

# Proinsulin Expression by Hassall's Corpuscles in the Mouse Thymus

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**The thymus expresses proinsulin, among many other tissue-specific antigens, and the inheritance of genetically determined low thymic proinsulin expression has been associated with impaired proinsulin-specific autoreactive T-cell tolerance and type 1 diabetes susceptibility. The cellular and molecular biology of proinsulin expression in the thymus remains unknown, and contradictory reports exist regarding the identity of proinsulin-producing cells. Using knock-in mice expressing  $\beta$ -galactosidase ( $\beta$ -Gal) under the control of an endogenous insulin promoter, we found that thymic proinsulin and  $\beta$ -Gal transcripts were detectable at high levels in purified thymic epithelial cells. Immunohistochemical analysis of  $\beta$ -Gal activity showed that most proinsulin expression can be accounted for by rare medullary epithelial cells of the Hassall's corpuscles. Moreover, flow cytometry analyses of  $\beta$ -Gal-positive cells showed that only 1–3% of all epithelial cells express proinsulin, and this technique will now provide us with a method for isolating the proinsulin-producing cells in mouse thymus. *Diabetes* 53:354–359, 2004**

**T**ype 1 diabetes results from autoimmune destruction of the insulin-producing pancreatic  $\beta$ -cells by autoreactive T-cells (1). The inherited immunologic defects responsible for this disease are polygenic in nature. Besides the major histocompatibility complex (HLA) on chromosome 6p21 (2–4), one of the few genetic loci that have been confirmed and functionally studied maps to the insulin gene regulatory region, containing a variable number of tandem repeats polymorphism. Variable number of tandem repeats alleles have little effect on pancreatic insulin expression, but in the thymus, the predisposing class I alleles correlate with low and the protective class III alleles with high levels of insulin expression (5,6). Low expression of insulin in the thymus of NOD mice has been reported (7) and may play a role in diabetes susceptibility. More important, we recently showed that mice with thymus-restricted insulin deficiency showed a strong peripheral proinsulin-specific

T-cell reactivity when compared with control animals (8). This corroborates our hypothesis (5) that genetically determined low thymic insulin expression levels predispose to human type 1 diabetes through less efficient insulin-specific autoreactive T-cell selection. In addition, it has recently been shown that NOD mice with drastically reduced insulin expression in the thymus present an accelerated type 1 diabetes development (9).

The thymus is a central lymphoid organ responsible for the generation of the T-cell repertoire, during which those T-cells that recognize self-antigens must be eliminated during T-cell development in the thymus by negative selection through clonal deletion (10–13). The importance of negative selection for tolerance to tissue-specific autoantigens such as insulin had, until recently, been in doubt. Such antigens were traditionally thought to be unavailable for presentation in the thymus, and tolerance to them was believed to occur by extrathymic peripheral mechanisms such as anergy, ignorance, or deletion (14–16). Growing evidence suggests that this may not be the case, and an increasing number of proteins previously considered to be strictly tissue-specific can be detected in the thymus. These include hormones, transcription factors, and secreted proteins. Many of the ectopically expressed antigens (proinsulin, thyroglobulin, myelin basic protein, and retinal S-antigen) are associated with organ-specific autoimmune disease (type 1 diabetes, thyroiditis, multiple sclerosis, and uveitis, respectively) (17–19). However, the expression of these self-antigens is limited to a very small subset of thymic cells, and studying how these specific cells result in the deletion of a wide variety of autoreactive T-cells has not been straightforward.

The thymus is anatomically divided into subcapsular, cortical, and medullary compartments. Thymic stroma contains a variety of professional antigen-presenting cells, including bone marrow-derived dendritic cells (DCs), macrophages, B-cells, endoderm-derived cortical epithelial cells, and medullary epithelial cells (mTECs) (20). A striking morphologic feature of the medulla is the presence of Hassall's corpuscles (HCs), which consist of concentric whorls of stratified keratinizing epithelium and share antigenic properties with ectodermic epithelium (21). Well developed in human thymus, they are small and sparse in murine thymus. The function of HC remains unknown, although recent studies in human thymus have demonstrated that they are the only regions in the medulla to contain apoptotic T-cells (22). In addition to this, the mTECs that make up the corpuscles have been shown to express a leukocyte and hematopoietic attractant, stromal-derived factor-1, and CD30-1, a membrane-associated glycoprotein related to tumor necrosis factor that is in-

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 $\beta$ -Gal,  $\beta$ -galactosidase; DC, dendritic cell; mTEC, medullary epithelial cell; HC, Hassall's corpuscle; TEC, thymic epithelial cell.  
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volved in T-cell signaling (23,24). Moreover, it has also been shown that CD30-deficient mice contain elevated numbers of thymocytes and show a gross defect in negative but not positive selection (25). Together, these results suggest that the HCs are central to negative T-cell selection.

