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SHORT COMMUNICATION

Autoimmunity associated with chemically induced thymic dysplasia

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Abstract

Autoimmune and inflammatory conditions are frequent complications in patients with reduced numbers of T cells. Here, we describe a mouse model of thymic stromal dysplasia resulting in peripheral T-cell lymphopenia. In *Foxn1:CFP-NTR* transgenic mice, the bacterial nitroreductase enzyme is expressed in thymic epithelial cells and converts the prodrug CB1954 into a cytotoxic agent. This strategy enables titratable and durable destruction of thymopoietic tissue in early embryogenesis. Our results indicate that the resulting low levels of thymic capacity for T-cell production create a predisposition for the development of a complex autoimmune syndrome, chiefly characterized by inflammatory bowel disease and lymphocytic organ infiltrations. We conclude that the *Foxn1:CFP-NTR* transgenic mouse strain represents a suitable animal model to optimize established clinical protocols, such as thymus transplantation, to correct various forms of thymic dysplasia and to explore novel treatment options.

Keywords: animal model, conditional cell ablation, *Foxn1*, inflammatory bowel disease, thymus

Introduction

In a recent retrospective analysis, autoimmune and inflammatory complications were noted in approximately a quarter of patients with primary immunodeficiency syndromes, particularly notable in situations of T lymphopenia (1). Interestingly, in a select group of patients, the phenotype of normal immunoglobulin levels with reduced or absent cellular immune reactivity was attributed to thymic dysplasia (2–6). A large fraction of such patients suffers from DiGeorge syndrome, first described as a cause of immunodeficiency about 50 years ago (7, 8); indeed, numerous reports have demonstrated a high prevalence of autoimmunity in these patients (1, 9–11). Based on the autoimmune phenotypes of other types of primary immunodeficiencies (1), it is tempting to speculate that the size of the residual thymic mass in the genetically heterogeneous group of thymic dysplasia patients determines whether or not autoimmunity develops. Considering the fact that rodents without thymopoietic activity lack autoimmunity (12), the presence of residual thymic masses of intermediate size might predispose patients to the development of autoimmunity as a result of immune dysregulation.

Here, we describe a mouse model that is based on a conditional cell ablation strategy to reduce the number of thymic epithelial cells (TECs) during embryogenesis, resulting in varying degrees of thymic hypoplasia. In our first report on this strategy, we concerned ourselves with the developmental distinction between thoracic and cervical thymus tissue (13). Here, we focus on the long-term immunological consequences of prenatally induced thymus hypoplasia/aplasia. Our results demonstrate that an intermediate level of residual thymopoiesis indeed predisposes mice to the development of autoimmunity, establishing a suitable animal model for the immunological perturbations resulting from similar immunodeficient states in patients.

Methods

Mice

Foxn1lacZ (14), *Foxn1:CFP-NTR* (13) and *Foxn1:eGFP* (15) transgenic mice were described earlier and kept in the animal facility of the Max Planck Institute of Immunobiology and Epigenetics under specific pathogen-free conditions. All animal experiments were performed in accordance with relevant guidelines and regulations, approved by the review committee of the Max Planck Institute of Immunobiology and Epigenetics and the Regierungspräsidium Freiburg, Germany (licence Az 35-9185.81/G-12/85).

Treatment with CB1954

Treatment of mice with CB1954 was done according to Corbeaux *et al*. (13). Pregnant mice were injected intraperitoneally on 3 consecutive days (E12.5, E13.5, E14.5 of gestation).

Tissue transplantation

Cervical thymi and thoracic thymus tissue were obtained from adult *Foxn1:eGFP* mice (15), as this facilitates the detection of the small cervical thymic lobes. In this strain (of mixed C57BL/6; FVB background), the frequency of cervical thymi is in the order of 75%, with an average of 1.25 cervical lobes per animal (range 1–3 lobes). Tissue fragments were transplanted under the kidney capsule of adult *Foxn1*$^{-/-}$ mice using a technique essentially equivalent to that described in Taguchi *et al*. (16).

