Published in final edited form as:

J Autoimmun. 2012 June ; 38(4): 322–331. doi:10.1016/j.jaut.2012.02.001.

A Novel Function of IL-2: Chemokine/Chemoattractant/Retention Receptor Genes Induction in Th Subsets for Skin and Lung Inflammation

Rahul Sharma^a, Sun-Sang J. Sung^a, Felicia Gaskin^b, Shu Man Fu^{a,c,d}, and Shyr-Te Ju^{a,c,d}

^aCenter for Immunity, Inflammation and Regenerative Medicine, Department of Medicine, Charlottesville, University of Virginia, VA 22908, USA

^bDepartment of Psychiatry and Neurobehavioral Sciences, Charlottesville, University of Virginia, VA 22908, USA

^cDepartment of Microbiology, Immunology, and Cancer Biology, School of Medicine, Charlottesville, University of Virginia, VA 22908, USA

Abstract

The Foxp3⁺CD4⁺ regulatory T-cell (Treg)-deficient Scurfy (Sf) mice rapidly develop severe inflammation in the skin and lungs with expanded Th subsets bearing increased expression of various chemokine/chemoattractant/retention receptor genes (CRG). Nine different double mutants were generated to elucidate their roles in the skin and lung inflammation. The expanded Th2 response and the increased expression of several CRG for the skin and lung inflammation were inhibited in Sf.*Il2*^{−/−} mice as previously described using microarray analysis. Herein in a reciprocal approach, we demonstrated that Sf.*Il4*^{−/−} and Sf.*Stat6*^{−/−} mice, despite lacking Th2 cytokines IL-4, IL-5, and IL-13, as well as the IL-4/STAT6-dependent CRG expression, the inflammation in the skin and lungs remained. The effect of the other Th1 cytokine IFN-γ was studied in Sf.*Ifng*^{−/−} mice in which the multi-organ inflammation (MOI) was delayed but fully developed afterward with enhanced CRG expression except for the IFN-γ-dependent *Cxcr3* in CD4⁺ T-cells. Similarly, a transient delay of MOI was observed for Sf.*Itgae*^{−/−} mice but their Th subsets and the critical CRG expansion remained. *Ltb4r1*^{−/−}, *Alox5*^{−/−}, *Cx3cr1*^{gfp/gfp}, or *Il10*^{−/−} mutant genes also failed to effectively block inflammation in the skin and lungs in Sf mice. Our study has identified a novel function of IL-2 as a powerful Th1 cytokine that induces a panel of CRG in Th subsets required for skin and lung inflammation in Sf mice. The CRG panel induced by IL-2 but not by IL-4 or IFN-γ explains the apparent “organ-specific” display of the skin and lung inflammation in Sf mice.

© 2012 Elsevier Ltd. All rights reserved.

Corresponding author: Dr. Shyr-Te Ju, 21 Hospital Drive, Old Medical School Building, Room 5777, University of Virginia, Charlottesville, VA 22908. Tel: 434-243-6358, sj8r@virginia.edu.

^dContributed equally to the work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

R. Sharma (rs3wn@virginia.edu), S.-S.J. Sung (sjs5c@virginia.edu), F. Gaskin (fg6p@virginia.edu), S.M. Fu (sf2e@virginia.edu)

Conflict-of-interest disclosure The authors declare no competing financial interests.

Keywords

Regulatory T-cell; Cytokine; Trafficking Receptor Gene; Skin and Lung Inflammation

Introduction

Peripheral tolerance is maintained by the CD4⁺Foxp3⁺ Treg [1, 2]. Foxp3 is an X-chromosome-linked transcription factor whose expression in CD4⁺ T-cells is essential for the suppressive function of Treg [3]. Mutations affecting Foxp3 expression and functions such as that present in the Sf mice and those in patients with “immune-dysregulation, polyendocrinopathy, enteropathy, X-linked” (IPEX) syndrome invariably cause early death resulting from MOI in skin, lungs, pancreas, small intestine and liver [4, 5].

A prominent Th2 response is associated with Sf mice and IPEX patients [6, 7]. In addition, IL-5-mediated eosinophilia, IL-13-mediated goblet cell metaplasia and IgE-mediated allergy are frequently observed [7]. Interestingly, the inflammation in the skin and lungs but not liver was strongly inhibited in Sf.*Il2*^{-/-} mice, indicating that IL-2 has a heretofore unrecognized novel function that is critically important in the inflammation in the former two organs [8, 9]. Our recent microarray analyses among B6, Sf, and Sf.*Il2*^{-/-} mice revealed that the Th2 response and the expression of a large panel of CRG in the Sf Th cells were inhibited too [8]. In adoptive transfer experiments, Sf.*Il2*^{-/-} lymph node (LN) cells did not induce inflammation in the skin and lungs whereas Sf LN cells did in *Rag1*^{-/-} recipients [8, 9]. The questions we are going to address are: whether inhibition of the Th2 response is sufficient to prevent the inflammation in the skin and lungs and why deficiency in IL-2 is so effective in providing lifelong protection against the inflammation in the skin and lungs.

In this study, we bred *Il2*^{-/-}, *Il4*^{-/-}, *Stat6*^{-/-}, *Ifng*^{-/-}, *Ltb4r1*^{-/-}, *Alox5*^{-/-}, *Itgae*^{-/-}, *Cx3cr1*^{gfg/gfp}, and *Il10*^{-/-} mutant genes into Sf mice and determined their effects on Th subsets, cytokine expression and organ inflammation. Importantly, the study of Sf.*Il4*^{-/-} and Sf.*Stat6*^{-/-} demonstrated in a reciprocal manner to Sf.*Il2*^{-/-} study that IL-4-, IL-5-, and IL-13-producing Th2 cells and IgE were not required for the inflammation in the skin and lungs. In a parallel study of another Th1 cytokine-deficient Sf.*Ifng*^{-/-} mice, the skin and lung inflammation was delayed but both Th2 and Th1 cells were present with increased expression of many of the IL-2-regulated CRG. Interestingly, Th17 response was not expanded in all cases. These observations indicate that CRG, but not Th2 response controlled by IL-2, are critical to the skin and lung inflammation in Sf mice. Our study has provided an apparent “organ-specific” mechanism for skin and lung inflammation in Sf mice. Importantly, the study firmly establishes a heretofore unrecognized novel function of Th1 cytokine IL-2 that induces a panel of CRG involved in skin and lung inflammation.

