

# Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity

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There has long been conceptual and experimental support for, but also challenges to, the notion that the initial period of the immune system's development is particularly important for the establishment of tolerance to self. The display of self-antigens by thymic epithelial cells is key to inducing tolerance in the T lymphocyte compartment, a process enhanced by the Aire transcription factor. Using a doxycycline-regulated transgene to target Aire expression to the thymic epithelium, complementing the Aire knockout in a temporally controlled manner, we find that Aire is essential in the perinatal period to prevent the multiorgan autoimmunity that is typical of Aire deficiency. Surprisingly, Aire could be shut down soon thereafter and remain off for long periods, with few deleterious consequences. The lymphopenic state present in neonates was a factor in this dichotomy because inducing lymphopenia during *Aire* turnoff in adults recreated the disease, which, conversely, could be ameliorated by supplementing neonates with adult lymphocytes. In short, Aire expression during the perinatal period is both necessary and sufficient to induce long-lasting tolerance and avoid autoimmunity. Aire-controlled mechanisms of central tolerance are largely dispensable in the adult, as a previously tolerized T cell pool can buffer newly generated autoreactive T cells that might emerge.

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Adaptive immune systems face the challenge of discriminating between foreign antigens, against which a response may have to be mounted, and self-constituents, which should be ignored or tolerated. Although there are multiple theoretical solutions to the challenge of self/nonself discrimination, Burnet and Fenner (1) proposed as a corollary to the clonal selection theory that the problem can be solved on the basis of time considerations; any antigen to which the immune system is exposed in the fetal or early neonatal stages is flagged as self and induces long-term tolerance. Lederberg (2) extended this notion by postulating that immature lymphocytes have a heightened sensitivity to tolerance induction. This "newborn privilege" was tested by introducing foreign molecules as surrogates for self, and, indeed, it did prove possible to induce tolerance by administration of allogeneic cells, viruses, or proteins to neonates but not in adults (3–6). However, neonatal exposure was not always tolerogenic, and the outcome was clearly dose dependent (7, 8). Although related observations already existed in the literature, the strongest challenge to the notion of neonatal

tolerance induction came from a trio of studies published in 1996 (9–11), which showed that neonatal lymphocytes could mount full immune responses under the appropriate conditions. Ridge et al. (9) pointed out that the differential effects of fetal versus adult exposure to allogeneic cells highlighted by the Medawar group, long a linchpin of neonatal tolerance, may have been due simply to the large disparities in doses (relative to body size) administered at the two stages. As a result, the notion of neonatal tolerance was questioned in the field, and more attention was given to the influence of concomitant triggering of the innate immune system by inflammatory and microbial ligands.

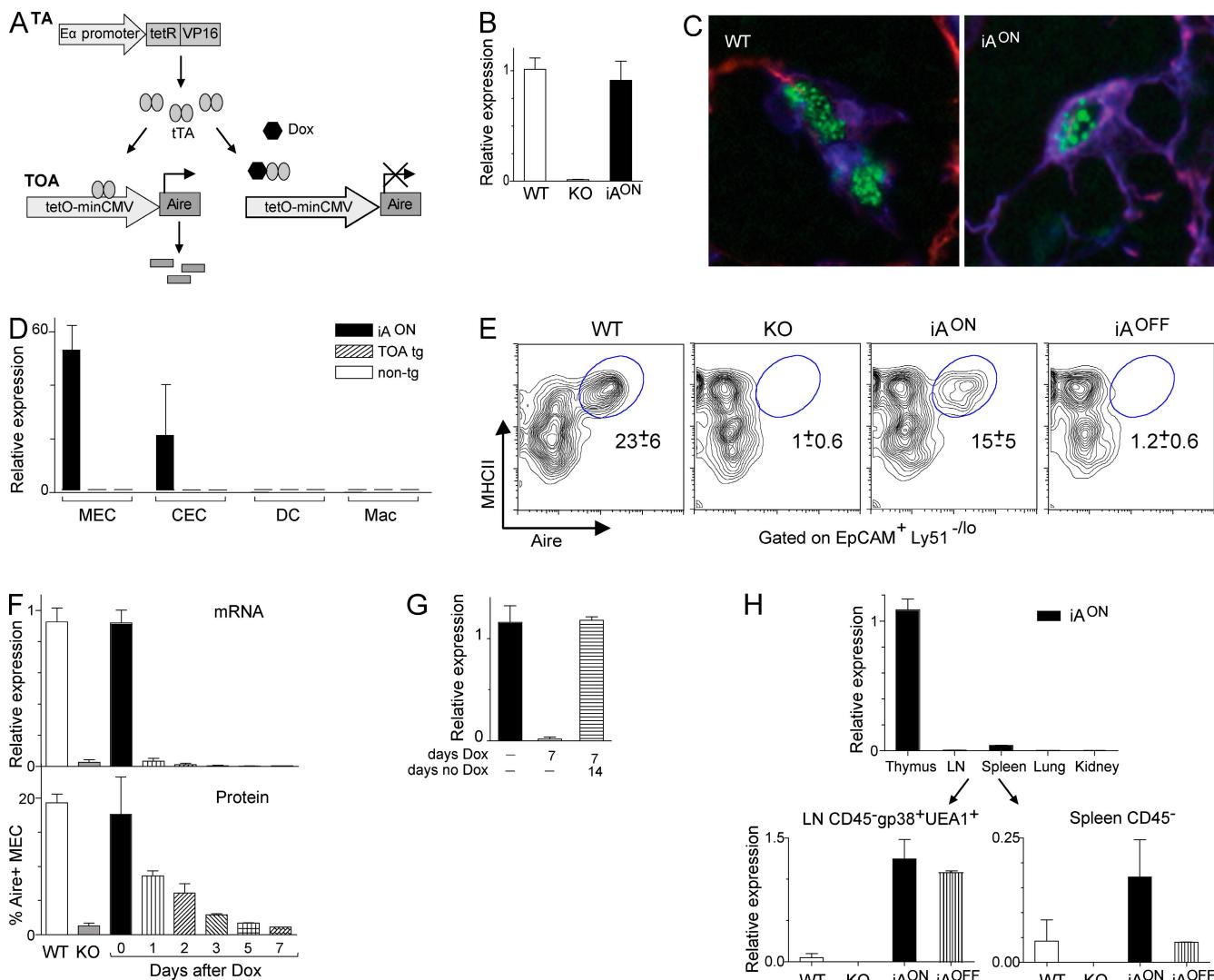
One issue with experimental testing of neonatal tolerance induction has been that, going back to the 1950s, investigations mainly relied on the administration of allogeneic or xenogeneic molecules in the tolerization and/or test steps but

