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Notch activation in thymic epithelial cells induces development of thymic microenvironments

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ABSTRACT

The development and maintenance of thymic microenvironments depends on sustained crosstalk signals derived from developing thymocytes. However, the molecular basis for the initial phase in the lymphoid dependent development of thymic epithelial cells (TECs) remains unclear. Here we show that similarly to regular thymocytes, developing B cells enforced to express the Notch ligand Delta-like-1 (DLL1) efficiently induce the non-polarized, three-dimensional (3D) meshwork architecture of cortical TECs in fetal thymic organ culture. Moreover, the DLL1-overexpressing B cells induce well-developed distinct medullae. Such medullae also arose in lobes reconstituted with Rag2$^{-/-}$ thymocytes overexpressing DLL1. Our present findings thus strongly suggest that Notch signaling from thymocytes to TECs induces TEC development at an early phase of thymic organogenesis. The present approach using non-T lineage cells for the in vitro construction of thymic environments may also provide a novel tool for thymus regeneration and T cell production in immunocompromised individuals.

1. Introduction

The thymic stroma comprises two major environments, the cortex and the medulla. Each region consists of distinct types of thymic epithelial cells (TECs): cortical TECs (cTECs) and medullary TECs (mTECs). It is now well accepted that both cTECs and mTECs arise from endodermal epithelial cells in the third pharyngeal pouch (Blackburn and Manley, 2004; Holländer et al., 2006; Rossi et al., 2007). In mice, the formation of the thymus anlage starts at 10 days post-coitum (dpc), when a cyst-like structure of polarized stratified epithelial cells is produced through invagination of the endoderm in the pharyngeal pouch. Colonization of the thymic anlage by T cell progenitors starts at 11 dpc, when the progenitors are seen juxtaposing the epithelial primordium (Itoi et al., 2001; Masuda et al., 2005a,b). The loss of polarity in epithelial cells leading to the induction of an epithelial cluster begins at 12 dpc, while the formation of an epithelial reticulum segregating in cortical and medullary environments proceeds from 13 dpc (Klug et al., 2002).

The induction of the non-polarized sponge-like architecture of TECs leading to the creation of thymic microenvironments supportive to thymopoiesis is critically regulated by developing thymocytes themselves and requires physical contact between the two cell populations. It has been shown that immature thymocytes at the CD4$^+$CD8$^+$ double positive (DP) stage play a critical role in the formation of the cTECs, whereas the medulla is formed through interaction of mTECs with mature single positive (SP) cells (Holländer et al., 1995; van Ewijk et al., 1994, 2000; Shores et al., 1994; Philpott et al., 1992). Such a unique mutual interdependency between thymocytes and TECs is often referred to as "thymic crosstalk" (van Ewijk et al., 1994).

Various types of ligand–receptor interactions have been implicated in signaling from TECs to thymocytes, which regulate the development of T cells. These include cytokines and chemokines and their receptors, such as SCF/c-kit, IL-7/IL-7R, CCL19/CCL21/CCR7, CXCL12/CXCR4, and MHC/TCR signaling (Anderson and Jenkinson, 2001; Petrie, 2002; Gray et al., 2005; Takahama, 2006). It has also been reported that Notch molecules expressed on early

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thymocytes play a crucial role in intrathymic T cell development (Radtke et al., 1999; Pui et al., 1999; Feyerabend et al., 2009). Although signals derived from thymocytes influencing the development of TECs remained elusive, recent studies have provided several examples for such signals. A first example in this context is LTβ/LTβR signaling, which has been shown to play a role in the functional maturation of mTECs (Boehm et al., 2003; Kajiura et al., 2004). Subsequently, involvement of RANKL/RANK signaling and CD40L/CD40, from so-called inducer cells and/or SP cells to mTECs, was demonstrated (Rossi et al., 2007; Akiyama et al., 2008; Hikosaka et al., 2008; Irla et al., 2008). However, these pathways appear not to be involved in the creation of the typical thymic architecture during ontogeny, because mice deficient for these ligands/receptors show a normal development in the basic structure of their medullary regions. Crosstalk signals influencing cTEC development remain at present unknown.

It is well known that thymic epithelial cells have a high regeneration potential to revive from serious damage caused by a loss of crosstalk signals. For example, when a fetal thymic (FT) lobe is treated with deoxyguanosine (dGuo), thymocytes are depleted from the lobe, resulting in the loss of cytoplasmic extensions of TECs, cluster formation of TECs, and appearance of thymic cysts lined by polarized epithelial cells (van Ewijk et al., 1999). Such a dGuo-treated lobe (dGuo-lobe) retains full potential to support T cell development, hence this in vitro system is widely used in studies on thymopoiesis (Anderson and Jenkinson, 2001). At the same time, this system can also be used to examine the development of TECs (Germeraad et al., 2003).

In order to identify key molecules involved in thymic crosstalk, we have employed a "gain-of-function" approach. For such a study, it is useful to employ the cells that can infiltrate into the thymic epithelial stroma but cannot influence TEC development. B lymphocytes seemed suitable candidates, because developing B cells, like T cells, localize in between epithelial cells, but unlike T cells, they minimally induce TEC development. We chose to examine whether Notch signaling on TECs is involved in thymic crosstalk. Several reasons motivate this choice. First, Notch–Delta interaction is involved in epithelial cell development in other organs like the skin and the gut (Lowell et al., 2000; Lefort and Dotto, 2004; Fre et al., 2005; Estrach et al., 2008). Second, previously reported studies argue that Notch receptors are expressed on TECs (Kaneta et al., 2000; Radtke et al., 2004). These reported data imply that a reciprocal Notch–Delta interaction between thymocytes and TECs might represent one example of molecular thymic crosstalk.