Materials and methods

Mice

All mice were obtained from the Jackson Laboratories, Bar Harbor, Maine, USA. B6.Cg-*Foxp3*^{sf/x}J mice were bred with male B6 mice to produce Sf mice [10]. Mice carrying both *Il2*^{-/-} and *Foxp3*^{sf/Y} genes (Sf.*Il2*^{-/-}) were generated and verified by PCR as described [9]. All other Sf double mutant mice were produced by the same breeding program. Mice were examined twice weekly for clinical signs of diseases, including inflammation of skin, ears, tails and areas around the eyes, body weight loss, lethargy, etc. The histological analyses and histological scores were conducted as previously described [8, 11]. Experiments involving animals were conducted in accordance with the protocols approved by the Animal Care and Use Committee (ACUC) of the University of Virginia.

Serum cytokines and IgE

Blood samples were collected from 3-week old mice and sera were collected after centrifugation. Individual sera were used to determine the level of various cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN- γ , and TNF- α) using the Procarta cytokine kits and the Luminex platform (Affymetrix Inc.). Serum IgE level was determined by ELISA (Bethyl laboratories, Inc.).

Flow cytometry

Axillary, brachial, inguinal, cervical, and facial LN of sex- and age-matched B6, Sf, and various double mutant mice were isolated and single cell suspensions were prepared from the pooled LN of individual mice [10]. Age-specified single cell suspensions from 3-week and 6-week old Sf.*Ifng*^{-/-} mice were also prepared. Cells were activated *in vitro* (4×10^6 cells/2 ml/24-well plate) for 4 hours in Phorbol 12-myristate 13-acetate (PMA) (20 ng/ml), ionomycin (1 μ M), and Monensin (2 μ M) (Sigma). Cells were then washed and suspended in 100 μ l of phosphate-buffered saline containing 4 mg bovine serum albumin and 1 μ g 2.4G2 anti-FcR monoclonal antibody and incubated with 0.2 μ g of PerCP-Cy5.5 anti-CD3 ϵ monoclonal antibody (145-2C11) and APC-eFluor780-labeled anti-CD4 (RM4.5) monoclonal antibody (eBioscience) for 30 minutes at 4°C. Cells were then fixed, permeabilized, and stained for 30 minutes using FITC-, PE- or APC-labeled antibodies against IL-2, IL-4, IL-5, IL-13, IL-10, IL-17, IFN- γ and TNF- α (eBioscience). Gated CD3⁺CD4⁺ cells were analyzed for intracellular cytokine production. 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).

Quantitative real-time PCR

CD4⁺ T-cells were FACS-sorted (>99% pure) from LN cells of 15-day old B6, Sf or various double mutant mice. Total RNA, prepared using RNEasy RNA isolation kit (Qiagen), was converted to cDNA using QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR analysis was performed using the iCycler iQ system (BioRad) that measures SYBR Green DNA binding. Based on our microarray study [8], 10 TRG that were highly differentially expressed in Sf and Sf.*Il2*^{-/-} CD4⁺ T-cells as compared with B6 samples were selected for analysis. All primer sequences for various genes examined in this study were obtained from PrimerBank website at <http://pga.mgh.harvard.edu/primerbank/>. Relative quantification of gene expression based on primer-efficiency correction was performed as described by M. W. Pfaffl [12]. Target gene expression level was normalized on *Gapdh* level and compared to the values obtained from B6 samples.

Histology

Tissues/organs from age-matched males of various strains were fixed with 10% neutral-buffered formalin (Fisher Scientific) and sections of paraffin-embedded tissue were stained with H&E. Tissues/organs examined included skin, ear, lung, and liver. Inflammation levels, based on the extent of leukocyte infiltration in 10 randomly selected fields, were scored as severe (4+), strong (3+), moderate (2+), mild (1+), and no inflammation (0).

Statistic analysis

Statistical analyses were performed using the Student's *t*-test function of Microsoft Excel software, employing heteroscedastic 2-tailed distribution. The *p* value of <0.05 is considered as a significant difference between the two compared groups.

Results

Fully developed skin and lung inflammation in the *Sf.II4^{-/-}* and *Sf.Stat6^{-/-}* mice

At two weeks old, Sf mice began to display clinical signs of skin inflammation including scaly and crusty skin on the eyelids, ears and tails, dermal thickening, and squinted eyes. Lethargy and lack of body weight gain soon followed. The clinical signs associated with skin inflammation were not observed in *Sf.II2^{-/-}* mice. The lethargy and lack of body weight gain were still observed but the lifespan was prolonged to 12-17 weeks old [9]. In *Sf.II4^{-/-}* and *Sf.Stat6^{-/-}* mice, these clinical signs of skin inflammation and lethargy appeared similar to that of Sf mice. Histological analysis revealed strong inflammation in the skin, lungs and liver in these mice even though their Th2 response was expected to be reduced (Fig. 1A and 1B). The total lymphocytes estimated in the LN were not significantly different among Sf, *Sf.II4^{-/-}* and *Sf.Stat6^{-/-}* mice (Fig. 1C). In contrast, inflammation in the skin and lungs but not liver was inhibited in *Sf.II2^{-/-}* mice even though their total lymphocytes were significantly higher than Sf mice. Thus, IL-4/STAT6-dependent response was not required for the skin and lung inflammation in Sf mice.

Comparison of cytokine-producing CD4⁺ T-cells

The frequency of cytokine-producing CD4⁺ T-cells was examined and compared among 3 weeks old B6, Sf, *Sf.II2^{-/-}*, and *Sf.II4^{-/-}* mice (Fig. 2). They were enumerated after *in vitro* incubation with PMA, ionomycin and Monensin for 4 hours. The cytokines examined were IL-2, IFN- γ , TNF- α , IL-10, IL-4, IL-5, IL-13, and IL-17. The compiled results of 4 mice in each group are presented in Fig. 2.

