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Involvement of T cell receptor Beta alterations in the development of otosclerosis linked to *OTSC2*

Running title: T cell receptor Beta alterations in otosclerosis

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Abstract

Otosclerosis is a common form of hearing loss, characterized by disordered bone remodeling. Within otosclerotic foci, several immunocompetent cells and immune modulating factors can be found. Different etiological theories involving the immune system have been suggested. However, a genetic component is clearly present. Seven autosomal dominant loci have been published, but none of the disease-causing genes has been identified. This study focused on the exploration of the second otosclerosis locus on chromosome 7q34-36 (*OTSC2*), holding the T cell receptor beta locus (*TRB* locus). A number of immunological analyses were carried out to seek differences related to T cell receptor Beta (TCRβ). A significantly lower TCRβ mRNA expression and percentage of blood circulating TCRαβ^{+} T cells was detected in *OTSC2* patients compared to controls and sporadic patients. Further analysis illustrated more significant disturbances in specific T cell subsets, including an increased CD28^{null} cell population, suggesting a disturbed T cell development and ageing in *OTSC2* patients. These disturbances could be associated with otosclerotic bone remodeling, given the known effects of immunocompetent cells on bone physiology. These data implicate the *TRB* locus as the causative gene in the *OTSC2* region and represent an important finding in the elucidation of the disease pathology.

Key words: otosclerosis, T cell receptor Beta, TCRB, CD28null cells, OTSC2, otic capsule

Introduction

Otosclerosis is a disorder of bone remodeling which may lead to impaired hearing. The disease exclusively affects the otic capsule, which is unique in its morphology and development. After development, growth, modeling, and remodeling are virtually absent in the otic capsule. In otosclerosis however, alternating stages of bone resorption, new bone formation and formation of new, dense, compact bone occur. This abnormal bone remodeling can lead to a fixation of the stapedial footplate and consequently a conductive hearing loss. This condition is called clinical otosclerosis and has a frequency of 0.3-0.4% in the European population.[1]

The etiology of the disease remains elusive, but genetic factors are certainly involved. Generally, otosclerosis is considered a complex disease involving different genes and environmental factors. Monogenic forms, caused by a single gene, also occur, but are rare. At this moment, 7 monogenic loci have been identified (*OTSC1-5, 7* and *8*),[2-8] but no disease-causing mutations could be found. For the complex form of otosclerosis, several association studies were performed to identify genes.[9-12]

Many different hypotheses for the etiology of otosclerosis have been proposed, including several theories involving a role for the immune system. It has been suggested that disease pathology can be induced by an auto-immune reaction to type II collagen present in embryonic cartilaginous remnants in the endochondral layer of the otic capsule. Elevated concentration of antibodies for type II collagen have been found in serum, otosclerotic foci and perilymph of patients.[13, 14] In addition, otosclerosis-like lesions were induced in rats by immunizing them with native type II collagen.[15] On the other hand, other studies could not confirm these observations or do not support this theory.[16-18] Furthermore, several studies describe a correlation of certain human leucocyte antigen (*HLA*) antigen/genes with

otosclerosis. This is of interest as the *HLA* system has a key role in the presentation of antigens and function of the adaptive immune system. In addition, certain *HLA* haplotypes have been associated with susceptibility to several autoimmune diseases.[19] However, conflicting data exist as the otosclerosis associated *HLA* genes vary in different studies[20, 21] or associations could not always be confirmed in other independent reports.[22]

Finally, the presence of various immunocompetent cells and immune modulating factors has been reported in and near active otosclerotic foci, suggesting a high local stimulation of the immune system and an inflammatory tissue reaction. Within otosclerotic lesions lymphocytes, macrophages, granulocytes, mast cells and plasma cells can be found. The great majority of lymphocytes present were T lymphocytes expressing T cell receptor alpha/beta (TCRαβ).[23, 24] Deposits of Ig (IgG, IgM and IgA) and complement C3 are present along the resorption lacunae in active otosclerotic lesions.[24, 25] Furthermore, TNFα mRNA expression and an increased production of IL-1α, IL-1β and IL-6 has been reported in otosclerotic bone.[26, 27] It has been suggested that this inflammatory process in otosclerosis is a response to persistent measles virus infection.[28]

Taken together, several observations support a role for an aberrant immune response in the development or progression of otosclerosis. However, it is still controversial whether these local immune responses are part of the true etiology or merely a consequence of disease.

In 2001, our group mapped a second locus for otosclerosis (*OTSC2*) to chromosome 7q34-36 in a large Belgian family.[3] The *OTSC2* region is located between markers D7S495 and D7S2426 and spans a region of about 11.4 Mb,[3] including the human T cell receptor beta locus (*TRB* locus). Given the central role of the TCR cluster in the immune system and given the presence of T cells in otosclerotic lesions, we hypothesized that defects in this gene might be associated with a potential immune pathogenesis underlying otosclerosis development in this family. Because of the large amount of coding elements and the considerable sequence

variation within this cluster it is an infeasible task to trace disease-causing mutations by means of DNA-sequencing techniques. For this reason, this study focused on the functional investigation of T cell receptor Beta (TCRβ) in the *OTSC2* linked family to potentially reveal a possible causative mechanism underlying the development of otosclerosis in the patients.

