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Large Functional Repertoire of Regulatory T Cell Suppressible Autoimmune T Cells in Scurfy Mice¹

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Abstract

Scurfy mice, a strain lacking functional Foxp3 transcription factor and CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells, spontaneously develop autoimmune responses against skin, lung, liver and tail. However, many organs/tissues are spared from autoimmune attack. Here, we demonstrate that scurfy mice contain dormant autoimmune T cells that induced new diseases such as sialoadenitis, dacryoadenitis, pancreatitis, gastritis, intestinal inflammation, colitis, and myositis in RAG-1 KO mice. Inflammation in as many as 12 organs/tissues was consistently induced in individual recipients with scurfy lymph node cells containing as few as 1.25×10⁶ CD4⁺ T cells. Moreover, transfer of the multiple organ autoimmune diseases could be suppressed by as little as 0.5×10⁶ CD4⁺CD25⁺ Treg cells, mediated by inhibiting autoimmune T cell expansion. Our study provides evidence for the presence of a large repertoire of autoimmune lymphocytes against various organs/tissues in scurfy mice as well as Treg cells in B6 mice capable of suppressing the expansion of these autoimmune lymphocytes. Various conditions that control the expression of autoimmune T cells are discussed.

Keywords

Autoimmunity; Inflammation; Tolerance/Suppression

1. Introduction

Thymocyte differentiation and selection impart distinct functions to different T cell populations. In the CD4⁺ T cell compartment, two subsets are generated: a CD25⁻ set that responds to antigens in the host and a CD25⁺Foxp3⁺ subset that suppresses these antigen-specific responses (reviewed in ¹⁻³). Autoimmune T cells with moderate affinities against self-antigens could escape negative selection and exit to the periphery (⁴⁻⁶). Also, some of the organ-specific antigens are not presented in the thymus during negative selection and are not

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negatively selected (7). These autoimmune T cells in the periphery are suppressed by the naturally occurring CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells (1⁻³).

Based on our current understanding of Treg cell function, a general defect in Treg cell expression should result in autoimmune responses against various organs in a systemic fashion. However, earlier pioneer studies either with the day-3 thymectomy mouse model in which Treg cell generation was interrupted or with the adoptive transfer model in which CD25⁻ lymphocytes were transferred into nude mice did not meet this expectation (8⁻¹¹). In the day-3 thymectomy model, leukocyte infiltration into various organs was limited. Even with the most sensitive targets of salivary and lacrimal glands and reproductive organs, leukocyte infiltration was observed only in a few strains and often not in every individual (9). In the adoptive transfer model, only gastritis and oophoritis were observed in 100% of the nude mouse recipients (10). The prevalence and frequency of organ-specific autoimmune diseases were higher in NOD-SCID recipients transferred with T cells depleted of glucocorticoid-induced TNF receptor family-related protein (11). However, these experimental systems used a large number of T cells for transfer and a long incubation time for disease development. In addition, there was a significant autoimmune antibody contribution to the systemic autoimmune manifestation (10, 11).

It is possible that both day-3 thymectomy and antibody treatments failed to completely eliminate Treg cells. However, even in hosts with a congenital defect in Treg cell expression, extensive autoimmune inflammation in multiple organs was rarely reported (12⁻¹⁷). In patients with the Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome (IPEX), a lethal autoimmune disorder resulted from Foxp3 mutation, organ involvement was often limited to a few (18). Even in Foxp3 mutant scurfy mice, which have no CD4⁺CD25⁺Foxp3⁺ Treg cells and die within 3-5 weeks after birth as a result of multi-organ autoimmune syndrome (19, 20), major organs that are inflamed are limited to skin, tails and lungs (21). Inflammation was moderate in the liver and at best borderline in the pancreas. No discernible inflammation was observed in salivary and lacrimal glands, gastrointestinal tract, central nervous system, joints, and other endocrine glands (21).

