How defects in central tolerance impinge on a deficiency in regulatory T cells

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Both central (thymic) and peripheral (nonthymic) mechanisms are important for the induction and maintenance of T cell tolerance. Mice with a defect in Foxp3, required for the generation and activity of $CD4^+CD25^+$ regulatory T cells, exhibit massive lymphoproliferation and severe inflammatory infiltration of multiple organs, in particular the lungs, liver, and skin. We have explored how this phenotype is influenced by an additional defect in central tolerance induction, generated by either crossing in a null mutation of the *Aire* gene or substituting the nonobese diabetic (NOD) genetic background. The double-deficient mice had fulminant autoimmunity in very early life and a gravely shortened lifespan vis-à-vis single-deficient littermates. They showed massive lymphoproliferation and exacerbated inflammatory damage, particularly in the lungs and liver. Yet, the range of affected sites was not noticeably extended, and, surprisingly, many organs, or regions of organs, remained untouched, suggesting additional important mechanisms to enforce immunological self-tolerance.

Aire | Foxp3 | central tolerance | peripheral regulation | autoimmunity

A variety of mechanisms have been proposed to explain how the immune system can tolerate self-constituents while effectively destroying foreign pathogens. For T lymphocytes, relevant processes include clonal deletion and/or anergy during T cell differentiation in the thymus or during encounter with antigens in the periphery, ignorance imposed by a variety of means, deviation to nonpathogenic T cell subsets, and extrinsic suppression of effector cells by regulatory T (Treg) cells. Which of these mechanisms dominate and which might be experimental exaggerations remain matters of debate, as does the relative importance of central tolerance induction vs. peripheral regulation (1, 2).

Central tolerance, also known as thymocyte negative selection, results in deletion or inactivation of potentially autoreactive T cells in the thymus. This process has long been viewed as a major mechanism of preventing autoimmunity (1, 3, 4), and important confirmation of this viewpoint has recently come from two mouse models. Mice lacking Aire serve as a model for a rare human disease, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (called APECED), characterized by mucocutaneous candidiasis and a broad spectrum of autoimmune disorders, directed mostly against endocrine organs. The autoimmune nature of this disease is indicated by lymphocytic infiltration of the target organs and circulating autoantibodies (autoAbs) against them (5, 6). APECED patients carry a defective *AIRE* (autoimmune regulator) gene (7, 8); mice bearing a mutation of the homologous *Aire* gene also show multiorgan autoimmunity manifested as lymphocytic infiltration and autoAbs (9–11). Aire was shown to exert its antiautoimmunity function at the level of thymic stromal cells, regulating the ectopic expression of a battery of peripheral-tissue antigens, e.g., insulin, fat acid-binding protein, and salivary protein-1 (10). Subsequent studies confirmed that Aire-null mice have a defect in the clonal deletion of autoreactive thymocytes (12, 13) and that they have an effective peripheral compartment of Treg cells (11, 13). Nonobese diabetic (NOD) mice are a model of type 1 diabetes (14, 15). They are best known for autoimmune attack on the pancreas but can also show several other autoimmune manifestations, arguing for a generalized defect (or defects) in immunological tolerance. Although much attention has been paid to putative problems of peripheral regulation [e.g., deficits in the number or activity of regulatory natural killer (NK)-T (16) or $CD4^+CD25^+$ T (17, 18) cell populations], several recent studies have demonstrated an important defect in central tolerance induction (19–23). This defect operates at the level of the thymocyte rather than the thymic stromal cell (20, 22, 23) and reflects the interplay of several genomic intervals (21, 23).

Even in ostensibly normal individuals, negative selection of thymocytes is not foolproof: autoreactive T lymphocytes can be quite easily detected in the periphery (24, 25). These potentially pathogenic cells must be kept in check by the diverse mechanisms of peripheral tolerance induction. One of these mechanisms, dominant suppression by Treg cells, has become a major focus, partially because of its potential in the treatment of autoimmune and other human diseases. An important population of Treg cells, discovered by Sakaguchi and colleagues (26), is the small $CD4^+CD25^+$ T cell subset: eliminating these cells by neonatal thymectomy resulted in autoimmune gastritis, colitis, thyroiditis, oophoritis, orchitis, etc., and reintroducing them reversed these autoimmune manifestations (26, 27). However, CD25 is not unique to Treg cells: it is also expressed by activated T lymphocytes. The most specific marker to date of $CD4^+$ Treg cells is the transcription factor Foxp3, which is required for the generation and activity of the $CD4^+CD25^+$ population (28–32). Humans lacking FOXP3 have an autoimmune disorder, immunodysregulation polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome, characterized by chronic diarrhea, type 1 diabetes, and eczema in early childhood and also by lymphadenopathy, hypothyroidism, anemia, thrombocytopenia, and respiratory distress. Pathological analysis reveals inflammatory damage in the lungs, liver, skin, thyroid gland, pancreas, intestines, and kidneys. In addition, many IPEX patients have autoAbs against a diversity of organs (33). Mice with a null mutation of *Foxp3* have massive lymphoproliferation and severe inflammatory infiltration of the skin and liver (34, 35).

