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A Transgenic Mouse Strain with Antigen-specific T Cells (RAG1KO/*sf*OVA) Demonstrates that the Scurfy (*sf*) Mutation Causes a Defect in T-Cell Tolerization

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The scurfy (*sf*) murine mutation causes severe lymphoproliferation, which results in death of hemizygous males (*sf*Y) by 22 to 26 days of age. The CD4+ T cells are crucial mediators of this disease. Recent publications have not only identified this mutation as the genetic equivalent of the human disease X-linked neonatal diabetes mellitus, enteropathy, and endocrinopathy syndrome, but also have indicated that the defective protein—scurfin—is a new forkhead/winged-helix protein with a frameshift mutation, resulting in a product without the functional forkhead. These results have lead to speculation that the scurfy gene acts by disrupting the T-cell tolerance mechanism, resulting in hyperresponsiveness and lack of down-regulation. The Rag1KO/*sf*Y OVA strain, with virtually 100% of its CD4+ T cells reactive strictly to ovalbumin (OVA) peptide 323-339, is an excellent model for determination of the *sf* mutation's ability to disrupt tolerance. We hypothesized that Rag1KO/*sf*OVA mice would not be tolerant to antigen at a dose that tolerizes control animals. We found that splenic cells from Rag1KO/*sf*Y OVA mice injected with the same dose of OVA peptide that induces tolerance in cells from control mice proliferate in vitro in response to OVA peptide. These results are consistent with a defect in the pathway responsible for peripheral T-cell tolerization.

Lesions of scurfy (*sf*) include hunched posture, runting, scaly skin, and cachexia. At necropsy, mice have marked splenomegaly, moderate hepatomegaly, enlarged lymph nodes, and a small, thin thymus (1-3). Microscopic lesions include severe lymphohistiocytic proliferation and infiltration, which effaces lymph node architecture, thickens the dermis, and forms nodular accumulations in portal areas of the liver (2). Recent work using the *sf* mouse indicated that *sf* T cells were hyperresponsive to T-cell receptor (TCR) ligation and had a decreased requirement for co-stimulation through the cell surface protein CD28, relative to that of normal controls. These cells also were less sensitive than normal controls to inhibitors of tyrosine kinase, indicating a defect in normal down-regulation of T-cell activation (4). Additionally, the gene in which the *sf* mutation lies (*Foxp3*), has been sequenced and determined to encode a transcription factor (scurfin) with a forkhead/winged-helix domain. The *sf* mutation causes a frameshift, producing a protein that lacks the functional forkhead domain (5). The *sf* mouse gained further interest with researchers when it was confirmed that this mutation is equivalent to the human disorder called X-linked neonatal diabetes mellitus, enteropathy, and endocrinopathy syndrome (IDEX) (6). Another author has speculated that, since *sf* is a homologue for X-linked autoimmunity-allergic disregulation syndrome (XLAAD), in which constitutively activated T cells cause disease, the *sf* mutation may result in T cells that are unable to undergo tolerance and so remain activated (7). However, since the antigenic stimulus for *sf* CD4+ T cells was (and still remains) unknown, it was difficult to evaluate the response of these T cells to antigens.

To control the T-cell antigenic determinants, the *sf* strain was bred to the DO11.10 strain, in which 75 to 95% of the mouse's TCRs are restricted to the chicken ovalbumin peptide 323-339 (OVA 323-339) (8). This produced *sf*Y OVA male mice that, compared with controls, had less severe disease; lived far longer than did the original *sf* strain; and yielded T cells that proliferated in culture in response to low concentrations of OVA peptide. However, the presence of 5 to 25% of T cells with TCRs still responsive to an unknown antigen made further studies of this model fruitless. Therefore, the recombinase-activating gene (RAG) knock-out (KO) mouse was then chosen for further breeding. Mice that lack the *RAG* gene are unable to produce either T or B cells without further genetic programming (9, 10) which, in our strain, was provided by the OVA transgene. The resultant Rag1KO/*sf*Y OVA male mice did not develop the scurfy disease despite the presence of genetically *sf* CD4+ T cells; their splenic cells have decreased proliferation at high doses of peptide in vitro, compared with Rag1KO/wt OVA cells; their thymus was capable of deletion of self-reactive thymocytes; and their CD4+ T cells activated in vivo when exposed long term to OVA peptide (11).

