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TGFβ Receptor Mutations Impose a Strong Predisposition for Human Allergic Disease

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Abstract

Transforming growth factor–β (TGFβ) is a multifunctional cytokine that plays diverse roles in physiologic processes as well as human disease, including cancer, heart disease, and fibrotic disorders. In the immune system, TGFβ regulates regulatory T cell (T_{reg}) maturation and immune homeostasis. Although genetic manipulation of the TGFβ pathway modulates immune tolerance in mouse models, the contribution of this pathway to human allergic phenotypes is not well understood. We demonstrate that patients with Loeys-Dietz syndrome (LDS), an autosomal dominant disorder caused by mutations in the genes encoding receptor subunits for TGFβ, *TGFBR1* and *TGFBR2*, are strongly predisposed to develop allergic disease, including asthma, food allergy, eczema, allergic rhinitis, and eosinophilic gastrointestinal disease. LDS patients exhibited elevated immunoglobulin E levels, eosinophil counts, and T helper 2 (T_H2) cytokines in their plasma. They had an increased frequency of $CD4^+$ T cells that expressed both Foxp3 and interleukin-13, but retained the ability to suppress effector T cell proliferation. T_H2 cytokine–producing cells accumulated in cultures of naïve $CD4^+$ T cells from LDS subjects, but not controls, after stimulation with TGFβ, suggesting that LDS mutations support T_H2 skewing in naïve lymphocytes in a cell-autonomous manner. The monogenic nature of LDS demonstrates that altered TGFβ signaling can predispose to allergic phenotypes in humans and underscores a prominent role for TGFβ in directing immune responses to antigens present in the environment and foods. This paradigm may be relevant to nonsyndromic presentations of allergic disease and highlights the potential therapeutic benefit of strategies that inhibit TGFβ signaling.

INTRODUCTION

Allergic diseases, including asthma, eczema, allergic rhinitis, and food allergy, are causes of tremendous morbidity and appear to be rising in prevalence in most of the developed world.

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Food and aeroallergens have also been increasingly recognized as prominent factors in the pathogenesis of eosinophilic gastrointestinal diseases (EGIDs), especially eosinophilic esophagitis (EoE) (1, 2). Although all these disorders demonstrate strong familial associations, their complex inheritance has frustrated efforts to elucidate their genetic basis. The inflammation associated with allergic conditions is promoted by $CD4^+$ T helper 2 (T_H2) cells that secrete interleukin-4 (IL-4), IL-5, and IL-13; however, the molecular pathways regulating these responses remain to be fully elucidated. Both epidemiologic and genetic studies have purported an important role for transforming growth factor–β (TGFβ) in allergic disorders, although how this multipotential cytokine with both potent pro- and anti-inflammatory activities contributes to allergic disease is not clear (3-6). Although T cell–specific deletion of TGFβR2 in mice was found to result in lethal inflammation as a result of uncontrolled T cell activation and differentiation, TGFβ can also promote the differentiation of proinflammatory T_H17 and T_H9 cells (7-12). Most information regarding the role of TGFβ in the immune system has been derived from engineered mouse models where the function of TGFβ ligands, receptor subunits, or signaling effectors has been disrupted. Few human correlates of these models exist, leaving in question the physiologic relevance of the observed phenotypes and inferred mechanisms in people with allergic disease. Here, we evaluate the immunophenotype of patients with Loeys-Dietz syndrome (LDS), who harbor naturally occurring mutations in the receptor for TGFβ and therefore provide an opportunity to examine the immunologic consequences of altered TGFβ signaling in human disease (13).

LDS has previously been associated with altered cardiovascular, craniofacial, and skeletal development, consistent with the known importance of TGFβ in these systems. Here, we demonstrate that affected individuals have a high prevalence of multiple immunologic phenotypes, including asthma, food allergy, eczema, allergic rhinitis, and EGID. The monogenic nature of LDS suggests that dysregulated TGFβ signaling is sufficient to predispose to allergic phenotypes in humans, and underscores the prominent role TGFβ plays in directing immune responses to mucosal antigens, including those present in the environment and foods.

RESULTS

Immunologic phenotype of LDS

Among 58 LDS patients, the median age was 13.3 years [interquartile range (IQR), 12.8], and 27 of 58 (47%) were male (Table 1). Of the 58 patients, 14 (24%) and 44 (76%) had a heterozygous mutation in *TGFBR1* and *TGFBR2*, respectively (Table 1). Specific mutations are listed in table S1. Thirty-one of the 58 (53%) participants reported an adverse reaction to food, and 23 of the 43 patients (53%) who had food allergen–specific testing were positive to ≥1 of the most common food allergens (median, 2; IQR, 0 to 5). Eighteen of 58 had a convincing history of an immediate reaction to a food (14 had a positive allergen-specific test to the same food; the other 4 never had testing to the suspect food), providing a conservative estimate of 31% for the prevalence of food allergy in LDS patients, compared to 6% of children and 2 to 4% of adults in the general population (Table 1 and table S2) (14, 15). The remaining 13 LDS patients who reported adverse food reactions did not meet our strict criteria for diagnosing food allergy. The most common food antigens were egg, milk, soy, peanut, and tree nuts (table S2). Eleven of 58 (19%) participants had been prescribed self-injectable epinephrine before their diagnosis with LDS.

Twenty-six of 58 (45%) respondents reported physician-diagnosed asthma compared to 8% of adults and 10 to 13% of children in the general population (Table 1) (16-18). Sixteen of 58 (28%) currently required asthma medication (12 of 58 daily inhaled corticosteroid alone or in combination with a long-acting β-agonist, 11 of 58 daily leukotriene antagonist, and 16 of 58 intermittent short-acting β-agonist). Twenty-eight of 58 (48%) had been diagnosed

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with allergic rhinitis, which affects between 10 and 40% of children and between 8 and 30% of adults in the United States (19-22). Eczema was diagnosed in 22 of 58 (38%) LDS subjects (Table 1) compared to 8 to 17% of children and 8 to 11% of adults in the general population (23-25). Thirty of 41 (64%) were sensitized to ≥1 of the seven aeroallergens tested (median, 2; IQR, 0.75 to 5). Thirty-eight of 58 (66%) reported gastrointestinal complaints (29 of 58 with poor growth, 6 of 58 with repetitive vomiting, 15 of 58 with chronic abdominal pain, 6 of 58 with dysphagia) that were potentially consistent with EGID. Ten of these patients had gastrointestinal biopsies performed, and of these, 6 (60%) showed overt histologic evidence of EoE (Fig. 1A and Table 1). The prevalence of EoE in the general population is about 0.05% (26, 27). Of the six LDS patients with biopsy-confirmed EoE, five were found to have eosinophilic gastritis (EoG) and four had eosinophilic colitis (EoC) (Fig. 1, B and C, and Table 1). Five of six individuals with EGID demonstrated clinical improvement with food avoidance diets. Children with LDS had body mass index (BMI) *z* scores significantly below normal, and the BMI *z* scores of LDS children with food allergy were significantly lower than those of LDS children without food allergy (fig. S1).

