A hypomorphic R229Q Rag2 mouse mutant recapitulates human Omenn syndrome

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Rag enzymes are the main players in V(D)J recombination, the process responsible for rearrangement of TCR and Ig genes. Hypomorphic Rag mutations in humans, which maintain partial V(D)J activity, cause a peculiar SCID associated with autoimmune-like manifestations, Omenn syndrome (OS). Although a deficient ability to sustain thymopoiesis and to produce a diverse T and B cell repertoire explains the increased susceptibility to severe infections, the molecular and cellular mechanisms underlying the spectrum of clinical and immunological features of OS remain poorly defined. In order to better define the molecular and cellular pathophysiology of OS, we generated a knockin murine model carrying the Rag2 R229Q mutation previously described in several patients with OS and leaky forms of SCID. These Rag2R229Q/R229Q mice showed oligoclonal T cells, absence of circulating B cells, and peripheral eosinophilia. In addition, activated T cells infiltrated gut and skin, causing diarrhea, alopecia, and, in some cases, severe erythrodermia. These findings were associated with reduced thymic expression of Aire and markedly reduced numbers of naturally occurring Tregs and NKT lymphocytes. In conclusion, Rag2R229Q/R229Q mice mimicked most symptoms of human OS; our findings support the notion that impaired immune tolerance and defective immune regulation are involved in the pathophysiology of OS.

Introduction
Omenn syndrome (OS) is a peculiar immunodeficiency in which a defect in immune response against pathogens coexists with signs of autoimmunity (1, 2). Clinically described for the first time in 1965 (3), OS has been characterized at the clinical level as a SCID with erythrodermia, hepatosplenomegaly, failure to thrive, susceptibility to infections, diarrhea, and a fatal outcome, unless treated with bone marrow transplantation (3–6). At the laboratory level, patients manifest eosinophilia and elevated IgE despite the absence of B cells, while T cell numbers are often normal or elevated. Although these T cells are oligoclonal, show a limited TCR repertoire, and express activation markers, they are not functional, thus justifying the combined nature of the immunodeficiency (7, 8). Mutations in either Rag1 or Rag2 are found in most, but not all, OS patients. Interestingly, these mutated proteins seem to maintain partial activity, as they are able to direct some recombination events that give rise to oligoclonal T cell populations, which could be responsible for peculiar manifestations of OS. Indeed, OS patients show an autoimmune phenotype that is not present in SCID patients carrying null Rag mutations (9). Although the presence of partial recombination activity is a prerequisite for OS due to Rag defects, it is likely that environmental factors such as exposure to specific pathogens could trigger the autoimmune response, since coexistence of SCID and OS in the same family has been described previously (10, 11). In particular, patients with typical OS-type Rag mutations who received bone marrow transplants very early did not manifest OS features, while their siblings who were diagnosed later did. More recently, other genes involved in OS have been described (12, 13), including a very rare Artemis mutation (14); this finding is also compatible with the same pathogenesis, since residual Artemis activity could allow a limited number of recombination events. Although the demonstration that the basic biochemical defect of OS lies in the process of recombination, this finding leaves several open questions. In particular, it is unclear whether selection of gene segments in V-to-J recombination is skewed in OS and whether V regions are selected for rearrangement based on their locations along the chromosome or on perfect matching of their recombination signal sequences. Furthermore, it is unknown whether secondary V(D)J rearrangements occur in OS. In addition, the mechanisms accounting for target tissue infiltration by activated lymphocytes are also largely unknown. Specifically, it is unclear whether defects in negative selection in the thymus or in generation and/or function of Tregs may play a role in the pathophysiology of peripheral tissue damage. Finally, the origin of elevated serum IgE remains to be defined, considering that IgE production requires normal V(D)J recombination and class switch, while few B cells are usually detected in OS patients.

Addressing these and other similar questions is challenging in humans, in which collection of samples is limited for ethical reasons. The availability of an animal model recapitulating the pathogenesis of OS would be important for understanding the cellular mechanism of this disease and might also provide useful insights.
R229Q/R229Q mice revealed marked growth impairment (data not shown). This study, which were screened by direct sequencing (Figure 1C).