Material and methods

Study population

Peripheral blood was collected from 12 patients from the previously described Belgian family segregating otosclerosis linked to *OTSC2*.[3] Only patients with the linked haplotype were included in the study. In addition, sporadic otosclerosis patients with no known affected family members (assumed to represent the complex form of otosclerosis) were ascertained through the St-Augustinus Hospital, Antwerp, Belgium. In all patients, the clinical diagnosis of otosclerosis was verified by stapes microsurgery. Unrelated healthy control individuals matched for age, sex and ethnicity for both otosclerosis patients groups were recruited through the Centre of Medical Genetics (Antwerp, Belgium) and the Biomedical Research Institute (Diepenbeek, Belgium). The following exclusion criteria were used in the selection of all individuals: known autoimmune or immune-related diseases, known viral or bacterial infections during blood donation, vaccination 3 weeks prior to blood donation, immunosuppressive or immune modulating medication, and chemotherapy. This study took place with approval of the local ethical committees.

Refinement of the *OTSC2* region

For refinement of the *OTSC2* region, 10 additional microsatellite markers were selected. At the proximal site two newly developed markers, OTSCm1 and OTSCm2, were analyzed between marker D7S495 and D7S684, because D7S2560 is not informative [3]. At the distal

side, 8 new markers were analyzed between D7S2513 and D7S2426: D7S2473, D7S661, OTSC2m3, D7S794, D7S676, D7S498, D7S2511, D7S2442. Information for all markers was taken from The Genome Database (http://www.gdb.org/). Marker OTSC2m1-3 are not in these databases and were developed on the basis of the human genome sequence (NCBI, build 36.3) (Supplementary Table S1). Polymerase chain reaction (PCR) was carried out under standard conditions. PCR products were fluorescently labeled. Capillary electrophoresis and pattern visualization were performed using a DNA analyzer (Model 4200, Li-Cor Inc, Lincoln, USA) or an ABI3130XL automatic DNA sequencer (Applied Biosystems Inc., Foster City, USA).

Mutation and CNV analysis

The coding regions and intron-exon boundaries of the selected genes were amplified in patient and control individuals. Direct sequencing of the PCR product was performed on an ABI3100 or ABI3130XL sequencer (Applied Biosystems Inc., Foster City, USA). To trace possible disease-causing copy number variants (CNVs) in the region, a patient with the linked haplotype from the *OTSC2* family was genotyped using the Illumina Human CNV370 Quad Beadchip, according to the manufacturer's protocol. Fluorescent signals were imported into the BeadStudio software version 3.3 (Illumina Inc., San Diego, USA) and intensities were normalized against a reference panel of 120 HapMap samples. Illumina's copy number analysis algorithm cnvPartition 1.2.0 (Illumina Inc., San Diego, USA) was used to detect aberrations. In addition, the QuantiSNP and PennCNV CNV-detection algorithms were also used.[29, 30]

***TCRBV* repertoire analysis and CDR3 spectratyping**

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation (Histopaque, Sigma-Aldrich, Bornem, Belgium). Total RNA was extracted from cells using the High Pure total RNA isolation kit (Roche, Basel, Switzerland) and reversed-transcribed into first-strand cDNA via oligo dT primers (Reverse transcriptase system, Promega, Madison, WI, USA) according to the manufacturer's instructions. The *TCRBV* usage of PBMCs was evaluated by means of quantitative RT-PCR (Lightcycler 2.0, Roche, Basel, Switzerland) using a specific forward and reverse primer for each of 21 functional *TCRBV* subfamily genes as described previously.[31] *TCRBV* gene primers were adapted to the current nomenclature.[32] The FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland) was used for PCR amplicon detection. PCR mixtures contained cDNA (100ng), forward/reverse primers (1µM), $MgCl_2$ (4mM) and LightCycler FastStart DNA Master SYBR Green I (Roche, Basel, Switzerland). The amplification protocol consisted of an initial 10 minutes at 95ºC, followed by 45 cycles of repeated denaturation (95ºC), annealing (3 sec at 60ºC) and elongation (10 sec at 72ºC). Samples were analyzed by means of the Second derivative Maximum Method using LightCycler Software v3.5 (Roche, Basel, Switzerland). The expression of each *TCRBV* gene was calculated relatively to the sum of the expression levels of all *TCRBV* genes.

