Foxp3^{+} Tregs require WASP to restrain Th2-mediated food allergy.

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The authors have declared that no conflict of interest exists.

ABSTRACT

In addition to the infectious consequences of immunodeficiency, patients with Wiskott-Aldrich syndrome (WAS) often suffer from poorly understood, exaggerated immune responses that can result in autoimmunity and elevated levels of serum IgE. Here we show that WAS patients and mice deficient in Wiskott-Aldrich syndrome protein (WASP) frequently develop IgE-mediated reactions to common food allergens. In *Was*$^{-/-}$ animals, this adjuvant-free IgE-sensitization to chow antigens was most pronounced for wheat and soy, and occurred under specific pathogen free as well as germ-free housing conditions. Allergic responses to food allergens in WASP deficiency critically depended upon Foxp3^{+} Tregs, as conditional deletion of WASP in this immune compartment resulted in more severe Th2-type intestinal inflammation than observed in *Was*$^{-/-}$ counterparts. While WASP-deficient Tregs efficiently contained Th1 and Th17-type effector differentiation *in vivo*, they failed to restrain Th2 effector responses that drive allergic intestinal inflammation. Loss of WASP was phenotypically associated with increased GATA3 expression by effector memory, but not naïve-like, Foxp3^{+} Tregs, which occurred independently of increased IL-4 signaling. Our results reveal a novel, Treg-specific role for WASP that is required for prevention of Th2 effector cell differentiation and allergic sensitization to dietary antigens.

INTRODUCTION

Type 2 immunity is involved in a variety of host-defense functions, ranging from protection against parasites and support of epithelial barrier integrity, to regulation of wound healing and control of metabolic homeostasis (1-3). Many of these functions are performed by cells of the innate immune system, including eosinophils, basophils and mast cells, which in turn are orchestrated by Th2 lymphocytes of the adaptive immune system through the production of type 2 cytokines such as IL-4, IL-5 and IL-13. Th2 help can furthermore instruct B cells to produce immunoglobulin E (IgE), which arms mast cells and basophils with antigen-specific effector functions through binding to their high-affinity IgE receptor Fc□RI on the cell surface. Despite the presence of multiple inhibitory pathways of type 2 immunity (1), dysregulation within this system is increasingly common in Westernized societies and can result in the production of allergen-specific IgE, type I hypersensitivity reactions, and allergic tissue inflammation (1-3). In order to better understand the recent surge in incidence as well as the pathogenesis of Th2-driven allergic diseases, the study of aberrant activation of Th2 effector responses is of critical importance.

As is true for Th1 responses, Th2-mediated immune reactions critically rely on the function of regulatory T cells (Tregs) for their containment. Functional defects in Foxp3, the lineage-defining transcription factor that identifies the best-characterized population of Tregs, results in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in human patients, and in the IPEX-related scurfy phenotype in mice (4). In both species, the severe lymphoproliferation that ensues shows concomitant Th1 and Th2 effector responses, which may counter-regulate each other (5), and manifests clinically as a loss of tolerance-to-self and autoimmunity in combination with elevated IgE levels and food allergies (5-7). Emerging data implicate functionally distinct subsets of $CD4^+Foxp3^+$ Tregs that exert differential control over Th1 and Th2 effector cell proliferation, which may be related to their anatomic location of origin. For instance, thymus-derived Tregs were recently shown to specifically control Th1 immunity (8), whereas selective loss of peripherally-induced (i)Tregs resulted in uncontrolled Th2, but not Th1 or Th17-type inflammation (9). These findings fit within the broader paradigm that lineage-committed $Foxp3^+$ Tregs are

responsive to environmental cues and can assume tissue-specific phenotypes by co-opting other transcription factors such as GATA3 or T-bet (10-12). The mechanisms and consequences of this phenotypic variation and functional plasticity are only beginning to be understood.

In addition to the human IPEX syndrome, dysregulated Th2 responses, atopy and elevated IgE levels occur in a range of primary human immunodeficiencies, some of which are phenocopied in the corresponding mouse models (13-18). The variety of genes that are affected in these disorders - e.g. *STAT3*, *DOCK8, PGM3* and multiple genes involved in TCR signaling such as *LAT, ZAP70* or *RAG* - suggests that hyper IgE phenotypes can result from alterations in a number of distinct immunological pathways. In most of these cases, however, the underlying mechanisms of increased IgE production or the functional consequences of elevated serum IgE have not been studied in detail.

One well-known primary immunodeficiency with elevated serum IgE is Wiskott-Aldrich syndrome (WAS) (13, 14). WAS is caused by mutations in the Wiskott-Aldrich syndrome (*WAS*) gene on the X-chromosome, which encodes the Wiskott-Aldrich syndrome protein (WASP) with expression restricted to hematopoietic lineages. WASP is the founding member of a family of actin regulators, capable of transducing a variety of signals to mediate changes in the actin cytoskeleton, and has been implicated in a great variety of cellular functions in both lymphocytes and non-lymphocytes (19, 20). More than 100 unique loss-of-function mutations in *WAS* have been reported, giving rise to a clinically heterogeneous group of patients (19). The most severely affected males present early in life with thrombocytopenia, eczema, autoimmune sequelae and recurrent infections, which can be fatal in the absence of bone-marrow transplantation or gene therapy. In contrast, milder loss-of-function mutations have been identified in patients who suffer from an attenuated form of the disease termed X-linked thrombocytopenia (XLT), which has excellent long-term survival with medical management alone (21). Despite the long-known association between *WAS* mutations and atopy, the antigenic specificity of the expanded IgE pool and the consequences of elevated IgE on the prevalence of allergic disease are only beginning to be investigated in human patients (22) and have not been addressed in WASP-deficient mice.