Crossed to produce the homozygous founder knockin mice in order to eliminate the heterozygous progeny was crossed with CMV-derived from ES cell clones were crossed with wild-type mice, and screening revealed that 1 ES cell clone underwent the expected mutation, and a neomycin resistance gene (PGK-neo cassette and thus obtain the desired expression of Rag2 during T and B cell differentiation.

This construct included about 3 kb of homology both upstream and downstream of the coding region containing the introduced mutation, and a neomycin resistance gene (PGK-neo) flanked by loxP sites was cloned downstream of the untranslated region (UTR) in the 3’ homology region.

Targeted replacement of Rag2 with the Rag2-R229Q/GFP vector was performed in 129Sv ES cells. PCR and Southern blot screening revealed that 1 ES cell clone underwent the expected homologous recombination event (Figure 1B). Chimeric mice derived from ES cell clones were crossed with wild-type mice, and the heterozygous progeny was crossed with CMV-Cre—expressing mice in order to eliminate the neo cassette and thus obtain the founder knockin Rag2+/R229QneoA mice. We confirmed the excision of neo by Southern blot analysis (Figure 1B). These mice were crossed to produce the homozygous Rag2R229Q/R229Q mice used in this study, which were screened by direct sequencing (Figure 1C). Heterozygous mice did not show any immunological defects or growth impairment (data not shown).

Analysis of the progeny obtained after breeding heterozygous Rag2+/R229Q mice showed that Rag2+/R229Q and Rag2R229Q/R229Q mice were viable and generated according to Mendelian inheritance. Homozygous Rag2R229Q/R229Q mice were fertile and were used to produce further generations, with an equal representation of male and female progeny.

Phenotypic analysis of Rag2R229Q/R229Q mice showed normal appearance at birth. However, at 8–10 weeks of age, 60% of the homozygous mutant mice developed substantial dorsal and facial hair loss. In addition, after an initial period of normal growth, a minority (4% of all Rag2R229Q/R229Q mice) started to develop severe alopecia, skin erythroderma, and wasting syndrome due to colitis (Figure 2A).

Histological analysis of Rag2R229Q/R229Q mice revealed marked infiltration by T lymphocytes and eosinophils in the skin and gut (Figure 2, B and C) that contrasted with the overall lymphoid depletion observed in the thymus, lymph nodes, and spleen. Similar to what we observed in Rag2−/− mice, immunostaining for cytokeratin 5, which detects medullary epithelium, revealed a diffuse network throughout thymic parenchyma with loss of corticomedullary differentiation in the thymi of Rag2R229Q/R229Q mice (Figure 2, D and E). Furthermore, unlike the thymic medullary region in Rag2−/− mice, the thymi in Rag2R229Q/R229Q mice were defective in forming Hassall corpuscle–like clusters (data not shown) (16). Abnormal architecture was also observed in lymph nodes, which revealed severe B cell depletion as well as a lack of B follicles (Figure 2F) and were composed of a diffuse population of pale histiocytes and dendritic cells admixed with T cells and eosinophils (Figure 2G). Moreover, scattered activated CD3+ and CD30+ immunoblasts were found in these areas (Figure 2G, right panels). Spleens from Rag2R229Q/R229Q mice showed similar morphological alterations, with severe depletion of the white pulp and particularly of B cells (data not shown). No Peyer patches were recognized in the gut. These pathological changes were consistently detected in all Rag2R229Q/R229Q mice at 6–8 weeks of age, regardless of the severity of clinical manifestations; however, the T cell infiltration and peripheral eosinophilia were more severe in mice that developed erythroderma and wasting syndrome.

**Results**

**Generation of the Rag2R229Q/R229Q model.** A targeting vector was designed to replace endogenous Rag2 with the R229Q mutation and a novel NheI site was engineered to use for homologous recombination in ES cells (see Figure 1A and Methods). EGFP was introduced as gene reporter to follow the proper expression of Rag2 during T and B cell differentiation. This construct included about 3 kb of homology both upstream and downstream of the coding region containing the introduced mutation, and a neomycin resistance gene (PGK-neo) flanked by loxP sites was cloned downstream of the untranslated region (UTR) in the 3’ homology region.

