Allergic dysregulation and hyperimmunoglobulinemia E in Foxp3 mutant mice

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Background: Regulatory T cells have been proposed to play an important role in regulating allergic inflammation. The transcription factor Foxp3 is a master switch gene that controls the development and function of natural and adaptive $CD4^+CD25^+$ regulatory T (T_R) cells. In human subjects loss-of-function Foxp3 mutations trigger lymphoproliferation, autoimmunity, and intense allergic inflammation in a disease termed immune dysregulation polyendocrinopathy enteropathy–X-linked syndrome.

Objective: We sought to examine the evolution and attributes of allergic inflammation in mice with a targeted loss-of-function mutation in the murine *Foxp3* gene that recapitulates a known disease-causing human *Foxp3* mutation.

Methods: *Foxp3* mutant mice were generated by means of knock-in mutagenesis and were analyzed for histologic, immunologic, and hematologic abnormalities. The role of signal transducer and activator of transcription 6 (Stat6) in disease pathogenesis was analyzed by using *Stat6* and *Foxp3* double-mutant mice.

Results: *Foxp3* mutant mice developed an intense multiorgan inflammatory response associated with allergic airway inflammation, a striking hyperimmunoglobulinemia E, eosinophilia, and dysregulated T_H1 and T_H2 cytokine production in the absence of overt T_H2 skewing. Concurrent Stat6 deficiency reversed the hyperimmunoglobulinemia E and eosinophilia and delayed mortality, which is consistent with a pathogenic role for allergic inflammation in Foxp3 deficiency.

Conclusion: Allergic dysregulation is a common and fundamental consequence of loss of $CD4^+CD25^+$ T_R cells caused by Foxp3 deficiency in different species. Abnormalities affecting T_R cells might contribute to a variety of allergic diseases. (J Allergy Clin Immunol 2005;116:1106-15.)

Key words: *Foxp3, dysregulation polyendocrinopathy enteropathy–X-linked, allergic inflammation, regulatory T cells, signal transducer and activator of transcription 6*

Compelling evidence indicates a key role for natural and adaptive $CD4^+CD25^+$ regulatory T (T_R) cells in the maintenance of immunologic tolerance to self-antigens and foreign antigens.[1] $CD4^+CD25^+$ T_R cells suppress the proliferation and cytokine production of conventional $CD4^+CD25^-$ T cells, as well as that of $CD8^+$ T cells and established T_H1 and T_H2 cells. Consistent with the role of T_R cells in maintaining tolerance, their loss of function caused by mutations in the Foxp3 transcription factor has been associated with lethal lymphoproliferative disease in the scurfy mouse. Affected scurfy male mice die at about the third week of age because of an unrelenting infiltrative lymphoproliferative disease that involves several organs.[2,3] Importantly, the scurfy phenotype can be reproduced by means of targeted *Foxp3* mutagenesis and is rescued by a *Foxp3* transgene, demonstrating a causative role of Foxp3 in pathogenesis.[4,5] We and others have shown that *Foxp3* mutations also underlie a homologous autoimmune lymphoproliferative disorder in human subjects, termed immune dysregulation polyendocrinopathy enteropathy–X-linked syndrome (IPEX) or X-linked autoimmunity–allergic dysregulation syndrome.[6-8]

One of the distinguishing features of IPEX is the presence of pronounced allergic inflammation, including increased IgE levels, eosinophilia, food allergy, and eczema. These observations are highly suggestive for a role of T_R cells in the regulation of the allergic response.[6] Such a role has been supported by studies examining regulatory mechanisms operative in allergic inflammation.[9] Mouse $CD4^+CD25^+$ T cells were found to suppress the *in vitro* differentiation of T_H2 cells from naive $CD4^+$ T cells.[10] In mice with severe combined immunodeficiency syndrome reconstituted with monoclonal populations of T and B cells, immunization with antigen induced increased serum IgE levels that were suppressed

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Abbreviations used
Hyper-IgE: Hyperimmunoglobulinemia E
IPEX: Immune dysregulation polyendocrinopathy enteropathy–X-linked syndrome
Stat6: Signal transducer and activator of transcription 6
T_R: $CD4^+CD25^+$ regulatory T
WT: Wild-type

by both $CD4^+CD25^+$ and $CD4^+CD25^-$ T_R cells, which is consistent with a critical role for T_R cells in tightly regulating IgE responses.[11] Murine adaptive T_R cells derived by T-cell receptor cross-linking in the presence of TGF-β suppressed antigen-induced allergic airway inflammation in a mouse model of asthma.[12] In human subjects acquired tolerance to cow's milk allergy is associated with the development of cow's milk allergen-responsive $CD4^+CD25^+$ T_R cells.[13] Approaches that induce tolerance to allergens, suppress allergic inflammation, or both have also been associated with induction of T_R cells. Repeated low-dose inhaled exposure to antigen suppressed allergic airway inflammation through a mechanism involving the induction of $Foxp3^+$ $CD4^+CD25^+$ T_R cells.[14] Corticosteroids, commonly used to suppress allergic inflammation, upregulate Foxp3 expression and the frequency of $CD4^+CD25^+$ T_R cells in the systemic circulation.[15] However, other studies have either failed to demonstrate an inhibitory function for $CD4^+CD25^+$ T_R cells in murine models of allergic airway inflammation or have demonstrated regulation of some but not all aspects of allergic inflammation.[16-18]

To better understand the role T_R cells in allergic inflammation, we analyzed mice with a loss-of-function mutation in Foxp3 that recapitulated an IPEX-causing human mutation associated with a premature stop codon proximal to the forkhead domain.[6]