Histology

Paraffin-embedded tissue sections were stained with haematoxylin/eosin according to standard techniques. Colitis score was determined following the procedure described in Izcue *et al*. (17).

Flow cytometry

Procedures for the analysis of haematopoietic cells in thymus, spleen and lymph nodes have been described in Swann *et al*. (18); see Supplementary Table 1, available at *International Immunology* Online, for list of antibodies.

Statistical analysis

T-tests (two-tailed) were used to determine the significance levels of the differences between the means of two independent samples, considering equal or unequal variances as determined by the *F*-test. For multiple tests, the conservative Bonferroni correction was applied.

Results and discussion

Near-total ablation of the thymic epithelium during embryogenesis

Our aim was to establish an animal model for the immunological consequences of thymic dysplasia. To this end, we employed a transgenic mouse model in which the bacterial nitroreductase gene (NTR) is expressed in TECs under the control of the mouse *Foxn1* promoter (13). During embryonic development, the large majority of TECs express *Foxn1* and thus also the bacterial enzyme from the transgenic expression cassette. NTR converts the CB1954 prodrug into a cytotoxic alkylating agent (19), thereby selectively killing expressing cells. To avoid the deleterious effects of CB1954 on pregnant mothers, crosses were used in which only the father was transgenic for the *Foxn1:CFP-NTR* transgene; note that NTR is expressed as a fusion protein with CFP to visualize expression of the transgene. When pregnant mothers (carrying both transgenic and non-transgenic embryos) are treated with CB1954 at the end of the second trimester, near-total ablation of the thoracic thymus can be achieved, as illustrated by the loss of CFP fluorescence (Fig. 1A). The timing of CB1954 treatment ensures that any cervical thymic tissue which develops at later stages of intrauterine development is unaffected by this treatment (13). Since not all individuals of our transgenic strain possess cervical thymi (15), our strategy was expected to result in cohorts of mice with variable thymic masses, ranging from no thymopoietic remnant (mice with total ablation of thoracic thymus and without cervical thymi) at one end of the spectrum, to mice with small pockets of thoracic thymus (Fig. 1B) and cervical thymic tissue at the other. The non-transgenic littermates served as controls for possible confounding effects resulting from the handling of pregnant mice. In conclusion, our strategy enables us to examine the consequences of variable degrees of thymic developmental dysplasia.

Phenotypic consequences of chemically induced thymic dysplasia

When we followed a large cohort of CB1954-treated non-transgenic and transgenic mice, we noticed that, beginning at 2–3 months of age, a progressively larger fraction of mice exhibited weight loss and diarrhoea (Fig. 1C), often associated with enlarged lymph nodes and splenomegaly (Fig. 1D). Inflammatory bowel disease was invariably associated with diarrhoea (Fig. 1E), as are lymphocytic infiltrations in the gut (Fig. 1F and G) and in other organs, such as liver, pancreas and salivary gland (Fig. 1H). All animals affected by this complex autoimmune syndrome were transgenic; no autoimmunity phenotype was observed in the non-transgenic (control) littermates despite their being exposed to the CB1954 prodrug during intrauterine development. Increased titres of autoantibodies were not detectable (data not shown).

The cohorts of mice described above were additionally examined for the degrees of thymopoietic activity by determining the number of CD4/CD8 double-positive thymocytes in the neck region and the thoracic cavity. The identification of small remnants of thymic epithelium was aided by the fluorescence emanating from the CFP-NTR fusion protein expressed in stromal cells (see Fig. 1A); however, because the CFP fluorescence signal is weak, it was not always possible to unambiguously identify all thymopoietic tissues in the mediastinum and, in particular, the neck regions. For the analysis of thymopoietic activity, we therefore removed large parts of the anterior upper mediastinal and neck tissues in order to comprehensively survey all sites of potential thymopoietic activity. As a consequence, this strategy necessitated that we focus on cells with the surface phenotype of CD4/CD8 double-positive thymocytes, to avoid confounding effects by co-sampling of non-thymic (i.e. lymph node) tissues. Overtly sick mice were euthanized at various ages; by the age of 35 weeks, most affected mice had developed diarrhoea, although the condition of some initially healthy mice deteriorated at later time points (Fig. 1C). Irrespective of the time point of analysis, we recovered fewer CD4/CD8 double-positive thymocytes (about 2–3 orders of magnitude) from the thoracic and neck regions in transgenic mice exposed to CB1954 than from control littermates, indicating the presence of persistent thymic hypoplasia (Fig. 2A).