We were curious as to the tolerance ability of this strain as the disease process in scurfy closely resembles that seen in a CTLA-4 knock-out mouse. The CTLA-4 molecule has been ex-

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tensively studied as a key for prevention of lymphoproliferation, long-term graft survival, and suppression of autoimmune disease (12-14). It has also been determined to be critical for induction of peripheral T-cell tolerance (15). It was, therefore, our hypothesis that Rag1KO/*sf*/Y OVA mice would not tolerize when exposed in vivo to the same high dose of peptide that caused tolerization in Rag1KO/wt OVA mice, nor would their splenic cells react in vitro to OVA peptide as would those of controls.

Materials and Methods

Mice. In 1949, the *sf* mutation appeared as a spontaneous mutation in the standard inbred strain 129/Rl at the Oak Ridge National Laboratory's (ORNL) Mouse House facility. This strain has been maintained by W. L. Russell at Oak Ridge as a forced heterozygote since that time. The strain chosen for this work also contained the mutation *Otc*^{spf}, which allowed for *sf* genotyping (see below). Female scurfy-sparse fur (*sf spf*/wt) mice were transported to the University of Tennessee for housing and breeding. Congenic OVA transgenic mice, derived from the DO11.10 hybridoma by Dennis Loh (Washington University, St. Louis, Mo.) (8), were obtained from Barry Rouse (with permission) at the University of Tennessee. Three founder Rag1 homozygous knock-out males (Rag1 -/-) on a B6;129S-Rag1^{tm1Mom} mixed background were purchased from the Jackson Laboratory (Bar Harbor, Maine). Mice that are homozygous deficient for either the *Rag1* or *Rag2* gene cannot produce mature T or B cells, the maturation process being halted at V(D)J recombination (9). This Rag1 mouse is similar to the SCID mouse, except that SCID mice have a defect in a double-stranded DNA break repair system; Rag1-deficient mice do not have this defect (9). Rag1-deficient mice proved to be quite hardy, and no special precautions were made in their husbandry, as needs to be the case with SCID mice.

All mice were housed in specific-pathogen-free, static microisolator cages at the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved Laboratory Animal Facility of the University of Tennessee College of Veterinary Medicine. The bedding was changed twice a week, and mice were fed Harlan Teklad 8640 rodent chow ad libitum and were given chlorinated water. All mice were routinely tested in house for pinworms and *Citrobacter* sp. (all test results were negative), and sentinel serum samples were submitted monthly to the University of Missouri Research Animal Diagnostic Laboratory to be tested using their "Basic" screen for 12 common pathogens; results of all tests were negative. All animal work was performed under the Institutional Animal Care and Use Committee (IACUC)-approved protocol No. 558, Immunology of the Scurfy Mouse.

Genotyping. The tail of all mice was clipped between four and six weeks of age, and DNA was extracted in routine manner (16). Genotyping for the *sf* mutant gene was conducted, using a modification of a published polymerase chain reaction (PCR) protocol (17), based on the observation that the *sf* gene is tightly linked to a mutation in the ornithine transcarbamylase gene (*Otc*) called sparse fur (*spf*). Primers for *spf* PCR analysis were 5'- CTA ACC CAT CAG AGT TTG AAA TAA AC -3' and 5'- CCC CTC TCA ATA CAT TCA CTG TCT -3', which flank a section of the *Otc* gene. Cycling conditions for this hot-start PCR were 94°C for four minutes, 30 cycles of 94°C for one minute, 63°C for one minute, and 72°C for one minute, followed by a final extension at 72°C for five minutes. The PCR amplification products were then directly digested overnight with 10 U of *Mse*1, which recognizes a unique cleavage site in the base-substitution *spf* mutation. Wild-type DNA, when separated on a standard 3% agarose gel and stained with ethidium bromide, yields an approximately 180-base pair (bp) band, but *spf* DNA yields two 90-bp fragments after *Mse*1 digestion. Heterozygous (carrier) females yield one of each band, homozygous *sf spf*/*sf spf* females and *sf spf*/Y males yield the 90-bp band, and wild-types of both sexes yield the 180-bp, intact band only.