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T cell differentiation in Rag2^{R229Q/R229Q} mice. Analysis of Rag2^{R229Q/R229Q}/GFP expression in thymocyte subsets revealed developmental regulation characteristic of Rag2 (17, 18) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI30928DS1).

Cell recovery from Rag2^{R229Q/R229Q} thymi was analogous to that of Rag2^{–/–} thymi and markedly reduced compared with that of Rag2^{+/–} thymi (Supplemental Figure 2). Staining with CD4 and CD8 antibodies revealed a dramatic depletion of the CD4^+CD8^– double-positive (DP) compartment, with relative enrichment of the CD4^+CD8^– double-negative (DN) subset, the predominant subset (Figure 3A).

Developmental progression of DN cells is characterized by an ordered sequence of phenotypes defined by CD44 and CD25 expression: CD4^+CD25^– (DN1) to CD4^+CD25^+ (DN2), CD4^–CD25^– (DN3), and CD4^–CD25^+ (DN4). The DN3 to DN4 transition is predominantly controlled by productive rearrangement of the TCR-β locus and expression of the pre-TCR in the plasma membrane. Analysis of DN cells from Rag2^{R229Q/R229Q} thymi with CD25 and CD44 antibodies revealed an almost complete arrest of thymocyte development at the DN3 stage, a hallmark of recombinase-deficient mice (Figure 3, A and B) (19). Nevertheless, a few DP cells were recovered and displayed progressive upregulation of TCR-α/β with concomitant expression of CD69, a feature of ongoing positive selection. CD4^+ as well as CD8^– single-positive (SP) cells expressed high levels of TCR-α/β, which suggests that minute amounts of thymocytes were positively selected and accumulated as SP cells (Figure 3A). Moreover, γδ T cells were not detected (data not shown).

Next, we examined the T cell compartment in secondary lymphoid organs. The spleen and the mesenteric, inguinal, and cervical lymph nodes showed a distribution of CD4^+ and CD8^+ lymphocytes (arrow), and eosinophils (arrowhead); most lymphoid cells were CD4^–CD8^– lymphocytes (top right) that expressed the activation antigen CD30 (bottom right). Original magnification, ×20 (left); ×40 (right).

Figure 2
Historical analysis of Rag2^{R229Q/R229Q} mice. (A) Phenotypic aspect of a 3-month-old Rag2^{R229Q/R229Q} mouse, showing severe alopecia and skin erythrodermia. (B) Skin biopsy revealed marked dermal inflammation (top) composed of CD3^+ lymphocytes (bottom left) and containing numerous eosinophils (bottom right). Original magnification, ×10 (top); ×20 (bottom). (C) Similarly, the gut showed dense inflammatory infiltration (left), mainly composed of CD3^+ cells (right). Original magnification, ×20. (D and E) Comparison between thymic tissue from age-matched 6-week-old Rag2^{–/–} and Rag2^{R229Q/R229Q} mice: the normal corticomedullary differentiation observed in Rag2^{–/–} thymus (D), as highlighted by the anti–cytokeratin 5 immunostaining (D, inset), was absent in the Rag2^{R229Q/R229Q} mouse (E and inset). Original magnification, ×4. (D and E). (F) B220 immunostaining showed B follicles (b) and paracortical areas (p) in Rag2^{–/–} mice (left) in contrast to the abnormal architecture and severe depletion of B cells observed in Rag2^{R229Q/R229Q} mice (right). Original magnification, ×4. (G) Nodal parenchyma from the Rag2^{R229Q/R229Q} mouse (left) shows admixture of histiocytes, large activated lymphoid cells (arrow), and eosinophils (arrowhead); most lymphoid cells were CD3^+ lymphocytes (top right) that expressed the activation antigen CD30 (bottom right). Original magnification, ×20 (left); ×40 (right).
CD28 (data not shown) or addition of IL-2 to the medium rescued T cell proliferation (Figure 4B). However, T cells stimulated with phorbol 12-myristate 13-acetate/ionomycin (PMA/ionomycin) showed increased intracellular production of IL-2, IFN-γ, and TNF-α in both CD4 and CD8 subsets (Supplemental Figure 3).