LDS patients also had significantly elevated peripheral eosinophil counts and total immunoglobulin E (IgE) levels (Fig. 1, D and E). Levels of IgG, IgA, and IgM were within the normal range, although IgG levels clustered at the upper end of normal and IgM levels at the lower limit (fig. S2). Total white blood cell counts were normal ($7087 \pm 2589/mm^3$). We found statistically higher levels of the T_H2 cytokines IL-5 and IL-13 in plasma from LDS patients compared to unaffected controls, as well as CCL2 (MCP-1), a chemokine important in recruiting inflammatory cells and promoting degranulation of mast cells and basophils (Fig. 1F). Serum levels of CCL5 (RANTES), a chemokine known to be down-regulated by TGFβ, were lower (Fig. 1F) (28). Cytokine profiles from LDS subjects were specific for a T_H2-dominated disorder because no differences in expression levels of 21 other cytokines were detected (table S3).

Regulatory T cell development in LDS

The tolerogenic functions of TGFβ are thought to be executed, at least in part, through its ability to promote the development and function of regulatory T cells (T_{regs}). Human T_{regs} were recently reported to consist of phenotypically and functionally distinct subpopulations, on the basis of their expression of CD45RA and the level of expression of CD25/Foxp3 (29). The three subpopulations that comprise the total T_{reg} population ($CD4^+CD25^+CD127^{lo}$ cells) include resting T_{regs} (rT_{regs}) ($CD45RA^+CD25^{inter}Foxp3^{inter}$), activated T_{regs} (aT_{regs}) ($CD45RA^-CD25^{high}Foxp3^{high}$), and a $CD45RA^-CD25^{inter}Foxp3^{inter}$ group. The number of total T_{regs} in the peripheral blood of LDS patients was significantly elevated compared to unaffected controls ($8.2 \pm 1.6\%$ in LDS and $5.8 \pm 2.0\%$ in controls; Fig. 2A), whereas no difference in the frequency of total $CD4^+$ lymphocytes was evident ($41.5 \pm 9.0\%$ in LDS and $40.2 \pm 6.1\%$ in controls). Further analysis revealed increased T_{regs} expressing intermediate levels of Foxp3 ($Foxp3^{inter}$), but no difference in the frequency of aT_{regs} (Fig. 2A) (29). Surprisingly, a significantly increased percentage of LDS rT_{regs} and aT_{regs}, which have previously been shown to secrete little cytokine (29), produced the T_H2 cytokine IL-13 compared to nonallergic controls (Fig. 2B). No difference in expression of IL-17 or interferon-γ (IFN-γ) was evident (Fig. 2, C and D), but IL-10 levels were higher in LDS rT_{regs} compared to nonallergic controls (fig. S3). Children with nonsyndromic allergic disease also demonstrated an increased frequency of rT_{regs} and $CD45RA^-Foxp3^{inter}$ T_{regs}, as well as $Foxp3^+$ cells that produced IL-13 (Fig. 2, A and B). A greater frequency of $Foxp3^{inter}$ T_{regs} in allergic children also produced IL-17, but not IFN-γ (Fig. 2, C and D). Despite their propensity to produce T_H2 cytokines, T_{regs} from LDS patients expressed normal levels of GATA3 and Foxp3, both of which can regulate effector cytokine expression by $Foxp3^+$ cells (fig. S4) (30, 31).

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To ascertain whether T_{regs} from LDS patients retain the ability to suppress effector T cell proliferation, purified T_{regs} were cultured at various ratios with responder T cells in the presence of a T cell receptor cross-linking stimulus. All three populations of T_{regs} from LDS patients effectively suppressed effector T cell proliferation (Fig. 3). We found no difference in expression of Helios, which has been reported to mark a population of T_{regs} with increased regulatory potential, by LDS T_{regs} compared to controls (fig. S5) (32).

The three subclasses of T_{regs} were also evaluated for expression of several T_{reg} markers, including CTLA-4 (cytotoxic T lymphocyte antigen–4), GITR [glucocorticoid-induced tumor necrosis factor receptor (TNFR)–related protein], and ICOS (inducible T cell costimulator), which may contribute to the immunosuppressive capacities of these cells. Increased levels of intracellular CTLA-4 were seen in rT_{regs} and $CD45RA^{-}Foxp3^{inter}$ T_{regs} from LDS patients compared to nonallergic and nonsyndromic allergic controls (Fig. 4). No differences were evident between allergic patients without LDS and controls (Fig. 4). Expression of all these markers was highest in aT_{regs} and lowest in rT_{regs}, as previously demonstrated (Fig. 4) (29).

Skewing potential of naïve $CD4^{+}$ lymphocytes in LDS

T_{regs} have been reported to exhibit phenotypic and functional plasticity under inflammatory conditions; therefore, it remained unclear whether the changes in T_{reg} function we observed in LDS were an indirect consequence of the general allergic milieu in these patients, or whether mutations in the TGFβ receptor directly affect the differentiation of naïve T cells. To assess whether LDS mutations influence the propensity for naïve lymphocytes to acquire T helper effector functions in response to TGFβ, we purified and cultured $CD4^{+}CD45RA^{+}CD45RO^{-}CD25^{-}$ naïve T cells in the presence of various doses of recombinant TGFβ1 for 4 days. Naïve T cells from both LDS patients and controls demonstrated an equal propensity to up-regulate expression of Foxp3 in response to TGFβ in a dose-dependent manner (fig. S6). Although the frequency of both $IL\text{-}13^{+}$ $Foxp3^{+}$ and $IL\text{-}13^{+}$ $Foxp3^{-}$ T lymphocytes decreased in both control and LDS cultures as the dose of added TGFβ1 increased, there was a higher percentage of $IL\text{-}13^{+}$ cells in LDS samples exposed to TGFβ (Fig. 5). No difference in IL-17 or IFN-γ expression was evident (fig. S7). The addition of a TGFβ-neutralizing antibody or a TGFβ receptor kinase inhibitor to the cultures suppressed the differentiation of $Foxp3^{+}$ $IL\text{-}13^{+}$ cells from naïve T lymphocytes in both LDS patients and controls, suggesting that TGFβ contributes to their development (fig. S8). No difference in IL-13 expression by naïve lymphocytes from LDS patients and controls was observed before culture (fig. S9).