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rarely addressed tolerance to true self-constituents, which were long impossible to manipulate experimentally. The *Aire* gene may provide such an experimental handle. *Aire* is a transcription factor expressed primarily in medullary epithelial cells (MECs) of the thymus, where it promotes the ectopic expression and presentation of peripheral tissue antigens (12). This presentation imparts deletional tolerance, and *Aire* deficiency results in multi-

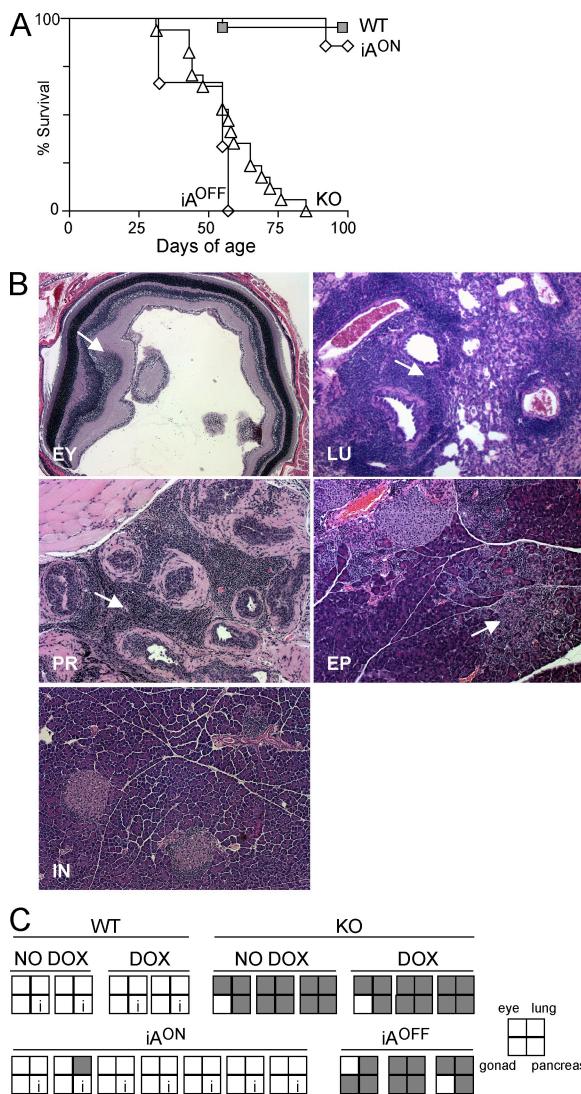
organ autoimmune disease in both human patients and KO mice. Importantly in the present context, the autoimmune disease that develops in *Aire*-deficient mice (hereafter *Aire* disease) is solely controlled by immunological tolerance and does not require microbial triggers (13). Thus, *Aire*-controlled presentation of self might provide a manipulable system that is more appropriate for investigating the temporal aspects of T cell tolerance.