We analyzed T cell repertoire by investigating TCRβ use among thymocytes and splenocytes obtained from 2 and 4 Rag2
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+/+ and R229Q/R229Q mice, respectively, and the results were compared with those in Rag2+/− mice. Figure 5A shows that in Rag2
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+/+ mice, use of some TCRβ family members increased in frequency within total T cells compared with that of Rag2+/− mice, both in the thymus and in the spleen. Interestingly, while use of some TCRβ family members, such as TCRβ3, expanded both in the spleen and in the thymus, other members showed a different distribution in the 2 organs. The immunoscope profiles in Figure 5B demonstrate the diversity of the TCR by showing the distribution of TCR third complementarity-determining region (CDR3) length for each TCRβ-TCRβ rearrangement. While the Rag2+/− mice showed a typical polyclonal distribution of CDR3 length, Rag2
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+/+ mice presented oligoclonsal patterns for most of the TCRβ family members.

Figure 3

Impaired T cell development and aberrant peripheral T cell activation in Rag2
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+/+ mice. (A) Left: Fluorescence-activated cell sorting analysis of thymocytes from the indicated mice stained with CD4 and CD8 antibodies. Top right: CD4+CD8+ cells were gated and analyzed for CD44 and CD25 expression. Bottom left: TCR-β and CD69 expression in electronically gated CD4+8+ cells. The percentage of TCR-βbrightCD69+ cells is indicated. Bottom right: Overlay of TCR-β surface expression in the indicated thymocyte subsets (gray histograms, Rag2+/+; open histograms, Rag2
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+/+). (B) Left: Percentage of cells during different T cell differentiation stages in the thymus. Right: Percentage of differentiating DN cells. Results are the mean and SD of 5 Rag2+/+ and 10 Rag2
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+/+ mice. P values were determined by unpaired Student’s t test.

B cell differentiation in Rag2
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+/+ mice. Most patients with OS show absence or severely reduced numbers of circulating B cells and profound hypogammaglobulinemia in spite of high levels of IgE. In Rag2
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+/+ mice, the number of B220+ cells was severely reduced in both bone marrow and spleen (Figure 6, A and B), suggesting that B cell development was impaired. In the bone marrow, productive rearrangement of the IgM heavy chain locus and expression of the membrane-bound pre-B cell receptor determines the developmental transition of B cell progenitors from the pro-B cell stage to the pre-B cell stage. We analyzed pro-B cell to pre-B cell transition by staining bone marrow cells with B220 and CD2 antibodies. As shown in Figure 6A, we observed a complete absence of B220lowCD2+ pre-B cells and B220highCD2+ cells that comprised immature and mature recirculating B cells, as was observed in Rag2+/− mice (20). Transitional B cells migrate to the spleen through the bloodstream and start to express membrane-bound IgM. IgM and IgD staining can distinguish transitional and mature recirculating B cells. In the bone marrow of Rag2
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+/+ mice, both these cell subsets were undetectable (Figure 6A). In the spleens of Rag2
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+/+ mice, few mature follicular IgM+IgD− B cells were detected, and transitional B cells were undetectable. Further staining of splenocytes for the complement receptor CD21 and the low-affinity receptor for IgE, CD23, revealed few CD21+CD23+ mature follicular B cells and a lack of CD21+CD23+ marginal zone B cells (21), showing that B cell development was severely compromised in Rag2
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+/+ mice (Figure 6B).

Along with the severe defect in B cell differentiation, Rag2
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+/+ mice showed hypogammaglobulinemia, with reduced levels of all IgG subclasses as well as of IgA and IgM. Although mean IgE serum levels were comparable to those of wild-type mice, some Rag2
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+/+ mice had markedly increased IgE (Figure 7), which correlated with more pronounced phenotypic manifestations. Despite the presence of low but detectable IgE levels, Rag2
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+/+ mice challenged with OVA were unable to produce OVA-specific antibodies (data not shown).