Dysregulated TGFβ signaling in LDS

The best-characterized TGFβ signaling pathway involves phosphorylation and heterodimerization of type I and II TGFβ receptors, resulting in phosphorylation and nuclear translocation of Smad2 and Smad3 proteins. LDS patients showed excessive nuclear accumulation of phosphorylated Smad2 (pSmad2) in thymic tissue, most prominent in the medulla, when compared to age-matched controls (Fig. 6, A and B). Additionally, $CD4^{+}$ lymphocytes in the peripheral blood of LDS patients demonstrated increased expression of pSmad2/3 after stimulation with TGFβ1 when compared to unaffected controls (Fig. 6C). However, no significant difference in expression of pSmad2/3 was found between LDS patients treated with the angiotensin II receptor blocker losartan, which has been shown to attenuate TGFβ signaling in other disorders (33-35), compared to controls (Fig. 6C). These results point to enhanced, rather than repressed, TGFβ signaling in the immune system of LDS patients.

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DISCUSSION

Here, we have demonstrated that mutations in either gene encoding TGFβ receptor subunits are sufficient to predispose to allergic phenotypes in humans. Although allergic diseases are known to have strong familial associations, our understanding of how genetic variants relate to specific dysfunction at the cellular level is often lacking (36-38). The alterations in TGFβ signaling that lead to T_H2 disease in LDS are complex. Most mutations in LDS patients are missense mutations that are predicted to disrupt the kinase activity of the receptors, and, in some cases, mutant receptors have been shown to lack the ability to propagate canonical TGFβ signaling (through pSmad2/3) when expressed in cells naïve for the corresponding receptor subunit (39-41). However, we found paradoxically enhanced TGFβ signaling in thymic tissue and $CD4^+$ lymphocytes from LDS patients, similar to what has been seen in the aorta (13). Decreased serum levels of CCL5 (RANTES) and increased expression of CTLA-4 by T_{regs} are consistent with excessive TGFβ signaling in LDS, because expression of these molecules is down- and up-regulated, respectively, by TGFβ (28, 42). Collectively, these data suggest that an increase in TGFβ signaling contributes to LDS immunologic pheno-types, but we cannot exclude a role for impaired TGFβ signaling in a critical developmental stage and/or cell type–specific manner. These issues will be best addressed using mouse models of LDS.

The increased frequency of peripheral $Foxp3^+$ T_{regs} in LDS patients is also an expected consequence of increased TGFβ signaling, but counterintuitive given the loss of tolerance. Although LDS T_{regs} expressed normal levels of typical T_{reg} markers including Helios and IL-10 and retained their ability to suppress effector T cell proliferation, they produced the T_H2 cytokine IL-13, a finding neither described nor observed in nonallergic controls but recapitulated by T_{regs} from children with nonsyndromic allergic disease (29). No alterations in T_H1 or T_H17 cytokine production were observed in LDS T_{regs}. Previous studies have suggested that T_{regs} may have functional and phenotypic plasticity in certain disease states, including multiple sclerosis where T_{regs} from affected individuals have been found to express T_H1 cytokines (43). Although additional studies are necessary to evaluate the role for T_H2 cytokine–producing $Foxp3^+$ cells in the pathogenesis of allergic disease, our data provide evidence for T_{reg} plasticity in these disorders.

The primary defect in TGFβ signaling caused by LDS mutations appears to confer lymphocytes with an intrinsic propensity to acquire and/or maintain T_H2 effector functions when stimulated with TGFβ. Whether this relates to loss of a suppressive effect of TGFβ on naïve T lymphocytes in LDS, or an off-setting positive influence in a subset of cells, remains to be determined. No difference in acquisition of T_H1 or T_H17 effector function was observed, suggesting that LDS mutations specifically influence T_H2 development. Although LDS mutations may promote T_H2 immunity through multiple mechanisms, potentially including effector cytokine production by cell types other than T cells, these data suggest that cell-autonomous changes in lymphocyte responses to TGFβ contribute to this phenotype.

Although other Mendelian disorders with a predisposition for allergic disease have been described, these disorders prominently feature overt evidence of immunodeficiency that is not seen in LDS (44, 45). The prevalence of allergic disease in LDS patients is significantly greater than that observed in the general population (14-17, 26, 27, 46, 47). Although our study was limited by our inability to directly challenge all patients with suspected food allergy or to do pulmonary function testing to confirm asthma diagnoses, the preponderance of evidence, including increased levels of total and allergen-specific IgE, serum and T_{reg}-produced T_H2 cytokines, peripheral eosinophilia, and propensity for lymphocyte skewing to T_H2 effector phenotypes in LDS patients, strongly suggests that this disease is dominated by

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a T_H2 immune response. Although EoE can be difficult to discern from gastroesophageal reflux disease both clinically and pathologically, the esophageal eosinophilic inflammation in most LDS patients failed to respond to treatment with proton pump inhibitors but markedly improved with food avoidance diets. Furthermore, nearly all LDS patients had eosinophilic inflammation in other parts of the gastrointestinal tract beyond the esophagus, suggesting that EGID is a bona fide feature of this disease. The pathogenesis of EGIDs is not completely understood, but growing evidence indicates that aeroallergens and food allergens play a central role (48). Recent studies have also emphasized a prominent role for T_H2 cytokines in the development of EGID, and children and adults with EoE often have other atopic diseases as well (49-51). The increased prevalence of EGID in LDS patients is therefore consistent with their propensity to develop other T_H2-mediated phenotypes.