Presence of the OVA transgene was also determined by use of PCR analysis with primers 5'- TGG CTC TAC AGT GAG TTT GGT -3' and 5'- CAG GAG GGA TCC ACT GCC AGC -3' and with cycling conditions of 94°C for four minutes, followed by 30 cycles of 94°C for one minute, 64°C for one minute, and 72°C for one minute, with a final extension at 72°C for five minutes. Samples were kept at 4°C before analysis, using a standard 1.5% agarose gel stained with ethidium bromide. These primers are specific for the vector used to insert the transgene, and will yield a 300-bp band in either a homozygote or a heterozygote, differentiating them from wild-type (no bands) but not from each other.

The *Rag1* gene fragment was amplified by PCR analysis, using primers that lie within the *Rag1* gene: 5'-CTC CCG AGC TTC TCA CTC AC-3' and 5'-AGC TAT CAC TGG GAG GCA GA-3' (primer sequences provided by Jackson Laboratory) and cycling conditions of 94°C for four minutes, 30 cycles of 94°C for one minute, 55°C for one minute, and 72°C for 30 sec., with a final extension at 72°C for five minutes. Samples were kept at 4°C before analysis, using a standard 2 to 3% agarose gel. At this annealing temperature, a non-specific band is consistently seen at 200 bp, which verifies that the PCR reaction was successful; a single band at 366 bp indicates either a wild-type or heterozygote; and no band except the PCR control indicates a homozygous knock-out.

In vivo injections. This protocol was a modification based on a previous study that assessed CTLA-4 function in peripheral T cells (15). Adult mice of similar age were allotted to four groups, one to five mice per group per experiment: Rag1KO/wt OVA mice were given 0.2 ml of incomplete Freund's adjuvant (IFA) alone (called the "naive group"), Rag1KO/wt OVA mice were given a stimulation dose of OVA peptide in IFA (the "stimulated group"), Rag1KO/wt OVA mice were given a tolerization dose of peptide in IFA (the "control tolerance group"), and Rag1KO/*sf*/Y OVA mice were given the tolerization dose in IFA (the "scurfy tolerance group"). The stimulated, control tolerance and scurfy tolerance groups were injected once with a dose of OVA 323-339 shown to cause stimulation in recipient DO11.10^{+} T cells (30 μg of peptide in IFA s.c.; final volume 0.1 to 0.2 ml), or a dose of peptide shown to cause tolerization (300 μg of peptide in IFA i.p., final volume 0.1 to 0.2 ml) (15). This experiment was performed on four occasions, and cells from all four groups were harvested each time.

Cell harvesting and plating. Mice were euthanized three days after injection by use of cervical dislocation. Spleens were aseptically harvested and manually strained through a 100-μm sterile nylon cell strainer (Falcon, Becton Dickinson, Mountain View, Calif.) into 5 ml of RPMI 1640 medium (Gibco, Grand Island, N.Y.). This medium was supplemented with 5% endotoxin-free fetal calf serum (FCS) (Intergen, Purchase, N.Y.), 10 ml of 100× antibiotic/antimycotic (Gibco), 190 μl of 2-β-mercaptoethanol (3.4×10^{-4} vol./vol.), 12.5 ml of 1*M* HEPES (Gibco), $NaHCO_3$ (0.1875%)

(Gibco), and 10 ml of solution 1 (600 mg of oxaloacetic acid, 40 mg of crystalline insulin, and 25 ml 100 μ*M* sodium pyruvate, dissolved with sterile water to a final volume of 50 ml). After centrifugation in an IEC Centra-8R centrifuge at 1,000 RPM for eight minutes, cell pellets were re-suspended in 5 ml of red blood cell (RBC) lysis buffer (filtered 0.144*M* ammonium chloride and 0.017*M* Tris base, pH 7.2) at room temperature. The RBCs were lysed by sample immersion in a 37°C water bath for two minutes and 30 sec. After a second, identical centrifugation, pelleted splenic cells were resuspended in 5 ml of medium, and an aliquot was diluted 1:10 and stained with 0.04% trypan blue in 1× phosphate-buffered saline (PBS; Gibco).