To weigh the relative roles of central tolerance and peripheral regulation and to explore their interdependence in controlling autoimmunity, we generated mice with deficiencies in both "arms" of immunological tolerance. The double-deficient animals had a mutation in Foxp3-derived Treg cells as well as a defect in thymocyte clonal deletion imposed by the *Aire*-null mutation or the NOD genetic background (or both).

Materials and Methods

Mice. B6.*Foxp3*^{sf} mice, carrying the "scurfy" null mutation of the *Foxp3* gene (35) and originally from The Jackson Laboratory,

Abbreviations: Treg cell, regulatory T cell; autoAb, autoantibody; NOD, nonobese diabetic; TCR, T cell antigen receptor; H&E, hematoxylin/eosin.

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were at a minimum of 10 generations of backcross onto the C57BL/6 (B6) background. To generate NOD.*Foxp3*^{sf} animals, we crossed the mutation more than nine generations onto the NOD/Lt background. The *Aire*^{o} mutation (10) has been backcrossed onto the B6 or NOD/Lt background for 10 generations. *Foxp3*^{sf} and *Aire*$^{o/o}$ mice were intercrossed to generate *Foxp3*^{sf}*Aire*$^{o/o}$ mice deficient in both *Foxp3* and *Aire*. B6.*Foxp3*^{sf} mice were crossed with mice carrying both the *OTII* (36) and *RIP-mOVA* (37) transgenes on the B6 background (OTII/RIP-mOVA/B6) to create OTII/RIP-mOVA/B6.*Foxp3*^{sf} mice and controls.

Histology. For a histological survey of the whole mouse, animals were killed, and the entire body was fixed in Bouin's solution (Sigma). Paraffin-embedded sections of all major organs and/or different regions of the whole body were stained with hematoxylin/eosin (H&E) and examined by microscopy. Inflammatory damage was scored as trace, 0.5; mild, 1; moderate, 2; severe, 3; or very severe, 4.

Tissue-reactive autoAbs were assayed as described before (10). Briefly, cryosections of the liver, pancreas, and lungs from recombination activating gene 1 (RAG1)-deficient B6 or NOD mice were fixed in acetone and used as substrates for immunostaining. Sera were diluted 1:20 and applied to the sections; bound IgG or IgM Abs were revealed by FITC-conjugated goat anti-mouse IgG or IgM (Jackson ImmunoResearch).

Flow Cytometry. Single-cell suspensions of lymphoid organs were prepared and blocked with anti-CD16/32 (2.4G2) and normal mouse serum (Jackson ImmunoResearch) against Fc-mediated nonspecific binding of Abs. The following Ab conjugates were used for immunostaining: FITC-conjugated anti-CD44, anti-TCRβ, and anti-TCR Vα2 (BD Pharmingen); phycoerythrin (PE)-conjugated anti-CD62L, anti-CD69, and anti-TCR Vβ5 (BD Pharmingen); anti-CD4-PE-Texas red and anti-CD8-Tricolor (Caltag, South San Francisco, CA). Stained samples were analyzed with an Epics XL flow cytometer (Beckman Coulter).

Results

A Double Deficiency in Peripheral Regulation and Central Tolerance Induction Gravely Shortens Lifespan. Mice lacking both Foxp3 and Aire on the B6 genetic background [B6.*Foxp3*^{sf}*Aire*$^{o/o}$ (n = 6)] had a normal appearance and growth rate until ≈10 days after birth; however, by ≈14 days of age, they were obviously runted and exhibited hair loss and skin damage on their ears and tail (data not shown). All B6.*Foxp3*^{sf}*Aire*$^{o/o}$ animals died or had to be killed before 28 days of age (Fig. 1 *Left*). Mice deficient in only Foxp3 [B6.*Foxp3*^{sf} (n = 5) or B6.*Foxp3*^{sf}*Aire*$^{+/o}$ (n = 6)] also suffered hair loss, a scaly tail, and ear damage but showed only minimal signs of weight loss at 14 days (data not shown). They survived up to ≈50 days (Fig. 1 *Left*). Single-deficient B6.*Aire*$^{o/o}$ animals (n = 6) or female *Aire*$^{o/o}$ animals carrying the *Foxp3*^{sf} mutation on one X chromosome [*Foxp3*$^{sf/+}$*Aire*$^{o/o}$ (n = 5)] remained overtly healthy for at least 120 days (Fig. 1 *Left* and data not shown).

