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CTLA4-Ig effectively controls immune activation and inflammatory disease in a novel murine model of leaky SCID

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ABSTRACT:

Background: Severe combined immunodeficiency (SCID) can be caused by loss-of-function mutations in genes involved in the DNA recombination machinery, such as *RAG1*, *RAG2* or *DCLRE1C*. Defective DNA recombination causes a developmental block in T cells and B cells, resulting in high susceptibility to infections. Hypomorphic mutations in the same genes can also give a partial loss of T cells, in a spectrum including leaky SCID (LS) and Omenn syndrome (OS). These patients not only develop life-threatening infections due to immunodeficiency, but also develop inflammatory/autoimmune conditions due to the presence of autoreactive T cells.

Objective: We sought to develop a preclinical model that fully recapitulates the symptoms of LS/OS patients including a model for testing therapeutic intervention.

Methods: We generated a novel mutant mouse (*Dclre1c^{leaky}*) that develops a LS phenotype. Mice were monitored for diseases and immune phenotype and immune function were evaluated using flow cytometry, ELISA and histology.

Results: *Dclre1c^{leaky}* mice present with a complete blockade of B cell differentiation, with a leaky block in T cell differentiation resulting in an oligoclonal TCR repertoire and enhanced cytokine secretion. *Dclre1c^{leaky}* mice also developed inflammatory symptoms including wasting, dermatitis, colitis, hypereosinophilia and high IgE levels. Development of a preclinical murine model for LS allowed the testing of potential treatment, with administration of CTLA4-Ig reducing disease symptoms and immunological disturbance, resulting in increased survival.

Conclusion: These data suggest that CTLA4-Ig should be evaluated as a potential treatment of inflammatory symptoms in LS and OS patients.

Key message:

- *Dclre1c*^{leaky} mouse recapitulates the symptoms and immunological features of LS/OS patients.
- CTLA4-Ig is efficiently controlling inflammation of these mice through a tight regulation of the CD4⁺ T cells.

Capsule summary: CTLA4-Ig controls immune dysregulation in an Artemis deficient mouse model that recapitulates leaky SCID/ Omenn Syndrome clinical manifestations.

Key words: leaky-SCID, Artemis, Treg, CTLA4, immune dysregulation.

Abbreviation: BCR, B cell receptor; CTLA4-Ig, Cytotoxic T-lymphocyte protein 4; DCLRE1C, DNA cross-link repair 1C; ENU, *N*-ethyl-*N*-nitrosourea; LIG4, DNA Ligase 4; LS, Leaky-SCID; OS, Omenn Syndrome; RAG, recombination activating gene; SCID, Severe combined immunodeficiency; TCR, T cell receptor; Treg, regulatory T cells.

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INTRODUCTION

Severe combined immunodeficiency disorders (SCID) are characterized by a profound defect in the adaptive immune system with a partial or total loss of function of T cells and B cells. Patients are highly susceptible to severe opportunistic infections and their survival expectancy is limited unless they receive early allogeneic hematopoietic stem cell transplantation. An important cause of SCID is mutation in genes such as *RAG1*, *RAG2*, *DCLRE1C*, *LIG4*, which produce proteins that are part of the DNA recombination machinery. During the V(D)J recombination of T cell receptor (TCR) or B cell receptor (BCR), *RAG1* and *RAG2* recognize the recombination-signal sequence (RSS) and cleave double stranded DNA, followed by the formation of hairpin loop at the end of the open DNA. In complex with other proteins, *ARTEMIS* (product of *DCLRE1C*) then cleaves these hairpin loops to allow the ligation of the newly formed DNA strand by DNA LIGASE IV (product of *LIG4*). In the absence of any of these components, defective V(D)J recombination blocks the formation of a functional TCR or BCR, leading to an early block of T and B cell differentiation¹.

In addition to the typical SCID phenotype, hypomorphic mutations in *RAG1*, *RAG2* and *DCLRE1C* genes can cause Omenn syndrome (OS) and leaky SCID (LS). OS is characterized by early and severe inflammatory symptoms, including generalized erythrodermia, diarrhea, hepatosplenomegaly, lymphadenopathy with hyperoesinophilia and elevated IgE levels. Unlike SCID, T cells are detected in the blood of patients in absence of maternal engraftment, and demonstrate peripheral activation and oligoclonal expansion of self-reactive T cells². LS patients show even higher levels of T cells in the blood (although still reduced relative to healthy

individuals), of an oligoclonal nature ². LS patients may present with a later clinical onset of the disease with combined immunodeficiency (CID) and immune dysregulation leading to autoimmune manifestations, mostly autoimmune cytopenia. Both OS and LS patients are highly susceptible to develop cancer from a lymphoid origin ³. In most OS and LS patients, residual activity of the protein is detected. Specifically, large deletions and mutations in the catalytically functional domains (β -lactamase and β -CASP domains) of the ARTEMIS protein lead to an absence of protein and SCID, while mutations in the C-terminal domain can result in the OS/LS phenotypes ⁴. OS and LS appear to lie on a spectrum of immunodeficiency, with potential for similar mutations to manifest with different clinical presentations ^{3,5}.

A diverse set of hypotheses have been formulated to explain the co-existence of autoimmunity and immunodeficiency in OS/LS ⁶. However the lack of preclinical models for LS/OS with Artemis deficiency has restricted both a functional understanding of the immune processes occurring, and also the testing of therapeutic tools. Here we developed a novel LS mouse model, caused by a hypomorphic point mutation in *Dclre1c*. Mutant mice develop a macroscopic phenotype similar to patients with LS, including the presence of peripheral T cell lymphocytes, the development of spontaneous inflammatory diseases and a high incidence of lymphoma development. Importantly, administration of the immunosuppressive drug CTLA4-Ig resulted in control of the inflammation and improved disease free-survival in comparison to control mice, identifying CTLA4-Ig as a potential treatment for leaky SCID patients.

METHODS

Mice

To generate the *Dclre1c*^{leaky} strain, founder C57BL/6 male mice were treated with 100 mg/kg ENU and bred to *Foxp3*^{GFP} females⁷. First-generation male offspring were backcrossed to *Foxp3*^{GFP} females to produce the second-generation offspring, which were in turn inter-crossed to produce the third generation for phenotype screening. Phenotypic screening involved flow cytometric analysis for CD4 and Foxp3 (GFP), and identification of mice with values for the percentage of GFP+ cells (within the CD4+ population) more than two standard deviations from the norm. Identified variant offspring were inter-crossed to produce the C57BL/6.*Foxp3*^{GFP}.*Dclre1c*^{leaky} mutant mouse strain. The *leaky* mutation identified by all-exome sequencing and confirmed by Sanger sequencing.

CTLA4-Ig (Abatacept, Oncia, Bristol-Myers Squibb) was administered to C57BL/6.*Foxp3*^{GFP}.*Dclre1c*^{leaky} and littermate controls starting at 8 weeks of age at a dose of 25mg/kg intraperitoneally every other week. Mice were maintained in specific pathogen-free facilities of University of Leuven. All experiments were approved by the University of Leuven ethics committee.

Western Blotting

Thymocytes were homogenized in lysis buffer consisting of 100 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 2 mM dithiothreitol, 1mM EDTA, Phosphatase inhibitor (Sigma-Aldrich) and Protease inhibitor (Pierce), then incubated on ice for 30 min. Cells were sonicated and lysate prepared. The NuPAGE® Precast Gel System was used for western blotting (Life technologies). 10-20 µg of lysate were separated on 4-12% bis-tris acrylamide gels and blotted on a PVDF membrane (GR Health care). Membranes were incubated using polyclonal IgG rabbit anti-mouse/human

ARTEMIS (1:1000, Thermo Scientific, PA5-26741) and mouse anti-GAPDH (1:10000, Thermo Scientific, GA1R) and developed using western Lightning Plus-ECL (Perkin Elmer) and the imaging system G:Box XRQ (Syngene). Quantification was performed using the AIDA software (Raytest, version 5.0).

TCR V β CDR3-size spectratype analysis

Total splenocytes were harvested and conserved in RNA Later stabilization reagent (Trizol, Thermo Fisher scientific). RNA was then extracted using the RNEasy mini kit (Qiagen) following the manufacturer's instructions. Genomic DNA was removed using recombinant RNase-free DNaseI (Roche). cDNA was synthesized from RNA (2 μ g) using oligo(dT)₁₈ primers with the "Transcriptor First Strand cDNA synthesis kit" (Roche). Semi-nested PCR was performed using sense primers for a panel of murine V β families and two C β anti-sense primers (IDT Technologies), as previously described. CDR3-size spectratype analysis was performed with GeneMapper version 4.0 Software (Applied Biosystems).

Flow cytometry

Single-cell suspensions were prepared from mouse thymus, bone marrow, spleen and pooled lymph nodes (cervical, inguinal, mesenteric, axillary and brachial). For intracellular cytokine staining, lymphocytes were plated at 5×10^5 cells/well in 96-well tissue culture plates in complete RPMI containing phorbol myristate acetate (50 ng/mL; Sigma-Aldrich), ionomycin (250 ng/mL; Sigma-Aldrich) and Monensin (1/1500; BD, Bioscience) for 4 hours at 37°C. All cells were fixed with BD Cytofix™ (BD, Biosciences) or fixed and permeabilized with the eBioscience Foxp3 staining kit (eBioscience/Affymetrix). Anti-murine antibodies included anti-CD4 (RM4-5), anti-

CD8 α (53-6.7), anti-Foxp3 (FJK-16s), anti-CD25 (PC61.5), anti-CTLA4 (UC10-4B9), anti-CD69 (H1.2F3), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-B220 (RA3-6B2), anti-CD19 (eBio1D3), anti-CD43 (eBioR2/60), anti-IgM (II/41), anti-IgD (11-26c), anti-NK1.1 (PK136), anti-Gr1 (RB6-8C5), anti-CD11b (M1/70), anti-CD127 (A7R34), anti-KLRG1 (2F1), anti-PD1 (J43), anti-CD3 (145-2c11), anti-CD24 (M1/69), anti-Qa2 (1-1-2), anti-NKG2D (CX5), anti-Ly49D (4E5), anti-CD122 (TM-b1), anti-IFN γ (XMG1.2), anti-IL4 (BVD6-24G2) and anti-IL17 (eBio17B7) from eBioscience and anti-Ki67 (B56) and anti-Siglec-F (E50-2440) from BD Biosciences.

Histology

Mouse tissues were preserved in 10% formalin and processed into paraffin-embedded tissue blocks by Histology Consultation Services. Each block had thin ($\sim 4\mu$) sections cut on a microtome, mounted on glass slides, and stained with hematoxylin and eosin (H&E). Pathological diagnosis was performed by Biogenetics Research Laboratories.

ELISA-MSD

IgE titers in individual serum samples were determined by a mouse IgE ELISA Ready-SET-Go! kit (eBioscience), according the manufacturer's protocol. Cytokine serum levels were quantified by the V-PlexTM mouse Pro-inflammatory panel MSD (Meso Scale Discovery) plates, according to the manufacturer's instructions

Statistical analyses

Single comparisons were analyzed using the non-parametric Mann-Whitney *U* test. All statistical analyses were carried out with GraphPad Prism. Data from mice

diagnosed with tumors were excluded from cell subsets and cytokines analysis.

