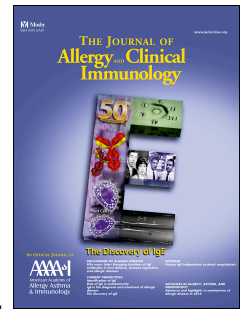


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Efficacy of lentiviral mediated gene therapy in an Omenn syndrome Rag2 mouse model is not hindered by inflammation and immune dysregulation

Valentina Capo, PhD, Maria Carmina Castiello, PhD, Elena Fontana, PhD, Sara Penna, MSc, Marita Bosticardo, PhD, Elena Draghici, MSc, Luigi P. Poliani, MD PhD, Lucia Sergi Sergi, MSc, Rosita Rigoni, MSc, Barbara Cassani, PhD, Monica Zanussi, BSc, Paola Carrera, PhD, Paolo Uva, PhD, Kerry Dobbs, MSc, Nicolò Sacchetti, BSc, Luigi D. Notarangelo, MD, Niek P. van Til, PhD, Gerard Wagemaker, PhD, Anna Villa, MD



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Title: Efficacy of lentiviral mediated gene therapy in an Omenn syndrome Rag2 mouse model is not hindered by inflammation and immune dysregulation

Valentina Capo, PhD¹; Maria Carmina Castiello, PhD¹; Elena Fontana, PhD²; Sara Penna, MSc¹; Marita Bosticardo, PhD¹; Elena Draghici, MSc¹; Luigi P. Poliani, MD PhD³; Lucia Sergi Sergi, MSc¹; Rosita Rigoni, MSc^{2,4}; Barbara Cassani, PhD^{2,4}; Monica Zanussi, BSc⁵; Paola Carrera, PhD⁵; Paolo Uva, PhD⁶; Kerry Dobbs, MSc⁷; Nicolò Sacchetti, BSc^{1,8}; Luigi D. Notarangelo, MD⁷; Niek P. van Til, PhD^{9,10}; Gerard Wagemaker, PhD^{9,11,12}; Anna Villa MD^{1,4}

Affiliation

¹San Raffaele Telethon Institute for Gene Therapy (TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy; ²Humanitas Clinical and Research Center, Rozzano, Milan, Italy; ³Institute of Molecular Medicine "A. Nocivelli", University Hospital "Spedali Civili", Brescia, Italy; ⁴Milan Unit, Istituto di Ricerca Genetica e Biomedica, Consiglio Nazionale delle Ricerche, Milan, Italy; ⁵Genomics for the diagnosis of human pathologies, San Raffaele Scientific Institute, Milan, Italy; ⁶CRS4, Science and Technology Park Polaris, Pula, Italy; ⁷Laboratory of Host Defenses, NIAID, National Institutes of Health, Bethesda, Maryland; ⁸Vita-Salute San Raffaele University, Milan, Italy; ⁹Department of Hematology, Erasmus University Medical Center, Rotterdam; ¹⁰Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands; ¹¹Hacettepe University, Stem Cell Research and Development Center, Ankara, Turkey; ¹²Raisa Gorbacheva Memorial Research Institute for Pediatric Oncology and Hematology, Saint Petersburg, Russia.

* Corresponding author: Anna Villa, MD; San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), Division of Regenerative Medicine, Stem Cells and Gene Therapy, San Raffaele Scientific Institute, Milan, Italy; Email: villa.anna@hsr.it

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Abstract

Background: Omenn syndrome (OS) is a rare severe combined immunodeficiency associated with autoimmunity, caused by defects of the lymphoid-specific V(D)J recombination. Most patients carry hypomorphic mutations in recombination activating genes (*RAG*) 1 or 2. Hematopoietic stem cell (HSC) transplantation is the standard treatment, however gene therapy (GT) may represent a valid alternative, especially for patients lacking a matched donor.

Objective: To determine the efficacy of lentiviral vector (LV) mediated GT in the murine model of OS (*Rag2*^{R229Q/R229Q}) in correcting immunodeficiency and autoimmunity.

Methods: OS Lin⁻ cells were transduced with a LV encoding the human *RAG2* gene and injected into irradiated OS recipients. Control mice were transplanted with wild-type or OS untransduced Lin⁻ cells. Immunophenotype, T-dependent and independent antigen challenges, immune spectratyping, autoantibodies detection and detailed tissue immunohistochemical analyses were performed.

Results: LV-mediated GT allowed immunological reconstitution, although suboptimal as compared to wild type bone marrow transplanted OS mice, in peripheral blood and hematopoietic organs, such as bone marrow, thymus and spleen. We observed *in vivo* variability in the efficacy of GT correlating with the levels of transduction achieved. Immunoglobulin levels and T cell repertoire normalized and gene corrected mice properly responded to challenges *in vivo*. Autoimmune manifestations, such as skin infiltration and

autoantibodies, dramatically improved in GT mice with a vector copy number/genome higher than 1 in the bone marrow and 2 in the thymus.

Conclusions: Our data show that LV-mediated GT for Omenn Syndrome significantly ameliorates the immunodeficiency even in an inflammatory environment.

Key messages:

- LV-mediated GT improves immune dysregulation and skin infiltration in Omenn syndrome

Capsule summary:

Gene therapy can represent a valid option for the treatment of Rag2 deficiency even in the presence of severe immune dysregulation and inflammation

Keywords:

Gene therapy, Omenn syndrome, autoimmunity, lentiviral vector, *Rag* genes

Abbreviations:

AIRE: autoimmune regulator
BAFF: B-cell activating factor
BM: bone marrow
CK: cytokeratin
DN: double negative
DP: double positive
GT: gene therapy
H&E: hematoxylin and eosin

- 98 HSCT: hematopoietic stem cell transplantation
99 Ig: immunoglobulin
100 IVIG: intravenous immunoglobulin
101 LV: lentiviral vector
102 M/C ratio: medulla/cortex ratio
103 MZ: marginal zone
104 OS: Omenn syndrome
105 PID: primary immunodeficiency
106 RAG: recombination activating genes
107 SCID: severe combined immunodeficiency
108 SD: standard deviation
109 SP: single positive
110 TCR: T cell receptor
111 TEC: thymic epithelial cell
112 TRA: tissue-restricted antigen
113 UCOE: ubiquitous chromatin opening element
114 UEA: Ulex europaeus agglutinin I
115 VCN: vector copy number/genome

INTRODUCTION

Genetic defects in recombination activating genes (*RAG*) 1 and 2 result in a spectrum of severe immune defects ranging from a profound block in T and B cell differentiation, the T⁻ B⁻ severe combined immunodeficiency (SCID) forms, to the presence of oligoclonal lymphocytes, a condition referred to as “atypical” or leaky SCID¹. In recent years, whole exome sequencing has identified *RAG* mutations in late childhood or young adulthood patients presenting a broad spectrum of clinical manifestations, including combined immunodeficiency associated with granulomas and/or autoimmunity or expansion of T cell receptor (TCR) $\gamma\delta$ T cells and variable antibody deficiency^{1–5}. Hypomorphic mutations also cause a distinct clinical phenotype, the Omenn syndrome (OS) in which immunodeficiency associates with the presence of eosinophilia and early-onset erythroderma due to the infiltration of oligoclonal activated T cells^{6–8}. Although circulating B cells are virtually absent, plasmablasts may be present in peripheral tissues and contribute to the pathogenesis of immune dysregulation, as revealed by the presence of autoantibodies^{9–11}. Since all these forms are fatal if untreated, hematopoietic stem cell transplantation (HSCT) is the standard of care, even in late diagnosed SCID. The average overall survival for SCID patients treated early in infancy is >90% at three years after transplantation^{12–17}. Poor overall survival has been reported after haploidentical HSCT with no or limited conditioning, clearly indicating that myeloablative conditioning is required to achieve long-term T and B cell recovery¹⁸. Given the encouraging results of gene therapy (GT) studies obtained in X-linked SCID^{19,20} and in adenosine deaminase deficiency^{21,22}, the GT approach represents an attractive therapeutic option for OS patients. Preclinical studies in *Rag1*^{-/-} model indicated that low RAG1 expression resulted in incomplete thymic reconstitution and consequent development of OS manifestations²³ or in very low levels of T and B cell counts²⁴. The preclinical model of *Rag2*^{-/-} GT provided more encouraging data, showing amelioration of the immunodeficiency in the absence of side effects with both retroviral and lentiviral vectors^{25,26}. Significant improvement in T and B cell reconstitution was observed using a lentiviral vector (LV) carrying codon-optimized human

RAG2 cDNA under the control of ubiquitous chromatin opening element (UCOE) promoter (UCOE-*RAG2co*). Nonetheless, it remains unclear whether the same therapeutic strategy could be effective in the presence of hypomorphic *RAG2* mutations, in which gene-corrected lymphoid progenitors compete with endogenous uncorrected lymphocytes in an inflammatory and autoimmune environment. Here we tested the UCOE-*RAG2co* LV in the OS preclinical model (*Rag2*^{R229Q/R229Q} mouse)²⁷ to evaluate the efficacy of a lentiviral mediated GT approach. We demonstrated that adequate transgene expression is required to overcome T and B cell differentiation block and restore thymic epithelial structure. GT treated mice showed dramatic amelioration in peripheral tissue infiltration particularly in the skin and antigen specific antibody production upon *in vivo* challenges. In summary, our data demonstrated the feasibility of lentiviral gene therapy approach even in the context of residual *RAG2* expression and, more importantly, in an inflammatory environment predisposing to autoimmunity.

METHODS

Lentiviral vector production. The 2.6kb UCOE-RAG2co LV, in which the human codon-optimized *RAG2* cDNA was driven by a 2.6kb UCOE, was produced as previously described²⁶. For the 2.2kb UCOE-RAG2co LV, 2.6kb UCOE was replaced by a shorter 2.2kb UCOE form²⁸.

Animals. Animal experimental procedures were approved by Institutional Animal Care and Use Committee of San Raffaele Hospital and Italian Ministry of Health (IACUC n. 710). C57Bl/6 WT mice were obtained from Charles River. Knock-in C57BL/6 *Rag2*^{R229Q/R229Q} colony was maintained onsite with heterozygous breeders²⁷.