Viable cells were counted by use of a hemocytometer. Enumerated cells were suspended in RPMI medium with 5% FCS at a concentration of 4×10^6 cells/ml. To determine lymphocyte proliferation, cell suspensions were plated in 96-well round-bottom plates, 100 μl/well in triplicate, plus 100 μl of OVA 323-339 in RPMI 1640/5% FCS medium to the following concentrations: 0 (control), 0.001, 0.01, 0.1, 1, 2, 5, and 10 μ*M*. Plates were incubated at 37°C in a 5% CO_2 incubator for 48 h. [^{3}H]Thymidine was then added in RPMI 1640/5% FCS medium at a concentration of 1 μCi/well, and incubation continued for six to eight hours.

After freezing at -20°C to stop incorporation, plates were thawed and harvested onto glass fiber filters (Packard BioScience, Meriden, Conn.) on a 96-well harvesting apparatus and counted for 10 min by use of a Matrix 96 Direct Beta Counter (Packard BioScience) interfaced with a computer. Mean counts for the triplicate wells at each peptide concentration for each group were entered into the GraphPad Prism program, which calculated mean and standard deviation for each treatment group at each concentration. Due to differences in splenocyte yield among groups, it was not always possible to plate three wells of each concentration for each group. Therefore, n did not equal four for all groups at each concentration for analysis.

Flow cytometry. Between 0.5 and 1×10^6 cells/sample (either freshly prepared or after 48 h in culture) were washed in a fluorescence-activated cell sorter (FACS) wash buffer (1% PBS, 1% bovine serum albumin (BSA), and 2% sodium azide [both from Sigma Chemical Co., St. Louis, Mo.], filtered) and stained first with purified rat anti-mouse CD32/CD16, to block non-antigen-specific binding of the F_c portion of antibodies to target cells. After this step, samples were stained with 1 μg each of one or more of the following (all from BD BioSciences Pharmingen, San Diego, Calif., and all rat anti-mouse antibodies except as noted): FITC-conjugated anti-CD8a (Ly-2); PE-conjugated anti-CD4 (L3T4); FITC-conjugated hamster anti-mouse anti-CD3e; PE-conjugated anti-CD45R/B220; biotinylated V_β 8.1, 8.2 TCR, a polyclonal antibody that binds (among other molecules) the OVA TCR; and KJ1-26 (gift of Barry Rouse and John Kappler, University of Tennessee), a monoclonal antibody specific for the αβ chains of the DO11.10 TCR (8). All samples were stained for 30 min at 4°C, washed in FACS buffer, resuspended in 1 ml of fresh FACS buffer, and analyzed by use of a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems) interfaced with a PowerMac 7600/120 PowerPC. Data analysis was performed, using Cellquest software, version 3.0f (Becton Dickinson).

Fluorescence data were collected for at least 10,000 ungated cells/sample and were logarithmically amplified. Postacquisition gating was done on the live lymphocyte population only. Live cells were determined by their forward and side scatter (FSC, SSC) characteristics, and on information provided by Becton Dickinson. Regions were manually drawn around stained cell populations. Populations were judged to be "dull" if their fluorescence intensity was below the average for the group, and "bright" if it was above the average intensity, and separate regions were created for each. Population percentages were computed for each region. Results are reported as percentage of positive staining of the viable cell population, excluding the non-viable portion.

Results

Tolerance induction assay, in vitro challenge, and stimulation counts. Mice were allotted to four treatment groups: Rag1KO/wt OVA (control mice), given a dose of OVA peptide that should induce stimulation of harvested T cells in vitro (stimulated); Rag1KO/wt OVA(control mice), given PBS (naive); Rag1KO/wt OVA (control mice), given a dose of OVA peptide that should induce tolerance (lack of response) by splenic cells in vitro (control tolerance); and Rag1KO/*sf*/Y OVA (experimental mice), given the same tolerance-inducing dose (scurfy tolerance) (Fig. 1). Splenic T cells from the naive group stimulated well in vitro at the four highest OVA peptide concentrations, as expected, since they had not received any signals to either increase or decrease their response. Cells from the control mice given the stimulation dose yielded high counts at ≥ 0.1 μ*M* peptide, which also was expected, because the in vivo doses stimulated or "primed" the cells for further proliferation. Due to experimental variation, however, these numbers were not significantly different from values for the naive group. Splenic cells from the control tolerance group scarcely responded to OVA peptide in vitro, as seen in other studies (15). In contrast, cells from the scurfy tolerance group proliferated significantly more in vitro than did cells from the control tolerized group at every concentration of peptide, and values were not significantly different from those for the highly proliferative naive group at all but the highest peptide concentration.