Homology alignment was performed using HomoloGene and BLAST.

RESULTS

An ENU-induced mutation in Artemis results in a leaky-SCID phenotype

In a screen for modulators of regulatory T cell (Treg) number, we exposed C57Bl/6 mice to the ENU mutagen and intercrossed with the *Foxp3*^{GFP} strain prior to flow cytometry-based screening for Treg numbers. One pedigree was isolated with the presentation of low CD4⁺ T cells and disproportionately low Tregs within the blood (**Fig. 1a**). All-exon sequencing identified a point mutation in *Dclre1c*, the gene encoding Artemis. This transition mutation (c.656T>C), named *leaky*, generated an amino acid substitution in position 219 coding for a proline instead of a leucine (**Fig. 1b**). The mutated residue is located between the β -lactamase and β -CASP domains, in a highly conserved region (**Fig. 1c-d**). Western blot analysis detected a strong presence of Artemis expression in thymus of *leaky* mice, indicating that the mutation did not affect the protein expression (**Fig. 1e**).

The discrepancy between *Dclre1c*^{KO} mice⁸, with near-complete T cell deficiency, and *Dclre1c*^{leaky} mice, with reduced but detectable numbers of T cells in all mice, spurred further characterization of T cell development in the mutant strain. *Dclre1c*^{KO} mice display a block in thymocyte development at the double negative stage (DN), during VDJ rearrangement⁸. *Dclre1c*^{leaky} mice also exhibited decreased absolute numbers of total thymic cells in comparison to wildtype mice (**Fig. 2a**). Further, percentages of DN cells were enhanced in Artemis mutant mice compared to wildtype mice, in both young and old animals (**Fig. 2b**). Double positive cells (DP) were severely reduced in

leaky mice (**Fig. 2c**), however, unlike knockout animals, both single positive CD8⁺ T cells (SP CD8⁺) and CD4⁺ T cells (SP CD4⁺) were detected in *Dclre1c^{leaky}* mice (**Fig. 2d-e**). This result indicates a hypomorphic allele allowing a fraction of thymocytes to rearrange competent T cell receptors (TCRs) for further progress to the next developmental stage. Detailed immunophenotyping of the thymus showed a dominant presence of recirculating cells within the SP T cell population in *Dclre1c^{leaky}* mice in comparison to wildtype animals (**Suppl Fig.1**). Interestingly, and in line with the initial phenotype screening, Foxp3⁺ Tregs were significantly decreased in both young and old mutant animals (**Fig. 2f**). In absolute numbers, all measured T cell subsets in the thymus were decreased in *leaky* mice, but substantial numbers of T cells were still able to rearrange their TCR and to mature to the periphery (**Suppl. Fig. 1**).

The LS phenotype in human has been described as a strongly reduced, but still present, number of CD3⁺ T cells in the peripheral blood, caused by hypomorphic mutation in known SCID genes, including *DCLRE1C*². In *Dclre1c^{leaky}* mice, both mature CD4⁺ and CD8⁺ T cells were present in the spleen, albeit at significantly lower numbers (**Fig. 3a-c** and **Suppl. Fig2d**). T cells were also present in the lymph nodes (**Suppl. Fig. 2**). Importantly, in almost all *leaky* mice analyzed, Treg numbers were disproportionately reduced (**Fig. 3d** and **Suppl. figure 2e**). Patients with a LS phenotype have a restricted T cell repertoire². This prompted us to analyze the diversity of TCR repertoire in the spleen of wildtype and *leaky* mice through TCR spectratyping. Consistent with the patient phenotype, we observed that *Dclre1c^{leaky}* mice displayed a very limited TCR repertoire compared to wildtype mice (**Fig. 3e**). Together, these results indicate that *leaky* mice recapitulate the oligoclonal nature of the T cell repertoire observed in LS due to restricted T cell differentiation. Regarding

other immune cell compartments, B cells were almost absent in *leaky* mice, with an early block between the pro-B cell and the pre-B cell stage during the bone marrow development with a conserved Pro-B cell compartment (median: 3.3% from total bone marrow cells for wildtype mice, versus 3.4% for *Dclre1c^{leaky}* mice) and a near-absent Pre-B cell compartment (median: 4.3% from total bone marrow cells for wildtype mice, versus 0.2% for *Dclre1c^{leaky}* mice, $p=0.003$) (**Fig. 3f-g**). The percentage of NK cells and myeloid cells were elevated in Artemis mutant mice due to the reduction in T and B lymphocytes, however absolute numbers were similar (**Fig. 3h-j**). An altered activation profile was, however, observed in NK cells from *Dclre1c^{leaky}* mice (**Suppl. figure 2f-g**). *Dclre1c^{leaky}* mice therefore fit the immunological criteria for leaky SCID, with severe but partial defects in TCR/BCR rearrangement, but otherwise intact immune systems.

Leaky mice develop inflammatory pathology

Published mice with mutations in Artemis phenocopy the classical SCID presentation of patients with *DCLRE1C* mutation⁸⁻¹¹, with defects in T cells and B cells causing immunodeficiency, but no spontaneous disease⁸⁻¹¹ (apart from an increased incidence of tumor under a specific backcross¹²). By contrast, *Dclre1c^{leaky}* mice recapitulate the LS phenotype present in a subset of *DCLRE1C* patients¹³, with spontaneous immune activation. The clonal T cells from *leaky* mice that underwent successful TCR rearrangement demonstrated high levels of oligoclonal peripheral expansion (**Fig. 3e**), with enhanced proliferation (**Fig. 4a** and **Suppl. Fig. 3a**). These expanded cells were mainly of an effector phenotype, with a significantly decreased naive T cell compartment (**Fig. 4b-c** and **Suppl. Data 3b-c**). Interestingly, CD122 expression was

increased in CD8⁺ central memory T cells (TCM), suggesting that *Dclre1c^{leaky}* T cells may have a higher reliance on IL15 for homeostasis (**Suppl. Data 3d**). This is further illustrated by an expansion over the time of this specific CD8⁺ TCM population in *Dclre1c^{leaky}* mice, while this population stays stable in wildtype animals (**Fig.4b-c** and **Suppl. Data 3b-c**). Indeed, splenic absolute number of CD8⁺ TCM cells increases 10-fold in old (median: 0.47×10^6) versus young (median: 0.04×10^6 , $p < 0.001$) *Dclre1c^{leaky}* mice, although the overall number remain below the wildtype cell number level (median: 3.1×10^6 and 2.7×10^6 , ns, in old and young mice respectively). The high T cell activation in *Dclre1c^{leaky}* mice was also demonstrated by the increased percentage of cells expressing CD69 at their cell surface, a marker of early T cell activation (**Fig.4d** and **Suppl. data 3e**). By contrast, no difference in the expression of CD127, KLRG1 or PD1 was observed in CD8⁺ T cell subsets (**Fig. 4e**), however a transient boost in short-lived effector cells (SLEC) was observed in *leaky* mice at young ages with resolution in older mice (**Suppl. data 4**). In order to determine whether the activated T cells in *leaky* mice were producing an inflammatory environment, we assessed cytokine release. Within *Dclre1c^{leaky}* mice, Th1 (IFN γ -secreting CD4⁺ T cells), Th2 (IL-4-secreting CD4⁺ T cells) and Th17 (IL-17-secreting CD4⁺ T cells) were all increased compared to wildtype animals (**Fig. 4f** and **Suppl. data 3f**), with serum increases in IFN γ also observed (**Fig. 4g**).

The inflammatory T cell phenotype of *Dclre1c^{leaky}* mice was associated with immune pathology. More than 50% of *leaky* mice developed a wasting disease with lower weight, poor grooming, an abnormal hunched posture or signs of severe dermatitis (**Fig. 5a-b** and **Suppl. Fig. 5a-c**). When evaluated by histology, all *leaky* analyzed mice showed signs of lymphoreticular infiltrates in different organs including lung,

liver, gastro-intestinal tract, pancreas, lymph nodes and skin with signs of severe vasculitis (**Fig. 5c**). Disease was accompanied by a high number of eosinophils in both spleen and peripheral blood (**Fig. 5d**), and substantial elevation of IgE (>50,000ng/ml) was detected in the serum of 26% of *leaky* mice (compared to 7% of age-matched controls). Consistently, IL-5 in the serum of *Dclre1c^{leaky}* mice was detected at a higher level. (**Suppl. Data 6**). In addition, tumors of a lymphoid origin developed in 15% of *Dclre1c^{leaky}* mice at 8-10 months of age (**Fig. 5a, e and Suppl. data 5d**), a level similar to that of spontaneous tumors reported in patients with *DCLRE1C* mutation and a LS phenotype^{3,4}. The combined effect was an increased premature mortality rate in Artemis mutant mice (18% at 8 months of age, compared to 0% in wildtype siblings at the same age). This immune pathology places the *Dclre1c^{leaky}* mouse strain on the spectrum of clinical immune pathology observed in *DCLRE1C* mutant patients with leaky SCID or Omenn Syndrome.

CTLA4-Ig treatment prevents immune pathology in Artemis mutant mice with leaky SCID

The development of a new animal model that recapitulated the key clinical features of LS allowed the testing of potential therapeutic interventions. The *Dclre1c^{leaky}* phenotype was associated with peripheral T cells activation including high IL-4 level, high IgE and increased level of eosinophils, which are characteristics of Treg-deficiency and Th2-mediated disease. These observations prompted us to treat *leaky* mice with CTLA4-Ig to investigate whether disease could be prevented by this treatment, previously shown to be effective in suppressing Th2 responses in mice with low Tregs¹⁴. *Dclre1c^{leaky}* and littermate control mice (including heterozygous mice) were left untreated or received 25mg/kg CTLA4-Ig every second week from the age of 8 weeks. Untreated *leaky* mice showed normal weight curves until 6 months of age,

at which point wasting (**Fig. 6a**) and premature mortality (27% of mice) were noted (**Fig. 6b**). By contrast, the CTLA4-Ig-treated *leaky* mice did not exhibit wasting, and no premature mortality was observed (**Fig. 6a,b**). Furthermore, none of the *Dclre1c^{leaky}* mice receiving CTLA4-Ig treatment developed any symptoms of skin inflammation (**Fig. 6c** and **Suppl. Fig. 7**) or hyper IgE (**Fig. 6d**). These observations indicate the efficacy of CTLA4-Ig for the autoimmune-related manifestations of LS with the exception. By contrast, however, colitis was not improved under CTLA4-Ig treatment and 15% of *Dclre1c^{leaky}* mice treated with CTLA4-Ig developed tumors, an incidence similar to the untreated group, indicating that CTLA4-Ig administration does not prevent the development of thymic lymphoma (**Fig. 6c**).