The limited involvement of multiple organs/tissues in scurfy mice raises several fundamental questions about Treg cell control of autoimmunity. Is there a lacunar defect in the thymic selection for autoimmune lymphocytes? Is the absence of autoimmune response in certain organs/tissues attributable to the presence of non-naturally occurring Treg cells? Or are there other mechanisms that prevent the development and expression of certain organ-specific autoimmune lymphocytes? Here, we provide evidence against the former two possibilities and favoring the third. We developed models that rapidly transfer multi-organ autoimmune responses in RAG-1 knockout (KO) mice. Surprisingly, lymph node cells of scurfy mice even transferred autoimmune diseases against organs that were not inflamed in the donor themselves, most notably in the salivary glands and organs associated with the gastrointestinal tract but other organs were also severely inflamed under appropriate condition. As many as 12 organs/tissues were inflamed in individual mice. These are Treg cell-regulated autoimmune responses because they are readily suppressed by the co-transfer of the CD4⁺CD25⁺ Treg cells from normal mice. The suppression is mediated by a mechanism that inhibits the expansion of autoimmune lymphocytes in the secondary lymphoid organs. By demonstrating the presence of Treg cell-suppressible autoimmune lymphocytes against 12 organs in individual mice, our study strongly suggests that there is no defect in the generation of a large repertoire of autoimmune lymphocytes in scurfy mice nor do scurfy mice use non-naturally occurring Treg cells to protect those organs spared from autoimmune attack. In addition, it suggests the presence of a large Treg cell repertoire capable of suppressing these autoimmune lymphocytes and maintaining peripheral tolerance in normal B6 mice.

2. Materials and Methods

Mice

C57BL/6 (B6) mice, B6.SJL-*PLprc^aPcp^b*/BoyJ (B6.CD45.1) and B6.129S7-*Rag1^{tm1Mom}*/J (RAG-1 KO) mice were obtained from the Jackson Laboratories, Bar Harbor, ME. Heterozygous female B6.Cg-*Foxp3^{sf/x}*/J mice (the Jackson Laboratories) were bred with male B6 mice to produce scurfy mice (*Foxp3^{sf/Y}*). The presence of the scurfy mutation was confirmed by PCR as detailed in the Jackson Laboratory's website. Mice were examined twice weekly for clinical signs of the autoimmune disease including manifestation of skin inflammation, body weight loss, and wasting etc.

Histology

Tissues/Organs were fixed with 10% neutral buffered formalin (Fisher Scientific) and sections of paraffin-embedded tissue were stained with H & E. Tissues/Organs examined included salivary glands, lacrimal glands, stomachs, small intestines, colons, lungs, hearts, thyroid glands, livers, kidneys, pancreas, skins, tails and skeletal muscles. We also scored inflammation levels by the extent of leukocyte infiltration in 10 randomly selected fields and categorized them as severe (4+), strong (3+), moderate (2+), mild (1+), and no inflammation (0+).

Adoptive transfer

Axillary, brachial, inguinal, cervical lymph nodes from 3 to 4 weeks old scurfy or B6 mice were isolated, pooled, and single cell suspensions were prepared in normal saline. Various numbers of cells were injected intravenously (IV), intraperitoneally (IP), or intramuscularly (IM) into the recipient mice. Both adult and 6 days old RAG-1 KO mice were used as recipients. In other experiments, CD4⁺ T cells were purified by positive selection using magnetic beads conjugated with anti-CD4 mAb (Miltenyi Biotec). The unbound fraction was used as CD4⁺ T cell-depleted population. The purity of both populations was determined with a Flow cytometer before transfer.

The CD4⁺CD25⁺ Treg cells and CD4⁺CD25⁻ T cells were isolated from lymph nodes of B6.CD45.1 mice using CD4⁺CD25⁺ Treg cell isolation kit (Miltenyi Biotec). The purity of the cells as determined by Flow cytometry was more than 85% in all cases. Enriched Treg cells (0.5×10^6 or 1.5×10^6) or CD4⁺CD25⁻ T cell control were co-transferred with 5×10^6 scurfy lymph node cells into RAG-1 KO mice. Mice were observed every other day for clinical signs of autoimmune syndrome. Histological determination of autoimmune responses against various organs/tissues was conducted at 3 to 5 weeks after transfer.

