly contaminated with parathyroid cells.

<table>
<thead>
<tr>
<th>Mice</th>
<th>$p$ Value</th>
<th>Genes in Pathway from RNAseq</th>
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<tr>
<td><strong>WT EAGD versus WT Ctrl</strong></td>
<td></td>
<td></td>
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<tr>
<td>5 wk</td>
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<td></td>
<td>Complement system</td>
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</tr>
<tr>
<td></td>
<td>VDR/RXR activation</td>
<td>$1.3 \times 10^{-3}$</td>
</tr>
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<td></td>
<td>IL-10 signaling</td>
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<tr>
<td>9 wk</td>
<td>Dendritic cell maturation</td>
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<tr>
<td></td>
<td>Complement system</td>
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<tr>
<td></td>
<td>IL-8 signaling</td>
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<td></td>
<td>VDR/RXR activation</td>
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<tr>
<td><strong>TG EAGD versus TG Ctrl</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 wk</td>
<td>Dendritic cell maturation</td>
<td>$2.7 \times 10^{-4}$</td>
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<td><strong>TG EAGD versus WT EAGD</strong></td>
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<td></td>
<td>VDR/RXR activation</td>
<td>$4.3 \times 10^{-2}$</td>
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To determine whether the transcriptome results in the TG mice reflected acute or chronic stimulation of CD40 on thyrocytes, we performed RNAseq and pathway analysis in human thyroid follicular cells exposed for 24 h to G28.5, a monoclonal CD40 stimulating Ab. Human thyroid follicular cells in primary cultures were exposed to G28.5 (1 μg) for 0 and 24 h, and RNA was isolated (see Materials and Methods). The samples for each time point were pooled. Not unexpectedly, this intervention, reflecting an acute stimulation of CD40, provoked a marked increase in the CD40 signaling pathway ($p = 6.16 \times 10^{-4}$). In addition, IPA showed other pathways that were significantly upregulated by acute stimulation of CD40 (Table III). Of interest to the pathogenesis of GD, CD40 stimulation in human primary cells triggered an increase in signaling pathways for the proinflammatory cytokines IL-17 ($p = 0.001$), IL-8 ($p = 0.01$), and IL-6 ($p = 0.02$). The IL-6 pathway is of particular interest, because it was also upregulated in the thyroids of TG mice with acute EAGD compared with WT mice with acute EAGD (Table II). In addition, the increase in transcripts for the cytokines IL-6, IL-8, and IL-1B indicate a role for thyroidal CD40 stimulation and communication between the innate and adaptive immune responses in the thyroid ($p = 0.017$) (Table III).

**Confirmation of transcriptome data: effects of thyroidal CD40 on cytokine/chemokine production by thyroid follicular cells**

Thyroid cells themselves are not capable of producing Abs. Thus, tissue-specific CD40 expression must be influencing TRAb production through other mechanisms. Because RNAseq analysis showed upregulation of IL-6 in thyroids of TG mice with acute EAGD compared with WT, we hypothesized that tissue-specific B cell responses are being activated through a bystander mechanism secondary to increased local inflammation in the thyroid triggered by IL-6. Increased thyroidal expression and stimulation of CD40 drives proinflammatory cytokine production and could augment this process.

**Analysis of cytokine expression in vivo in EAGD thyroids.** To confirm RNAseq data, mRNA was purified from AdTSHR- and AdLacZ-immunized TG and WT mice. Quantitative RT-PCR analysis showed that both TG and WT mice that developed EAGD had significantly increased IL-6 expression compared with

<table>
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<th>Pathway</th>
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<td>IL-6 signaling</td>
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<td>IL8, MAP3K14, IL1B, IL6</td>
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<tr>
<td>Communication between innate and adaptive immune cells</td>
<td>1.8 x 10^{-2}</td>
<td>IL8, IL1B, IL6, CSF2</td>
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</table>

**FIGURE 3.** Cytokine production in EAGD thyroid and human primary thyroid cultures. (A) Thyroidal IL-6 relative quantification in WT and transgenic (TG) control (AdLacZ controls), non–EAGD-immunized (healthy), and EAGD mice. There was an increase in thyroidal IL-6 expression between healthy and EAGD mice in both WT and TG mice ($p = 0.037$ and $p = 0.023$, respectively). In addition, there was a further increase in IL-6 expression in TG EAGD compared with WT EAGD mice ($p = 0.047$). Cytokine secretion in CD40 stimulated human primary thyroid cultures. (B) IL-6 production was increased compared with unstimulated controls after 1 d of treatment, but it did not reach statistical significance ($p = 0.054$); however, after 3 d, TNF-α secretion was significantly increased ($p = 0.007$).
CD40 IN THYROID AUTOIMMUNITY

CD40 augments EAGD via increased IL-6 secretion

We have shown in multiple ways that IL-6 is an important downstream molecule of CD40 signaling in EAGD. Therefore, we hypothesized that increased IL-6 production in CD40 transgenic mice played a role in the increased Ab production seen in these mice. To test this hypothesis, we treated immunized TG and WT mice with anti–IL-6 Ab and analyzed TRAb and T4 levels at various time points in these mice.

