

## ORIGINAL ARTICLE

# Involvement of T-cell receptor- $\beta$ alterations in the development of otosclerosis linked to OTSC2

I Schrauwen<sup>1,8</sup>, K Venken<sup>2,5,8</sup>, K Vanderstraeten<sup>1</sup>, M Thys<sup>1</sup>, J-J Hendrickx<sup>1,6</sup>, E Franssen<sup>1</sup>, L Van Laer<sup>1,7</sup>, PJ Govaerts<sup>3</sup>, M Verstreken<sup>4</sup>, I Schatteman<sup>4</sup>, P Stinissen<sup>2</sup>, N Hellings<sup>2</sup> and G Van Camp<sup>1</sup>

<sup>1</sup>Department of Medical Genetics, University of Antwerp, Antwerp, Belgium; <sup>2</sup>Hasselt University, Biomedisch Onderzoeksinstituut and Transnationale Universiteit Limburg, School of Life Sciences, Diepenbeek, Belgium; <sup>3</sup>The Eargroup, Antwerp-Deurne, Belgium and

<sup>4</sup>University Department of Otolaryngology, St-Augustinus Hospital Antwerp, Antwerp, Belgium

Otosclerosis is a common form of hearing loss, characterized by disordered bone remodeling in the otic capsule. Within the 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. In large otosclerosis families, seven autosomal-dominant loci have been found, 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 significantly lower T-cell receptor- $\beta$  (TCR- $\beta$ ) mRNA expression and percentage of blood circulating TCR- $\alpha\beta^+$  T cells was detected in OTSC2 patients compared with controls and patients with the complex form of the disease. Further analysis illustrated more significant disturbances in specific T-cell subsets, including an increased CD28<sup>null</sup> 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.

Genes and Immunity (2010) 11, 246–253; doi:10.1038/gene.2010.3; published online 25 February 2010

**Keywords:** otosclerosis; TCR- $\beta$ ; TCRB; CD28<sup>null</sup> cells; OTSC2; otic capsule

## Introduction

Otosclerosis is a disorder of bone remodeling that 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.<sup>1</sup>

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, seven monogenic loci have been identified (OTSC1-5, 7 and 8),<sup>2–8</sup> but no disease-causing mutations could be found. For the complex form of otosclerosis, several association studies were performed to identify genes.<sup>9–12</sup>

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 autoimmune 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 the serum, otosclerotic foci and perilymph of patients.<sup>13,14</sup> In addition, otosclerosis-like lesions were induced in rats by immunizing them with native type II collagen.<sup>15</sup> On the other hand, other studies could not confirm these observations or do not support this theory.<sup>16–18</sup> Furthermore, several studies describe a correlation of certain human leukocyte 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

Correspondence: Dr G Van Camp, Department of Medical Genetics, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium. E-mail: guy.vancamp@ua.ac.be

<sup>5</sup>Current address: Laboratory for Molecular Immunology and Inflammation, Department of Rheumatology, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium.

<sup>6</sup>Current address: Department of Otolaryngology & Head and Neck Surgery, UZ Brussel, Vrije Universiteit Brussel (VUB), Laarbeeklaan 101, 1090 Brussel, Belgium.

<sup>7</sup>Current address: Center for Medical Genetics Ghent, Ghent University, De Pintelaan 185–9000 Ghent, Belgium.

<sup>8</sup>The authors contributed equally to this work.

Received 12 August 2009; revised 19 October 2009; accepted 23 October 2009; published online 25 February 2010

several autoimmune diseases.<sup>19</sup> However, conflicting data exist as the otosclerosis-associated *HLA* antigens vary in different studies<sup>20,21</sup> or associations could not always be confirmed in other independent reports.<sup>22</sup>

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 (TCR- $\alpha\beta$ ).<sup>23,24</sup> Deposits of Ig (IgG, IgM and IgA) and complement C3 are present along the resorption lacunae in active otosclerotic lesions.<sup>24,25</sup> Furthermore, tumor necrosis factor- $\alpha$  mRNA expression and an increased production of interleukin (IL)-1 $\alpha$ , IL-1 $\beta$  and IL-6 has been reported in otosclerotic bone.<sup>26,27</sup> It has been suggested that this inflammatory process in otosclerosis is a response to persistent measles virus infection.<sup>28</sup>

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.<sup>3</sup> The *OTSC2* region is located between markers D7S495 and D7S2426 and spans a region of about 11.4 Mb,<sup>3</sup> 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 TCR- $\beta$  in the *OTSC2*-linked family to potentially reveal a possible causative mechanism underlying the development of otosclerosis in the patients.