Flow cytometry. At euthanasia, samples of splenic T cells were taken for flow cytometry, and compared with other samples taken at 48 h after plating for the stimulation assays. We examined the percentages of CD4+ and KJ1-26+ in the groups, presuming the OVA to have activated $CD4^+KJ1\text{-}26^+$ T cells. We focused on two populations: $CD4^{+bright}KJ1\text{-}26^{+bright}$ cells, which represent T cells proliferating the most in response to peptide, and $CD4^{+dull}KJ1\text{-}26^{+dull}$ cells, which represented those cells not as strongly stimulated.

In general (Table 1), we determined that cells from the scurfy tolerance group, which do not tolerize in vivo and yield cells that proliferate in the presence of peptide ex vivo, are different from controls of these two cell groups. At euthanasia, the percentage of $CD4^{+bright}KJ1\text{-}26^{+bright}$ cells in the Rag1KO/*sf*/Y OVA group was noticeably lower than values for all other groups, although variation in the naive and control tolerance groups over several experiments did not result in significant difference. This would seem to indicate that the $CD4^+KJ1\text{-}26^+$ transgenic T cells from these mice are not proliferating maximally. After 48 h in vitro stimulation in the presence of OVA peptide, it was evident that the increase in proliferation in the stimulated group was due principally to an increase in $CD4^{+bright}KJ1\text{-}26^{+bright}$ cells, with some input from the increased $CD4^{+dull}KJ1\text{-}26^{+dull}$ cells. Cells from the naive group did not have such increase in $CD4^{+bright}$KJ1-