Treatment of mice with anti–IL-6. We induced EAGD as before and treated mice with either 1 mg anti–IL-6 blocking Ab or isotype control. The mice were sacrificed on day 35 (Fig. 4A). There was no difference in the production of TRAb over time in WT mice treated with anti–IL-6 Ab compared with isotype control (Fig. 4B, left panel). In addition anti–IL-6 treatment did not affect T4 levels in WT mice immunized with TSHR (Fig. 4B, right panel) In contrast, there was a significant delay in TRAb production in TG mice treated with anti–IL-6 Ab compared with mice treated with isotype controls (p = 0.04 on day 26) (Fig. 4C, left panel). Moreover, T4 levels in anti–IL-6-treated TG mice were significantly lower than in the isotype control-treated mice at days 31 and 35 (p = 0.033 and 0.041, respectively). In fact, the average T4 level in TG mice treated with anti–IL-6 did not rise above the upper limit of normal (Fig. 4C, right panel). This suggests that anti–IL-6 mAb specifically blocked the effects of thyroidal CD40 in promoting EAGD development. Overall, TG mice treated with anti–IL-6 had a significant decrease in the frequency of EAGD compared with WT isotype control, WT anti–IL-6, and TG isotype control-treated mice (Table IV).

cAMP bioassay of serum from anti–IL-6-treated mice. To confirm that anti–IL-6 treatment inhibited the formation of TSHR-stimulating IgG (TSI) in TG mice, we tested TSHR-stimulating IgG activity in their serum using a cAMP bioassay. HEK–TSHR cells transfected with a PGl4.29 [luc2P/CRE/Hygro] vector, containing a CRE that drives transcription of a luciferase reporter gene, were treated with purified mouse IgG

FIGURE 4. Effect of anti–IL-6 treatment on TSHR Abs in EAGD. (A) Modified Nagayama mouse model used to test IL-6 action in EAGD. (B) EAGD WT and transgenic (TG) mice were treated with either anti–IL-6 or isotype control Abs, and then, TRAb and T4 levels in each group of mice were analyzed. (B) TRAb and T4 levels in anti–IL-6 and isotype control-treated WT mice (isotype, n = 8; anti–IL-6, n = 8). There was no significant difference in TRAb or T4 levels in WT mice treated with anti–IL-6 compared with those treated with isotype control. (C) TRAb and T4 levels in anti–IL-6 and isotype control-treated TG mice (isotype, n = 7; anti–IL-6, n = 7). TG mice treated with anti–IL-6 had delayed Ab production compared with mice treated with isotype control (left panel). Although TRAb levels in anti–IL-6-treated TG mice eventually caught up to those of isotype-treated controls, there was no increase in T4 levels in these mice (right panel), suggesting that Abs produced by TG anti–IL-6-treated mice were blocking or neutralizing, rather than stimulating in nature. Stars, p < 0.05. Points where anti–IL-6 mice were significantly different from the isotype controls. (D) cAMP bioassay results. HEK–TSHR cells transfected with a PGl4.29 [luc2P/CRE/Hygro] vector, containing a CRE that drives transcription of a luciferase reporter gene, were treated with purified mouse IgG from TG mice treated with anti–IL-6 Abs or isotype control. TSI activity was decreased in anti–IL-6-treated TG mice (p = 0.02), which is concordant with the decrease seen in TRAb and T4 levels (C) Serum samples are pooled from five mice from each treatment group, at each time point. Samples were run in duplicate. Dotted lines represent the upper limit of normal of TRAb and T4 levels.
from TG mice treated with anti–IL-6 or isotype control Abs. cAMP is produced upon TSHR stimulation; therefore, luciferase output is an indicator of levels of TSHR-stimulating IgG present. The activity of TSI was significantly decreased in anti–IL-6-treated TG mice (p = 0.02) (Fig. 4D), concordant with the decrease seen in T4 levels in the previous experiment (Fig. 4C).