Lin- transduction and transplant. Six to 10-week-old donor WT or *Rag2*^{R229Q/R229Q} (OS) mice were sacrificed and femurs and tibias were flushed to collect bone marrow (BM). Lineage-negative (Lin-) cells were enriched from total BM with Lineage Cell Depletion kit and autoMACS separator (Miltenyi Biotec), according to manufacturer's instructions. OS Lin- were transduced overnight with LVs at multiplicity of infection (MOI) 5-10 (vector titer 1.3-3.8x10⁸ TU/ml) in StemSpan SFEM medium (STEMCELL Technologies) supplemented with 2% FCS (Euroclone), 1% Pen/Strep, 1% Glutamine (Gibco) and the following cytokines (Peprotech): rmTPO 20ng/ml, rmSCF 50ng/ml, rhFLT3L 10ng/ml, rmIL3 10ng/ml and rhIL6 20ng/ml. Untransduced OS and WT Lin- were cultured in parallel in the same medium. Recipient mice were conditioned by lethal total body irradiation (900 RAD, split dose) at least 2 hours before transplant and then were injected in the caudal vein with 0.5x10⁶ Lin- cells. Donors and recipients were mismatched by sex or CD45 alleles to follow up chimerism. Gentamicin sulfate (Italfarmaco) was administered in drinking water (8µg/ml) for the first 2 weeks after transplant to prevent infections.

Flow cytometry analysis. Single cell suspensions from tissues were obtained as previously described²⁹. Cells were stained with the following antibodies (BD Pharmingen, Miltenyi Biotec, Biolegend or eBioscience): CD3, CD4, CD8, CD11b, CD19, CD21, CD23, CD24, CD25, CD43, CD44,

CD45.1/2, CD62L, CD69, CD117, B220, IgM, IgD, NKp46, NK1.1, Lin+ cocktail, Sca1. Viability was determined with Live/dead fixable dead cell stain kit (ThermoFisher Scientific). Samples were acquired on FACS Canto II (BD) and analysed with FlowJo software.

Real-time quantitative PCR. Genomic DNA was extracted with QIAamp DNA Blood mini kit (QIAGEN), according to manufacturer's instructions. Vector copy number/genome (VCN) was quantified by quantitative Real-Time PCR on Vii7 PCR system (Applied Biosystems) as previously described³⁰. For digital droplet PCR, 20ng of genomic DNA were analysed using the QX200 Droplet Digital PCR System (Bio-Rad), according to manufacturer's instructions, with the same set of primers and probe used for Real-Time PCR and *Rpp30* gene (Bio-Rad) as normalizer. Chimerism in sex mismatched transplants was evaluated by quantification of cells carrying Y chromosome, as previously described³¹.

Gene expression analysis. Total RNA was obtained from spleen cells with RNeasy mini kit (QIAGEN) and reverse-transcribed with High-capacity cDNA Reverse Transcription kit (Applied Biosystems), following manufacturer's instructions. Vector-driven human recoded *RAG2* gene expression was analysed with Custom Plus TaqMan RNA assay and normalized to HPRT gene expression (Single Tube TaqMan Gene Expression Assay, Applied Biosystems).

Immunoglobulin and cytokine quantification. Total IgA, IgG and IgM levels were quantified on serum samples obtained at sacrifice using MILLIPLEX MAP mouse immunoglobulin isotyping magnetic bead panel and MAGPIX system (Merck Millipore), according to manufacturer's instructions. IgE and BAFF were analysed with mouse IgE ELISA Set (BD Biosciences) and BAFF Quantikine ELISA (R&D systems), respectively, following manufacturer's instructions. Pneumovax-specific IgM and TNP-KLH-specific IgM and IgG were quantified in the serum after immunization by ELISA assay as previously described³².

Histological analysis. Tissue samples were collected at sacrifice and processed as previously described²⁹. In addition, Fezf2/Fez1 expression was analysed by immunohistochemistry (F441, IBL America). Arbitrary skin infiltration score was calculated, scoring from 0 to 4 (where 0 is absent and 4 is severe) the following manifestations: abnormalities of keratinocytes, immune cell infiltration, alterations of the epidermis and presence of cutaneous abscesses.

***In vitro* T cell proliferation.** For T cell proliferation assays, 5×10^5 spleen cells were labelled with CellTrace Violet or FarRed cell proliferation kits (Thermo Fisher) according to the manufacturer's instructions. Labelled cells were stimulated with anti-mouse CD3e antibody (1 μ g/ml, BD Pharmingen), IL-2 (100U), PMA + Ionomycin (50ng/ml and 1 μ g/ml respectively, Calbiochem) Concanavalin A (2.5 μ g/ml, Sigma) and cultured in RPMI medium supplemented with 10% FBS, 1% Pen/Strep, 1% Glutamine and 50 μ M β -mercaptoethanol (Gibco). Cells were analysed after 3 days by flow cytometry. Proliferation was expressed as frequency of cells that diluted the cell proliferation dyes.

TCR spectratyping. cDNA was amplified using constant and variable primers detailed in Online Repository Table E1. FAM labelled products were analysed by capillary electrophoresis.

***In vivo* immunizations.** Four months after GT, mice were intraperitoneally injected with 115 μ g of Pneumovax vaccine (Sanofi Pasteur). Serum samples were collected weekly for 3 weeks. For TNP-KLH challenge, mice were intravenously injected with 100 μ g of TNP-KLH (Biosearch Technologies). Three weeks later, animals were intraperitoneally injected with 100 μ g of TNP-KLH to boost immune response. Antigen specific IgM and IgG antibodies were evaluated in serum by ELISA as previously described³².

Autoantibodies assays

Anti-double-strand DNA (dsDNA) antibodies were evaluated by ELISA as previously described³². Screening for a broad panel of IgM and IgG

autoantibodies was performed using autoantibody arrays (UT Southwestern Medical Center, Genomic and Microarray Core Facility)³³.

Statistical analysis. Statistical analyses were performed with GraphPad Prism, using non parametric one-way ANOVA with Dunns post-test, Spearman's correlation and non-parametric combination test. Significance levels are: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

RESULTS

Gene therapy improves peripheral B and T cell counts in Omenn

Syndrome mice. To study the efficacy of GT, OS mice were lethally irradiated and transplanted with Lin⁻ cells, previously transduced with LV 2.6kbUCOE-RAG2co or 2.2kbUCOE-RAG2co (GT mice)^{26,28}. Additional mice were treated with untransduced WT or OS Lin⁻ cells, as positive (BMT WT) and negative (BMT OS) controls, respectively. Starting at 6 weeks post treatment, we observed the appearance of B lymphocytes in the peripheral blood of GT mice with both vectors, in marked contrast to BMT OS mice in which this subset was virtually absent, as expected (Fig. 1a). B cell absolute counts increased over time for 13 weeks post GT and then remained stable up to the time of sacrifice (28 weeks), even though at levels significantly lower than BMT WT controls. After GT, we also observed an improvement in T lymphocytes, which were present in low counts in BMT OS mice (Fig. 1a and 1b). Moreover, the CD4⁺/CD8⁺ T cell ratio, which is often inverted in BMT OS mice, returned to normal levels after treatment (Fig. 1c). Nonetheless, leukopenia was not fully resolved by GT (Fig. 1d), demonstrating a partial immune reconstitution.

Overcoming the B cell differentiation block. Mice were sacrificed 7 months after treatment and tissues were analysed to assess immune reconstitution. Full donor chimerism (>85%, data not shown) was established in bone marrow, spleen and thymus. BM was analysed to study B cell development, since OS mice present a block in the initial B cell differentiation steps due to very low levels of RAG2 activity in precursors¹¹. GT mice showed a reduction of early B cell precursors with the appearance of recirculating mature B cells in the BM (Fig. 2a). Although GT mice displayed a statistically significant increase in mature B cell counts as compared to BMT OS animals, only a few of them exhibited counts comparable to BMT WT animals (Fig 2b). The results obtained were in accordance to the VCN in BM of GT mice (VCN = 1.0 ± 1.0 for 2.6kbUCOE-RAG2co GT mice and VCN = 0.3 ± 0.2 for 2.2kbUCOE-RAG2co GT mice) and a statistically significant correlation between VCN and mature recirculating B cell counts was found (Online Repository Fig. E1a). In the spleen, absolute counts were normal in some GT

mice, but low or similar to BMT OS in others (Fig. 2c). Consistently, variability in the splenic B cell counts and vector-driven RAG2 expression positively correlated with VCN, underscoring the importance of sufficient RAG2 expression for proper immune reconstitution (Online Repository Fig. E1a and b) (VCN in spleen = 1.8 ± 1.0 and 1.4 ± 0.2 for GT 2.6kbUCOE-RAG2co and GT 2.2kbUCOE-RAG2co, respectively). In particular, BMT OS mice had few immature transitional cells, whereas GT allowed normal maturation of marginal zone (MZ) and follicular B cells with a relative increase in the frequency of marginal zone cells (Fig. 2d and Online Repository Fig. E1c), likely due to the lymphopenic environment³⁴. To evaluate the functionality of B cells after therapy, we measured immunoglobulin (Ig) levels in the serum of our cohorts of mice at the time of sacrifice. We achieved normalization in IgM, IgA and IgG production, thus indicating that even low B cell numbers were able to produce adequate Ig levels. Furthermore, IgE, usually elevated in OS mice^{27,35}, returned to low levels of BMT-WT mice (Fig. 2e).

Amelioration of thymic architecture and output. GT was able to partially restore thymic structure and function. Histology and immunostaining revealed restoration of corticomedullary demarcation. The medulla area was variable in GT mice and consistent with VCN retrieved in thymocytes (Fig3a). GT mice, in particular those treated with LV 2.6kbUCOE-RAG2co, showed a marked improvement in the medulla/cortex (M/C) ratio, even if they did not reach the levels of BMT WT controls (Fig. 3b). Furthermore, thymic recovery was found to significantly correlate with VCN (Fig. 3c). Since defects in central tolerance have been described in OS patients and in the mouse counterpart^{27,36}, we evaluated the expression of AIRE, the master gene regulator of the promiscuous expression of tissue restricted antigens (TRA) in the thymus³⁷. AIRE⁺ cells were dramatically reduced in BMT OS controls, as expected, and were increased only in the minority of GT mice, suggesting incomplete maturation of medullary thymic epithelial cells (mTEC) particularly in GT mice with lower VCN (Fig. 3d and Online Repository Fig. E2a,b). We also measured the expression of Fezf2, recently identified as key regulator of AIRE-independent TRA gene expression³⁸. Similarly to AIRE, the low frequency of Fezf2⁺ cells was partly corrected in the group treated with LV

2.6kbUCOE-RAG2co (Fig. 3e and Online Repository Fig. E2a,c). Finally, we investigated the thymocyte compartment. GT mice showed improved thymic cellularity (Fig. 3f), with a high variability explained by strong differences in the huRAG2 expression (Fig. 3g and Online Repository Fig. 1d) (VCN = 2.1 ± 1.9 and 1.7 ± 1.7 for GT 2.6kbUCOE-RAG2co and GT 2.2kbUCOE-RAG2co, respectively). CD4⁻CD8⁻ double negative (DN) precursors were indeed predominant in the poorly immune reconstituted BMT OS thymi. After GT, this subpopulation dramatically diminished with an expansion of CD4⁺CD8⁺ double positive (DP) cells, re-establishing a subset distribution similar to that of BMT WT thymus (Fig. 3h). On the other hand, mature single positive (SP) CD4⁺CD8⁻ and CD8⁺CD4⁻ T cells presented similar frequency in all groups analysed.