Here we show that patients with mutations in *WAS* demonstrate an increased frequency of sensitization to food allergens as measured by serum specific IgE and skin prick testing and increased prevalence of clinically relevant food allergy in childhood when compared to the general population. Similarly, *Was*$^{-/-}$ mice spontaneously develop IgE-mediated immune responses and intestinal mast cell expansion. Using conditional WASP-deficient mice, we identified that WASP deficiency limited to Foxp3^{+} Tregs results in a strongly Th2-skewed, allergic inflammation of the small intestine, which was exacerbated compared to complete *Was*$^{-/-}$ counterparts. Phenotypically, WASP-deficient Tregs displayed elevated levels of Th2 transcription factor GATA3 in the effector-like, but not naïve subset of Foxp3^{+} Tregs, which occurred independently of IL-4. These findings demonstrate that WASP is required for Foxp3^{+} Tregs to exert selective control over Th2-type immune responses.

RESULTS

Sensitization to food antigens and food allergy are enriched among patients with* WAS *mutations. We assessed the overall burden of clinical food allergy within a cohort of 25 patients with mutations in the *WAS* gene. Results from three patients were excluded from the analysis as they could not be tested prior to hematopoietic stem cell transplantation. A variety of *WAS* mutations were observed in this cohort (**Figure 1A**), which consequently consisted of both patients with WAS (n=15) as well as XLT (n=10). Elevated IgE was observed in 59% of patients (13/22), and food allergen-specific IgE was detected in 33% (4/12) of WAS and 20% (2/10) of XLT patients (**Figure 1B**) (23). Compared to the general population, individuals with *WAS* mutations were more likely to demonstrate serum sensitization to peanut, milk, and egg (**Figure 1C**) (23). The prevalence of physician-diagnosed food allergy among WAS and XLT patients in childhood (20% and 30%, respectively) was also increased compared to rates reported among children in NHANES (6.5%) (24) and approached levels reported among children with moderate-severe atopic dermatitis (37±13%) (**Figure 1C**) (25), although none of the patients reported a history of anaphylaxis. Food sensitization was generally detected with greater sensitivity using serum IgE (sIgE) testing than by SPT (**Figure 1D**, left panel): peanut and egg white allergens failed to elicit positive SPT responses in two physician-diagnosed food allergic patients who were found positive for sIgE against these two allergens (**Figure 1D**, right panel). One patient with a clinical history consistent with food allergy was negative both by SPT and by serum-specific IgE to all tested food antigens. Five additional patients who underwent sIgE testing could not have SPT performed and were not included in evaluating the sIgE/SPT concordance rates. Taken together, our data demonstrate a marked enrichment of clinically relevant food-antigen-specific IgE in patients with mutations in *WAS.*

Was$^{-/-}$ *mice spontaneously develop IgE-mediated immune responses to food allergens.* Following the observation that WAS patients frequently develop IgE antibodies against food antigens, we investigated the occurrence of IgE-mediated responses to allergens in chow in WASP-deficient mice. *Was*$^{-/-}$ females or hemizygous *Was*$^{-}$ males (henceforth both referred to as *Was*$^{-/-}$ mice) on BALB/c, C57BL/6, and 129SvEv backgrounds developed elevated levels of total

serum IgE and IgG1 in comparison to background-matched wild-type (WT) controls (**Figure 2A** and **Supplemental Figure 1A, 1C**). Serum IgE increased with age and correlated positively with the density of surface-bound IgE on circulating basophils, indicating that binding of IgE to its high-affinity receptor FcεRI was unperturbed in *Was*$^{-/-}$ animals (**Supplemental Figure 1A, B**). We next developed ELISA-based assays to screen for antibodies against the five main components of mouse chow. Food-specific antibodies were not detected in WT mice while soy and wheat-specific IgE and IgG1 were readily detected in the serum of *Was*$^{-/-}$ mice (**Figure 2B**). For all investigated food extracts, a strong positive correlation between ingredient-specific titers of IgE and IgG1 was observed (**Supplemental Figure 1D**). The observation that food-specific IgE could be detected in *Was*$^{-/-}$ mice on three different genetic backgrounds indicated that sensitization to ingested antigens did not result from colonic inflammation since, in contrast to *Was*$^{-/-}$ mice on the colitis-prone 129SvEv background (26, 27), animals on the BALB/c and C57BL6 background are resistant to colitis (**Supplemental Figure 1E, F**).

To assess whether food-specific serum IgE from *Was*$^{-/-}$ mice was functional in mediating type I hypersensitivity reactions, bone marrow-derived WT mast cells were loaded with serum from *Was*$^{-/-}$ mice previously identified by ELISA to be sensitized to food antigens (FA Sens) or with serum from *Was*$^{-/-}$ mice of comparable IgE titer that had been found to not be sensitized to food (FA Non-Sens, **Figure 2C**). The appearance of lysosomal-associated membrane protein 1 (LAMP-1) at the cell surface of mast cells is an established surrogate marker for antigen/IgE-mediated histamine release (28, 29), and we found that WT mast cells loaded with serum from food-sensitized *Was*$^{-/-}$ mice degranulated in response to stimulation with an antigen extract from conventional chow (CCh), but not in response to an extract from a protein-free, amino-acid-based elemental diet (ECh, **Figure 2C**). In contrast, mast cells loaded with serum from non-food-sensitized animals were not activated by either food extract, which further demonstrated that IgE-dependent degranulation was antigen-specific and established that screening of antibody reactivity against the five main chow components is a reliable marker for the overall anti-food IgE reactivity.