While the precise mechanism of action of CTLA4 is still under debate, there is strong evidence suggesting that reducing ligand access to the co-stimulation molecule, CD28, constitutes one of its principal functions¹⁵. An immunological analysis of the effects of CTLA4-Ig treatment on control and *leaky* mice found that the main immune phenotype caused by CTLA4-Ig treatment was a pronounced and sustained decrease in CD4⁺ T cells, including further reduction of the Treg subset (**Fig. 7a-c** and **Suppl. Fig. 8a-b**). This is in accordance with the fact that CD28 co-stimulation is critical for T cell peripheral survival¹⁶, with a more profound requirement present in Tregs^{17,18}. Further, we observed a lower percentage of CD4⁺ T cell proliferation (**Fig. 7d** and **Suppl. Fig. 8c**) and activation (**Fig. 7e** and **Suppl. Fig. 8d**) in the spleen and lymph nodes following CTLA4-Ig treatment. Regarding cytokine secretion, there was a strong reduction of the absolute number of cytokine-secreting T cells due to the generic blockade on T cell activation (**Fig. 7f-h**), specifically in Th2 as a reflect of better control of Th2 over Th1 by CTLA4-Ig¹⁴. In addition, IFN γ and TNF α were

significantly lower in the serum of *Dclre1c*^{leaky} mice receiving CTLA4-Ig (**Fig. 7i-j**).
Finally, it is worth noting that all significant immune differences induced by CTLA4-Ig treatment were observed in both littermate control and *Dclre1c*^{leaky} mice, indicating that the successful ablation of immune pathology in *leaky* mice is driven by the same immunological processes initiated by CTLA4 in control mice.

DISCUSSION

While complete T cell deficiency presents as profound immunodeficiency, incomplete forms, prototypically Omenn syndrome and leaky SCID, manifest as a paradoxical co-presentation of immunodeficiency and immune dysregulation with spontaneous inflammatory/autoimmune symptoms. Numerous hypotheses have been put forward to explain this paradox at the immunological level, including a mismatch between available effector and regulatory T cell clones after oligoclonal expansion, reduced efficiency of thymic tolerance processes in the abnormal thymic tissue present, or a hyper-stimulatory phenotype of dendritic cells created by the peripheral T cell deficiency^{6,19}. However a limited range of pre-clinical models has hampered the ability to test these models and develop therapeutic strategies.

Mutations in ARTEMIS drive clinical phenotypes that fall in the SCID-OS-LS spectrum. Mouse models, by contrast, show a much more restricted phenotypic range, owing to the concentration on “knockout” mutations. Standard Artemis knockout mice possess a SCID-like phenotype, although the presence of T cells in periphery suggesting a partial redundancy of Artemis for TCR rearrangement in mice⁸. Mutations in the C-terminal domain of Artemis, more likely to promote a leaky phenotype in patients⁴, have only a partial block in B and T cell development¹⁰,

however none of these models present with clinical symptoms analogous to those experienced by leaky SCID patients. In the Artemis mouse model reported here, point mutation L219P, results in a clinical phenotype that is very similar to patient with LS/OS, with inflammatory disorder in multiple organs. Specifically, *Dclre1c^{leaky}* recapitulates key features of LS/OS patients including dermatitis, wasting disease, splenomegaly, inflammatory cytokines, high IgE level and hyperoesinophilia. With symptomatic development in older mice, leaky mice fit within the LS clinical range, albeit with similarities to OS. While no mutation in 219 position has been described in a SCID patient, a mutation in the nearby residue 228, within the same conserved region, has been reported in a patient with a SCID phenotype and partial VDJ recombination and DNA repair activity *in vitro*⁴.

The serendipitous development of a LS pre-clinical model allowed for a dissection of potential tolerance break-points and, thus, testing of intervention strategies. The most striking observation was the low amount of Tregs found in both thymus and in periphery. One could speculate that the only allowed V(D)J rearrangement in LS condition is detrimental to the Treg formation and/or maintenance due to a selection of TCR with a specific affinity²⁰. This low Treg phenotype can account for the inflammatory Th1/Th2 profile observed in *Dclre1c^{leaky}* mice, owing to the asymmetry in Treg control over the two immune arms¹⁴. Additional homeostatic “space filling” pressures can be extrapolated from the oligoclonal expansion and adoption of the TEM phenotype observed in T cells. The logical extension of this immunological presentation is that therapeutic intervention requires: substitution for Treg-deficiency, enhanced suppression of the Th2 response and a counter-action of the enhanced CD80/CD86 stimulatory profile of lymphopenic dendritic cells.

417

418 Current treatment for inflammatory manifestation in LS/OS patients are based on non-
419 specific immunosuppressive drugs such as corticosteroids and cyclosporine A ^{13,21}.
420 While these treatments show some limited success, the combination of limited
421 efficacy and undesirable side-effects leave room for clinical improvement. The new
422 therapeutic approaches are directing towards better targeted and specific treatment
423 with less sides effects, as illustrated with the use of TpoR agonist in the case of
424 thrombocytopenia ²¹. The immunology-directed rationale of changes identified in our
425 pre-clinical model identified CTLA4-Ig, a soluble version of CTLA4, as a key
426 therapeutic candidate. CTLA4-Ig meets all of the intervention criteria listed above:
427 first, as a potent suppressive molecule used by Tregs for immune suppression ²²,
428 CTLA4-Ig can substitutive for Treg-deficiency; second, CTLA4-Ig is highly effective
429 at quenching Th2 responses ¹⁴; and third, CTLA4-Ig effectively competes with
430 CD80/CD86 for CD28 binding ¹⁵ countering the elevated expression of these
431 molecules in lymphophenic dendritic cells. As bi-monthly administration of CTLA4-
432 Ig to *Dclre1c*^{leaky} mice resolved inflammation, prevented tissue damage and improved
433 overall survival, the immunological rationale of CTLA4-Ig selection for murine LS
434 treatment can be regarded as validated. While abatacept demonstrates high efficiency
435 in rodent studies, when considering clinical translation additional options become
436 available, with belatacept demonstrating higher affinity towards human CD86²³.

437

438 Overall our study identified a novel mutation of Artemis resulting in a murine pre-
439 clinical model of leaky SCID. Consistent with CD4+ T cells being the driver of
440 inflammatory disease in LS ²⁴, and based on assessment of the immunological
441 disturbances observed, CTLA4-Ig proved an effective drug to prevent disease

manifestation and increase survival. By contrast, no improvement was observed in the elevated incidence of lymphoma in these mice, which mirrors the patient presentation⁴, indicating a mechanistically independent origin for the oncological components of disease. In addition, colitis seems to be resistant to CTLA4-Ig treatment. This observation is in accordance with clinical trials that showed failure of CTLA4-Ig in the treatment of patients with Crohn's disease or ulcerative colitis²⁵, secondary to the resistance of inhibition of Th17 by CTLA4²¹. Together these results call for a carefully-controlled trial of CTLA4-Ig treatment of OS/LS patients, to investigate the potential for treating the autoimmune/inflammatory components of disease.

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566

FIGURE LEGENDS

Figure 1. New point mutation identified in *Dclre1c*^{leaky}. Using ENU mutagenesis, *leaky* mice with a point mutation in Artemis were identified. **(A)** *Leaky* mice were identified with a low level of peripheral Treg in the blood. **(B)** Sanger sequencing of *Dclre1c*, encoding for Artemis, in wildtype and *leaky* mice confirmed a T to C mutation resulting in a lysine to proline mutation in amino acid change. **(C)** Mutation confers a L219P substitution (star) between the 2 active domains of the protein (β -lactamase, SM00849 and β -CASP domain, PF07522). **(D)** L219P mutation is located in a region conserved across species. **(E)** Western blot (left) and relative expression (right) of Artemis protein in wildtype (n=5) and *Dclre1c*^{leaky} thymocytes (n=5).

Figure 2. A partial T cell developmental block in *Dclre1c*^{leaky} mice. The thymus from wildtype and *Dclre1c*^{leaky} mice was evaluated at 8 weeks and 8-10 months of age. Thymic cell subsets were analyzed by flow cytometry. **(A)** Thymus absolute cell numbers. **(B)** Percentage of double negative cells (DN) from total cells. **(C)** Percentage of double positive cells (DP) from total cells. **(D)** Percentage of CD8⁺ single positive cells (SP CD8⁺) from total cells. **(E)** Percentage of CD4⁺ single positive cells (SP CD4⁺) from total cells. **(F)** Percentage of regulatory T cells (Treg) from SP CD4⁺ cells. Median and individual data points shown. **(A-F)** Pooled data from up to 11 experiments, wildtype 8 weeks old mice (n=3), *Dclre1c*^{leaky} 8 weeks old mice (n=8), wildtype 8-10 months old mice (n=14) and *Dclre1c*^{leaky} 8-10 months old mice (n=27).

Figure 3. *Dclre1c*^{leaky} mice have a leaky-SCID phenotype in the periphery. Spleens from wildtype and *Dclre1c*^{leaky} mice were evaluated at 8 weeks old and

between 8 to 10 months old. Splenic cell subsets were analyzed by flow cytometry. (A) Absolute numbers of CD8⁺ and CD4⁺ T cells in the spleen at 8-10 months. (B) Percentage of CD4⁺ T cells, (C) CD8⁺ T cells and (D) Treg (CD4⁺Foxp3⁺ cells). (E) Spectratyping CDR-3 analysis of TCR V β diversity. Representative mice and V β families are presented. Black bars indicate saturation of signal intensity. (F) Representative bone marrow B cell maturation flow cytometry plots (left) and percentage of early B cell progenitors Pro-B cells and Pre-B cells graph (right). (G) Percentage of splenic B cells and (H) NK cells. (I) Absolute numbers of splenic NK cells and (J) neutrophils (Gr-1^{high}/CD11b^{high} cells). Median and individual data points shown. (A-D and F-J) Pooled data from up to 11 experiments wildtype 8 weeks old mice (n=3), *Dclre1c*^{leaky} 8 weeks old mice (n=from 7 to 8), wildtype 8-10 months old mice (n=from 3 to 14) and *Dclre1c*^{leaky} 8-10 months old mice (n=from 8 to 27).

Figure 4. *Dclre1c*^{leaky} have increased T cell activation and pro-inflammatory cytokines in serum. Splenic T cells from wildtype and *Dclre1c*^{leaky} mice were evaluated at 8 weeks and 8-10 months of age. T cell subsets were analyzed by flow cytometry. (A) Percentage of CD4⁺ and CD8⁺ T cells expressing Ki67. (B) T cells were characterized as CD62L⁺CD44⁻ naïve cells, CD62L⁺CD44⁺ central memory T cells (TCM) and CD62L⁻CD44⁺ effector memory T cells (TEM) for CD4⁺ and (C) CD8⁺ subsets. (D) Percentage of CD4⁺ and CD8⁺ T cells expressing CD69. (E) Percentage of CD8⁺ T cells expressing CD127, KLRG1 and PD1. (F) Splenocytes were stimulated in vitro for 4 hours with PMA/Ionomycin and CD4⁺ T cells evaluated for cytokine secretion including IFN γ , IL-4 and IL-17. (G) Serum levels of IFN γ and IL-4. Median and individual data points shown. Pooled data from up to 11 experiments, wildtype 8 weeks old mice (n=3), *Dclre1c*^{leaky} 8 weeks old mice (n=8),

618 wildtype 8-10 months old mice (n=from 4 to14) and *Dclre1c*^{leaky} 8-10 months old
 619 mice (n=from 6 to 27).

