A missense mutation in TFRC, encoding transferrin receptor 1, causes combined immunodeficiency

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Patients with a combined immunodeficiency characterized by normal numbers but impaired function of T and B cells had a homozygous p.Tyr20His substitution in transferrin receptor 1 (TfR1), encoded by TFRC. The substitution disrupts the TfR1 internalization motif, resulting in defective receptor endocytosis and markedly increased TfR1 expression on the cell surface. Iron citrate rescued the lymphocyte defects, and expression of wild-type but not mutant TfR1 rescued transferrin uptake in patient-derived fibroblasts. TfRCY20H/Y20H mice recapitulated the immunological defects of patients. Despite the critical role of TfR1 in erythroid development and function, patients had only mild anemia and only slightly increased TfR1 expression in erythroid precursors. We show that STEAP3, a metalloreductase expressed in erythroblasts, associates with TfR1 and partially rescues transferrin uptake in patient-derived fibroblasts, suggesting that STEAP3 may provide an accessory TfR1 endocytosis signal that spares patients from severe anemia. These findings demonstrate the importance of TfR1 in adaptive immunity.

Combined immunodeficiencies (CID) are characterized by life-threatening infections due to genetic defects resulting in impaired T- and B-lymphocyte development or function1. The 41 documented monogenic causes of CID have identified pathways and molecules important for adaptive immunity, but many patients with CID remain without a genetic diagnosis1. We report the first human immunodeficiency caused by defective iron transport.

Fourteen Kuwaiti children in family A (Supplementary Fig. 1) had severe childhood infections, leading to the death of six individuals (Supplementary Table 1). Three patients (A1, A2 and A3) followed at our center had hypogammaglobulinemia, normal lymphocyte counts, intermittent neutropenia and intermittent thrombocytopenia (Supplementary Tables 2 and 3). Hematological parameters were normal, except for borderline-low hemoglobin levels in two patients and low mean corpuscular volume (MCV) in all three (Supplementary Table 2). Data on six additional patients showed severe hypogammaglobulinemia and mild anemia resistant to iron supplementation (data not shown). Eight patients received early hematopoietic stem cell transplantation (HSCT) from matched siblings, with resolution of clinical and laboratory abnormalities.

Patient 1 of family B from western Saudi Arabia is the 5-year-old son of consanguineous parents, with early-onset chronic diarrhea and recurrent infections (Supplementary Table 1). He had agammaglobulinemia, normal lymphocyte counts, intermittent thrombocytopenia, mildly low hemoglobin levels and low MCV (Supplementary Tables 2 and 3). He was treated with antibody against CD20 for presumed autoimmune thrombocytopenia, resulting in loss of circulating B cells without clinical improvement.

The numbers of circulating total (CD3+), helper (CD4+) and cytotoxic (CD8+) T cells, natural killer (NK; CD3−CD16+CD56+) cells and B (CD19+) cells in patients were normal or nearly normal. However, percentages of CD19+CD27+ memory B cells, important for antibody production, were reduced among CD19+ B cells (Supplementary Table 3). Proliferation of peripheral blood mononuclear cells (PBMCs) in
response to the mitogen phytohemagglutinin (PHA), cross-linking of the T cell receptor (TCR) with antibody against CD3, and combined phorbol 12-myristate 13-acetate (PMA) and ionomycin, which bypasses the TCR, was significantly decreased for cells from all four patients (Fig. 1a). T cell co-stimulation using antibody against CD28 or addition of interleukin (IL)-2 growth factor did not correct the defective TCR-driven proliferation, which was not associated with increased incidence of apoptosis (data not shown). These observations demonstrate a global defect in T cell proliferation.

Ligation of CD40 on B cells by CD40 ligand expressed on activated T cells in the presence of IL-4 causes proliferation-dependent immunoglobulin class-switch recombination from IgM to IgG and IgE, reflective of the production of high-affinity, protective antibodies. Proliferation and secretion of IgG and IgE in response to combined exposure to antibody against CD40 and IL-4 were significantly decreased in patient-derived PBMCs in comparison to control cells (Fig. 1b). IgE switching requires expression of Iε-Cε germline transcripts, which are early products of class-switch recombination, and activation-induced cytidine deaminase (AICDA), which initiates deletional switch recombination followed by expression of mature Iε-Cε transcripts. The patients had normal expression of immature Iε-Cε germline transcripts and AICDA mRNA in their B cells, but mature Iε-Cε transcripts were undetectable (Fig. 1c). Collectively, these data demonstrate impaired T cell proliferation as well as defective B cell proliferation and class-switching, which in combination constitute the mechanism underlying the susceptibility to severe infections characteristic of CID.