Expansion of intestinal mast cells is a common symptom in food allergic patients (30, 31) and has been demonstrated to correlate with disease severity in experimental mouse models of

food allergy (32-34). In line with these findings, we observed expansion of the small intestinal mast cell pool in *Was*$^{-/-}$ mice as detected by chloroacetate staining on jejunal tissue sections (**Figure 2D**). In addition, mRNA expression levels of the mucosal mast cell marker *Mcpt1* was elevated in *Was*$^{-/-}$ animals and positively correlated with the cumulative anti-food IgE titer (**Supplemental Figure 1G**). Since *Was*$^{-/-}$ mice cannot eliminate food antigens from their diet, we hypothesized that persistent oral allergen exposure would result in continuous IgE/FcεRI-mediated degranulation of intestinal mast cells. Indeed, *Was*$^{-/-}$ animals had elevated serum levels of the mast cell protease MCPT1 (**Figure 2E**), which is released from mucosal mast cells in response to IgE-mediated detection of food antigens (32, 35). Moreover, anti-food IgE titers positively correlated with serum MCPT1 levels (**Supplemental Figure 1H**). As expected, elimination of the dietary allergen via switching *Was*$^{-/-}$ mice from conventional chow to an elemental amino acids-based chow resulted in a decline in serum MCPT1 after 7 days, with the most pronounced therapeutic effect (defined as ΔMCPT1) observed in mice with the highest cumulative anti-food IgE titers (**Figure 2F**). Oral rechallenge with soy extract after more than a week of allergen elimination led to rapid activation of mucosal mast cells indicated by increased levels of serum MCPT1, but did not result in signs of anaphylactic shock as measured by a reduction in core body temperature (**Figure 2G**). Combined, these data demonstrate that *Was*$^{-/-}$ mice develop food-specific IgE, which effectively mediates mast-cell degranulation *in vitro* and *in vivo.*

The microbial flora is dispensable for spontaneous oral sensitization to chow, but shapes the humoral anti-food response in Was*$^{-/-}$ *mice. Since commensal microbes play a dominant role in regulating IgE-mediated responses to food antigens (36-38), we assessed the contribution of the microbiome to the induction of food-specific IgE responses in *Was*$^{-/-}$ animals. When compared to specific pathogen free-housed (SPF) WT mice, both SPF and germ-free (GF) *Was*$^{-/-}$ mice showed elevated total IgE and IgG1 levels. Although total IgE was lower in GF *Was*$^{-/-}$ than in SPF *Was*$^{-/-}$ animals (**Figure 3A**), detailed food-specificity profiling of IgE and IgG1 revealed that sensitization to food occurred efficiently in mice housed under either condition (**Figure 3B**) with MCPT1 titers comparable between SPF and GF *Was*$^{-/-}$ mice (**Figure 3C**). Further comparative analysis of the isotype composition of the humoral anti-food response in GF *Was*$^{-/-}$ and SPF *Was*$^{-}$

$^{-/-}$ mice showed that anti-food IgG1 and IgG2b titers were elevated in the absence of microbes, whereas food-specific IgG3 and IgA levels were diminished (**Figure 3D and Supplemental Figure 2**). Since the chow used in SPF and GF setting differed only by one additional cycle of high-dose irradiation in the latter, it is reasonable to assume that all *Was*$^{-/-}$ animals were exposed to food of nearly identical antigenicity. Consequently, food-specific IgE responses in *Was*$^{-/-}$ mice do not require microbial modifications or co-signals that could potentially confer allergenic properties to food antigens. Moreover, these results demonstrate that food allergy in *Was*$^{-/-}$ animals does not require alterations in the commensal flora that may possibly occur as a consequence of WASP deficiency.

Conditional deletion of WASP in Foxp3^{+} regulatory T cells results in exacerbated Th2-type intestinal inflammation. WASP is expressed in all hematopoietic lineages, and its deficiency in dendritic cells, B cells, effector T cells or regulatory T cells (Tregs) has been described to have a variety of consequences for immune responses (19, 27, 39, 40). Unlike *Was*$^{-/-}$ mice, *Was*$^{-/-}$*Rag2*$^{-/-}$ double knockout mice presented with serum MCPT1 levels comparable to WT or *Rag2*$^{-/-}$ animals (**Supplemental Figure 3A**), indicating that intestinal mast cell expansion and activation in WASP deficiency does not occur in the absence of an adaptive immune system. We next analyzed strains with conditional deletion of WASP in B cells *(Was*$^{fl/fl}$*Mb1*-Cre), CD11c^{+} dendritic cells (*Was*$^{fl/fl}$*Itgax*-Cre) or Foxp3^{+} Tregs (*Was*$^{fl/fl}$*Foxp3*^{fl}*Foxp3*-Cre). Elevated serum MCPT1 was not found in animals harboring conditional deletions of WASP in B cells or dendritic cells, but was present in *Was*$^{fl/fl}$*Foxp3*-Cre females or *Was*^{fl}*Foxp3*-Cre males (henceforth also referred to as *Was*$^{fl/fl}$*Foxp3*-Cre mice) on both the C57BL/6 and 129SvEv backgrounds (**Figure 4A**). Histological analysis confirmed a profound expansion of mucosal mast cells in the small intestine of *Was*$^{fl/fl}$*Foxp3*-Cre mice but not in their *Was*^{wt}*Foxp3*-Cre littermates (**Figure 4B**). Mucosal mast cell infiltration occurred in the absence of gross histological changes and we observed no evidence of colitis in *Was*$^{fl/fl}$*Foxp3*-Cre mice on the C57BL/6 background (**Figure 4B**).