**Figure 1. Conditional *Aire* transgenic system.** (A) Schematic of the Dox-controlled TA and TOA dual transgenes (together referred to as iA). (B) Real-time PCR analysis of *Aire* transcripts in whole thymus of *Aire* WT ( $n = 8$ ), *Aire* KO ( $n = 2$ ), and *iA*<sup>ON</sup> ( $n = 8$ ) mice. Values are expressed relative to WT thymus as a standard. (C) *Aire* staining (green) thymic epithelial cells (counterstained with anti-keratin5 [blue] and -keratin8 [red]) of WT and *iA*<sup>ON</sup> mice (100 $\times$  objective). No *Aire* staining was detectable in KO mouse samples or WT samples stained with secondary antibody only. (D) Real-time PCR analysis of *Aire* reporter expression in thymic MEC (CD45<sup>-</sup>Ly51<sup>-/-</sup>EpCam<sup>+</sup>), CEC (CD45<sup>-</sup>Ly51<sup>+</sup>EpCam<sup>+</sup>), DC (CD3<sup>-</sup>CD19<sup>-</sup>CD45<sup>+</sup>CD11c<sup>+</sup>), and macrophages (CD3<sup>-</sup>CD19<sup>-</sup>CD45<sup>+</sup>CD11c<sup>-</sup>CD11b<sup>+</sup>) of *iA*<sup>ON</sup>, reporter-only TOA<sup>+</sup>, and WT mice ( $n = 2$ ). Expression level is normalized to RNA from whole *iA*<sup>ON</sup> thymus. (E) Flow cytometry analysis of MHC class II and intracellular *Aire* in EpCAM<sup>+</sup>Ly51<sup>-/-</sup>-gated MECs of one representative WT, KO, *iA*<sup>ON</sup>, and *iA*<sup>OFF</sup> sample. The mean percentage  $\pm$  SD of the MHCII<sup>hi</sup>*Aire*<sup>+</sup> population for groups of 5–11 mice is shown. (F) Real-time PCR analysis of whole thymus *Aire* transcript (top) and percentage of *Aire*<sup>+</sup> cells within thymic MECs (bottom) at different times after *iA* turnoff by Dox treatment in *iA* mice (means of  $n = 2$ –3 per time point). (G) Real-time PCR of *Aire* transcripts in thymus of *iA* mice that were untreated, treated with 10  $\mu$ g/ml Dox for 1 wk, or weaned off Dox for 2 wk after a 1-wk Dox treatment ( $n = 2$ ). Values are relative to *iA*<sup>ON</sup> thymus. (H) Real-time PCR analysis of *iA* transcripts in organs of *iA*<sup>ON</sup> mice (top), *Aire* transcripts of sorted LN CD45<sup>-</sup>gp38<sup>+</sup>UEA-1<sup>+</sup> (bottom left), or spleen CD45<sup>-</sup> (bottom right) stromal cells ( $n = 2$ ). Error bars show SD. Results shown are representative of two (D, G, and H) or three or more (B, C, E, and F) experiments.

## RESULTS AND DISCUSSION

To this end, we attempted to develop a regulable *Aire* transgenic system that could complement an *Aire* deficiency in a time- and dose-controlled manner. The TA transgene drives expression of the tetTA (tTA) transcriptional activator in thymic epithelium (14); its product activates the tetO*Aire* (TOA) reporter transgene, which carries the mouse *Aire* complementary DNA (cDNA) under the dictates of a tTA-responsive promoter (Fig. 1 A). When both transgenes are present, *Aire* is expressed in thymic epithelium, but administration of doxycycline (Dox) blocks the interaction of tTA with the reporter gene promoter, turning *Aire* off. The transgenes were crossed to the *Aire* KO mutation (15) on the nonobese diabetic (NOD) genetic background to generate *Aire*<sup>−/−</sup>TA<sup>+</sup>TOA<sup>+</sup> mice, which are referred to as inducible-*Aire* (iA) mice.



**Figure 2.** The iA transgenic system rescues *Aire* disease. (A) Survival curve of WT ( $n = 7$ ), *Aire*<sup>−/−</sup>TOA<sup>+</sup> reporter-only ( $n = 29$ ), iA<sup>ON</sup> ( $n = 7$ ), and iA<sup>OFF</sup> ( $n = 3$ ) mice. Mice were sacrificed upon 15–20% body weight loss. KO versus iA<sup>ON</sup>,  $P = 0.0002$  by Kaplan-Meier survival analysis. (B) Representative histopathology of tissues affected in *Aire* KO NOD mice performed at 14–17 wk, or earlier for mice that had to be sacrificed as a result of wasting disease. EY, eye (retinal degeneration); LU, lung; PR, prostate; EP, exocrine pancreas; IN, pancreatic islets (insulitis). White arrows point at areas of infiltration/retinal degeneration. (C) Histopathology analysis in WT, KO, iA<sup>ON</sup>, and iA<sup>OFF</sup> mice at 14 wk of age. Filled squares represent diseased tissue, as indicated (i, insulitis typical of *Aire*-proficient NOD mice). Results in A–C are representative of more than three independent experiments.

RT-PCR analysis of *Aire* transcripts in the thymus showed that the iA system rescued *Aire* expression to values close to those of WT mice (Fig. 1 B). Similarly, the usual punctate staining of *Aire* in nuclei from thymic epithelial cells was recovered in iA mice (Fig. 1 C). Transgene-encoded *Aire* was expressed in medullary and cortical epithelial cells, with little to no expression in DCs and macrophages, as expected from the properties of the driver construct (Fig. 1 D). Expression in these thymic populations was dependent on the presence of both the driver and reporter transgenes, as the reporter alone yielded no detectable expression (Fig. 1 D). Flow cytometry after intracellular staining confirmed the presence of *Aire* in MECs from iA mice, in quantities slightly higher than those of WT littermates and of an *Aire*<sup>+</sup> MEC population in close to normal proportions (Fig. 1 E).