CDR3 spectratyping analysis was performed using a TCRβ gene clonality assay (*InVivo*Scribe Technologies) on genomic DNA samples. This assay includes a multiplex PCR assay to detect the vast majority of clonal TCRβ gene rearrangements. The multiplex mastermixes target conserved regions within the V-D-J regions that flank the unique hypervariable antigen-binding region 3 (CDR3).[33] The first mastermix contains 23Vb primers, 6 Jb1 and 3 Jb2 primers. The second mastermix contains 23 Vb and 4 Jb2 primers and the

third mastermix contains 2 Db and 13 Jb primers. Analysis of PCR products was performed on an ABI3100 automatic DNA sequencer (Applied Biosystems Inc., Foster City, USA).

TCRβ mRNA expression analysis

Total RNA was extracted from blood using the PAXgene^{tm} blood RNA System (PreAnalytiX, Hombrechtikon, Germany). DNAse treatment was performed with the DNA-free kit (Ambion Inc, Austin, USA). cDNA synthesis was done in duplicate with random hexamer primers using the Superscript™ III First-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). For quantitative real-time PCR, TaqMan Gene Expression Assays (ABI, Foster City, CA, USA) for TCRβ and CD3 and three housekeeping genes (*TBP*, *UBC* and *YWHAZ*) were used. The assay for TCRβ was developed to target a region that is identical in *TRBC1* and *TRBC2* (F-primer: 5-CCCAGGATAGGGCCAAACC-3; R-primer: 5-TCATAGAGGATGGTGGCAGACA-3; reporter sequence: CCACAGTCTGCTCTACCC). CD3 was also investigated as it is associated on the cell surface with TCRαβ or TCRγδ to form a functional TCR complex. The assay for CD3 was developed to recognize the epsilon chain which associates with both TCRαβ and TCRγδ (F-primer: GAGAGGCCACCACCTGTTC; R-primer: GTCTCTGATTCAGGCCAGAATACAG; reporter sequence: TTTCCGGATGGGCTCATAG). Mixtures consisted of 2µl cDNA, 5µl LightCycler® 480 Probes Master (2x, Roche, Basel, Switzerland), 0.5 µl TaqMan Assay and 2.5 µl water (PCR grade, Roche, Basel, Switzerland) and were run on a LightCycler® 480 Instrument (Roche, Basel, Switzerland). The cycling conditions were as follows: 10 min 95 °C and 40 cycles at 95 °C for 10 s and 60 °C for 1 min. Analysis was done using qBase Software v1.3.5.[34] Normalized values were calculated using stably expressed housekeeping genes.

Flow cytometric analysis

PBMCs were suspended in staining buffer (PBS, 2% FBS and 0.09% NaN_3) and incubated for 30 min (at 4°C) with Fluorescein isothiocyanate (FITC), phycoerythrin (PE) and/or peridinin chlorophyll protein (PerCP) conjugated mAbs. The following mAbs were used: anti-human CD3 (clone SK7), CD4 (clone SK3), CD8 (clone SK1), CD19 (clone 4G7), CD25 (clone 2A3), CD16/56 (clone B73.1/MY31), TCRαβ (clone R73), TCRγδ (clone 11F2), CD28 (clone CD28.2) (all from BD Biosciences), anti-humanTCRVα24 (clone C15) and TCRVβ11 (clone C21) (purchased from Beckman coulter, Analis, Suarlée, Belgium). For intracellular staining of FOXP3, cell surface staining was first completed and cells were subsequently fixed and permeabilized according to the FOXP3 staining buffer set protocol (eBioscience, San Diego, USA) before adding anti-human FOXP3 (clone PCH101, eBioscience) for 30 min at 4° C. Subsequently, cells were washed twice (400g, 5 min) and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Quadrants and cut-off boundaries were set based on IgG1/IgG2a control staining (BD Biosciences) or negative/positive control populations. Data acquisition and analysis were performed using CellQuest software (BD Biosciences). Lymphocyte populations were defined according to their phenotype: T cells ($CD3^+CD19^-$), B cells ($CD19^+CD3^-$) and NK cells ($CD16/CD56^+CD3^-$). T cells were subsequently subdivided according to specific lineage markers: $CD4^+$ T cells ($CD4^+CD8^-$), $CD8^+$ T cells ($CD4^-CD8^+$), double negative T cells ($CD4^-CD8^-$), double positive T cells ($CD4^+CD8^+$), TCRαβ T cells ($TCR\alpha\beta^+TCR\gamma\delta^-$), TCRγδ T cells ($TCR\alpha\beta^-TCR\gamma\delta^+$), activated T cells ($CD3^+CD25^{int}$), NKT cells ($TCRV\alpha24^+V\beta11^+$), Tregs ($CD4^+CD25^+FOXP3^+$) and CD28null T cells ($CD3^+CD28^-$ and $CD4^+CD28^-$).

