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Selectin dependence of allergic skin inflammation is diminished by maternal atopy

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Abstract

Allergic skin inflammation requires the influx of inflammatory cells into the skin. Extravasation of leukocytes into the skin requires interactions between endothelial selectins and their glycan ligands on the surface of leukocytes. Selectin ligand formation requires the activity of several glycosyltransferases, including *Fut7*. In this report we tested the importance of *Fut7* for the development of allergic skin inflammation in the Stat6VT transgenic mouse model. We observed that *Fut7*-deficiency was protective but did not eliminate disease. Segregation of the data by gender of the parent that transmitted the Stat6VT transgene, but not by gender of the pups which were analyzed for disease, revealed that the protective effects of *Fut7*-deficiency were significantly greater when dams were Stat6VT-negative. In contrast, in mice from litters of Stat6VT+ dams, *Fut7*-deficiency resulted in only modest protection. These findings indicate that pups from atopic dams exhibit a greater propensity for allergic disease, similar to observations in humans, and that the effect of maternal atopy is due to enhanced selectin-independent mechanisms of leukocyte recruitment in their offspring. Together, these results demonstrate that *Fut7*-deficiency can be protective in a model of AD, but that maternal atopy diminishes these protective effects, suggesting alternative pathways for leukocyte recruitment in the absence of *Fut7* enzyme activity. These observations have implications for understanding how the environment in utero predisposes for the development of allergic disease.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease presenting with a variety of clinical manifestations, including blepharitis and eczema (1, 2). Asthma, allergic rhinitis, and food allergy are common comorbidities associated with AD. Children from parents with a history of allergic disease exhibit an increased risk of allergies, asthma, and AD

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(3), demonstrating the significance of the early life environment and genetic risk factors in developing AD. The predisposition to allergic disease is even more pronounced in offspring of allergic mothers (4, 5).

This role for the early life environment has been recapitulated in mouse models of allergic disease. Dams sensitized to egg albumin prior to mating produce mice that are prone to develop allergic inflammation. Inhibition of allergic responses in the mother by anti-IL-4 prior to conception reduces the risk of allergic responses in offspring (6), demonstrating the importance of the Th2 response to maternal transmission of allergic potential. However, the effects of the maternal environment have not been extensively examined specifically in the setting of AD models.

The Stat6VT transgenic mouse was developed to define the effects of a hyperactive Th2 response on allergic inflammation (7). These mice show a strong predisposition to developing allergic inflammation in lungs, skin, and eyes (8–10), suggesting that this constitutively active Stat6 mutant is sufficient to induce a pathologic Th2 environment, including production of the cytokine IL-4, which is implicated in AD pathogenesis. Stat6VT mice respond to aggravating stimuli in an exaggerated manner and exhibit scratching behaviors similar to patients (10–12). Together, these findings suggest that the Stat6VT mouse model is a useful tool for exploration of AD-like allergic skin inflammation.

Analysis of AD skin lesions show increased infiltration by $CD4^+$ T cells, dendritic cells, mast cells, and eosinophils (13). Selectins play an important role in leukocyte traffic into tissues, especially skin, during the inflammatory process, and therefore are a potential target to impair leukocyte infiltration. E- and P- selectin are particularly important for rolling during extravasation as a prelude to leukocyte adhesion and transmigration (14, 15). Selectins recognize glycan ligands on the leukocyte surface that require the α1,3 fucosyltransferase, FucT-VII for synthesis(16). Induction of FucT-VII, encoded by *Fut7,* in activated T cells is repressed by IL-4 (17), but in the context of allergic disease, can be induced by IL-9 or IL-25 through a common p38 MAPK pathway (18). T cells are highly dependent on selectin-dependent migration, and in *Fut7*-deficient mice, T cell migration into sites of inflammation is severely impaired (16, 19). However, the effects of disrupting selectin-ligand formation, and the role of maternal atopy in determining the relevance of specific pathways of inflammatory cell infiltration, have not been examined in a model of spontaneous AD.

This study focused on defining the role of FucT-VII-dependent fucoslyation in the Stat6VT model of AD. While we observed diminished development of AD-like disease in mice lacking *Fut7*, the magnitude of the protection conferred by *Fut7*-deficiency differed as a function of whether or not the mother of the mice analyzed expressed the Stat6VT Tg. These studies define a role for *Fut7* and selectin ligands in AD-like disease, document a contribution of the fetal microenvironment to the development of AD, and show for the first time that the fetal microenvironment can influence the relative importance of distinct pathways of leukocyte recruitment.