## 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 markers 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, 14 genes in the refined region were also candidate genes based on their function described in the literature. The coding regions

	patient III:11	patient III:16
<b>Centromere</b>		
<b>D7S495</b>	3	2
<i>OTSC2m1</i>	3	2
<b>D7S2560</b>	1	1
<i>OTSC2m2</i>	2	2
<b>D7S684</b>	3	3
<b>D7S2505</b>	1	1
<b>D7S2513</b>	6	6
<i>D7S2473</i>	1	1
<i>D7S661</i>	2	2
<i>OTSC2m3</i>	3	1
<i>D7S794</i>	3	2
<i>D7S676</i>	1	1
<i>D7S498</i>	4	5
<i>D7S2511</i>	5	5
<i>D7S2442</i>	2	1
<b>D7S2426</b>	2	4
<b>Telomere</b>		

**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.

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 copy number variants (CNVs) using the Illumina Infinium single-nucleotide polymorphism genotyping platform (Illumina Inc., San Diego, CA, USA). Different detection algorithms were used, but no aberrations were detected in the region.

### No disturbed *TCR $\beta$* profile but a reduced *TCR- $\beta$* mRNA expression in *OTSC2* patients

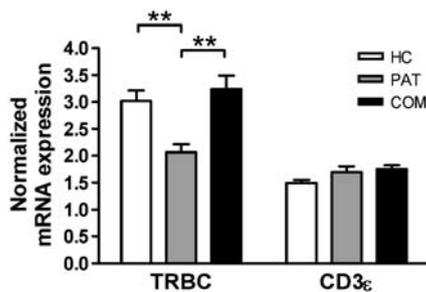
To investigate whether an alteration in the *TRB* locus is associated with otosclerosis in the *OTSC2*-linked family, a quantitative analysis of *TCR $\beta$*  gene usage was performed. The relative mRNA expression of *TCR $\beta$*  subfamily genes in peripheral blood mononuclear cells (PBMCs) of 7 otosclerosis 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 *TCR $\beta$*  usage, although some restrictions could be found potentially associated with the high age of patients and controls.<sup>29</sup> Patients of the Belgian *OTSC2* family showed a *TCR $\beta$*  profile comparable to that of age-matched controls. As no specific *TCR $\beta$*  profile could be found in *OTSC2* patients, *TCR $\beta$*  analyses were not performed for patients with the complex form of otosclerosis. In addition, CDR3 spectratyping was performed on DNA samples of four *OTSC2* patients and two unaffected controls (one individual related by marriage and one familial control). Results were in line with the *TCR $\beta$*  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 (TCR- $\beta$ ) 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 otosclero-

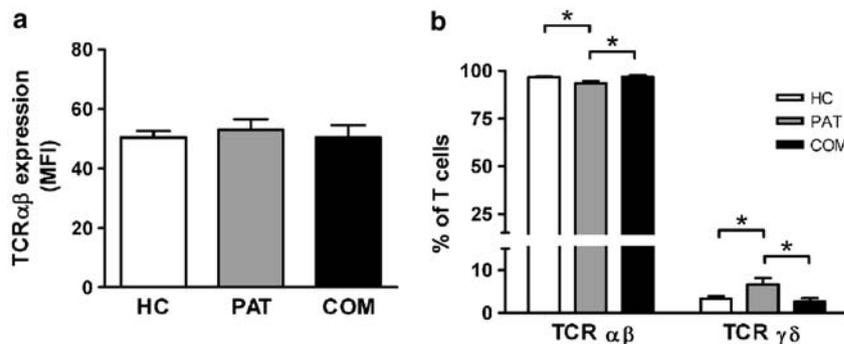
sis and 26 control individuals were analyzed (Figure 2). A significant difference of TCR- $\beta$  mRNA expression was found between the three groups ( $P=0.002$ , analysis of variance (ANOVA)). *Post hoc* tests showed a significantly lower expression of TCR- $\beta$  mRNA in *OTSC2* patients compared with controls and patients with the complex form of otosclerosis ( $P=0.004$  and  $0.005$ , respectively). No significant differences for TCR- $\beta$  mRNA expression were noted between controls and patients with the complex form of otosclerosis ( $P=1.000$ , *post hoc*). For *CD3*, no significant differences were found between the three groups ( $P=0.058$ , ANOVA).