Recovery of T cell phenotype and function. We examined T cell numbers and immunophenotype in the spleen 7 months post-treatment. Similar to B lymphocytes, GT provided a statistically significant rise of T cell counts, even though they remained notably lower than BMT WT values (Fig. 4a and Online Repository Fig. E1d). Moreover, we detected the re-establishment of a correct CD4⁺/CD8⁺ T cell ratio (Fig. 4b), similarly to the peripheral blood. Detailed investigation of T cells in GT mice revealed the appearance of naïve CD4⁺ and CD8⁺ lymphocytes. Indeed, in OS mice the vast majority of T cells showed an activated phenotype (Fig. 4c). Again, frequency and counts of naïve T cells after therapy remained lower as compared to WT-transplanted controls. Hence, we moved to analyse the T cell functionality *in vitro*. Spleen cell proliferation was induced with different combinations of TCR-dependent (α CD3 and IL-2) or -independent (PMA+Ionomycin and concanavalin A) stimuli. While CD8⁺ T lymphocytes from BMT OS mice were unresponsive, GT mice behaved similarly to BMT WT controls in response to all stimuli (Fig. 4d). The same results were obtained in CD4⁺ T cells (Online Repository Figure E3). Furthermore, we analysed TCR V β repertoire diversity by fragment length analysis (spectratyping) to assess TCR rearrangements as an indirect measure of the restoration of RAG2 activity. As expected, BMT OS presented a skewed oligoclonal TCR repertoire. GT mice with an adequate reconstitution level revealed a predominantly polyclonal spectratype,

superimposable to those of BMT WT samples (Fig. 4e, GT3 mouse). Conversely, GT mice in which the VCN was low and immune reconstitution poor showed an oligoclonal repertoire, comparable to BMT OS mice (Fig. 4e, GT4 mouse with low VCN and GT5 mouse with intermediate VCN).

Efficient response to *in vivo* challenges. To understand whether B and T lymphocytes were functional *in vivo*, three months after transplantation, we challenged GT mice with antigens to induce T-dependent or T-independent immune responses. The first group was injected intraperitoneally with the P23 Pneumovax vaccine that elicits a T-independent B cell response. Three weeks after vaccination, we quantified antigen-specific IgM production, revealing the incapacity of BMT OS mice to mount an immune response. On the contrary, B cells of GT mice generated a P23-specific IgM response superimposable to that of WT transplanted mice (Fig. 5a). The variability of immune response to Pneumovax depended on the extent of immune reconstitution, since it statistically correlated with B cell counts in the spleen (Fig. 5b). The second group of mice was challenged with TNP-KLH antigens that induce a T-dependent B cell response in order to evaluate the crosstalk between T and B cells. Three weeks after injection, antigen-specific IgM production was absent in BMT OS, but properly recovered in GT mice (Fig. 5c). A second boost dose was administered to previously immunized mice, to study the T-dependent Ig class switching. One week later, antigen-specific IgG levels were measured in the serum of treated mice. As predicted IgG were undetectable in BMT OS sera, whereas GT-treated mice presented an IgG stimulation index comparable to that of BMT WT controls (Fig 5d). Similar to Pneumovax immunization, the extent of the immune response to TNP-KLH correlated with the absolute counts of B and T cells in the spleen (Fig. 5e).

Partial improvement of autoimmune manifestations. Inflammation and autoimmune-like manifestations are peculiar in OS, so we investigated factors associated with autoimmunity. We examined B-cell activating factor (BAFF), which is required for B cell survival and homeostasis and linked to the emergence of autoimmunity when overproduced³⁹. BAFF was elevated in the BMT OS sera, probably due to the lack of its target B lymphocytes. GT mice

exhibited a reduction in serum BAFF levels, although statistically higher than BMT WT (Fig. 6a). Circulating anti-dsDNA autoantibodies were present in 22% of GT mice, while absent in BMT OS likely due to the complete absence of B cells after total body irradiation (Fig. 6b). Furthermore, to extend the analysis to a broader panel of autoantigens, we tested a high-throughput autoantigen microarray platform containing 123 different autoantigens. We did not observe any significant changes in IgM and IgG autoantibodies production in GT treated mice as compared to BMT WT mice (Fig.6c). Conversely, positivity for a broad panel of autoantigens was detected in the sera obtained from untreated Omenn mice tested (Fig. 6d and Online Repository Table E2). A typical OS hallmark is skin erythroderma caused by infiltration of CD3⁺ T lymphocytes and eosinophils and consequently dermal inflammation²⁷. Alteration of keratin layer, immune cell infiltration, thickness of the epidermis and presence of cutaneous abscesses were graded to calculate an arbitrary inflammatory skin score (the higher, the more severe). At the time of sacrifice, BMT OS mice displayed severe skin manifestations. GT mice showed mitigation of the symptoms, although strong variability was observed, as some mice exhibited skin alterations and immune infiltration, whereas others had a completely normal skin phenotype (Fig. 7a,b). These findings were explained by the variability in immune reconstitution achieved with GT. Since VCN could not be calculated in the skin, we correlated the infiltration score with the B and T cell counts in the blood at the time of sacrifice as a measure of post-treatment reconstitution and observed a statistically significant inverse correlation (Fig 7c), indicating that the mice in which the skin inflammation score was higher were those in which T and B cell reconstitution was lower. Overall our findings indicate a substantial, although incomplete, correction of the Omenn phenotype as shown in Table 1.

DISCUSSION

Mutations in *RAG* genes are responsible for a broad spectrum of clinical manifestations. Allogeneic HSCT is the standard of care, ideally performed prior to onset of life-threatening complications and organ damage, but its overall outcome remains unsatisfactory and can be recommended only when a matched donor is available^{12,17,18,40}. In this scenario, GT may represent a powerful alternative approach, as demonstrated by encouraging results obtained in recent clinical primary immunodeficiency (PID) trials⁴¹. Preclinical studies in the *Rag2*^{-/-} model indicated UCOE as the most promising promoter to drive the expression of human *RAG2* transgene²⁶ and, on this basis, we tested whether this approach could rescue defects with residual RAG activity. OS represents a paradigmatic model because of severe inflammation and dysregulated immune environment. We exploited the *Rag2*^{R229Q/R229Q} mouse, faithfully recapitulating the human Omenn syndrome disease. Indeed, *Rag2*^{R229Q/R229Q} mice have an expansion of oligoclonal autoreactive T cells infiltrating organs, including skin and gut, and a virtual absence of circulating B cells in the presence of elevated IgE serum levels^{42,43}. Dramatic alterations in thymic architecture with consequent impairment in negative selection of thymocytes were reported both in mice and in OS patients⁴⁴. Here we used the 2.6kbUCOE-RAG2co vector tested in the *Rag2*^{-/-} murine model²⁶ and in parallel we tested the shortened 2.2 kb version of UCOE²⁸. GT follow-up lasted 28 weeks during which a minor fraction of treated mice died because of lack of immune reconstitution and disease progression, similarly to BMT OS mice (Online Repository Figure E4 and Table E3). Of note, a thymic mass was observed at autopsy in one out of 35 GT treated mice. Immunohistochemical analysis of the thymus revealed a lymphoproliferative expansion of CD3 positive cells in the absence of peripheral CD3 expansion. B cell development in OS mice is partially arrested at the B cell progenitor stage, with consequently reduction in splenic B cells and relative expansion of immature transitional B cells. In the BM, partial B cell subset redistribution was observed in both GT groups. In the periphery, splenic B cells were lower than BMT WT controls, although increased in number as compared to BMT OS mice. Importantly, GT induced a marked reduction in the immature

transitional B cell fraction and relative expansion of MZ B cells that in a lymphopenic environment might indicate increased exposure to blood-borne antigens^{34,45}. In the peripheral blood, transduced B cells appeared and increased over time, remaining stable up to seven months, although at lower frequencies and counts than the BMT WT group. Nonetheless, these results are in line with data reported in the *Rag2* knock-out model²⁶. The 2.2kb UCOE vector showed slower B cell reconstitution, but reached counts comparable to the 2.6kb LV at the latest time point. However, the analysis of serum Ig production indicated a normalization of IgM subtype, while IgG levels were more variable and increased as compared to BMT OS group. Serum IgE levels were reduced in both GT groups and reached levels similar to those of BMT WT mice. Of note, serum BAFF levels were elevated in BMT OS, as well as in OS mice and patients^{9,11}, and diminished in the majority of GT treated mice, although not reaching the BMT WT serum levels. However, the IgM and IgG serum autoantibodies profile did not reveal any gross alterations in GT treated mice. *In vivo* challenges demonstrated that GT mice responded to T cell-dependent and -independent antigens by producing antigen specific antibodies, indicating that the B cells, although reduced in numbers, are able to mount a proper response.

Analysis of VCN in both BM and in spleen indicated a significant correlation between B cell recovery and expression of the transgene. However, VCN higher than 2 did not always correlate with faster B cell reconstitution and higher peripheral B cell counts, suggesting that tightly regulated *Rag2* expression might be required to obtain an adequate B cell development. To overcome this hurdle, gene editing strategies mediated by various nucleases are currently in early-phase development to achieve precise genetic correction and avoid unregulated gene expression⁴⁶. Furthermore, the human *RAG2* expressed by UCOE vectors might prevent complete immune reconstitution in the GT mice. Indeed, species-specific differences might hamper the ability of the human *RAG2* to bind the murine *RAG1* zinc finger regions and form the fully functional *RAG1*-*RAG2* recombinase complex, thus limiting the complex enzymatic activity⁴⁷. Overall, data observed in this preclinical model show partial reconstitution of the B cell compartment, similarly to SCID patients who are functionally agammaglobulinemic and

require intravenous immunoglobulin (IVIG) replacement therapy due to poor B-cell reconstitution, despite successful T cell reconstitution after HSCT and pre-transplant conditioning^{48,49}.