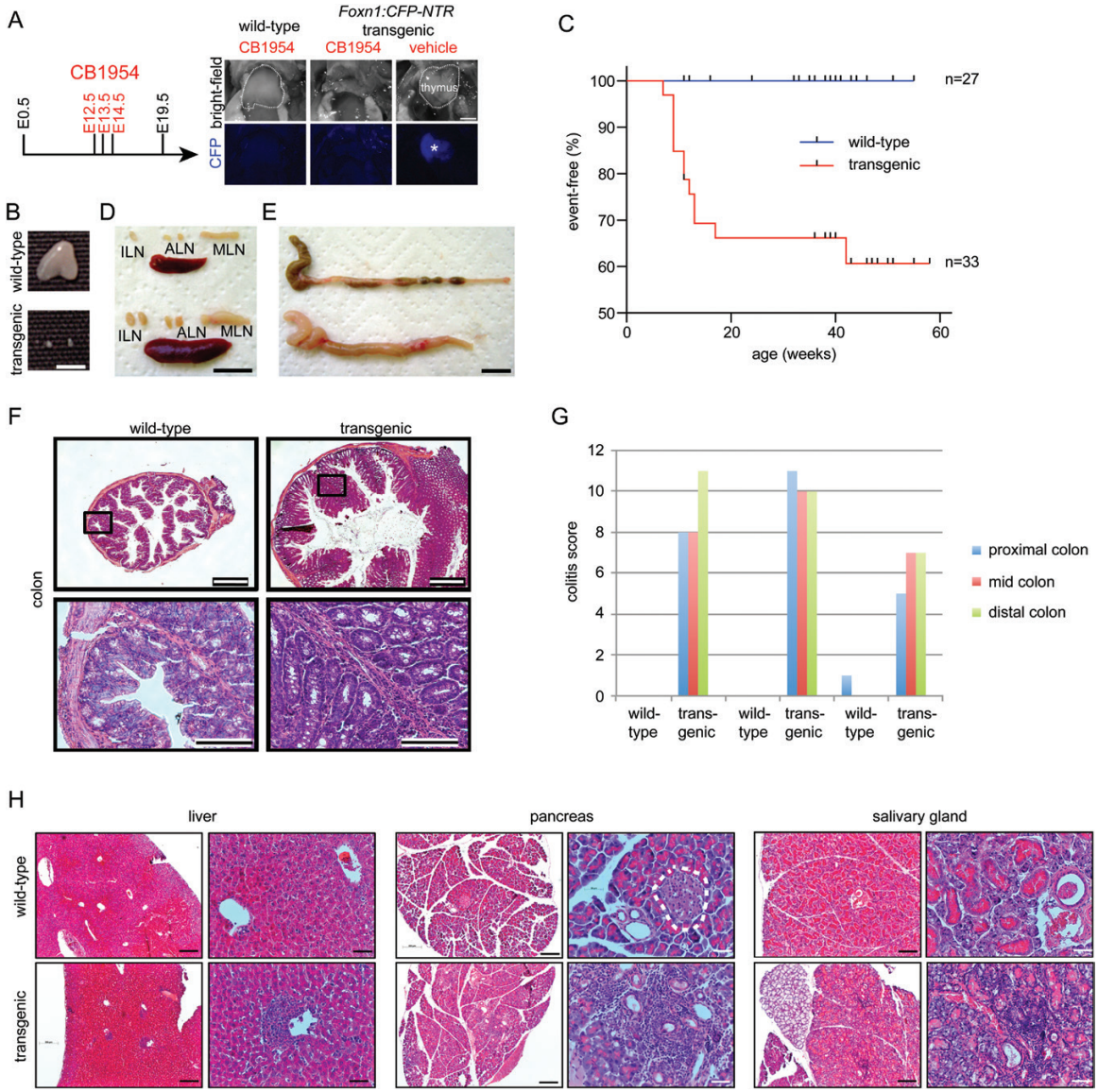

Fig. 1. Phenotypic consequences of chemically induced thymic dysplasia. (A) Schematic representation of the CB1954 prodrug treatment regimen during gestation (far left). In contrast to wild-type mice (left panel), treatment of transgenic embryos with CB1954 causes permanent loss of thymic tissue (middle; absence of blue fluorescence); vehicle-treated embryos exhibit a normal thymus (far right; blue fluorescence emanating from TECs in the thymus). Scale bar = 2.5 mm. (B) Presence of small thymic tissue remnants in transgenic mice after CB1954 treatment (lower panel); the thymus of a wild-type sibling is shown in the upper panel for reference. Scale bar = 2.5 mm. (C) Kaplan–Meier analysis of wild-type and transgenic mice after intrauterine CB1954 treatment; the occurrence of diarrhoea is recorded over age. $P = 0.0011$; log-rank (Mantel–Cox) test. (D) Enlarged lymph nodes and spleen in adult mice with thymic dysplasia (bottom group of organs) compared to healthy wild-type siblings; ALN, axillary lymph nodes; ILN, inguinal lymph nodes; MLN, mesenteric lymph nodes. Scale bar = 10 mm. (E) Example of an inflamed colon from a transgenic mouse with diarrhoea (bottom), compared to the colon from a healthy wild-type sibling (top). Scale bar = 10 mm. (F) Histological analysis of inflamed colon tissue. In the upper panels (scale bar = 0.5 mm), the enlarged regions shown in the lower panels (scale bars = 0.1 mm) are indicated. (G) Colitis scores for three wild-type and three transgenic mice measured in different regions of the colon. (H) Lymphocytic infiltrations in parenchymatous organs of transgenic mice exhibiting diarrhoea; an intact islet of Langerhans is indicated in the pancreatic high-power section. Scale bars = 0.2 mm for low power views and 0.05 mm for high power views.

Peripheral lymphopenia correlates with thymopoietic activity

Compared to control mice, all transgenic mice exhibited peripheral T-cell lymphopenia (Fig. 2B), with a prominent phenotypic signature of activation indicative of homeostatic proliferation (Fig. 2C). Interestingly, healthy transgenic mice did not have higher numbers of T cells in the periphery than sick transgenic mice (Fig. 2B), indicating that the overall number of peripheral T cells is a poor predictor of outcome. By contrast, the fraction of peripheral T cells expressing CD44 ($CD3^+CD44^+CD25^-$) correlates with the presence of diarrhoea (Fig. 2C). Since the expression of CD44 is an indicator of homeostatic proliferation (20), it appears that the peripheral T-cell compartment in mice eventually developing autoimmunity was initially smaller than that of mice maintaining immune homeostasis. Hence, homeostatic proliferation of