Flow cytometry

The samples analyzed include lymph node cells, purified CD4⁺ T cells, cells depleted of CD4⁺ T cells, and splenic cells of RAG-1 KO recipients. Cells (10^6) were suspended in 100 μ l of PBS solution (containing 4 mg with of bovine serum albumin and 1 μ g of anti-FcR mAb 2.4G2) and incubated with 0.2 μ g of various fluorescent antibodies for 30 minutes at 4°C. FITC, PE, or PE-Cy5 conjugated anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-CD44 (IM7), anti-CD62L (MEL-14) and anti-CD69 (H1.2F3) mAb were obtained from BD Biosciences. At least 10^4 stained cells were analyzed using a FACScan equipped with CellQuest (BD Biosciences). Post acquisition analyses were carried out using FlowJo™ software (Tree Star, Inc, OR).

3. Results

Scurfy mice developed autoimmune response against a few target organs

We examined the H & E stains of many different organs to determine the extent of the multi-organ autoimmune response in scurfy mice. Scurfy mice of 3 to 4 weeks old were used because they developed severe clinical signs of autoimmune syndrome. The organs examined were the ear, skin, tail, lung, liver, brain, joint, salivary gland, lacrimal gland, stomach, small intestine, colon, muscle, kidney, pancreas, thyroid and adrenal glands. Autoimmune response is defined by leukocyte infiltration into target organ using age- and sex-matched B6 mice as control. No inflammation was observed for these tissues/organs of B6 mice. A strong and extensive inflammatory response was observed in the ears, the tail, the skin and the lung (A-D in Figure 1). A moderate infiltration of leukocytes was also observed in the liver (E in Figure 1). Surprisingly, little or no inflammation was observed for the rest of the organs examined (not shown).

Transfer of autoimmune responses against 12 organs/tissues: ear, skin, lung, liver, pancreas, salivary gland, lacrimal gland, stomach, small intestine, colon, kidney, and muscle

To determine if there were autoimmune lymphocytes against organs that were spared from autoimmune attack in scurfy mice, we transferred (IV) 25×10^6 or 5×10^6 lymph node cells (25% CD4⁺ T cells) from scurfy mice into RAG-1 KO mice and determined inflammation in various organs 3 weeks and 5 weeks later, respectively. The results are shown in Figure 2. With the exception of tail, organs that were inflamed in the donor scurfy mice were also inflamed in the RAG-1 KO recipients. Their extent of inflammation was similar or only slightly less severe than that observed in the donor mice. Organs in this category include ear, skin, lung, and liver (A-D in Figure 2). By contrast, inflammation with varied intensities was seen in muscle, kidney, pancreas, salivary gland, lacrimal gland, stomach, small intestine, and colon of the recipient mice, although inflammation in these organs was not observed in the donor scurfy mice. The histology results showed mild inflammation in the pancreas (peri-islets) and the muscle (L and E in Figure 2), moderate inflammation in the kidney interstitium, moderate to severe inflammation in the salivary and the lacrimal glands (F-I in Figure 2), and severe inflammation in the stomach, small intestine, and colon (I-K in Figure 2). Organs that remained free of inflammation in RAG-1 KO recipients were thyroid gland, adrenal gland, and joint (data not shown).