Coupling the Foxp3 deficiency with the NOD, rather than the B6, genetic background also led to more rapid mortality: all NOD.*Foxp3*^{sf} (n = 6) or NOD.*Foxp3*^{sf}*Aire*$^{+/o}$ (n = 7) mice died or had to be killed before 28 days of age (Fig. 1 *Right*); there was no significant difference in the survival rate between these two groups. In contrast, the *Aire* deficiency on the NOD background [NOD.*Aire*$^{o/o}$ (n = 5) and NOD.*Foxp3*$^{sf/+}$*Aire*$^{o/o}$ (n = 7)] had no evident impact on early mortality (Fig. 1), although these animals did show severe disease by 70–90 days of age (data not shown). *Foxp3*-deficient mice endowed with both defects in central tolerance [i.e., NOD.*Foxp3*^{sf}*Aire*$^{o/o}$ mice (n = 7)] had a further shortened lifespan (Fig. 1 *Right* and data not shown).

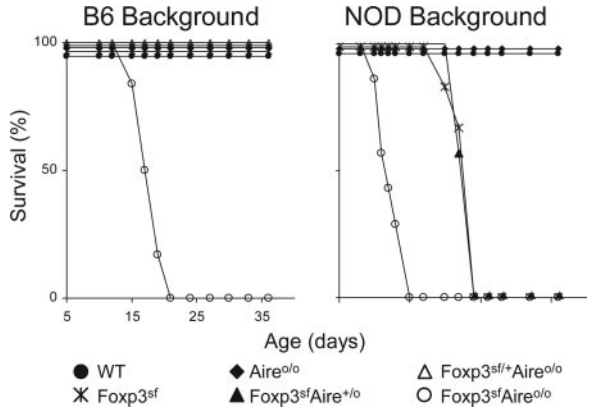

Fig. 1. A gravely shortened lifespan in mice deficient in both central and peripheral tolerance. Survival rates of normal, *Aire*$^{o/o}$, *Foxp3*$^{sf/+}$*Aire*$^{o/o}$, *Foxp3*^{sf}, *Foxp3*^{sf}*Aire*$^{+/o}$ and *Foxp3*^{sf}*Aire*$^{o/o}$ mice on either the B6 (*Left*) or NOD (*Right*) genetic background (n = 5–7 for each group).

Autoimmune Pathology. To ascertain the cause of early death in B6.*Foxp3*^{sf}*Aire*$^{o/o}$ mice, we performed a histological survey of the whole body of animals of different ages. We did not find inflammatory infiltrates in any organs of 6-day-old animals of any genotype. At 9 days of age, at a time when mice lacking just *Foxp3* exhibited no signs of inflammation in any of these organs, some regions of the lungs in littermates deficient in both *Foxp3* and *Aire* (n = 3) were infiltrated by lymphocytes and other inflammatory cells (Fig. 2*a*). The other organs of the double-deficient mice remained free of inflammation at this age. By 14 days, most regions of the lungs of B6.*Foxp3*^{sf}*Aire*$^{o/o}$ mice (n = 4) were invaded by inflammatory cells. B6.*Foxp3*^{sf} and B6.*Foxp3*^{sf}*Aire*$^{+/o}$ (n = 5) littermates also presented with inflammatory infiltrates in the lungs, comparable in these two single-deficient strains but far less extensive than in the double-deficient animals.

Another internal organ subject to severe damage at 2 wk was the liver. In both B6.*Foxp3*^{sf}*Aire*$^{o/o}$ and B6.*Foxp3*^{sf} mice, the hepatic portal areas were heavily surrounded by polymorphic mononuclear cells and lymphocytes. Whereas most hepatocytes in *Foxp3* single-deficient animals appeared intact, up to 50% of those in their double-deficient littermates were necrotic (Fig. 2 *b–d*). We did not see a significant difference in the liver pathology of B6.*Foxp3*^{sf} and B6.*Foxp3*^{sf}*Aire*$^{+/o}$ single-deficient mice.

B6.*Foxp3*^{sf}*Aire*$^{o/o}$, B6.*Foxp3*^{sf}, and B6.*Foxp3*^{sf}*Aire*$^{+/o}$ mice all showed moderate dermal infiltration by lymphocytes, macrophages, and granulocytes, leading to the trademark scurfy cutaneous lesions. We did not notice any significant differences between single- and double-deficient mice in this regard (Fig. 2*d*).

Foxp3^{sf}*Aire*$^{o/o}$ mice also exhibited mild pancreatitis, but the infiltration was limited to interstitial areas, leaving the islets and the vast majority of the exocrine tissue intact. We also detected mild interstitial infiltration and occasional glomerular damage in the kidneys and trace-level inflammation in the fat, skeletal muscles, stomach, and colon in the double-deficient animals. In B6.*Foxp3*^{sf} or B6.*Foxp3*^{sf}*Aire*$^{+/o}$ mice, the pancreas, kidneys, fat, muscles, stomach, and colon also had some inflammation, but it was reduced compared with that of double-deficient animals (Fig. 2*d* and data not shown).

