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X-linked Foxp3 (Scurfy) Mutation Dominantly Inhibits Submandibular Gland Development and Inflammation Respectively through Adaptive and Innate Immune Mechanisms

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Sjögren’s syndrome is an autoimmune disease affecting the salivary and lacrimal glands, causing dry eyes and dry mouth (1). The mechanism that triggers leukocyte infiltration into these organs remains largely unknown. We observed Sjögren’s-syndrome-like disease in Il2rα−/− and Il2rα−/− mice; both are partially deficient in the naturally occurring CD4+ Foxp3+ regulatory T cells (Treg), but only the latter two develop inflammation in the submandibular gland (SMG), a critical target of Sjögren’s syndrome. In this study, we investigated the reason that strong inflammation in SMG, and that oral application of LPS induced SMG inflammation, but not GCT expression. LPS treatment induced up-regulation of several chemokines in SMG with little effect on the chemokine receptors on CD4+ T cells in Sf mice. Our study demonstrates that Sf mutation affects SMG development through adaptive immunity against accessory reproductive organs, and the manifestation of SMG inflammation in Sf mice is critically controlled through innate immunity. The Journal of Immunology, 2009, 183: 3212–3218.

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1 Abbreviations used in this paper: Treg, regulatory T cell; GCT, granular convoluted tubule; Sf, Scurfy; SMG, submandibular gland.

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X-linked Foxp3 (Scurfy) Mutation Dominantly Inhibits Submandibular Gland Development and Inflammation Respectively through Adaptive and Innate Immune Mechanisms

Sjögren’s syndrome is an autoimmune disease affecting the salivary and lacrimal glands, causing dry eyes and dry mouth (1). The mechanism that triggers leukocyte infiltration into these organs remains largely unknown. We observed Sjögren’s-syndrome-like disease in Il2rα−/− and Il2rα−/− mice; both are partially deficient in the naturally occurring CD4+ Foxp3+ regulatory T cells (Treg), but only the latter two develop inflammation in the submandibular gland (SMG), a critical target of Sjögren’s syndrome. In this study, we investigated the reason that strong inflammation in SMG, and that oral application of LPS induced SMG inflammation in RagI−/− recipients. A strong correlation was observed between the development of the granulotubular convoluted tubules (GCT) of the SMG in these mice and SMG resistance to inflammation. Moreover, GCT development in Sf.Rag1−/− mice was not impeded, indicating a role of adaptive immunity. In the Sf.Fas−/− mice, this block was linked to atrophy and inflammation in the accessory reproductive organs. Testosterone treatment restored GCT expression, but did not induce SMG inflammation, indicating GCT is not required for inflammation and additional mechanisms were controlling SMG inflammation. Conversely, oral application of LPS induced SMG inflammation, but not GCT expression. LPS treatment induced up-regulation of several chemokines in SMG with little effect on the chemokine receptors on CD4+ T cells in Sf mice. Our study demonstrates that Sf mutation affects SMG development through adaptive immunity against accessory reproductive organs, and the manifestation of SMG inflammation in Sf mice is critically controlled through innate immunity. The Journal of Immunology, 2009, 183: 3212–3218.

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SL.II2<sup>−/−</sup> mice were generated, as previously described (8, 9). Mice (Sl.II2ra<sup>−/−</sup>) carrying both Il2ra<sup>−/−</sup> and Foxp3<sup>−/−</sup> genes were generated by breeding B6.II2ra<sup>−/−</sup> males with B6.II2ra<sup>−/−</sup>Foxp3<sup>−/−</sup> females. Mice carrying both of the Foxp3<sup>−/−</sup> and Rag1<sup>−/−</sup> genes (Sl.Rag1<sup>−/−</sup>) were generated by breeding Rag1<sup>−/−</sup> male with B6.Cg-Foxp3<sup>−/−</sup>/J mice, followed by breeding Rag1<sup>−/−</sup>Foxp3<sup>−/−</sup> female progeny with Rag1<sup>−/−</sup> male. Presence of the II2<sup>−/−</sup>, Il2ra<sup>−/−</sup>, Foxp3<sup>−/−</sup>, Fas<sup>−/−</sup>, and Rag1<sup>−/−</sup> mutation was confirmed by PCR, as detailed in The Jackson Laboratory’s website. Mice were examined twice weekly for clinical signs of disease, including skin inflammation, body weight loss, wasting, etc. All animal experiments were approved by the Animal Care and Use Committee of the University of Virginia.