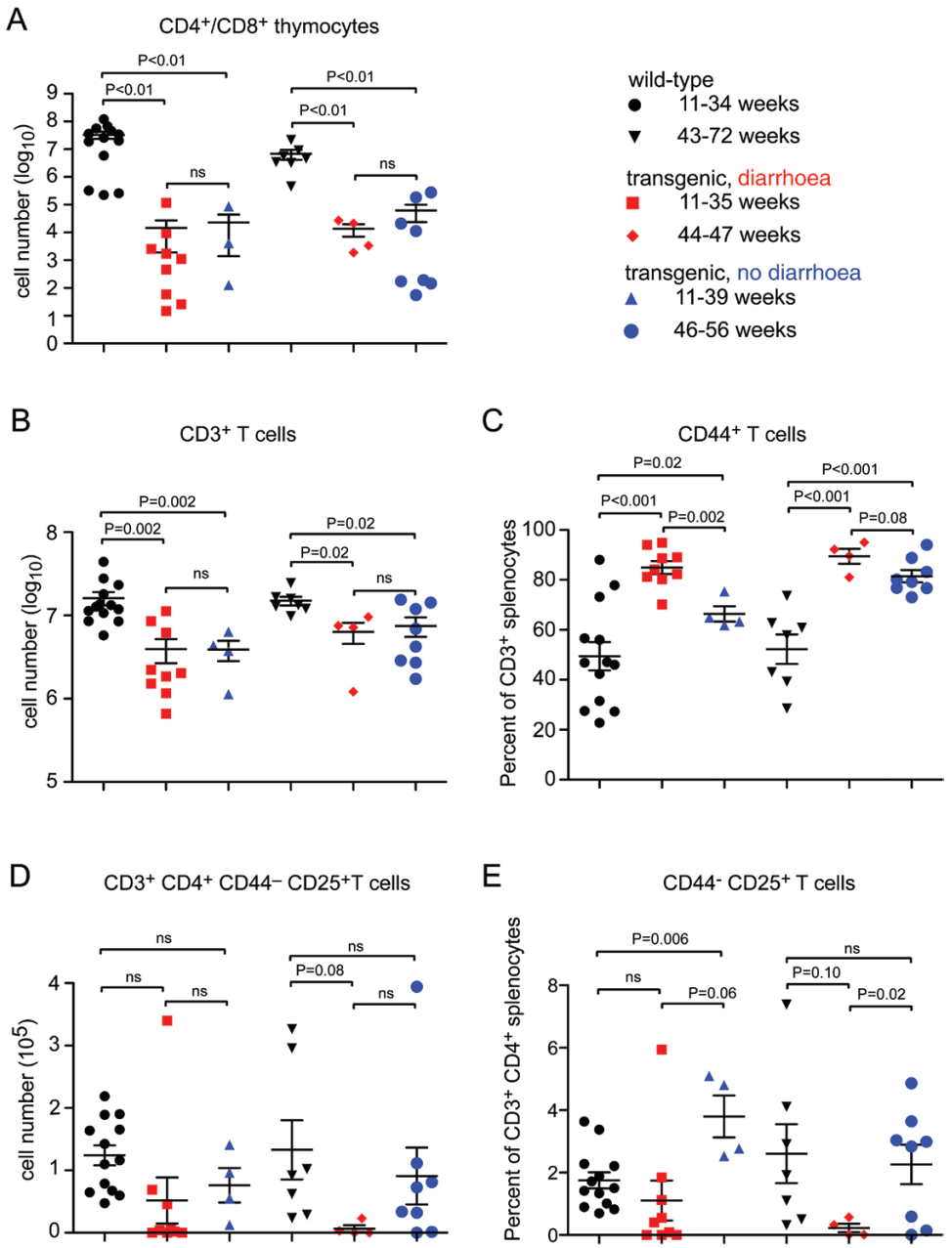

Fig. 2. Peripheral lymphopenia as a consequence of thymic dysplasia. (A) Number of CD4/CD8 double-positive thymocytes in combined mediastinal and cervical tissues. Mice are grouped according to age, genotype and clinical condition (absence/presence of diarrhoea); note that wild-type mice never developed diarrhoea. Since there is a constant albeit age-dependent relationship between the number of thymocytes and the number of TECs (26, 27), it is possible to estimate the fraction of thymopoietic tissue remaining in the transgenic mice. Because sick mice tend to have lower numbers of CD4/CD8 double-positive thymocytes, we calculated the fraction of thymopoietic tissue only for the apparently healthy cohorts of transgenic mice. The thymopoietic capacity of the younger group of mice (11–39 weeks of age) amounts to ~0.1% of control mice, and the thymopoietic capacity of the cohort of older mice corresponds to ~1% of controls. Note that in one of the four transgenic mice of the 11–39-week-old cohort of apparently healthy mice, no $CD4^+/CD8^+$ double-positive thymocytes could be found; this data point cannot be displayed in the logarithmic representation of the results chosen for this panel. (B) Number of $CD3^+$ T cells in the spleen. (C) Percentage of splenic $CD3^+$ T cells expressing CD44. (D) Absolute numbers of T cells with regulatory phenotype ($CD3^+CD4^+CD44^-CD25^+$). (E) Proportion of $CD44^-CD25^+$ cells among $CD3^+CD4^+$ splenocytes. *P* values exceeding 0.20 are categorized as not significant (ns).