IFN- γ is not sufficient for the development of skin and lung inflammation

Under the assay conditions, B6 LN cells expressed a high proportion of IL-2-producing T cells (33%). They were detected due to the high sensitivity of IL-2 assay. However, they were CD44^{low} and lacked IFN- γ (Fig. 2a and 2b). In contrast, the great percentage of IL-2⁺ T-cells in Sf and Sf double mutants were CD44⁺ (not shown). B6 mice expressed little IFN- γ ⁺CD4⁺ T-cells, which were increased significantly in Sf mice (Fig. 2b) and the fraction was comparable to that of IL-2⁺ T-cells in the same samples. In *Sf.II2^{-/-}* mice, IL-2⁺CD4⁺ T-cells were absent but the frequency of IFN- γ ⁺CD4⁺ T-cells was similar to Sf samples, indicating that IL-2 deficiency did not affect IFN- γ production in Th1 cells (Fig. 2a and 2b). The data suggest that IFN- γ is not as critical as IL-2 to the skin and lung inflammation in the Sf mice.

Absence of Th2 response in *Sf.II4^{-/-}* mice does not inhibit skin and lung inflammation

B6 mice had few IL-4⁺CD4⁺, IL-5⁺CD4⁺, and IL-13⁺CD4⁺ T-cells, which were significantly increased in Sf mice (Fig. 2e-2g). The expression of these CD4⁺ T-cells was strongly inhibited in *Sf.II2^{-/-}* mice. In *Sf.II4^{-/-}* mice, IL-4⁺CD4⁺ T-cells were rarely detected (Fig. 2e). IL-5⁺CD4⁺ T-cells were strongly inhibited (Fig. 2f). A significant but moderate inhibition was observed for IL-13 (Fig. 2g). Interestingly, the level of IFN- γ ⁺CD4⁺ T-cells (~60%) in the *Sf.II4^{-/-}* mice was significantly higher than Sf and *Sf.II2^{-/-}* samples (Fig. 2b). In addition, this value was higher than the 30% of IL-2⁺CD4⁺ T-cells in the same samples. This could be caused by the transient expression of IL-2 [13], the compensation from Th0 cells [14], and/or the absence of negative regulation of IFN- γ production by STAT6 [15]. By contrast, a significant expression of IL-10⁺CD4⁺ T-cells was observed in *Sf.II4^{-/-}* and *Sf.II2^{-/-}* samples, suggesting IL-10 expression in Sf CD4⁺ T-cells is not controlled by IL-2 and IL-4 (Fig. 2d). Similarly, the frequency of TNF- α ⁺CD4⁺ T-cells was also increased in Sf mice as compared to B6 control and the strong expression was not diminished in *Sf.II2^{-/-}* and *Sf.II4^{-/-}* samples (Fig. 2c).

Minor roles of Stat6 in skin and lung inflammation

The levels of Th subsets and cytokine-producing CD4⁺ T-cells were also determined in Sf.*Stat6*^{-/-} mice. The percentage of CD4⁺ T-cells that produced IL-2, IFN- γ , or TNF- α was comparable between Sf.*Il4*^{-/-} and Sf.*Stat6*^{-/-} mice (Fig. 2a-2c). The expression of Th2 cells in Sf.*Stat6*^{-/-} mice was somewhat different from Sf.*Il4*^{-/-} mice. Although IL-4-producing Th2 cells were significantly reduced as compared with Sf samples, they were still significantly more than B6 samples (Fig. 2e). This suggests that IL-2 is more critical than STAT6 in regulating the development of Th2 cells but STAT6 is still needed for the optimal expansion of the IL-4⁺CD4⁺ T-cells in Sf mice.

Low expression of IL-17⁺ CD4⁺ T-cells

Th17 cells have also been shown to be an important effector Th subset in certain autoimmune diseases but how pervasive and contributing of this subset to Sf MOI has not been determined. In Sf mice, despite losing Treg and developing severe MOI, Th17 cells were few as compared with Th1 and Th2 subsets. This low value was maintained in Sf.*Il2*^{-/-} and Sf.*Il4*^{-/-} mice in which the Th2 cell expansion was prevented (Fig. 2h). Moreover, this value was low regardless of the level of IFN- γ ⁺CD4⁺ T-cells (Fig. 2b). Th17 cells express IL-10R and Th17 expansion could be inhibited by IL-10 produced by non-Treg [16]. This mechanism is consistent with the fact that high levels of IL-10-producing T-cells were presented in Sf, Sf.*Il2*^{-/-}, Sf.*Il4*^{-/-}, and Sf.*Stat6*^{-/-} mice and despite the additional presence of IL-6 level that favors Th17 generation from naïve CD4⁺ T-cells [17], Th17 cell levels remained low in the Sf and many of the double mutant mice.

Delayed but fully developed MOI in Sf.*Ifng*^{-/-} mice

IFN- γ is the principal marker for Th1 cells. IFN- γ inhibits Th2 response under the *in vitro* induction condition skewed against Th2 development. IFN- γ can activate macrophages and neutrophils, particularly in the presence of LPS, to become potent inflammatory cells in the inflamed tissues. IFN- γ induces CXCL9, CXCL10, and CXCL11 from various cells to attract leukocytes to target organs [18, 19]. IFN- γ induces strong MHC expression on various antigen-presenting cells and as such exacerbates the ongoing immune response. Less is known about its effect on CRG expression in CD4⁺ T-cells. Because both Sf.*Il2*^{-/-} and Sf mice had high serum IFN- γ and IFN- γ -producing CD4⁺ T-cells [8], yet only the Sf mice developed inflammation in the skin and lungs (Fig. 1a and 1b), the question as to what extent IFN- γ influenced the inflammation was addressed.