620

621 **Figure 5. *Dclre1c*^{leaky} mice are affected by inflammatory diseases and lymphoma.**

622 Mice were evaluated at 8-10 months of age for disease incidence and markers of
 623 inflammation. **(A)** 34 *leaky* mice were evaluated for pathological symptoms, each
 624 circle represents an individual mouse. **(B)** Mouse weight. **(C)** Histology of lung, liver,
 625 skin and colon of both wildtype and *Dclre1c*^{leaky} mice. Arrows show lymphoreticular
 626 infiltrates. **(D)** Percentage of eosinophils into the spleen and the blood. **(E)**
 627 Representative histology of a lymphocytic lymphoma from the thymus, infiltrating the
 628 liver and lung. **(A)** Pooled data up to 11 experiments, **(B)** pooled data from 5
 629 experiments, wildtype mice (n=8) and *Dclre1c*^{leaky} mice (n=17), **(C and E)**
 630 representative histology image, scale bars = 50µm, **(D)** represents 1 experiment,
 631 wildtype mice (n= from 3 to 9) and *Dclre1c*^{leaky} mice (n=from 4 to 7).

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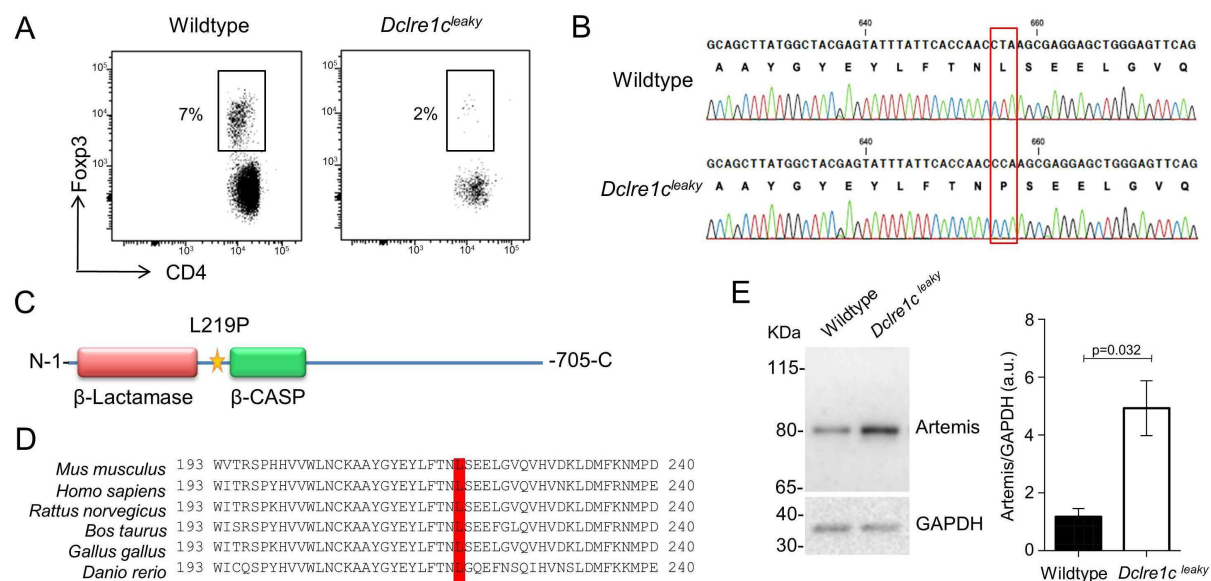
633 **Figure 6. CTLA4-Ig treatment prevents immune pathology in *Dclre1c*^{leaky} mice.**

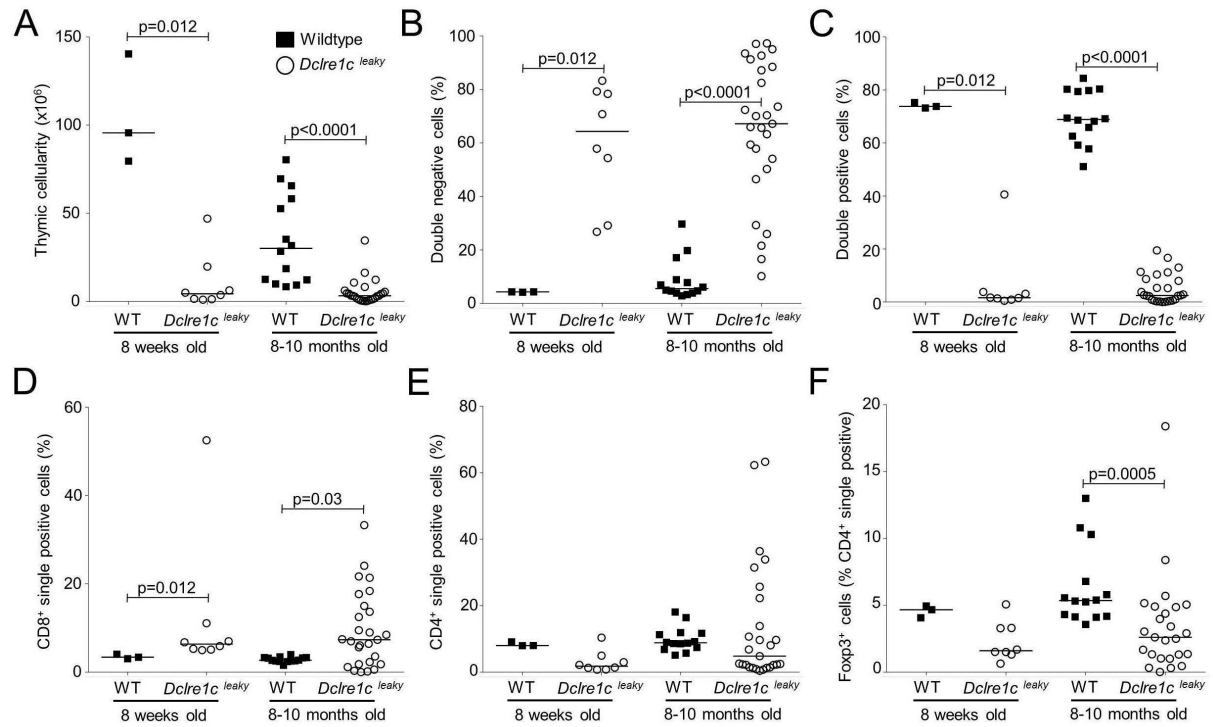
634 *Dclre1c*^{leaky} mice and littermate controls (Ctrl) were followed up for a longitudinal
 635 study with and without received CTLA4-Ig treatment (25mg/kg every two weeks
 636 starting at 8 weeks). Mice were evaluated at 40 weeks of age. **(A)** Weight curve. **(B)**
 637 Survival curve. **(C)** Adverse events/pathological observations were monitored in all
 638 mice at the time of sacrifice, each circle represents an individual mouse. **(D)** IgE
 639 concentration in the serum. Mean and SEM are shown for A, and median and
 640 individual data points shown for D. At the start of the study (A and B) untreated
 641 control mice (n=11) and untreated *Dclre1c*^{leaky} mice (n=11), CTLA4-Ig treated control
 642 mice (n=12) and CTLA4-Ig treated *Dclre1c*^{leaky} mice (n=13). At the time of analysis

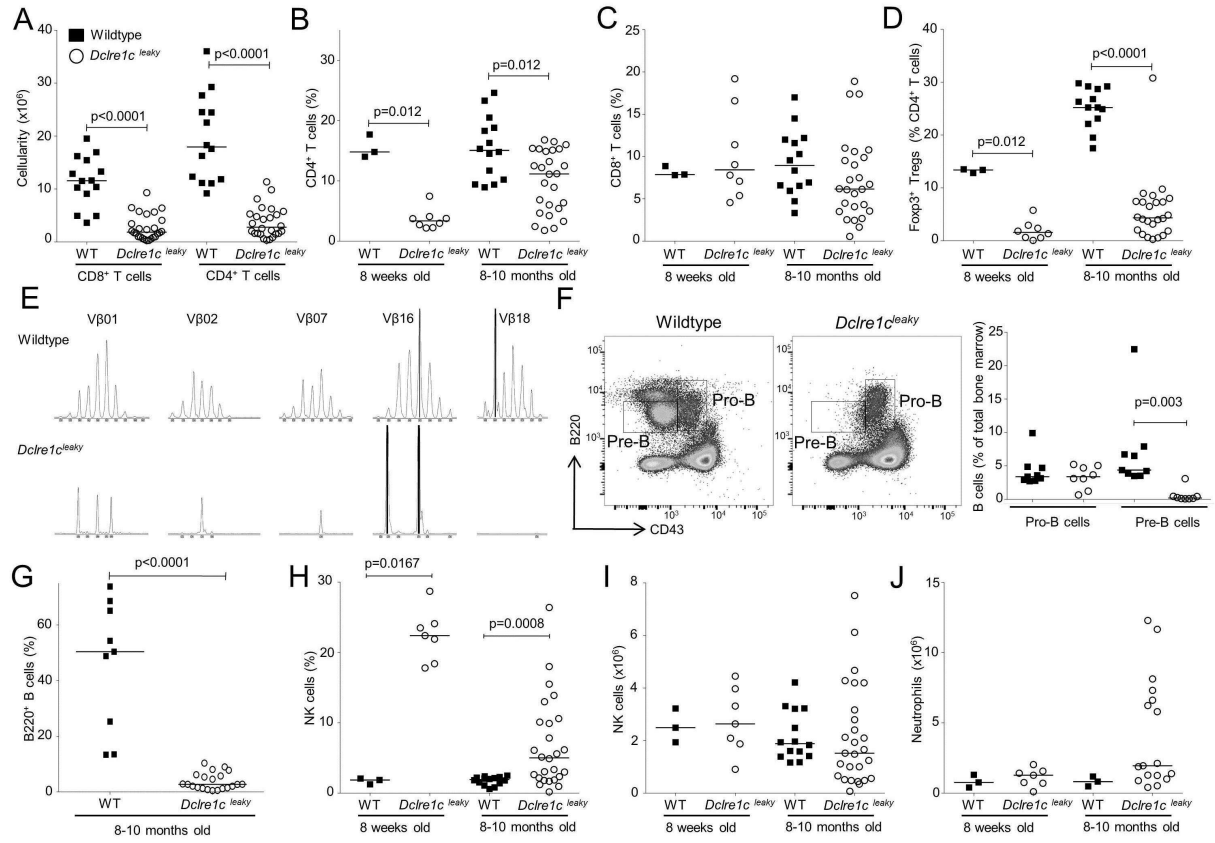
643 (C and D) untreated control mice (n=10) and untreated *Dclre1c^{leaky}* mice (n=9),
 644 CTLA4-Ig treated control mice (n=12) and CTLA4-Ig treated *Dclre1c^{leaky}* mice
 645 (n=13).

