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Rapid thymectomy of NSG mice to analyze the role of native and grafted thymi in humanized mice

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Humanized mice are invaluable tools to study various disease pathologies while allowing experimental manipulation of human cells *in vivo*, especially in the context of autoimmunity and transplantation. Humanized mice generated with transplantation of human fetal thymus and fetal liver-derived CD34+ cells to immunodeficient mice, one version of which is termed the “Bone marrow, Liver, Thymus (BLT)” mouse, are widely used to study the human immune system [1]. NOD-scid common gamma chain knockout (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) (NSG) mice are immunodeficient mice most often used to generate humanized mice. NSG mice have a vestigial thymus (Figure 1A), reflecting the absence of interaction of developing thymocytes with thymic epithelial cells (TECs), which is required for normal development of TECs [2]. Even after human immune systems are generated with human fetal liver CD34+ hematopoietic stem cells (HSCs), the size of the native NSG mouse thymus is much smaller and its cell count is much lower than that of a human thymus grafted under the kidney capsule (Figure 1B). However, the NSG mouse native thymus supports a low level of human thymopoiesis (Figure 1C), with similar ratios of double positive (DP) and single positive (SP) CD4 and SP CD8 cells compared to grafted human thymi (Figure 1D). Regulatory T cells (Tregs), however, are generated in lower proportions in native mouse thymus compared to grafted human thymus (Figure 1D). These observations complicate humanized mouse models that include a grafted human thymus, as the two pools of peripheral human T cells developing in native mouse and grafted human thymus may have different functional properties and antigen recognition capabilities. To address this limitation and develop a model allowing assessment of peripheral T cells developing only from a grafted thymus, we devised a rapid method for removing the native thymus of NSG mice (Figure 1F).

Thymectomy has been shown to effectively prevent the development of mouse T cells in immunocompetent mice. In 1960, Donald Metcalf performed thymectomy on 4–6-week-old C57BL mice and found that lymphocyte counts declined and lymphoid organs became

atrophic [3]. The function of the thymus was not known at that time and he concluded that thymus secretes a lymphocytosis-stimulating factor [3]. Jacques Miller, who discovered the immunological function of the thymus, showed that neonatally thymectomized mice (1–16h after birth) fail to reject allogeneic and xenogeneic skin grafts [4] and fail to mount antibody responses to *Salmonella typhi* H antigen [5]. When he transplanted a thymus to neonatally thymectomized mice, immune function was restored [5]. Miller described a wasting syndrome in neonatally thymectomized mice and described it as an infectious disease [6], while other groups later characterized it as an autoimmune disease, due to insufficient Treg development [7] and/or lack of efficient negative selection at the neonatal stage [8]. Miller later showed that thymectomy in adulthood, in contrast to the neonatal stage, had no immediate effect on immune function [9], though some subtle deficiencies were manifested later [9]. Studies with thymectomized and bone marrow transplanted mice eventually led to the discovery of two types of lymphocytes, B and T cells [10]. Therefore, thymectomy has played a key role in forming our understanding of the immune system.

The published protocols for adult and neonatal thymectomy rely on cutting the ribs in order to open a hole in the chest allowing removal of thymi that are of normal size in immunocompetent mice [11]. As the size of the NSG mouse thymus is much smaller than that in immunocompetent mice, we developed a method (Figure 1F) that does not require cutting the ribs and therefore is less invasive and associated with less risk of pneumothorax (detailed in supplementary section). Additionally, while most protocols use a suction system to remove the thymus, we use forceps, which diminishes the risk of rupturing the vessels. As the thymus is surrounded by large vessels, a bleeding incident could be fatal. The surgery for each mouse takes less than five minutes, allowing large groups of mice to be prepared in one session. The survival rate is greater than 90% and the rate of failure (incomplete thymectomy) is less than 5%.

Thymectomized mice receiving human hematopoietic stem cells (HSCs) develop other human hematopoietic lineages but no T cells (Figure 2A). Our model therefore allowed us to compare the behavior of these human immune cells in the presence or absence of human T cells. The number of human B cells, monocytes and dendritic cells were significantly reduced in the absence of a thymus and hence T cells (Figure 2A-C).

We observed that grafted human thymus (in thymectomized NSG mice) results in higher levels of human T cell reconstitution in the periphery compared to that achieved from a native mouse thymus, as shown by significantly increased T cell counts and greater proportions of recent thymic emigrants (defined as CD31⁺ CD45RA⁺) in the periphery of animals with a human compared to a native mouse thymus (Figure 2A).

B cells, monocytes and DC counts were higher in mice with a human thymus graft compared to mice with a native mouse thymus. These observations might reflect a supporting influence of T cells on maintenance of HSCs, as suggested by Geerman et al. [12], or a direct impact of T cells and/or their secreted cytokines on proliferation and/or survival of other hematopoietic lineages. T cells are a major source of IL-3, a key and highly species-specific hematopoietic cytokine that might play a role in these observations [13]. It is also possible

that the thymus itself has some supporting effect on HSCs and/or other hematopoietic lineages.