In contrast to B cells, transduced T cell progenitors show a higher selective advantage. A detailed analysis of thymic epithelium indicates a substantial improvement in cortico-medullary demarcation and maturation of mTEC compartment with the expression of AIRE and Fezf2, two independent factors controlling tissue-restricted antigens expression and governing central tolerance mechanisms, defective in OS, leading to the survival of autoreactive T cells^{27,42}. Of note, VCN values indicate a positive correlation with the formation of medulla compartment and RAG2 expression. Consistently, gene-corrected thymocytes increased in numbers, overcoming the block at the DN3 stage and expanding the DP and SP thymocyte populations, demonstrating the occurrence of normal V(D)J recombination. The analysis of VCN in the thymus showed higher values than those detected in total BM, suggesting the existence of a selective advantage for corrected T lymphocyte precursors (Online Repository Fig. E5a and c). Comparable results were obtained in *Rag2*^{-/-} mice, in which a partial correction of the thymic defect was, however, sufficient to substantially improve lymphopenia, peripheral T phenotype and compartmentalization of thymic epithelium²⁶. These findings suggest that additional pharmacological pre-treatments might be needed to improve thymic reconstitution, in particular in the case of OS²⁹. In this regard, it is important to emphasize that the *Rag2*^{-/-} mouse does not present inflammatory and autoimmune features typical of OS that can further affect thymic microenvironment. Important alterations in central tolerance have been described in the murine OS model and in patients, indicating that both the correction of thymic epithelial function and the immunological reconstitution are critical to obtain a significant improvement in peripheral immunopathology. Indeed, lower survival and a higher rate of clinical problems after transplantation have been observed in SCID patients treated after 3.5 months of age, when the thymus is more severely affected¹⁴.

Despite lower absolute counts of circulating and splenic T cells, CD4/CD8 T cell ratio normalized in the periphery. Importantly, T cells, characterized by an effector phenotype in BMT OS, showed a redistribution of subpopulations with

the appearance of naïve CD4⁺ and CD8⁺ T cells. Immunoscope revealed a V β polyclonal distribution that correlated with VCN. *In vitro* T cell proliferation confirmed the functionality of gene corrected T cells, which was similar to those isolated from the BMT WT spleen.

Finally, GT decreased the severity of skin damage, the hallmark of OS. Epidermal and dermal infiltration were reduced in GT mice and the improvement was more evident in those mice with higher B and T cell counts. These findings are clinically relevant as they provide additional proof that T cells with sufficient transgene expression are properly corrected and no longer auto reactive. Our results offer hope for a prospective evaluation of GT intervention in other PIDs characterized by autoimmunity and inflammation, particularly in immune defects caused by hypomorphic mutations in genes of the V(D)J recombination process. In many of these PIDs, several preclinical studies using improved viral vectors are currently ongoing with the ultimate goal to determine their clinical applicability^{50–53}. These preclinical studies will allow understand to what extent inflammation and competition between endogenous uncorrected and corrected progenitors affect immune reconstitution, and will also clarify the effects of deregulated protein expression on the immune system. To overcome problems related to the ectopic, non physiological expression of therapeutic genes^{54–56}, genome editing strategies in hematopoietic stem cells are now emerging. However, gene addition currently remains a valid option for several genetic disorders, as a result of technical advances in vector production, improved transduction, and in maintaining hematopoietic stem cell stemness and fitness^{50,57}. In this regard, the availability of *in vivo* models, such as our *Rag2* hypomorphic mutant, represents a valuable tool for the systematic study of efficacy and safety of novel medical interventions.

In conclusion, our findings provide the rationale to further develop gene therapy and editing approaches even in the presence of inflammation and autoimmunity.

Author contributions

VC, MCC, MB and AV designed and performed research, analysed data, and wrote the paper; EF, SP, ED, RR, BC and NS contributed to perform the experiments; LSS produced the lentiviral vectors; MZ and PC performed spectra typing analysis; PU, KD and LDN contributed to data interpretation; NvT and GW provided the 2.6kb UCOE-RAG2co LV and the 2.2kb UCOE-RAG2co lentiviral vectors and contributed to data interpretation.

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Conflict of interests

We do not have any conflict of interest to declare.

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Table list

Table I. Main outcomes of Omenn Syndrome gene therapy

Online Repository Table E1. Primers for TCR spectratyping

Online Repository Table E2. Statistical analysis of autoantigen arrays.

Online Repository Table E3. Adverse events in GT mice.

Figure legends

Figure 1. Peripheral blood Immune reconstitution. (a) Absolute counts of B (left) and T (right) lymphocytes at the indicated time points after treatment. UT (untreated) CTRL WT and CTRL OS n=17; BMT WT and BMT OS n=16; GT 2.6kbUCOE n=25; GT 2.2kbUCOE n=7. (b) Absolute counts of CD4+ (left) and CD8+ (right) T cells in peripheral blood at sacrifice in untreated (CTRL WT and CTRL OS) or transplanted (BMT WT, BMT OS and GT) mice. (c) Ratio between CD4+ and CD8+ T cells at sacrifice. (d) Absolute counts of the white blood cells (WBC) at the indicated time points after treatment. UT (untreated) CTRL WT and CTRL OS n=17; BMT WT and BMT OS n=16; GT 2.6kbUCOE n=25; GT 2.2kbUCOE n=7. All data show mean $\pm$ SD. Statistical significance was determined by non-parametric one-way ANOVA with Dunns post-test excluding untreated groups. * p<0.05, ** p<0.01, *** p<0.001.

Figure 2. B cell development and functionality. (a) Relative frequencies of pre-B, immature and mature recirculating B populations in bone marrow (BM). BMT WT n=16, BMT OS n=11; GT 2.6kbUCOE n=21; GT 2.2kbUCOE n=7 (b) Counts of mature recirculating B cells in BM. (c) Counts of B lymphocytes in the spleen. (d) Relative frequencies of transitional, marginal and follicular B populations in the spleen. BMT WT n=16, BMT OS n=10; GT 2.6kbUCOE n=21; GT 2.2kbUCOE n=7. (e) Levels of IgM, IgA, IgG and IgE in the sera of transplanted mice at sacrifice. Statistical significance was determined by non-parametric combination test (panels a and d) or non-parametric one-way ANOVA with Dunns post-test (panels b, c and e). * p<0.05, ** p<0.01, *** p<0.001. All data depicted are mean $\pm$ SD.

Figure 3. Thymic characterization. (a) Histological analysis of thymic sections stained with hematoxylin and eosin, cytokeratin 5 and cytokeratin 8 of representative BMT WT, BMT OS and GT 2.6kbUCOE mice. Vector copy number/genome (VCN) in the thymus is indicated for GT mice. Scale bar: 500 μ m. (b) Ratio between thymic medullary and cortical areas. (c) Correlation between the medulla/cortex ratio and VCN of GT 2.6kbUCOE (squares) and GT 2.2kbUCOE (triangles). Spearman r value is indicated. (d) Counts of

845 AIRE-positive cells in the analysed thymic sections (in mm²). (e) Counts of
 846 Fexf2-positive cells in the analysed thymic sections (in mm²). (f) Thymic
 847 cellularity (cell counts). (g) Correlation between vector copy number/genome
 848 (VCN) and vector-induced human codon-optimized (co) Rag2 expression in
 849 thymocytes of GT 2.6kbUCOE (squares) and GT 2.2kbUCOE (triangles).
 850 Spearman r value and statistical significance are indicated. (h) Distribution of
 851 double negative (DN), double positive (DP), single positive CD4 and CD8
 852 cells in thymus (mean $\pm$ SD). BMT WT n=16, BMT OS n=10; GT 2.6kbUCOE
 853 n=20; GT 2.2kbUCOE n=6. Statistical significance was determined by non-
 854 parametric one-way ANOVA with Dunns post-test (panels b, d, e and f), by
 855 Spearman correlation (panels c and g) or non-parametric combination test
 856 (panel h). * p<0.05, ** p<0.01, *** p<0.001. Error bars represent standard
 857 deviation.

858
 859 **Figure 4. T cell development and functionality.** (a) Absolute counts of T
 860 cells. (b) Ratio between CD4+ and CD8+ T cells. (c) Distribution of naïve,
 861 effector and memory subpopulations in CD4+ (left) and CD8+ (right) T cells.
 862 BMT WT n=16, BMT OS n=9; GT 2.6kbUCOE n=21; GT 2.2kbUCOE n=7.
 863 (d) Percentage of CD8+ T cells that proliferate in response to the indicated
 864 stimuli. (e) TCR repertoire analysis in spleen cells. Eleven V β families of
 865 representative BMT WT, BMT OS and GT 2.6kbUCOE mice (with low,
 866 medium and high VCN) are shown. Statistical significance was determined by
 867 non-parametric one-way ANOVA with Dunns post-test (panels a and b) or
 868 non-parametric combination test (panel c). * p<0.05, ** p<0.01, *** p<0.001.
 869 All data show mean $\pm$ SD.

870
 871 **Figure 5. In vivo challenges.** (a) Stimulation index of Pneumovax-specific
 872 IgM, calculated as the ratio of IgM produced at day 21 after vaccination and
 873 before immunization. (b) Correlation between stimulation index of
 874 Pneumovax-specific IgM and absolute count of splenic B cells in BMT WT
 875 (black dot), BMT OS (empty circle) and GT 2.6kbUCOE (squares). Spearman
 876 r value is indicated. (c) Stimulation index of TNP-KLH-specific IgM, calculated
 877 as the ratio of IgM produced 21 days after immunization and before
 878 immunization. (d) Stimulation index of TNP-KLH-specific IgG, calculated as
 879 the ratio of specific IgG produced at day 28 and before the boosting dose. (e)
 880 Correlation between stimulation index of TNP-KLH-specific IgG and absolute
 881 count of splenic B (left) or T cells (right). Spearman r value is indicated for
 882 each graph. Statistical significance was determined by non-parametric one-
 883 way ANOVA with Dunns post-test (panels a, c and d) or by Spearman
 884 correlation (panels b and e). * p<0.05, ** p<0.01, *** p<0.001. All data show
 885 mean $\pm$ SD.

886
 887 **Figure 6. Autoimmune manifestations.** (a) Levels of BAFF (B-cell activating
 888 factor) in the sera at sacrifice. Statistical significance was determined by non-

parametric one-way ANOVA with Dunns post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(b)** Frequency of mice with presence (white) or absence (black) of anti-dsDNA autoantibodies in sera (BMT WT $n=14$, BMT OS $n=10$, GT 2.6kbUCOE $n=18$, GT 2.2kbUCOE $n=5$) **(c)** Heatmap representation of relative autoantibody IgM and IgG reactivity against 123 antigens tested in BMT WT, GT and Systemic lupus erythematosus (SLE) mice. Colours correspond to normalized (z-score) expression levels. **(d)** Heatmap representation of relative autoantibody IgM and IgG reactivity against 86 antigens tested in untreated WT and Omenn Syndrome mice. Colours correspond to normalized (z-score) expression levels.