METHODS

Generation and characterization of *Foxp3* mutant mice

The original scurfy mutation was found in linkage disequilibrium with another mutation, the closely linked sparse-fur (*Otc*spf) mouse.[19] To avoid this and other confounding mutations, to recapitulate the effects of human disease causing mutation, and to facilitate experiments with wild-type (WT) control animals on an identical inbred background, a Foxp3-deficient mouse strain was derived by means of targeted knock-in mutagenesis, whereby an A→T substitution was introduced in the first base position of codon 276 in exon 8 of the *Foxp3* gene. This substitution, which results in the codon specificity changing from a lysine (AAG) to a stop (TAG) signal, is functionally analogous to a human mutation (IVS9 +4 A→G) that results in a frame shift at codon 273 of the human protein and gives rise to a premature stop signal at codon 286. Two base pair substitutions were introduced in codon 277 (AGC→ATA) to facilitate analysis of the targeted gene, resulting in the creation of a novel *Eco*RV restriction site (Fig 1). The targeting construct included a neomycin-resistance gene (*Neo*) for positive selection of successfully targeted clones and a diphtheria toxin gene (*DT*) for negative selection against randomly inserted targeting constructs. The *Neo* gene was flanked by *LoxP* sequences to allow its removal by the Cre recombinase after successful targeting. Targeting plasmids were introduced by means of electroporation into RW4 embryonic stem cells and subjected to G418 (Calbiochem, San Diego, Calif) selection. Resistant clones were screened for homologous recombination by means of southern blotting with a probe corresponding to exon 1 of *Foxp3*, which lies 5′ to the homology sequence. Successful targeting was associated with a decrease in the size of the genomic *Eco*RV fragment that normally hybridizes with the probe from 11 kb to 7.7 kb (Fig 1). A successfully targeted clone was injected into C57BL/6 blastocysts to create male chimeras. Founder male chimeras were mated with WT BALB/c mice and C57BL/6 mice, and heterozygous F1 females were mated with Cre-deleter mice (The Jackson Laboratory, Bar Harbor, Me) to remove the floxed *Neo* cassette.[20] The female heterozygotes were asymptomatic, whereas *Foxp3* mutant male mice experienced an aggressive lymphoproliferative and myeloproliferative disease and died within the first few weeks of life. Female heterozygotes were further backcrossed up to 8 generations on BALB/c (Taconic, Germantown, NY) and C57BL/6 (Charles River Laboratories, Wilmington, Mass) backgrounds to provide nearly homogenous genetic backgrounds. Heterozygous females on BALB/c background were also backcrossed to signal transducer and activator of transcription 6 (Stat6) homozygous mutant (knockout) males on the same background (The Jackson Laboratory) to create double-mutant mice.

Details of the following methods are found in the Online Repository in the online version of this article at www.jacionline.org: *Foxp3* allele-specific PCR analysis; quantitative real-time PCR analysis of *Foxp3*, *T-bet*, *GATA-3*, and hypoxanthine phosphoribosyl-transferase transcripts; automated complete blood cell count and differential analysis; flow cytometry (including intracellular cytokine staining); and immunoglobulin and cytokine assays (including serum cytokine levels and cytokine production by purified $CD4^+$ splenocytes).

RESULTS

Blood and tissue eosinophilia and allergic airway inflammation in $Foxp3^-$ mice

Introduction of a nonsense mutation at codon 276 of *Foxp3* resulted in a profound decrease in *Foxp3* mRNA levels (>10-fold) and the complete loss of Foxp3 protein expression, as detected by intracellular staining of $CD4^+$ splenocytes with an anti-murine Foxp3 mAb (Fig 1). This was most likely a consequence of nonsense-mediated degradation of the mutant mRNA induced by the presence of a premature stop codon.[21] Analysis of $CD4^+CD25^+$ cells in 9-day-old WT and $Foxp3^-$ mice before the onset of phenotypic disease revealed their profound deficiency in mutant pups compared with WT littermates (see Fig E1 in the Online Repository in the online version of this article at www.jacionline.org) and consistent failure of $CD4^+CD25^+$ T_R cell development in agreement with observations made in another $Foxp3^-$ model.[5] As the disease progressed and became phenotypic, there was an expansion in mutant lymphoid tissues of activated $CD4^+$ T cells that expressed CD25 as an activation marker, as is normal for such nonregulatory populations (data not shown). Together, these observations indicate the complete loss of $Foxp3^+$ $CD4^+CD25^+$ T_R cells because of the introduced mutation.

Gross morphologic examination and histologic analysis of *Foxp3* mutant ($Foxp3^-$) male mice on the BALB/c