Breeding *Ifng*^{-/-} into Sf mice decreased the cytokine response of CD4⁺ T-cells that produced IL-2, TNF- α , IL-4, IL-5, and IL-13 (Fig. 3A). The separate presentation of the Sf.*Ifng*^{-/-} data is for clarity purpose. The clinical signs of inflammation in the skin, eyes, ears and tail were reduced and delayed by 1-3 weeks. Histological analysis of the MOI indicated a significant inhibition of inflammation in the skin and ears. Although the trend of inhibition in the lungs and liver was observed, the difference was not significant when compared with Sf samples (Fig. 3C). The reduced inflammation in ears, skin, lungs and liver in the 3 weeks old Sf.*ifng*^{-/-} mice is still significant statistically as compared with normal B6 controls. The lifespan of Sf.*Ifng*^{-/-} mice was prolonged to 6-7 weeks old and at that time the MOI was fully developed as shown by both histology and histological scores of inflammation (Fig. 3C). The total number of CD3⁺, CD4⁺ T-cells, or total lymphocytes in the LN (3 weeks old) was comparable to Sf samples (Fig. 3D). The results are in contrast to Sf.*Il2*^{-/-} mice in which the inflammation in the skin and lungs was inhibited for the entire lifespan even in the presence of increased IFN- γ level and significant expansion of lymphocytes (Fig. 1, 2 and 4).

Only without IL-2, no other serum cytokines correlated with lifelong protection of skin and lung inflammation in Sf mice

Serum levels of various cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IFN- γ , and TNF- α) and IgE of age-matched mice were determined (Fig. 4). Sera from Sf mice contained high levels of cytokines associated with Th1 and Th2 responses. Great variability was observed because some cytokines were also produced by non-Th cells during inflammation. Nevertheless, one cytokine that was not increased was IL-17. The lack of significant increase in IL-17 was also observed in Sf.*Il2*^{-/-}, Sf.*Il4*^{-/-}, Sf.*Stat6*^{-/-} and Sf.*Ifng*^{-/-} mice. Serum IFN- γ level was dramatically increased in Sf.*Stat6*^{-/-} mice over and above that in Sf sera, consistent with the idea that STAT6 is a negative regulator for IFN- γ production [15].

IgE is not required for the skin and lung inflammation in Sf mice

High serum IgE levels have always been associated with allergic skin inflammation and certain lung inflammation conditions. The serum IgE level was dramatically increased in Sf mice as compared with B6 samples. The increase was completely inhibited to undetectable levels in the Sf.*Il4*^{-/-} and Sf.*Stat6*^{-/-} mice whereas it remained high in Sf.*Il2*^{-/-} and Sf.*Ifng*^{-/-} mice (Fig. 4B). Interestingly, Sf.*Il2*^{-/-} mice expressed a significant serum level of IgE. These data demonstrate that increase in serum IgE is not essential to the inflammation in the skin and lungs in Sf and the Sf double mutant mice examined herein.

Th cytokines regulate CRG expression: mechanism and specificity

IL-2 regulates not only the Th2 response but also CRG expression in CD4⁺ T-cells that are capable of transferring inflammation to the skin and lungs of *Rag1*^{-/-} recipients [8]. Therefore, it becomes important to determine whether the over-expression of these CRG is also restricted to a specific Th subset or regulated by a specific Th cytokine. By determining the CRG expression and its regulation by specific cytokines, the individual CRG could be implicated or eliminated for its involvement in the inflammation of skin and lungs.

The expression of several CRG in CD4⁺ T-cells that have been implicated in the inflammation of skin and lungs was determined by quantitative PCR (Fig. 5A). In Sf.*Il4*^{-/-} mice, the marked increase in the expression of *Cysltr1*, *Ltb4r1*, and *Il1rl1* was not observed in CD4⁺ T-cells but the inflammation in the skin and lungs remained. The IL-4-dependent expression of these CRG was confirmed with the results of Sf.*Stat6*^{-/-} samples (Fig. 5B). In this experiment, a larger panel of CRG was examined so that the specificity of CRG regulation could be better addressed. The CRG selected for examination were: *Cysltr1*, *Ltb4r1*, *Ptgir*, *Il1rl1*, *Ccr1*, *Ccr3*, *Ccr4*, *Ccr8*, *Cxcr3* and *Cxcr6*. They were chosen because some of them were selectively increased in Sf CD4⁺ T-cells as compared with Sf.*Il2*^{-/-} samples whereas others were enhanced in both samples as compared with B6 CD4⁺ T-cells [8]. As shown in Fig. 5B, the expression of *Cysltr1*, *Ltb4r1*, and *Ptgir* was significantly inhibited in Sf.*Stat6*^{-/-} mice as compared with Sf samples. The expression of *Il1rl1* was also reduced, but the level of inhibition was not statistically significant. In contrast, the expression of *Ccr1*, *Ccr3*, *Ccr4*, *Ccr8*, *Cxcr3* and *Cxcr6* remained high in Sf.*Stat6*^{-/-} mice. These observations indicate that the *Cysltr1*, *Ltb4r1*, *Ptgir*, and perhaps *Il1rl1* genes are preferentially regulated by IL-4/STAT6 and that the Th1 cells do not need their expression to induce inflammation in the skin and lungs in the Sf mice.

Our previous study showed that when compared with B6 CD4⁺ T-cells, *Ccr1* and *Ccr8* were selectively enhanced in Sf but not Sf.*Il2*^{-/-} samples whereas *Ccr3*, *Ccr4*, *Cxcr3* and *Cxcr6* were up-regulated in both samples [8]. The data obtained with Sf.*Ifng*^{-/-} and Sf.*Stat6*^{-/-} mice demonstrated that the expression of *Cxcr3* among the CRG examined was critically dependent on IFN- γ in Sf mice (Fig. 5B). The Sf.*Stat6*^{-/-} mice had the highest levels of

serum IFN- γ among all samples examined (Fig. 4A) and this correlated with an even higher expression of *Cxcr3* and *Cxcr6* (Fig. 5A). The expression *Cxcr6* is also dependent on IFN- γ (20, 21). Increased expression of Th *Cxcr6* in *Sf.Stat6*^{-/-} mice may require IFN- γ levels higher than that expressed in Sf mice. Overall, these data demonstrate that inhibiting the CRG specifically regulated by IL-4/STAT6 or IFN- γ is not sufficient to effectively prevent inflammation in the skin and lungs in Sf mice as in *Sf.II2*^{-/-} mice in which IL-2 production is completely eliminated.