**Treatment of mice**

Adoptive transfer experiments were conducted by i.v. injection of 15 × 10<sup>6</sup> lymph node cells into adult Rag1<sup>−/−</sup> male recipients. Various organs and tissues were harvested at 4–12 wk after transfer. Testosterone treatment was conducted on 4-wk-old Sl.Fas<sup>−/−</sup> mice by twice weekly s.c. injection of 25 μl of testosterone oenanthate (Sigma-Aldrich) solution (2 mg/ml in corn oil) or vehicle control. Various organs and tissues were harvested after 3 wk of treatment. Sl.Fas<sup>−/−</sup> mice and control littermates (4 wk old) were also treated by daily oral feeding of LPS or poly(I:C) solution (10 μl of 1 mg/ml, one in the morning and one in the evening; Sigma-Aldrich). Various organs and tissues were harvested 3 wk later, H&E stained, and examined under microscope.

**Histology**

Tissues/Organs were fixed with 10% neutral buffered formalin (Fisher Scientific). Sections of paraffin-embedded samples were stained with H&E and examined under microscope.

**Semiquantification of inflammation and GCT expression**

The extent of leukocyte infiltration in tissue sections was examined and categorized into five groups, from 0 to 4, to indicate, respectively, the degree of inflammation as none (0 infiltration lesion), mild (1 lesion), moderate (2 lesions), strong (3–4 lesions), and severe (>4 lesions). The overall inflammation index of samples of a group of mice was calculated as mean ± SD. For quantification of GCT, images of H&E-stained SMG sections were acquired using Olympus BX51 microscope equipped with a digital camera at ×100 magnifications. Images of two random optical fields from at least three different mice of each strain were captured. The images were then analyzed using Kodak 1D image analysis software (Eastman Kodak), and the area occupied by the GCT was marked using the region of interest tool. The total area occupied by the GCT in each image was expressed as percentage of area occupied by GCT.

**Chemokines and chemokine receptor analyses**

SMG were removed from Slf mice treated either with LPS or PBS and collected in RNA later reagent (Qiagen). Pieces of SMG were suspended in RLT buffer (Qiagen) and homogenized using TissueLyser system (Qiagen). Total RNA from homogenate was purified using RNAeasy Mini kit (Qiagen) followed by first-strand cDNA synthesis using the QuantiTect reverse transcription kit (Qiagen). The expression levels of CCL2, CCL3, CCL4, CCL5, CCL9, CXCL2, CXCL10, and CXCL12 were determined by real-time PCR on MyiQ machine (Bio-Rad) using the Taqman gene expression assays (Applied Biosystems). A RNA sample from the SMG of a normal C57BL/6 mouse was used as a calibration control. This sample was chosen because its cytokine expression pattern closely represents the average pattern obtained from five normal samples. The data are represented as fold change in gene expression over the B6 calibrator. Statistical significance of variance was determined by the nonparametric Mann-Whitney U test using GraphPad Prism software. A value of p<0.05 was considered statistically significant.

**Flow cytometric analysis**

Flow cytometric staining for various chemokine receptors was conducted on the draining lymph nodes of SMG in Slf mice treated with PBS and LPS. Age-matched, male B6 mice were included for comparison. Lymphocytes were stained with anti-CD4 mAb along with Abs specific to CCR3 (TG14/CCR3), CCR5 (HM-CCR5), CXCR2 (TG11/CXCR2), and CXCR3 (CXCR3-173) (Biolegend). The expression of chemokine receptors on gated CD4<sup>+</sup> T cells was presented.