Materials & Methods

Generation of Stat6VT and Stat6VT x *Fut7−/−* mice

The generation of Stat6VT transgenic mice on a C57BL/6 background was previously described (7). To obtain Stat6VT mice lacking α1,3 fucosyltransferase, Stat6VT mice were mated to *Fut7*−/− mice. Mice were weaned at 28 days of age. All experiments used littermates as controls. All mice were maintained in pathogen-free conditions.

Quantification of incidence and mortality in Stat6VT and Stat6VT x *Fut7−/−* mice

Mice with ages ranging from 9 to 20 weeks were monitored weekly for the onset and development of characteristic features of atopic dermatitis consistent with the Stat6VT model. Severity of disease was scored using the mouse EASI scale. Briefly, scores were generated by evaluating individual regions of the body including the head and neck, extremities, back and abdomen, and tail. The severity index was calculated by evaluating the erythema, infiltration/papulation, excoriations, and lichenification within a single body region on a scale of 0–3. The percent area affected by one or more of the key signs within a body region was estimated. The severity index was multiplied by the percent area and multiplied by 10 to make whole numbers. Scores from different body regions were summed for a final disease score for the animal. Mice were photographed from consistent positions using a camera affixed to a stable stand at fixed height. Disease scoring was blinded from genotype to minimize bias.

Histology

Ear tissues were preserved in fixative 10% buffered formalin at room temperature. After 1 week formalin was replaced with 70% ethanol and stored at room temperature until processing. All samples were submitted and processed with hematoxylin and eosin by the IU Histology core facility.

Tissue processing

Cells from inflamed ear tissues were identified using flow cytometry. Briefly, inflamed ear tissues were cut in half along the cartilage to expose the dermis and incubated for 1.5 hours at 37°C with the dermis side in contact with serum free Dulbecco's Modified Eagle Medium (Gibco) containing 100 μg/mL Liberase (Roche) and 10 μg/mL DNase (ThermoFisher). Ears were then cut into small pieces using scissors before being transferred into a C tube where they were processed on the GentleMACS (Miltenyi). Samples were then filtered through a 100 μm filter screen and cells were purified using a 40% Percoll (Sigma-Aldrich) gradient. Cells were counted and subsequently identified using flow cytometry analyzing for both granulocyte and CD4 T cell cytokine production.

Flow cytometry analysis

The frequency of cytokine-producing T cells was determined by intracellular cytokine stimulation. Briefly, 0.2–1.0×10^6 cells were stimulated in media containing PMA (50ng/mL) and ionomycin (1g/mL). After 4 hours monensin (2μM) was added to stimulated cells and 2 hours later cells were washed with FACS buffer (PBS with 0.5% BSA). All

cell populations were stained for surface markers and a fixable viability dye (eBioscience) and fixed with 4% formaldehyde at room temperature for 10 minutes. After fixation, cells were permeabilized with permeabilization buffer (eBioscience) and stained for intracellular cytokines in the same buffer.

Granulocyte gating strategy: granulocytes > single cells > viable cells > CD45+*. CD45 was only used where labeled. From either viable or CD45+ cell populations neutrophils were defined as Ly6G+CD11b+ and eosinophils were defined as Ly6G-CD11c-SiglecF+. Lymphocyte gating strategy: lymphocytes > single cells > viable cells. From the viable population CD4+ T lymphocytes were defined as CD3+CD4+. Cytokine production was measure from the CD4+CD4+ population after intracellular cytokine stimulation. Antibodies used included those from BioLegend (anti-CD3-PerCP-Cy5.5, clone 17A2; anti-CD4-PE, clone GK1.5; anti-CD11b-PerCP-Cy5.5, clone ICR44; anti-CD11c-PE-Cy7; clone N418; anti-CD45-FITC, clone 104; anti-IL-4-PE-Cy7, clone 11B11; anti-IL-17A-APC, clone eBio17B7; anti-IFNg-FITC, clone XMG1.2; anti-Ly6G-APC, clone 1A8), from BD (anti-SiglecF-PE, clone E50–2440) and Invitrogen (eFluor 780 Viability Dye).

Statistics and data analysis

All statistics were done using Prism software version 9 (GraphPad). Welch's t test was used for the comparisons between groups. An area under the curve analysis was used to compare disease score time courses. All data is representative of 2–3 experiments. Flow cytometry data was collected using an Attune flow cytometer (Life Technologies) and was analyzed using FlowJo version 10 (Tree Star).