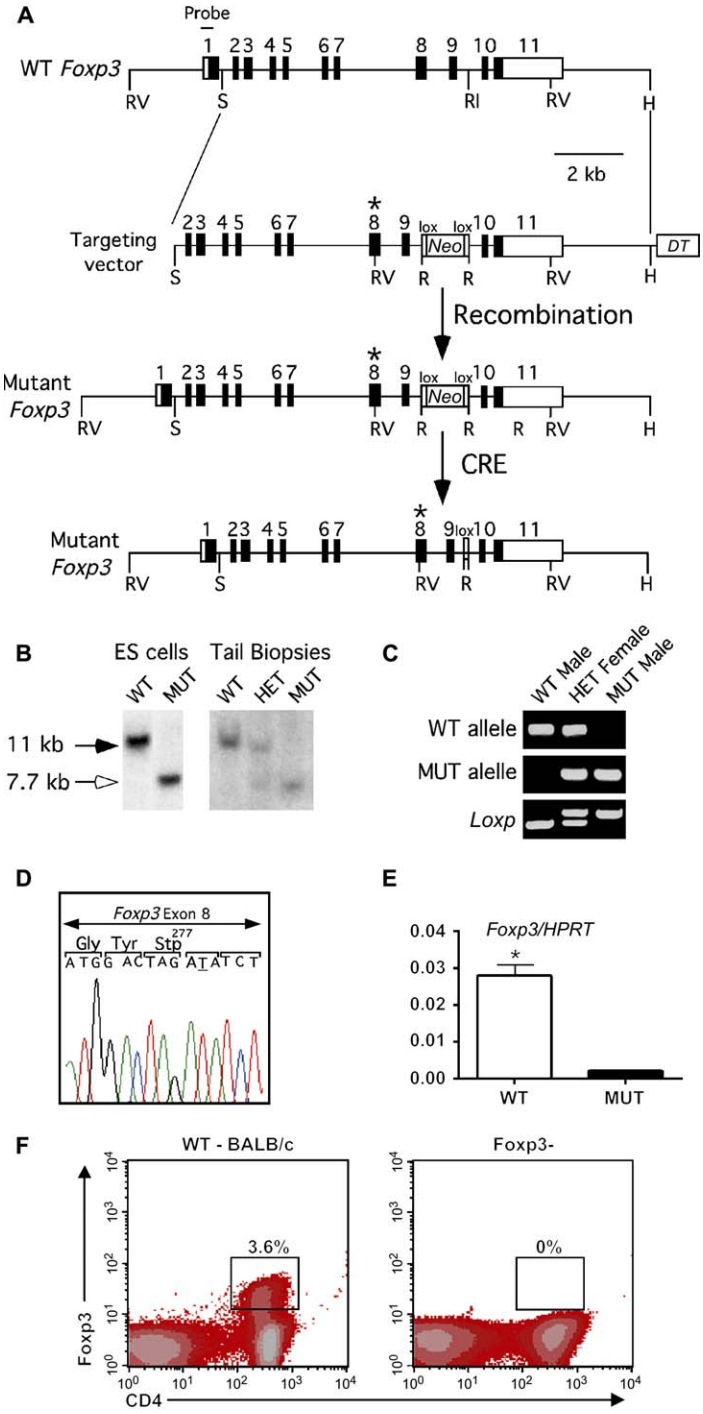

FIG 1. Targeted knock-in mutagenesis of murine *Foxp3*. **A**, Targeting strategy. Codon K276, located in exon 8 of the WT allele, was changed into a stop codon *(*)*. A novel *Eco*RV restriction site was also created immediately downstream. **B**, Southern blot analysis of *Eco*RV-digested embryonic stem cell and mouse tail genomic DNA. An 11-kb WT genomic fragment detected with an exon 1 probe is reduced to 7.7 kb in the mutant allele. **C**, PCR analysis of mouse tail DNA using WT and mutant allele-specific primers (*upper* and *middle panels*, respectively) or primers spanning the residual 34-bp *LoxP* site left in intron 9 after Cre-mediated excision of the floxed Neo cassette. **D**, Sequence analysis of *Foxp3* genomic DNA of mutant mice demonstrating the premature stop codon. **E**, Real-time PCR analysis of *Foxp3* mRNA expression in WT and mutant splenocytes. Results are expressed as a ratio of *Foxp3* transcripts to hypoxanthine phosphoribosyl-transferase *(HPRT)*, which was used as an endogenous control gene (*$P = .0017$, n = 4). **F**, Intracellular staining WT and mutant $CD4^+$ splenocytes with anti-murine Foxp3 mAb. *MUT*, Mutant; *HET*, heterozygous.