Following the observation that Treg-specific WASP deletion was sufficient for expansion of intestinal mast cells, we next asked whether WASP deficiency in other immune compartments contributed further to the pathogenesis of food allergic sensitization. Comparison of co-housed,

age and sex-matched *Was*$^{fl/fl}$*Foxp3*-Cre, *Was*$^{-/-}$ and WT animals revealed that *Was*$^{fl/fl}$*Foxp3*-Cre mice developed higher levels of total and soy-specific IgE and IgG1 (**Figure 4C**), as well as higher serum MCPT1 levels and increased intestinal *Mcpt1* mRNA expression (**Figure 4D**) than mice with complete deletion of WASP. More severe allergic intestinal inflammation was confirmed by digital mRNA expression profiling on jejunal tissue, which revealed a type 2 immune cluster that contained multiple mast-cell markers in addition to *Mcpt1*, including *Mcpt2, Fcer1a, Fcer1b,* and *Cpa3,* together with Th2-type cytokines *Il4, Il13* and *Il5* that were more prominently expressed in *Was*$^{fl/fl}$*Foxp3*-Cre than *Was*$^{-/-}$ mice (**Figure 4E and Supplementary Figure 3B**). This analysis indicated that jejunal inflammation was Th2-specific since mRNA levels of *Ifng*, *Il17a* and *Tnfa* in the small intestine were equivalent to WT levels in both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 4F and Supplementary Figure 3B**). Taken together, these results demonstrate that conditional deletion of WASP in Foxp3^{+} Tregs resulted in increased development of IgE-mediated immune responses to food antigens and Th2-specific small intestinal tissue inflammation.

***WASP-deficient Tregs fail to selectively suppress Th2 effector responses* in vivo.** *Was*$^{-/-}$ animals are known to have reduced Foxp3^{+} Treg numbers, and WASP-deficient Tregs exhibit aberrant proliferation and suppression in response to TCR stimulation *in vitro* (39, 41, 42). These abnormalities, which can be partly attributed to reduced levels of IL-2, have previously been associated with the occurrence of autoimmunity and colitis in *Was*$^{-/-}$ mice (39, 41). The sequelae of Treg-conditional WASP deficiency on intestinal immune homeostasis, however, have thus far remained undefined.

In sharp contrast to *Was*$^{-/-}$ mice, both relative and absolute Foxp3^{+} Treg numbers in the MLNs and Peyer's patches (PPs) of *Was*$^{fl/fl}$*Foxp3*-Cre were equal to or higher than those of WT counterparts (**Figure 5A and Supplemental Figure 4A**). This increase in Treg numbers compared to *Was*$^{-/-}$ mice could have been due to higher levels of IL-2, since CD4^{+} T cells from the MLNs of *Was*$^{fl/fl}$*Foxp3*-Cre mice were found to produce WT levels of IL-2 upon anti-CD3/CD28 stimulation (**Figure 5B**). We reasoned that a relative underrepresentation of peripherally-induced Foxp3^{+} Tregs (iTregs), which particularly control tolerance to foreign antigens (9), could potentially underlie the occurrence of IgE-mediated immune responses to food antigens. However, cell surface staining of

neuropilin-1, the absence of which specifically defines iTregs (43, 44), revealed that iTregs made up equivalent fractions of total Foxp3^{+} Tregs in WT, *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Supplemental Figure 4B**). Similarly, we found no differences in the activation-status of Tregs as *Was*$^{fl/fl}$*Foxp3*-Cre and WT mice showed equal fractions of CD44^{hi}CD62L^{lo} effector-memory Tregs (**Supplemental Figure 4C**).

Despite these normal numerical and phenotypical characteristics of WASP-deficient Foxp3^{+} Tregs, we observed that both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice developed a mild CD4^{+} lymphoproliferation in MLNs (**Figure 5C**). Within this expanded CD4^{+} T cell pool in MLNs of *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice, an increased fraction of cells displayed the CD44^{hi}CD62L^{lo} effector-memory T cell phenotype. Within this latter fraction, we observed an increase in Th2-skewed effector T cells as determined by their co-expression of ICOS and the Th2 transcription factor GATA3 (45, 46) (**Figure 5C and Supplemental Figure 4D**). In sharp contrast, the fraction of Th1-skewed, T-bet^{+} effector T cells was decreased in *Was*$^{fl/fl}$*Foxp3*-Cre mice, whereas the abundance of Th17-skewed, RORγt^{+} T cells was equivalent amongst all three genotypes (**Figure 5D**). This selective Th2 skew in the CD4 effector compartment was corroborated further by analysis of cytokine production from CD4^{+} mesenteric lymphocytes cultured *ex vivo*, which showed significantly increased production of IL-4 and IL-13, whereas IFN-γ and IL-17 production was similar to WT mice in both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre animals (**Figure 5F**). These results thus demonstrated that WASP-deficient Tregs are capable of normally regulating Th1 or Th17 differentiation, but fail to specifically contain Th2 effector responses *in vivo*.

To assess the pertinence of the Th2 cytokine IL-4 on the pathogenesis of food allergy in *Was*$^{-/-}$ mice, we then analyzed the extent to which food allergic sensitization occurred in *Was*$^{-/-}$*Il4*$^{-/-}$ animals. Anti-soy, as well as total IgE and IgG1 responses were completely abrogated in the absence of IL-4, and mucosal mast cell expansion and activation did not occur as assessed by serum MCPT1 levels (**Figure 5G and Supplemental Figure 4E**). Furthermore, anti-soy titers of the IL-4-independent IgG2b isotype were also significantly lower in *Was*$^{-/-}$*Il4*$^{-/-}$ mice (**Figure 5H**), signifying a critical role for Th2-mediated inflammation in the induction of anti-food humoral immune responses in WASP-deficient mice.