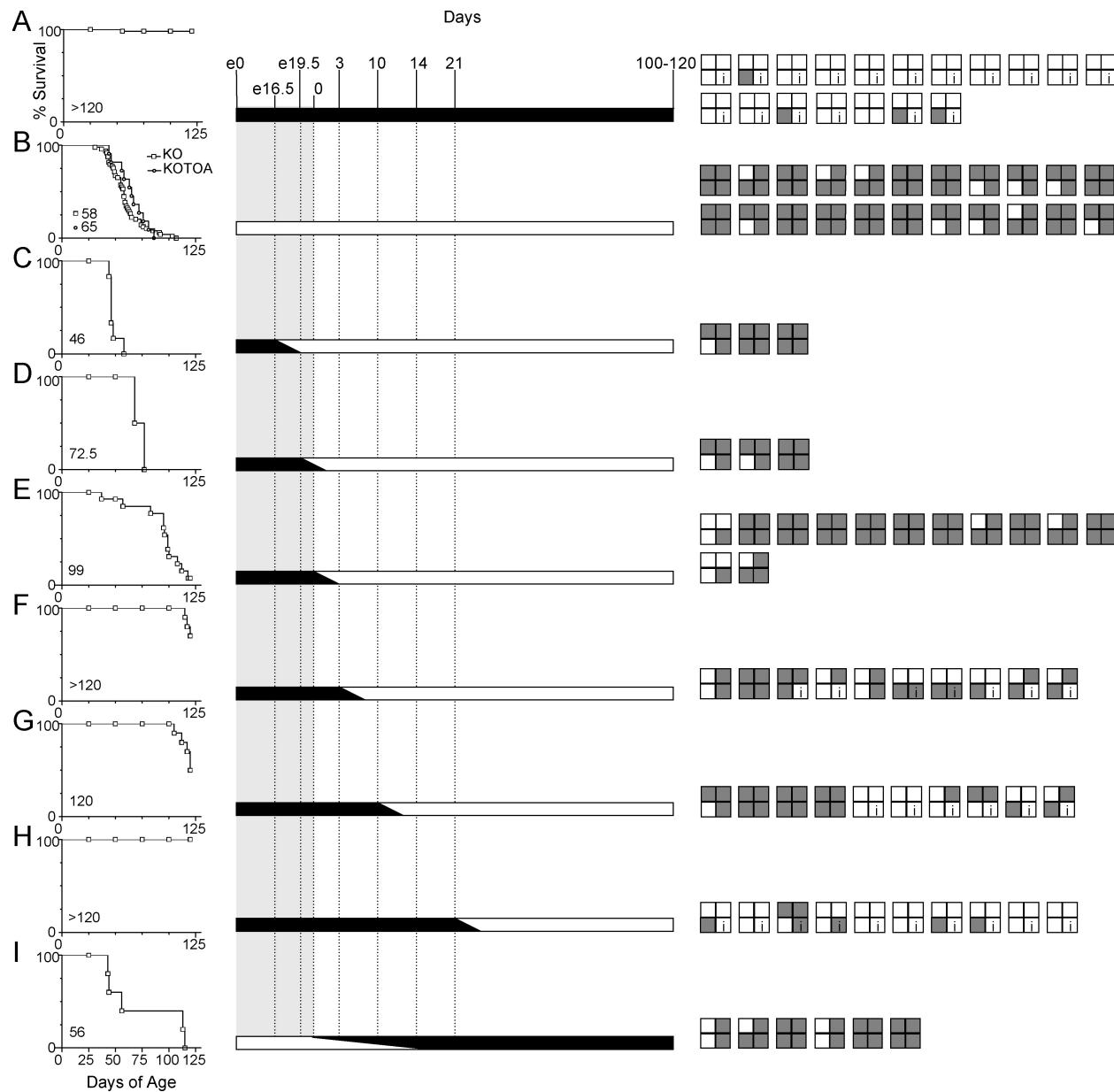
*Aire* expression in the thymus of iA mice proved sensitive to Dox, with a complete loss of *Aire* transcripts within the first 24 h of Dox treatment and a disappearance of *Aire*<sup>+</sup> MECs over the subsequent 3 and 5 d (Fig. 1 F), which is consistent with the rapid turnover of *Aire*<sup>+</sup> MHCII<sup>hi</sup> MECs (16). Reexpression of *Aire* after a turnoff period was more sluggish but could be achieved after 1–2 wk of Dox withdrawal, particularly when low doses were initially used (Fig. 1 G and not depicted). Because the turnoff with Dox treatment was sharper than the turnon in this model, as in other Dox-controlled transgenic settings, regulated *Aire* extinction was the strategy primarily used in the studies described in the subsequent paragraphs.