Central and peripheral tolerance in Rag2
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+/+ mice. The appropriate development of the thymic epithelial component is particularly relevant for T cell homeostasis because medullary epithelium expresses tissue-specific self-antigens under the control of the transcription factor autoimmune regulator (AIRE). Rag2
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+/+ mice displayed atrophic thymus, which were macroscopically indistinguishable from those of Rag2−/− mice, and did not reveal a medullary compartment in histochemical study. Analysis of Aire expression in Rag2
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+/+ thymi revealed low levels of Aire mRNA, as
observed in Rag2−/− mice, which are devoid of the medullary component of the organ (Figure 8A). In keeping with a possible defect in central tolerance related to reduced Aire expression, abnormal and severe lymphocyte infiltration in lungs and livers was observed in Rag2R229Q/R229Q mice (Figure 8, B–E). Finally, we evaluated the presence of naturally occurring Tregs (nTregs; defined here as CD25highFoxp3+) in our murine model. This subset has been shown to be crucial to prevent autoimmune manifestations (22). In order to determine whether nTregs could be reduced in Rag2R229Q/R229Q mice, Foxp3 expression was analyzed in thymus, spleen, and

Figure 5
Immunoscope analysis of TCR repertoire. (A) Quantitative TCRβ repertoire determined by real-time PCR analysis on T cells from the 2 thymi and 4 spleens from Rag2R229Q/R229Q mice (designated as 1–4) and from 2 representative Rag2−/− mice (designated as 1 and 2). (B) Representative immunoscope profiles of TCRβ analysis in the thymus and spleens of the mice as in A. The x axes represent CDR3 length, and y axes represent arbitrary fluorescence intensity of the runoff products.
The number of nTregs in all tissues examined was significantly decreased in Rag2<sup>R229Q/R229Q</sup> mice compared with Rag2<sup>+/+</sup> mice (Figure 9).

**Discussion**

Hypomorphic mutations in Rag1 and Rag2 genes in humans have been associated with a spectrum of clinical and immunological manifestations that range from typical and severe T-B-SCID to leaky SCID (with residual production of T cells and occasionally B cells) to OS, a disorder characterized by the presence of autologous, activated T cells that infiltrate target organs (3). The reasons underlying this variable phenotype are unclear. Along with the primary genetic defect, both genetic background and environmental factors have been postulated to play a role in the pathophysiology of the disease (23, 24). In addition, defective thymopoiesis has been shown to impair Aire expression in the thymic medulla, thus disturbing negative selection of autoreactive T cells that may escape to the periphery and damage target organs (25).

In agreement with these observations, a variety of genetic defects that affect early T cell development have been recently shown to cause OS in humans (12–14). In order to develop a model that recapitulates OS and could therefore be used to better dissect the mechanisms behind the various phenotypic manifestations of the disease, we have developed a homozygous R229Q mutation. We chose to investigate the effects of this mutation, as it was originally described in a patient mouse carrying the R229Q mutation. We picked to investigate the contribution of several patients with OS and in infants with leaky SCID (15), and we and others subsequently found this amino acid change in several patients with OS and in infants with SCID (10, 11). In this regard, the R229Q mutation offers a unique setting in which to evaluate the contributory roles of genetic background and environmental factors in determining the phenotype. Biochemical investigation of this mutant form of Rag2 has previously been shown to reduce V(D)J recombination activity by over 150-fold (10, 15).