The immunologic phenotype in LDS provides valuable insight into TGFβ's intricate role in directing immune responses to mucosal antigens and suggests that alterations in TGFβ signaling are sufficient to promote T_H2 immunity and allergic disease in humans. Our findings in LDS further demonstrate that mutations in a single gene can predispose to the complex phenotypes associated with allergic disorders, and therefore would suggest that genes involved in the TGFβ signaling pathway should be prioritized when evaluating data from large genome-wide association studies aimed at elucidating the genetic contributions to allergic disease. Finally, identification of a single pathway that can strongly predispose to allergic disease may have tremendous therapeutic implications. Although excessive TGFβ signaling is already known to be pathogenic in remodeling of the lung in asthmatics, the esophagus of patients with EoE, and the skin of patients with eczema, LDS provides the first direct evidence that altered TGFβ signaling is sufficient to incite these disorders (52-55). This observation may facilitate the development of therapeutic strategies for these common conditions. Losartan is a U.S. Food and Drug Administration–approved drug, which has known efficacy in reducing excessive TGFβ signaling in several disorders (33, 34). Studies in mice with Marfan syndrome, an aortic aneurysm syndrome closely related mechanistically and phenotypically to LDS, have revealed a remarkable ability of this drug to prevent the major cardiovascular complications associated with this disease (35). Whether losartan can modify allergic disease remains to be tested, but our finding that losartan mitigates TGFβ signaling alterations in lymphocytes of LDS patients suggests that this or related treatment approaches may hold promise.

MATERIALS AND METHODS

Study design and patients

All patients with confirmed mutations in TβRI or TβRII, encoded by *TGFBR1* or *TGFBR2*, respectively, who were seen in the Johns Hopkins Connective Tissue Disorders clinic through 31 December 2008, living in the United States, not deceased, and able to be contacted were asked to participate in the study. Seventy-one patients met criteria, and 65 agreed to participate. Detailed questionnaires regarding allergic and gastrointestinal disease, including biopsy reports, skin and/or allergen-specific in vitro IgE testing, types of food reactions, medication use, and type and duration of gastrointestinal and allergic symptoms, were sent to each participant. Questionnaires were returned from 58 of 71 subjects (82%). Information was also confirmed and/or retrieved by review of the patients' medical records. Cases of asthma, eczema, and allergic rhinitis were identified by self-report of doctor-diagnosed disease, review of medical records, and/or clinical examination by the authors. The diagnosis of food allergy was based on a convincing history of acute reaction after exposure to the implicated food. In all but four cases (where no testing to the suspected food had been performed), subjects also had an allergen-specific IgE level to the implicated food of >0.35 kU_A/liter (ImmunoCAP, Phadia) or positive skin prick test administered by a

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board-certified allergist. Nonallergic controls in this study had no clinical features of LDS and no history of symptoms suggestive of allergic disease. Children with nonsyndromic food allergy were recruited from the Johns Hopkins Pediatric Allergy Clinic. Nine of these 26 children had also been diagnosed with EoE based on esophageal biopsy findings, 16 with asthma, 18 with eczema, and 18 with allergic rhinitis. This study was approved by the Institutional Review Board of the Johns Hopkins University School of Medicine.

Laboratory data

Complete blood counts and quantitative Ig levels were performed for 50 of 58 (86%) subjects by a commercial laboratory. Allergen-specific IgE levels to the seven most common food (egg white, milk, peanut, soybean, sesame, wheat, and codfish) and aeroallergens (alternaria, *Dermatophagoides farinae*, timothy grass, ragweed, dog, cat, and white oak) were measured in 41 of 58 (71%).

Measurement of plasma cytokines

Plasma cytokines were measured with multiplex bead immunoassay (human 27-plex panel; Bio-Plex, Bio-Rad) according to the manufacturer's directions. Limits of detection for the Bio-Rad assay are as follows: IL-1β, 0.6 pg/ml;IL-1Ra, 5.5 pg/ml;IL-2, 1.6 pg/ml;IL-4, 0.7 pg/ml; IL-5, 0.6 pg/ml; IL-6, 2.6 pg/ml; IL-7, 1.1 pg/ml; IL-8, 1.0 pg/ml; IL-9, 2.5 pg/ml; IL-10, 0.3 pg/ml; IL-12 (p70), 3.5 pg/ml; IL-13, 0.7 pg/ml; IL-15, 2.4 pg/ml; IL-17, 3.3 pg/ml; CCL11 (eotaxin), 2.5 pg/ml; bFGF (basic fibroblast growth factor), 1.9 pg/ml; G-CSF (granulocyte colony-stimulating factor), 1.7 pg/ml; GM-CSF (granulocyte-macrophage colony-stimulating factor), 2.2 pg/ml; IFN-γ, 6.4 pg/ml; CXCL10 (IP-10), 6.1 pg/ml; CCL2 (MCP-1), 1.1 pg/ml; CCL3 (MIP-1α), 1.6 pg/ml; CCL4 (MIP-1β), 2.4 pg/ml; PDGF-BB (platelet-derived growth factor–BB), 2.9 pg/ml; CCL5 (RANTES), 1.8 pg/ml;TNF-α, 6.0 pg/ml; and VEGF (vascular endothelial growth factor), 3.1 pg/ml. Concentrations of CCL3 (MIP-1α) and CCL4 (MIP-1β) for both LDS and nonallergic subjects were above the upper limit of the assay and therefore were not included in our analysis.

Isolation of PBMCs

Blood was collected in EDTA tubes. Blood for analysis of T_{reg} subsets was subjected to double Percoll (density, 61 and 55%; GE Healthcare) centrifugation and fixed in 4% paraformaldehyde and frozen at −80°C (56). Blood for analysis of surface marker expression, cytokine production, and isolation of naïve T cells was processed by Ficoll (GE Healthcare) or single Percoll gradients.

Flow cytometry

To determine the percentage of $CD4^+$ cells present as T_{reg} subsets, fixed cells were stained with CD4-PerCP, CD25-APC (allophycocyanin), CD127-FITC (fluorescein isothiocyanate), and CD45RA–PE (phycoerythrin)–Cy7 (all BD Biosciences), and subsets were identified as previously described (29). Expression of T_{reg} markers was determined by surface staining of fresh PBMCs using the cocktail of antibodies described above to define T_{regs} along with GITR-PE, ICOS-PE, CTLA-4–PE (all from BD Biosciences), or Helios-PE (BioLegend), followed by intracellular staining with Foxp3 (BioLegend, clone 259D) and/or GATA3 (eBioscience) using the Foxp3 Fixation/Permeabilization kit (eBioscience) according to the manufacturer's directions. T_{reg} subsets were identified by first gating on $CD4^+CD25^{high}CD127^{lo}$ cells. rT_{regs} were defined as $CD45RA^+$ with intermediate expression of Foxp3, $CD45RA^-Foxp3^{inter}$ T_{regs} as $CD45RA^-$ with intermediate expression of Foxp3, and aT_{regs} as $CD45RA^-$ with high expression of Foxp3. Intracellular cytokine production was detected by culturing PBMCs in AIM V medium (Invitrogen) with phorbol 12-myristate 13-acetate (25 ng/ml) (Sigma-Aldrich) and ionomycin (250 ng/ml) (Sigma-

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Aldrich) in the presence of brefeldin (3 μg/ml) (eBioscience) for 4 to 4.5 hours. Cells were then treated with IC fixation buffer (eBioscience) according to the manufacturer's instructions and then stained with CD4-PerCP, CD25-APC, CD127-PE, CD45RA-PE-Cy7, and either IL-13–FITC (eBioscience), IFN-γ–FITC (BD Biosciences), IL-10–FITC (Caltag), or IL-17–FITC (BD Biosciences).