#### Reduced frequency of blood-circulating TCR- $\alpha\beta^+$ cells in *OTSC2* patients

The reduced TCR- $\beta$  expression in total PBMCs could be due to either a reduced number of TCR- $\alpha\beta$ -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 TCR- $\beta$  protein expression per cell (mean fluorescence intensity) between otosclerosis patient groups and controls ( $P=0.561$ , ANOVA, Figure 3a). However, a significantly reduced percentage of TCR- $\alpha\beta$ -positive T cells was demonstrated in the *OTSC2* patients ( $P=0.019$ , ANOVA) as compared with the complex patients ( $P=0.041$ , *post hoc*) and controls ( $P=0.038$ , *post hoc*). These results indicate that



**Figure 2** mRNA expression of *CD3* and TCR- $\beta$  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 s.e.m.  $**P<0.01$ .



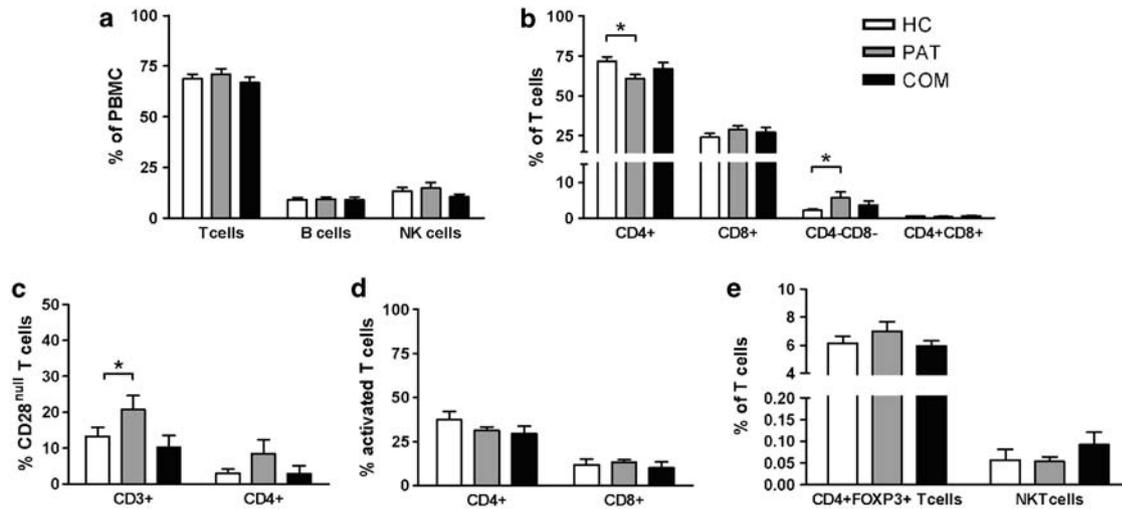
**Figure 3** TCR- $\beta$  protein expression and frequency of TCR- $\alpha\beta$ - and TCR- $\delta\gamma$ -positive cells. (a) TCR- $\beta$  protein expression per cell (mean fluorescence intensity, MFI). (b) Percentage of TCR- $\alpha\beta$ - and TCR- $\delta\gamma$ -positive T cells. PAT: *OTSC2* patients ( $n=12$ ), HC: controls ( $n=18$ ), COM: patients with the complex form of otosclerosis ( $n=12$ ). Error bars indicate s.e.m.  $*P<0.05$ .

the reduced expression of TCR mRNA in *OTSC2* patients is associated with a lower number of blood circulating TCR- $\alpha\beta^+$  T cells. In parallel with the reduced percentage of TCR- $\alpha\beta^+$  T cells, a significantly higher percentage of TCR- $\gamma\delta^+$  T cells was observed in *OTSC2* patients ( $P=0.010$ , ANOVA) compared with controls ( $P=0.033$ , *post hoc*) and complex patients ( $P=0.015$ , *post hoc*) (Figure 3b).

#### Analysis of T-cell subsets

To further evaluate the difference in TCR- $\alpha\beta$ -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, natural killer (NK) cells or B cells (the major lymphocyte populations) between otosclerosis patients and controls (ANOVA  $P=0.557$ ,  $0.413$  and  $0.982$ , 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*<sup>+</sup>*CD8*<sup>-</sup> T cells in *OTSC2* patients compared with controls, which just missed significance ( $P=0.051$ , *post hoc*) in the first measurement, but did reach significance in the second measurement taken at another time point ( $P=0.039$ , *post hoc*; Figure 4b). In addition, an increased percentage of double-negative (DN) (*CD4*<sup>-</sup>*CD8*<sup>-</sup>) T cells was found in *OTSC2* patients as compared with controls ( $P=0.039$ , *post hoc*; Figure 4b). T cells that had lost their *CD28* expression (*CD28*<sup>null</sup> T cells), defined as senescent T cells, were present at a higher percentage in *OTSC2* patients compared with controls. The difference was significant for the total *CD28*<sup>null</sup> T cells ( $P=0.033$ , *post hoc*), but not for the *CD4*<sup>+</sup>*CD28*<sup>null</sup> subset alone ( $P=0.117$ , *post hoc*; Figure 4c). Finally, no differences in the percentage of activated T cells (*CD3*<sup>+</sup>*CD25*<sup>int</sup>) could be seen between the study groups (*CD4*<sup>+</sup>:  $P=0.100$  and *CD8*<sup>+</sup>:  $P=0.657$ , ANOVA; Figure 4d). Percentages of specialized regulatory T-cell subsets, *CD4*<sup>+</sup>*CD25*<sup>+</sup> *FOXP3*<sup>+</sup> T cells and natural killer T cells (NKT), 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),