Results

FucT-VII-deficiency reduces Stat6VT-induced dermatitis

To elucidate the role of FucT-VII in AD, we utilized the Stat6VT transgene (Tg) model of AD with mice either wildtype (*Fut7*+/+) or deficient for FucT-VII (*Fut7*−/−). Mice carrying the Stat6VT Tg that were heterozygous for FucT-VII-deficiency (*Fut7*+/−) were crossed to *Fut7*+/− mice to obtain littermate controls (Fig. 1A). Mean disease scores were analyzed in control or *Fut7*-deficient Stat6VT transgenic mice every week beginning at 9 weeks of age (Fig. 1B). Severity of AD was scored using a modified Eczema Area and Severity Index (EASI) scoring method (20). This quantitative scoring system incorporates both degree of severity and percentage of total body area affected and was modified for this study to account for anatomical differences between mice and humans.

We observed significantly reduced disease progression in Stat6VT *Fut7*−/− mice (Fig 1C). At 17 weeks of age, mice were euthanized, and the severity of inflammation was assessed as moderate, mild, or lacking disease. Mice lacking the Stat6VT Tg were uniformly free of disease (Fig 1D). Approximately half of the Stat6VT+ *Fut7*+/+ mice showed moderate disease, with the remainder showing mild disease. In sharp contrast, ~50% of Stat6VT+ *Fut7*−/− *mice* showed no disease, with the remainder showing only mild disease (Fig 1B). These data demonstrate a strong protective effect of Fut7 deletion on development of AD-like disease. Interestingly, Stat6VT+ Fut7+/− mice exhibited an intermediate disease

phenotype, with a significant population free of disease and a ~50% reduction in the fraction of mice with moderate disease, suggesting an influence of *Fut7* gene dosage (Fig 1D). Together, these decreases in disease scores and disease incidence in *Fut7*−/− Stat6VT mice show that deficiency in *Fut7* gene expression leads to significantly decreased Stat6VT-induced allergic skin inflammation.

Maternally expressed Stat6VT decreases the protective effect of *Fut7*-deficiency

Previous studies stressing the importance of the in utero environment on subsequent development of allergic disease (21, 22) led us to assess disease after grouping mice based on whether or not dams expressed the Stat6VT Tg. This segregation revealed that *Fut7*−/− mice born to Stat6VT-negative dams exhibited the lowest disease scores (Fig. 2A). Importantly, there was no difference in disease scores by gender of offspring. This difference in disease incidence and severity as a function of whether or not the dam expressed the Stat6VT Tg was clearly evident at a representative time point of 15 weeks of age (Fig. 2B). To account for variation of scores over time, incidence of moderate disease or greater severity (score of 3 or higher) was examined throughout the lifetime of the mice. Stat6VT+ *Fut7*−/− mice that were born to Stat6VT-negative dams had the lowest lifetime incidence of moderate disease (Fig. 2C). Differences in inflammation could also be appreciated visually by blepharitis and skin inflammation on the ear and in histological examination of ear tissues stained with hematoxylin and eosin (Fig. 2D–E). In the histological examination the protective effect of Fut7-deficiency can be seen in diminished epidermal thickening and overall inflammation and swelling in the tissue, but only in mice from a Stat6VT-negative dam (Fig. 2D). Thus, the protective effect seen by impairing leukocyte traffic in Stat6VT+ *Fut7*−/− mice was diminished in mice born to atopic mothers.

Maternal Stat6VT Tg expression promotes increased infiltration and altered CD4 T cell cytokine production in *Fut7*-deficiency

To further investigate leukocyte infiltration in lesional skin, we isolated cells from inflamed ear tissue and analyzed them using flow cytometry. Both Stat6VT *Fut7*−/− and *Fut7*+/+ progeny from Stat6VT+ *Fut7*+/− dams demonstrated higher eosinophil (defined as $Ly6G^{-}SiglecF^{+}CD11c^{-}$) infiltration in inflamed ear tissue, compared to *Fut7*−/− progeny of Stat6VT-negative *Fut7*+/− transgenic dams (Fig. 3A–B). While in some mice neutrophil frequency was increased in progeny from Stat6VT+ *Fut7*+/− dams there was variation with some mice having little or no neutrophil infiltrate. There were similar observations when examining the frequency of eosinophils among the CD45+ cellular compartment (Fig. 3C). These data further document the effects of maternal transgene expression on allergic skin inflammation in the progeny.