peripheral T cells predisposes to autoimmunity, a well-known clinical phenomenon (1). Indeed, when compared to an autoimmunity-stricken sibling, transgenic mice without diarrhoea exhibit a larger fraction of naive $CD3^+CD62L^+CD44^-$ splenic T cells, and a smaller fraction of activated $CD62L^-CD44^+$ T cells (Supplementary Figure 1, available at *International Immunology* Online). In addition, in transgenic mice with autoimmunity, we observed low numbers of splenic T cells with a regulatory phenotype ($CD3^+CD4^+CD44^-CD25^+$), whereas apparently healthy transgenic mice had two to three times

more of such cells (Fig. 2D). This difference was also seen when we compared the fractions of $CD44^{-}CD25^{+}$ cells among $CD4^{+}$ splenic T cells (Fig. 2E). Collectively, these findings support the notion that a state of immune dysregulation underlies the autoimmune phenomena observed in our model.

Cervical thymi are not solely responsible for the autoimmunity phenotype

In its present form, our transgenic model is tailored to specifically destroy the thoracic but not the cervical thymic tissues (13). Therefore, it was important to rule out the possibility that the observed autoimmunity was caused by abnormal T-cell differentiation and/or selection in the residual cervical thymi *per se* in (at least) some treated transgenic animals (13). To address this issue, we transplanted cervical thymi and/or fragments of thoracic thymi under the kidney capsule of *Foxn1*-deficient *nude* mice. Since nude mice entirely lack thymopoietic activity, they are protected from any T-lymphopenia-associated autoimmunity (21); hence, any autoimmunity arising after transplantation must be caused, directly or indirectly, by the grafted tissues. A total of eight nude mice received transplants of thoracic or cervical thymi, four of which developed an autoimmune syndrome, chiefly characterized by diarrhoea and/or splenomegaly, mirroring the frequency of autoimmunity in mice with dysplastic thymus (Fig. 1C); sick mice were euthanized between 7 and 13 weeks after transplantation, whereas the apparently healthy mice were monitored for up to 37 weeks after transplantation (Supplementary Table 2, available at *International Immunology* Online). The number of peripheral T cells varied, although no correlation existed between peripheral T cell numbers and clinical outcome; hence, as observed after chemically induced thymic aplasia (Fig. 2B), a low cell count was not predictive of diarrhoea (Fig. 2B). Hence, we conclude that in our system, and in accordance with previous results (22), T cells produced by cervical thymi *per se* are not associated with autoimmunity (Supplementary Table 2, available at *International Immunology* Online). Therefore, rather than being caused by a qualitatively different population of T cells maturing within cervical thymi, the autoimmune phenomena observed in our model can be attributed to the chronic lymphopenic state of and the ensuing imbalance in immunoregulatory mechanisms in these mice.

Conclusion

Collectively, our present results indicate that *Foxn1:CFP-NTR* transgenic mice represent a useful model of thymic dysplasia. As shown previously, the degree of TEC ablation can be adjusted by modifications of the intensity of the pharmacological treatment during pregnancy (13). We anticipate that this transgenic system will find its chief use in modelling congenital thymic dysplasia and in exploring and optimizing suitable treatment options, such as thymus transplantation (23). Future technical modifications might render this model applicable in the examination of the effect of postnatal insults (infectious or iatrogenic) to the thymic epithelial compartment. At present, one of the major obstacles for this type of application is the activity of the *Foxn1* promoter also in the suprabasal keratinocyte layer of skin (24); treatment of mice with the prodrug after the onset of *Foxn1* expression in keratinocytes (after E.14.5) causes severe damage to the skin, with only few embryos surviving this treatment. Hence, efforts are underway to modify the 28 kb promoter (25) currently used in the expression cassette to render it specific for the thymic epithelium, in order to be able to induce thymic dysplasia during late gestation and after birth.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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