***WASP-deficient effector Tregs assume a Th2-like phenotype*.** Recent data indicate a role for Th2-type-programmed Tregs in an experimental model of murine food allergy as well as human children (47). Similarly, a Th2-promoting fraction of Tregs that depends on TCR signaling was identified in asthmatic mice and patients (48). Since aberrant responses to TCR activation are a feature of WASP-deficient T cells (49), we analyzed the extent to which WASP-deficient Tregs display a Th2-like phenotype. In both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice, we observed increased fractions of GATA3^{+}ICOS^{+} Tregs in MLNs compared to WT counterparts (**Figure 6A and Supplemental Figure 5A**). These cells were confined to the CD44^{hi}CD62L^{lo} effector-like Treg subset as GATA3 expression in the naïve-like fraction of Tregs was equivalent to WT CD4^{+}Foxp3^{+}CD44^{lo}CD62L^{hi} Tregs (**Figure 6B**). Th2-type reprogrammed Tregs in the context of food allergy had previously been observed in a genetic model that relies on increased signaling through the IL-4 receptor (47). We therefore asked whether WASP-deficient CD44^{lo}CD62L^{hi} Tregs exhibit an increase GATA3 expression pattern in response to increased availability of IL-4 in a Th2-skewed inflammatory setting. However, analysis of *Was*$^{-/-}$*Il4*$^{-/-}$ animals revealed that while Th2 skewing of effector T cells and IL-4 production from CD4^{+} mesenteric lymphocytes was abrogated (**Figure 6C**), an equivalent increase in the fractions of GATA3^{+}ICOS^{+} memory-effector Tregs was found when compared to *Was*$^{-/-}$ IL-4 competent counterparts, while relative Treg numbers were similar between groups (**Figure 6D and Supplemental Figure 5B**). Additional analysis revealed that increased expression of transcription factors did not extend to T-bet or RORγt, as memory-effector Tregs co-expressing these markers were found in similar frequencies amongst all genotypes (**Figure 6D**). These data demonstrate that WASP-deficient effector memory Tregs, but not naïve-like Tregs, assume a Th2-like phenotype independently from increased IL-4 signaling.

DISCUSSION

The Wiskott-Aldrich syndrome is an illustrative example of how investigating a monogenetic human disease can advance our understanding of immune pathways in health and disease (16, 19). Data from *Was*$^{-/-}$ mice (26) revealed that WASP-dependent functions are highly conserved between mouse and man and that many aspects of the human disease are faithfully mimicked in these animals. WASP is best characterized for its role as stimulator of ARP2/3-mediated actin polymerization, which enables a variety of downstream effector functions including tissue migration and filopodia stability. A vast body of literature has accumulated that addresses the molecular and immunological consequences of deficiency of WASP in not just T and B lymphocytes, but also iNKT cells, platelets, NK cells, mast cells, dendritic cells, monocytes and neutrophils (50). Because many of these WASP-dependent functions have been defined in either patients and mice lacking WASP in all hematopoietic lineages, or in isolated cell systems *ex vivo* or *in vitro*, the systemic sequelae of cell-specific perturbations in WASP-mediated signals has remained largely unknown (50).

Here we report an increased prevalence of allergic responses against common food antigens as a feature of WAS in humans. Although elevated levels of serum IgE and eczema are well-described characteristics of the disease, the burden of IgE-mediated allergic disorders among patients with WAS or XLT has remained largely uncharacterized. Our studies demonstrate that the elevated serum IgE pool in these patients is functional and has the capacity to mediate allergic reactions to common food antigens, which should prompt treating physicians to be vigilant for food allergy among these patients and to obtain a careful history of adverse events after food ingestion.

The connection between WASP deficiency and the occurrence of IgE-mediated reactions against foods was further corroborated by studies in *Was*$^{-/-}$ animals. WASP-deficient mice produce IgE and IgG1 antibodies with specificity for components of chow, most prominently soy and wheat, which are also two allergens commonly implicated in IgE-mediated food allergy in human patients (51, 52). These responses were observed in *Was*$^{-/-}$ mice on three different genetic backgrounds, independent of colonic inflammation, and occurred similarly in mice that were housed under germ-

free settings when compared to those colonized with an SPF-flora. As such, our findings suggest that mutations in *Was* predispose to allergic disease independently from microbe-derived signals or differences in strain-specific immune constitution.

Using a loxP/Cre-based system permitting cell-specific deletion of WASP (53), we demonstrated that Foxp3$^+$ Tregs are critically dependent on WASP-mediated signals for their ability to maintain tolerance to ingested food antigens. These results indicate that WASP deficiency in other immune compartments such as antigen presenting cells, B cells, or effector CD4$^+$ T cells is dispensable for the pathogenesis of food allergic responses in *Was*$^{-/-}$ mice and, potentially, WAS patients. In fact, the absence of WASP in non-Foxp3$^+$ immune cells is likely to have a net dampening effect on IgE sensitization to food antigens since both food-specific IgE titers and allergic intestinal tissue inflammation were significantly lower in *Was*$^{-/-}$ mice when compared with *Was*$^{fl/fl}$*Foxp3*-Cre mice. In support of this conclusion, work by Morales-Tirado et al. previously identified a Th2-specific dysfunction of WASP-deficient CD4$^+$ effector T cells, manifesting as a reduced production of IL-4 and compromised expulsion of *N. Brasiliensis* (54). Indeed, following *ex vivo* TCR stimulation, we confirmed reduced production of IL-4 and IL-13 by CD4$^+$ mesenteric lymphocytes obtained from *Was*$^{-/-}$ mice when compared to cells from *Was*$^{fl/fl}$*Foxp3*-Cre mice. In human WAS patients, which are more likely to resemble *Was*$^{-/-}$ mice than *Was*$^{fl/fl}$*Foxp3*-Cre animals, reduced effector function in Th2 lymphocytes may account for the observation that only 20-30% of WAS and XLT patients in our cohort suffer from clinically relevant food allergy. Notably, none of the food allergic patients suffered from anaphylaxis upon allergen exposure, which may be related to reduced mast-cell histamine secretion in response to IgE-mediated activation, as has been described for WASP-deficient mice (55). The discordance between sIgE levels and SPT in patients with mutations in *WAS*, with several false-negative SPT results, support the hypothesis of a certain degree of mast cell dysfunction in WAS patients.