Statistical analysis

Differences in mRNA expression and flow cytometric measurements between the three groups were tested using ANOVA. Subsequently, we traced which intergroup difference(s) contributed to the significance with a posthoc test using Bonferroni correction for multiple testing. All ANOVA models were constructed in a stepwise backwards way. First, a model was fitted with sex and age as covariates, to account for influences of age and sex on expression or lymphocyte frequency. If the covariates were not significant, meaning that there was no effect of age and sex, they were removed from the model. To test the fit of the ANOVA model, a one-sample Kolmogorov-Smirnov test and QQplot were performed to check normality of the residuals. In addition, a Levene's Test on the residuals was carried out to test equality of error variance. For both tests a cut-off of 0.001 was taken. Outcome variables for which the model check failed, were tested using a non-parametric Kruskal-Wallis test. All tests were performed using SPSS 15.0 (SPSS, Chicago, IL, USA). Figures were made with Prism software v4.0 (Graphpad, San Diego, CA, USA). Results are expressed as mean value ± standard error of the mean (SEM) unless otherwise indicated. For *OTSC2* patients, flow cytometric parameters were repeated at a different time point. A Pearson correlation coefficient was calculated to test reproducibility of these measurements.

Results

Refinement of the *OTSC2* region and mutation analysis

Genotyping of 10 additional microsatellite markers was used to refine the *OTSC2* candidate region. The haplotypes of the key recombinant persons are shown in figure 1. In patient III-16

a recombination occurs that localizes the otosclerosis-causing gene in this family distal of marker OTSC2m1. At the telomeric site a recombination occurs between marker D7S661 and OTSC2m3 in patient III:11. Consequently, the otosclerosis-causing gene is mapped to a candidate region of 5.6 Mb, flanked by the markers OTSC2m1 (centromeric) and OTSC2m3 (telomeric). This region contains a total of 193 genes/gene predictions, including the *TRB* locus with 93 genes. Other than the *TRB* locus, fourteen genes in the refined region were also candidate genes based on their function described in the literature. The coding regions and intron-exon boundaries of all 14 genes (*ATP6V0A4*, *CLEC5A*, *EPHA1*, *EPHB6*, *HIPK2*, *KLRG2*, *LUC7L2*, *MKRN1*, *PIP*, *PRSS2*, *SSBP1*, *TRIM24*, *TRPV5* and *TRPV6*) were subjected to DNA-sequencing. No disease-causing mutation was revealed. In addition, the complete *OTSC2* region was screened for CNVs using the Illumina Infinium SNP genotyping platform. Different detection algorithms were used, but no aberrations were detected in the region.

No disturbed *TCRBV* profile but a reduced TCRβ mRNA expression in *OTSC2* patients

To investigate whether an alteration in the *TRB* locus is associated with otosclerosis in the *OTSC2* linked family, a quantitative analysis of *TCRBV* gene usage was performed. The relative mRNA expression of *TCRBV* subfamily genes in PBMCs of 7 otosclerosis patients of the family and 10 age-matched random healthy controls is given in Supplementary Figure S1. In general, PBMCs of patients and controls showed a relatively broad *TCRBV* usage, although some restrictions could be found potentially associated with the high age of patients and controls.[35] Patients of the Belgian *OTSC2* family showed a *TCRBV* profile comparable to that of age-matched controls. Because no specific *TCRBV* profile could be found in *OTSC2* patients, *TCRBV* analyses were not performed for patients with the complex form of

otosclerosis. In addition, CDR3 spectratyping was performed on DNA samples of 4 *OTSC2* patients and 2 unaffected controls (one individual related by marriage and one familial control). Results were in line with the *TCRBV* repertoire analysis, as no specific profile was detected that was present only in *OTSC2* patients (data not shown).

Subsequently, a quantitative analysis of the total expression of the TCR beta chain (TCRβ) and CD3 mRNA in PBMCs was performed to investigate a possible effect on TCR expression. A total of 11 *OTSC2* patients, 13 patients with the complex form of otosclerosis and 26 control individuals were analyzed (Figure 2). A significant difference of TCRβ mRNA expression was found between the three groups ($p = 0.002$, ANOVA). Posthoc tests showed a significantly lower expression of TCRβ mRNA in *OTSC2* patients compared to controls and compared to patients with the complex form of otosclerosis ($p = 0.004$ and $p = 0.005$, respectively). No significant differences for TCRβ mRNA expression were noted between controls and patients with the complex form of otosclerosis ($p = 1.000$, posthoc). For *CD3* no significant differences were found between the 3 groups ($p = 0.058$, ANOVA).