As for the NOD background, 2-wk-old NOD.*Foxp3*^{sf} or NOD.*Foxp3*^{sf}*Aire*$^{+/o}$ mice (n = 6, total) exhibited more severe inflammatory pathology in the lungs, liver, skin, pancreas, kidneys, stomach, colon, fat, and muscles than did their counterparts on the B6 background. In general, the pathological

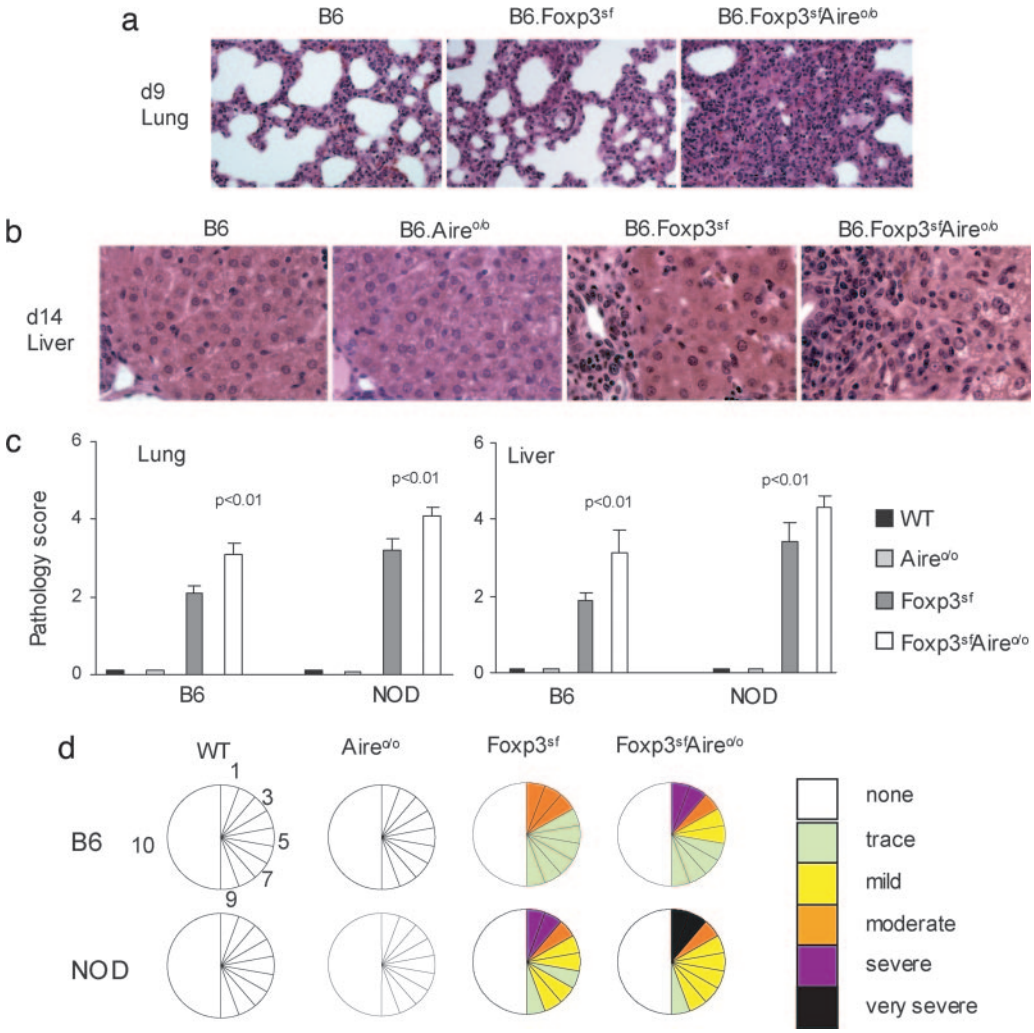

Fig. 2. Histopathology. (*a*) H&E sections of the lungs from 9-day-old mice on the B6 background, showing accelerated inflammatory infiltration in the lungs of *Foxp3*^{sf}*Aire*$^{o/o}$ mice. (Original magnification: ×200.) (*b*) H&E sections of the liver from 14-day-old mice on the B6 background, showing exacerbated inflammatory damage in *Foxp3*^{sf}*Aire*$^{o/o}$ mice. (Original magnification: ×200.) (*c*) Pathology score of the lungs and liver of mice on the B6 or NOD background. (*d*) A summary of histological surveys of 2-wk-old mice: 1, lung; 2, liver; 3, skin; 4, pancreas; 5, kidney; 6, fat; 7, muscle; 8, stomach; 9, colon; and 10, noninfiltrated organs including the central nervous system, the joints, the lachrymal, salivary, prostate, adrenal, thyroid, and parathyroid glands, the eye, and the small intestine (n = 3–6, with the lower ns representing WT and *Aire*$^{o/o}$ mice).

differential between single-deficient *Foxp3*^{sf} and double-deficient *Foxp3*^{sf}*Aire*$^{o/o}$ mice (n = 5) was diminished compared with that noted on the B6 background, although it was still quite discernible (Fig. 2 *c* and *d* and data not shown).