Study of *Sf.Ltb4r1*^{-/-}, *Sf.Alox5*^{-/-}, *Sf.Itgae*^{-/-}, *Sf.Cx3cr1*^{gfp/gfp} and *Sf.II10*^{-/-} mice

This series of studies included other double mutant mice from which additional information were obtained with respect to the effect of cytokines and CRG on Th subset expression and MOI, respectively. The data are summarized in Table 1.

The *Sf.Ltb4r1*^{-/-} mice had expanded Th1 and Th2 responses and inflammation in ears, skin, lungs and liver. With only 4 mice examined, there was some variability in inflammation scores of ears, skin and lungs. Two mice died at 24 days old and two died at 31 days old. On the other hand, *Sf.Alox5*^{-/-} mice displayed severe MOI. Their Th1 cells did not change much but their Th2 cells were twice as much as that in the Sf mice, and they died within 3 weeks after birth. This could be due to the fact that 5-lipoxygenase produces pro-inflammatory leukotrienes that target Th2 cells, neutrophils, monocytes, and macrophages [22]. Collectively, the results indicate that leukotrienes and leukotriene receptors could contribute to but are not as critical as IL-2 to the MOI in Sf mice.

The *Cx3cr1* gene encodes *Cx3cr1* in lymphocytes, monocytes and macrophages [23]. Its deletion in *Sf.Cx3cr1*^{gfp/gfp} mice (*gfp* replaces the *Cx3cr1*) had no significant effect on the MOI. This is consistent with the lack of *Cx3cr1* up-regulation in Sf CD4⁺ T-cells as compared with B6 samples [8].

The absence of CD103 did not affect Th subset expression, but did delay inflammation in the skin and lungs and slightly prolonged the lifespan [11]. CD103-independent and lethal MOI were fully developed soon afterward [11]. This is consistent with the fact that E-cadherin on epithelial cells retains CD103⁺CD4⁺ T-cells to prolong tissue inflammation. It is important to note that CD103 up-regulation in CD4⁺ T-cells requires IL-2, suggesting that CD103 expression regulated by IL-2 contributes in part to the strong inflammation [11]. Finally, the MOI was not inhibited in *Sf.II10*^{-/-} mice, indicating that IL-10 had little effect on the Sf MOI, most likely due to the expression by Sf mice of multiple inflammatory effector mechanisms.

Discussion

Our recently microarray analyses among B6, Sf, and *Sf.II2*^{-/-} CD4⁺ T-cells has shown that IL-2 is required for the induction of Th2 response and expansion of a panel of CRG and these functions coincided with the development of skin and lung but not liver inflammation in the Sf mice [8]. In a reciprocal manner, the present study using *Sf.II4*^{-/-} and *Sf.Stat6*^{-/-} mice confirmed and ruled out the possibility that the inflammation in the skin and lungs is solely dependent on Th2 response/cytokines and the CRG regulated by IL-4 and STAT6. We also investigated the role of the other Th1 cytokine IFN- γ in *Sf.Ifng*^{-/-} mice and identified that IL-2-but not IFN- γ -regulated CRG in CD4⁺ T-cells were the critical components for the inflammation in the skin and lungs. These results demonstrate that the novel role of IL-2 as a heretofore under-appreciated CRG-inducing Th1 cytokine that controls the expression of CRG critical to the inflammation in the skin and lungs of Sf mice. This novel function is surprising given the fact that *II2*^{-/-} mice themselves spontaneously develop organ inflammation, although they don't develop skin and lung inflammation.

IL-2 also regulates the IL-4/STAT6-dependent CRG expression by affecting Th2 expansion and IL-4 production. Many of the CRG associated with Th2 response (*Cysltr1*, *Ltb4r1*, *Ptgir*, and *Il1rl1*) are dependent on IL-2 for expression [8]. The present study demonstrated that this was due to the IL-2-dependent induction of IL-4 and IL-4-activated STAT6. On the other hand, the enhanced expression of *Ccr1*, *Ccr3*, *Ccr4*, *Ccr8*, *Cxcr3* and *Cxcr6* was not inhibited in Sf.*Stat6*^{-/-} samples. The strong inflammation in the skin and lungs in Sf.*Il4*^{-/-} and Sf.*Stat6*^{-/-} mice indicate that Th2 response and the CRG selectively regulated by IL-4/STAT6 are not required for the inflammation in the skin and lungs in Sf mice. On the other hand, the expression of these critical CRG in Sf.*Ifng*^{-/-} mice was not inhibited albeit delayed due to the absence of IFN- γ stimulation on general immune response. These observations demonstrate the specificity and complexity of IL-2 in controlling CRG expression.

Inflammation in the skin and lungs of Sf mice has been attributed to a prominent Th2 response as a result of Treg deficiency. Our study demonstrates that IL-2 controlled by Treg is an important cytokine for skin and lung inflammation. Treg suppression of Th2 response has been attributed to multiple mechanisms including those mediated by *Ccr4* for Treg trafficking to the skin and lungs and *Irf4* expression within Treg for Th2 response suppression [1, 24, 25]. Our study suggests that Treg control of Th2 response and the inflammation in the skin and lungs is mediated by its consumption and inhibition of IL-2 production. Various forms of inflammation in the skin and lungs regulated by Treg deficiency, although showing prominent Th2 response, may contain a Th1 response that controls and participates in the inflammation [26-28]

The inflammation process in Sf mice represents a complex cellular and cytokine interacting network not easily observed in experimentally induced inflammation models, which are often transient and short-lived. In this regard, the nearly complete blockade of skin and lung inflammation in Sf.*Il2*^{-/-} mice contrasts sharply with Sf.*Il4*^{-/-}, Sf.*Stat6*^{-/-} or Sf.*Ifng*^{-/-} mutants that provide little or partial protection. Additional breeding experiments showed that the *Ltb4r1*, *Cx3cr1*, *Alox5*, *Itgae* and *Il10* genes were also not as critical as IL-2 to the inflammation in the skin and lungs in Sf mice. Our observations do not disagree with the roles of these genes in the inflammation in the skin and lungs under specific inflammation conditions but rather emphasize the critical role of IL-2 in controlling the development and progression of the inflammation under the unique un-manipulated *in vivo* condition in Sf mice.