**Results**

Foxp3<sup>−/−</sup> gene dominantly suppressed SMG inflammation in male II2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice

II2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice, but not Slf mice developed spontaneous inflammation in SMG even though Slf mice contained T cells capable of inducing SMG inflammation in Rag1<sup>−/−</sup> recipients (2). To determine whether the resistance is inherently associated with Foxp3<sup>−/−</sup> mice, we introduced Foxp3<sup>−/−</sup> gene into male II2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice to generate Sf.II2<sup>−/−</sup> and Sf.Il2ra<sup>−/−</sup> mice, respectively. In all cases, these mice lived significantly longer than Slf mice, but their SMG remained free from inflammation (8). By contrast, inflammation in colon normally observed in II2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice remained (Fig. 1A).

We transferred lymph node cells from Slf and Sf.II2<sup>−/−</sup> mice to determine whether they contained competent cells capable of inducing SMG inflammation in Rag1<sup>−/−</sup> recipients. In addition, we also tested Sl.Fas<sup>−/−</sup> mice because these mice lived more than 15 wk and their SMG remained free of inflammation (see Fig. 4A). All induced moderate/strong inflammation in the SMG of the recipients (Fig. 1B). Taken together, these results indicate that the SMG resistance to inflammation in Slf, Sl.II2<sup>−/−</sup>, and Sl.Fas<sup>−/−</sup> mice occurs even in the presence of competent inflammation-inducing T cells, and this resistance is inherently and dominantly associated with Foxp3<sup>−/−</sup> in male mice in an organ-specific manner, i.e., inflammation is inhibited in SMG, but not colon.

Dominant resistance coincided with inhibition of SMG development

We compared the SMG expression level between male and female II2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice that were 8 wk old using the semiquantification method. B6 mice were used as control. As shown in Fig. 2A, a strong age-dependent expression of GCT was observed in B6 male as opposed to the weak expression of GCT with less granule content in B6 female. The SMG of II2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice also displayed sexual dimorphism, but the GCT in male II2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice was less than age-matched B6 male. This could be caused by SMG inflammation that induces GCT atrophy (Fig. 2B) (2). By contrast, the SMG of Sl.II2<sup>−/−</sup> and Sl.Il2ra<sup>−/−</sup> mice was severely underdeveloped with fewer and smaller GCT than male II2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice (Fig. 2B). Indeed, highly statistically significant differences in GCT expression levels between II2<sup>−/−</sup> and Slf.II2<sup>−/−</sup> mice and between II2ra<sup>−/−</sup> and Slf.Il2ra<sup>−/−</sup> mice were observed (Fig. 2C). Unlike II2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice, the SMG growth arrest in Slf.II2<sup>−/−</sup> and Slf.Il2ra<sup>−/−</sup> mice occurred in the absence of SMG inflammation, indicating that the inhibition of GCT growth in this case is not caused by local inflammation, but rather the consequence of the early systemic inflammatory response. In support of this, we observed SMG sexual dimorphism between Slf.Rag1<sup>−/−</sup> male and Slf.Rag1<sup>−/−</sup> female; both lacked a functional adaptive immune response (Fig. 2D). Moreover, transfer of Slf lymph node cells into male Slf.Rag1<sup>−/−</sup> recipients readily induced SMG inflammation (Fig. 2E). These results indicate that Foxp3<sup>−/−</sup>F<sup>−/−</sup> indirectly controls SMG development and SMG inflammation through the adaptive immune system.

Foxp3 expression has been demonstrated in the epithelial cells of mammary and prostate glands (10). Foxp3 expression in the SMG has not been determined. The facts that SMG of Slf.Rag1<sup>−/−</sup> mice displays sexual dimorphism for GCT expression and becomes inflamed upon transfer of Slf lymph node cells indicate that Foxp3, if expressed in SMG per se, does not play a role in the regulation of SMG development and the SMG resistance to inflammation-inducing T cells.
null
poly(I:C). A strong inflammation was induced by both treatments, but the GCT expression of the treated mice remained inhibited (Fig. 4B and data not shown). The two approaches provided reciprocal evidence that dissociates GCT growth inhibition from resistance to inflammation attack.