At 14 days of age, as described above, Aire deficiency on neither the B6 nor the NOD background (n = 4) resulted in observable pathological changes, but a lack of Foxp3 manifested as autoimmune damage in a particular set of organs. The accelerated mortality in the double-deficient animals might reflect one of two distinct pathological scenarios. First, an Aire deficiency on top of a Foxp3 deficiency could provoke pathology in a wider range of target organs. Second, the absence of Aire could, instead, accelerate and exacerbate pathology in the same, and only the same, organs targeted by the Foxp3 deficiency. Our findings suggested the latter scenario, and so we think that the accelerated death of *Foxp3*^{sf}*Aire*$^{o/o}$ mice was caused primarily by failure of the lungs and liver.

Strikingly, despite massive lymphoproliferation, many of the organs of the young mice deficient in both Foxp3 and Aire on either the B6 or NOD background showed no evident leukocytic infiltration. The spared organs included the central nervous system; the joints; the lachrymal, salivary, prostate, adrenal, thyroid, and parathyroid glands; the eyes; and the small intestine (Fig. 2*d*). For example, as illustrated in Fig. 3, in the small intestine of both *Foxp3*^{sf} and *Foxp3*^{sf}*Aire*$^{o/o}$ mice, even with a highly lymphoproliferative Peyer's patch, the adjacent intestinal

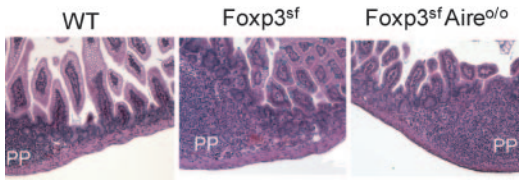

Fig. 3. Absence of significant tissue damage in the small intestine despite massive lymphoproliferation in the Peyer's patch (PP). Sections of the small intestine from 2-wk-old mice were stained with H&E. Only a portion of the Peyer's patch is shown. Note the intact intestinal villi and glands adjacent to the hyperproliferative Peyer's patch in *Foxp3*^{sf} and *Foxp3*^{sf}*Aire*$^{o/o}$ mice. Data are representative of 4–6 mice in each group on either the B6 or NOD background. (Original magnification: ×100.)

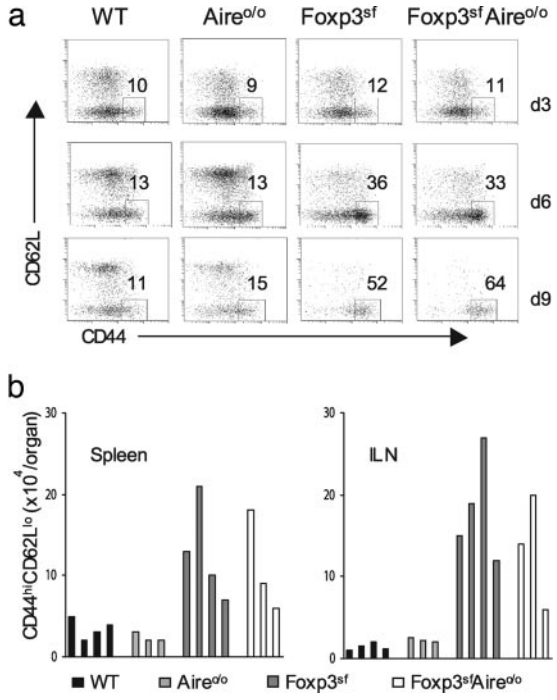

Fig. 4. T cell activation in *Foxp3^{sf}Aire$^{o/o}$* mice was not further increased over that in *Foxp3^{sf}* animals. The lymphoid organs of 3-day-, 6-day- or 9-day-old mice were analyzed by flow cytometry for activated T cells identified as CD44^{hi}CD62L^{lo}. (*a*) Similar frequencies of activated T cells in the spleen of *Foxp3^{sf}* and *Foxp3^{sf}Aire$^{o/o}$* mice. The number in the plot is the percentage of gated cells in the CD4$^+$ subset. Patterns also reflect CD8$^+$ T cells. Data are from 2–4 mice in each group on either the B6 or NOD background. (*b*) Total numbers of CD4$^+$CD44^{hi}CD62L^{lo} in the spleen and inguinal lymph nodes (ILN) of 9-day-old mice. Each bar represents one animal.

villi and glands remained virtually free of inflammatory involvement.

No Evident Role for AutoAbs in Pathogenesis. Aire-deficient mice produce autoAbs against a multiplicity of organs (9–11), which prompted us to examine sera from the 14-day-old Foxp3/Aire double-mutants. Cryosections of the liver, lungs, and pancreas were evaluated as targets. All four B6.*Foxp3^{sf}Aire$^{o/o}$* animals that we tested were negative for IgG reactive to these tissues; only two of them had trace or low-level IgM against the pancreas, but these sera were negative for IgM against the liver or lungs. We also tested three B6.*Foxp3^{sf}* littermates. Again, we did not find IgG reactive to any of the organs; all three had trace or low-level IgM against the pancreas but neither the liver nor the lungs. Thus, the existence (or nonexistence) of detectable autoAbs did not seem to correlate with inflammatory injury, suggesting that the pathology in *Foxp3^{sf}Aire$^{o/o}$* mice might not be very dependent on autoAbs.