Figure 7. Skin infiltration. **(a)** Histological analysis of skin sections stained with hematoxylin and eosin (left) of representative BMT WT, BMT OS and GT 2.6kbUCOE mice. In the upper right picture, eosinophils are visible at higher magnification; in the lower right picture, T cell infiltration in CD3-stained sections. Scale bars represent 200 μm in left picture and 50 μm in right pictures. **(b)** Arbitrary score of skin manifestations, calculated as described in methods. **(c)** Correlation between skin infiltration score and absolute count of B (top) and T cells (bottom) in the peripheral blood of BMT WT (black dot), BMT OS (empty circle) and GT 2.6kbUCOE (squares). Spearman r value is indicated for each graph. Statistical significance was determined by non-parametric one-way ANOVA with Dunns post-test (panel b) or by Spearman correlation (panel c). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Online Repository Figure E1. VCN and cell counts correlations. **(a)** Correlation between vector copy number/genome (VCN) and absolute counts of mature recirculating B cells in the bone marrow (left) and in the spleen (right). Spearman r value and statistical significance are indicated for each graph. **(b)** Correlation between vector copy number/genome (VCN) and vector-induced human codon-optimized (co) Rag2 expression in spleen cells of GT 2.6kbUCOE (squares) and GT 2.2kbUCOE (triangles). Spearman r value and statistical significance are indicated. **(c)** Absolute counts of marginal zone (left) and follicular (right) B cells in the spleen. **(d)** Correlation between vector copy number/genome (VCN) and absolute counts of thymocytes (left) and splenic T cells (right) of GT 2.6kbUCOE (squares) and GT 2.2kbUCOE (triangles). Spearman r value and statistical significance are indicated for each graph (ns, not statistically significant). Statistical significance was determined by Spearman correlation (panels a, b and c) or by non-parametric one-way ANOVA with Dunns post-test (panel c). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Online Repository Figure E2. Thymic reconstitution. **(a)** Histological analysis of thymic sections stained with Ulex europaeus agglutinin I (UEA), AIRE and Fezf2 of representative BMT WT, BMT OS and GT 2.6kbUCOE

mice (VCN range in the thymus is indicated for each representative GT group). Scale bar: 100 μ m. **(b)** Correlation between medulla/cortex ratio and AIRE-positive (left) of Fexf2-positive (right) cells in the analysed thymic sections (mm^2). Spearman r value is indicated for each graph. Statistical significance was determined by Spearman correlation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Online Repository Figure E3. T cell proliferation. Representative FACS plot overlays show proliferation of CD4⁺ (left) and CD8⁺ (right) splenic cells of BMT WT, BMT OS and GT mice, after stimulation with anti-CD3, antiCD3+IL-2, PMA+Ionomycin and Concanavalin A.

Online Repository Figure E4. Survival. Kaplan-Meier survival curves for BMT WT (n=16), BMT OS (n=16), GT 2.6kbUCOE (n=27) and GT 2.2kbUCOE (n=8) mice.

Online Repository Figure E5. Vector copy number. **(a)** VCN in the bone marrow (BM), spleen and thymus of GT 2.6kbUCOE (squares) and GT 2.2kbUCOE (triangles). **(b)** VCN obtained in Lin⁻ cells transduced with 2.6kb UCOE LV (n=6) or with 2.2kbUCOE (n=1) after 10 days of *in vitro* culture. **(c)** VCN in sorted B, T and myeloid cells of the BM (black squares) and spleen (empty squares) of GT 2.6kbUCOE mice, calculated by digital droplet PCR. Statistical significance was determined by non-parametric one-way ANOVA with Dunns post-test. * $p < 0.05$, ** $p < 0.01$.

Table I. Main outcomes of Omenn Syndrome (OS) gene therapy

Outcomes	Untreated OS mice	OS mice after GT
<i>B cell counts</i>	Absent or very low	Improved but lower than BMT WT mice
<i>B cell phenotype</i>	Blocked at early stage of differentiation	Normal maturation
<i>T cell counts</i>	Strongly Reduced	Improved but lower than BMT WT mice
<i>T cell phenotype</i>	Highly activated and exhausted with oligoclonal skewed repertoire	Presence of naïve cells, able to respond to stimuli and with skewed to polyclonal phenotype
<i>IgM and IgG levels</i>	Very low	Normal
<i>IgE levels</i>	Normal to high	Normal
<i>Thymus</i>	Absence of medulla; very low thymocyte counts	Low to normal medullary area, in correlation with VCN; improved thymocyte counts
<i>Vaccination response</i>	Unable to respond to vaccination	Able to mount proper antigen response
<i>Autoantibodies</i>	Low to high	Low or absent
<i>Skin infiltration</i>	Moderate to severe	Absent to moderate