Reduced frequency of blood circulating TCRαβ$^+$ cells in *OTSC2* patients

The reduced TCRβ expression in total PBMCs could be due to either a reduced number of TCRαβ positive T cells or a reduced expression of TCR at the cellular level. To distinguish between these possibilities, PBMC samples of 12 *OTSC2* patients, 12 complex patients and 18 healthy controls were analyzed for the expression of TCR by means of flow cytometry (Figure 3). No significant differences were observed in TCRβ protein expression per cell (mean fluorescence intensity, MFI) between otosclerosis patient groups and controls ($p = 0.561$, ANOVA, Figure 3A). However, a significantly reduced percentage of TCRαβ positive T cells was demonstrated in the *OTSC2* patients ($p = 0.019$, ANOVA) as compared to the

complex patients (p = 0.041, posthoc) and controls (p = 0.038, posthoc). These results indicate that the reduced expression of TCR mRNA in *OTSC2* patients is associated with a lower number of blood circulating TCRαβ^{+} T cells. In parallel with the reduced percentage of TCRαβ^{+} T cells, a significantly higher percentage of TCRγδ^{+} T cells was observed in *OTSC2* patients (p = 0.010, ANOVA) compared to controls (p = 0.033, posthoc) and complex patients (p = 0.015, posthoc) (Figure 3B).

Analysis of T cell subsets

To further evaluate the difference in TCRαβ positive T cells between *OTSC2* patients and controls, a detailed investigation of the frequency of different T cell subsets was performed by additional flow cytometric analyses of blood samples from the same subjects (Figure 4). First, no significant differences were noted regarding the percentages of T cells, NK cells or B cells (the major lymphocyte populations) between otosclerosis patients and controls (ANOVA p= 0.557; 0.413; 0.982, respectively, Figure 4A). Next, different T cell subsets were analyzed. When analyzing CD4 and CD8 expression on T cells, we detected a reduced percentage of CD4^{+}CD8^{-} T cells in *OTSC2* patients compared to controls, which just missed significance (p = 0.051, posthoc) in the first measurement, but did reach significance in the second measurement taken at another time point (p = 0.039, posthoc; Figure 4B). In addition, an increased percentage of double negative (CD4^{-}CD8^{-}) T cells was found in *OTSC2* patients as compared to controls (p = 0.039, posthoc; Figure 4B). T cells which had lost their CD28 expression (CD28^{null} T cells), i.e. senescent T cells, were present at a higher percentage in *OTSC2* patients compared to controls. The difference was significant for the total CD28^{null} T cells (p = 0.033, posthoc), but not for the CD4^{+}CD28^{null} subset alone (p = 0.117, posthoc; Figure 4C). Finally, no differences in the percentage of activated T cells (CD3^{+}CD25^{int}) could

be seen between the study groups ($CD4^+$: p = 0.100 and $CD8^+$: p = 0.657, ANOVA; Figure 4D). Percentages of specialized regulatory T cell subsets, $CD4^+CD25^+FOXP3^+$ T cells and NKT cells, were comparable between the three groups (ANOVA p = 0.373 and p = 0.478, respectively; Figure 4E).

For 10 *OTSC2* patients, flow cytometric analyses of 10 parameters were repeated at a separate time point. Statistical analysis revealed that the data correlated very well between both measurements (data not shown), indicating a high reproducibility of the results. Differences between *OSTC2* patients and controls were significant for the same parameters as in the first measurement (data not shown).

Discussion

Several hypotheses have been proposed that suggest a role for the immune system in the development of otosclerosis. Although the suggested theories remain controversial, the observations that immune cells are present in active otosclerotic foci, do point towards a role for the immune system in otosclerosis pathology. Here we present data that implicate the involvement of the *TRB* locus in the development of otosclerosis in the family linked to *OTSC2*.

The human TCR complex comprises integral membrane proteins with a fundamental role in the adaptive immune system, participating in the activation of T cells in response to antigens presented by HLA molecules on antigen presenting cells. For the majority of T cells, the TCR heterodimer consists of a TCR α and β chain, whereas only a small subset of T cells express the TCR γ and δ chain. As the *OTSC2* region on chromosome 7 contains the complete TCRβ locus, it was considered a major candidate for the development of otosclerosis in the *OTSC2* linked family. Genotyping extra microsatellite markers proximally and distally could reduce

the original *OTSC2* candidate region from 11.4 Mb to only 5.6 Mb, still including the *TRB* locus. DNA-sequencing of 14 other possible candidate genes in the region did not reveal a disease causing mutation in the exons or intron-exon boundaries. In addition, CNV-analysis did not reveal aberrations in the *OTSC2* region in the patient analyzed.

During the development of T cells in the thymus, an extensive somatic DNA recombination of variable and joining region segments of the α and β TCR gene segments takes place to guarantee a highly diverse TCR repertoire required for reactivity to a large arsenal of potential antigens. We found no significant changes in the *TCRBV* profile of peripheral blood T cells from *OTSC2* patients as compared to age-matched controls. In addition, CDR3 spectratyping analyses did not reveal a specific *TCRBV CDR3* length profile present only in *OTSC2* patients. These results are therefore not supportive for a selective expansion of specific *TCRBV* T cell subsets during T cell development in the thymus or as a consequence of an antigenic stimulation or depletion of T cell clones in the periphery as a result of a genetic defect at the *OTSC2* locus. However, as about 80% of the lymphocytes present in the otosclerotic footplate were shown to be T-lymphocytes,[23] it cannot be excluded that T cells within the otosclerotic lesions can have a biased TCR expression profile reactive towards tissue specific antigens.