Because Stat6VT-mediated AD is defined by a Th2 pathology (9), we next analyzed if there were alterations in CD4+ lymphocytes as well as cytokines produced by CD4+ T cells. Stat6VT+ *Fut7*−/− progeny from Stat6VT+ *Fut7*+/− dams had around a 5-fold increase in CD4+ T cell (defined as $CD3^{+}CD4^{+}$) infiltration in inflamed ear tissue, compared to Stat6VT+ *Fut7*−/− progeny from Stat6VT-negative *Fut7*+/− dams (Fig. 4A–B). There was not a significant increase in CD3+CD4- population that are likely CD8 T cells (Fig. 4B). Among CD4+ T cells, we observed no significant difference in expression of the key Th2

cytokine IL-4 in mice from atopic vs non-atopic dams (Fig. 4C). However, due to the higher degree of inflammation and consequent increase in recruited T cells in mice from atopic mothers, there was a significant increase in the fraction of all cells which expressed IL-4 in mice from atopic moms compared with that of mice from non-atopic dams (Fig. 4D). In contrast, there was a striking increase in the proportion of IFNγ-producing T cells among CD4+ T cells of mice from atopic dams compared to non-atopic dams, and this difference was even greater among total cells (Fig, 4C–D). These findings show that mice which develop in atopic mothers have much greater T cell infiltration with greater production of IFNγ than mice which develop in a non-atopic environment.

Discussion

Selectin ligand expression is critical for the ability of leukocytes to infiltrate tissues, particularly the skin (15, 23–26). *Fut7* is critical for selectin ligand formation, as mice deficient in FucT-VII have negligible L-, E-, or P- selectin ligand activity (16, 27). In this report we have defined an important role for FucT-VII in allergic skin inflammation associated with Stat6VT transgene expression. We observed that allergic skin inflammation was significantly diminished in Stat6VT+ *Fut7*−/− mice. Unexpectedly, we observed that this reduction in allergic skin inflammation as a result of *Fut7*-deficiency was closely linked to whether the Stat6VT transgene was expressed by the pregnant dam or not. Our results document a switch in the importance of distinct leukocyte adhesion pathways induced by an atopic maternal environment.

Maternal history of atopic disease is a significant risk factor for the development of atopy in children (5, 28, 29). In mouse models it has also been documented that sensitized dams will produce pups with a greater propensity for the development of allergic inflammation (6). Consistent with this, when data were segregated according to whether or not the pregnant dams were atopic due to expression of the Stat6VT transgene, we observed increased disease severity in offspring of both sexes from litters from atopic mothers. Because selectins are essential for leukocyte, especially T cell traffic to skin, we then examined the effect of deletion of *Fut7* on development of AD in Stat6VT+ mice. We found that deletion of *Fut7* largely protected pups of non-atopic mothers from development of AD, whereas this protective effect of *Fut7* deletion was much smaller in pups from atopic mothers. While we saw increased infiltration of CD4+ lymphocytes and eosinophils in progeny from atopic dams, additional immune populations are most likely playing a role in this process. Indeed, we have observed changes in a number of populations in lesional skin of Stat6VT+ mice (30). While we did not see a difference in CD3+CD4- infiltrate in the progeny from Stat6VT+ *Fut7*+/− dams more detailed characterization of potential changes in CD8+ or γδ T lymphocytes as well as monocyte/macrophage recruitment are important future directions of investigation. Given the well-documented critical role for Fut7 in selectin ligand biosynthesis, this finding clearly implies that an atopic environment leads to the induction of an alternative pathway of leukocyte recruitment.

We have previously established that there are systemic effects of Stat6VT transgene expression in T cells (8–10). Indeed, we previously observed that dendritic cells in neonatal mice from Stat6VT Tg dams exhibited altered cytokine production that was similar to

patterns observed in infants from atopic mothers (5, 31). This suggests that although FucT-VII-deficiency is protective for allergic skin inflammation in the absence of additional pro-atopic signals, the effects of an atopic environment on the infant's immune system can overcome that protection. How cells migrate into tissues in the absence of selectin ligands is still unclear but likely involves compensatory effects of additional adhesion molecules, particularly VLA4/VCAM-1(32–38). Consistent with this, VCAM-1 expression on endothelium is strongly enhanced by both IL-4 and IFNγ (39–44), both of which we show here are produced at high levels by T cells in offspring of atopic mothers but not non-atopic mothers. This difference in cytokine profiles could be linked to the development of type I cells in chronic AD lesions(1, 12). Other pathways have been shown to influence leukocyte rolling velocity such as moesin. Lacking this moiety was associated with deficient slow rolling necessary for effective leukocyte recruitment into tissues (38). Other proposed pathways include the concept of "the path of least resistance" in which strong barrier function and deficient crawling, as is seen in *Fut7*−/− mice, are associated with increased transcellular migration (37). This would be associated with strengthened adhesion likely via ICAM and invasive podosomes (45, 46). Another mechanism alternative to the selectin pathway is through endothelial expressed scavenger receptors that may pose a similar purpose and are a field of burgeoning study (47). All these may be the focus of future studies trying to discern this alternative route for leukocyte recruitment into tissues.