Our study has demonstrated that IL-2 but not IFN- γ of the Th1 response is the cytokine that induces the expression of CRG critical to the inflammation in the skin and lungs. In addition, IL-2 is critical to the expanded Th2 response in Sf mice [8]. As IFN- γ is inhibitory against Th2 response, the facilitating role of IL-2 for the Th2 response underscores the complexity of interplay between Th1 and Th2 responses that occurs spontaneously *in vivo* in Sf mice. In Sf.*Il2*^{-/-} mice in which the inflammation in the skin and lungs was strongly inhibited, the expression of IFN- γ mRNA, IFN- γ -producing CD4⁺ T-cells and serum IFN- γ level was not different from that observed in the Sf mice that displayed severe inflammation in the skin and lungs [8]. Therefore, the reason that inflammation in the skin and lungs was inhibited in Sf.*Il2*^{-/-} mice was not due to a lack of IFN- γ expression but rather the lack of expression of CRG that was crucial for migration of the Th cells to the skin and lungs. The results obtained with the Sf.*Ifng*^{-/-} mice demonstrate in a reciprocal manner that the absence of IFN- γ not only failed to up-regulate the Th2 response but in fact was suppressive against both Th1 and Th2 cytokine production in the early phase of inflammation.

In conclusion, the present studies using 9 different Sf double mutant mice have clearly established that IL-2 has a heretofore unnoticed novel function that promotes the expression

of a panel of CRG in CD4⁺ T-cells during the MOI process. Moreover, in *Sf.II4^{-/-}* and *Sf.Stat6^{-/-}* in which the Th2 cytokines of IL-4, IL-5 and IL-13 were greatly inhibited, the skin and lung inflammation remained, indicating the participation of Th1 response in the skin and lung inflammation. Although IL-2 regulates the Th2 response but the expression of CRG specific to leukotrienes and prostacyclins is controlled by IL-2-dependent IL-4 production and mainly through the IL-4-dependent activation of STAT6. Similarly, most of the CRG in Th1 cells critical to the inflammation in the skin and lungs of Sf mice are regulated by IL-2 but not IFN- γ . Despite the complete absence of IL-4, STAT6 or IFN- γ , the MOI still developed in the respective Sf double mutants, suggesting that MOI in Sf mice is controlled by multiple effector mechanisms and that inhibiting a single component (such as IL-4, IFN- γ , CD103, etc.) that is usually perceived as critical to many inflammation diseases is insufficient to completely prevent the MOI in Sf mice. This is in contrast to *Sf.II2^{-/-}* mice in which the CRG critical to skin and lung inflammation was inhibited so that the lifelong protection of the organ inflammation was achieved. Our study has provided genetic, cellular and molecular mechanisms as to how the inflammation in the skin and lungs in Sf mice is controlled by specific Th cytokines with the highly significant finding that IL-2 has a heretofore unrecognized novel function that provides powerful induction force for the expression of a panel of CRG that are critical to the skin and lung inflammation during inflammation response in Sf mice. The present studies underscore the possibility that IL-2 may be targeted therapeutically for skin and lung inflammation.

Acknowledgments

The excellent technical assistance of Mr. CE Abaya is greatly appreciated. This work was supported by National Institutes of Health Grants AR-051203 (STJ), DE-017579 (STJ), AI-079906 (SJS), AR-047988 (SMF), AR-049449 (SMF) and an Institute grant-in-aid (RS).

References

1. Shevach EM. Mechanisms of Foxp3⁺ T regulatory cell-mediated suppression. *Immunity*. 2009; 30:636–45. [PubMed: 19464986]
2. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. Regulatory T cells: how do they suppress immune responses? *Int Immunol*. 2009; 21:1105–11. [PubMed: 19737784]
3. Fontenot JD, Rasmussen JP, Williams LM, Dooley L, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor Foxp3. *Immunity*. 2005; 22:329–41. [PubMed: 15780990]
4. Brunkow ME, Jeffery EW, Hjerrild KA, Paepers B, Clark LB, Yasayko SA, Wilkinson JE, et al. Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet*. 2001; 27:68–73. [PubMed: 11138001]
5. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova J-L, Buist N, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*. 2001; 27:18–20. [PubMed: 11137992]
6. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *J Clin Invest*. 2000; 106:R75–81. [PubMed: 11120765]
7. Lin W, Truong N, Grossman WJ, Haribhai D, Williams CB, Wang J, Martin MG, et al. Allergic dysregulation and hyperimmunoglobulinemia E in Foxp3 mutant mice. *J Allergy Clin Immunol*. 2005; 116:1106–15. [PubMed: 16275384]
8. Sharma R, Sharma PR, Kim Y-C, Leitingner N, Lee JK, Fu SM, et al. IL-2-controlled expression of multiple T cell trafficking genes and Th2 cytokines in the regulatory T cell-deficient Scurfy mice: Implication to multiorgan inflammation and control of skin and lung inflammation. *J Immunol*. 2011; 186:1268–78. [PubMed: 21169543]
9. Zheng L, Sharma R, Gaskin F, Fu SM, Ju ST. A novel role of IL-2 in organ-specific autoimmune inflammation beyond regulatory T cell checkpoint: both IL-2 knockout and Fas mutation prolong