It was recently reported that *Aire* is expressed in the stromal cells of peripheral lymphoid organs (17, 18). *Aire*-encoding transcripts from the iA transgenes were detected almost exclusively in the thymus, with very low expression in the spleen and LNs (<3–5% of thymus values; Fig. 1 H, top). In the CD45<sup>−</sup>gp38<sup>+</sup>UEA-1<sup>+</sup> stromal cell population described by Lee et al. (17), *Aire* transcripts were 10 $\times$  more abundant in iA<sup>ON</sup> than in WT mice but were maintained in iA<sup>OFF</sup> animals, likely because of the leakiness occasionally observed with Dox-controlled systems (Fig. 1 H, bottom left). Similarly, significant transgene-driven *Aire* transcripts were detected in CD45<sup>−</sup> spleen cells that correspond to the eTAC cells of Gardner et al. (18), but this expression was also only partially affected by Dox, with residual expression in treated iA mice comparable to that of WT (Fig. 1 H, bottom right). No expression was detected in other tissues, including the lung, kidney, heart, or brain (Fig. 1 H, top, and not depicted).

The key test of complementation was whether the iA transgenes could protect, in a Dox-sensitive manner, from *Aire* disease. Mice with the *Aire*-null mutation on the NOD background develop wasting disease, with a median survival time of 55 d (19) because of multiorgan lymphocytic infiltration that

has particular predilection for the lung, exocrine pancreas, eye, and ovary/prostate. In contrast, NOD.Aire<sup>-/-</sup> mice are protected from the insulitis that normally affects the NOD strain (19, 20). These observations were reproduced in our study (Fig. 2). Aire deficiency was indeed complemented by the transgenes, as iA mice in which the reporter was continuously on (iA<sup>ON</sup>) survived to 100 d of age and beyond, developing the typical insulitis of NOD mice, rather than

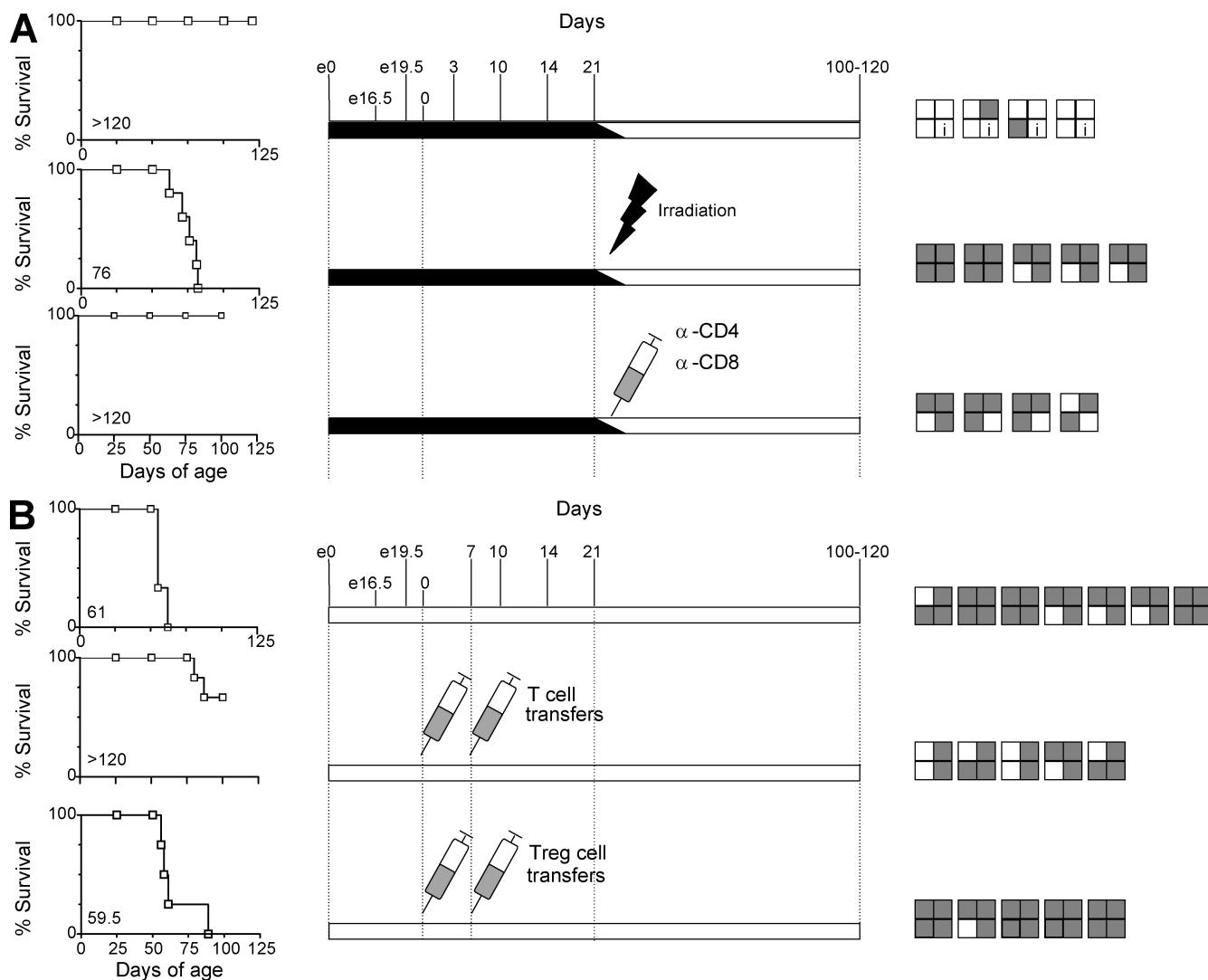
exocrine pancreatitis. Only rare isolated and mild infiltration of the lung or gonads was detected over the course of multiple experiments, comparable with that observed in similarly aged WT mice. Aire turnoff by continuous Dox exposure (iA<sup>OFF</sup>) restored the full array of disease manifestations, confirming that Aire disease depends on regulated Aire expression. As expected, Dox treatment of either WT or KO mice had no effect.