Our results highlight the critical importance of selectin ligands in recruitment of Th2 cells and other inflammatory leukocytes which underlie the development of allergic skin inflammation. They further suggest that an atopic in utero environment can alter or induce other potential pathways of leukocyte traffic which can play a critical role in induction of AD.

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Abbreviations:

AD	atopic dermatitis
Stat6VT	human Stat6 mutant where the V_{547} and T_{548} are replaced by alanines

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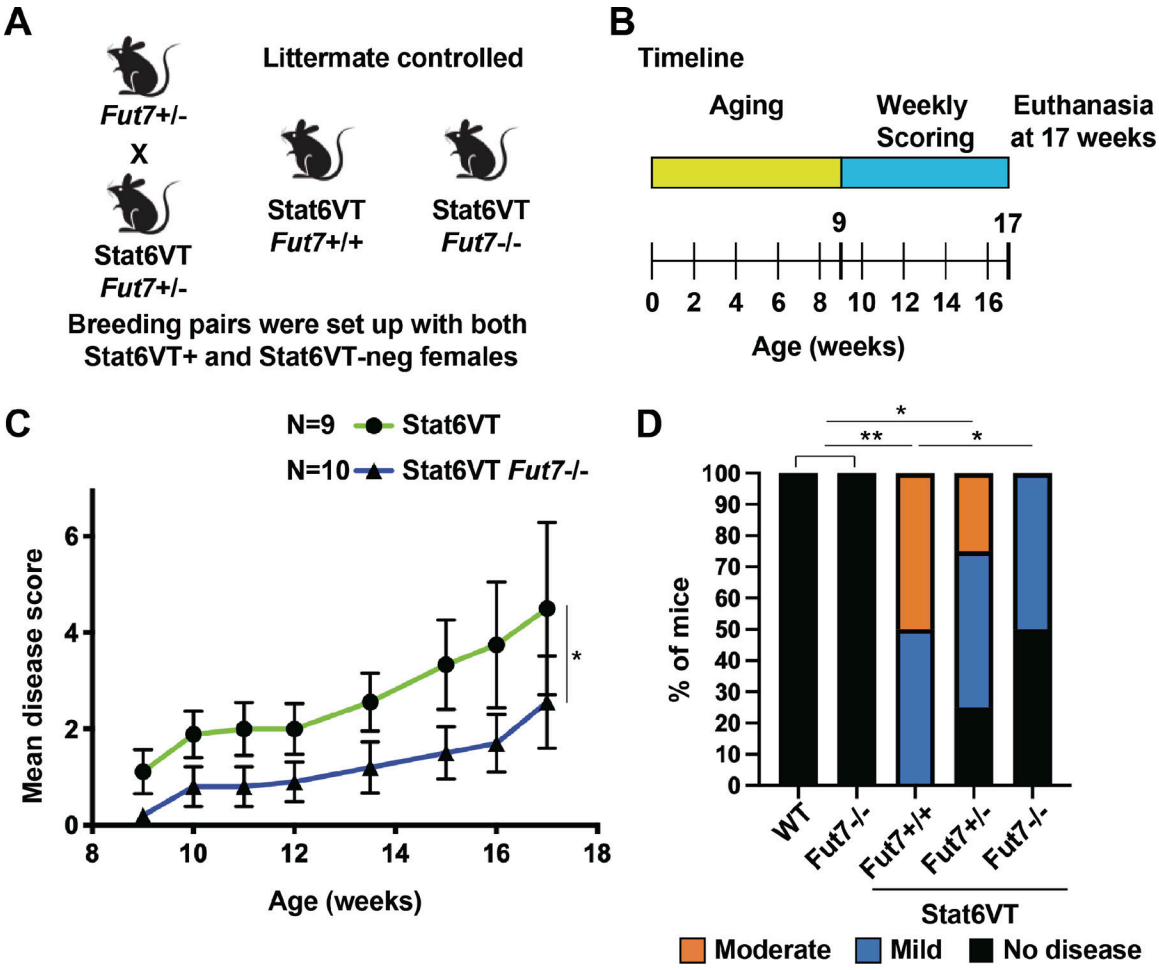