Exacerbated Diseases Not a Reflection of Enhanced Lymphoproliferation. A deficiency in Foxp3 leads to early activation and proliferation of lymphocytes (38). The exacerbated pathology in *Foxp3^{sf}Aire$^{o/o}$* mice could reflect an enhancement of this phenomenon, manifested either earlier or more robustly. To test this possibility, we analyzed the cells in the lymphoid organs of animals on either the B6 or NOD background. At 3 days of age, the cellularity of the thymus and spleen of *Foxp3^{sf}Aire$^{o/o}$* mice ($n = 3$) was similar to that of *Foxp3^{sf}* ($n = 4$), *Aire$^{o/o}$* ($n = 2$), or normal control ($n = 4$) animals, and the percentages of activated CD4$^+$ and CD8$^+$ T cells displaying CD44, CD62L, or CD69 in *Foxp3^{sf}Aire$^{o/o}$* mice and all control groups were identical (Fig. 4*a Top* and data not shown). By day 6, although the total numbers of cells in the spleen and thymus in all groups remained comparable, the frequencies of CD69$^+$ or CD44^{hi}CD62L^{lo} activated T cells in mice deficient in Foxp3 ($n = 3$) or in both Foxp3 and Aire ($n = 2$) were elevated to ≈3-fold greater than those of Aire single-deficient ($n = 2$) or normal control ($n = 2$) animals (Fig. 4*a Middle* and data not shown). By day 9, most of the CD4$^+$ and CD8$^+$ T lymphocytes in the spleens of mice deficient in Foxp3 ($n = 4$) or in both Foxp3 and Aire ($n = 3$) exhibited an activated phenotype; the difference between these two groups, if there was any, was small (Fig. 4 *a Lower*). At this age, a Foxp3 deficiency led to an increase in total cell numbers in the spleen and lymph nodes, ranging from 50% to 5-fold above controls, but an additional deficiency in Aire did not further augment the lymphoproliferation. The total numbers of CD4$^+$CD44^{hi}CD62L^{lo} cells in the spleen and inguinal lymph nodes were markedly increased in both *Foxp3^{sf}* and *Foxp3^{sf}Aire$^{o/o}$* mice above those of normal or *Aire$^{o/o}$* mice, but there was no further elevation in the *Foxp3^{sf}Aire$^{o/o}$* over the *Foxp3^{sf}* animals (Fig. 4*b*). Therefore, the exacerbated autoimmunity in *Foxp3^{sf}Aire$^{o/o}$* vis-à-vis *Foxp3^{sf}* mice was not due to enhanced lymphoproliferation in the former.

No Apparent Role for Foxp3 in Clonal Deletion of Thymocytes. There is robust evidence that Aire controls autoimmunity through influencing central tolerance rather than peripheral regulation (10–13). On the other hand, data clearly establish that Foxp3 is essential for the peripheral regulation mediated through CD4$^+$CD25$^+$ Treg cells (28–30), and there is evidence for an exclusive role for Foxp3 in these cells (31). However, whether Foxp3 additionally participates in negative selection of autoreactive thymocytes has never been rigorously tested, and some role for Foxp3 in central tolerance induction was recently proposed (39). To address this possibility, we took advantage of the OTII/RIP-mOVA double-transgenic system that has been used to demonstrate the role of Aire in clonal deletion of thymocytes (13). OTII is an MHC class II-restricted transgenic mouse line carrying a rearranged Vα2$^+$Vβ5$^+$ T cell antigen receptor (TCR) specific for an ovalbumin peptide presented by I-A^b (36). Introducing cognate antigen via the *RIP-mOVA* transgene leads to deletion of Vα2$^+$Vβ5$^+$ clonotypic T cells, a process greatly impaired in the absence of Aire (13). As shown in Fig. 5*a*, WT OTII ($n = 3$) and *Foxp3^{sf}* OTII ($n = 2$) mice exhibited similar profiles of CD4$^+$ and CD8$^+$ T cells in the thymus and spleen; when the *RIP-mOVA* transgene was crossed into these lines, CD4/CD8 ratios were similarly reduced in WT OTII/RIP-mOVA ($n = 3$) and *Foxp3^{sf}* OTII/RIP-mOVA ($n = 4$) mice. Furthermore, the introduction of mOVA reduced the number of Vα2$^+$Vβ5$^+$ clonotypic T cells in the thymus of Foxp3-sufficient and -deficient OTII transgenic mice with an identical efficacy ($53 \pm 12 \times 10^5$ cells in a WT OTII thymus to $2.0 \pm 0.6 \times 10^5$ cells in a WT OTII/RIP-mOVA thymus, and $44 \pm 1 \times 10^5$ cells in a *Foxp3^{sf}* OTII thymus to $1.6 \pm 0.5 \times 10^5$ cells in a *Foxp3^{sf}* OTII/RIP-mOVA thymus), and virtually eliminated the Vα2$^+$Vβ5$^+$ clonotypic T cells in the spleen of both WT OTII/RIP-mOVA and *Foxp3^{sf}* OTII/RIP-mOVA mice ($11 \pm 3 \times 10^5$ cells in a WT OTII spleen to $0.11 \pm 0.11 \times 10^5$ cells in a WT OTII/RIP-mOVA spleen, and $5 \pm 0.2 \times 10^5$ in a *Foxp3^{sf}* OTII spleen to $0.03 \pm 0.02 \times 10^5$ cells in a *Foxp3^{sf}* OTII/RIP-mOVA spleen) (Fig. 5*b*). Therefore, in contrast to Aire, Foxp3 does not affect the clonal deletion of OTII thymocytes mediated by its cognate antigen. Noteworthily, the *OTII* transgene did not completely rescue *Foxp3^{sf}* mice from lymphoproliferation, as evidenced by an over-representation of T cells expressing nonclonotypic TCRs in the spleen of *Foxp3^{sf}* OTII mice compared with WT OTII animals (Fig. 5*b*), as has been observed with another TCR transgene (40).