Genome-wide linkage scans of family A implicated a single locus at chromosome 3q28-29, but no pathogenic mutation was found within...
Figure 3 Correction of lymphocyte defects in patients A1–A3 with iron citrate. (a–c) Effect of addition of iron citrate on T cell proliferation in response to three stimuli (a), B cell proliferation and IgE synthesis in response to stimulation with antibody against CD40 and IL-4 (b), and molecular events in IgE isotype switching (c). In graphs, bars represent means ± s.e.m. from three independent experiments; *P < 0.05, ***P < 0.001.

this linkage peak (Supplementary Note). Therefore, whole-genome sequencing was performed on patient A1, his unaffected father and patient A2. A missense mutation in TFRC (c.58T>C; NM_003234.2), which encodes transferrin receptor 1 (TfR1; also known as CD71), was the only rare nonsynonymous or splice-site mutation homozygous in both patients and heterozygous in the obligate carrier father (Fig. 2a). TFRC is located 919 kb downstream of the distal boundary of the linkage peak; this unexpected location can be explained by recent de novo occurrence of the linkage and segregation of both the mutant and non-mutant copies of the disease-associated haplotype within the family (Supplementary Note). The c.58T>C mutation segregated perfectly with the disease phenotype in 34 available family members and was absent from multiple variant databases and 731 genotyped controls (Supplementary Table 4). The resulting p.Tyr20His substitution (NP_003225.2) disrupts the TfR1 intracellular internalization motif4 (Fig. 2b), and the Tyr20 residue is perfectly conserved in the 81 non-human vertebrate species surveyed (Supplementary Fig. 2).

Because of the similarities among patients from families A and B, we performed Sanger sequencing for the c.58T>C mutation in family B; the mutation was homozygous in patient B1 and heterozygous in his parents and his sister (Fig. 2a). Although the families were from different geographic regions and not known to be related, patient B1 shares a homozygous haplotype with the five genotyped patients from family A across a 3.3-Mb interval at chromosome 3q29-ter that

Figure 4 Lymphocyte defects and impaired TfR1 internalization in TfrcY20H/Y20H mice. (a) Representative T cell proliferation measured by CellTrace Violet dye dilution (left) and pooled data from three mice studied in three independent experiments (right). KI, TfrcY20H/Y20H knock-in mice; α-CD3 and α-CD28, antibodies against CD3 and CD28; M, medium. (b) Effect of addition of iron citrate on T cell proliferation in three mice studied in three independent experiments. Dashed lines represent the normal values for T cell proliferation in response to stimulation with antibody against CD3 and combined treatment with PMA and ionomycin. cpm, counts per minute. (c) B cell proliferation and IgG1 and IgE secretion following stimulation from three mice of each genotype in two independent experiments; ND, not detected. (d) Cell surface expression of TfR1, determined by FACS analysis, on unstimulated splenic T (CD3+) and B (B220+) cells, representing five mice of each genotype in five independent experiments. (e) Representative (left) and pooled (right) FACS analysis of TfR1 expression on T cells activated with PMA plus ionomycin after TfR1 cross-linking in three mice from each genotype in three independent experiments. Results for all graphs represent means ± s.e.m.; *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 5 Partial rescue of defective transferrin uptake in patient-derived fibroblasts by STEAP3 expression. (a) Cell surface expression of TfR1 on CD235a− EPCs for early (R1) and intermediate (R2) normoblasts from patients A1 and A3 and three controls. (b) Internalization of TfR1 after 30 min of TfR1 cross-linking on EPCs from wild-type and Tfrc<sup>Y20H/Y20H</sup> mice. (c) mRNA expression of STEAP genes in human cells from three controls. Fib, fibroblasts. (d) Coimmunoprecipitation (IP) and immunoblotting (WB) of Myc-tagged wild-type human TfR1 and FLAG-tagged wild-type mouse STEAP3 or Pyk2 (as a negative control) in cotransfected HEK293T cells. Immunoblotting of lysates without immunoprecipitation served as a positive control. (e) Coimmunoprecipitation and immunoblotting of Myc-tagged Tyr20His mutant human TfR1 (Mut-TfR1-Myc) and FLAG-tagged wild-type mouse STEAP3 or Tyr288His mutant STEAP3 (Mut-STEAP3-FLAG) in cotransfected HEK293T cells. (f) Uptake of Alexa Fluor 568–labeled transferrin by patient-derived fibroblasts transfected to express wild-type or Tyr288His mutant mouse STEAP3 (left) and quantification of uptake in comparison to untransfected patient and control fibroblasts, assayed in parallel (n = 3 per group) (right). Scale bars, 10 μm. Mut, Tyr288His mutant mouse STEAP3.