**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<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>-</sup>CD8<sup>-</sup> and CD4<sup>+</sup>CD8<sup>+</sup> cells of T cells, the CD4<sup>+</sup> measurement just missed significance ( $P=0.051$ , *post hoc*) in the first measurement, but did reach significance in the second measurement ( $P=0.039$ , *post hoc*). (c) Percentage of CD28<sup>null</sup> (senescent) cells of total T cells (CD3 and CD4<sup>+</sup> T cells (CD4)). (d) Percentage of CD25<sup>int</sup> (activated) cells of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. (e) Percentage of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T cells and NKT cells. PAT: *OTSC2* patients ( $n=12$ ), HC: controls ( $n=18$ ), COM: patients with the complex form of otosclerosis ( $n=12$ ). Error bars indicate s.e.m. \* $P < 0.05$ .

indicating a high reproducibility of the results. Differences between *OTSC2* 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 toward 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- $\alpha$  and - $\beta$  chain, whereas only a small subset of T cells express the TCR- $\gamma$  and - $\delta$  chain. As the *OTSC2* region on chromosome 7 contains the complete TCR- $\beta$  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  $\alpha$  and  $\beta$  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 with 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,<sup>23</sup> it cannot be excluded that T cells within the otosclerotic lesions can have a biased TCR expression profile reactive toward tissue-specific antigens.

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

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<sup>+</sup>)CD4<sup>-</sup>CD8<sup>-</sup> or DN cells and subsequently become double-positive (CD3<sup>+</sup>)CD4<sup>+</sup>CD8<sup>+</sup> thymocytes.<sup>30</sup> Finally, after positive and negative selection, these T-cell precursor cells differentiate into single-positive T cells (CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup> or CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>) 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 TCR- $\gamma\delta$ <sup>+</sup> T cells show either a DN or CD8<sup>+</sup> phenotype, an increase in these cells as observed in our study, could also account for differences in CD4<sup>+</sup> and DN T cells.

Furthermore, several specialized T-cell subsets, either regulatory or effector cells that have 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.<sup>31</sup> In this study, we focused on two important naturally occurring (thymic developing) regulatory T cells, that is, NKT cells and CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells (Tregs). For various human autoimmune diseases, it was shown that peripheral Treg and NKT cell function or frequencies are disturbed.<sup>32</sup> 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<sup>+</sup>CD25<sup>int</sup>).

Significant higher percentages of CD28<sup>null</sup> T cells were shown in *OTSC2* patients. CD28 is constitutively expressed on T cells, but its expression is transiently downregulated following T-cell activation and declines progressively with immune senescence.<sup>33</sup> CD28<sup>null</sup> T cells are clonally expanded cells, which are highly pro-inflammatory and express killer Ig-like receptors. An expanded population of CD28<sup>null</sup> T cells (CD8<sup>+</sup> or CD4<sup>+</sup>) can be found in patients with autoimmune diseases.<sup>34,35</sup> Because of their cytotoxic capacities and decreased susceptibility to immunoregulation, CD28<sup>null</sup> T cells might facilitate or sustain chronic autoreactive immune responses.<sup>36</sup>

On the basis of the location of the TCR- $\beta$  gene cluster in the candidate *OTSC2* region, and the significant immune alterations that were found in TCR- $\alpha\beta$ <sup>+</sup> T cells and other specific T-cell subsets, we suggest that this gene harbors the disease-causing mutation 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.<sup>37</sup> 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.<sup>38</sup> What is striking is the increased frequency of CD28<sup>null</sup> T cells in *OTSC2* patients. CD28<sup>null</sup> 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 toward 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 are 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 have 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.<sup>39</sup>

How specific mutations could affect T-cell development or ageing is unclear. Possibly, mutations could affect the efficiency of V-D-J recombination and in this way influence T-cell development. TCR gene rearrangements can impact TCR- $\alpha\beta/\gamma\delta$  lineage choice during T-cell development and the transition from DN to double-positive cells in the differentiation of TCR- $\alpha\beta$ <sup>+</sup> cells,<sup>40,41</sup> which would explain our current observations. Changes in, for instance, the activity of regulatory elements can affect TCR- $\beta$  gene rearrangement.<sup>42</sup> 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.<sup>43</sup>

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, 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 toward 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.