Several chemokine receptors/integrins are strongly up-regulated on the CD4+/H11001 T cells in Sf mice (12). Yet, these T cells failed to enter the SMG. We hypothesize that LPS can directly act on SMG by increasing their chemokine production. This change sensitizes the SMG to the inflammation-inducing T cells already present in the Sf mice. We analyzed the chemokine expression pattern of the SMG of control Sf mice and those treated with LPS using quantitative RT-PCR. We chose eight chemokines, including those that are known to be up-regulated upon LPS stimulation (13). Four chemokines, CCL3, CCL5, CXCL2, and CXCL10, were significantly increased in the LPS-treated Sf mice as compared with PBS-treated control (Fig. 4C). Although the expression levels of CCL2, CCL4, CCL9, and CXCL12 were not significantly up-regulated, additional samples are needed to firmly determine whether this is really the case with CCL4. These data demonstrated that LPS induced a direct change in the SMG of Sf mice and the underdeveloped SMG in Sf mice is capable of responding to LPS by producing chemokines.

We then analyzed CD4+/H11001 T cells of the SMG-draining lymph nodes of these mice for the expression of chemokine receptors specific to the up-regulated chemokines. The expression of chemokine receptors targeted by CCL3, CCL5, CXCL2, and CXCL10 showed considerable variability, and the differences between PBS-treated and LPS-treated Sf mice were not significant (Fig. 4D). Although a strong trend of increase in their expression was observed when compared with B6 controls, the difference was not significant, except for CXCR2. Apparently, the overall mild increase in these trafficking/retention receptors on CD4+ T cells of Sf mice was not sufficient to induce inflammation unless increase in chemokine production in SMG was achieved by LPS treatment. These observations provided evidence that LPS acted on SMG...
chemokine production and induced inflammation without affecting GCT development.

**Discussion**

Our study provides evidence for the following scheme for SMG development and inflammation response. The genetic defect in Sf mice results in the inhibition of two critical components of the process: one required for GCT expression, and one for the susceptibility to inflammation; both depended on a functional immune system. A consequence of the severe and early systemic inflammatory response in Sf mice is the inhibition of GCT-inducing agents such as testosterone by the adaptive immunity. Testosterone treatment induced GCT overexpression, but not the component controlling susceptibility to inflammation-inducing T cells. The reciprocal result, obtained with LPS and poly(I:C) treatments, demonstrated that TLR agonist induced SMG inflammation, but not GCT development in Sf mice. Evidence provided strongly suggests that the inflammation in the underdeveloped SMG is limited by its innate immune response that can be overcome by TLR agonists through chemokine induction.

We have previously shown that daily LPS application induces SMG inflammation in Sf mice (2). In the present study, daily LPS application to the long-lived Sf Fas<sup>+/+</sup> mouse was also able to induce SMG inflammation, indicating that although the key defect in the induction of SMG inflammation is long-lasting, the ability to break it by LPS remained as well. In addition, an agonist for a different TLR also induced SMG inflammation in these mice. Although multiple i.p. injections of these agonists into adult (New Zealand White × New Zealand White)F<sub>1</sub> mice activate innate immunity in the SMG (14), we chose oral applications to neonates for ease of application and less injury. It has been shown that LPS directly affects various organs through circulation (15). We showed that LPS treatment up-regulated the production of several chemokines in the SMG of Sf mice, thereby indicating that oral applications of LPS can act on the SMG of Sf mice and induce biological changes. In contrast, the corresponding chemokine receptors on CD4<sup>+</sup> T cells were not up-regulated upon LPS treatment. These observations strongly suggest that the neonatal and underdeveloped SMG in Sf mice is limited in its ability to produce sufficient chemokines required for the attraction of inflammation-inducing CD4<sup>+</sup> T cells to cause SMG pathology and salivation dysfunction.