Results in a, b and f represent means ± s.e.m.; *P < 0.05, ***P < 0.001.

includes Tfrc, suggesting identical-by-descent inheritance of the mutation from an unknown common ancestor.

TfR1 is important for cellular iron uptake. Circulating apotransferrin binds two Fe<sup>3+</sup> ions to form holotransferrin, which binds to TfR1 (ref. 5). The TfR1-holotransferrin complex is internalized by receptor-mediated endocytosis, which requires an aromatic residue at the Tyr20 position mutated in the patients<sup>6</sup>. When intracellular iron levels are low, iron- regulatory proteins bind to the 3′ UTR of Tfrc mRNA, increasing its stability<sup>7</sup>. Tfrc mRNA and TfR1 protein levels were higher in PBMCs from patients than in controls (Fig. 2<sup>c,d</sup>), consistent with this feedback mechanism. TfR1 was minimally expressed on the cell surface of unstimulated control T and B cells but was expressed on a large percentage of patient-derived T and B cells, at levels 13- and sevenfold higher, respectively, than on controls (Fig. 2<sup>e</sup>). Likewise, TfR1 expression was sixfold higher on the cell surface of patient-derived fibroblasts in comparison to control cells (Supplementary Fig. 3a). Levels of soluble TfR1, generated by cleavage of surface-bound TfR1, were elevated in patient sera (Supplementary Table 2), consistent with increased TfR1 expression on the cell surface. We examined TfR1 endocytosis in Epstein-Barr virus (EBV)-transformed B cells and activated T cells, both of which highly express TfR1 on the cell surface. Cross-linking of TfR1 dramatically decreased its cell surface expression on control but not patient-derived cells, demonstrating that the p.Tyr20His substitution impairs TfR1 internalization (Fig. 2f and Supplementary Fig. 3b). Furthermore, transduction of cells to express wild-type TfR1 but not TfR1 or GAPDH1 corrected defective transferrin uptake by patient-derived fibroblasts (Fig. 2g,h). HSCT corrected TfR1 expression on patient T and B cells and fully corrected in vitro T and B cell function (Supplementary Fig. 4).

TfR1-mediated iron uptake is essential for lymphocyte development and proliferation<sup>8,9</sup>. Lymphocytes can also internalize iron through pathways involving non-transferrin-bound iron<sup>10,11</sup>. Addition of iron citrate, which supersaturates transferrin so that excess free iron is internalized independently of TfR1, corrected the proliferation, IgE secretion and Il-4 transcript expression of patient-derived lymphocytes (Fig. 3). These findings demonstrate that insufficient iron uptake is the cause of the defective T and B cell activation underlying the CID in these families. Moreover, the nature of this CID phenotype indicates that pathways involving non-transferrin-bound iron cannot compensate for impaired TfR1 internalization <em>in vivo</em>, thus suggesting an essential role for TfR1 in host immunity.