This thymectomized NSG model allows comparison of the developmental features and antigen recognition patterns of human T cells in autologous human, allogeneic human and xenogeneic thymus without contamination by T cells generated in a mouse thymus, which may otherwise confound results. For instance, T cells originating from NSG native thymus could not be distinguished from those developing in transplanted neonatal human thymus in a recent study, calling into question the role of grafted neonatal thymus tissue in the T cell reconstitution observed [14]. To assess the reconstituting ability of pediatric thymus without the confounding effect of a native mouse thymus, we grafted 3–5 pieces of thymus tissue from 4 y/o or 13 y/o donors under the kidney capsule of thymectomized NSG mice and intravenously injected fetal liver-derived CD34⁺ cells. With the exception of one mouse, the recipient mice did not demonstrate human T cells in periphery (Figure S2A). Grafted post-natal thymus did not grow (Figure S2B) and the harvested small grafts, even in the mouse with peripheral human T cells, did not contain a robust population of CD4⁺ CD8⁺ cells, suggesting minimal or no active thymopoiesis (Figure S2C). Most probably, T cells that showed up in the periphery of that mouse originated from pre-existing thymocytes in the donor thymus and escaped depletion with anti-CD2 antibody. Likewise, mouse thymectomy is required for the valid assessment of the efficacy of ES- and iPS-derived thymic epithelial cells in supporting human thymopoiesis in vivo. The simplified method for NSG mouse thymectomy described here will facilitate such studies.

Study approval.

Protocols involving the use of human tissues and animals were approved by the Institutional Review Board and the Institutional Animal Care and Use Committee of Columbia University (New York, NY), and all of the experiments were performed in accordance with the protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of native mouse thymus and grafted human thymus in NSG, NOD and humanized mice.

A, Appearance of NOD vs NSG (non-humanized) mouse thymi. B, Appearance of native NSG thymus vs human grafted thymus 20 weeks after human HSC administration. Thymic cell counts are also shown for grafted human thymus (n=51, pooled from 8 independent experiments) and native mouse thymus (n=6, pooled from 3 independent experiments) groups. C, Flow cytometry (FCM) dot plot of human thymocytes in a representative NSG native thymus at 20 weeks post-transplantation. D, Percentage of DP, SP-CD4 and SP-CD8 cell subsets among human CD45⁺ cells in humanized mice with a native mouse thymus

(n=7, pooled from 3 independent experiments) vs a grafted human thymus (n=11, pooled from 3 independent experiments). E, Percentage of thymic Tregs (defined as CD25⁺ FOXP3⁺ CD4 T cells) among SP-CD4 T cells in humanized mice with a native mouse thymus (n=7, pooled from 3 independent experiments) versus a grafted human thymus (n=8, pooled from 3 independent experiments). Each symbol in plots in panels B, D and E represents one mouse. Means±SEM are also shown. Unpaired t-tests with Bonferonni multiple-comparisons correction was used for comparisons between each group. F, Steps of the protocol for removing the native NSG mouse thymus.

Figure 2. Human immune cell reconstitution in humanized mice with grafted human thymus, native mouse thymus and no thymus.

In 3 separate experiments (A-C), humanized mice were generated with human fetal liver CD34⁺ HSCs and different thymi (autologous human fetal thymus vs native mouse thymus vs no thymus). The native NSG mouse thymus was removed in the “human thymus” and “no thymus” groups. In Experiment 1, all three groups were compared in a single experiment. In Experiments 2 and 3, only the two indicated groups were generated. In Experiment 1, the number of mice for no thymus, human thymus and mouse thymus groups were 3, 5 and 11, respectively. In Experiment 2, the number of mice for human thymus and mouse thymus

groups were 12 and 13, respectively. In Experiment 3, the number of mice for no thymus and human thymus groups were 4 and 3, respectively. All groups in each experiment were generated with the same HSCs. At different times post transplantation, human immune cell reconstitution was evaluated using flow cytometry (FCM). The cell count per μl of blood is reported for T and B cells, conventional dendritic cells (cDCs) and monocytes, defined as CD3^+ , CD19^+ , CD11c^+ and CD14^+ cells, respectively. The percentage of recent thymic emigrants (RTEs), defined as $\text{CD31}^+ \text{CD45RA}^+$ cells among CD4 and CD8 T cells is reported for Experiment 1. All data are shown as means $\pm$ SEM. Unpaired t-tests with Bonferonni multiple-comparisons correction or one-way ANOVA were used for comparisons between different groups at each time point. Statistically significant differences are indicated with * (* $0.01 < \text{p-value} < 0.05$, ** $0.001 < \text{p-value} < 0.01$, *** $\text{p-value} < 0.001$).