As compared with normal B6 male, the SMG of the sex- and age-matched Il2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice are underdeveloped, but not severely growth arrested as the SMG of Sf Il2<sup>−/−</sup> and Sf Il2ra<sup>−/−</sup> mice. Unlike Sf or Sf Fas<sup>+/+</sup> mice, Il2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice still contain a significant level of Treg. In addition, they lack IL-2 and IL-2Rα, respectively. Thus, the inflammation process in Il2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice is reduced and delayed as compared with the corresponding Sf Il2<sup>−/−</sup> and Sf Il2ra<sup>−/−</sup> mice. Like Sf Il2<sup>−/−</sup> and Sf Il2ra<sup>−/−</sup> mice, Il2<sup>−/−</sup> and Il2ra<sup>−/−</sup> male are reproductive incompetent with light body weight and probably lack normal level of testosterone, but they live significantly longer than Sf Il2<sup>−/−</sup> and Sf Il2ra<sup>−/−</sup> mice. These factors could account for the weak inhibition of SMG development in male Il2<sup>−/−</sup> and Il2ra<sup>−/−</sup> mice. In this regard, the relatively more advanced SMG development in these mice with appropriate environmental changes may allow sufficient chemokine production to attract inflammation-inducing CD4<sup>+</sup> T cells to SMG and induce inflammation as the mice lived beyond weaning to adult age.

Several hormones can induce dominant GCT overexpression in female or castrated male (5, 6). We examined the long-lived Sf Fas<sup>+/+</sup> mice and observed dramatic atrophy and significant inflammation in many of the accessory reproductive organs. Specifically, severe atrophy was observed in the seminal vesicle, prostate, epididymis, and testis with varying degree of inflammation in different TLR also induced SMG inflammation with varying degree of inflammation in many of the accessory reproductive organs. Specifically, severe atrophy was observed in the seminal vesicle, prostate, epididymis, and testis with varying degree of inflammation. Spontaneous inflammation in multiple male accessory reproductive organs is rare, and our study showed for the first time that it does indeed develop in Sf Fas<sup>+/+</sup> mice that lived beyond weaning and reached adult age. This finding strongly suggests that testosterone is the main reason that Sf mice fail to display a normal male dominant presentation of the GCT. Indeed, our testosterone treatment fully restored GCT development, but the SMG remained free of inflammation. Conversely, testosterone treatment also restored the development of the accessory reproductive organs, but the inflammation remained. This result dissociates the testosterone-dependent organ development from inflammation induced by Treg deficiency in both directions, i.e., inflammation in accessory reproductive organs or lack of inflammation in SMG was maintained regardless of testosterone-induced organ development. This result also suggests that lacking GCT is not the reason for the inability of Sf mice to develop SMG inflammation.

Despite totally lacking Treg, many of the organs/tissues such as CNS, joints, endocrine, and mucosal organs are free of inflammation in Sf mice despite the fact they have a large functional inflammatory repertoire (16, 17). In this regard, the present study has implication toward the genetic control of regulatory mechanisms involved in the multiorgan inflammation in Sf mice. In contrast to the Foxp3<sup>+/+</sup>-dependent regulation of SMG inflammation in Sf Il2<sup>−/−</sup> mice, the Il2<sup>−/−</sup> defect conversely inhibited the skin and lung inflammation in Sf mice, but...
not the liver inflammation and colitis associated with $\text{IL2}^{-/-}$ mice, i.e., $\text{SI.Fas}^{-/-}$ mice do not develop skin and lung inflammation (12). This apparent organ-specific control of inflammation is in fact mediated by different mechanisms. It turns out that IL-2 is required for the expression of a panel of receptors involved in the trafficking/homing and retention of the pathogenic CD4$^+$ T cells to skin and lungs. Among the receptors that are highly up-regulated are cysteinyl leukotriene receptor 1, leukotriene B$_4$ receptor 1, CCR8, CXCR6, IL-1R-like1, and CD103 of the E$_7$ integrin (12). We have extensively characterized the role of IL-2 in the up-regulation of CD103 of the $\alpha_6\beta_2$ integrin on CD4$^+$ T cells from the SMG-draining lymph nodes of LPS-treated and PBS-treated SI mice ($n = 3$). Age-matched B6 male mice were included for comparison. * Indicates $p < 0.02$; **, $p < 0.05$.

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References