**Figure 3. A neonatal time window for Aire-mediated tolerance.** Survival curve analysis (left; the numbers are the mean survival times in days) and histopathology (right) for iA mice in which Aire turnoff was initiated at different times or initiated on embryonic day (e) 0 and then allowed to be reexpressed from birth (day 0), as depicted in the central diagram (black fill, Aire on; white fill, Aire off). Tissues were harvested for histology after a 14–17-wk follow up, or earlier if they had to be sacrificed as a result of wasting disease. WT (A;  $n = 53$ ), KO (B;  $n = 49$ ), and Aire<sup>-/-</sup>TOA<sup>+</sup> reporter-only (B;  $n = 11$ ) mice were included as controls in individual experiments and are pooled. iA<sup>ON(e0-e16.5)</sup> (C;  $n = 3$ ), iA<sup>ON(e0-e19.5)</sup> (D;  $n = 3$ ), iA<sup>ON(e0-D0)</sup> (E;  $n = 13$ ), iA<sup>ON(e0-D3)</sup> (F;  $n = 10$ ), iA<sup>ON(e0-D10)</sup> (G;  $n = 10$ ), iA<sup>ON(e0-D21)</sup> (H;  $n = 10$ ), and iA<sup>OFF(e0-D0)</sup> (I;  $n = 6$ ) are shown.  $P < 0.002$  when comparing KO to iA mice in E–H (Kaplan-Meier test). The histopathology diagram is as described for Fig. 2. All experiments were performed at minimum of three times per condition.

The functional importance of Aire in stromal cells of peripheral lymphoid organs remains conjectural (17, 18), although the transferability of the autoimmune disease by grafts of Aire-deficient thymi (15) suggests that it is not relevant for Aire disease. Consistent with this notion, there was no correlation between peripheral Aire expression, which was largely insensitive to Dox (Fig. 1 H), and protection from disease, which was fully sensitive (Fig. 2). This peripheral expression is thus not relevant to the present study, although it may not be a coincidence that the same peripheral cells that normally express low levels of Aire are also those that show leaky expression of the transgenic reporter.

Thus, the inducible Aire system provided us with a means to vary the expression of and tolerance to Aire-controlled self-antigens. To dissect the influence of *Aire* expression on tolerance throughout life, we examined the incidence of wasting disease and autoimmune infiltration in iA mice in which Aire was expressed over different time intervals (Fig. 3). Dox treatment should result in clearance of peripheral tissue antigens within a matter of days, from their own molecular turnover and because Aire-positive cells turn over rapidly, with a half-life of 3–4 d (16). Turning *Aire* off before birth resulted in full-blown wasting disease and multiorgan autoimmunity (Fig. 3, C and D), but keeping it on for an increasing number of days after birth resulted in



**Figure 4. Aire is required to prevent autoimmunity during lymphopenia.** Survival curve analysis (left) and histopathology (right) in supplemented Aire-deficient mice (WT, KO, and additional control mice were included in each experiment and are not shown here for clarity but are explained in detail in Figs. S1–S3). (A) iA<sup>ON(e0-D21)</sup> mice were otherwise untreated (top), lethally irradiated and bone marrow reconstituted (middle), or anti-CD4/CD8 treated at 3 wk of age (bottom) for a 2-wk period. Irradiated/reconstituted iA<sup>ON(e0-D21)</sup> versus KO (P = NS) or WT (P = 0.0024) was calculated by Kaplan-Meier survival analysis. (B) Aire<sup>-/-</sup> mice were untreated (top; n = 6), received 10 × 10<sup>6</sup> T cell-enriched peripheral LN and spleen cells (middle; n = 5), or received 3 × 10<sup>5</sup> T reg cells (bottom; n = 5) on days 1 and 7. T cell-supplemented KO mice versus KO (P = 0.005; Kaplan-Meier) or versus WT (NS) and T reg cell-supplemented KO mice versus KO (NS) or versus WT (P = 0.0001) were compared. Experimental and histopathology diagrams are as in Fig. 3. All results in A and B are representative of two or more independent experiments.