Fig1. Peripheral blood immune reconstitution

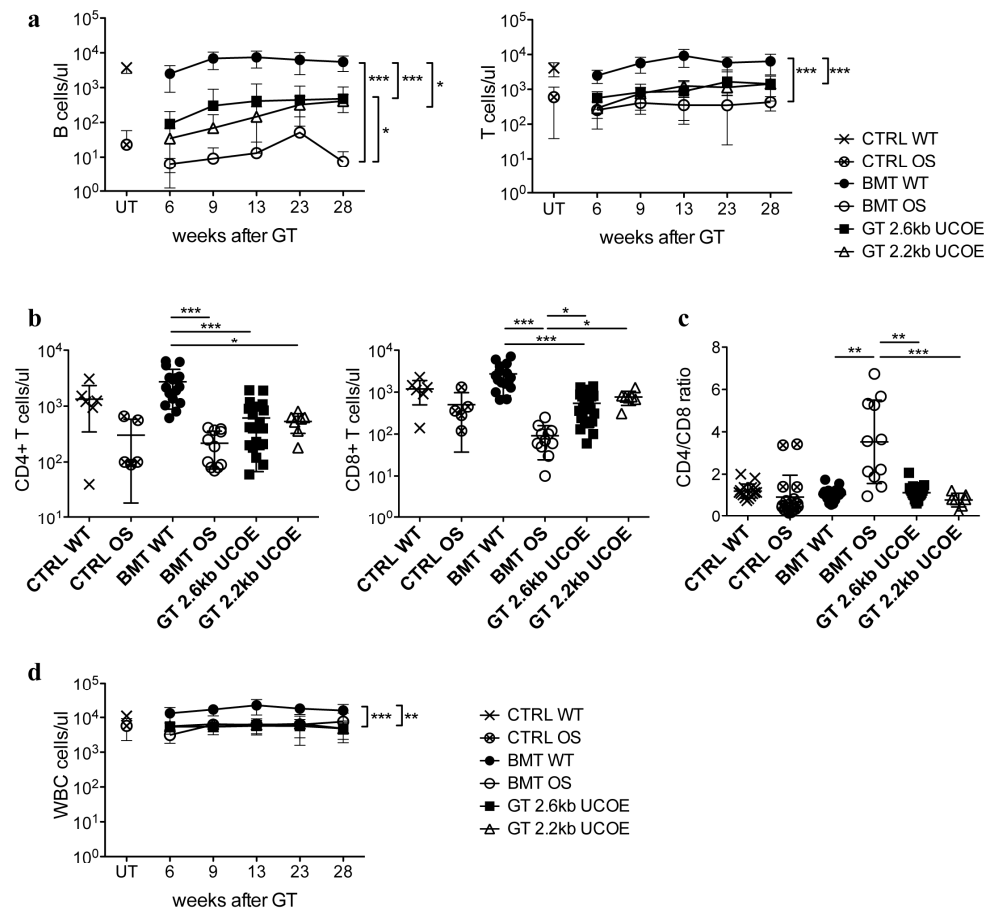


Fig2. B cell development and functionality

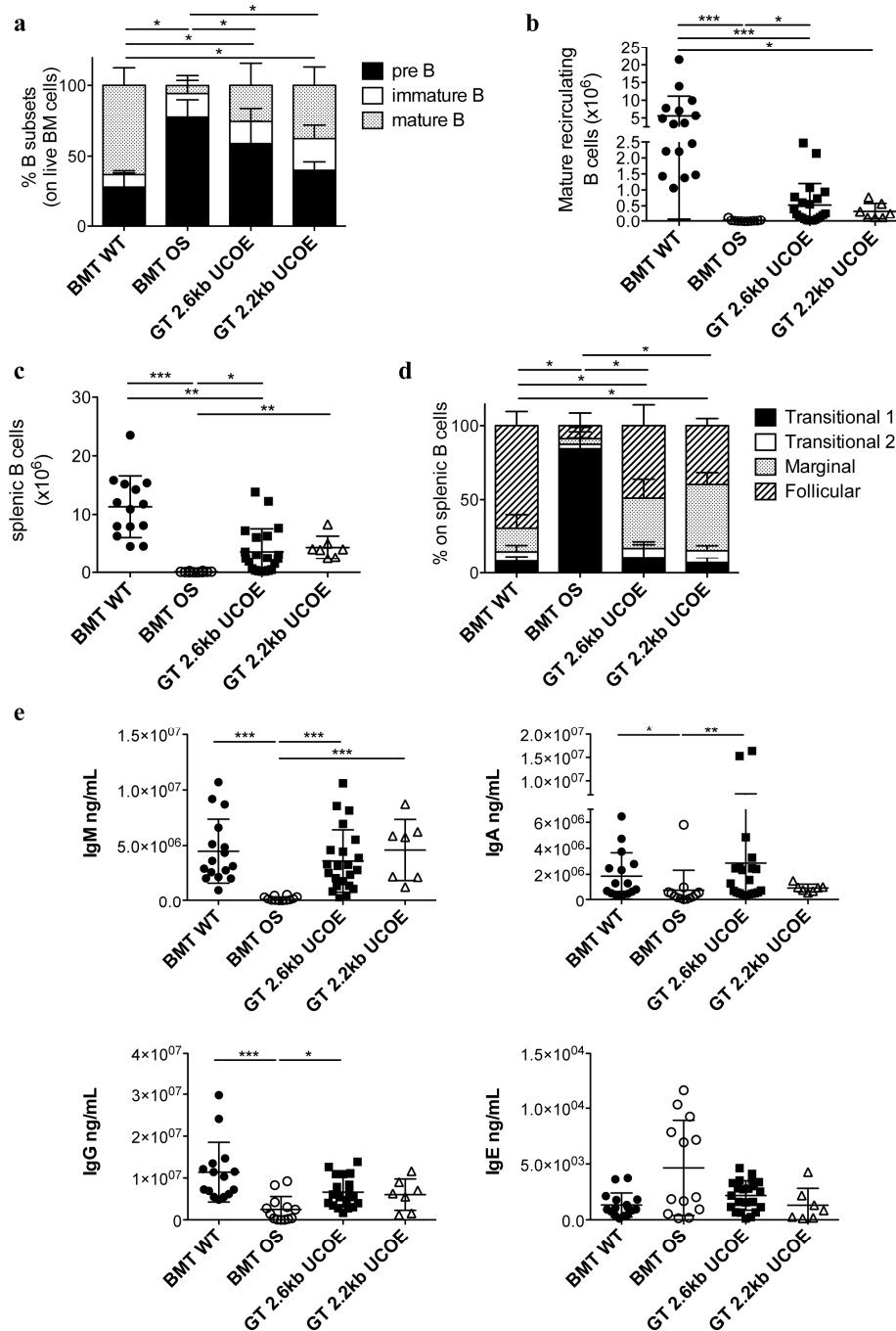


Fig3. Thymic characterization

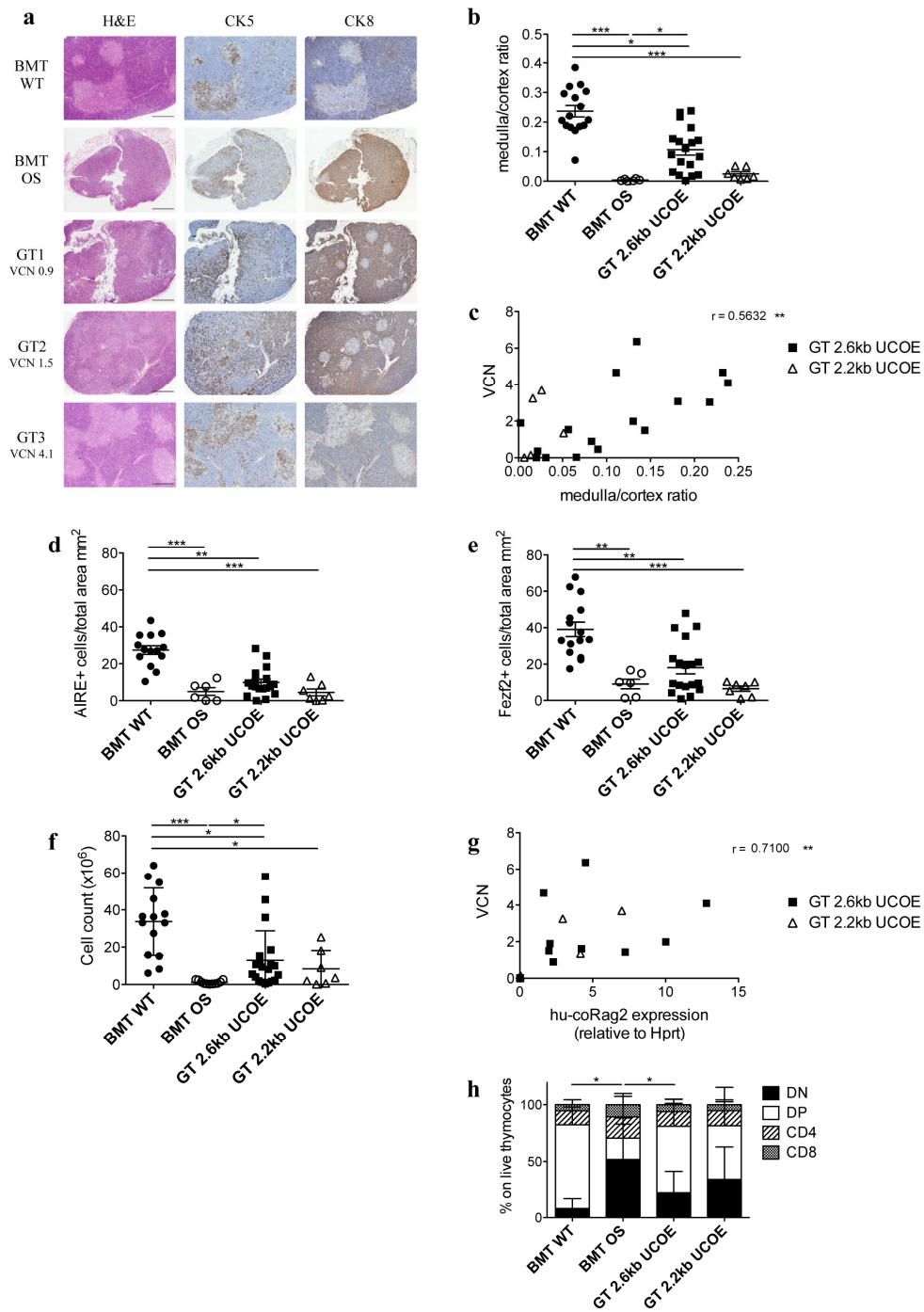


Fig4. T cell development and functionality

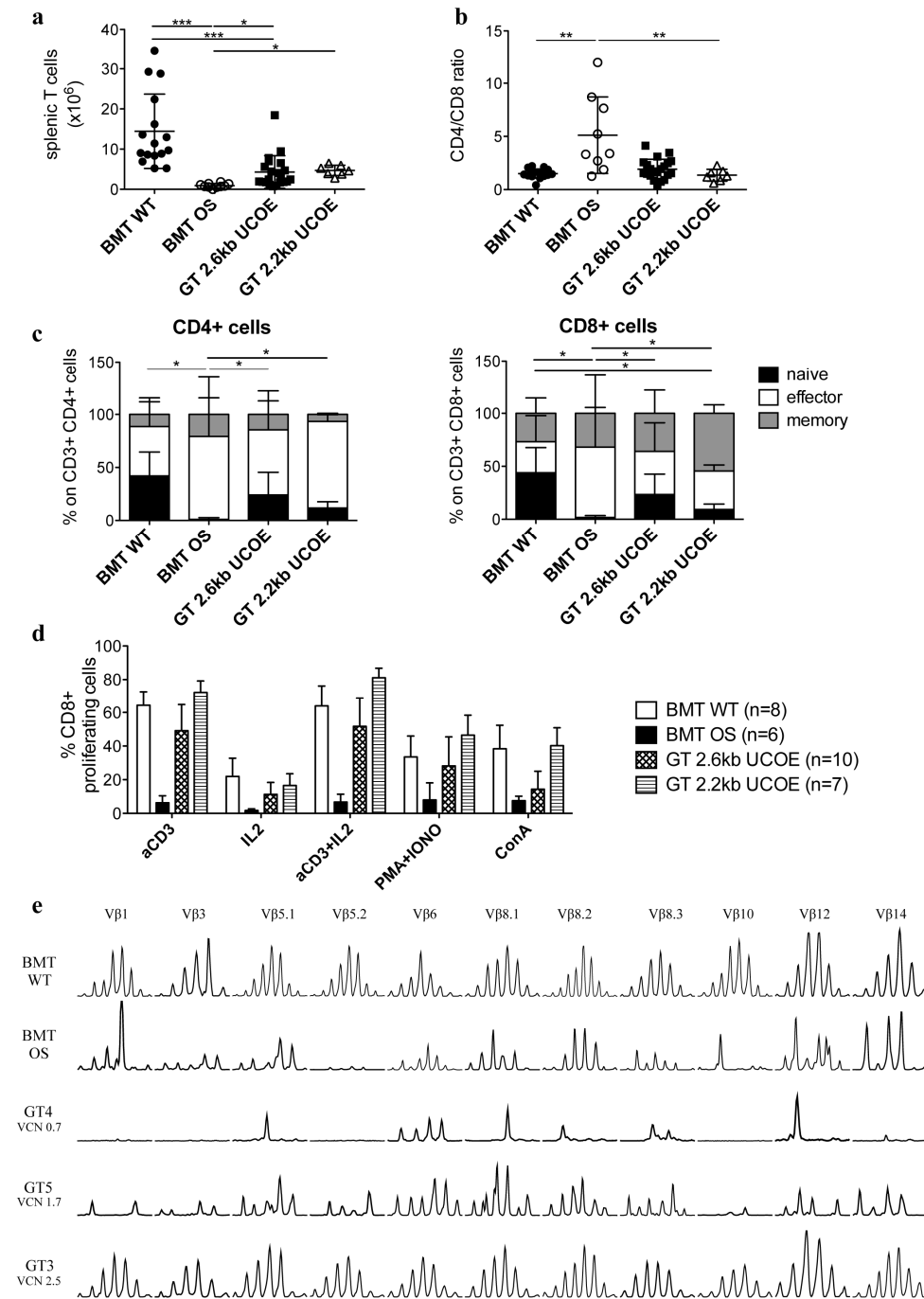


Fig5. *In vivo* challenges

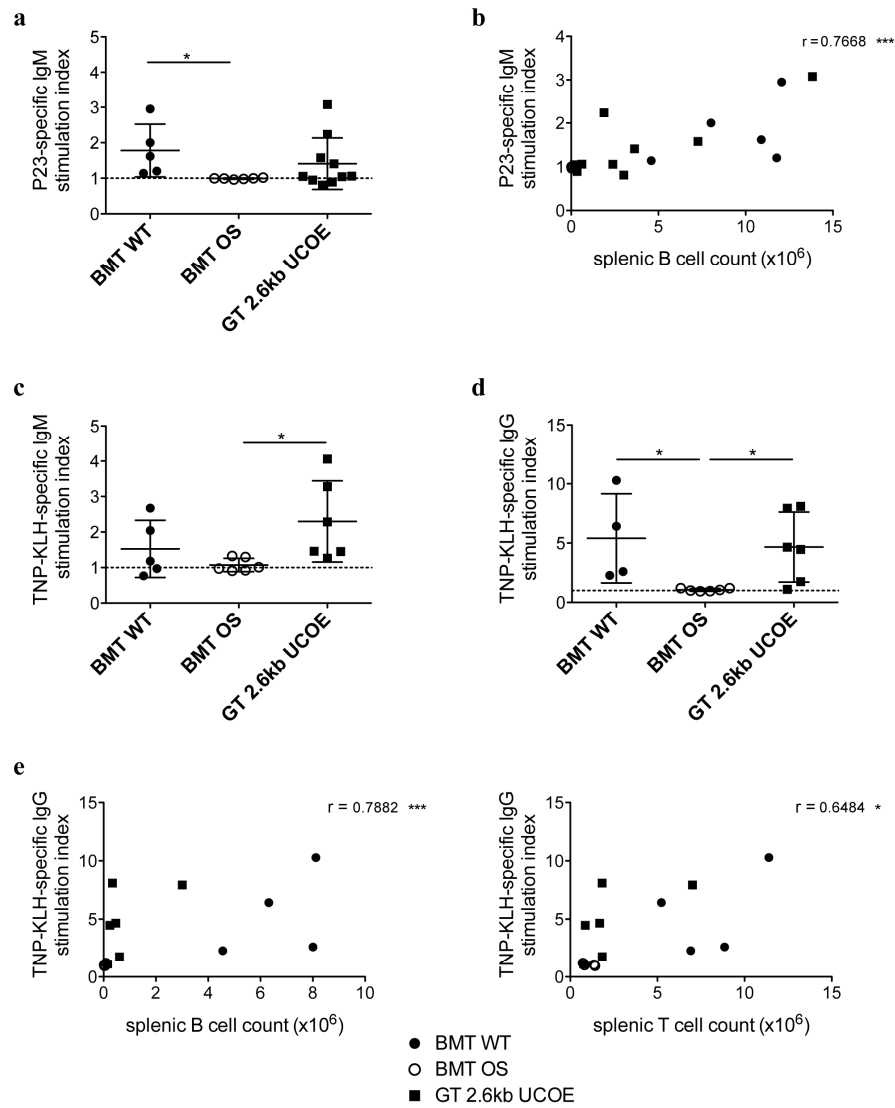


Fig6. Autoimmune manifestations

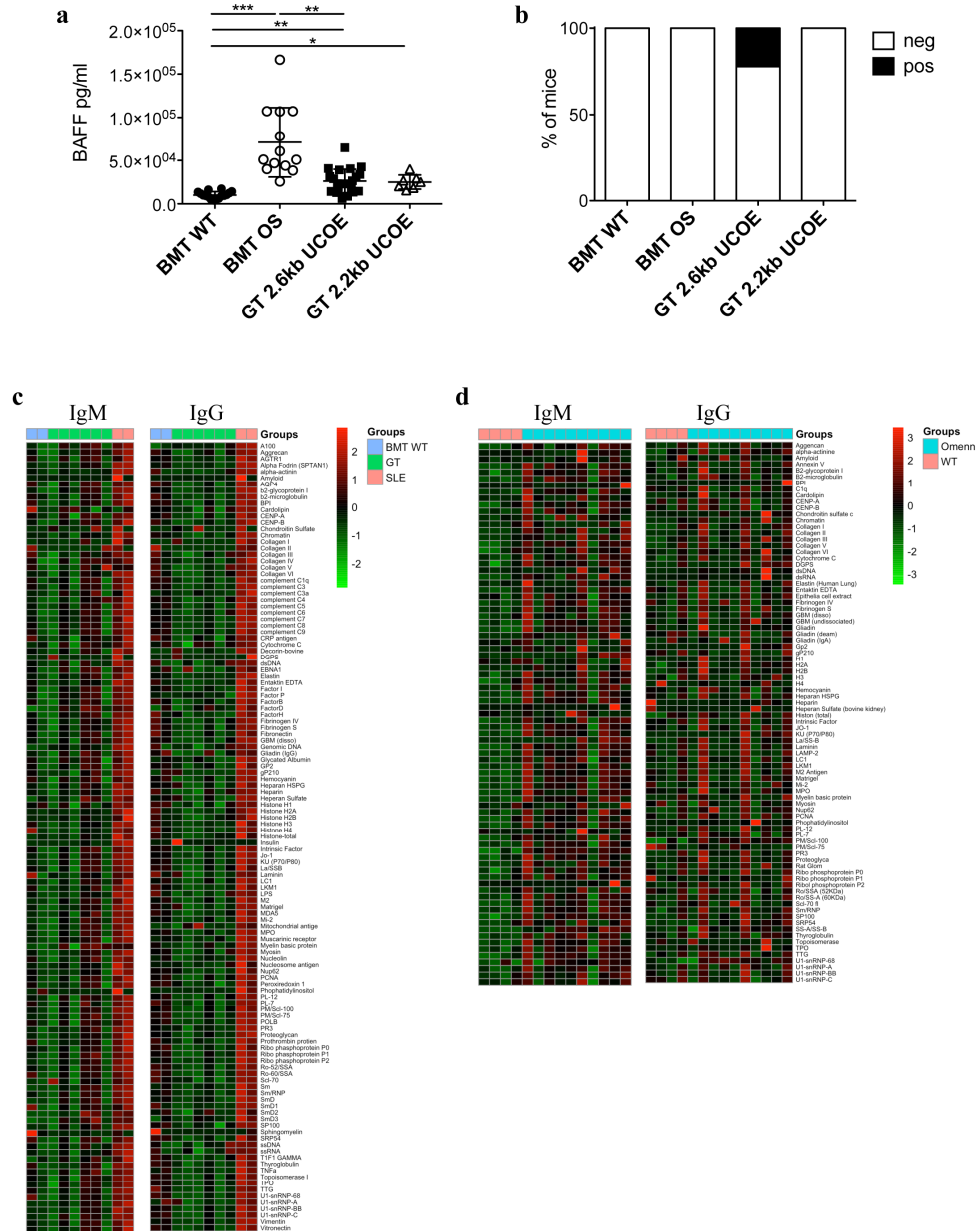
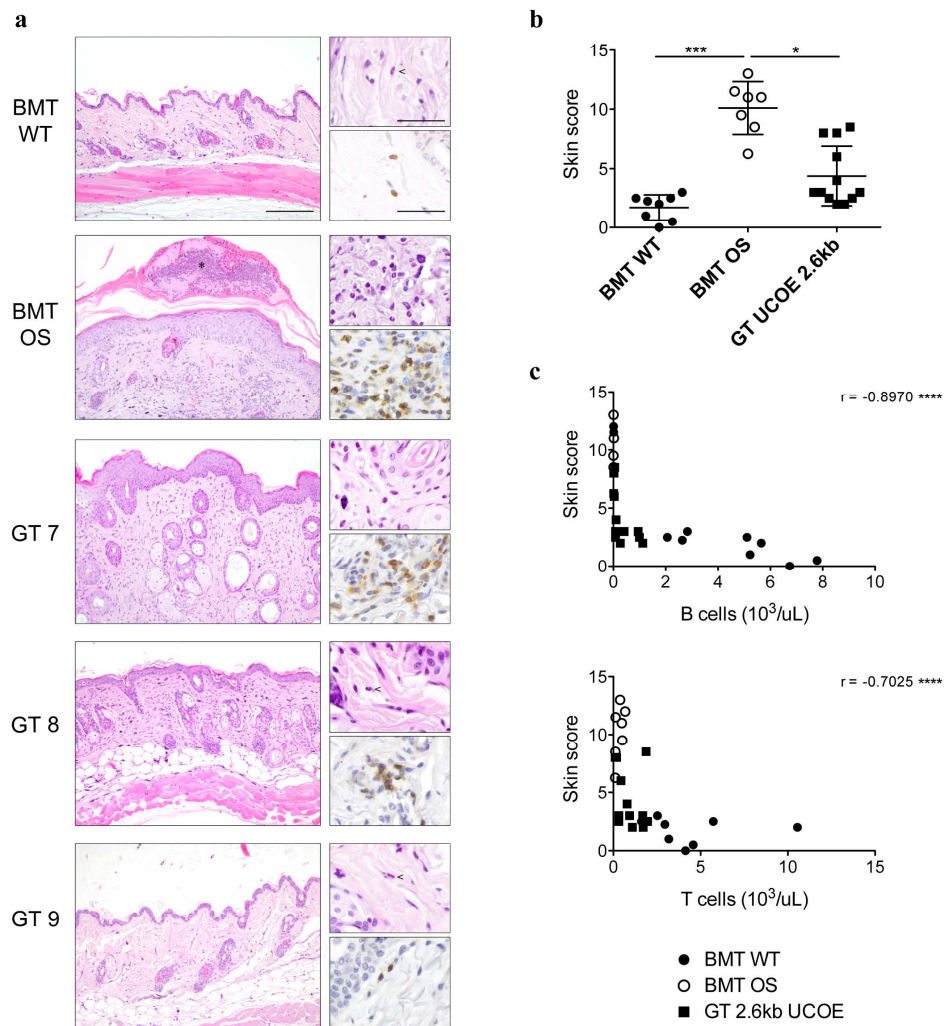


Fig7. Skin infiltration



Online Repository Table E1. Primers for TCR spectratyping

Name	Sequence (5'-3')	Modification
CB FAM	TTG GGT GGA GTC ACA TTT CTC	5' 6-FAM
muVb1	CAG ACA GCT CCA AGC TAC TTT TAC	
muVb2	ATG AGC CAG GGC AGA ACC TTG TAC	
muVb3	GAA ATT CAG TCC TCT GAG GCA GGA	
muVb4	CTA AAG CCT GAT CAC TCG GCC ACA	
muVb5.1	CCT TGG AGC TAG AGG ACT CTG CCG	