## Materials and methods

### Study population

Peripheral blood was collected from 12 patients from the previously described Belgian family segregating otosclerosis linked to *OTSC2*.<sup>3</sup> 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 before blood donation, immunosuppressive or immune-modulating medication and chemotherapy. This study took place with the approval from 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.<sup>3</sup> At the distal side, eight 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/>). Markers 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). 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, NE, USA) or an ABI3130XL automatic DNA sequencer (Applied Biosystems Inc., Foster City, CA, 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.). To trace possible disease-causing 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.) and intensities were normalized against a reference panel of 120 HapMap samples. Illumina's copy number analysis algorithm *cnvPartition* 1.2.0 (Illumina Inc.) was used to detect aberrations. In addition, the QuantiSNP and PennCNV CNV-detection algorithms were also used.<sup>44,45</sup>

#### TCRBV repertoire analysis and CDR3 spectratyping

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 reverse transcribed into first-strand cDNA using 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 reverse transcriptase PCR (Lightcycler 2.0, Roche) using a specific forward and reverse primer for each of 21 functional TCRBV subfamily genes as described previously.<sup>36</sup> TCRBV gene primers were adapted to the current nomenclature.<sup>46</sup> The FastStart DNA Master SYBR Green I kit (Roche) was used for PCR amplicon detection. PCR mixtures con-

tained cDNA (100 ng), forward/reverse primers (1  $\mu$ M), MgCl<sub>2</sub> (4 mM) and LightCycler FastStart DNA Master SYBR Green I (Roche). The amplification protocol consisted of an initial 10 min at 95 °C, followed by 45 cycles of repeated denaturation (95 °C), annealing (3 s at 60 °C) and elongation (10 s at 72 °C). Samples were analyzed by means of the Second derivative Maximum Method using LightCycler Software v3.5 (Roche). 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 TCR- $\beta$  gene clonality assay (*InVivoScribe* Technologies, La Ciotat, France) on genomic DNA samples. This assay includes a multiplex PCR assay to detect the vast majority of clonal TCR- $\beta$  gene rearrangements. The multiplex mastermixes target conserved regions within the V-D-J regions that flank the unique hypervariable antigen-binding region 3 (CDR3).<sup>47</sup> The first mastermix contains 23 Vb 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.).

#### TCR- $\beta$ mRNA expression analysis

Total RNA was extracted from blood using the PAXgene blood RNA System (PreAnalytiX, Hombrechtikon, Germany). DNase treatment was performed with the DNA-free kit (Ambion Inc., Austin, TX, USA). cDNA synthesis was carried out in duplicate with random hexamer primers using the Superscript III First-strand synthesis system for reverse transcriptase PCR (Invitrogen, Carlsbad, CA, USA). For quantitative real-time PCR, TaqMan Gene Expression Assays (Applied Biosystems Inc.) for TCR- $\beta$  and CD3 and three housekeeping genes (*TBP*, *UBC* and *YWHAZ*) were used. The assay for TCR- $\beta$  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: 5'-CCACAGTCTGCTCTACC C-3'). CD3 was also investigated, as it is associated with the cell surface with TCR- $\alpha\beta$  or TCR- $\gamma\delta$  to form a functional TCR complex. The assay for CD3 was developed to recognize the  $\epsilon$ -chain, which associates with both TCR- $\alpha\beta$  and TCR- $\gamma\delta$  (F-primer: 5'-GAGAGGC CACCACCTGTTC-3'; R-primer: 5'-GTCTCTGATTCAG GCCAGAATACAG-3'; reporter sequence: 5'-TTTCCGG ATGGGCTCATAG-3'). Mixtures consisted of 2  $\mu$ l cDNA, 5  $\mu$ l LightCycler 480 Probes Master (2  $\times$ , Roche), 0.5  $\mu$ l TaqMan Assay and 2.5  $\mu$ l water (PCR grade, Roche), and were run on a LightCycler 480 Instrument (Roche). The cycling conditions were as follows: 10 min at 95 °C and 40 cycles at 95 °C for 10 s and 60 °C for 1 min. Analysis was carried out using qBase Software v1.3.5.<sup>48</sup> Normalized values were calculated using stably expressed housekeeping genes.

#### Flow cytometric analysis

PBMCs were suspended in staining buffer (phosphate-buffered saline, 2% fetal bovine serum and 0.09% NaN<sub>3</sub>) and incubated for 30 min (at 4 °C) with fluorescein isothiocyanate, phycoerythrin and/or peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibodies.