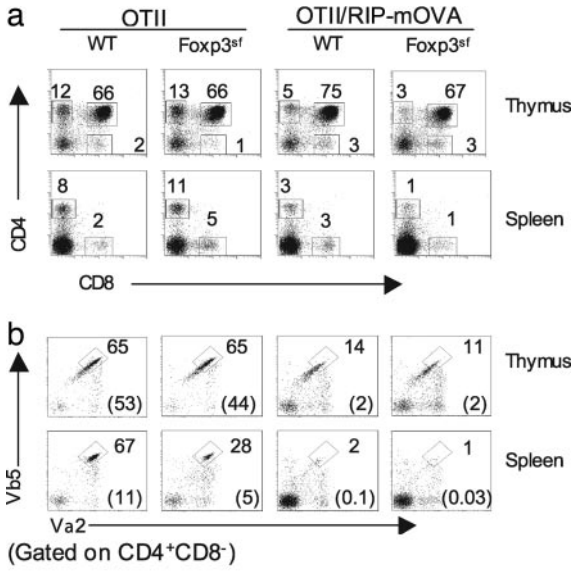

Fig. 5. No role of Foxp3 in clonal deletion of autoreactive T cells. Flow cytometric analyses of the thymus and spleen of the OTII TCR transgenic mice, with or without the cognate antigen expressed by the *RIP-mOVA* transgene, in the presence or absence of Foxp3. (*a*) CD4 and CD8 profiles in the lymphoid gate. (*b*) The clonotypic $V\alpha2^+V\beta5^+$ expression by $CD4^+CD8^-$ cells. Numbers in plots indicate the percentage of the gated clonotype^{hi} T cells, and numbers in parentheses are the average total number ($\times 10^5$) of the gated cells per organ. Data represent two experiments. Each group had two to four mice between 2 and 3 wk of age.

Discussion

The massive lymphoproliferation and multiorgan inflammatory infiltrates resulting from FOXP3/Foxp3 deficiencies, compared with the relatively milder phenotypes in the absence of AIRE/Aire, have raised questions about whether peripheral regulation is more important than central tolerance in keeping autoimmunity in check, and whether FOXP3/Foxp3 might not play a role in both processes (39). In the absence of evidence arguing otherwise, the currently prevailing hypothesis has limited the function of Foxp3 to the generation and/or functioning of $CD4^+CD25^+$ Treg cells (31). However, removal of $CD4^+CD25^+$ T cells through neonatal thymectomy resulted in rather mild autoimmune manifestations in a few specific organs, without apparent signs of lymphoproliferation (41). One possibility for this discrepancy is a role for Foxp3 in central tolerance induction (39). Here, we have demonstrated efficient clonal deletion of autoreactive T cells in the absence of this transcription factor. Taken together with an earlier report that Foxp3-deficient DO11.10 TCR transgenic thymocytes were effectively deleted by soluble cognate peptide (40), these results suggest that Foxp3 is unlikely to play a role in deleting self-reactive T cells from the maturing repertoire.