**Discussion**

In this study, we used a mouse model of GD (EAGD) to test the hypothesis that thyroidal overexpression of CD40, driven by a CD40 Kozak SNP, plays a role in the etiology of GD. However, in contrast to humans, where it is well documented that CD40 is expressed and functional on thyroid follicular cells, both in normal and Graves’ glands (16, 23), we found that WT mice express very low levels of CD40 in the thyroid. Therefore, to test this hypothesis, we created a transgenic mouse overexpressing CD40 in the thyroid (Supplemental Fig. 1). Our data demonstrated that thyroid-specific overexpression of CD40 plays a role in the pathogenesis of GD. TG mice had significantly higher levels of TSHR-stimulating Abs resulting in more severe thyrotoxicosis. This was a specific effect and not the result of global increase in Igs, because the levels of total Igs and their isotypes were not different between TG and WT mice (Supplemental Fig. 4).

Mechanistically, in thyroids from EAGD mice, IL-6 expression was found to be augmented in the setting of overexpression of CD40 in the thyroid, suggesting a role for IL-6 secretion, triggered by thyroidal CD40 stimulation, in the production of tissue-specific Abs. Indeed, IL-6 has been shown to play an important role in adaptive immunity, specifically in inducing B cell differentiation into Ab-producing plasma cells (31–33). To confirm the role of IL-6 in Ab production in EAGD, we blocked IL-6 in TG and WT EAGD mice and analyzed the production of thyroid-specific Abs. Although there was no difference in TRAb and T4 levels in WT mice treated with anti–IL-6 Ab, this treatment delayed production of TRAb in TG mice. Moreover, it appears that blocking IL-6 restricted the production of pathogenic (stimulating) TRAbs, because T4 levels in anti–IL-6-treated CD40-TG mice remained within the normal range, even though the titers of Abs eventually returned to the levels of isotype control-treated mice. Because this effect was seen only in the TG mice, it suggests that IL-6 plays a role in augmenting pathogenic TRAb production in EAGD in the setting of high levels of CD40 in the thyroid. Supporting this notion are the data showing that stimulation of CD40 on primary human thyroid cells resulted in the secretion of proinflammatory cytokines including IL-6.

The data presented in this study help to provide a novel mechanism for the association of CD40 with GD as well as other autoimmune diseases. Previously, we and others (9, 34–36) have shown an association of a SNP in the Kozak sequence of the CD40 gene with GD. The association was significantly stronger in a subset of Graves’ patients having high titers of thyroid-specific Abs, suggesting that CD40 played a role in the production of Abs that mediate GD (8–10). Functionally, the CC genotype (associated with disease) has been shown to cause increased CD40 protein expression (8). However, it was not clear whether increased CD40 expression on B cells, thyroid cells, or both, conferred susceptibility to GD. In this study, we show that thyroidal expression of CD40 plays an important role in disease etiology as overexpression of CD40 in the thyroid augmented disease and deletion of CD40 in non–BM-derived cells including thyroid cells attenuated disease. Because our chimeric-KO mice had CD40 deletion in all non–BM-derived cells and not only in thyroid cells, it is possible that CD40 expression in other non-BM cells in addition to thyroid cells may also contribute to disease etiology. Moreover, EAGD itself caused increased thyroidal expression of CD40 in WT mice, which can additionally help perpetuate the disease (Supplemental Fig. 3F).

Our findings showing a role of target tissue expression of CD40 in the etiology of GD may be relevant to other autoimmune diseases. Indeed, in addition to being expressed in the human thyroid (15, 22), CD40 has been shown to be expressed in many tissues.

**Figure 5.** Proposed mechanism of the function of CD40 in the pathogenesis of GD. During times of local inflammation (e.g., induced by excess iodine or infection), thyroidal CD40 stimulation results in local cytokine secretion and bystander activation of T and B cells and increased B cell tissue-specific responses, which leads to thyroid-specific Ab production. When other predisposing factors are present, either environmental or genetic, this autoimmune reaction can result in the production of pathogenic Abs to thyroid-specific Ag and the onset of clinical GD.
that are targets for other organ-specific autoimmune conditions such as β cells (17), the target in type 1 diabetes, spinal cord (37), tissue affected by MS, keratinocytes (19), cells affected in psoriasis, colon fibroblast and intestinal epithelial cells (20, 38), cells effected by inflammatory bowel disease, and synovial cells from RA patients (21). Moreover, genetic associations with the CD40 gene locus have been reported in conditions besides GD, including RA, MS, and asthma, suggesting that tissue-specific CD40 expression may also play a role in these autoimmune diseases. In MS, CD40 expression is increased in the spinal cord during acute relapses of disease, and deleting CD40 in the CNS compartment in experimental autoimmune encephalomyelitis has been shown to result in a less severe disease (39, 40), similar to our observation in mice lacking CD40 in the thyroid.