muVb5.2	CCT TGG AAC TGG AGG ACT CTG CTA	
muVb5.3	CCT TGG ACC TAG AGG ACT TTA CTG	
muVb6	GCC CAG AAG AAC GAG ATG GCC GTT	
muVb7	GGA TTC TGC TAA AAC AAA CCA GAC ATC	
muVb8.1	GCT TCC CTT TCT CAG ACA GCT GTA	
muVb8.2	GCT ACC CCC TCT CAG ACA TCA GTG	
muVb8.3	GGC TTC TCC CTC TCA GAC ATC TT	
muVb9	CTC TCT CTA CAT TGG CTC TGC AGG	
muVb10	CTT CGA ATC AAG TCT GTA GAG CCG G	
muVb11	TGA AGA TCC AGA GCA GCG GGC CCC	
muVb12	CCA CTC TGA AGA TTC AAC CTA CAG AAC CC	
muVb13	CAA GAT CCA GTC TGC AAA GCA GGG	
muVb14	GCA CGG AGA AGC TGC TTC TCA GCC	
muVb15	GCA TAT CTT GAA GAC AGA GGC	
muVb16	CTC TGA AAA TCC AAC CCA CAG CAC TGG	
muVb17	TCT GAA GAA GAC GAC TCA GCA CTG	
muVb18	GCA AGG CCT GGA GAC AGC AGT ATC	

Online Repository Table E2. Statistical analysis of autoantigen arrays

Pair	IgM p-value	IgG p-value
BMT WT vs GT	0,3129	0,0204
BMT WT vs SLE	0,0647	0,0588
GT vs SLE	0,0357	0,0000
WT vs Omenn	0,0000	0,0400