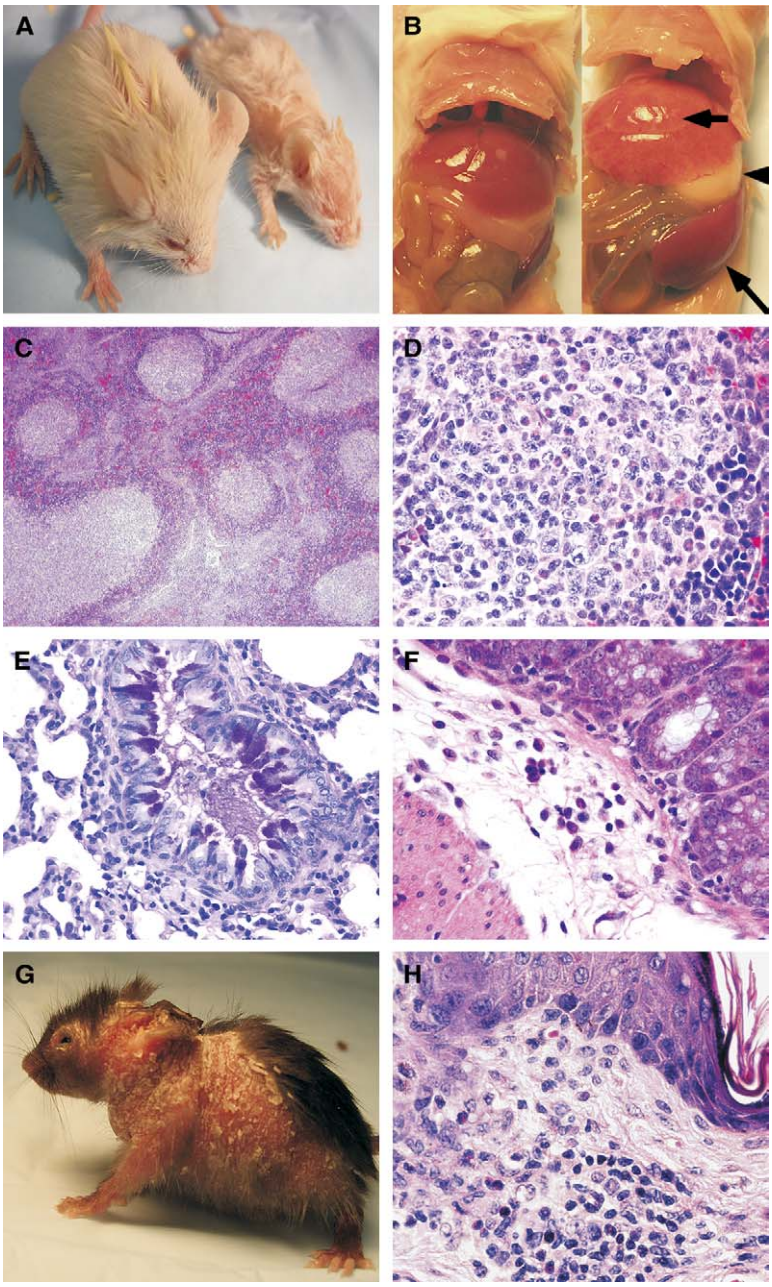

FIG 2. Lymphoproliferation, myeloproliferation, and allergic inflammation in Foxp3-deficient mice: **A-F**, BALB/c background; **G** and **H**, C57BL/6 background. **A**, Compared with its 18-day-old unaffected littermate *(left)*, the Foxp3-deficient mouse *(right)* is runted, has blepharitis caused by periorbital inflammation, and has fur loss caused by dermatitis. **B**, Gross abdominal pathology. Compared with its normal littermate *(left)*, the Foxp3-deficient mouse *(right)* has massive splenomegaly *(long arrow)*. The liver *(short arrow)* is intensely infiltrated with lymphoid and myeloid elements, and the stomach is persistently engorged with milk *(arrowhead)*, which is reflective of gastroparesis caused by inflamed stomach walls. **C** and **D**, Spleen. Fig 2, *C*, shows extensive lymphoproliferation with a lymphosarcoma-like picture and intense extramedullary hematopoiesis (original magnification 100×). Fig 2, *D*, shows cellular composition within the periarteriolar lymphoid sheath showing lymphoid and myeloid elements and prominent eosinophilia (original magnification 400×). **E**, Lung. Goblet cell metaplasia (pink-staining cells) and mucin impaction in the lumen of a bronchiole (original magnification 400×). **F**, Colon. Submucosal edema with mucosal and submucosal eosinophilic infiltration (original magnification 100×). **G**, Intense dermatitis with scaling and fur loss in a 5-week-old affected mouse on the C57BL/6 background. **H**, Histopathology of the skin showing inflammation with a prominent eosinophilic component (hematoxylin and eosin staining, original magnification 400×). Fig 2, *C*, *D*, *F*, and *H*, Hematoxylin and eosin staining; Fig 2, *E*, periodic acid–Schiff staining.

TABLE I. Peripheral blood parameters of WT and Foxp3$^-$ mice

Cohort (n)	WBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Platelets	Hemoglobin
WT (28)	6.46 ± 1.00	0.89 ± 0.09	5.059 ± 0.303	0.34 ± 0.04	0.17 ± 0.02	1031 ± 92	8.60 ± 0.16
Foxp3$^-$ (38)	21.07 ± 0.38	5.811 ± 0.40	11.02 ± 0.68	2.2 ± 0.17	1.58 ± 0.15	737 ± 98	6.98 ± 0.36

Complete peripheral blood counts and differential white blood cell counts were determined in 15- to 19-day-old WT and Foxp3$^-$ BALB/c mice (n = 28 and 38, respectively). White blood cell and platelet populations are expressed as multiples of 10^9/L, whereas hemoglobin concentrations are in grams per deciliters. Results were analyzed by using a 2-tailed, unpaired Student *t* test: $P < .0001$ for all white cell populations examined, $P = .0005$ for hemoglobin, and $P = .0384$ for platelets.
WBC, White blood cell count.

background at 2 weeks of age revealed an intense lymphoproliferative and myeloproliferative disorder (Fig 2, *A-F*). There was massive splenomegaly (Fig 2, *C* and *D*) and a mixed inflammatory infiltrate composed of lymphocytes, neutrophils, monocytes, and a prominent eosinophilic component that involved several organs, including the liver, stomach, pancreas, and skin (data not shown). Of particular interest were several findings consistent with spontaneous ongoing allergic airway inflammation, including marked peribronchial infiltrates, goblet cell metaplasia, and mucus impaction reminiscent of severe asthma (Fig 2, *E*). The gastrointestinal tract was also involved with eosinophilic and neutrophilic infiltration and crypt abscesses (Fig 2, *F*). These changes were not unique to mutant mice on the BALB/c background because mice bearing the same mutation on the C57BL/6 background also suffered a multisystem lymphoproliferative and myeloproliferative disease associated with allergic inflammatory changes with particularly prominent dermatitis and allergic airway inflammation (Fig 2, *G* and *H*, and data not shown). The intense lymphoproliferative and myeloproliferative disorder of *Foxp3* mutant mice was associated with leukocytosis, lymphocytosis, monocytosis, anemia, and thrombocytopenia (Table I). There was also marked blood eosinophilia, which is consistent with previous observations made in patients with IPEX and with the observation of tissue eosinophilia in the mutant mice (Table I).

Foxp3$^-$ mice exhibit hyperimmunoglobulinemia E and T_H1 and T_H2 cytokine dysregulation

Immunologic analysis of 15-day-old Foxp3$^-$ BALB/c mice revealed marked expansion of the CD4 and CD8 populations, a significant percentage of which were activated (data not shown). There was a striking increase in the serum IgE and IgG1 levels, which is consistent with the action of T_H2 cytokines in inducing isotype switching to IgG1 and IgE (Fig 3, *A-C*). There was a reciprocal decrease in IgG2a levels, which might reflect both suppression of endogenous synthesis of IgG2a by T_H2 cytokines and decreased absorption of maternal IgG2a. The increase in IgE and IgG1 levels was not unique to the BALB/c background because a similarly striking increase was noted on the C57BL/6 background (Fig 3, *D-I*). The capacity of Foxp3$^-$ mice to survive longer on the C57BL6 compared with the BALB/c background (data not shown) allowed the observation of an age-dependent increase in the serum IgE levels in Foxp3$^-$ C57BL/6 mice that reached upward of 1 mg/mL by ages 23 to 30 days and persisted at older ages. These IgE levels were close to 4 orders of magnitude greater than the levels of WT littermate control animals.