We found that introduction of the Rag2 R229Q mutation did not affect viability and fertility in mice, but severely impaired T and B cell development and recapitulated most of the leaky features of OS. In particular, thymocyte development was predominantly arrested at the DN3 stage, during which expression of RAG is required to proceed along the differentiation pathway. The few SP thymocytes generated in Rag2<sup>R229Q/R229Q</sup> mice expressed TCR-α/β at the cell surface and were exported to the periphery, where they infiltrated target organs. Interestingly, the pattern of T cell infiltration observed in Rag2<sup>R229Q/R229Q</sup> mice mimicked that observed in patients with OS, with preferential localization in the skin and in the gut. Furthermore, the infiltrating T lymphocytes express activation markers and are oligoclonal, suggesting selective peripheral expansion (8). Competition within an appropriate number of clonal specificities upon microbial infection could ensure self-tolerance; thus the reduced diversity of the T cell repertoire in Rag2<sup>R229Q/R229Q</sup> mice could favor the expansion of T cell clones with inappropriate reactivity toward self-peptide/MHC complexes and development of immunopathology. In addition, because compensatory peripheral T cell expansion in lymphopenia was previously shown to produce autoimmune disease (26), the reduced thymic output in Rag2<sup>R229Q/R229Q</sup> mice could predispose animals to autoimmunity. A defective expression of tissue-specific self antigens controlled by AIRE contributes to the pathogenesis of autoimmune manifestations (27, 28). Rag2<sup>R229Q/R229Q</sup> mice have atrophic thymus and barely detectable Aire expression, suggesting a defect in negative selection of potentially self-reactive T cells (25, 29). The defective Aire expression might lead to the severe lymphocyte infiltration in target organs observed in the livers, lungs, and skin of affected mice. In Rag2<sup>R229Q/R229Q</sup> mice, few DP cells were detected and TCR-β chains were undetectable by intracellular staining in DN cells (data not shown), implying severe impairment of pre-TCR expression and signaling. Because signaling by the pre-TCR could influence thymic epithelium development (30), we speculate that
The observation that the leukopenia of the immunological phenotype of Rag2\textsuperscript{R229Q/R229Q} mice was more apparent in T than in B lymphocytes, while consistent with observations in patients with OS, may reflect stronger pressure for peripheral expansion within the T lymphocyte lineage.

A remarkable finding of the present study was the variability of the phenotypic manifestations observed in Rag2\textsuperscript{R229Q/R229Q} mice, with 60% of mice developing skin and hair abnormalities and 4% developing severe erythrodermia and weight loss. This variability mimicked that observed in humans and may be due to different factors, including variability in the genetic background.

The analysis of intracellular cytokine production after stimulation with PMA/ionomycin revealed increased production of IL-2, IFN-γ, and TNF-α, indicating Th1 profile production. These findings are in contrast with observations in OS patients. We speculate that environmental factors and antigen overload could play a role in the induction of increased IL-4 production and thereby favor IgE synthesis in patients with OS. Moreover, genetic background has been shown to influence Th1/Th2 balance and IgE serum levels (32). The Rag2\textsuperscript{R229Q/R229Q} mice generated in our laboratory carried the hypomorphic Rag2 mutation on a mixed C57BL/6 genetic background. In addition, these mice were kept under specific pathogen-free conditions. Therefore, we cannot exclude a possible effect of antigen encounter on the complexity of phenotypic manifestations. Future experiments with pathogen exposure and antigen challenge will help clarify this issue. The hypothesis that infiltration of target organs by oligoclonal T cells in patients with OS could be a result of defective negative selection of autoreactive T cell clones can now be explored by crossing Rag2\textsuperscript{R229Q/R229Q} mice with TCR transgenic mice, followed by exposure to the specific antigen.

In summary, we have shown that introduction of a homozygous Rag2 R229Q mutation in mice caused disturbed lymphoid development and phenotypic changes that largely recapitulated the spectrum of clinical manifestations associated with hypomorphic RAG mutations seen in humans with OS. Therefore, we propose this mouse model may be a useful tool to gain further insight into the pathophysiology of this complex syndrome.

### Methods

#### Construction of Rag2\textsuperscript{R229Q/GFP} targeting vector

QuikChange XL Site-Directed mutagenesis kit (Stratagene) was used to introduce nucleotide changes in the SalI/AseI fragment containing the coding region (GenBank accession number AC084753). The first nucleotide exchange was GC→AG at position 841–842, which led to the R229Q amino acid change. In addition, in order to perform an easier identification of the targeted ES clones, we introduced 2 silent mutations at positions 823 (A→G) and 826 (T→A), creating a new Nhel site. The mutated SalI/AseI fragment was subsequently cloned into the SacI site of the Clontech pEGFP-N1 vector and sequenced in order to perform an easier identification of the targeted ES clones, we introduced 2 silent mutations at positions 823 (A→G) and 826 (T→A), creating a new Nhel site. The mutated SalI/AseI fragment was subsequently cloned into the SacI site of the Clontech pEGFP-N1 vector and sequenced in order to verify that the RAG2 GFP fusion gene was in frame (pRAG2-EGFP-N1 construct). This step was performed according to the strategy previously described by Monroe et al. (18). A 750-bp AseI/KpnI fragment containing the 3′ UTR of Rag2 was cloned into the NotI site of the pEGFP-N1 vector containing a floxed neo cassette. Finally, a 5′ homology fragment of 3.0 kb (Clal/Sall) was cloned upstream the mutated Rag2-GFP segment, while a 2.2 kb BamHI/3′ homology fragment was inserted downstream of the neo cassette.