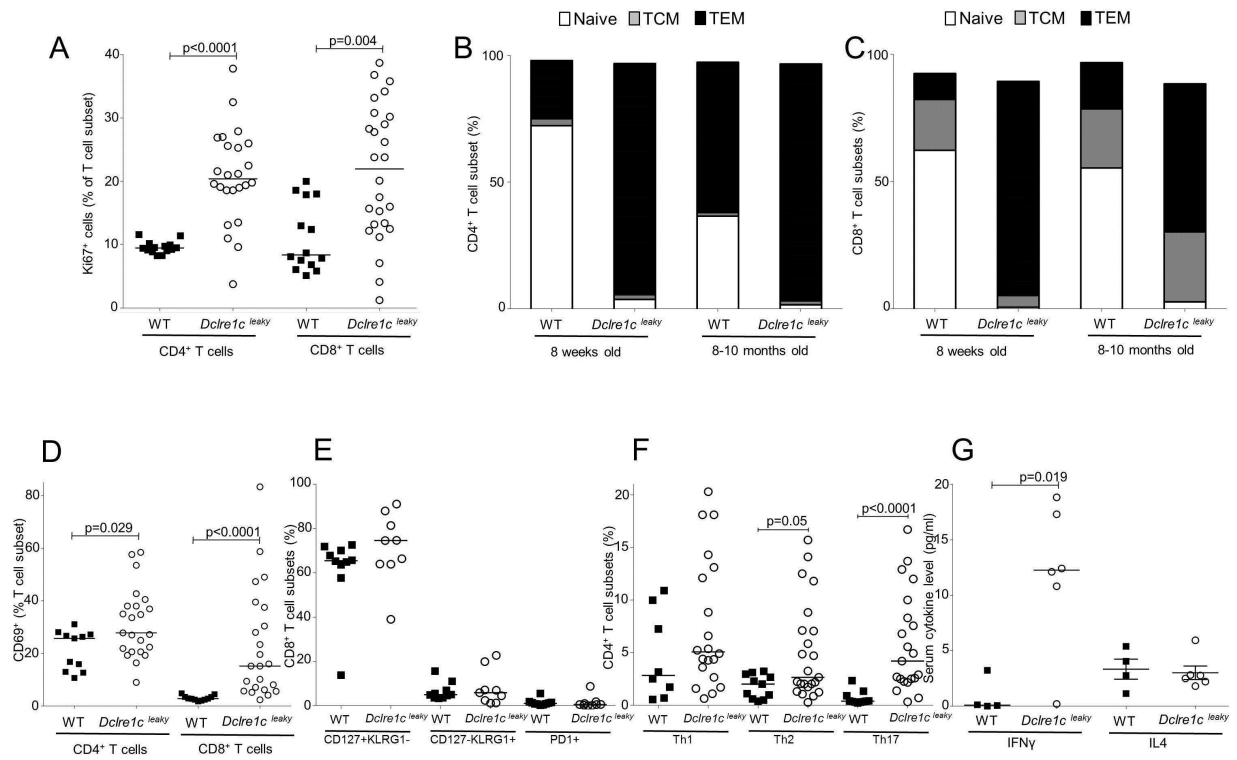
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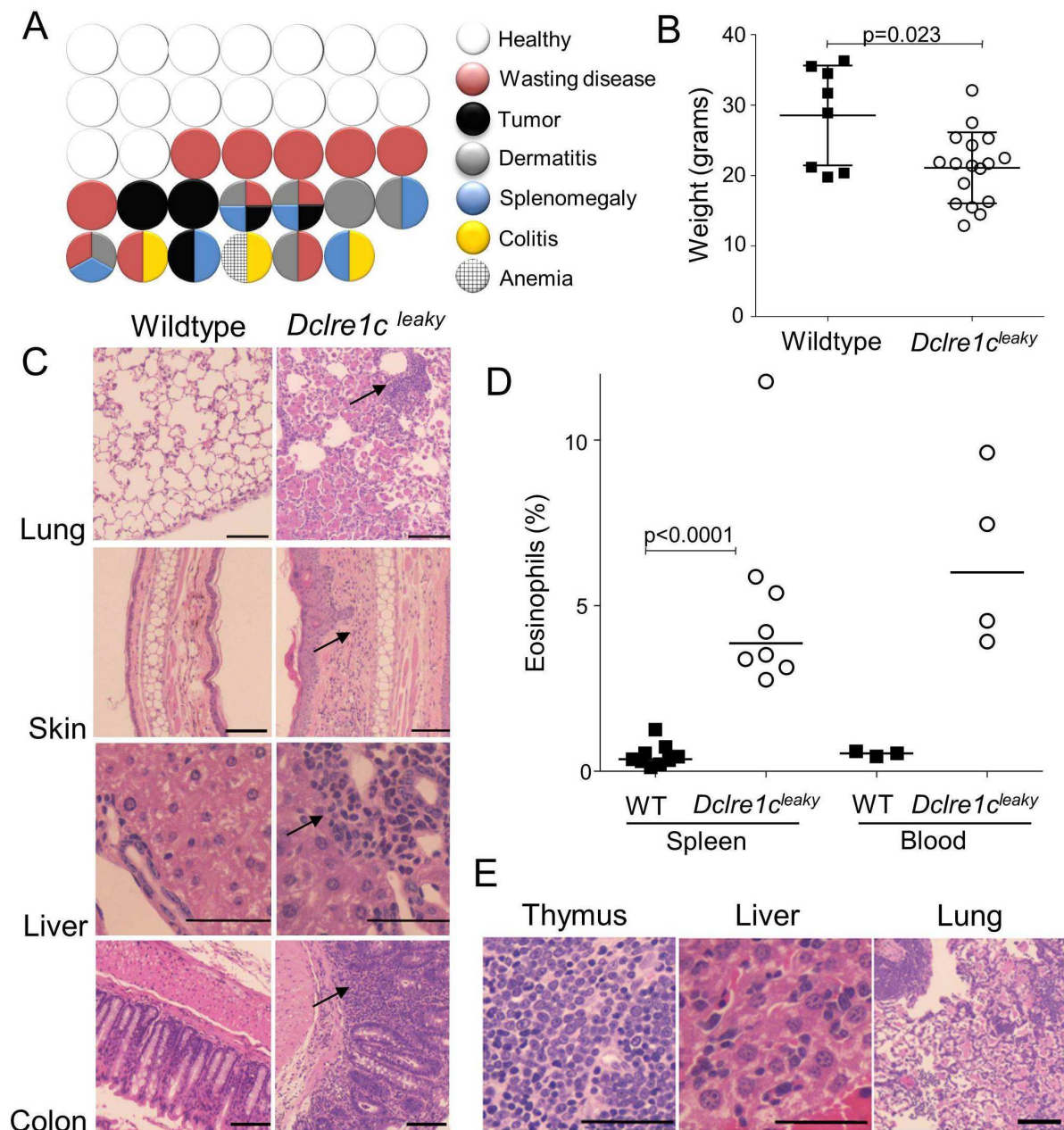
647 **Figure 7. CTLA4-Ig treatment effectively decreases inflammation in *Dclre1c^{leaky}***
 648 **mice.** *Dclre1c^{leaky}* mice and littermate controls (Ctrl) received CTLA4-Ig (25mg/kg)
 649 every two weeks starting at 8 weeks old. Mice were evaluated at 40 weeks old for the
 650 immune cell compartment in the spleen. **(A)** Kinetic of CD4⁺ T cells in the blood at 3,
 651 6 and 9 months old mice. **(B)** Percentage of total CD4⁺ T cells. **(C)** Within CD4⁺ T
 652 cells, percentage of Treg (CD4⁺Foxp3⁺ cells). **(D)** Ki67⁺ cells, and **(E)** CD69⁺ cells.
 653 **(F)** Absolute number of Th1 (IFN γ secreting CD4⁺ T cells), **(G)** Th2 (IL4 secreting
 654 CD4⁺ T cells) and **(H)** Th17 cells (IL17 secreting CD4⁺ T cells). **(I)** Serum level of
 655 IFN γ (one very high value for *Dclre1c^{leaky}* is not shown) and **(J)** TNF α . Median and
 656 individual data points shown. (A, I and J) represents one experiment, **(B to H)**
 657 representative data of 2 experiments. Untreated control mice (n=from 3 to 9) and
 658 untreated *Dclre1c^{leaky}* mice (n=from 3 to 9), CTLA4-Ig treated control mice (n=from
 659 5 to 12) and CTLA4-Ig treated *Dclre1c^{leaky}* mice (n=from 5 to 11).