To determine the effect of neonatal condition on the expression of autoimmune lymphocytes, we transferred 5×10^6 (IP) lymph node cells of 3.5 weeks old scurfy mice into 6 days old RAG-1 KO mice and determined organ inflammation when they were 21 days old (last day before weaning) and 5 days after weaning. At 15 days after transfer and before weaning, the recipients developed severe inflammation in the lung and moderate inflammation in the skin, pancreas, liver, salivary glands and lacrimal glands. Moreover, a mild inflammation was observed in the kidney and gastrointestinal organs (Table 1). Interestingly, organs with direct exposure to external environment such as skin, salivary gland, lacrimal gland and gastrointestinal organs, but not pancreas and kidney, rapidly became severely inflamed at just 5 days after weaning (Table 1). Skeletal muscle remained free of inflammation in this transfer model.

We observed a moderate inflammation often extended into the adjacent muscle region layer in various organs that were strongly inflamed. However, such inflammation in skeletal muscle tissue was seen neither in scurfy mice nor in the recipients of adoptive transfer of scurfy lymph node cells by IV or IP routes. To determine if scurfy mice contain autoimmune lymphocytes against skeletal muscle, we transferred various numbers (1×10^6 , 5×10^6 , or 25×10^6) of their lymph node cells into RAG-1 KO mice intramuscularly. Surprisingly, this rarely used route of cell transfer is as efficient as IV for the induction of multi-organ autoimmune syndrome.

Multiple inflammations in 12 organs were observed even in the recipients of 10^6 transferred cells. Unlike IV transfer, severe inflammation was observed in the skeletal muscle tissues in the area of injection (Figure 3A), in the thigh opposite to the transfer (Figure 3B), and in the upper limbs (Figure 3C). Both peri-vascular leukocyte infiltration and leukocytes surrounding the muscle cells were observed. In addition, disintegrated and damaged muscle cells were evident. This pattern of muscle pathology resembles the histology seen in dermatomyositis. This finding is significant as animal models for polymyositis and dermatomyositis are rare. The reason for the successful induction of inflammation in skeletal muscle is under investigation.

To determine whether $CD4^+$ T cells are sufficient to induce autoimmune responses, we transferred (IV) purified lymph node $CD4^+$ T cells (2×10^6 , >97% $CD4^+$ staining) of scurfy mice to RAG-1 KO recipients. Lymph node cells depleted of $CD4^+$ T cells (5×10^6 , <1% $CD4^+$ staining) were transferred for comparison. With the $CD4^-$ cells, no organ inflammation was observed at 5 weeks after transfer (not shown). Under identical conditions, $CD4^+$ T cells alone were sufficient to transfer the autoimmune diseases against various organs as described above in the IV transfer model. This transfer of autoimmune disease is significant because unlike earlier systems in which autoreactive antibodies may have played a significant role in the multi-organ inflammation, the present study establishes that $CD4^+$ T cells alone are sufficient to induce the multi-organ autoimmune inflammatory responses in RAG-1 KO recipients.

Treg cells suppress the transfer of multiple autoimmune responses

Transfer of the $CD4^+CD25^+$ Treg cells into scurfy mice has generated variable results in previous studies (22, 23). One group transferred 10^6 $CD4^+CD25^+$ Treg cells into scurfy mice of 3 days or 7 days old and this resulted in partial prolongation of lifespan (22). The other group transferred 4×10^5 $CD4^+CD25^+$ Treg cells to 1-2 days old scurfy mice and these mice became disease-free as determined by disease phenotype (wasting and colitis) and lymph node cellularity (23). In both reports, the protection from multi-organ inflammation was not studied, thus, leaving the issue open as to whether B6 $CD4^+CD25^+$ Treg cell repertoire is large enough to protect mice from autoimmune inflammation in multiple organs. The adoptive transfer model provides a more efficient and better controlled system to address this issue. We transferred 5×10^6 lymph node cells of scurfy mice either alone or with 0.5×10^6 $CD4^+CD25^+$ Treg cells from B6.CD45.1 mice into RAG-1 KO recipients. The mice were examined for inflammation in various organs at 5 weeks after transfer. Without co-transfer of $CD4^+CD25^+$ Treg cells, the ear, the lung, the liver, the stomach, the small intestine, and the colon were strongly inflamed (Figure 4; A-F and M). The histology of these organs was selectively presented to show the extent of Treg cell-mediated suppression (Figure 4; G-L and M). Co-transfer $CD4^+CD25^+$ Treg cells and scurfy lymph node cells into RAG-1 KO mice completely protected these organs from autoimmune attack in two of the three recipients (Fig. 4). This suppression is specific because co-transfer of the $CD4^+CD25^-$ T cells did not suppress these autoimmune responses (data not shown). Interestingly, the remaining one recipient was not protected at all, showing multi-organ inflammation as severe as the RAG-1 KO mice that had received scurfy lymph node cells. We detected $CD4^+CD25^+Foxp3^+$ T cells in the suppressed but not the unsuppressed recipients (data not shown). In subsequent experiments, 1.5×10^6 $CD4^+CD25^+$ Treg cells were found to strongly suppress the multi-organ autoimmune responses in all four recipients. A mild leukocyte infiltration was only observed in the gut and lung and only in two of the four recipients. The compiled histological data are presented in Fig. 4M. Thus, the repertoire of Treg cells in normal B6 mice is large enough to suppress the autoimmune lymphocytes present in the scurfy mice against 12 organs/tissues in individual mice⁴.