Figure 1:

Mice lacking FucT-VII show decreased Stat6VT-induced dermatitis. A, *Fut7+/−* mice were crossed to generate litter matched experimental groups. The Stat6VT Tg was present in either the sire or the dam. B, Mice were monitored weekly from 9 to 17 weeks of age for development of AD and disease was blindly scored according to a modified EASI scale. C, Average modified EASI score +/− SEM for Stat6VT *Fut7+/+* vs Stat6VT *Fut7−/−* groups over the time course. An area under the curve analysis was used for comparison. D, Final disease scores were recorded and categorized into no disease (scores 0), mild disease (score of 1–2), and moderate disease (score of 3–6). Data are representative of two independent experiments with 8–10 mice per group. A chi-squared test was used to generate p values. *$p < 0.05$, **$p < 0.01$.

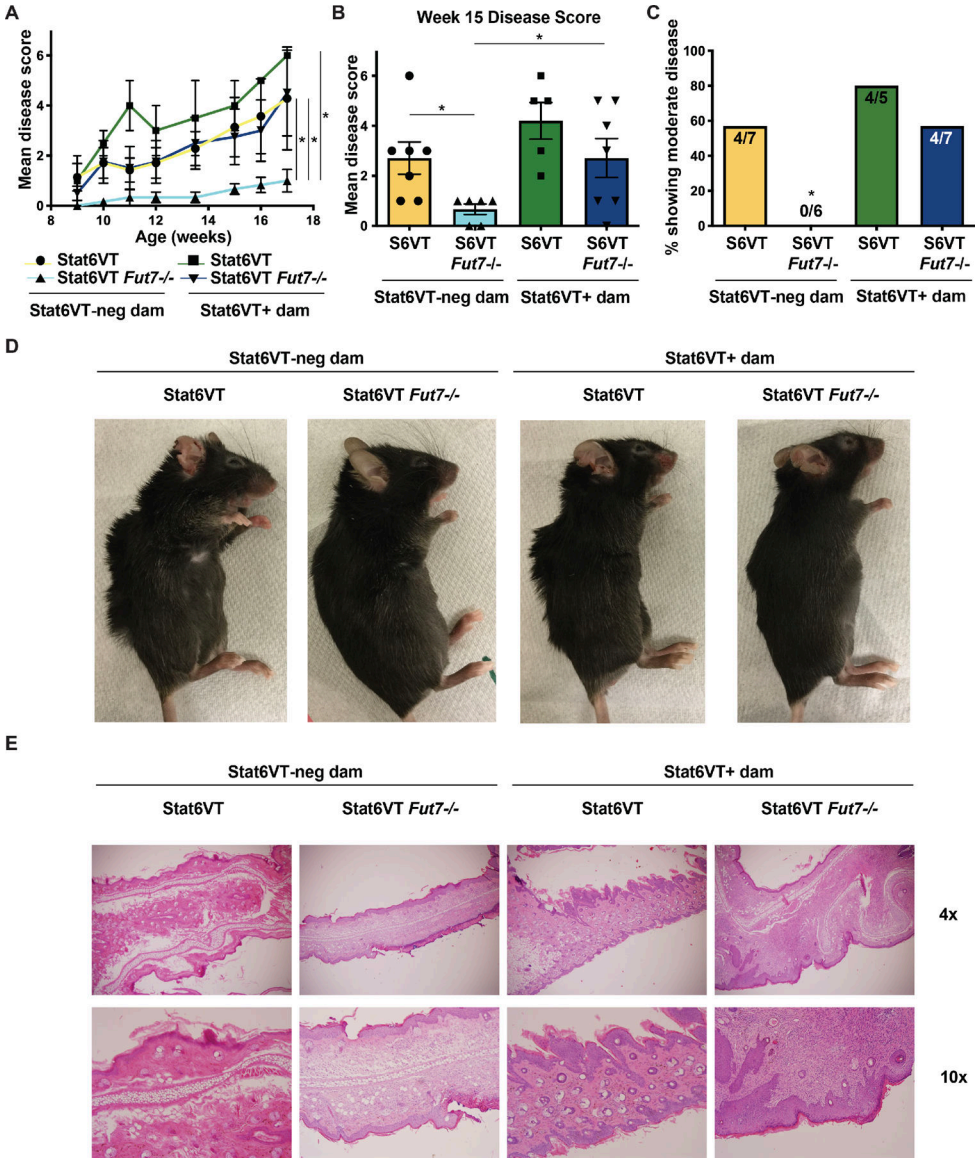

Figure 2:

The protective effect associated with *Fut7*-deficiency is mitigated when Stat6VT Tg is inherited from a transgenic mother. A, Average modified EASI score +/− SEM for mice were segregated according to parental Stat6VT expression throughout time course. An area under the curve analysis was used to compare among groups. B, Mice at the representative age of 15 weeks were scored for disease. Student's unpaired two-tailed *t* test was used to generate *p* values. C, the percent of mice with scores of 3+ were recorded as having moderate disease. Fisher's Exact test was used to generate P values. D, At 17 weeks, mice were euthanized for analysis. Representative photos of disease in Stat6VT-induced dermatitis are shown. E, Representative ear tissue stained with hematoxylin and eosin at 5x and 10x magnifications. Data are representative of two independent experiments with 5–7 mice per group. $*p < 0.05$.

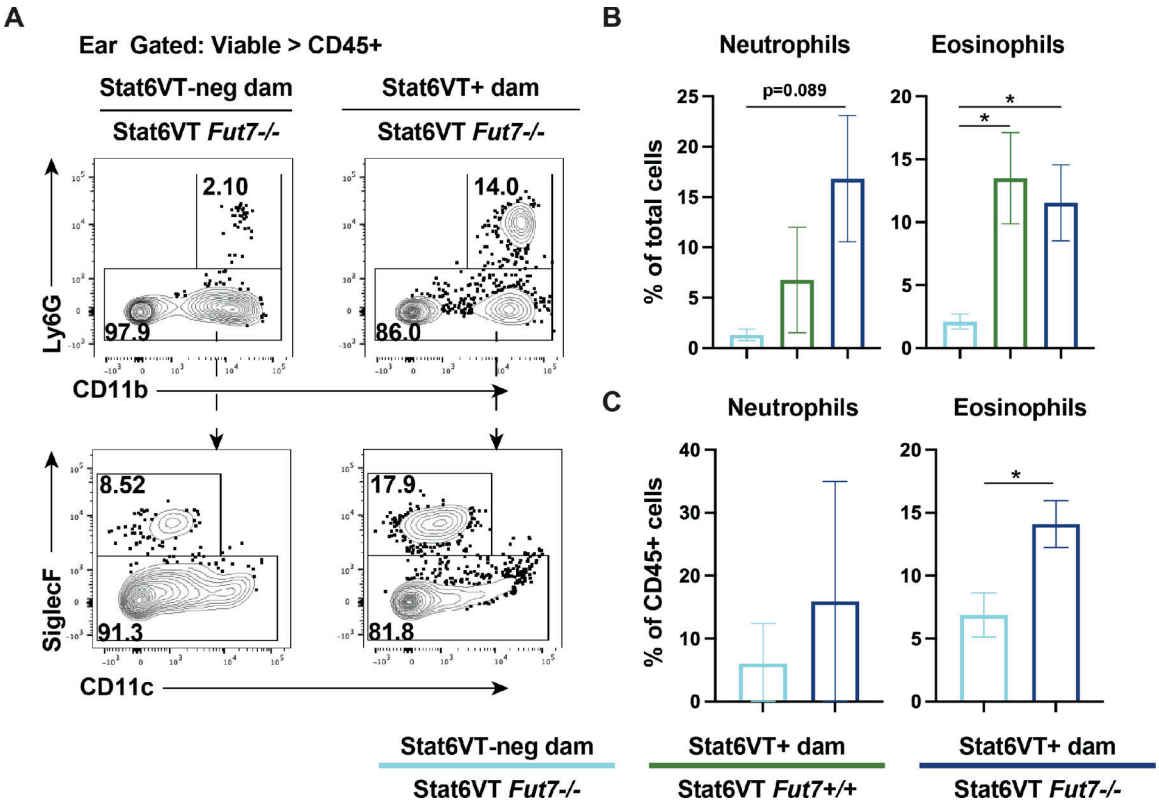

Figure 3:

Progeny from Stat6VT+ dams in the context of *Fut7*-deficiency is associated with increased eosinophil cell infiltrate. A, Representative flow cytometry dot plots from cells isolated from inflamed ears. B, Bar graphs showing frequency of neutrophil, eosinophil populations from total cells. C, Bar graphs showing frequency of neutrophil, eosinophil populations from CD45+ cells. Data are representative of two independent experiments with 3–4 mice per group. Welch's t test was used with a $*p < 0.05$.