To determine whether the hyperimmunoglobulinemia E (hyper-IgE) and allergic inflammatory response in Foxp3$^-$ mice reflected biased T_H2 cytokine production, we examined the *in vivo* production of T_H1 and T_H2 cytokines in 15-day-old WT and *Foxp3* mutant mice on the BALB/c background, as reflected by the serum concentrations of the respective cytokines. There was a dramatic increase in the serum concentrations of both T_H1 (IFN-γ and TNF-α) and T_H2 (IL-4, IL-5, and IL-10) cytokines in mutant mice compared with those seen in WT littermates (Fig 4). Sera of mutant mice also had increased concentrations of several proinflammatory factors, including IL-6 (Fig 4) and the chemotactic factor monocyte chemotactic protein 1 (data not shown). Analysis by means of intracellular cytokine staining revealed that the IL-4–producing T cells were predominantly $CD4^+$, whereas both $CD4^+$ and $CD8^+$ T cells expressed IFN-γ (see Fig E2 in the Online Repository in the online version of this article at www.jacionline.org). Together these data indicated that there was a nonselective dysregulated production of both T_H1 and T_H2 cytokines in Foxp3$^-$ mice.

Real-time PCR measurement of *GATA-3* and *T-bet* mRNA levels was undertaken in splenocytes of WT and Foxp3$^-$ mice to further verify that the dysregulated allergic inflammation in Foxp3$^-$ mice was not exclusively caused by enhanced T_H2 cell skewing. Both GATA-3 and T-bet are master switch factors that determine T_H cell differentiation toward the T_H2 or T_H1 subset, respectively. Analysis at postnatal days 3, 9, and 17 showed a progressive and age-dependent increase in the levels of *T-bet* and *GATA-3* transcripts in splenocytes of mutant mice compared with that seen in WT littermates, which is consistent with the expansion of T-cell populations expressing both T_H1 and T_H2 cytokines in mutant Foxp3$^-$ mice (Fig 5, *A* and *B*). The ratio of their mRNA levels has been verified as a surrogate measure of the T_H1/T_H2 cytokine polarization in mixed cell populations *in vivo*.[22,23] The ratio of *GATA-3* to *T-bet* transcripts in WT BALB/c male pups increased in an age-dependent manner, which is consistent with the well-known T_H2 bias of the BALB/c strain. In contrast, the ratio of *GATA-3*/*T-bet* transcripts in Foxp3$^-$ splenocytes failed to increase over time (Fig 5, *C*). Furthermore, analysis of *GATA-3* and *T-bet* transcript levels in different tissues (eg, liver and lung) failed to reveal any preferential

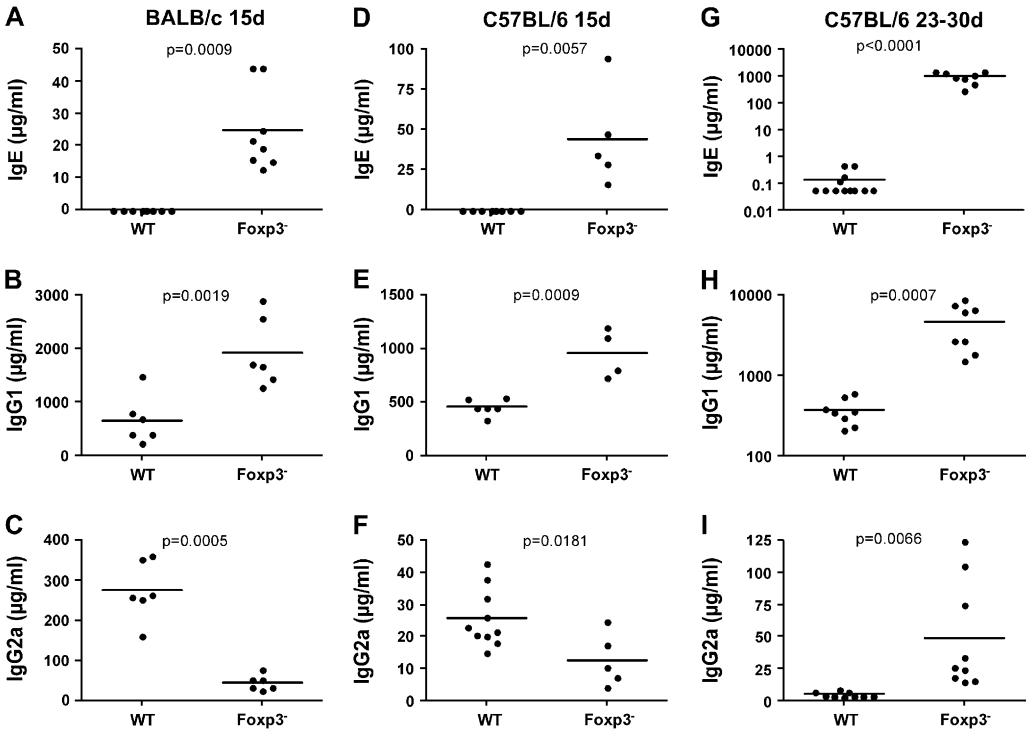

FIG 3. Hyper-IgE levels in Foxp3$^-$ mice on BALB/c and C57BL/6 backgrounds. **A-C**, Fifteen-day-old BALB/c mice; **D-F**, 15-day-old C57BL/6 mice; **G-I**, 23- to 30-day-old C57BL/6 mice. Sera of WT and Foxp3$^-$ littermate male pups were analyzed for IgG1, IgG2a, and IgE levels by means of ELISA. Results were analyzed by using the unpaired, 2-tailed Student *t* test.

expansion of the *GATA-3* transcript pool relative to that of *T-bet* (data not shown). These results argue against skewed T_H2 cell polarization as the underlying mechanism of dysregulated allergic inflammation in Foxp3$^-$ mice and suggest instead that the combined effects of dysregulated T_H1 and T_H2 cytokine expression underlies the inflammatory phenotype.