To further investigate potential TCR alterations, global TCRβ mRNA expression was investigated in PBMCs. These analyses revealed a lower TCRβ expression in *OTSC2* patients, compared to controls and patients with the complex form of otosclerosis. Subsequent flow cytometric analysis demonstrated that this lower TCRβ expression in total PBMCs is due to a lower frequency of TCR$\alpha\beta^+$ T cells, as no difference in TCRβ protein expression per cell was found. In contrast, a higher number of TCR$\gamma\delta^+$ T cells was found in *OTSC2* patients. These findings could point towards a potential disturbance in the developmental phase/checkpoint that drives T-cells to mature into either TCRαβ or TCRγδ positive T cells. CD3 mRNA

expression in total PBMCs was similar, which is consistent with the fact that CD3 associates with both TCRαβ and TCRδγ.

As T cells can be classified into different subsets based on phenotypical and functional characteristics, it was of interest to further investigate the prevalence of these T cell subsets in *OTSC2* patients. Thymocyte precursor cells begin their differentiation and education process as ($CD3^+$)$CD4^-CD8^-$ or double negative (DN) cells and subsequently become double positive (DP) ($CD3^+$)$CD4^+CD8^+$ thymocytes.[36] Finally, after positive and negative selection these T cell precursor cells differentiate into single positive T cells ($CD3^+CD4^-CD8^+$ or $CD3^+CD4^+CD8^-$) and migrate to the periphery. In the peripheral blood of otosclerosis *OTSC2* patients, there was a significantly increased frequency of DN T cells and a decreased percentage of CD4 single positive T cells. This could suggest that alterations in T cell development occur in *OTSC2* patients resulting in an altered constitution of T cell subsets in the peripheral circulation. As TCRγδ$^+$ T cells show either a DN or $CD8^+$ phenotype, an increase in these cells as observed in our study, could also account for differences in $CD4^+$ and DN T cells.

Furthermore, several specialized T cell subsets, either regulatory or effector cells that play a role in autoimmune diseases, were analyzed. It is known that regulatory T cells actively downregulate the activation and expansion of self-reactive T cells.[37] Here, we focused on two important naturally occurring (thymic developing) regulatory T cells, i.e. natural killer T cells (NKT) and $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs). For various human autoimmune diseases, it was demonstrated that peripheral Treg and NKT cell function or frequencies are disturbed.[38] In contrast, in *OTSC2* patients we did not detect significant differences in the prevalence of these regulatory T cells. In addition, no differences were detected in activated T cells ($CD3^+CD25^{int}$).

Significant higher percentages of $CD28^{null}$ T cells were shown in *OTSC2* patients. CD28 is constitutively expressed on T cells, but its expression is transiently down-regulated following T cell activation and declines progressively with immune senescence.[39] $CD28^{null}$ T cells are clonally expanded cells, which are highly pro-inflammatory and express killer Ig-like receptors. An expanded population of $CD28^{null}$ T cells ($CD8^+$ or $CD4^+$) can be found in patients with autoimmune diseases.[40, 41] Because of their cytotoxic capacities and decreased susceptibility to immunoregulation, $CD28^{null}$ T cells might facilitate or sustain chronic autoreactive immune responses.[31]

Based on the location of the TCRβ gene cluster in the candidate *OTSC2* region, and the significant immune alterations that were found in $TCR\alpha\beta^+$ T cells and other specific T cell subsets, we suggest that this gene harbors the disease causing mutations in this family. However, it remains difficult to understand how such mutations may lead to the immune alterations and pathology of the disease. By release of soluble factors, T cells can communicate with bone cells and in this way alter bone remodeling.[42] Why there is a specific localized immune reaction in the otic capsule is unclear, but the otic capsule is a unique structure in a way that it retains calcified cartilage known as 'globuli interossei' throughout life, being the only site in the body where this occurs.[43] Striking is the increased frequency of $CD28^{null}$ T cells in *OTSC2* patients. $CD28^{null}$ T cells may have a direct pathologic role, but could also affect immune homeostasis and in this way promote the development of an autoimmune attack on, for instance, the unique embryonic cartilaginous remnants in the otic capsule. It is possible that in *OTSC2* patients, T cells reactive towards specific epitopes of the otic capsule could become activated by viral infections (through bystander activation or molecular mimicry) and mediate or sustain an immune response resulting in bone remodeling as seen in otosclerotic lesions.