- lifespan of Scurfy mice but by different mechanisms. *J Immunol.* 2007; 179:8035–41. [PubMed: 18056343]
10. Sharma R, Jarjour WN, Zheng L, Gaskin F, Fu SM, Ju ST. Large functional repertoire of regulatory T-cell suppressible autoimmune T cells in scurfy mice. *J Autoimm.* 2007; 29:10–19.
 11. Sharma R, Sung SS, Abaya CE, Ju AC, Fu SM, Ju ST. IL-2 regulates CD103 expression on CD4⁺ T cells in Scurfy mice that display both CD103-dependent and independent inflammation. *J Immunol.* 2009; 183:1065–73. [PubMed: 19553521]
 12. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001; 29:e45–26. [PubMed: 11328886]
 13. Villarino AV, Tato CM, Stumhofer JS, Yao Z, Cui YK, Hennighausen L, O'Shea JJ, et al. Helper T cell IL-2 production is limited by negative feedback and STAT-dependent cytokine signals. *J Exp Med.* 2007; 204:65–71. [PubMed: 17227909]
 14. Miner KT, Croft M. Generation, persistence, and modulation of Th0 effector cells: Role of autocrine IL-4 and IFN- γ . *J Immunol.* 1998; 160:5280–87. [PubMed: 9605125]
 15. Metwali A, Blum A, Elliott DE, Weinstock JV. Interleukin-4 α chain and STAT6 signaling inhibit gamma interferon but not Th2 cytokine expression within Schistosome granulomas. *Infect Immunity.* 2002; 70:5651–58. [PubMed: 12228294]
 16. Huber S, Gagliani E, Esplugues E, O'Connor W Jr, Huber FJ, Chaudhry A, Kamanaka M, et al. Th17 cells express interleukin-10 receptor and are controlled by Foxp3⁺ and Foxp3⁺ regulatory CD4⁺ T cells in an interleukin-10-dependent manner. *Immunity.* 2011; 34:554–65. [PubMed: 21511184]
 17. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal development pathways for the generation of pathogenic TH17 and regulatory T cells. *Nature.* 2006; 441:235–38. [PubMed: 16648838]
 18. Mach F, Sauty A, Iarossi AS, Sukhova GK, Neote K, Libby P, et al. Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. *J Clin Invest.* 1999; 104:1041–50. [PubMed: 10525042]
 19. Cole KE, Strick CA, Paradis TJ, Ogborne KT, Loetscher M, Gladue RP, et al. Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med.* 1998; 187:2009–21. [PubMed: 9625760]
 20. Nakajima C, Mukai T, Yamaguchi N, Morimoto Y, Park WR, et al. Induction of the chemokine receptor CXCR3 on TCR-stimulated T cells: dependence on the release from persistent TCR-triggering and requirement for IFN-gamma stimulation. *Eur J Immunol.* 2002; 32:1792–01. [PubMed: 12115663]
 21. Calabresi PA, Yun SH, Allie R, Whartenby KA. Chemokine receptor expression on MBP-reactive T cells: CXCR6 is a marker of IFN-gamma-producing effector cells. *J Neuroimmunol.* 2002; 127:96–05. [PubMed: 12044980]
 22. Duroudier NP, Tulah AS, Sayers I. Leukotriene pathway genetics and pharmacogenetics in allergy. *Allergy.* 2009; 64:823–39. [PubMed: 19416143]
 23. Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, Kakizaki M, et al. Identification and molecular characterization of fractalkine receptor CXCR1, which mediates both leukocyte migration and adhesion. *Cell.* 1997; 91:521–30. [PubMed: 9390561]
 24. Sather BD, Treuting P, Perdue N, Miazgowiec M, Fontenot JD, Rudensky AY, et al. Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. *J Exp Med.* 2007; 204:1335–47. [PubMed: 17548521]
 25. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu TT, et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature.* 2009; 458:351–56. [PubMed: 19182775]
 26. Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest.* 1999; 103:175–83. [PubMed: 9916129]

27. Randolph DA, Stephens R, Carruthers CJ, Chaplin DD. Cooperation between Th1 and Th2 cells in a murine model of eosinophilic airway inflammation. *J Clin Invest.* 1999; 104:1021–29. [PubMed: 10525040]
28. Oyoshi MK, He R, Kumar L, Yoon J, Geha RS. Cellular and molecular mechanisms in atopic dermatitis. *Adv Immunol.* 2009; 102:135–226. [PubMed: 19477321]

Highlights

- IL-2 induces trafficking related genes in Sf Th-cells for skin and lung inflammation.
- Sf.*Il4*^{-/-} and Sf.*Stat6*^{-/-} mice develop skin and lung inflammation.
- Sf.*Ifng*^{-/-} mice develop MOI without inhibition of IL-2-controlled trafficking genes.
- IL-2 has a newly identified function for Sf Th-cell trafficking to skin and lungs

Figure 1. Histological comparison of organ inflammation among Sf, Sf.II2^{-/-}, Sf.II4^{-/-}, and Sf.Stat6^{-/-} mice

(A) Ear, skin, lung, and liver of indicated mice of 3 weeks old were processed for H&E stain as described [9]. As compared to B6, Sf, Sf.II4^{-/-}, and Sf.Stat6^{-/-} mice developed severe inflammation in ear, skin, lung and liver whereas inflammation in ear, skin and lung but not liver was inhibited in Sf.II2^{-/-} mice. Arrows indicate leukocyte infiltration. Arrow heads indicate goblet cell metaplasia. (B) The degrees of inflammation in various organs of indicated mice are presented as histological scores. (*) indicates significant difference as compared with B6 samples (N=4, p<0.05). (C) The total number of cells, CD3⁺ T-cells, and CD4⁺ T-cells obtained from pooled LN of indicated mice. Only Sf.II2^{-/-} samples showed a significant increase as compared to Sf samples.

Figure 2. Th subset distribution and cytokine-producing profile in B6, Sf, Sf.II2^{-/-}, Sf.II4^{-/-}, and Sf.Stat6^{-/-} mice

The experiments were conducted as described in “Materials and Methods” and the data are presented as % stained cells of the gated CD4⁺ T-cells. At least 4 samples of each group were analyzed. Approximately 33% of B6 samples displayed IL-2⁺ due to high sensitivity of the assay (*). However, they lacked IFN-γ (Fig. 2b). (**) indicates a significant difference in cytokine-producing Th cells as compared with Sf samples.