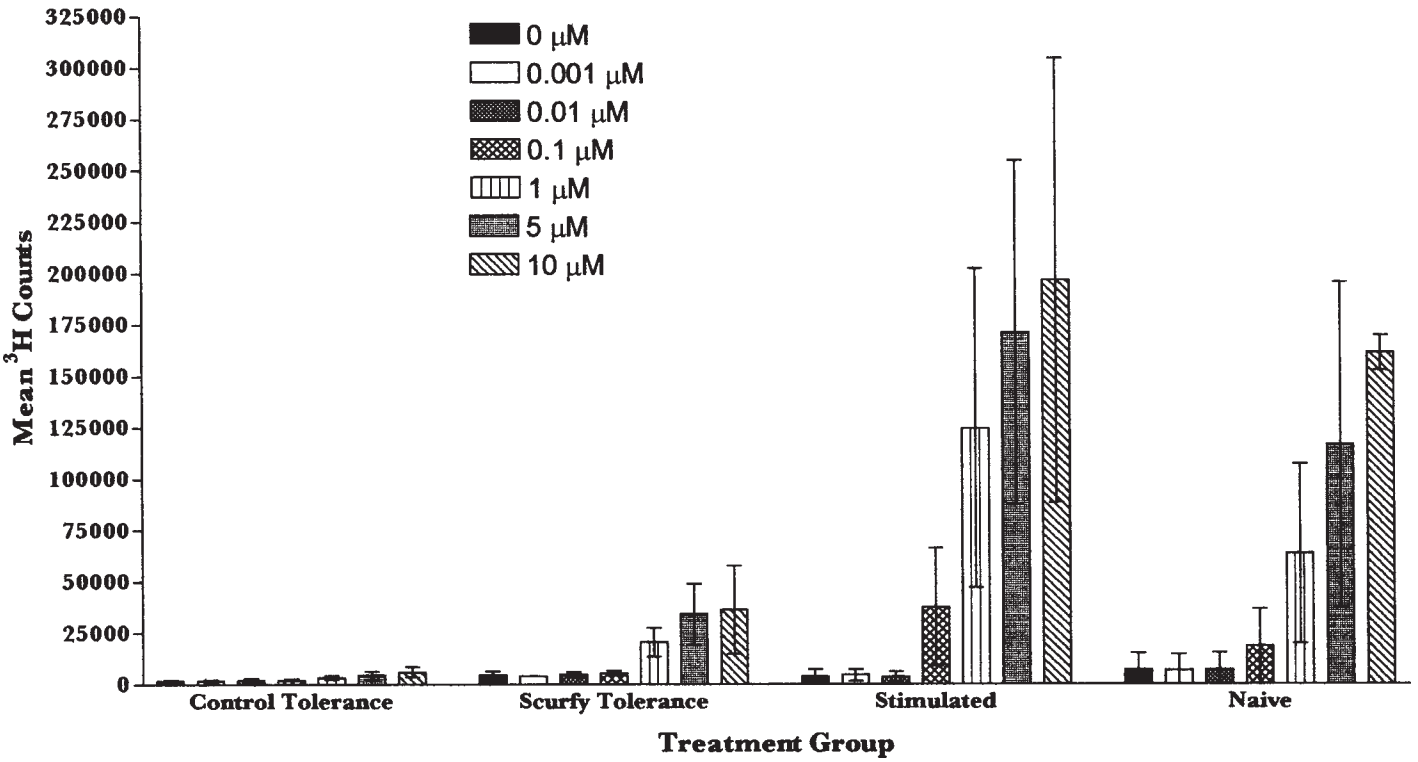

Figure 1. Means and standard deviations of [^{3}H]thymidine incorporation counts (10-min collection time) for all splenic cells harvested from the four treatment groups (control tolerance, scurfy tolerance, stimulated, and naive control) and challenged in vitro for 48 h with variable concentrations of ovalbumin (OVA) peptide (from 0 to 10 μ*M*). At each concentration, the data are represented from between 1* to 4 experiments for each treatment group, with each group containing between 1 and 5 mice/experiment. The results from the scurfy tolerance group were not significantly different from the high proliferation seen in the stimulated and naive control groups, whereas they were significantly different from the low proliferation seen in the control tolerance group. These results indicate that cells from the scurfy tolerance group failed to tolerize, proliferating after being exposed to the same amount of OVA peptide that caused virtually no proliferation in the control tolerance group.
*Only one experiment with two mice of the scurfy tolerance group for the 0.001 μ*M* OVA concentration is shown; all other points are for at least two experiments of at least two mice each.

Table 1. Flow cytometric data from tolerance induction assays. Cells from whole splenic preparations were taken at euthanasia (ex vivo) and after 48 h in ovalbumin (OVA) peptide culture. Each treatment group consisted of pooled cells from two to three mice (n = 1 to 3 for each group). Numbers represent percentage total of live splenocytes only. *Data from only one treatment group of three (pooled) animals could be used for this parameter, due to unexpected high background levels for the other two groups; therefore standard deviation computation was not possible

Group/treatment	Ex vivo				After 48 h stimulation			
	CD4+ KJ1-26-	CD4- KJ1-26+	CD4+^{bright} KJ1-26+^{bright}	CD4+^{dull} KJ1-26+^{dull} *	CD4+ KJ1-26-	CD4- KJ1-26+	CD4+^{bright} KJ1-26+^{bright}	CD4+^{dull} KJ1-26+^{dull}
Rag1KO/wt OVA, stimulated	3.5 +/- 2.0	13.4 +/- 9.1	16.4 +/- 8.8	43.5	12.2 +/- 5.8	11.6 +/- 8.4	25.5 +/- 0.5	46.5 +/- 2.9
Rag1KO/wt OVA, naive	9.7 +/- 7.1	4.6 +/- 0.7	4.4 +/- 4.3	54.0	23.0 +/- 3.8	4.9 +/- 1.8	3.5 +/- 2.0	64.8 +/- 3.2
Rag1KO/wt OVA, control tolerance	3.1 +/- 2.3	5.5 +/- 4.1	6.2 +/- 6.1	45.3	6.1 +/- 1.6	5.1 +/- 2.0	9.8 +/- 0.6	69.2 +/- 1.6
Rag1KO/*sf*/Y OVA, scurfy tolerance	2.5 +/- 0.2	11.1 +/- 3.7	2.8 +/- 0.3	59.8	2.0 +/- 0.2	10.1 +/- 1.7	2.2 +/- 0.1	79.1 +/- 2.1

26$^{+bright}$ percentage, but, rather an increase in CD4^{+dull}KJ1-26^{+dull} cells. Cells from the control tolerance group also had an increase in CD4$^{+bright}$ KJ1-26$^{+bright}$ cells after 48 h; these cells were presumably beginning the process of tolerization and apoptosis, which begins with an initial stimulation phase (18), rather than proliferating. The scurfy tolerance group cells, although they indicated increase in proliferation by [H^3]thymidine incorporation, did not have a significant increase in percentage of the total cell population of CD4$^{+bright}$KJ1-26$^{+bright}$ cells, relative to the concentrations seen ex vivo. However, the percentage of the CD4^{+dull}KJ1-26^{+dull} cells increased, relative to values at euthanasia, contributing to the proliferation seen in the stimulation assays.