Mechanism of suppression

The mechanism of the suppression of multi-organ inflammation was addressed. At 5 weeks after transfer, there is a great expansion of transferred cells not only in the inflamed organs but also in the spleen and lymph nodes. Spleens of RAG-1 KO mice that received 5×10^6 scurfy lymph node cells (in which 10^6 are $CD4^+$ T cells) 5 weeks earlier expanded to $52 \pm 17 \times 10^6$ cells (in which 4×10^6 are $CD4^+$ T cells) (Figure 5A). In sharp contrast, RAG-1 KO mice that had received both scurfy donor cells and B6.CD45.1 $CD4^+CD25^+$ Treg cells had few and nearly undetectable leukocytes in various target organs. The spleens of the suppressed mice contained on the average of $11 \pm 4 \times 10^6$ leukocytes in which 0.2×10^6 were $CD4^+$ T cells of the donor origin (Figure 5A). The scurfy donor cells contain a significant portion of memory $CD4^+$ T cells (63% $CD44^{high}$), recently activated $CD4^+$ T cells (46% $CD69^{high}$), and effector memory cells (54% $CD62L^{low}CD44^{high}$) (Figure 5B and 5C). In contrast, the great majority of B6 lymph node T cells express naïve phenotype of $CD44^{low}CD62L^{high}CD69^{low}$. In the RAG-1 KO recipients of scurfy donor cells, 95% of the splenic $CD4^+$ T cells were $CD44^{high}$ and 43% of the $CD4^+$ T cells were $CD69^{high}$. The co-transfer of $CD4^+CD25^+$ Treg cells from B6.CD45.1 mice reduced the percentage of $CD69^{high}$ expression to 10%, which is close to that of B6 control (Figure 5C). The suppressed $CD4^+$ T cells remained $CD44^{high}$. These results indicate that the $CD4^+CD25^+$ Treg cells suppress the multi-organ autoimmune inflammation mainly by inhibiting the activation and expansion of the transferred autoimmune memory $CD4^+$ T cells in the secondary lymphoid organs.

4. Discussion

Considering the potentially extremely large lymphocyte repertoire and self antigens relative to the small number of target organs in a host, it is reasonable to expect that in hosts with a genetic defect in the generation of Treg cells, autoimmune lymphocytes against various organs should be relieved from autoimmune suppression, expand and infiltrate many organs/tissues. Nevertheless, such a wide and systemic autoimmune response has been reported neither in IPEX patients nor in mice with congenital Treg cell deficiency (12⁻²⁰). The major point of the present study is that we have developed adoptive transfer models to demonstrate that autoimmune responses against as many as 12 organs/tissues could be induced by lymphocytes of individual scurfy mice, thereby providing evidence that there is no defect in the generation of a large autoimmune repertoire in the scurfy mice, that there are no non-naturally-occurring Treg cells in scurfy mice responsible for the sparing of certain organs from autoimmune attack, and that congenital Treg cell-deficient mice contain autoimmune lymphocytes against the great majority if not all of the organs of the host. The second major point is the demonstration of an equally effective Treg cell population in normal mice capable of suppressing these autoimmune responses in individual mice.