Previous studies on Tregs in *Was*$^{-/-}$ mice have hypothesized that the immunopathology in these animals results in part from reduced numbers of Foxp3$^+$ CD4$^+$ lymphocytes in secondary lymphoid organs (39, 41, 42). However, we found that conditional deletion of WASP in *Was*$^{fl/fl}$*Foxp3*-Cre mice did not lead to lower Treg numbers in PP's or MLNs when compared to WT

animals. These findings implicate functional abnormalities in WASP-deficient Tregs as the principal cause of immune dysregulation in *Was*$^{-/-}$ mice. In further support of a Treg-intrinsic role for WASP in controlling aberrant immune responses, we observed increased expression of the Th2 transcription factor GATA3 in activated WASP-deficient Tregs from either *Was*$^{fl/fl}$*Foxp3*-Cre or *Was*$^{-/-}$ hosts when compared to WT animals.

Although the molecular mechanism of Treg-specific dysfunction in the absence of WASP remains to be elucidated, emerging data suggest aberrant signaling downstream of TCR ligation as a likely candidate. Increased Th2 responses and elevated IgE also result from loss of function of the TCR-associated scaffold protein LAT (56, 57). WASP is rapidly recruited to the TCR upon ligation (58), and is required for internalization of the TCR complex (59). Furthermore, WASP is indispensible for the formation of immune synapses, acting reciprocally with PKCtheta to maintain immune synapse stability (60). Interestingly, mice deficient in PKCtheta are resistant to allergic asthma, have reduced levels of serum IgE and show intact Th1 but aberrant Th2 responses (61), suggesting that unopposed activity of WASP at the immune synapse may give rise to the opposite phenotype we observed in *Was*$^{-/-}$ animals. The failure to maintain effective immune synapses between T cells and APCs has also been demonstrated in human WAS patients and was associated with reduced Ca^{2+} signaling and aberrant signal integration in WASP-deficient T cells (62). Since Tregs have been shown to require continuous TCR-dependent signaling for optimal suppressive function (63, 64), such alterations in downstream events following TCR-mediated activation in the absence of WASP may be responsible for the loss of Th2-suppressive capacity. In this regard, unrestrained Th2 responses, elevated serum IgE and allergic airway inflammation were reported in mice with Treg-conditional deletion of the protein kinase CK2 (48). Moreover, deficiency of the TCR-associated phosphatase SHP-1 results in an increased population of GATA3^{+} Tregs and food allergy similar to WASP-deficiency (47). Together, these findings support a link between aberrant TCR signaling in WASP-deficient Tregs and unrestrained Th2 pathology *in vivo*.

In summary, we identified an increased prevalence of IgE-mediated food allergy amongst a cohort of patients with mutations in *WAS*. Using *Was*$^{-/-}$ mice, we showed that IgE sensitization occurs both in the presence and absence of an intestinal flora and that the deficiency of WASP in

Foxp3^{+} Tregs alone is sufficient to drive allergic intestinal inflammation. While capable of fully containing Th1 and Th17 effector responses *in vivo,* WASP-deficient Tregs exhibit a Th2-like phenotype and fail to selectively restrain Th2 effector differentiation resulting in intestinal mast cell expansion and activation. Our findings demonstrate that in WAS, defective Foxp3^{+} Tregs promote Th2-type immune hyperactivation and allergic disease.

METHODS

Patients. 25 consecutive patients with Wiskott-Aldrich syndrome (WAS) or X-linked thrombocytopenia (XLT) followed by NHGRI on an active clinical protocol (NCT00006319), provided informed consent on an NIH IRB-approved research protocol designed to study atopy (NCT01164241). Prior to enrollment, a clinical diagnosis of WAS or XLT was confirmed by mutation analysis of the WAS gene and WASP protein expression as previously described (65). Comprehensive allergic histories, review of all available outside records pertaining to prior allergic or immunologic evaluation and therapeutic intervention, as well as physical examinations were performed at the Clinical Center of the National Institutes of Health (NIH). Total and allergen-specific serum IgE levels were quantified by ImmunoCAP (Uppsala, Sweden) from all patients (n = 25). Skin prick testing to a panel of common allergens was performed in patients with sufficient intact / non-inflamed skin (n = 14) and compared to standard clinical positive and negative controls. Because three individuals had previously undergone hematopoietic stem cell transplantation, data from sera and skin prick tests were excluded from analysis.

Animals. The generation of *Was*$^{-/-}$*, Was*$^{-/-}$*Il4*$^{-/-}$*, and Was*$^{-/-}$*Rag2*$^{-/-}$ mice has been previously described (26, 27) and *Was*$^{-/-}$ animals have since been made commercially available from The Jackson Laboratory (Bar Harbor, ME). All mice used in this study were bred in house and maintained in accordance with institutional guidelines in specific pathogen-free conditions at the Boston Children's Hospital. 129SvEv *Was*$^{-/-}$ animals were re-derived in germ-free conditions at Boston Children's Hospital. C57BL/6 mice harboring floxed *Was* alleles (*Was*$^{fl/fl}$ females *or Was*^{fl} males*)* have been previously described (53) and were backcrossed for 10 generations onto the 129SvEv background. *Itgax*-Cre mice and *Foxp3*-Cre mice on the C57BL/6 background were obtained from Jackson laboratory and backcrossed for at least 10 generations onto the 129SvEv background. Serum from *Was*^{fl}*Mb1*-Cre and *Was*^{wt}*Mb1*-Cre animals was kindly provided by Drs. Stefano Volpi and Luigi Notarangelo at Boston Children's Hospital.