Taken together, our data indicate that T cell development and ageing is disturbed or altered in *OTSC2* patients. Whether this could potentially reflect an ongoing autoimmune process in *OTSC2* patients is difficult to determine. It is well known that genetic factors play a role in the development of many autoimmune diseases. However, multiple cooperating events, including genetic factors and environmental factors (like perhaps measles virus infection), may be needed before a self-reactive clone bypasses sequential tolerance checkpoints resulting in the emergence of autoimmune disease.[44]

How specific mutations could affect T cell development or ageing is unclear. Possibly, mutations could affect the efficiency of VDJ recombination and in this way influence T cell development. TCR gene rearrangements can impact TCRαβ/γδ lineage choice during T cell development and the transition from DN to DP cells in the differentiation of TCRαβ$^+$ cells,[45, 46] which would explain our current observations. Changes in for instance activity of regulatory elements can affect TCRβ gene rearrangement.[47] In addition, changes in T cell development could also increase or accelerate replicative senescence of T cells in the periphery resulting in altered competence of the immune system and increased vulnerability for autoimmune responses.[48]

An important point in this study is that differences in T cell parameters found for *OTSC2* patients seem to be specific to this family, as they were generally not observed in patients with the complex form of otosclerosis. This implies that this is not a consequence of the otosclerosis disease pathology in se, but rather supports a causative role of the *TRB* locus in the pathology in this family.

In conclusion, we here present evidence that implicates the *TRB* locus as the causative gene in the *OTSC2* region. Although the precise mechanism is unclear, we propose that a genetic defect in the *TRB* locus leads to a disturbed T cell development and ageing, potentially influencing T cell reactivity towards unique structures within the otic capsule. Given the

effect of immunocompetent cells on bone homeostasis, these disturbances can lead to a disorganized bone remodeling as seen within otosclerotic lesions.

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Conflict of Interest

All authors have no conflict of interest.

Supplementary information is available at the Genes and Immunity's website.

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Figure Legends

Figure 1: Refinement of the *OTSC2* region by the analysis of additional genetic markers.

Markers in bold were analyzed previously. The disease causing-haplotype is indicated with a rectangle.

Figure 2: mRNA expression of CD3 and TCRβ in total PBMCs.

PAT: *OTSC2* patients (n = 11), HC: controls (n = 26), COM: patients with the complex form of otosclerosis (n = 13). Error bars indicate SEM.

**: $p<0.01$

Figure 3: TCRβ protein expression and frequency of TCRαβ and TCRδγ positive cells.

(A). TCRβ protein expression per cell (mean fluorescence intensity, MFI). (B) Percentage of TCRαβ and TCRδγ positive T cells. PAT: *OTSC2* patients (n = 12), HC: controls (n = 18), COM: patients with the complex form of otosclerosis (n = 12). Error bars indicate SEM. *: $p<0.05$

Figure 4: Analysis of T cell subsets.

(A)Frequency of T cells, B-cells and NKT cells within the PBMC population, (B) percentage of $CD4^{+,}$ $CD8^{+,}$ $CD4^{-}CD8^{-}$ and $CD4^{+}CD8^{+}$ cells of T cells, the $CD4^{+}$ measurement just missed significance ($p = 0.051$, posthoc) in the first measurement, but did reach significance in the second measurement ($p = 0.039$, posthoc) (C) percentage of $CD28^{null}$ (senescent) cells of total T cells (CD3) and $CD4^{+}$ T cells (CD4), (D) percentage of $CD25^{int}$ (activated) cells of $CD4^{+}$ and $CD8^{+}$ T cells, (E) percentage of $CD4^{+}CD25^{+}FOXP3^{+}$ T cells and natural killer T cells (NKT).

PAT: *OTSC2* patients (n = 12), HC: controls (n = 18), COM: patients with the complex form of otosclerosis (n = 12). Error bars indicate SEM. *: $p<0.05$

Figure legends supporting information

Supplementary Figure S1: TCRBV repertoire analysis in OTSC2 patients (PAT, n = 7) and age-matched healthy controls (HC, n = 10).

The expression of 21 TCRBV genes was determined by real-time PCR for PBMCs of (A) OTSC2 patients (PAT, n = 7) and (B) age-matched healthy controls (HC, n = 10). For each sample, the expression of each TCRBV gene was calculated relatively as compared to the sum of expression levels of all TCRBV genes. Data are shown as the mean TCRBV gene expression (± SEM) determined of two independent PCR analyses.