Generation of Rag2\textsuperscript{R229Q/R229Q} mice. Animal experiments protocol has been approved by the Ministry of Health and Local authorities (Institutional Ani-
The Rag2 targeting vector was transfected into 129Sv ES cells according to standard electroporation procedures using 40 μg of linearized NotI plasmid, and ES clones were selected by G418/gancyclovir resistance. Targeted ES clones were screened by PCR, using a 5′ primer located upstream of the targeting vector insertion site and a 3′ primer downstream of the NheI site, and subsequently sequenced to verify the presence of nucleotide changes. Positive clones were further confirmed by Southern blot analysis using a probe located in the 3′ region (a 520-bp BamHI/EcoRV fragment) on EcoRV-digested genomic DNA. Targeted ES clones were injected in blastocysts, the resulting chimeras were bred with C57BL/6 mice, and heterozygous animals were identified with the same approach used to identify ES clones. These mice were crossed to CMV-Cre transgenic mice in order to achieve neo cassette excision. Southern blot analysis performed on EcoRV-digested genomic DNA using the previously described probe revealed neo cassette excision: the heterozygous floxed mice showed a wild-type band together with a slightly shorter band as a result of the introduction of an additional (polylinker-derived) EcoRV site during the vector construction. We intercrossed Rag2+/R229Q mice to produce homozygous knockin Rag2R229Q/R229Q mice.

**Histology.** Mice were sacrificed at different time points after birth, and tissue samples were partially fresh-frozen in cryostat embedding medium (Bio-Optica) and partially fixed in 10% neutral-buffered formalin for both paraffin embedding and cryoprotection in 30% sucrose/PBS before freezing into cryostat embedding medium. Sections from both paraffin and frozen blocks were submitted for histological and immunohistochemical evaluation. H&E staining was used to study basic histopathological features. Frozen sections were air-dried for 18–24 hours, then fixed in acetone and used for immunohistochemistry by an indirect immunoperoxidase technique. The following primary antibodies were applied: rat anti-CD3ε (clone CT-CD3; Valter Occhiena), rat anti-CD4 (clone GK1.5; Southern Biotechnology Associates), rat anti-CD8 (clone CT-CD8a; Valter Occhiena), rat anti-B220 (clone RA3-6B2; Valter Occhiena), and goat anti-CD30 (clone TNFRSF8; R&D Systems). Immunostains on paraffin sections for CD30 and CD3 were performed upon antigen retrieval with microwave treatment in 1.0 mM EDTA buffer, pH 8.0, for 15 minutes. Primary antibodies were applied for 2 hours and followed by 30 minutes’ incubation in buffer containing the biotinylated specific secondary antibodies (Vector Laboratories). Immunolabeling was then performed by incubation for

**Figure 8**
AIRE expression analysis and cellular infiltration in target organs. (A) Amplification of Aire cDNAs obtained from Rag2+/+, Rag2R229Q/R229Q, and Rag2–/– thymic mRNAs. As an internal control, Gapdh was used. (B–E) Inflammatory infiltration in the lungs and livers of Rag2+/+ and Rag2R229Q/R229Q mice. Compared with the Rag2+/+ mouse (B), histological analysis of a representative 4-month-old Rag2R229Q/R229Q mouse revealed dense peribronchioral and perivascular inflammatory infiltration in the lung (C) composed mainly of CD3+ lymphocytes (C, inset). Similarly, in contrast to the control mouse (D), dense inflammatory infiltration composed of CD3+ T cells was found in liver portal tracts (E and inset). Original magnification, ×10 (B–E); ×40 (insets).