The allergic dysregulation of Foxp3$^-$ mice is Stat6 dependent

Foxp3$^-$/Stat6 knockout double-mutant mice were derived and were examined for cytokine production, serum immunoglobulin concentrations, eosinophilia, and T_H2-dependent histopathologic changes to determine whether the allergic inflammation and the hyper-IgE proceeded in a Stat6-dependent mechanism. Concurrent Stat6 deficiency profoundly impaired the production of T_H2 cytokines by CD4$^+$ T cells of Foxp3$^-$ mutant mice while simultaneously enhancing the production of the T_H1 cytokine IFN-γ (Fig 6, *A*). The eosinophilia associated with Foxp3 deficiency was markedly decreased in the double-mutant mice (Table I vs Fig 6, *B*), whereas the increased serum IgE concentrations were normalized (Fig 3 vs Fig 6, *C*). The goblet cell metaplasia observed in the lungs of Foxp3$^-$ mice disappeared in the double-mutant mice (Fig 6, *D*). These results are consistent with the progression of the allergic inflammatory response in Foxp3$^-$ mice by a T_H2 cytokine– and Stat6-dependent mechanism.

The effect of Stat6 deficiency on the survival of Foxp3$^-$ mice on the BALB/c background was analyzed by using Kaplan-Meier survival curves. Whereas Foxp3$^-$ mice on the BALB/c background had a median survival of 16.5 days, concurrent Stat6 deficiency prolonged the median survival to 23 days ($P = .0001$, log-rank test; Fig 7). More specifically, Stat6 deficiency appeared to protect against early mortality of Foxp3$^-$ mice on the BALB/c background, which is consistent with early lethality being precipitated by Stat6-dependent causes. There was no significant difference, however, in the upper limit of the survival span between Foxp3$^-$ and Foxp3$^-$/Stat6 knockout double-mutant mice, suggesting that this is set by the action of Stat6-independent pathways.

DISCUSSION

Severe allergic inflammation is a cardinal manifestation of loss-of-function *Foxp3* mutations in human subjects. Affected human subjects experience atopic dermatitis, food allergy, asthma, increased IgE levels, and eosinophilia. Although reports on the naturally occurring scurfy mouse and induced Foxp3$^-$ mutant mice have not specifically commented on the presence of dysregulated allergic inflammation, the data presented herein demonstrate that this is indeed the case. Furthermore, allergic dysregulation in Foxp3$^-$ mutant mice evolved independent of the

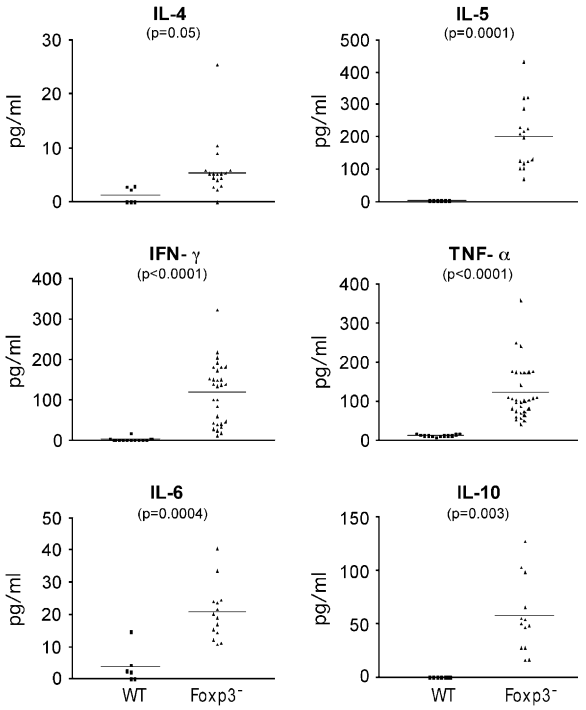

FIG 4. Serum concentration of T_H1 (IFN-γ and TNF-α), T_H2 (IL-4, IL-5, and IL-10) and proinflammatory (IL-6) cytokines in Foxp3$^-$ mice relative to a littermate control animal. Cytokine levels were determined by using the Cytoplex Bead Array. Results were analyzed by means of the unpaired, 2-tailed Student *t* test.