a progressively marked increase in the median survival age and in the protection from multiorgan infiltration (Fig. 3, E–H). Mice expressing *Aire* only until 21 d (iA<sup>ON(0–21)</sup>) were indistinguishable from iA<sup>ON</sup> controls, including the appearance of “normal” insulitis (Fig. 3 H). Several iA<sup>ON(0–21)</sup> mice that were observed for >250 d remained similarly free of disease (unpublished data). These results demonstrate that *Aire* expression during the neonatal period is essential for self-tolerance but that it is dispensable beyond weaning age. This conclusion was confirmed by the reverse experiment, in which *Aire* was turned on only after birth; in spite of continued *Aire* expression for the remainder of their lives, these animals showed wasting and autoimmune pathology (Fig. 3 I). Thus, exposure to self-antigens during the neonatal period is both necessary and sufficient to prevent the devastating autoimmunity associated with *Aire* disease.

These data imply that escape of autoreactive lymphocytes into the periphery is more tolerable after weaning age than during the neonatal period, and they raise two connected but different questions: what is special about the neonatal period, and why don’t the intolerant T cells emerging from the adult Aire<sup>OFF</sup> thymus induce pathology? Neonatal T cells are peculiar in several respects, any of which could be relevant in the present context: their TCRs are largely devoid of N-region diversity and have a different intrinsic affinity for major histocompatibility complex molecules (21–23); they include relatively few CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory (T reg) cells (24), and they are able to access the parenchymal tissues (25). Finally, neonatal thymocytes emerge into peripheral T cell compartments that are largely empty and, thus, show lymphopenia-driven proliferation, a state which can provoke effector-like potentialities (26–30). Several experiments were set up to explore some of these explanations. First, iA<sup>ON(0–21)</sup> mice were lethally irradiated and reconstituted with syngeneic bone marrow while *Aire* was turned (and maintained) off (Fig. 4 A), a setting which should recreate in an adult mouse the lymphopenic conditions of the neonate. In this case, iA<sup>ON(0–21)</sup> mice did develop wasting disease and multiorgan lymphocytic infiltration (Fig. 4 A). Unmanipulated iA<sup>ON(0–21)</sup> mice remained free of disease, and all other controls behaved as expected (Fig. S1). Similarly, partial T cell lymphopenia was induced in iA<sup>ON(0–21)</sup> mice by injection of depleting anti-CD4/CD8 mAbs at 3 wk of age at the time of *Aire* turnoff. Multiorgan pathology ensued, albeit in the absence of wasting disease (Fig. 4 A; and Fig. S2 shows additional controls). Third, Aire<sup>−/−</sup> neonates were infused with boluses of adult spleen and LN cells (10 × 10<sup>6</sup> T-enriched cells at days 1 and 7), thus reducing their lymphopenic state. Distinct, albeit not complete, protection was achieved with this treatment, with less frequent and delayed wasting and a reduced intensity of histological lesions. Such protection could not be achieved by the transfer of 3 × 10<sup>5</sup> FoxP3<sup>+</sup> T reg cells alone, which is equivalent to the number of T reg cells present within the 10 × 10<sup>6</sup> T cell-enriched cells’ bolus (Fig. 4 B and Fig. S3). These results are not compatible with a major influence of the neonatal TCR repertoire or with an intrinsic “openness”

of neonatal tissues. Neither are they compatible with an explanation based solely on the relative dearth of T reg cells at birth because after irradiation and reconstitution from bone marrow precursors, T cell populations are restored with largely normal proportions of T reg cells (31, 32). An explanation which is purely based on T reg cell deficiency would not account for the autoimmune pathology in reconstituted iA<sup>ON(0–21)</sup> mice. Rather, they indicate that lymphopenia provides a permissive environment for the initiation of autoimmunity, because T cells spontaneously acquire effector functions and/or are able to enter tissues, in which escape of autoreactive cells from the thymus has deleterious consequences.

The autoimmune manifestations of *Aire* disease have striking similarities to those induced by neonatal thymectomy (d3Tx): widespread but slowly progressing autoimmunity, interindividual variability, and a clear parallel in the target organs preferentially affected on different genetic backgrounds (19, 33). The autoimmunity induced by d3Tx was initially thought to reflect defective T reg cells, but it has since been realized that the numbers and activity of T reg cells are normal in d3Tx mice (34, 35). A unifying interpretation is that the T cell compartment requires exposure to self-antigens in the thymus during the postnatal period. Eliminating this exposure by either thymectomy or shutdown of *Aire* expression results in incomplete tolerance. This tolerogenic exposure can be achieved only in the thymus and not in the periphery, a notion which is also supported by the classical studies of embryonic quail/chick embryonic grafts (36); pre/postnatal exposure to peripheral tissues, even as massive as an entire limb, cannot replace the contribution of thymic epithelium. In addition, the lymphopenic state inherent to a growing T cell pool exacerbates this dependency, which is consistent with the autoimmune manifestations often observed in lymphopenic situations, such as the “immune reconstitution inflammatory syndromes” observed after T cell depletion in HIV or other viral infections (37). In contrast, a fully established adult T cell pool can provide enough control, via regulatory cells or other mechanisms of peripheral tolerance, to buffer incoming self-reactive cells arising from an *Aire*-deficient thymus. But these buffers can be established only if thymic exposure to self has occurred previously in the formative stages.