Inflammatory destruction caused by a Foxp3-deficiency on the autoimmunity-prone NOD background was worse than that on the B6 background, resulting in a much-shortened lifespan of NOD.*Foxp3^{sf}* compared with B6.*Foxp3^{sf}* mice (Figs. 1 and 2). What element(s) of the NOD background might aggravate the disease caused by the Foxp3 deficiency? A number of defects in both central and peripheral tolerance in the NOD mouse have been observed (42, 43). Discounting $CD4^+CD25^+$ Treg cells, which would be gone in the absence of Foxp3, the defective NK-T cell population in NOD mice could be a factor in the more severe scurfy disease in this strain. However, it is currently unclear to what extent this subset plays a pathogenic or a protective role, or both, in different disease contexts (44). On the other hand, evidence gathered by a number of groups using a variety of strategies has demonstrated a defect in central tolerance induction in the NOD mouse (19–23). Thus, the severe autoimmune phenotype in NOD.*Foxp3^{sf}* vis-à-vis B6.*Foxp3^{sf}* animals can perhaps best be explained by the combined effects of ineffective central tolerance and abrogation of peripheral regulation by $CD4^+CD25^+$ T cells.

Indeed, abolishing Aire-mediated central tolerance induction along with Foxp3-promoted peripheral regulation led to fulminant autoimmune disease and early death even in mice on the B6 background. Reminiscent of the pathological difference between NOD.*Foxp3^{sf}* and B6.*Foxp3^{sf}* mice, autoimmunity targeted the same set of organs in animals deficient in Foxp3 and those lacking both Foxp3 and Aire, but the damage was worse in the latter group. At 14 days of age, the Aire deficiency did not lead to signs of autoimmunity, but combining it with the Foxp3 deficiency resulted in earlier and very profound autoimmune damage in the lungs and liver and worse inflammatory infiltration into the pancreas, kidneys, muscles, fat, stomach, and colon (Figs. 1 and 2 and data not shown). Aire regulates ectopic thymic transcription of peripheral-tissue antigens expressed in the lungs, liver, pancreas, etc. (10), and, presumably, autoreactive T lymphocytes capable of responding to those antigens would escape to the periphery in its absence. Hence, the muted disease in young *Aire$^{o/o}$* mice suggests that such potentially pathogenic T cells can be kept in check by Treg cells. Once they emerge into the periphery, they can be revealed at an early age by removal of Foxp3-expressing Treg cells. Hence, the *Aire^{0}* and *Foxp3^{sf}* mutations define the two distinct and critically important phases of immune regulation, central and peripheral tolerance.

Interestingly, there was still a detectable difference in the phenotype of NOD.*Foxp3^{sf}* and NOD.*Foxp3^{sf}Aire$^{o/o}$* mice, although the difference was less than that between the B6 counterparts. If the exacerbated phenotype of NOD.*Foxp3^{sf}* vs. B6.*Foxp3^{sf}* animals can be attributed to a defect in central tolerance induction in NOD mice, the difference between NOD.*Foxp3^{sf}* and NOD.*Foxp3^{sf}Aire$^{o/o}$* or between B6.*Foxp3^{sf}Aire$^{o/o}$* and NOD.*Foxp3^{sf}Aire$^{o/o}$* animals (Figs. 1 and 2) might suggest that the central tolerance mechanism exposed by the NOD defect acts through a pathway different from that in which Aire participates. This would not be surprising, given that Aire functions in thymic stromal cells (10, 11) and that the central tolerance defect in the NOD strain is intrinsic to the thymocytes (20, 22, 23).

The autoimmune damage in the liver and lungs of *Foxp3^{sf}Aire$^{o/o}$* mice was striking, highlighting the importance of both central tolerance and peripheral regulation in protecting these vital organs. However, equally impressive was the lack of any detectable inflammatory infiltration in many organs, including the central nervous system, joints, endocrine glands, salivary glands, eyes, lachrymal glands, prostate glands, and small intestine. This resistance to T cell invasion is particularly impressive given that most T cells in these mutant animals were activated (Fig. 4) and that all secondary lymphoid tissues exhibited massive lymphoproliferation. In the small intestine, for example, even the tissue adjacent to a hyperproliferative Peyer's patch appeared intact (Fig. 3). Aire does regulate ectopic thymic transcription of peripheral-tissue antigens belonging to the brain, intestine, salivary glands, lachrymal glands, etc. (10). Presumably, in Foxp3/Aire double-deficient mice, pathogenic T cells capable of recognizing constituents of these organs emerge into the periphery and are free from suppression by $CD4^+CD25^+$ Treg cells. One is provoked, then, to question what mechanism protects these organs. Is it an Aire-independent mode of central tolerance induction? For example, Aire is not involved in clonal deletion mediated by thymic dendritic cells, and, interestingly, a set of Aire-independent peripheral-tissue antigens has been identified (10, 45), suggesting the possibility of an "Aire-2." Is it a Foxp3-independent form of peripheral regulation (e.g., via NK-T or $CD8^+$ T cells)? Is it simply some means of anatomical/physiological restraint, e.g., lack of access due to immature vasculature? The last

possibility could explain why the liver and lungs are prominent targets, but it is unlikely to be the explanation for the lack of damage in the small intestine.

In summary, rather analogous to the situation with cancer, the development of an autoimmune disease reflects "multiple hits" in breaking down a net of tolerance induction mechanisms. In a normal individual, an exquisite collaboration among these diverse mechanisms is responsible for preventing the immune system from attacking self-tissue.

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