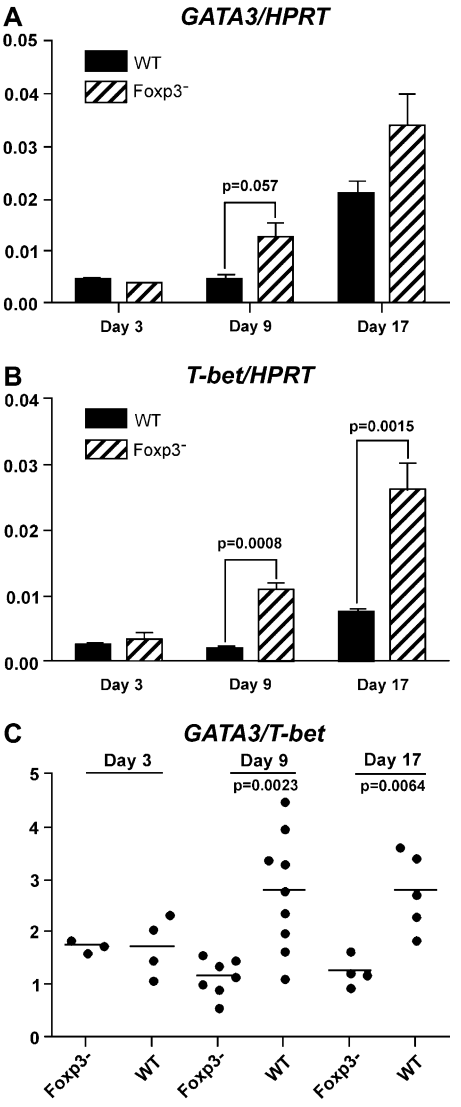

FIG 5. Quantitation of *GATA-3* and *T-bet* transcripts in splenocytes of WT and Foxp3$^-$ littermate pups. **A** and **B**, *GATA-3* and *T-bet* transcripts were quantitated by means of real-time PCR in unfractionated splenocytes of WT and Foxp3$^-$ littermate pups at ages 3, 9, and 17 days. Results are normalized to transcript levels of hypoxanthine phosphoribosyl-transferase *(HPRT)*. **C**, Ratio of *GATA3/T-bet* mRNA levels at the different age groups. Results were analyzed by using the unpaired, 2-tailed Student *t* test.

genetic background of affected mice. This phenotypic convergence in mice and human subjects indicates that allergic dysregulation is a fundamental attribute of Foxp3 deficiency.

Several pathologic features present in Foxp3$^-$ mice recall human allergic disorders, including spontaneous allergic airway inflammation, atopic dermatitis–like skin inflammation, and blood and tissue eosinophilia. Of particular interest is the extraordinary increase of serum IgE levels, one of the highest in the recorded literature. Remarkably, the dysregulated allergic inflammation in Foxp3$^-$ mice occurs in the setting of a concurrent and vigorous T_H1- and T_H2-type response and in the absence of biased T_H2 cell skewing *in vivo*. There was no evidence of preferential production of T_H2 cytokines relative to T_H1 cytokines either *in vitro* or *in vivo* because both types of cytokines were produced at very high levels by *in vitro*–stimulated Foxp3$^-$ lymphocytes and were detected at high levels in the sera of mutant mice. Also, there was no evidence of skewed expression of *GATA-3* transcripts relative to those of *T-bet* in the splenocytes, lung, or liver of Foxp3$^-$ mice compared with that seen in littermate control animals. These results are consistent with the expectation that Foxp3 deficiency would be associated with dysregulated T_H1 and T_H2 cell function given that $CD4^+CD25^+$ T_R cells can effectively suppress both the T_H1 and T_H2 lineages. Previous studies have indicated that rather than being counterregulatory, T_H1 and T_H2 cells act in concert to promote allergic inflammation.[24-26] Such synergy might well underlie the severity of the inflammatory response in Foxp3$^-$ mice.

The development of intense allergic inflammation and hyper-IgE responses in the absence of overt T_H2 skewing contrasts with observations made in other mouse models, including cytotoxic T lymphocyte–associated antigen (CTLA-4),[27] inhibitor of DNA binding 2 (Id2),[28] B cell CLL/lymphoma 6 (BCL-6),[29,30] and T-bet[31] knockout mice, in which the development of allergic inflammation occurs in the context of biased T_H2 skewing. Of note, disruption of CTLA-4, which is expressed on $CD4^+CD25^+$ T_R cells and implicated in their mechanism of action, is also associated with increased IgE levels.[27] However,