To obtain further support for the pathogenicity of the p.Tyr20His substitution, we generated Tfrc<sup>Y20H/Y20H</sup> knock-in mice (Supplementary Fig. 5a,b). In contrast to Tfrc<sup>−/−</sup> null mice<sup>12</sup>, Tfrc<sup>Y20H/Y20H</sup> mutant mice were viable, indicating that the mutation results in a hypomorphic allele. Tfrc<sup>Y20H/Y20H</sup> mice had significantly decreased serum IgG levels, hemoglobin levels and MCV in comparison to controls but normal percentages of splenic T and B cells, naive and memory T cells, NK cells and invariant NK T (iNKT) cells (Supplementary Table 5). However, Tfrc<sup>Y20H/Y20H</sup> T cells proliferated poorly in response to antibody against CD3 and treatment with PMA plus ionomycin, with proliferation significantly improved by the addition of iron citrate (Fig. 4a,b). Tfrc<sup>Y20H/Y20H</sup> B cells proliferated poorly in response to stimulation with an antibody against CD40 and IL-4 and secreted minimal IgG1 and IgE in response to stimulation with lipopolysaccharide (LPS) and IL-4 (Fig. 4c). TfR1 cell surface expression on mutant T and B cells was significantly increased, reflecting impaired internalization (Fig. 4d,e).

The stringent requirement of mouse TfR1 for hemoglobin synthesis in erythroblasts and erythrocyte development accounts for the embryonic lethality observed in Tfrc<sup>−/−</sup> mice<sup>12</sup>. Therefore, the mild anemia in patients was unexpected. Cell surface expression of TfR1 on glycolyporphorin A<sup>+</sup> (CD235a<sup>+</sup>) erythroid precursor cells (EPCs) from bone
This motif is critical for STEAP3 internalization, and the highly conserved Tyr288 residue in STEAP3 mRNA was highly expressed in normal human EPCs, whereas all four STEAP genes were poorly expressed in normal human fibroblasts, T cells and B cells. Wild-type STEAP3 and STEAP3 co-immunoprecipitated from lysates of cotransfected HEK293T cells, and both wild-type STEAP3 and the internalization-defective Tyr288His STEAP3 mutant associated with the Tyr20His TfR1 mutant (Fig. 5). Transfection to express wild-type STEAP3 but not Tyr288His mutant STEAP3 partially rescued defective transferrin uptake in patient-derived fibroblasts (Fig. 5f). These findings suggest that, through interaction with TfR1, STEAP3 facilitates TfR1 endocytosis in EPCs, and this mechanism may protect patients with this CID from severe anemia.

Hereafter, the role of TfR1 in host defense had not been established, and TFRC mutations had not been implicated in human disease. We describe a new CID caused by a homozygous mutation in TFRC that hinders TfR1-mediated iron internalization, resulting in defective T and B cell proliferation as well as impaired class-switching, which is necessary for antibody production. To our knowledge, this is the first human mutation implicating impaired iron transport in the pathogenesis of an immunodeficiency. We demonstrate that insufficient iron internalization constitutes the mechanism underlying this CID through in vitro correction of the lymphocyte defects in patients with iron citrate, which is internalized independently of TfR1. Furthermore, the relative importance of transferrin-dependent and non-transferrin-bound iron to host immunity was previously unknown. Both the patients and Tfrc<sup>Y20H/Y20H</sup> mouse model demonstrate the essential role of iron internalization via TfR1 in immune function. Our data also suggest a new pathway for TfR1 endocytosis mediated by its interacting partner STEAP3, which may account for the mild effect of the mutation on erythropoiesis. Of clinical relevance, we have taken advantage of elevated TfR1 cell surface expression on lymphocytes to diagnose two patients at birth, thereby facilitating early cure by HSCT.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Clinical variant data are accessible via ClinVar under accession SCV000255595. Microarray data are available via the Gene Expression Omnibus ( GEO) under accession GSE75358.