The following monoclonal antibodies 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), TCR- $\alpha\beta$  (clone R73), TCR- $\gamma\delta$  (clone 11F2), CD28 (clone CD28.2) (all from BD Biosciences, Erembodegem, Belgium), anti-human TCRV $\alpha$ 24 (clone C15) and TCRV $\beta$ 11 (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, CA, USA) before adding anti-human FOXP3 (clone PCH101, eBioscience) for 30 min at 4°C. Subsequently, cells were washed twice (400 g, 5 min) and analyzed on a FACSCalibur flow cytometer (BD Biosciences). Quadrants and cutoff 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<sup>+</sup>CD19<sup>-</sup>), B cells (CD19<sup>+</sup>CD3<sup>-</sup>) and NK cells (CD16/CD56<sup>+</sup>CD3<sup>-</sup>). T cells were subsequently subdivided according to specific lineage markers: CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD8<sup>-</sup>), CD8<sup>+</sup> T cells (CD4<sup>-</sup>CD8<sup>+</sup>), DN T cells (CD4<sup>-</sup>CD8<sup>-</sup>), double-positive T cells (CD4<sup>+</sup>CD8<sup>+</sup>), TCR- $\alpha\beta$  T cells (TCR $\alpha\beta$ <sup>+</sup>TCR $\gamma\delta$ <sup>-</sup>), TCR $\gamma\delta$  T cells (TCR $\alpha\beta$ <sup>-</sup>TCR $\gamma\delta$ <sup>+</sup>), activated T cells (CD3<sup>+</sup>CD25<sup>int</sup>), NKT cells (TCRV $\alpha$ 24<sup>+</sup>V $\beta$ 11<sup>+</sup>), Tregs (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>) and CD28<sup>null</sup> T cells (CD3<sup>+</sup>CD28<sup>-</sup> and CD4<sup>+</sup>CD28<sup>-</sup>).

#### 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 *post hoc* test using Bonferroni correction for multiple testing. All ANOVA models were constructed in a stepwise backward 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 Q–Q-plot 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 cutoff of 0.001 was taken. Outcome variables for which the model check failed were tested using a nonparametric 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  $\pm$  s.e.m. unless otherwise indicated. For OTSC2 patients, flow cytometric parameters were repeated at a different time point. A Pearson's correlation coefficient was calculated to test reproducibility of these measurements.

#### Conflict of interest

The authors declare no conflict of interest.

#### Acknowledgements

We thank all family members and other participants of this study. We also thank Geert Vandeweyer and Edwin Reyniers for the technical assistance with CNV analysis and Kris Van Den Bogaert for help with this project. This work was supported by EuroHear by grants from the European Commission (FP6 Integrated project EuroHear LSHG-CT-20054-512063), the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO) (grant G.0138.07), the University of Antwerp (TOP grant) and the Belgian Charcot foundation. IS has a predoctoral position with the FWO. KV was financed by the Hasselt University.