For T_{reg} suppression assays, PBMCs were isolated from LDS patients or nonallergic controls and enriched for $CD4^+$ T cells (Miltenyi) using negative selection, and then stained with CD4-APC, CD25-PE, CD45RA-PE-Cy7 (all from BD Pharmingen), and CD127-FITC (eBioscience). Cells retained on the column were retrieved and irradiated at 32 Gy for use as antigen-presenting cells. T_{regs} were first identified as being $CD4^+CD25^{high}$ and $CD127^{lo}$. The three subpopulations of T_{regs} were then sorted on the basis of their expression of CD45RA and CD25. T responder cells were identified as $CD4^+CD45RA^+CD25^-CD127^+$ and were labeled with CellTrace Violet according to the manufacturer's instructions (Life Technologies). Labeled T responder cells (5×10^4) were cultured with 5×10^4 (1:1), 2.5×10^4 (2:1), or 1×10^4 (5:1) of each T_{reg} subset in duplicate in 96-well round-bottom plates in the presence of 1×10^5 irradiated antigen-presenting cells and soluble anti-human CD3 (0.7 mg/ml) (eBioscience) for 4 days. Cells were subsequently stained with 7-aminoactinomycin D (BD Biosciences) and CD4 APC to assess for dilution of the CellTrace Violet dye in viable T responder cells.

To assess the skewing potential of naïve $CD4^+$ T cells in LDS patients and nonallergic controls, enriched $CD4^+$ T cells were prepared as described above. These cells were stained with CD4-APC, CD25-PE, CD45RA-PE-Cy7 (all from BD Biosciences), and CD45ROFITC (eBioscience) and then flow-sorted; naïve cells, defined as $CD4^+CD25^-CD45RA^+CD45RO^-$, were collected. The naïve cells were cultured at about 1×10^5 cells/ml in 200 ml of Iscove's modified Dulbecco's medium (Life Technologies) containing 5% fetal bovine serum in 96-well flat-bottom plates that had been coated with anti-human CD3ε (10 μg/ml) (eBioscience). Cultures were stimulated with anti-human CD28 monoclonal antibody (1 μg/ml), along with recombinant TGFβ1 (0, 0.01, 0.1, 1, or 10 ng/ml) (R&D Systems) for 4 days. In some experiments, TGFβ-neutralizing antibody (100 μg/ml) (Genzyme) or 5 μM SD-208 kinase inhibitor (Tocris) was added to the cultures. Cultures were then stimulated to induce cytokine expression with 1× Stimulation Cocktail (eBioscience) for 4.25 hours, and subsequently stained with CD4-APC (BD Biosciences), Foxp3–Pacific Blue (BioLegend), IL-13–PE (BD Biosciences), IFN-γ–AF700 (BioLegend), and IL-17–PerCP–Cy5.5 (eBioscience) using the Transcription Factor Buffer kit (BD Biosciences) according to the manufacturer's instructions. Lymphocytes were analyzed by first gating on $CD4^+$ cells and then evaluating expression of Foxp3 and each cytokine listed above on the $CD4^+$ population.

For phosflow experiments, whole blood was collected into EDTA tubes from patients with LDS and unaffected relatives. Blood was aliquoted and incubated in the presence or absence of recombinant human TGFβ1 (5 ng/ml) (R&D Systems) at 37°C for 0, 15, 60, and 240 min. Blood was then processed with the Perm Buffer III Phosflow kit (BD Biosciences) per the manufacturer's instructions. Cells were stained with CD4-PerCP (BD Biosciences) and unlabeled pSmad2/3 (Cell Signaling) followed by staining with anti-rabbit IgG AF488 (Cell Signaling).

Data were acquired with an LSRII (BD Biosciences) and analyzed with FACSDiva (6.5, BD Biosciences) and FlowJo (9.3.1, Tree Star Inc.) software. Cells were sorted with MoFlo (Beckman Coulter). Appropriate isotype and/or secondary antibody controls were included in all experiments.

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Immunohistochemistry

Thymic tissue from LDS patients obtained at the time of aortic surgery was stained with antibodies directed against pSmad2 (13). Control samples were obtained from age-matched individuals without LDS, another connective tissue disorder, or primary syndrome whose thymus had been removed during cardiac surgery. The number of positively stained nuclei in three high-powered fields per slide was scored by a blinded observer. Gastrointestinal biopsies were obtained for clinically indicated reasons and processed per clinical standards of the institution at which they were obtained.

Statistics

Statistics were performed with Prism 5.0 (GraphPad Software) or Stata 12.1 (Stata Corp.). Evaluation of pSmad2/3 was by longitudinal analysis fitting to a linear plus quadratic term. For analysis of Ig levels, the 95th percentile for Ig concentration for age was subtracted from the patient's Ig concentration (57, 58). Student's *t* test was then performed and compared to a value of zero. Statistical analysis was similarly performed to evaluate for significantly low levels of Ig, where the 5th percentile for age was used as the reference. The proportion of nuclei that stained positively for pSmad by immunohistochemistry was compared by Fisher's exact test. All other comparisons were by Mann-Whitney/Wilcoxon test. The box in box plots defines the 25 and 75% quartiles, the division within the box defines the median, and the whiskers define the range.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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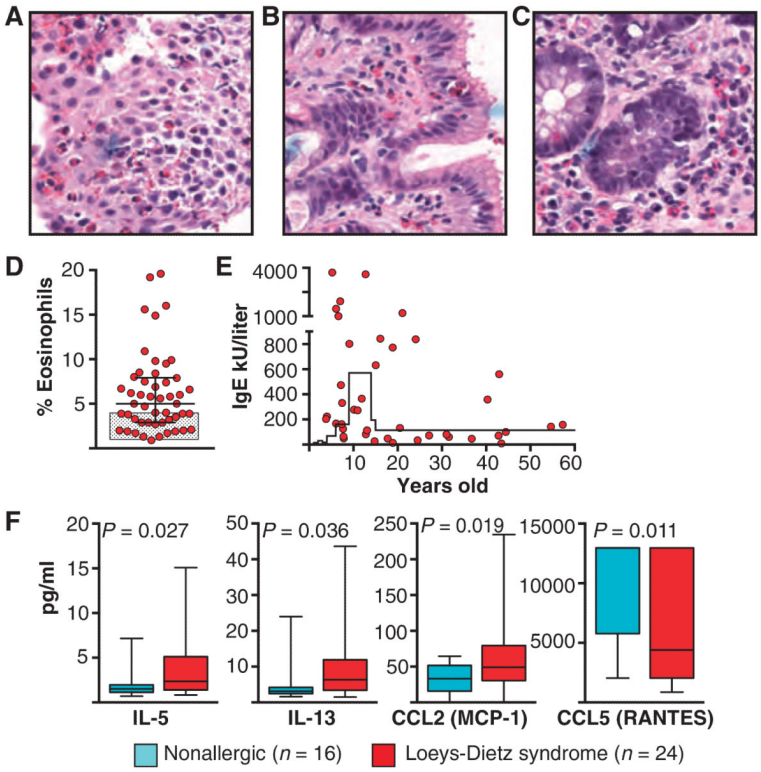