Chow and chow extracts. All mice were kept on irradiated Prolab® Isopro® RMH 3000 (LabDiet, St. Louis, MO) throughout their lives. Crude samples of the five main (%w/w) components (in order of abundance: ground wheat, soybean meal, wheat middlings, ground yellow corn, and fish meal) were obtained directly from LabDiet. Protein extracts for *in vitro* studies were generated by soaking approximately 10 gram of homogenized chow pellets or meal component in 40 ml of phosphate buffered saline (PBS) in a 50 ml Falcon® tube, which was placed in a shaking incubator at 37 °C for 4 hours, and then spun at 5000 x g for 10 minutes. The supernatant containing solubilized food antigens was then ultra-centrifuged (100,000 x g) for 1 h and sterile-filtered using a 20-micron syringe filter (Corning, Tewksbury, MA). Protein concentration of extracts was quantified using a colorimetric protein assay (Bio-Rad, Hercules, CA). For allergen elimination experiments, mice were transferred to a clean cage containing a protein-free, amino acid-based formula (Research Diets Inc, New Brunswick, NJ). An elemental chow extract was obtained from mortar-and-pestle-homogenized pellets as described for regular chow.

ELISAs. Total and OVA-specific IgE and IgG1 ELISAs were performed as previously described (34) using the following reagents: capture antibodies: anti-mouse IgE (southern biotech 1110-01) and anti-mouse IgG1 (1070-01); protein standards: mouse IgE and IgG1 κ isotype controls (BD Pharmingen), and mouse anti-ovalbumin IgE (MCA2259, AbD Serotec); detection reagents: horseradish peroxidase-conjugated anti-mouse IgE (southern biotech 1110-05) and IgG1 (southern biotech 1070-05), biotin-conjugated ovalbumin (US Biologicals), and streptavidin-conjugated horseradish peroxidase (BD Pharmingen).

For food-specific Ig ELISAs, 96-well polystyrene plates (Costar assay plates, Corning) were coated overnight with 100 µl of food extract in PBS (100 $\mu g ml^{-1}$ protein concentration). Plates were subsequently washed 6 times with 300 µl of 0.05% tween in PBS (washing buffer) and blocked with 10% fetal calf serum (FCS) in PBS for a minimum of 2 h at room temperature. Serum dilutions ranging from 1:30 to 1:5000 were prepared in 2% FCS in PBS buffer and transferred to wells in a volume of 100 µl. Plates were incubated at room temperature for 3 h and then washed again 6 times in washing buffer, after which anti-food Ig antibodies were detected with horseradish

peroxidase-conjugated antibodies to IgE (1110-05), IgG1 (1070-05), IgG2a (1080-05), IgG2b (1090-05), IgG3 (1100-05), or IgA (1040-05, all from Southern Biotech) in 1:1000 dilutions in 2% FCS in PBS for 1 h at room temperature. Following 6 additional washes, plates were developed in the dark for 3-10 minutes with 100 µl of tetramethylenbenzidine per well (KPL, Gaithersburg, MD). This reaction was stopped by the addition of 50 µl of 2M H_2SO_4 prior to spectrophotometric analysis at 450nm (Perkin Elmer). To allow semi-quantitative comparison of reactivity against different food extracts, all five investigated extracts were coated in equivalent concentrations in adjacent wells on the same 96-well plate. For each sample, a sixth well was included that was coated with PBS alone and the OD of this blank well was subtracted from the OD of the extract-coated wells to correct for any non-specific binding and thus obtain the Ig reactivity against any of the five components. Cumulative anti-food Ig titers were defined as the sum of the blanked OD values of all five investigated extracts. For some experiments, wells were coated with combined wheat and soy extracts, and 2-fold serial dilutions of a positive serum sample were used to quantify the anti-food IgG1 reactivity as arbitrary units/ml (U/ml).

Serum MCPT1, as well as IL-4, IL-13, IL-2, IL-17a and IFN-γ ELISAs were performed using a commercially available kit (eBioscience) according to the manufacturer's instructions.

Flow cytometric analysis. To assess basophil-surface IgE loading, whole blood samples were collected in EDTA. Following lysis of erythrocytes in RBC lysis buffer (eBioscience), cell pellets were blocked with anti-mouse CD16/CD32 (clone 2.4G2, BD Pharmingen) and stained with anti-mouse CD45 (clone 30-F11), anti-mouse CD49b (clone DX5), anti-mouse FceRIa (clone MAR-1) and anti-mouse IgE (clone RME-1, all from Biolegend). Basophils were identified within peripheral blood mononuclear cells as $CD45^{Mid}CD49b^{+}FceRIa^{+}IgE^{+}$, and IgE surface loading was determined from the mean fluorescence intensity of the anti-IgE stain. Immune profiling of MLN and PP lymphocytes was performed using the following fluorochrome-conjugated antibodies: antio-CD16/CD32 (clone 2.4G2, BD Pharmingen), anti-CD4 (clone GK1.5), anti-Foxp3 (clone FJK-16s, eBioscience), anti-GATA3 (clone TWAJ, eBioscience), anti-CD44 (clone IM7), anti-CD62L (clone MEL-14), anti-ICOS (clone C398.4A), anti-T-bet (clone 4B10), anti-RORγt (clone B2D,

eBioscience) anti-neuropilin-1 (product number FAB556A, R&D systems) and Fixable Viability Dye eFluor® 450 (eBioscience). Transcription factors were stained using the Foxp3/Transcription Factor Staining kit (eBioscience).

Mast cell degranulation assays. Bone marrow-derived cells from WT BALB/c mice were differentiated to mast cells *in vitro* by culturing cells in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 100 U/ml penicillin and 100 $\mu g ml^{-1}$ streptomycin, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 10 $ng ml^{-1}$ interleukin-3 and 20 $ng ml^{-1}$ stem cell factor (SCF) (henceforth referred to as mast cell medium). Experiments were performed using cultures that were at least 8 weeks old and had a purity of >90% as assessed by cell surface co-expression of mast cell markers c-kit (clone 2B8) and FceRIa (clone MAR-1, both from Biolegend) by flow cytometry. Prior to degranulation assay, mast cells were resuspended in 10^6/ml mast cell medium and loaded overnight with dilutions of mouse serum in concentrations as indicated per experiment. The next day, cells were washed twice in medium, plated in a 96-well tissue culture plate (10^5 cells in 100 µl per well) and stained with viability dye. Cells were then challenged for 10 minutes with an antigen cocktail containing solubilized chow antigens (protein concentration 5 $\mu g ml^{-1}$) or OVA (500 $ng ml^{-1}$), in combination with anti-mouse LAMP-1 antibody (clone 1D4B, Biolegend). Following two washes with cold flow cytometry buffer (0.01% NaN_3 in PBS), cells were acquired on a FACSCanto II flow cytometer (BD Bioscience) and further analyzed using FlowJo software (Treestar).