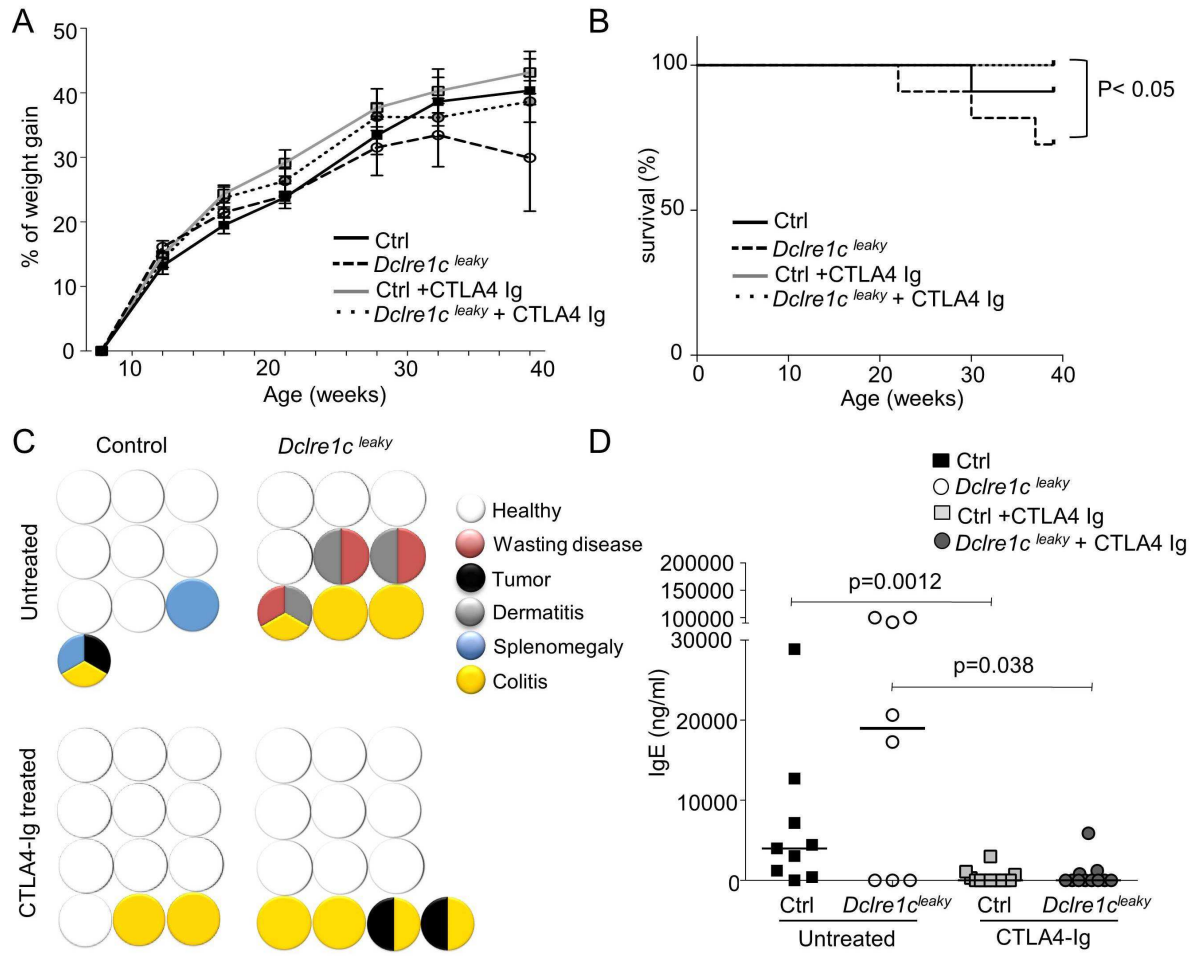


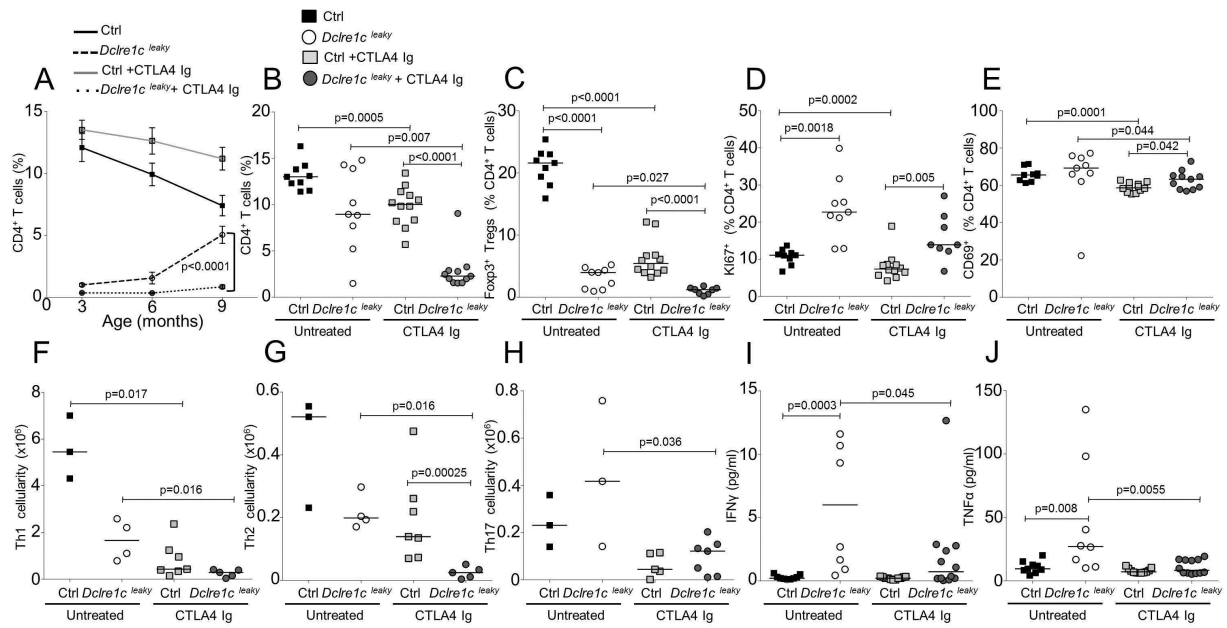












SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. T cell developmental defects in *Dclre1c*^{leaky} mice. Thymus from 8-10 months old wildtype and *Dclre1c*^{leaky} mice (A-C) or 10 weeks old (D-F) were analyzed for cell numbers and T cells subsets by flow cytometry. **(A)** Representative flow cytometry plot from thymus, including two mice within the *Dclre1c*^{leaky} phenotype spectrum. **(B)** Thymocyte subset absolute cell number for double negative (DN), double positive (DP) and **(C)** the CD4⁺ and CD8⁺ single positive (SP) and Treg subsets. **(D)** Representative flow cytometry plot for mature (CD3+CD69-) and immature (CD3+CD69+) cells within CD4+ and CD8+ SP subsets. **(E)** Representative flow cytometry plot for the presence of recirculating cells (CD24-Qa2+) within CD3+CD69- or CD3+CD69+ cells in CD4+ and CD8+ SP subsets. **(F)** Percentage of recirculating T cells in the mature CD3+CD69- subsets of CD4+ and CD8+ SP compartement. Median and individual data points shown. **(B-C)** Pooled data from up to 11 experiments, wildtype mice (n=14) and *Dclre1c*^{leaky} mice (n=from 24 to 26). **(D-F)** Represent a single experiment, wildtype mice (n=4) and *Dclre1c*^{leaky} mice (n=4).

Supplementary Figure 2. *Dclre1c*^{leaky} mice have a leaky-SCID phenotype in periphery. Lymph nodes from wildtype and *Dclre1c*^{leaky} mice were evaluated at 8 weeks old and between 8 to 10 months old. T cell subsets were analyzed by flow cytometry (A-E). **(A)** Representative flow cytometry plot from the lymph nodes, including two mice within the *Dclre1c*^{leaky} phenotype spectrum. **(B)** Percentage of total CD8⁺ T cells, **(C)** CD4⁺ T cells. **(D)** Absolute numbers of CD8⁺ and CD4⁺ T cells in the spleen at 8 weeks old. **(E)** Percentage of Tregs (CD4⁺Foxp3⁺ cells) in the lymph nodes. **(F)** NKG2D geometric mean fluorescence expression (gMFI) in splenic NK cells in 10 weeks old mice. **(G)** CD69 and Ly49D percentage in splenic NK cells in 10 weeks old mice.

Median and individual data points shown. **(B-E)** Pooled data from up to 11 experiments, wildtype 8 weeks old mice (n=3), *Dclre1c^{leaky}* 8 weeks old mice (n=8), wildtype 8-10 months old mice (n=14) and *Dclre1c^{leaky}* 8-10 months old mice (n=from 26 to 27). **(F-G)** Represent a single experiment, wildtype mice (n=4) and *Dclre1c^{leaky}* mice (n=4).

Supplementary Figure 3. *Dclre1c^{leaky}* have increased T cell activation and pro-inflammatory cytokines in serum. Lymph nodes from wildtype and *Dclre1c^{leaky}* mice were evaluated at 8 weeks old and between 8 to 10 months old (A-C, E, F). T cell subsets were analyzed by flow cytometry. **(A)** Percentage of CD4⁺ and CD8⁺ T cells expressing Ki67. **(B-C)** CD4⁺ and CD8⁺ T cell subpopulations were characterized as CD62L⁺CD44⁻ naïve cells, CD62L⁺CD44⁺ central memory T cells (TCM) and CD62L⁻CD44⁺ effector memory T cells (TEM) by flow cytometry. **(B)** Percentage of CD4⁺ naïve, TCM and TEM cells. **(C)** Percentage of CD8⁺ naïve, TCM and TEM cells. **(D)** Percentage of CD122⁺ cells within the CD8⁺ TCM cell subsets in 10 weeks old mice. **(E)** Percentage of CD4⁺ and CD8⁺ T cells expressing CD69. **(F)** Cells from lymph nodes were stimulated *in vitro* for 4 hours with PMA/Ionomycin and CD4⁺ T cells evaluated for cytokine secretion including IFN γ , IL-4 and IL-17. Median and individual data points shown. **(A-C, E,F)** Pooled data from up to 11 experiments, wildtype 8 weeks old mice (n=3), *Dclre1c^{leaky}* 8 weeks old mice (n=8), wildtype 8-10 months old mice (n=14) and *Dclre1c^{leaky}* 8-10 months old mice (n=from 26 to 27). **(D)** Represents a single experiment, wildtype mice (n=4) and *Dclre1c^{leaky}* mice (n=4).

Supplementary Figure 4. Increased short-lived effector cells in young *Dclre1c^{leaky}* mice. CD8⁺ T cells were analyzed in cohort of 10 weeks old mice by flow cytometry in spleen and lymph nodes and compared to older mice (8-10 months old). **(A)** Percentage of CD127⁺KLRG1⁻ memory precursor effector cells (MPEC) and CD127⁻KLRG1⁺ short lived

effector cells (SLEC) within CD44⁺CD8⁺ subsets in spleen and **(B)** lymph nodes. **(C)** Percentage of Eomes⁺ and T-bet⁺ cells within CD8⁺CD44⁺CD62L⁻ subsets, effector memory T cells (TEM), in spleen and **(D)** lymph nodes of 10 week old mice. **(E)** Percentage of LAG3⁺ and TIM3⁺ cells within CD8⁺CD44⁺CD62L⁻ subsets, effector memory T cells (TEM), in spleen and **(F)** lymph nodes of 10 week old mice. Median and individual data points shown. **(A-F)** Represent a single experiment, wildtype mice (n=4) and *Dclre1c*^{leaky} mice (n=4).

Supplementary Figure 5. *Dclre1c*^{leaky} are affected by inflammatory diseases and are higher risk for lymphoma. Mice from 8 to 10 months old were evaluated at the time of sacrifice for disease incidence. **(A)** Representative picture of a wildtype mouse. **(B)** Representative picture of *Dclre1c*^{leaky} mouse, including hunched posture and dermatitis of the ear (arrow), **(C)** and tail (arrow). **(D)** Representative picture of *Dclre1c*^{leaky} mouse with a thymic tumor (arrow).