**Note: Any Supplementary Information and Source Data files are available in the online version of the paper.**

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**AUTHOR CONTRIBUTIONS**

H.H.J. performed functional experiments on the index family and on the Tfrc<sup>Y20H/Y20H</sup> mouse model. S.E.B. identified the TFRC mutation in the index family and performed genetic experiments and genome-wide linkage and whole-genome sequencing analyses. J.C. generated and analyzed the Tfrc<sup>Y20H/Y20H</sup> mouse model together with W.B. and D.F., performed functional experiments on patient B1 and provided clinical care to the patients in the index family. N.R., M.J.M., B.C.S. and Z.-J.L. performed functional experiments on the index family. F.R. performed genetic experiments. S.H.A. and B.K.-A.-R. provided ancestry-matched control DNA samples. H.A.-D., R.A., M.A.-S., A.V., E.S. and S.A. identified and provided clinical care for patient B1. E.G.D. provided tissue specimens from the affected patients in family A who had undergone HSCT. T.K.O. performed bioinformatics analysis. M.-V., N.C.A., L.D.N., M.D.F. and W.A.-H. gave critical advice. W.A.-H. ascertained and provided clinical care to the index family. H.H.J., S.E.B., J.C., L.M.K. and R.S.G. wrote the manuscript. R.S.G. and L.M.K. designed and coordinated the investigations. The final version of the manuscript was approved by all authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS
Study participants. We enrolled 34 members of a consanguineous Kuwaiti family (family A) and four members of a consanguineous family from western Saudi Arabia (family B) in the study. Genomic DNA was prepared from saliva, whole-blood samples or skin fibroblasts cells. Informed consent was provided by adult donors or by the children's parents or guardian on forms approved by local ethics committees and by the Institutional Review Board at Boston Children's Hospital. DNA samples from 479 healthy donors of Middle Eastern ancestry and 252 subjects of European ancestry were used as controls. Protocols used in the human studies have been approved by the Committee on Clinical Investigations at Boston Children's Hospital.

Genetic analysis. Genomic DNA from 32 subjects (five affected and 27 unaffected) from family A and the proband from family B was genotyped at 909,622 SNPs on the Genome-Wide Human SNP 6.0 Array (Affymetrix). Genotype calling for linkage analysis in family A was performed using Birdseed v2 with a stringent confidence threshold of 0.02. To enrich for the highest performing and most informative SNPs, we then filtered the panel to include only 145,751 SNPs with an across-sample SNP Call Rate of 1.00, a Hardy-Weinberg equilibrium P value $\geq 1 \times 10^{-3}$ and a minor allele frequency $>0$.

Because of the high sample numbers and multiple consanguinity loops, the full family A pedigree was much larger than could be analyzed by any program that computes an exact logarithm of the odds ratio (LOD) score. Genome-wide linkage scans were therefore conducted with Merlin v1.12 (ref. 18) using a series of overlapping subfamilies. A focused linkage analysis of 127 SNPs on chromosome 3q28-ter and using all available subjects was conducted with SimWalk2 v2.91, which can analyze pedigrees of arbitrary size using a stringent confidence threshold of 0.02. To enrich for the highest performing and most informative SNPs, we then filtered the panel to include only 145,751 SNPs with an across-sample SNP Call Rate of 1.00, a Hardy-Weinberg equilibrium P value $\geq 1 \times 10^{-3}$ and a minor allele frequency $>0$.

Protocols used in the human studies have been approved by the Committee on Clinical Investigations at Boston Children's Hospital.
the TFRC mutant encoding p.Tyr20His in the same vector. The vector expresses TurboGFP as a transfection marker independently through an internal ribosome entry site. The vector was packaged into lentiviral particles using the self-assembling DNA nanoarray technique developed by Drmanac et al. (2010). TFRC expression levels were determined by both Western blotting and flow cytometry using anti-TFRC antibody and isotype control antibodies. The mean red fluorescence intensity was calculated as described previously (Drmanac et al., 2010). Statistical analysis was performed using Student’s t test for comparing two groups, and one-way ANOVA was used for comparing more than two groups. For mouse experiments, the sample size was determined using a conservative estimate of a 50% decrease in lymphocyte proliferation, immunoglobulin secretion, T cell expression and T cell internalization in TfrcY20H/Y20H cells in comparison to controls, with a standard deviation of 10%. To detect this difference with 95% power, we would need at least two mice of each genotype for each experiment. All experiments were performed a minimum of two times unless otherwise indicated.