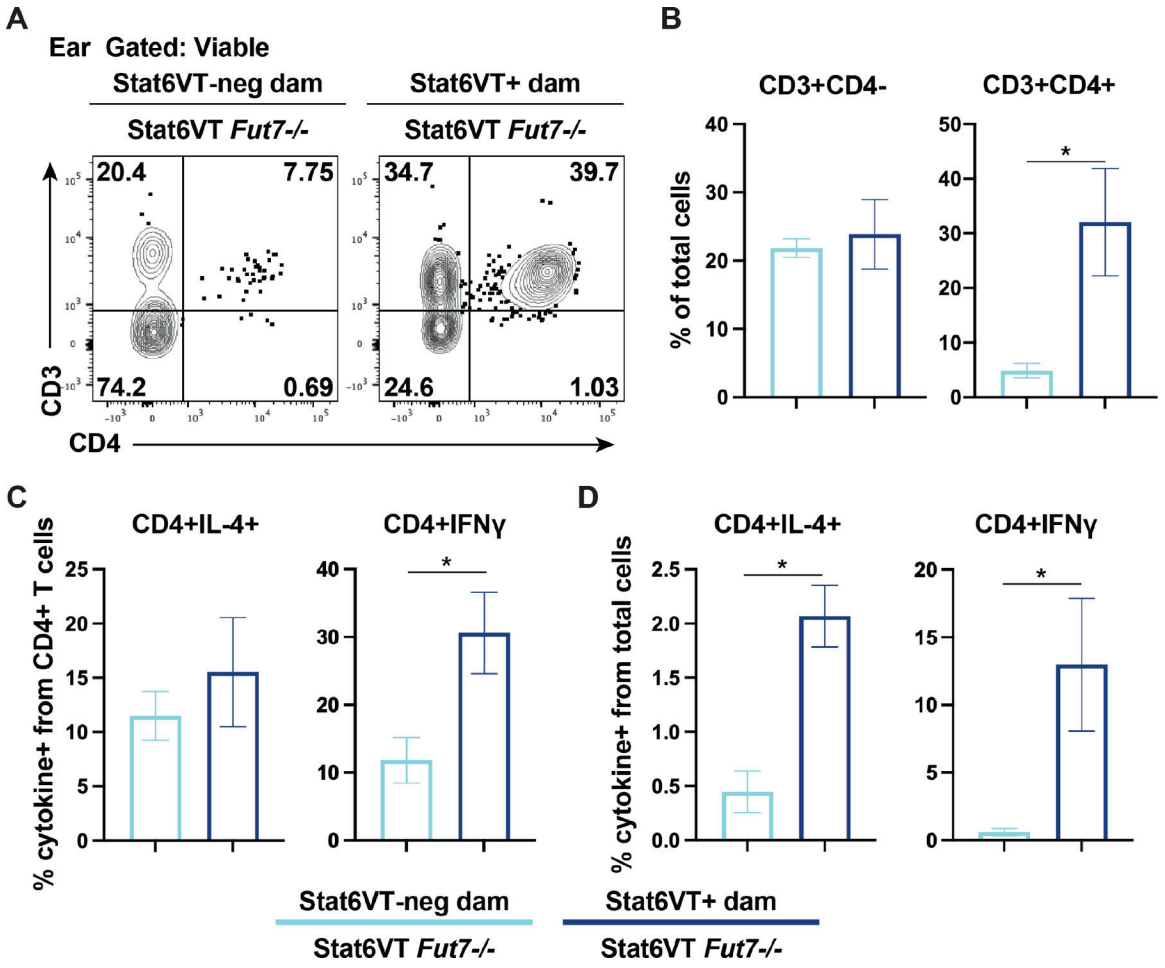

Figure 4:

Maternal expression of the Stat6VT Tg in the context of Fut7 deficiency is associated with increased CD4+ T cell infiltrate and an inflammatory cytokine profile. A, Representative flow cytometry dot plots from cells isolated from inflamed ears. B, Bar graphs showing frequency CD3+CD4- and CD3+CD4+ T cell population. C, Frequency of cytokine positive cells among CD4+ T cells in the ear. D, Frequency of cytokine positive cells from total cells measured via flow cytometry from the ear. Data are representative of two independent experiments with 3–4 mice per group. Welch's t test was used with a $*p < 0.05$.