Histology and mast cell staining. Following euthanasia, intestinal sections were isolated and flushed with cold PBS. Three jejunal sections per animal were fixed in formalin for 24h and then transferred to 70% ethanol. Tissue samples were paraffin-embedded and sectioned by the Histology Core facility of Beth Israel Deaconess Medical Center (Boston, MA), and mast cell quantification was performed as described (33). In brief, slides were deparaffinized in sequential xylene baths and rehydrated in graded alcohol solutions. Chloroacetate esterase staining solution consisted of 0.04% naphtol AS-D chloroacetate (Sigma), 0.04% pararosaniline (Sigma), and 0.04% sodium nitrite (Sigma) in PBS, and was used to stain slides for 30 minutes at room temperature. Slides

were counterstained with modified Harris hematoxylin solution (Sigma) and mounted in Permount (Fisher). For statistical analysis, a blinded investigator counted mast cells in 4 randomly selected high-power fields. Images were captured using an Olympus DP70 microscope equipped with DP Controller software (Olympus corporation).

Quantitative RT-PCR. Three jujunal tissue sections were pooled and stored in RNAlater (Qiagen). Following tissue disruption and homogenization, total RNA was extracted using an RNeasy Plus mini kit (Qiagen) and reverse transcribed using iScript Supermix (Bio-Rad). *Mcpt1* gene expression was assessed with iQ SYBR Green Supermix (Bio-Rad) using the following primers: FOR 5'-GAG GAC AGA TGT GGT GGG TTT-3' and REV 5'-AGG AGT CAA CTC AGC TTT CTC TT-3', and normalized against expression of housekeeping gene *Hprt* (FOR 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3' and REV 5'-GAG GGT AGG CTG GCC TAT AGG CT-3'). Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method, in which the ΔCt of a WT sample was set as reference value.

In vivo *allergen challenge.* To assess the occurrence of oral anaphylaxis, mice were starved for 3 hours and then challenged with 12.5 mg soy protein in 300 µl PBS by gavage. Body temperature was registered with the use of implantable temperature transponders (IPTT-300, Biomedic Data Systems, Seaford, Del). Baseline temperature was defined as the average of three measurements prior to challenge, and severity of anaphylaxis was assessed by calculating the ΔT from this average every 10 minutes after challenge.

Ex vivo *culture and stimulation of mesenteric lymphocytes.* Following isolation of MLNs, single cell suspensions were purified using a MagniSort Mouse $CD4^+$ T cell Enrichment Kit (eBioscience) according to the manufacturer's protocol and resuspended and plated in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 100 U/ml penicillin, 100 $\mu g ml^{-1}$ streptomycin, 2 mM L-glutamine and 50 µM -βmercaptoethanol. Cytokine production was determined by ELISA in supernatants obtained in triplicates from 200,000 cultured cells per well that were stimulated for 18-24h with anti-CD3/CD28 using Dynabeads® (Life Technologies).

Nanostring assay and hierarchical cluster analysis. Digital gene expression profiling was performed on isolated whole tissue jejunal RNA as previously described using a customized Nanostring® codeset consisting of 86 inflammatory targets and five housekeeping genes (34). 57 out of 86 investigated genes that were differentially expressed in any pair-wise 2-sided t-test comparison ($p<0.05$) were subjected to hierarchical cluster analysis based on absolute Pearson correlation value with pairwise average linkage using the GenePattern algorithm (http://genepattern.broadinstitute.org).

Statistical analysis. All results were analyzed and visualized using GraphPad Prism version 6.0c for Mac (GraphPad Software). Details pertaining the use of statistical tests are provided in the Figure legends.

Study approval. All patients provided informed consent prior to inclusion in the study. Clinical studies were performed under an NIH IRB-approved research protocol (NCT01164241). All animal experiments were performed in accordance with Institutional Animal Care and Use Committee-approved protocols number 13-06-2415R and 14-04-2677R (IACUC, Boston Children's Hospital), and adhered to the National Research Council's 'Guide to the care and Use of Laboratory Animals'.

AUTHOR CONTRIUBTIONS

Designed and conducted the clinical patient study: JJL, MGL, EG, PS, CCN, NJ, KDS, FC, JDM; Conceived and designed the mouse experiments: WSL, JAG, SBS, EF; Performed mouse experiments: WSL, JAG, JJ, MD; Acquired and analyzed data: WSL, JJL; Obtained funding: WSL, EHHMR, JDM, SBS, EF; Provided reagents: AJT; Wrote the manuscript: WSL; Edited the paper: JJL, JAG, JDM, SBS, EF. All authors approved the final version of the manuscript.

ACKNOWLEDGEMENTS

We thank members of the Fiebiger laboratory and the Snapper laboratory for discussion and technical assistance. This work was supported by grants from the National Institutes of Health: DK106311 (to JAG), DK034854 (to SS), and AI075037 (to EF). This work was further supported by a senior research grant of the Crohn's and Colitis Foundation of America (to EF), an unrestricted gift from the Mead Johnson Nutrition Company (to WSL and EF), the Harvard Digestive Diseases Center Grant P30DK034854. Additional support was provided by the Helmsley Charitable Trust, and the Wolpow Family Chair in IBD Treatment and Research (to SBS). Funding for clinical patient studies was provided in part by the Division of Intramural Research of the NIAID and NHGRI, NIH.

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