Fig. 1. Evidence for allergic predilection in LDS patients.

(**A** to **C**) Biopsies stained with hematoxylin and eosin demonstrating an eosinophilic infiltrate in the esophagus (A), stomach (B), and colon (C) of a child with LDS. Magnification, ×40. (**D**) Percentage of eosinophils in the peripheral blood of LDS patients (n = 50). Levels were significantly increased (P = 0.009) compared to the norm (shaded box) by Wilcoxon test. Line and whiskers indicate mean and SD, respectively. (**E**) Total serum levels of IgE (kU/liter) from LDS patients versus age (n = 41). Levels were elevated (P = 0.016; Student's t test, two-tailed) above the 95% confidence interval for age as indicated by the solid line. Each point represents an individual patient in (D) and (E). (**F**) Levels of IL-5, IL-13, CCL2 (MCP-1), and CCL5 (RANTES; pg/ml) in plasma from patients with LDS (n = 24) and age-matched nonallergic controls (n = 16). Significant P values are indicated; comparisons were done by Wilcoxon test.

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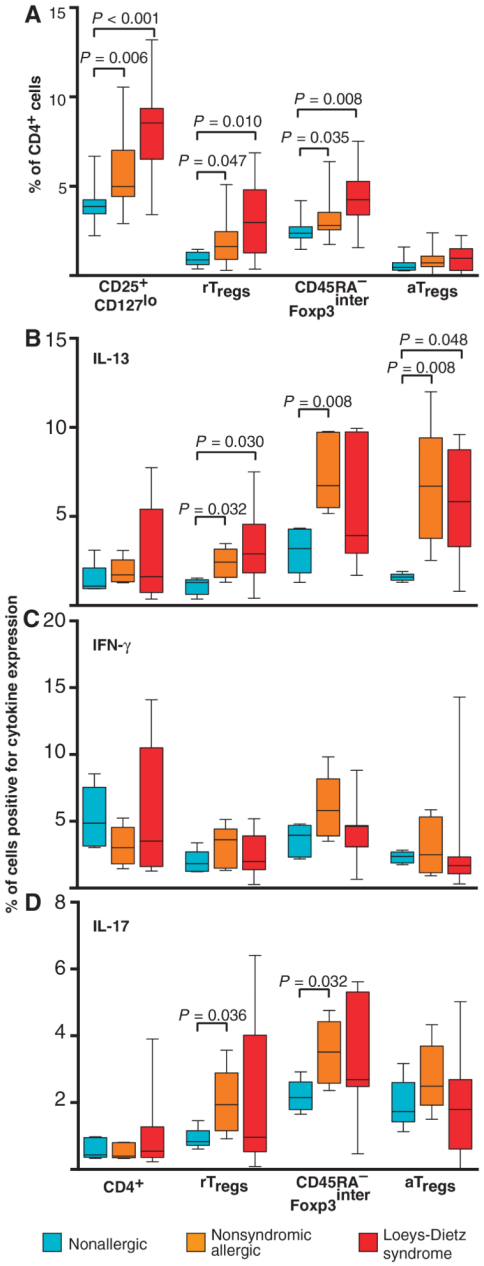

Fig. 2. Frequency and function of Foxp3$^+$ cells in patients with LDS and nonsyndromic allergic disease.

(**A**) Number of total T_{regs} ($CD4^+CD25^+CD127^{lo}$) in individuals with LDS (n = 16) and nonsyndromic allergic disease (n = 26) compared to age-matched nonallergic controls (n = 8). Further analysis revealed increases in rT_{regs} and $CD45RA^-Foxp3^{inter}$ cells, but no difference in the frequency of aT_{regs}, in LDS (n = 16) and nonsyndromic allergic subjects (n = 26) compared to nonallergic controls (n = 8). (**B** to **D**) Percentage of $CD4^+$ lymphocytes as well as each T_{reg} subset that expresses the cytokines IL-13 (B), IFN-γ (C), and IL-17 (D) in LDS patients (n = 7), children with non-syndromic allergic disease (n = 5), and nonallergic controls (n = 5). Significant P values are indicated; all comparisons were done by Wilcoxon test.

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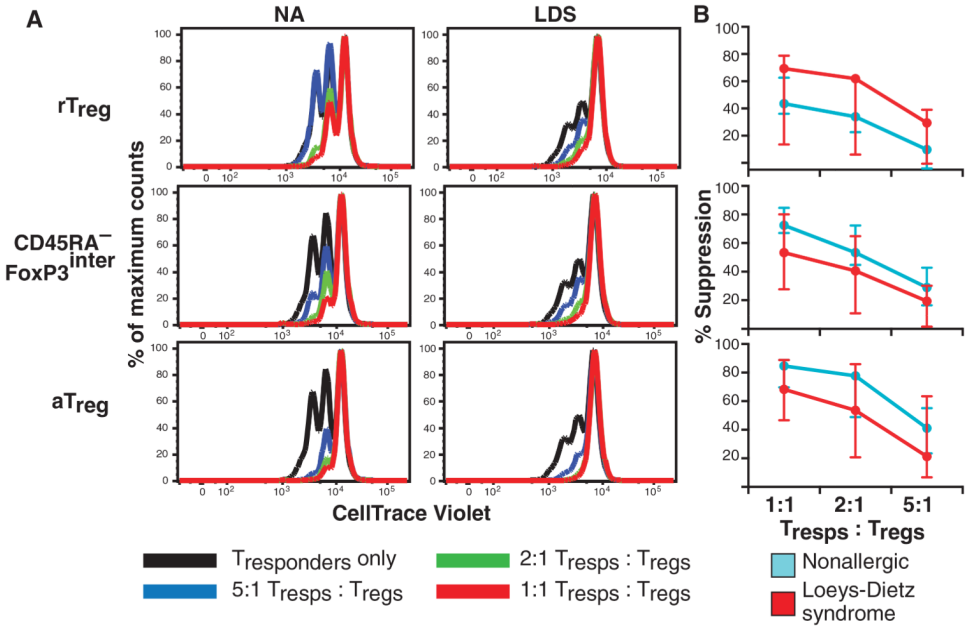

Fig. 3. Suppressive activity of LDS T_{regs}.