There are several possibilities that can explain why scurfy mice display autoimmune responses against only a few target organs but transfer of scurfy lymphocytes to RAG-1 KO mice induces severe inflammation in additional organs/tissues. Scurfy mice live a short life so that autoreactive lymphocytes against certain organs may be of low frequencies and do not have sufficient time to expand, resulting in lack of autoimmune manifestation. Scurfy mice remain free of inflammation before 9 days old (21), a period in which lymphopenic expansion is critical (24). After this period, there are approximately 19 days for autoimmune diseases to develop before death. Other factors such as organ development (salivary gland, prostate gland, etc) and environmental factors (immune status differences between scurfy and RAG-1 KO mice, food intake before and after weaning, etc) are likely possibilities as well. The adoptive transfer of

⁴Using the IM transfer approach, co-transfer of B6 $CD4^+CD25^+$ Treg cells with scurfy lymph node cells also suppressed myositis (unpublished observation).

5×10^6 lymph node cells (containing 1.25×10^6 CD4⁺ T cells) from scurfy mice into RAG-1 KO neonates or adults resulted in autoimmune responses against multiple organs including those that were not inflamed in the donor mice. This indicates that organ development is not an important factor. Moreover, the total number of CD4⁺ T lymphocytes in the secondary lymphoid organs of RAG-1 recipients (4×10^6) was significantly smaller than that in scurfy mice ($\sim 60 \times 10^6$). Thus, it seems unlikely that scurfy mice have insufficient numbers of autoimmune lymphocytes against those organs that are spared from autoimmune attack. It appears that the neonatal condition in scurfy mice is inhibitory toward the expression of these autoimmune responses. Before weaning, scurfy mice live on mother's milk that contains antibodies and cytokines that may create an environment inhibitory toward autoimmune responses against certain organs. Both neonates and adults of RAG-1 KO mice lack and/or are deficient in these immune modulators, allowing the development of multi-organ autoimmune syndrome in these recipients upon adoptive transfer.

Nevertheless, the ability of a small number of CD4⁺ T cells (2×10^5 CD4⁺ T cells in IM transfer and 1.25×10^6 CD4⁺ T cells for IV and IP transfers) to transfer multi-organ autoimmune syndrome suggests the presence of a large functional autoimmune repertoire. The biochemical and molecular information for a large autoimmune T cell repertoire is just beginning to get appreciated (25). Particularly in scurfy mice, the repertoire may become more diversified because T cells with self-reactive TCRs may divert into the pool of autoimmune population of non-Treg cells (25). Additionally, targeted knockout of Foxp3 or even weakened Foxp3 may change Treg cells into autoimmune T cells (26-28).

Our study also suggests a requirement for lymphocyte expansion before disease induction in RAG-1 KO recipients. The hypothesis is supported by the experiment in which B6 CD4⁺CD25⁺ Treg cells were co-transferred with scurfy lymph node cells and the multi-organ autoimmune response was inhibited. The suppression correlated with the inhibition of the expansion of scurfy CD4⁺ T cells. In this case, there was little or no infiltration of leukocytes in the target organs and the number of scurfy CD4⁺ T cells in the secondary lymphoid organs was greatly reduced. The data indicate that suppression occurred in the secondary lymphoid organs through inhibition of expansion of autoimmune T lymphocytes. Treg cells have been shown to suppress both at the activation phase and expansion/effector phase of an immune response (29-31). The nature of our co-transfer experiments, however, does not allow us to determine if Treg cells could effectively suppress at the expansion/effector phase of the multi-organ syndrome.