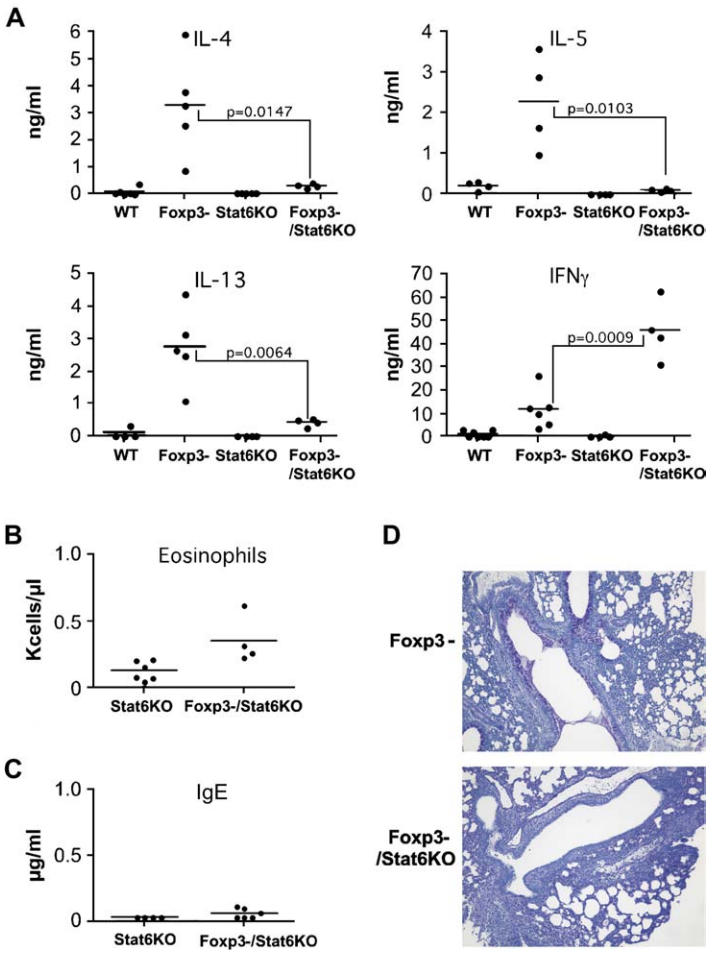

FIG 6. Concurrent Stat6 deficiency reverses attributes of allergic inflammation in Foxp3$^-$ mice. **A**, Production of IL-4, IL-5, IL-13, and IFN-γ by purified CD4$^+$ splenic T cells of 15-day-old WT, Foxp3$^-$, Stat6 knockout *(KO)*, and Foxp3$^-$/Stat6 knockout mice after *in vitro* stimulation with anti-T-cell receptor β *(TCRβ)* plus anti-CD28 mAbs. **B** and **C**, Peripheral blood eosinophil count (Fig 6, *B*) and serum IgE concentrations (Fig 6, *C*) in Stat6 knockout and Foxp3$^-$/Stat6 knockout mutant mice. **D**, Concurrent Stat6 deficiency abolishes goblet cell metaplasia of Foxp3$^-$ mutant mice (periodic acid–Schiff staining, original magnification 100×).

unlike Foxp3$^-$ mice, CTLA-4 knockout mice have been reported to exhibit biased T_H2 skewing but normal CD4$^+$CD25$^+$ T_R cell development and function, suggesting a distinct mechanism for increased IgE response in CTLA-4 knockout mice.[27,32] The lack of T_H2 skewing in Foxp3$^-$ mice differs from previous observations on *in vitro*–activated T cells of Foxp3-deficient human subjects, which were found to preferentially express T_H2 cytokines. The difference could reflect the evolution of human Foxp3 deficiency in the context of immunosuppressive therapy, might reflect an outcome of Foxp3 deficiency in the context of a human neonatal environment that strongly favors T_H2 responses, or both.

Foxp3$^-$ mice exhibited strikingly high levels of IgE that increased in an age-dependent manner to exceed the IgG1 levels of WT mice. Such levels raise the question of the mechanisms by which IgE is being induced. Potential mechanisms contributing to the hyper-IgE response of Foxp3$^-$ mice include enhanced bystander-type switching to IgE driven by local high-level production of IL-4, antigen-specific IgE responses driven by dysregulated T_H2 cell clones, and derepression of a direct inhibitory effect of T_R cells on B-cell production of IgE. The identity of antigens driving the IgE response remains unknown. Previous observations on subjects with IPEX have noted the presence of food allergy, which is consistent with reactivity to exogenous antigens driving at least part of the IgE response. Notwithstanding the fact that the mice were housed in a specific pathogen-free environment, environmental agents, such as microbial flora, might also act to exacerbate the allergic inflammation of Foxp3$^-$ mice. The role of food antigens and microbial flora in disease pathogenesis is the subject of ongoing investigation.

The allergic inflammatory stigmata observed in Foxp3$^-$ mice evolved through a Stat6-dependent mechanism, as evidenced by the abrogation of the hyper-IgE and goblet cell metaplasia and mucus production in the airway and the decrease of the eosinophil counts to near-normal

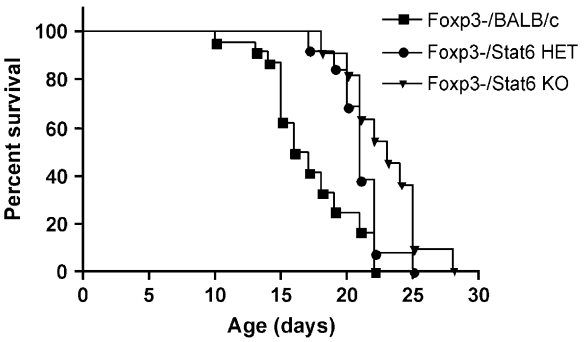

FIG 7. Effect of concurrent Stat6 deficiency on the survival of Foxp3$^-$ mice on BALB/c background. Kaplan-Meier survival curves generated on 24 Foxp3$^-$/Stat6 WT, 13 Foxp3$^-$/Stat6 heterozygous *(HET)*, and 11 Foxp3$^-$/Stat6 knockout *(KO)* male pups, respectively, all on BALB/c background. Median survival was 16.5 days (range, 10-22 days) for Foxp3$^-$/Stat6 WT mice, 21 days (range, 17-25 days) for Foxp3$^-$/Stat6 heterozygous mice ($P < .0045$ vs Foxp3$^-$/Stat6 WT mice, log-rank test), and 23 days (range, 18-28 days) for Foxp3$^-$/Stat6 knockout mice ($P < .0001$ vs Foxp3$^-$/Stat6 WT mice, log-rank test). All Foxp3$^+$/WT littermates survived normally (data not shown).

levels. A pathogenic function of the allergic inflammatory response in Foxp3 deficiency was evidenced by the protection of Foxp3$^-$/Stat6 knockout double-mutant mice against an early wave of mortality but not against a second later phase. The dissociation by Stat6 deficiency of the mortality curve of Foxp3$^-$ mice into early and late phases points to distinct sets of mechanisms underlying the lethality of Foxp3 deficiency at different developmental stages. Identifying these mechanisms might help to elucidate the immunoregulatory functions of T_R cells during development.

The observation that Foxp3$^-$ mice spontaneously exhibit several features associated with allergic diseases in human subjects, including allergic airway inflammation, atopic dermatitis–like skin disease, and exceedingly increased IgE levels, implicates abnormalities in T_R cell function in the pathogenesis of allergic disorders. Several human allergic disorders, including food allergy, atopic dermatitis, and asthma, have already been associated with abnormal antigen-specific T_R cell function. Such abnormalities might relate to the failure to develop, expand, or both antigen-specific T_R cells; impaired tissue homing of circulating T_R cells; and/or failure to upregulate suppressor functions of T_R cells once they enter the target tissue. Delineating the role of abnormal T_R cell function in common human allergic disease would have significant clinical and therapeutic implications.

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