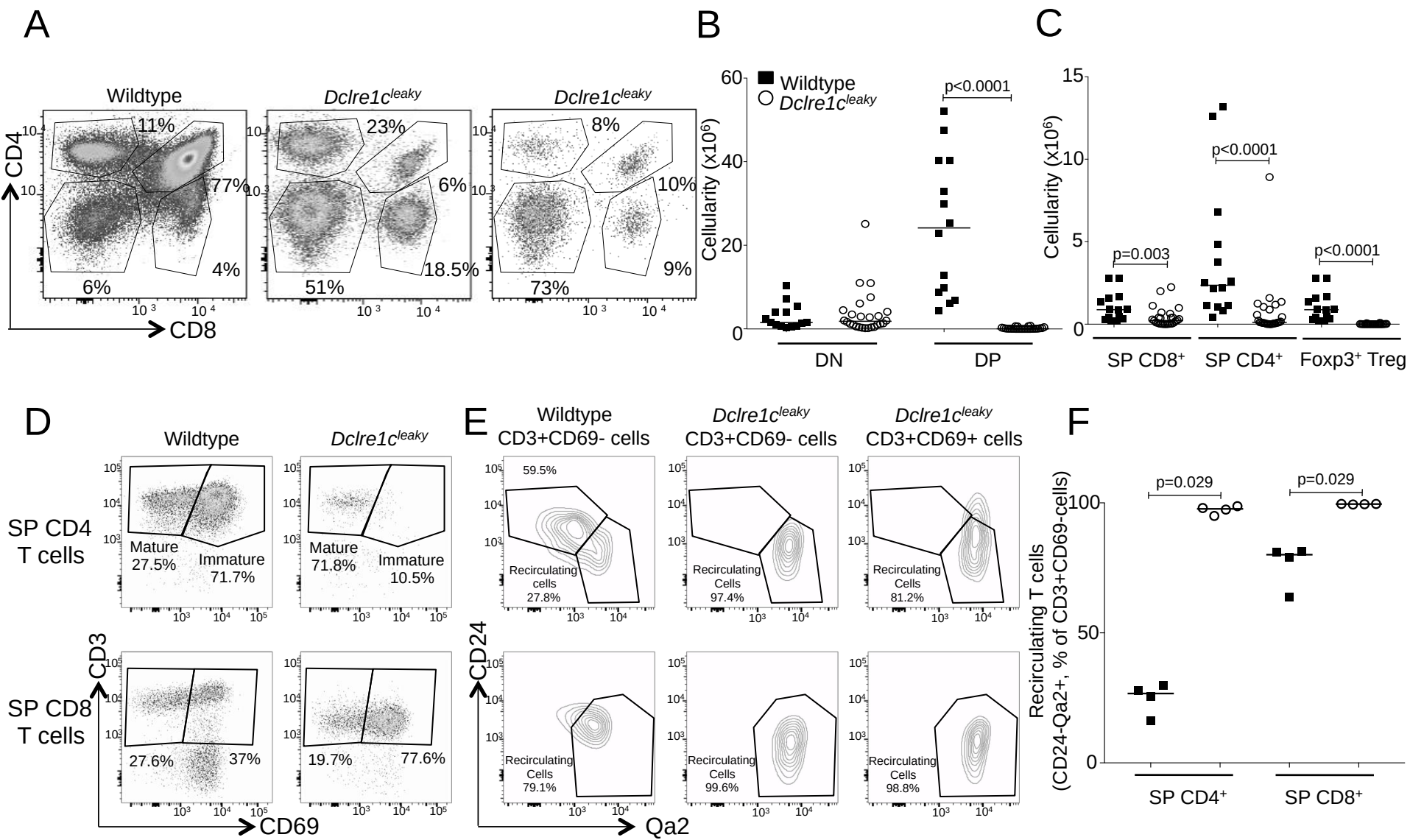
Supplementary Figure 6. IL-5 is elevated in *Dclre1c*^{leaky} mouse serum. Serum from mice from 8 to 10 months was evaluated for the presence of IL-5 using MSD assay. Median and individual data points shown. Pooled data from 2 experiments, wildtype (n=4) and *Dclre1c*^{leaky} mice (n=6).

Supplementary Figure 7. CTLA4-Ig treatment prevents immune pathology in *Dclre1c*^{leaky} mice. *Dclre1c*^{leaky} mice and littermate controls (Ctrl) were followed up for a longitudinal study with or without CTLA4-Ig treatment (25mg/kg) every two weeks starting at 8 weeks old. Mice were evaluated at 40 weeks old. Representative picture of an **(A)**

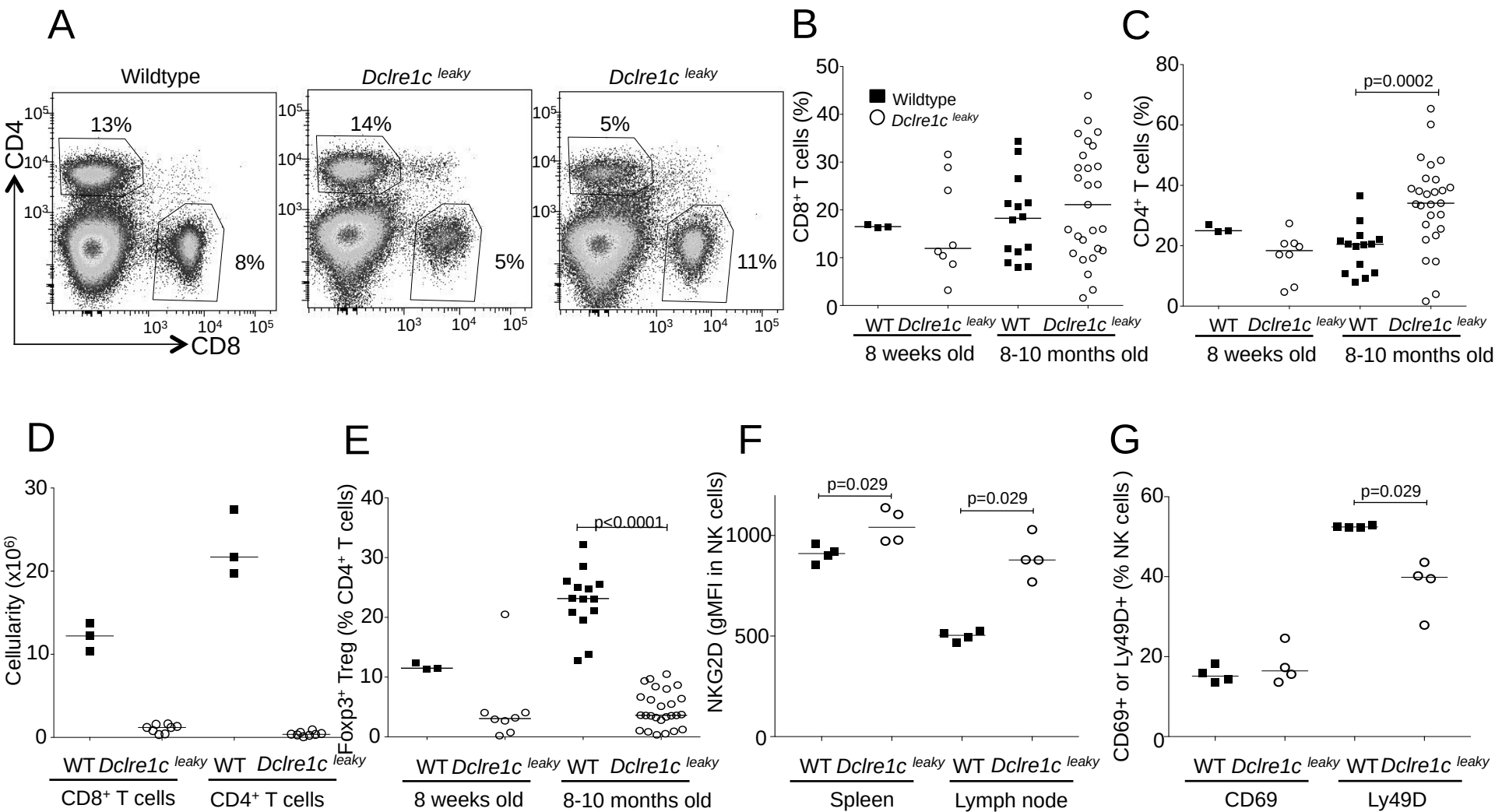
untreated control mouse, **(B)** control mouse after CTLA4-Ig treatment, **(C)** untreated *Dclre1c^{leaky}* mouse, and **(D)** *Dclre1c^{leaky}* mouse after CTLA4-Ig treatment.

Supplementary Figure 8. CTLA4-Ig treatment decreased total CD4⁺ T cells in *Dclre1c^{leaky}* mice and the overall inflammatory cytokine environment. *Dclre1c^{leaky}* mice and littermate controls (Ctrl) received CTLA4-Ig (25mg/kg) every two weeks starting at 8 weeks old. Mice were evaluated at 40 weeks old for the immune cell compartment in the lymph nodes. **(A)** Percentage of total CD4⁺ T cells. **(B)** Percentage of Treg from total CD4⁺ T cells. **(C)** Ki67⁺ cells and **(D)** CD69⁺ cells from total CD4⁺ T cells. Median and individual data points shown. **(A to D)** representative data of 2 experiments, untreated control mice (n=from 5 to 10) and untreated *Dclre1c^{leaky}* mice (n=9), CTLA4-Ig treated control mice (n=11) and CTLA4-Ig treated *Dclre1c^{leaky}* mice (n=from 7 to 11).