One of the major effects of CD40 stimulation is induction of cytokine secretion. Cytokine secretion after CD40 stimulation has been previously studied in target tissues of other autoimmune conditions. In the β cell, CD40 stimulation led to the secretion of IL-6, IL-8, MCP-1, and MIP-1β (41). On keratinocytes, stimulation of CD40 with sCD154 and IFN-γ resulted in the upregulation of cellular adhesion molecules, the antiapoptotic gene Bcl-x, IL-8, IL-6, TNF-1α as increased levels of adhesion molecules and IL-6, GM-CSF, MIP-1β in synovial fibroblasts, CD40 stimulation led to proliferation as well (42). Finally, autoimmune conditions, where target tissue CD40 has also been previously studied in target tissues of other autoimmune conditions.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1: Characterization of Thyroidal CD40 Over-expressing TG Mice.

(A) bTg-CD40 cDNA construct used to generate CD40 transgenic mice. (B) PCR genotyping results from CD40 transgenic and non-transgenic mice. Primers for the TSHβ gene were used as controls for DNA present in the reaction; this product was 383 bases long. CD40 positive mice produced a band 533bp. (C) Southern blot confirmation of the PCR results. Genomic DNA was digested with Spel and Sall, and run on an agarose gel. DNA was transferred and probed, and CD40 positive mice displayed a band at 3.8kb. (D) Quantitative PCR of CD40 expression levels from thyroids of wild type and CD40 transgenic mice. Transgenic mice over-expressed CD40 in the thyroid 7-23 times more than wild type mice. (E) Immunohistochemistry of wild type (left) and transgenic (right) thyroids. Thyroids from mice were stained with anti-CD40 followed by DAB. While WT mice showed minimal expression of CD40, both at mRNA and protein levels, TG mice had significant levels of CD40. Please note that CD40 transgene expression looks patchy, due to the angle of sectioning and moderate transgene expression.
Supplemental Figure 2: Comparison of EAGD in BALB/c vs. BALB/cBy mice.

To confirm the BALB/cBy strain of mice had similar disease susceptibility and parameters as BALB/c, EAGD was induced in each strain, and disease was analyzed. There was no difference between the two mouse strains in the frequency of mice that developed disease (6/8 (75%) BALB/c mice and 5/8 (62.5%) BALB/cBy mice (p>0.05)). Both BALB/c and BALB/cBy mice that were immunized were able to produce TRAb, with no differences in the levels produced between the two strains (avg. TRAb 1056.92 U/L in BALB/c and 1013.34 U/L in BALB/cBy; p=0.45) (right panel). Additionally, analysis of the T4 levels of BALB/c and BALB/cBy mice developing EAGD demonstrated that there was no significant difference in the level of T4 produced between the two strains (avg. 12.006 ug/dL in BALB/c and 14.443 ug/dL in BALB/cBy; p=0.26) (left panel). Dotted lines represent the upper limit of normal of TRAb and T4 levels.
**Supplemental Figure 3:** Macroscopic and microscopic features of EAGD. (A) Normal mouse thyroid showing two small lobes on both sides of the trachea. (B) Large goiter from mouse with EAGD. (C) H&E stain of thyroid section from EAGD mouse (X200) showing proliferation and invagination of thyroid follicular cells into the lumen of the follicle. (D-E) Higher magnification (X400) of normal follicular epithelium (D) and hyperplastic epithelium (E). It should be noted that the EAGD model is not a perfect model of Graves’ disease since no lymphocytic infiltration is seen in the thyroid, as observed in human GD (27). (F) CD40 mRNA levels in muscle, thyroid, and pancreas from EAGD and control (non-immunized or AdLacZ immunized) mice. There was no difference in CD40 mRNA expression levels in muscle and pancreas between EAGD and control mice. In contrast, CD40 expression was increased in thyroid tissues of EAGD mice albeit with borderline significance (p=0.07). This suggests a role for increased thyroidal expression of CD40 in augmenting and sustaining the high TRAb levels during the course of EAGD.
Supplemental Figure 4: IgG isotype levels in TG and WT mice immunized with AdTSHR or AdLacZ. IgG isotype levels were measured using the Millipore luminex kit according to the manufacturer instructions. As expected all immunized mice had significantly increased levels of IgG1 (A), IgG2a (B), and IgG2b (C) without any change in IgG3 (D) levels compared to non-immunized mice. These data show that both Th1 and Th2 responses are important in the EAGD model. However, there was no difference in Ig isotype levels between immunized WT and TG mice. This shows that the increased TSHR stimulating antibodies in the TG mice is a specific effect and not due to global increase in any Ig subclass.