Discussion

The generation of the Rag1KO/*sf*/OVA strain, where all CD4$^+$ T cells are strictly OVA peptide 323-339-dependent, set the stage for more detailed examination of the behavior of the *sf* mutation and scurfy disease-causing mature CD4$^+$ T cells. Self-tolerance is achieved principally by elimination or inactivation of self-reactive T and B cells (18), and a functioning CTLA-4 pathway has been found to be crucial to achieve this (15). Because we hypothesized that the *sf* mutation is either in the CTLA-4 pathway, or in a similar signalling cascade in the tolerance-induction mechanism of the T cell, we decided to use this new strain to test the *sf* mutation's effect on CTLA-4 function,

using a modification of a published protocol (15) for evaluating CTLA-4 function.

As evidenced by 48-h in vitro stimulation assays after euthanasia, Rag1KO/*sf*/Y OVA T cells did not tolerize at the same dose of OVA peptide as did cells from control Rag1KO/wt/Y OVA mice. In fact, in one experiment, twice the dose was inadvertently administered to the experimental mice, and T cells still did not tolerize on the basis of in vitro [H^3]thimidine incorporation (data not shown). These data also indicated that cells from Rag1KO/*sf*/Y OVA mice given the tolerizing dose proliferated differently in response to OVA peptide than did cells from the control-stimulated and naive groups. As seen in the *sf*/Y OVA line, cells of the Rag1KO/*sf*/Y OVA group given the tolerizing dose proliferated higher at lower doses of peptide. At higher doses, however, this proliferation leveled off, perhaps indicating that there may still be some negative control mechanisms acting on the T cells.

In examining flow cytometric data, it is evident that the population of reactive T cells in vivo was different between that of the control tolerance and experimental tolerance groups on the basis of percentages seen at euthanasia. This apparent difference was also present at 48 h after in vitro stimulation. Flow cytometric results also indicated that the proliferation we observed in vitro for the scurfy tolerance group was not due to the same cell type ($CD4^{+bright}KJ1\text{-}26^{+bright}$) as observed in stimulated controls, but rather to $CD4^{+dull}KJ1\text{-}26^{+dull}$ cells, which was more similar to the changes seen in the naive group.

Additionally, limited enzyme-linked immunoassays were performed to determine whether the scurfy tolerance group splenic T cells produced interleukin 2 (IL-2) in culture, indicating active proliferation, as opposed to those cells undergoing early apoptosis, which, although perhaps containing $CD4^{+bright}KJ1\text{-}26^{+bright}$ cells, would not be producing IL-2. It was clear that cells from the stimulated and naive groups produced high amounts of IL-2 after 24 h in culture (average of 270 (n = 3) and 155 (n = 3) pg/ml, respectively). Cells from the control tolerized groups produced low amounts (19 [n = 2] pg/ml averaged values). The average from scurfy tolerance group cells was 139 (n = 3) pg/ml, just below the average for the naive group and far above that for the control tolerance group. Large experimental variation between the same experimental groups on different days did not allow statistically significant comparisons over all the groups; however, the same relative relationships in IL-2 production among the groups were consistent for any single day (data not shown).

On the basis of this tolerance-induction study, the peripheral T cells of Rag1KO/*sf*/Y OVA mice do not tolerize normally, even when twice the dose that causes tolerance in control mice is administered. The response seen was not purely stimulatory; however; it is evident that either some type of negative control is still functional in the peripheral *sf* T cells, or the population of *sf* T cells that is proliferating is distinct from those in the stimulated and control groups. It remains to be determined how the *sf* mutation, with its truncated, non-functional protein, specifically causes this effect.

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