Figure 3. Comparison of Th subset expression and cytokine-producing profile among B6, Sf, and *Sf.Ifng*^{-/-} samples

(A) The experiments were conducted as described in the “Materials and Methods”. The results of B6 and Sf mice from Fig. 2 were included for comparison. Because the infrequent production of double mutants (about 1 in 16 progeny) and the limited time frame to complete the study before fatality, they were used whenever they were available. The results were compiled from many such independent and individual experiments. The separate presentation of the results obtained from *Sf.Ifng*^{-/-} mice is for the purpose of clear data presentation. (*) indicates a significant difference in cytokine production of *Sf.Ifng*^{-/-} CD4⁺ T-cells as compared with Sf samples ($p < 0.05$). (B) Histological comparison of the MOI among B6, Sf, and *Sf.Ifng*^{-/-} mice: The MOI of the 6-7 weeks old *Sf.Ifng*^{-/-} mice were included to demonstrate that the delayed inflammation eventually progressed to severe inflammation at this age. Arrows indicate leukocyte infiltration. Arrow heads indicate goblet cell metaplasia. (C) Inflammation scores of target organs of 4 mice are presented. A moderate and significant inhibition of MOI was observed for the ear and skin in the 3-week old *Sf.Ifng*^{-/-} samples as compared to that of Sf mice (*). At this stage, the inflammation scores in the lung and liver were also inhibited but did not reach significance statistically. When compared with B6 samples, the inflammation scores in the 3 weeks old *Sf.Ifng*^{-/-} mice were still significantly induced (**, $p < 0.05$). The inflammation scores of the 6 weeks old *Sf.Ifng*^{-/-} mice were as high as that in the 3 weeks old Sf mice. (D) Total lymphocytes, CD3⁺ T-cells and CD4⁺ T-cells in the pooled LN of mice (N=4) of 3 weeks old Sf and 3 weeks old *Sf.ifng*^{-/-} samples are shown. N.S. indicates not significant.

Figure 4. Serum levels of cytokines and IgE

Serum levels of cytokines (A) and IgE (B) were determined as described in “Materials and Methods”. Each dot represents an individual sample of the indicated mice. (*) indicates a significant difference (N=4 to 5, p<0.05) as compared to Sf samples. Because many of the cytokines are also produced by non-CD4⁺ T-cells, these changes may not correlate well with the cytokine profiles observed for CD4⁺ Th subsets.

Figure 5. Expression of CD4⁺ T-cell CRG in B6, Sf, Sf.II2^{-/-}, Sf.II4^{-/-}, Sf.Stat6^{-/-} and Sf.Ifng^{-/-} mice

The LN CD4⁺ T-cells from individual mice (N=3) of indicated groups were analyzed for CRG by quantitative PCR as described in “Materials and Methods”. The results are expressed as fold-change using B6 control values as one. (A) The up-regulation of *Cysltr1*, *Ltb4r1*, and *Il1r1* in Sf mice was inhibited in Sf.II2^{-/-} samples but the statistical difference between *Il1r1* expression levels in Sf and Sf.II4^{-/-} samples was slightly greater than 0.05, perhaps due to limited sample size. (B) The effect of *Ifng*^{-/-} and *Stat6*^{-/-} mutations on the expression levels of 10 different CRG in Sf mice: In the 5-6 weeks old Sf.Ifng^{-/-}, the expression of the nine up-regulated CRG in Sf mice remained high with the exception of *Cxcr3*, the expression of which is specifically controlled by IFN- γ . In Sf.Stat6^{-/-} mice, the expression of *Cysltr1*, *Ltb4r1* and *Ptgir* were significantly reduced as compared with Sf samples. The expression of *Cxcr3* and *Cxcr6* was significantly higher than that in the Sf samples, consistent with the high IFN- γ levels in these mice. (*) indicates p<0.05.

Table 1

MOI, Th subset, and lifespan of various Sf double mutant mice.

Mice ^{d)}	Number of mice	Inflammation score (mean $\pm$ S.D.) ^{b)}				CD3 ⁺ T-cells ($\times 10^6$)	CD4 ⁺ T-cells ($\times 10^6$)	Th1 ^{c)} %	Th2 ^{c)} %	Lifespan (weeks)
		Ear	Skin	Lung	Liver					
B6	>4	0 $\pm$ 0 *	0 $\pm$ 0 *	0 $\pm$ 0 *	0 $\pm$ 0 *	10 $\pm$ 2 *	5 $\pm$ 1 *	1 $\pm$ 1 *	1 $\pm$ 1 *	>24
Sf	>4	4 $\pm$ 0.1	3.5 $\pm$ 0.5	3.5 $\pm$ 0.6	2.5 $\pm$ 0.6	32 $\pm$ 7	16 $\pm$ 5	20 $\pm$ 7	12 $\pm$ 3	3 - 4
Sf.Lth4 ^{-/-}	4	2 $\pm$ 1.4	2 $\pm$ 0.6	2.5 $\pm$ 0.6	3 $\pm$ 0.6	32 $\pm$ 5	14 $\pm$ 3	12 $\pm$ 5	13 $\pm$ 5	4 - 5
Sf.Lox ^{5/-}	4	4 $\pm$ 0.1	4 $\pm$ 0.1	4 $\pm$ 0.1	4 $\pm$ 0.1 *	38 $\pm$ 7	17 $\pm$ 4	19 $\pm$ 2	24 $\pm$ 2 *	< 3
Sf.Cx3cr1 ^{gfp/gfp}	3	4 $\pm$ 0.6	3.5 $\pm$ 0.6	3.5 $\pm$ 0.6	3 $\pm$ 0.6	N.D ^{e)}	N.D.	N.D.	N.D.	3 - 4
Sf.Ilgae ^{-/-}	4	1.5 $\pm$ 0.6 *	1 $\pm$ 0.6 *	2 $\pm$ 0.6 *	3 $\pm$ 0.5	29 $\pm$ 8	14 $\pm$ 5	16 $\pm$ 5	12 $\pm$ 2	6 - 7
Sf.IH10 ^{-/-}	3	4 $\pm$ 0.6	3.5 $\pm$ 0.6	3.5 $\pm$ 0.6	3 $\pm$ 0.6	N.D.	N.D.	N.D.	N.D.	3 - 4

a) At least 3 male mice were examined and analyzed at 3 weeks of age for each group. Sf.Lox^{5/-} mice were examined at 18 days after birth.

b) Inflammation scores were determined by histology as described in "Materials and Methods".

c) IFN- γ ⁺ Th1 and IL-4⁺Th2 cells were determined by intracellular staining as described in "Materials and Methods" and expressed as % of the CD4⁺T-cells.

d) Total number ($\times 10^6$) of CD3⁺CD4⁺T-cells and IL-4⁺CD4⁺T-cells in the pooled peripheral LN cells.

e) Not determined.

* p value < 0.05 compared with Sf mice.