**Generation of TfrcY20H/Y20H knock-in mice.** A gene-targeting construct was generated to introduce the mutation encoding p.Tyr20His into exon 3 of mouse Tfrc. DNA fragments 3.5 kb and 3.3 kb in length were PCR amplified from the Tfrc gene in C57BL/6 genomic DNA and cloned into a gene-targeting vector upstream and downstream, respectively, of a neomycin resistance gene under the control of the PGK1 (phosphoglycerate kinase 1) promoter, used as a positive selectable marker. A gene fragment encoding diphtheria toxin A, located outside the homology arms, was used as a negative selectable marker. The c.587>T mutation was introduced into the construct by site-directed mutagenesis, and the mutant clone was sequenced to ensure the absence of off-target PCR-induced mutations. The construct was then linearized and electroporated into C57BL/6 embryonic stem (ES) cells, which were selected for neomycin resistance using standard techniques (Jabara, 2001). ES cell clones that had successful integration of the targeting construct, as determined by Southern blot and PCR, were injected into C57BL/6 blastocysts, which were implanted into foster mothers. Offspring were genotyped by PCR and Sanger sequencing. Chimeric mice were backcrossed to generate germine TfrcY20H/Y20H heterozygous mice, which were then intercrossed to produce TfrcY20H/Y20H homozygous-mutant mice. All mice were kept in a pathogen-free environment. Retro-orbital bleeds were performed to obtain peripheral blood for quantification of mouse hematological parameters (Hemavet 950FS) and serum immunoglobulins, which were quantified using standard ELISA techniques.

**Mouse cell cultures and reagents.** Anti-mouse monoclonal antibodies to the following molecules and the appropriate isotype controls were used for flow cytometry: CD4 (GK1.4), CD8 (53-6.7) and CD62L (MEL-14) from BioLegend and CD44 (IM7), CD25 (PC61.5), FOXP3 (FJK-16S), NK1.1 (PK3D), CD136 (DX5) and Ter119 (TER-119) from eBioscience. Mouse CD16 (α-GalCer analog) was from the NIH Tetramer Facility–Emory University Vaccine Center. Mouse splenic T and B cells were purified by negative selection (Pan-T Cell Isolation Kit II and CD43 Kit, respectively, Miltenyi Biotec). Purified mouse T cells were labeled with CellTrace Violet (Invitrogen) according to the manufacturer’s protocol and stimulated with plate-bound monoclonal antibody against CD3ε (5 μg/ml; 145-2C1), soluble monoclonal antibody against CD28 (2 μg/ml; 37.51) (both from eBioscience), recombinant mouse IL-2 (40 ng/ml; PeproTech), and PMA plus ionomycin for 96 h before FACS analysis. T cell proliferation was measured using the proliferative index (PI), calculated as described previously (Drmanac et al., 2010).

Purified B cells were stimulated with recombinant mouse IL-4 (50 ng/ml; R&D Systems) plus either antibody to mouse CD40 (1 μg/ml; HM40-3, Pharmingen) or LPS (10 μg/ml; Sigma). Proliferation was assayed at day 4 by measuring the incorporation of [3H]thymidine, which was added for the last 16–20 h of culture. On day 7, supernatants were collected and assayed for IgG and IgG1 by ELISA. Standard flow cytometry methods were used for staining of cell surface proteins, and data were collected and analyzed as described for human cell staining. Fluorochrome-labeled monoclonal antibodies to the following mouse molecules and the appropriate controls were used for staining: TR1 (R71217) from eBioscience and CD3 (17A2) and B220 (RA3-682) from BioLegend. For TR1 internalization, resting mutant T cells and purified wild-type or mutant T cells stimulated with PMA and ionomycin for 16–18 h or bone marrow isolated from mouse femurs was used, following the same procedure performed on human EBV-transformed B cells. Erythroid precursors were identified using fluorochrome-labeled monoclonal antibodies to CD44 (IM7) and Ter119 (Ter-119) from eBioscience. Purified rat anti-mouse TR1 (R71217) was used for cross-linking and phycoerythrin-conjugated goat anti-rat IgG was used for secondary detection of surface TR1 expression. The percentage of TR1 internalized was calculated as described previously. Internalized TR1 in mouse erythroblasts was calculated as follows: MFI0 at 0 min – MFI after 30 min of TR1 cross-linking.

**Statistical analysis.** Student’s t test was used to compare groups with equal variances, and two-way ANOVA was used for comparing more than two groups. For mouse experiments, the sample size was determined using a conservative estimate of a 50% decrease in lymphocyte proliferation, immunoglobulin secretion, T cell expression and T cell internalization in TfrcY20H/Y20H cells in comparison to controls, with a standard deviation of 10%. To detect this difference with 95% power, we would need at least two mice of each genotype for each experiment. All experiments were performed a minimum of two times unless otherwise indicated.

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