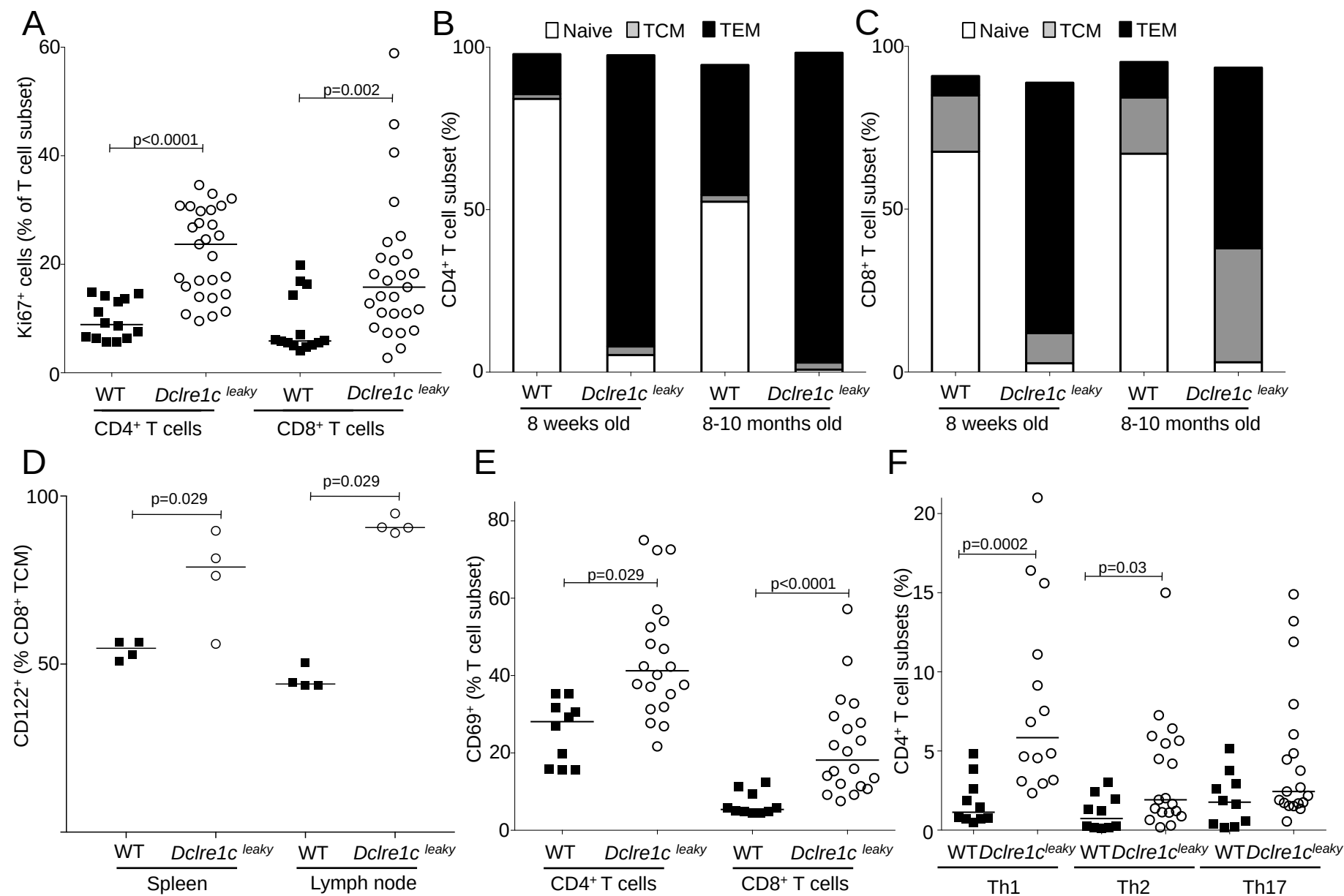
Supplementary Figure 1



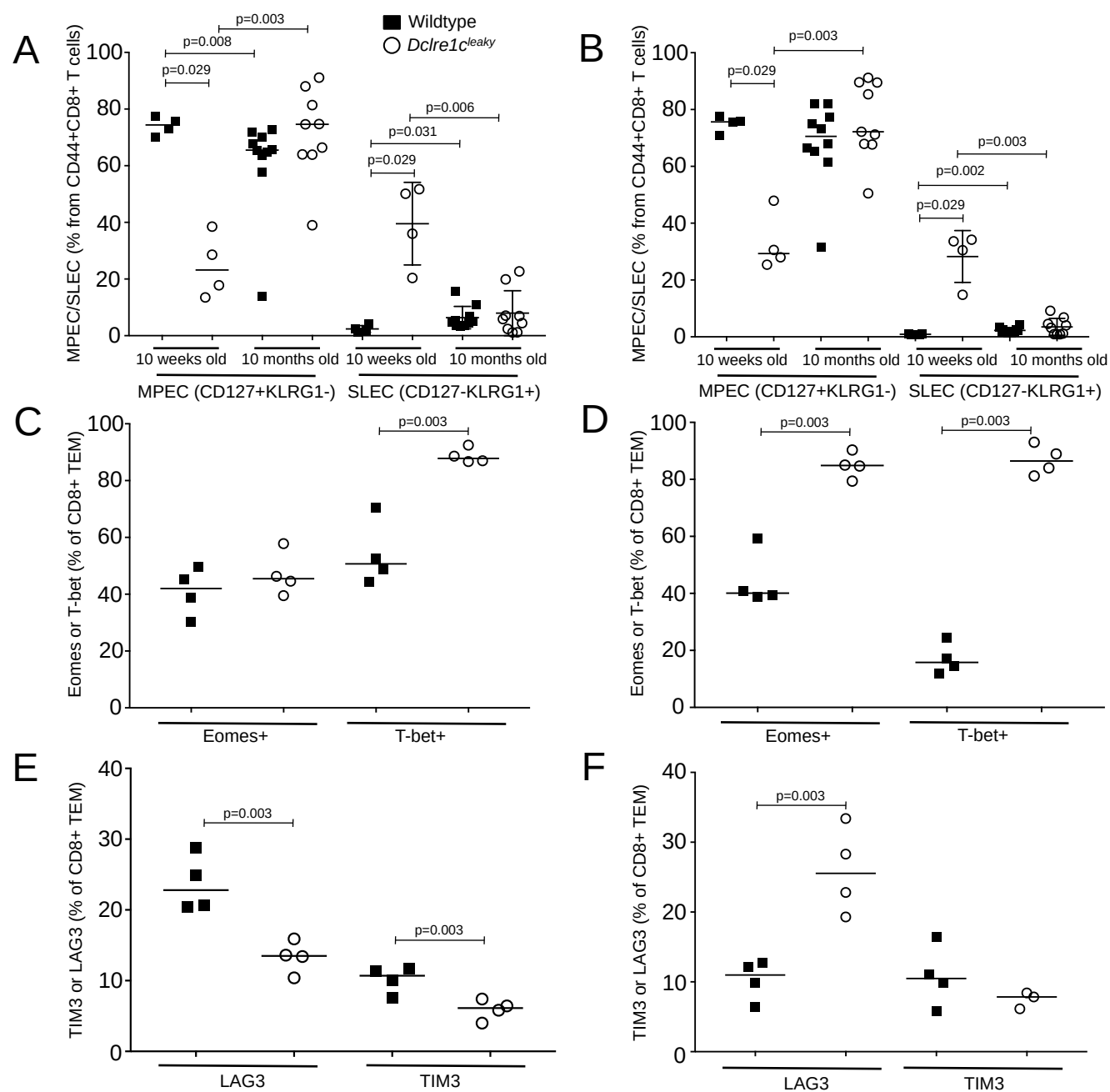
Supplementary Figure 2



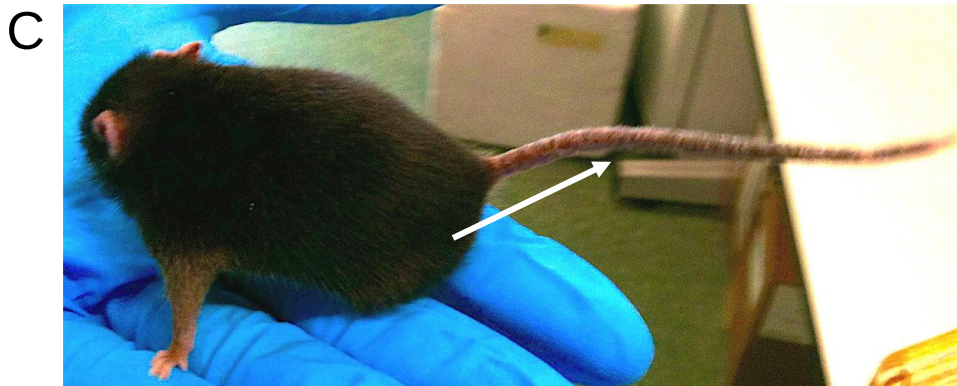
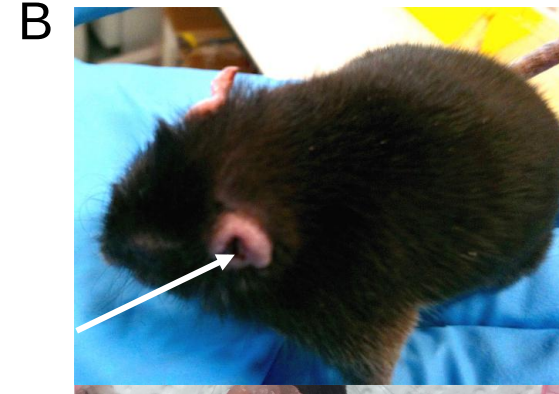
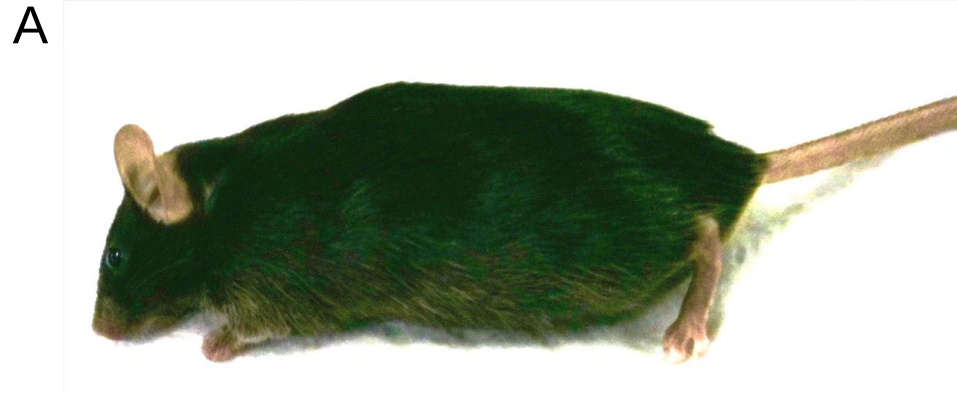
Supplementary Figure 3



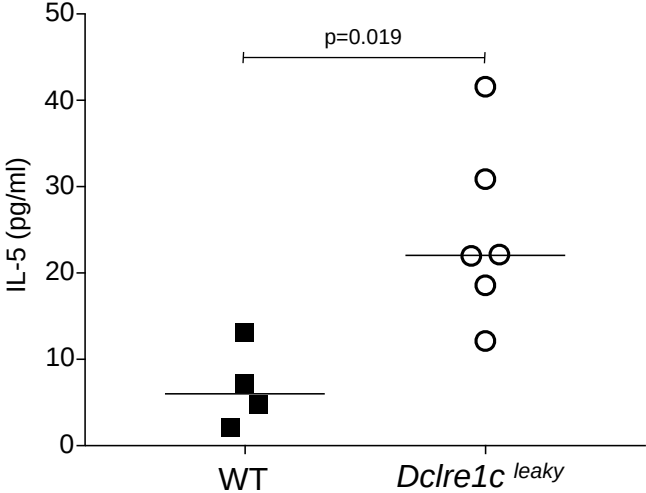
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6

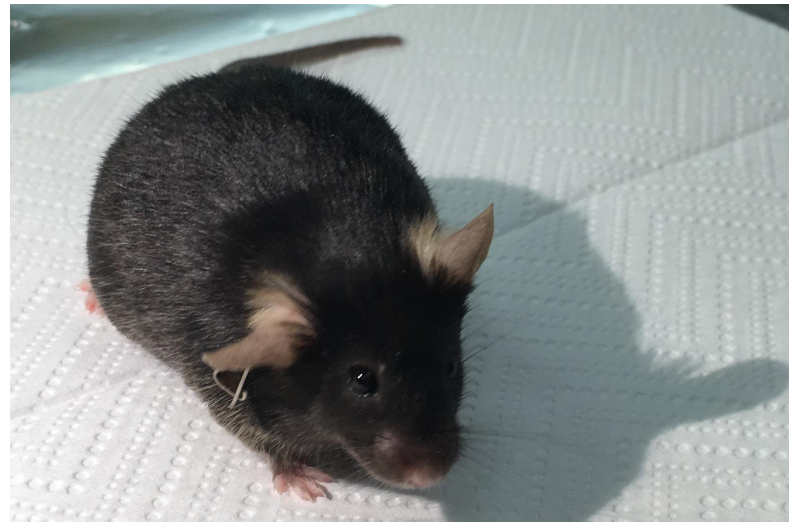


Supplementary Figure 7

A



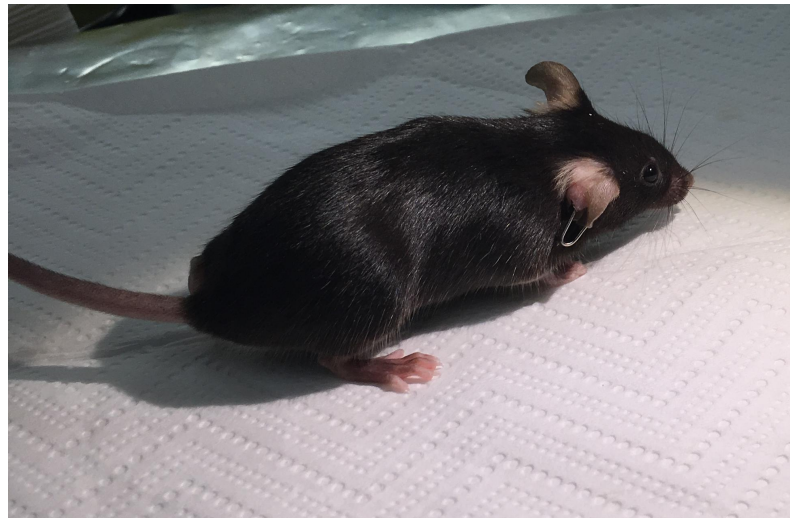
B



C



D



Supplementary Figure 8