In the present study we show that, upon enforced expression of the Notch ligand Delta-like-1 (DLL1) in developing B cells in an in vitro gain of function approach, these cells integrate in the epithelial stroma and efficiently induce development of the non-polarized phenotype of both cTECs and mTECs. These results indicate that the Notch signaling pathway from thymocytes to TECs is involved in the lymphoid-dependent phase of TEC maturation, as well as in TEC-dependent lymphoid development. Thus the Notch pathway is a molecular example of thymic crosstalk.

2. Experimental

2.1. Materials and methods

2.1.1. Mice

C57BL/6 (B6) and Balb/c mice were purchased from CLEA Japan Inc (Tokyo, Japan). B6Ly5.1 mice and Rag2$^{-/-}$ mice were maintained in our animal facility. Embryos at various stages of gestation were obtained from time-mated pregnant mice. The day of finding the vaginal plug was designated as 0 dpc.

2.1.2. Antibodies

For flow cytometric studies, the following antibodies were used: anti-Ly5.1 (A20), anti-c-kit (2B8), anti-erythroid lineage cells (TER119), anti-Gr-1 (RB6-8C5), anti-B220 (RA3-6B2), anti-Thy1.2 (53-2.1), anti-CD19 (1D3), anti-CD45 (30-F11), anti-CD8 (53-6.7), anti-CD4 (H129.19), anti-mouse IgM (II/41), anti-EpCAM (G8.8), anti-BP-1 (6C3). All were purchased from BD PharMingen (San Jose, CA). Goat anti-rat IgG was purchased from Molecular Probes (Eugene, OR). TER119, anti-Gr-1, anti-B220, anti-CD19 and anti-Thy-1.2 were used as Lin for cell sorting of progenitor cells. For immunohistochemistry, the following antibodies were used: anti-Thy-1, anti-B220, rat anti-cytokeratin (K)8 (PROGEN, Heidelberg, Germany), rabbit anti-cytokeratin (Dako, Glostrup, Denmark), rabbit anti-IKAROS (Hattori et al., 1996), ER-TR4 (van Vliet et al., 1984), ER-TR5 (van Vliet et al., 1984), and biotinylated UEA-1 (Vector, Burlingame, CA), as primary antibody or reagent, followed by Alexa Fluor488 goat anti-rat IgG (H+L) conjugate, Alexa Fluor488 goat anti-rabbit IgG conjugate, goat anti-rabbit IgG-Texas Red conjugate, Alexa Fluor546 goat anti-rat IgG (H+L) conjugate, Alexa Fluor546 streptavidin conjugate, and goat anti-rabbit IgG-Texas Red conjugate (all from Molecular Probes), as secondary reagents. For flow-cytometric separation of cTECs and mTECs, the following antibodies were used: biotinylated UEA-1, anti-EpCAM (G8.8), anti-CD45, anti-Ly51 (clone 6C3/BP1).

2.1.3. Preparation of fetal hematopoietic and thymic epithelial cells

Embryos were separated from the placenta using fine forceps. Embryos were placed in a Petri dish containing tissue culture medium while the FT and the fetal liver (FL) were isolated by dissection. A single cell suspension of FL was prepared by gently pipetting the FL lobes with a capillary pipette. To obtain a cell suspension of FT, lobes were minced between glass slides using the frosted portion of the slide. All fetal cells were then passed through 40 μm nylon mesh and rinsed with RPMI-1640 medium. Viable cells were counted using trypan blue dye exclusion.

2.1.4. Preparation of fetal TECs

Thymuses for 17 dpc fetuses were dissected and trimmed of fat and connective tissue. Small thymic fragments were gently stirred in 50 ml of RPMI-1640 on a magnetic stirrer at 4 °C for 30 min to remove the majority of thymocytes. Next, thymic fragments were placed into 30 ml of fresh RPMI-1640 and stirred for another 30 min at 4 °C. Then, thymic fragments were incubated in 40 ml of collagenase (Wako, Osaka, Japan) (1 mg/ml) supplemented with DNase (Sigma, St. Louis, MO) (10 mg/ml) in RPMI-1640 for 15 min at 37 °C. Thymic fragments were further teased to be reduced in size using a Pasteur pipette. Fragments were then repeatedly incubated with 12 ml of freshly prepared enzyme mixtures at 37 °C for 15 min. Single stromal cells were prepared by gently pipetting fragments using a 21G needle followed by a 26G needle. Cells were washed twice with PBS containing FCS (2%) and EDTA (5 mM) and then passed through 40 μm nylon mesh. In cell sorting, CD45$^-$EpCAM$^+$Ly51$^+$UEA-1$^-$ cells and CD45$^-$EpCAM$^+$Ly51$^-$UEA-1$^+$ cells were collected as cTECs and mTEcs, respectively.

2.1.5. Fetal thymic organ culture, reconstitution experiments

To prepare thymocyte-depleted FT lobes, FT from 15 dpc embryos were cultured on polycarbonate filters (pore size 8.0 μm) (Nucleopore Co., Pleasanton, CA) floating on culture medium containing 1.35 mM dGuo (Nacalai Tesque, Kyoto, Japan) for a period of 6 days. For reconstitution experiments, the lobes were washed and single dGuo-treated lobes were placed individually into wells of a 96-well V-bottom plate, to which progenitors were added. The plates were centrifuged at 150 × *g* for 5 min. To culture the lobes under high oxygen submersion (HOS) conditions, placed