**Figure 9**
Analysis of nTregs in Rag2R229Q/R229Q mice. (A) Fluorescence-activated cell sorting analysis of thymus and spleen cells stained with CD25 and Foxp3 antibodies electronically gated on CD4+ cells. Numbers indicate the percentages of total live cells. (B) Histograms show percentage of CD25hiFoxp3+ cells in thymi, spleens, and lymph nodes of Rag2+/+ and Rag2R229Q/R229Q mice. P values were determined by unpaired Student’s t test.
20 minutes with streptavidin-peroxidase amplification system (BioGenex) and revealed using 3-amin-9-ethylcarbazole (Lab Vision). Slides were counterstained with hematoxylin. Images were acquired with an Olympus DP70 digital camera mounted on an Olympus BX60 microscope, using AnalySIS imaging software (version 3.2; Olympus).

Lymphocyte proliferation and intracellular cytokine staining. CD90+ T cells were purified from splenocytes of Rag2β2β2/R229Q/R229Q mice using anti-CD90 MACS microbeads (Miltenyi Biotec); resuspended in RPMI 1640 containing 10% heat-inactivated FCS, 2 mM glutamine and penicillin/streptomycin (100 IU/ml), and 10 μM 2-mercaptoethanol, and placed in flat-bottomed 96-well plates in the presence of surface-bound anti–mouse CD3 mAb (0.2, 2, and 20 μg/ml) alone or combined with 100 IU/ml IL-2 (Proleukin [aldesleukin]; Chiron) and 2 μg/ml anti-CD28. After 72 hours, cells were pulsed for 14–16 hours with 0.037 MBq (1 μCi) per well of [3H]-thymidine (Amersham Pharmacia Biotech). Cells were then harvested, and proliferation (in cpm) was measured as a scintillation counter. For intracellular cytokine staining, 1 × 10⁶ cells obtained from lymph nodes were stimulated for 2 hours with 1 mM ionomycin and 100 μM PMA. After addition of 5 μg/ml brefeldin A for 2 hours, cells were stained with FITC-conjugated CD8 and PerCP-conjugated CD4 antibodies. Cells were fixed in 2% formaldehyde and, after washing in permeabilization buffer (PBS containing 2% FCS and 0.5% saponin), were stained with allophycocyanin-conjugated (APC-conjugated) anti–IFN-γ, PE-conjugated anti–IL-4, APC-conjugated anti–TNF-α, and APC-conjugated anti–IL-2 antibodies.

Fluorescence-activated cell sorting analysis and antibodies. Single cell suspensions from thymi, bone marrow, spleens, and lymph node nodes were prepared and stained with specific fluorescent-conjugated antibodies. Thymocytes were incubated with the following antibodies: APC- or PerCP-conjugated anti-CD4, PE- or CyChrome-conjugated anti-CD8, FITC-conjugated anti-CD44, PE-conjugated anti-CD25, FITC-conjugated anti-CD69, PE-conjugated anti–TCR-α, and FITC-conjugated anti–TCR-β. Bone marrow, spleen, and lymph node nodes were stained with PE-conjugated anti-IgB, biotin-conjugated anti-CD43, PE-conjugated anti-IgM, FITC-conjugated anti-IgD, FITC-conjugated anti-IgA, and PE-conjugated anti-CD23. Samples stained with biotin-conjugated antibodies underwent an additional incubation with CyChrome-conjugated streptavidin. PE-conjugated anti–CD41 tetramer antibodies were kindly provided by C. Terhorst (Harvard Medical School, Boston, Massachusetts, USA). The staining with anti-Foxp3 mAbs (FJK-16s; eBioscience) was performed following the manufacturer’s instructions. All the antibodies used were from BD. A minimum of normal TCRVβ repertoire was analyzed and separated by a 373A sequencer (Applied Biosystems). The size and intensity of each band were analyzed by the quantitative Immunoscope approach (33, 34). The Gaussian distribution of the different CDR3 lengths was characteristic of normal TCRVβ repertoire.

Statistics. For comparison between groups, an unpaired 2-tailed Student’s t test was used. Mann-Whitney test (nonparametric analysis) was used to evaluate the significance of Ig differences. A P value less than 0.05 was considered significant.

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