(**A** and **B**) The three subsets of T_{regs} (rT_{regs}, $CD45RA^{-}Foxp3^{inter}$ T_{regs}, and aT_{regs}) were purified and cocultured at various ratios (1:1, 2:1, and 5:1) with $CD4^{+}$ effector T cells that had been labeled with CellTrace Violet. Cultures were stimulated with anti-CD3 and irradiated antigen-presenting cells, and dilution of the dye, a marker of proliferation, was assessed 4 days later. (A) Results from a representative experiment ($n = 3$). (B) Percent suppression of effector T cell proliferation at the different ratios (median and range are indicated). LDS ($n = 3$) and nonallergic (NA) control ($n = 3$) T_{regs} effectively suppress effector T cell proliferation.

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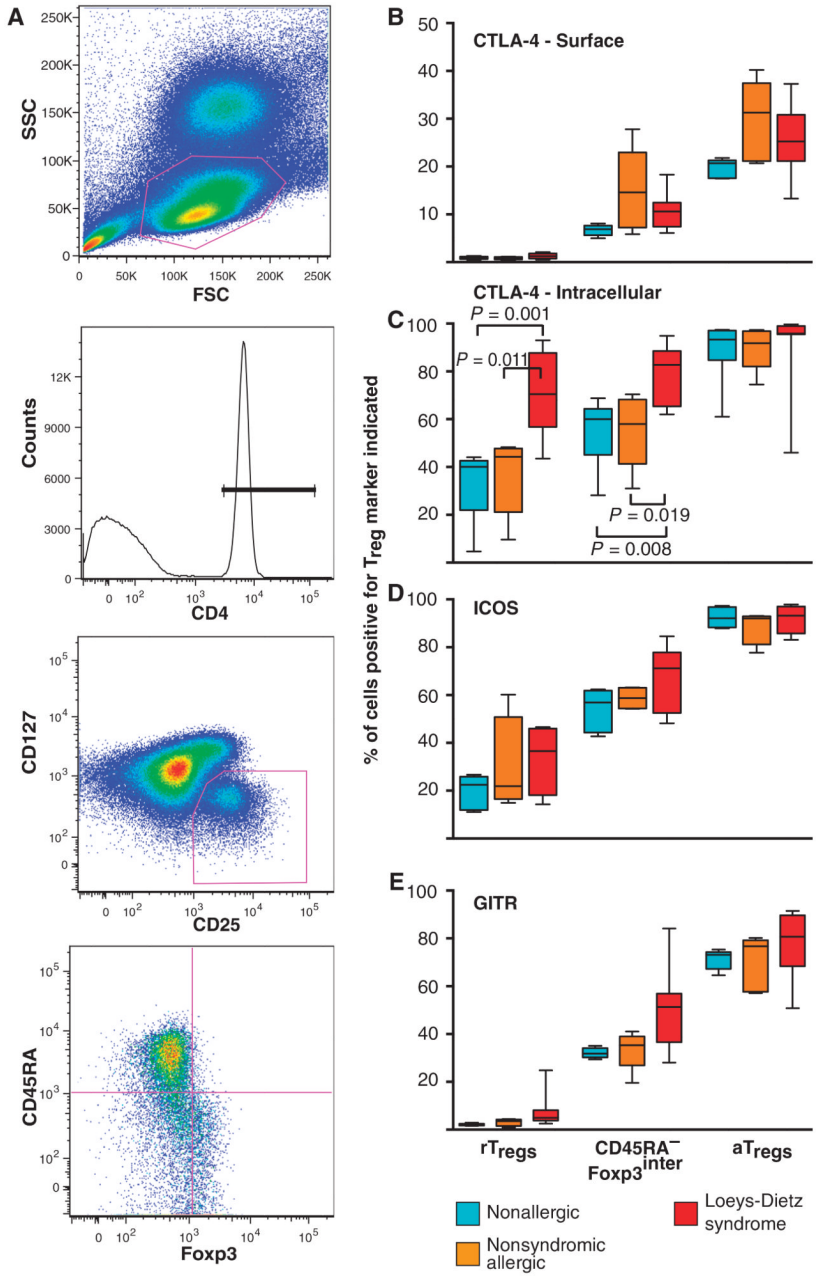

Fig. 4. Expression of T_{reg} markers by Foxp3$^+$ cells from patients with LDS, nonsyndromic allergic disease, and nonallergic controls.

(**A**) Gating scheme used to identify the three distinct subsets of Foxp3$^+$ cells. Peripheral blood mononuclear cells (PBMCs) from LDS patients (n = 8), age-matched subjects with nonsyndromic allergic disease (n = 5), and nonallergic controls (n = 5) were stained with CD4, CD25, and CD127 antibodies. T_{regs} were defined as CD25$^+$CD127^{lo} cells after first gating on lymphocytes [based on forward scatter (FSC) and side scatter (SSC)] that were CD4$^+$. CD4$^+$CD25$^+$CD127^{lo} cells were then divided into rT_{regs}, CD45RA$^-$Foxp3^{inter} T_{regs}, and aT_{regs} based on their expression of CD45RA and Foxp3. (**B** to **E**) Percentage of each T_{reg} subset that expresses the indicated T_{reg} marker (CTLA-4, ICOS, and GITR). Boxes define the 25 and 75% quartiles, divisions within the boxes the medians, and whiskers the range. No difference in expression of any T_{reg} marker was found except that a significantly

greater percentage of rT_{regs} and $CD45RA^{-}Foxp3^{inter}$ T_{regs} from LDS patients expressed intracellular CTLA-4 compared to individuals with nonsyndromic allergic disease and nonallergic controls. Significant *P* values are indicated; comparison by Wilcoxon test.