## MATERIALS AND METHODS.

**Mice.** The TOA construct contains *Aire* cDNA (positions 36–1879 from GenBank/EMBL/DDJB under accession no. NM\_009646.1) and an intron and polyadenylation site from the rabbit β-globin gene in the tetO-SB plasmid. The fragment was prepared for microinjection into NOD embryos by AatII and Eco47III digestion and purification as previously described (38). Progeny were screened by a PCR assay specific for transgenic *Aire* (primers: 5'-GCAACGTGCTGGTTGTG-3' and 5'-TGACTCCAAGTTGC-CATCTG-3'). TA mice (14) were backcrossed more than seven times onto NOD, fixing NOD alleles at *Atg7*, *Idd3*, and *Idd5.2*. Aire<sup>−/−</sup> mice on the NOD background have been previously described (19). Dox hydrochloride (Sigma-Aldrich) was provided in drinking water at 100 µg/ml, unless otherwise indicated. Equal sweetener was added to the water to mask Dox’s bitter flavor, and water was changed three times per week. For prenatal or neonatal treatment, Dox-supplemented water was provided to pregnant or lactating mothers. To follow wasting disease, mice were weighed three times per week and sacrificed when they lost 15–20% body weight relative to littermates. For

bone marrow chimeras, recipients were irradiated (9 Gy), reconstituted with  $10 \times 10^6$  bone marrow cells from a syngeneic WT mouse, and kept on with sulfatrim for the remainder of the experiment. For mAb depletion of CD4 and CD8 T cells, mice were treated i.p. with 0.5 mg each of anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) every 3 d for a 2-wk period. All mice were maintained under specific pathogen-free conditions at the Harvard Medical School facility (protocol 02954 approved by Harvard Medical School's Institutional Animal Care and Use Committee). For lymphocyte transfer experiments, spleen and LN cells were depleted of non-T cells with the negative selection T cell isolation reagent (Miltenyi Biotec) and  $10 \times 10^6$  cells were injected i.p. into neonates. For T reg cell transfer experiments, T reg cells from spleen and LN of female Foxp3-GFP (NOD) transgenic mice were isolated by sorting (B220 $^-$ CD4 $^+$ GFP $^+$ ) and  $3 \times 10^5$  T reg cells were injected i.p. into neonates.

**Real-time PCR.** RNA was prepared from tissues or sorted cells with Trizol (Invitrogen), treated with DNase (Applied Biosystems) for 30 min at 37°C to remove contaminating DNA, and cDNA synthesized with random or gene-specific priming (GSP). For GSP, the 5'-TCATCTCTACCAGGTATAAGT-GAC-3' primer was used. cDNA was used as a template for amplification by Taqman RT-PCR (Mx3000P; Agilent Technologies). Both WT and transgenic *Aire* transcripts were detected using the 5'-GTACAGCCGCCTG-CATAGC-3' and 5'-CCCTTCCGGGACTGGTT-3' primers and the Fam-5'-CTGGACGGCTCCAAAGATGT-3'-Tmra probe. For detection of *Aire* transcripts only from the TAO transgene, the 5'-TGAGA-ACATCAGGCTCC-3' and 5'-GGAAAGGCAGTGTCTATGGC-3' primers and the Fam-5'-CGTCTGAGGCTGCACCG-3'-Tmra probe were used. Whole thymus RNA from WT or iA $^{\text{ON}}$  (depending on the experiment) was used as a reference. Data were visualized and analyzed with MxPro software (Agilent Technologies), using the standard curve method.

**Stromal cell isolation and intracellular *Aire* staining.** Suspensions of thymic stromal cells were prepared and stained for intracellular *Aire* as previously described (16). For stromal cells from peripheral LNs and spleen, small incisions were made in the tissues, which were agitated in RPMI supplemented with 5% FBS to release free lymphocytes. The remaining tissue was digested in RPMI 5% FBS with 0.5 mg/ml collagenase (Roche), 1 mg/ml dispase (Roche), and 1 mg/ml DNase (Sigma-Aldrich), and the released cells were stained for FACS analysis.

**Antibodies and flow cytometry.** Anti-mouse gp38-Alexa Fluor 647 and rat anti-mouse *Aire* (5H12 clone) were gifts from S. Turley (Dana Farber Cancer Institute, Boston, MA) and H. Scott (Walter and Elisa Hall Institute, Melbourne, Australia), respectively. Anti-keratin-5 and -8 antibodies were obtained from Covance. Flow cytometric analysis was performed on an LSRII, sorting on a FACSAria (BD). Data were analyzed with FlowJo software (Tree Star, Inc.).

**Online supplemental material.** Figs. S1–S3 show additional curves for control animals corresponding to Figs. 3 and 4. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20090300/DC1>.

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