#### References

- 1 Declau F, Van Spaendonck M, Timmermans JP, Michaels L, Liang J, Qiu JP *et al*. Prevalence of otosclerosis in an unselected series of temporal bones. *Otol Neurotol* 2001; **22**: 596–602.
- 2 Tomek MS, Brown MR, Mani SR, Ramesh A, Srisailapathy CR, Coucke P *et al*. Localization of a gene for otosclerosis to chromosome 15q25–q26. *Hum Mol Genet* 1998; **7**: 285–290.
- 3 Van Den Bogaert K, Govaerts PJ, Schatteman I, Brown MR, Caethoven G, Offeciers FE *et al*. A second gene for otosclerosis, OTSC2, maps to chromosome 7q34–36. *Am J Hum Genet* 2001; **68**: 495–500.
- 4 Chen W, Campbell CA, Green GE, Van Den Bogaert K, Komodikis C, Manolidis LS *et al*. Linkage of otosclerosis to a third locus (OTSC3) on human chromosome 6p21.3–22.3. *J Med Genet* 2002; **39**: 473–477.
- 5 Brownstein Z, Goldfarb A, Levi H, Frydman M, Avraham KB. Chromosomal mapping and phenotypic characterization of hereditary otosclerosis linked to the OTSC4 locus. *Arch Otolaryngol Head Neck Surg* 2006; **132**: 416–424.
- 6 Van Den Bogaert K, De Leenheer EM, Chen W, Lee Y, Nurnberg P, Pennings RJ *et al*. A fifth locus for otosclerosis, OTSC5, maps to chromosome 3q22–24. *J Med Genet* 2004; **41**: 450–453.
- 7 Thys M, Van Den Bogaert K, Iliadou V, Vanderstraeten K, Dieltjens N, Schrauwen I *et al*. A seventh locus for otosclerosis, OTSC7, maps to chromosome 6q13–16.1. *Eur J Hum Genet* 2007; **15**: 362–368.
- 8 Bel Hadj Ali I, Thys M, Beltaief N, Schrauwen I, Hilgert N, Vanderstraeten K *et al*. A new locus for otosclerosis, OTSC8, maps to the pericentromeric region of chromosome 9. *Hum Genet* 2008; **123**: 267–272.
- 9 Thys M, Schrauwen I, Vanderstraeten K, Janssens K, Dieltjens N, Van Den Bogaert K *et al*. The coding polymorphism T263I in TGF- $\beta$ 1 is associated with otosclerosis in two independent populations. *Hum Mol Genet* 2007; **16**: 2021–2030.
- 10 Schrauwen I, Thys M, Vanderstraeten K, Franssen E, Dieltjens N, Huyghe JR *et al*. Association of bone morphogenetic proteins with otosclerosis. *J Bone Miner Res* 2008; **23**: 507–516.
- 11 Schrauwen I, Ealy M, Huentelman MJ, Thys M, Homer N, Vanderstraeten K *et al*. A genome-wide analysis identifies genetic variants in the RELN gene associated with otosclerosis. *Am J Hum Genet* 2009; **84**: 328–338.
- 12 Schrauwen I, Thys M, Vanderstraeten K, Franssen E, Ealy M, Cremers CW *et al*. No evidence for association between the renin-angiotensin-aldosterone system and otosclerosis in a large Belgian-Dutch population. *Otol Neurotol* 2009; **30**: 1079–1083.
- 13 Yoo TJ. Etiopathogenesis of otosclerosis: a hypothesis. *Ann Otol Rhinol Laryngol* 1984; **93**: 28–33.
- 14 Reshetnikov NN, Popova TI. IgA, IgM and IgG levels and antibodies to native DNA and collagen type II in the perilymph of patients with otosclerosis. *Vestn Otorinolaringol* 1992; **1**: 14–16.