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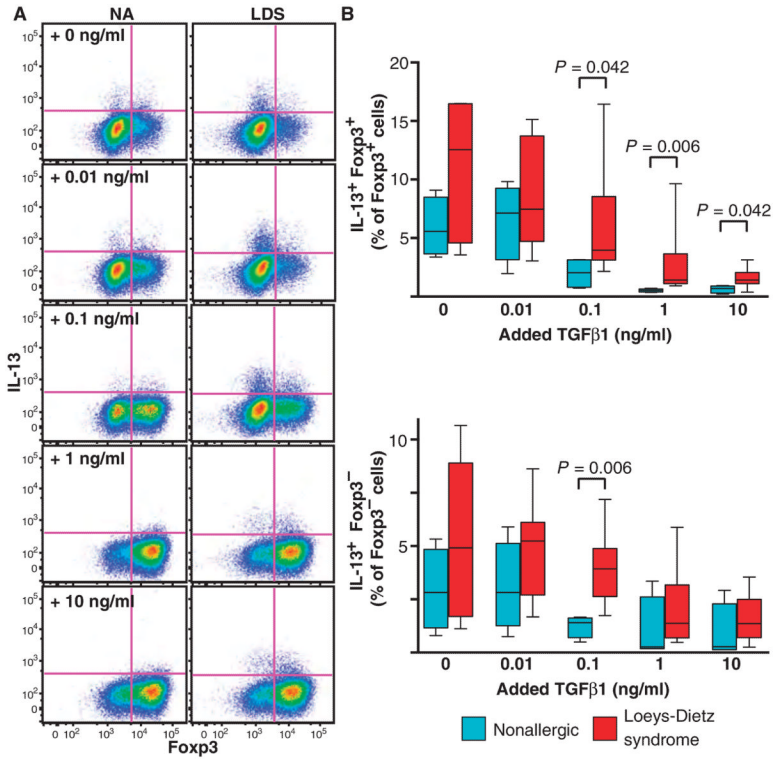

Fig. 5. Skewing potential of naïve CD4$^+$ lymphocytes from LDS subjects and nonallergic controls after TGFβ stimulation.

(**A**) Representative dot plot depicting expression of Foxp3 and IL-13 in LDS (n = 7) and nonallergic (NA; n = 4) CD4$^+$ lymphocytes 4 days after naïve CD4$^+$ T cells were cultured with increasing doses of recombinant TGFβ1 as indicated. (**B**) Percentage of IL-13–expressing Foxp3$^+$ and Foxp3$^-$ cells in cultures from LDS patients (n = 7) compared to nonallergic controls (n = 4) after treatment with the indicated doses of recombinant TGFβ1. Significant P values are indicated; comparisons by Mann-Whitney test.

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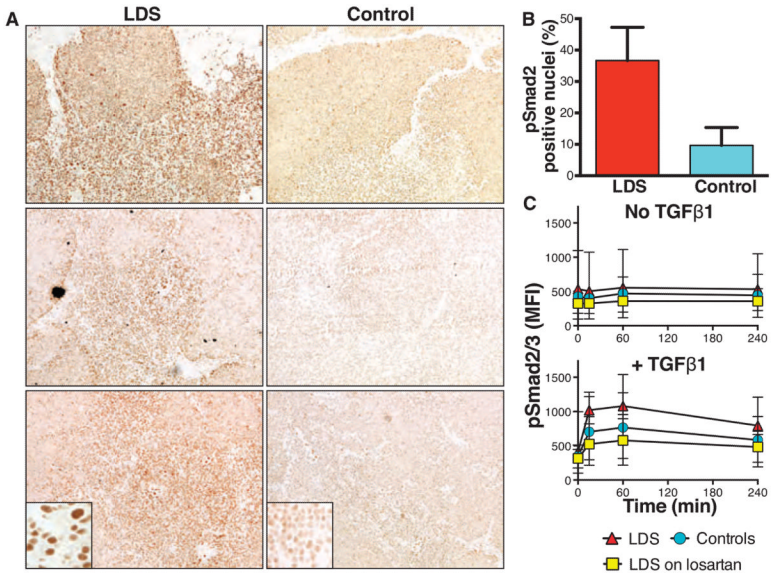

Fig. 6. Status of TGFβ signaling in the immune system of LDS patients.

(**A**) Immunostaining for pSmad2 in thymic tissue from three patients with LDS and age-matched controls ($n = 3$) with increased intensity of nuclear pSmad2 in LDS patients. (**B**) Percentage of nuclei that stained positively for pSmad2 in LDS versus control thymi from (A) as evaluated by a blinded observer. $P < 0.001$, Fisher's exact test. (**C**) Expression of pSmad2/3 before (No TGFβ1) and at various time points after (+ TGFβ1) treatment of whole blood with recombinant TGFβ1 from patients with LDS ($n = 4$) and unaffected relatives ($n = 4$; Controls), and LDS patients receiving therapeutic doses of losartan ($n = 4$; LDS on losartan). Mean fluorescence intensities (MFI) of pSmad2/3 staining in gated $CD4^+$ lymphocytes are plotted. Levels of pSmad2/3 were increased ($P = 0.050$; comparison by longitudinal analysis) after stimulation in LDS versus controls and LDS on losartan. Medians and IQRs (whiskers) are shown.

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Table 1

Characteristics of LDS patients with mutations in TβR1 and TβR2. Number of subjects (%) for each category is reported, except for age, where median age in years (25th to 75th percentile) is listed. EGID, eosinophilic gastrointestinal disease, diagnosed by biopsy; EoE, eosinophilic infiltrate in the esophagus; EoG, eosinophilic infiltrate in the gastric mucosa; EoC, eosinophilic infiltrate in the colon.

	TβR1	TβR2	All
Number of subjects	14 (24)	44 (76)	58
Male	8 (57)	19 (43)	27 (47)
Age at enrollment (years)	12.9 (9.8–22.2)	13.6 (7.2–19.2)	13.3 (7.3–20.1)
Food allergy	4 (29)	14 (32)	18 (31)
Asthma	7 (50)	19 (43)	26 (45)
Allergic rhinitis	6 (43)	22 (50)	28 (48)
Eczema	7 (50)	15 (34)	22 (38)
EGID	2 (14)	4 (9)	6 (10)
EoE	2 (14)	4 (9)	6 (10)
EoG	2 (14)	3 (7)	5 (9)
EoC	2 (14)	2 (5)	4 (7)