It is generally accepted that environmental factors are important regulators for autoimmune responses. The fact that scurfy mice lack autoimmune diseases against the salivary gland, lacrimal gland and gastrointestinal organs is particularly relevant. Scurfy mice die before or soon after weaning. The oral and gastrointestinal organs of newborns prior to weaning are not populated by the flora that commonly resides in the oral cavity and gastrointestinal tract in the adults. As a result, the innate immune activity of the mice before weaning is significantly weaker than that in adult mice (32). As innate immune response is a critical regulator of adaptive immune response, a weak innate immune response does not favor the development of autoimmune disease. Indeed, mice with a defect in innate immunity failed to develop colitis under conditions that readily induce colitis in control mice (33). In this regard, we have been able to induce salivary gland inflammation by daily feeding of one week old scurfy mice with 10 μ l of 1 mg/ml of LPS solution for 17 days (33). Transfer of multi-organ autoimmune diseases into adult RAG-1 KO mice and the rapid development of autoimmune responses against salivary glands and gastrointestinal organs soon after weaning are consistent with this interpretation.

The tails of donor scurfy mice were severely inflamed. Unlike other organs, transfer of tail inflammation was not observed in IV and IM transfer systems. A weak inflammation was observed in the IP transfer of scurfy cells into RAG-1 KO neonates (data not shown). The reason remains unclear. Similarly, myositis was observed by the IM transfer approach but not IV and IP transfers. These results suggest that additional dormant autoimmune lymphocytes may be present and may not be detected by these adoptive transfer approaches. In this respect, it should be noted that a number of organs/tissues were not inflamed despite the induction of autoimmune inflammation in multiple organs/tissues in the recipients of adoptive cell transfer. Transfer of inflammation response may be difficult to organs that are not easily accessible to lymphocytes such as brain, spinal cord, and joints. Absence of B cells in RAG-1 KO mice may also affect manifestation of autoimmune responses that are dependent wholly or partly on autoantibody production.

Finally, the present study also demonstrated for the first time that a single transfer of lymph node cells of individual scurfy mice induces multiple autoimmune responses against at least 12 organs of the RAG-1 KO recipients, an observation that reflects the large autoimmune repertoire and the true meaning of multi-organ autoimmune syndrome. Interestingly, some of them may be considered animal models for human diseases. For example, the salivary gland inflammation observed herein is associated with acinar cell destruction, ductal cell hypertrophy and reduced salivation function (34). Salivary gland malfunction is a typical manifestation of Sjögren's syndrome. The data suggest that Treg cell deficiency may be one underlying factor for Sjögren's syndrome. Similar arguments could be made for other organ-specific autoimmune diseases such as myositis, gastritis, colitis, etc. Whether these diseases could be caused by a defect in organ/tissue-specific Treg cells is interesting and remains to be addressed. Organ-specific Treg cells that suppress organ-specific autoimmune diseases have been described (35–38). Treg cells that recognize self antigens commonly expressed by various organs/tissues could also explain the results of the present study (7, 25). Recent studies have demonstrated that Treg cell repertoire is very large (25, 39) and a sizable part of it recognizes ubiquitously presented self antigens (25). In this respect, it is remarkable that a co-transfer of as little as 0.5×10^6 CD4⁺CD25⁺ Treg cells suppressed the autoimmune responses against all of these target organs, demonstrating the power of Treg cells and suggesting the presence of Treg cells specific to many if not all organs/tissues in B6 mice for the maintenance of periphery tolerance. The simplicity, rapidity and effectiveness of the system suggest that it will be an extremely useful animal model to study the specificity, regulation and mechanisms of the multi-organ autoimmune syndrome at the levels of organ-specific autoimmune diseases, autoimmune T cells and Treg cells that control these autoimmune T cells.