Figure 1

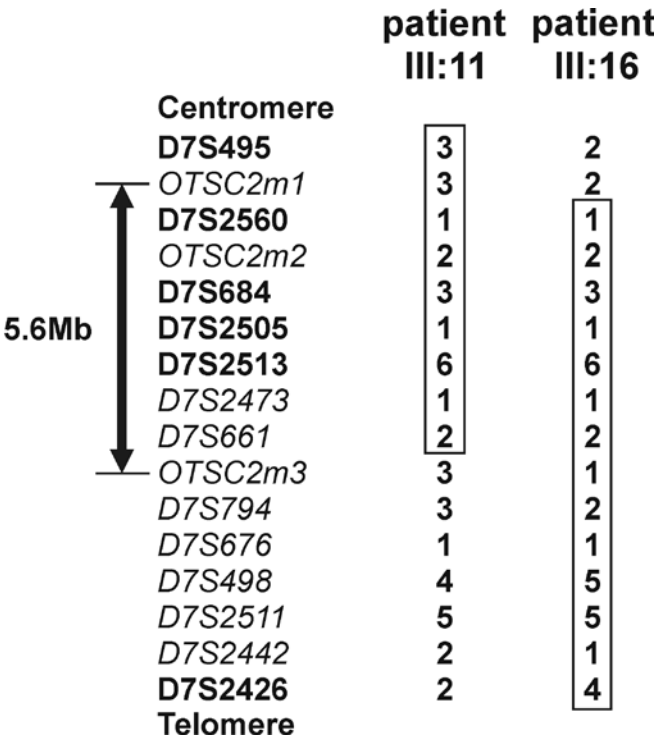


Figure 2

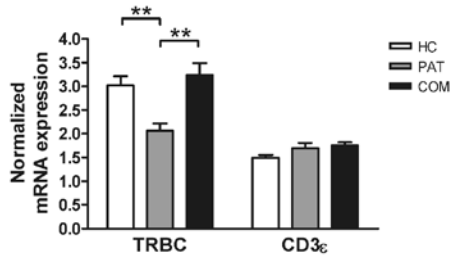


Figure 3

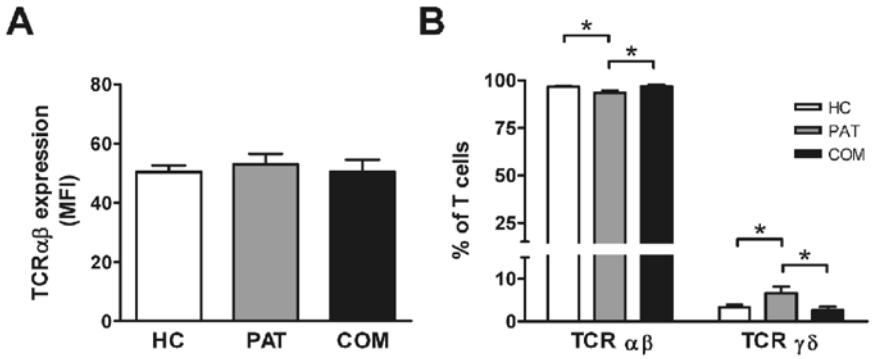
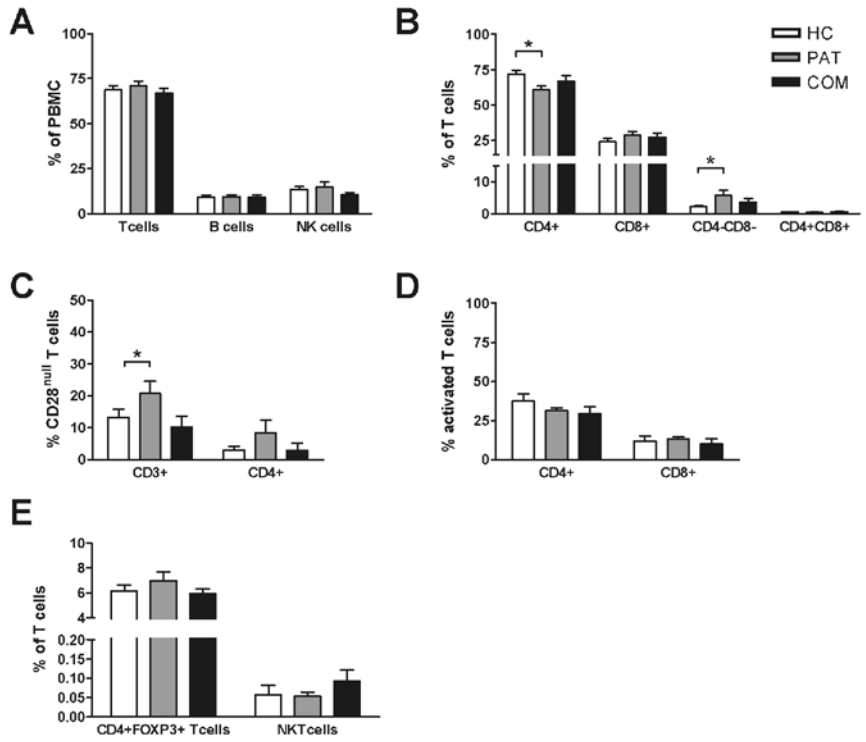


Figure 4



Supplementary Figure 1