- 15 Yoo TJ, Tomoda K, Stuart JM, Kang AH, Townes AS. Type II collagen-induced autoimmune otospongiosis. A preliminary report. *Ann Otol Rhinol Laryngol* 1983; **92**: 103–108.
- 16 Lolov SR, Edrev GE, Kyurkchiev SD, Kehayov IR. Elevated autoantibodies in sera from otosclerotic patients are related to the disease duration. *Acta Otolaryngol* 1998; **118**: 375–380.
- 17 Solvsten Sorensen M, Nielsen LP, Bretlau P, Jorgensen MB. The role of type II collagen autoimmunity in otosclerosis revisited. *Acta Otolaryngol* 1988; **105**: 242–247.
- 18 Harris JP, Woolf NK, Ryan AF. A reexamination of experimental type II collagen autoimmunity: middle and inner ear morphology and function. *Ann Otol Rhinol Laryngol* 1986; **95**: 176–180.
- 19 Burton PR, Clayton DG, Cardon LR, Craddock N, Deloukas P, Duncanson A *et al*. Association scan of 14 500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. *Nat Genet* 2007; **39**: 1329–1337.
- 20 Gregoriadis S, Zervas J, Varletzidis E, Toubis M, Pantazopoulos P, Fessas P. HLA antigens and otosclerosis. A possible new genetic factor. *Arch Otolaryngol* 1982; **108**: 769–771.
- 21 Singhal SK, Mann SB, Datta U, Panda NK, Gupta AK. Genetic correlation in otosclerosis. *Am J Otolaryngol* 1999; **20**: 102–105.
- 22 Pedersen U, Madsen M, Lamm LU, Elbrond O. HLA-A, -B, -C antigens in otosclerosis. *J Laryngol Otol* 1983; **97**: 1095–1097.
- 23 Arnold W, Friedmann I. Immunohistochemistry of otosclerosis. *Acta Otolaryngol Suppl* 1990; **470**: 124–128; discussion 128–129.
- 24 Altermatt HJ, Gerber HA, Gaeng D, Muller C, Arnold W. Immunohistochemical findings in otosclerotic lesions. *Hno* 1992; **40**: 476–479.
- 25 Arnold W, Friedmann I. Otosclerosis—an inflammatory disease of the otic capsule of viral aetiology? *J Laryngol Otol* 1988; **102**: 865–871.
- 26 Bodo M, Carinci P, Venti G, Giammarioli M, Donti E, Stabellini G *et al*. Glycosaminoglycan metabolism and cytokine release in normal and otosclerotic human bone cells interleukin-1 treated. *Connect Tissue Res* 1997; **36**: 231–240.
- 27 Karosi T, Jokay I, Konya J, Szabo LZ, Pytel J, Jori J *et al*. Detection of osteoprotegerin and TNF-alpha mRNA in ankylosed stapes footplates in connection with measles virus positivity. *Laryngoscope* 2006; **116**: 1427–1433.
- 28 Niedermeyer HP, Arnold W. Otosclerosis and measles virus—association or causation? *ORL J Otorhinolaryngol Relat Spec* 2008; **70**: 63–69; discussion 69–70.
- 29 Naylor K, Li G, Vallejo AN, Lee WW, Koetz K, Bryl E *et al*. The influence of age on T cell generation and TCR diversity. *J Immunol* 2005; **174**: 7446–7452.
- 30 Haynes BF, Denning SM, Singer KH, Kurtzberg J. Ontogeny of T-cell precursors: a model for the initial stages of human T-cell development. *Immunol Today* 1989; **10**: 87–91.
- 31 Jiang H, Chess L. Regulation of immune responses by T cells. *N Engl J Med* 2006; **354**: 1166–1176.
- 32 Venken K, Hellings N, Hensen K, Rummens JL, Medaer R, D'Hooghe MB *et al*. Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4+CD25+ regulatory T-cell function and FOXP3 expression. *J Neurosci Res* 2006; **83**: 1432–1446.
- 33 Vallejo AN, Brandes JC, Weyand CM, Goronzy JJ. Modulation of CD28 expression: distinct regulatory pathways during activation and replicative senescence. *J Immunol* 1999; **162**: 6572–6579.
- 34 Thewissen M, Somers V, Venken K, Linsen L, van Paassen P, Geusens P *et al*. Analyses of immunosenescent markers in patients with autoimmune disease. *Clin Immunol* 2007; **123**: 209–218.
- 35 Raffener B, Dejaco C, Duftner C, Kullich W, Goldberger C, Vega SC *et al*. Between adaptive and innate immunity: TLR4-mediated perforin production by CD28null T-helper cells in ankylosing spondylitis. *Arthritis Res Ther* 2005; **7**: R1412–R1420.
- 36 Thewissen M, Somers V, Hellings N, Fraussen J, Damoiseaux J, Stinissen P. CD4+CD28null T cells in autoimmune disease: pathogenic features and decreased susceptibility to immunoregulation. *J Immunol* 2007; **179**: 6514–6523.
- 37 Lorenzo J, Horowitz M, Choi Y. Osteoimmunology: interactions of the bone and immune system. *Endocr Rev* 2008; **29**: 403–440.
- 38 Milroy CM, Michaels L. Pathology of the otic capsule. *J Laryngol Otol* 1990; **104**: 83–90.
- 39 Goodnow CC. Multistep pathogenesis of autoimmune disease. *Cell* 2007; **130**: 25–35.
- 40 Mombaerts P, Clarke AR, Rudnicki MA, Iacomini J, Itohara S, Lafaille JJ *et al*. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* 1992; **360**: 225–231.
- 41 Joachims ML, Chain JL, Hooker SW, Knott-Craig CJ, Thompson LF. Human alpha beta and gamma delta thymocyte development: TCR gene rearrangements, intracellular TCR beta expression, and gamma delta developmental potential—differences between men and mice. *J Immunol* 2006; **176**: 1543–1552.
- 42 Bouvier G, Watrin F, Naspetti M, Verthuy C, Naquet P, Ferrier P. Deletion of the mouse T-cell receptor beta gene enhancer blocks alphabeta T-cell development. *Proc Natl Acad Sci USA* 1996; **93**: 7877–7881.
- 43 Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. *J Pathol* 2007; **211**: 144–156.
- 44 Colella S, Yau C, Taylor JM, Mirza G, Butler H, Clouston P *et al*. QuantiSNP: an Objective Bayes Hidden-Markov Model to detect and accurately map copy number variation using SNP genotyping data. *Nucleic Acids Res* 2007; **35**: 2013–2025.
- 45 Wang K, Li M, Hadley D, Liu R, Glessner J, Grant SF *et al*. PennCNV: an integrated hidden Markov model designed for high-resolution copy number variation detection in whole-genome SNP genotyping data. *Genome Res* 2007; **17**: 1665–1674.
- 46 Rowen L, Koop BF, Hood L. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science* 1996; **272**: 1755–1762.
- 47 van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL *et al*. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003; **17**: 2257–2317.
- 48 Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 2007; **8**: R19.

Supplementary Information accompanies the paper on Genes and Immunity website (<http://www.nature.com/gene>)