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Figure 1. Scurfy mice developed autoimmune response against a few target organs

Various organs/tissues of scurfy mice (left column) and sex-, age-matched B6 mice (right column) were stained (H&E) and examined under microscope. Strong inflammation was observed in ear (A), neck skin (B), tail (C) and lung (D). A moderate inflammation was observed in liver (E). The corresponding organs of B6 mice did not display any inflammation (F-J). Magnification-100X.

Figure 2. Transfer of autoimmune responses against multiple organs/tissues

Adult RAG-1 KO male were transferred (IV) with 25×10^6 scurfy lymph node cells. Three weeks later, the histologic slides of ear (A), skin (B), lung (C), liver (D), muscle (E), kidney (F), salivary gland (G), lacrimal gland (H), stomach (I), small intestine (J), colon (K), and pancreas (L) were examined for inflammation. Corresponding organs/tissues of age-matched RAG-1 KO male mice that received B6 lymph node cells did not show detectable inflammation and were not presented for simplicity. Similar results were obtained in RAG-1 KO recipients transferred with 5×10^6 scurfy lymph node cells and examined 4 weeks later (not shown). Final magnification is 100x, except L which is 200x.

Figure 3. Transfer of myositis

Scurfy lymph node cells (25×10^6) were transferred (IM) into the right thigh of RAG-1 KO mice and skeletal muscle of site of injection (A), left thigh (B) and upper limb (C) were examined for inflammation at 3 weeks after transfer. Similar results were obtained in RAG-1 KO recipients of 10^6 or 5×10^6 scurfy lymph node cells (data not shown). Magnification-100x.

Figure 4. Treg cells suppress the transfer of autoimmune responses against multiple organs/tissues Adult RAG-1 KO mice were transferred (IV) with 5×10^6 scurfy lymph node (LN) cells alone (A-F) or with 0.5×10^6 CD4⁺CD25⁺ T cells (G-L) purified from B6.CD45.1 mice (Treg). Five weeks later, ear (A & G), lung (B & H), liver (C & I), stomach (D & J), small intestine (E & K), and colon (F & L) were examined for inflammation by histology (Magnification-100x). Adult RAG-1 KO mice that had received 5×10^6 scurfy lymph node cells showed comparable degree of inflammation. Histological scores (Mean $\pm$ standard deviation) of suppressed mice and control (n=6) were presented in the bar diagram (M). The H & E stained sections of various organs/tissues were scored on a five-point scale from 0 to 4 depicting leukocyte infiltration as none, mild, moderate, strong, and severe, respectively. Complete suppression was observed in

most organs/tissues and more than 80% suppression was observed in lungs, liver, stomach, small intestine, and colon.

Figure 5. Treg cells inhibit autoimmune memory T cell expansion

The donor cells and the spleens of both the suppressed and unsuppressed mice in Figure 4 were examined for the expression of CD44, CD62L and CD69. The total number of leukocytes in the spleen was determined (A). The circles represent measurements of individual mice. The bars represent the means. The expression of CD44 and CD62L on gated CD4⁺ T cells is shown in (B). The CD69 expression on gated CD4⁺ T cells is shown in (C).

Table IEffect of weaning on inflammation in RAG-1 KO neonates transferred (IP) with scurfy lymph node cells^a

Organ/Tissue	Scurfy donor	neonate RAG-1 KO recipients ^b	
		Not weaned	5-day weaned
Skin	3+	2+	3+
Salivary gland	-	2+	4+
Lacrimal gland	-	2+	4+
Stomach	-	1+	3+
Small intestine	-	1+	3+
Colon	-	1+	3+
Lung	4+	3+	3+
Liver	2+	2+	2+
Skeletal muscle	-	-	-
Kidney	2+	1+	1+
Pancreas	1+	2+	2+

^a Mean histological score on a 4-point scale (n=3 for before weaning and n=2 for after weaning) ; (-) denotes no infiltrates, (+) – denotes inflammation ranked between mild (1+), moderate (2+), moderate-severe (3+), and severe (4+).

^b Determined before weaning or at 5 days after weaning.