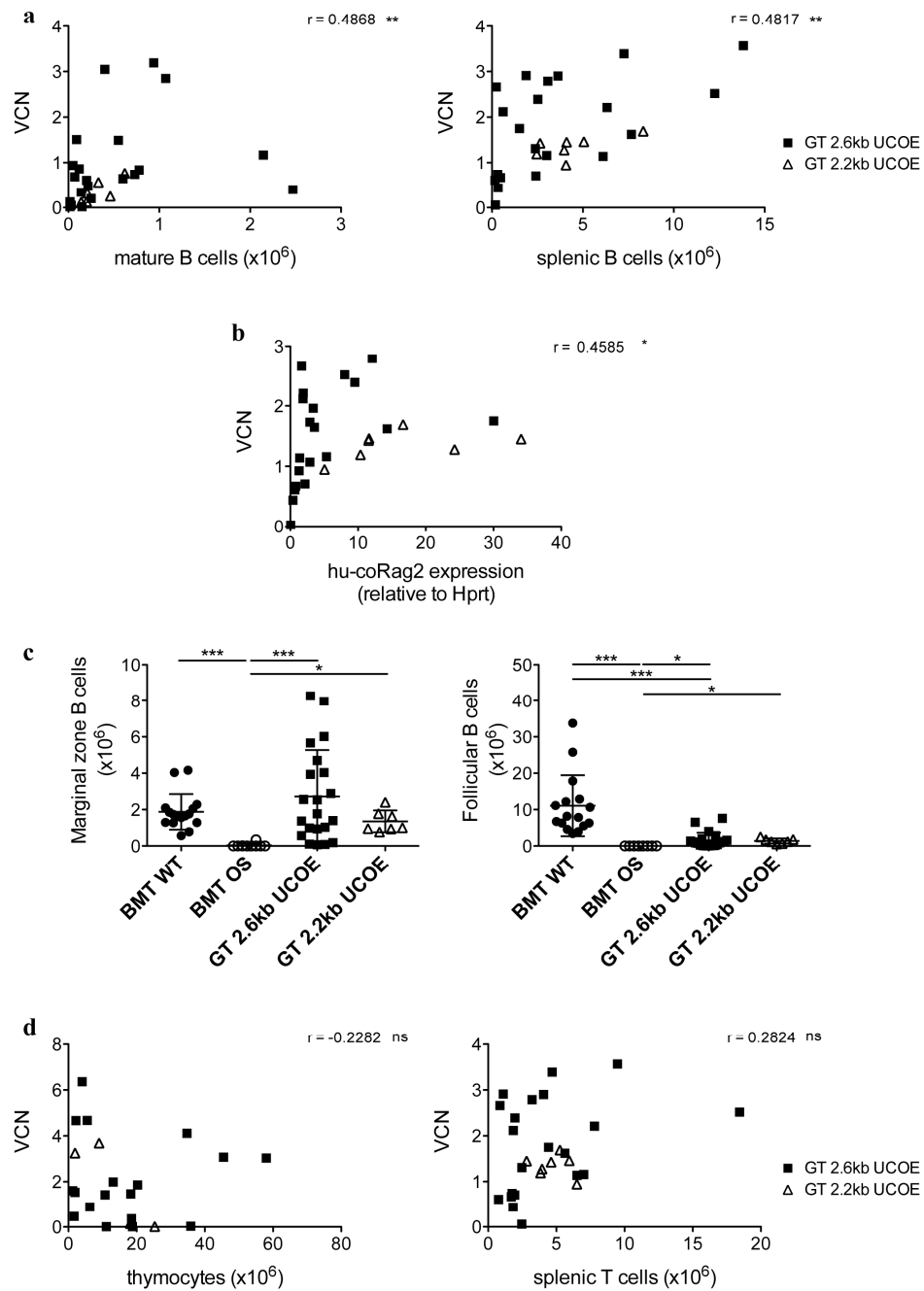
Note: To assess global differences in expression between groups we computed p-values using the global ANCOVA method (Hummel M, Meister R, Mansmann U. GlobalANCOVA: exploration and assessment of gene group effects. Bioinformatics. 2008;24(1):78–85. doi: 10.1093/bioinformatics/btm531. pmid:18024976)

For groups with n<4, approximate p-values were used. For larger groups, p-values were calculated permutation-based.

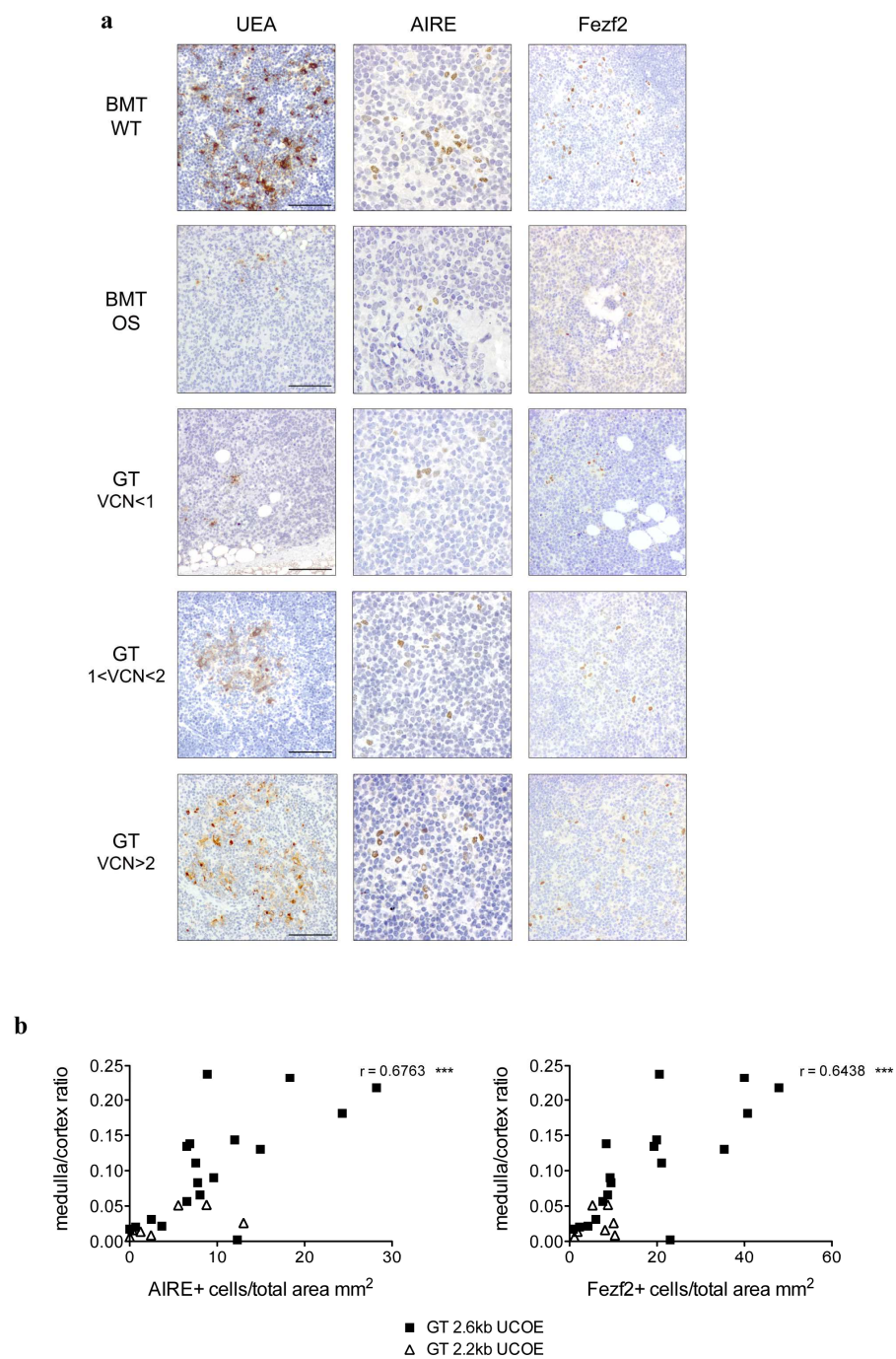
Online Repository Table E3. Adverse events in GT mice.

Mouse no	Vector	Death (weeks post GT)	Observations	Immune reconstitution
1	2.2kb UCOE	2	Probable lack of engraftment	Not evaluated
2	2.6kb UCOE	2	Probable lack of engraftment	Not evaluated
3	2.6kb UCOE	5	Dental malocclusion	Not evaluated
4	2.6kb UCOE	14	Thymic mass	Good (VCN 1.3 in BM, 2.0 in spleen)
5	2.6kb UCOE	17	No major findings	Poor (VCN 0.3 in BM, 0.4 in spleen)
6	2.6kb UCOE	27	No major findings	Intermediate

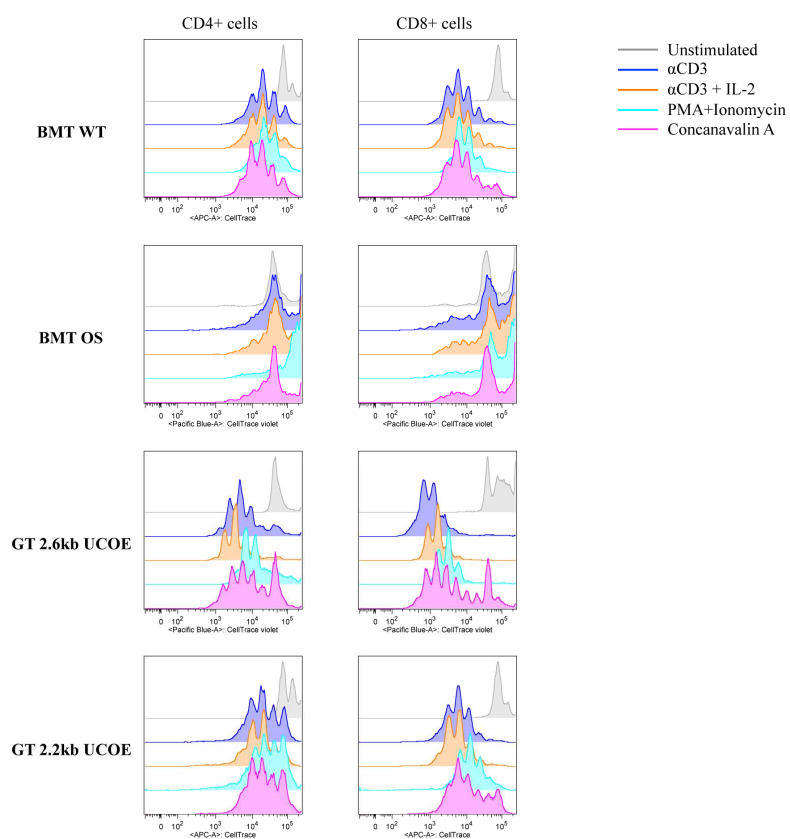
Online Repository Figure E1. VCN and cell counts correlations



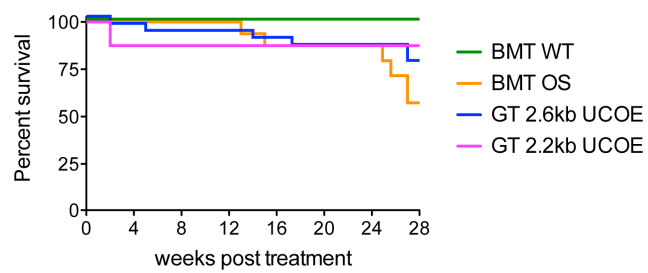
Online Repository Figure E2. Thymic reconstitution



Online Repository Figure E3. T cell proliferation



Online Repository Figure E4. Survival



Online Repository Figure E5. Vector copy number