In mice, conflicting reports exist regarding the identity of proinsulin-producing cells in the thymus (19,26). To provide a definite answer, we used knock-in mice expressing  $\beta$ -galactosidase ( $\beta$ -Gal) under the control of endogenous *Ins2* promoter. We show that most of insulin promoter-directed transcription can be accounted for by epithelial cells in HC, which can be isolated by flow cytometry using a fluorescent  $\beta$ -Gal substrate.

## RESEARCH DESIGN AND METHODS

*Ins1*-KO and *Ins2*-KO mice were a gift of Dr. J. Jami (Institut Cochin, Paris, France) and were generated as described by Duveillie et al. (27). *Ins2*-KO mice have their *Ins2* gene replaced by a functional *LacZ* gene that is under the regulation of the endogenous *Ins2* gene promoter, whereas *Ins1* was disrupted by introducing a neomycin cassette against the same genetic background. All mice were bred at our animal facility under conditions specified by the Canadian Council of Animal Care.

**RNA preparation and cDNA synthesis.** Pancreatic and thymic RNA from 2- to 4-week-old mice was isolated by Trizol (Gibco, Rockville, MD) and treated with DNase (Gibco). RNA was isolated from enriched thymic cell populations with RNeasy Mini Kit (Qiagen, Mississauga, Ontario, Canada) with typical yields of RNA between 50 and 400 ng. An equal amount of each RNA (2.5  $\mu$ g of tissue-extracted and 50–100 ng of single cells or more as with the case of DCs) was reverse-transcribed using random primers and Superscript II Reverse-Transcriptase (Gibco). Parallel samples in which reverse transcriptase was omitted were always included to confirm the absence of DNA contamination.

**Quantitative and semiquantitative RT-PCR.** Insulin expression in the thymus- and pancreas-extracted tissues was analyzed by quantitative RT-PCR. An equal amount of each cDNA sample (2  $\mu$ l from thymic and 2  $\mu$ l from 1:300 diluted pancreatic cDNA) was then added to serial dilutions of the insulin competitor (56, 28, 14, 7, 3.5, 1.7, or 0 amol/tube) or with the cyclophilin competitor (2.5, 1, 0.5, 0.25, 0.1, or 0  $\times 10^9$  copies/tube) for loading control. The insulin competitor is an internally deleted cloned insulin sequence amplified with the same primers but 45 bp shorter, constructed as described by Forster (28). For cyclophilin analysis, we used the PCR Kit for cyclophilin (Quantum mRNA; Ambion, Austin, TX) as described by the manufacturer. The PCR products were resolved using PAGE, and bands were quantified using Geldoc Software (Bio-Rad, Hercules, CA). The insulin concentrations were normalized for the amount of starting RNA using cyclophilin measurement as described (8).  $\beta$ -Gal expression in pancreas and thymus was analyzed by semiquantitative RT-PCR. An equal amount of cDNA (2  $\mu$ l of diluted 1:1, 1:10, and 1:100 cDNA samples from tissues and undiluted amount for cell preparations) was amplified by PCR. The thermal cycler conditions for  $\beta$ -Gal and insulin amplification were 150 and 180 s at 92–95°C followed by 40 cycles of 92–94°C for 30–60 s, 53–55°C for 30–60 s, and 74–72°C for 60–90 s, respectively. The following primer pairs were used (forward and reverse primers respectively): insulin-specific primers: 5'-GGCTTCTTCTACACCCC CA-3' and 5'-TACCAGCTGGAGAAGACTACTG-3'; and bacterial-specific  $\beta$ -Gal primers: 5'-TGGCGGACCGCTAT-CAGGAC-3' and 5'-TGTATCATCTGGTTCGCTGG-3'.

**Immunohistochemistry.** Organs from mice were harvested, embedded in Tissue-Tek (Sakura, Finetech, Torrance, CA; OCT compound), snap-frozen in isopentane precooled in dry ice, and sectioned in 10- $\mu$ m-thick sections.

For  $\beta$ -Gal activity, the sections were rinsed in 0.1 mol/l PBS, fixed with 0.2% glutaraldehyde, 5 mmol/l EGTA, and 2 mmol/l  $MgCl_2$  in PBS for 10 min at room temperature, washed three times (5, 10, and 15 min) in PBS, then stained with color solution (1 mg/ml X-gal, 5 mmol/l  $K_3Fe [CN]_6$ , 5 mmol/l  $K_3Fe [CN]_6$ , and 2 mmol/l  $MgCl_2$  in PBS) for 24–48 h at 37°C. The sections were rinsed in PBS then counterstained with Nuclear Fast Red (DAKO, Mississauga, Ontario, Canada) as described by the manufacturer.

For Immunohistochemistry, the sections stained with X-gal were rinsed and endogenous peroxidase activity was quenched for 5 min in 0.3%  $H_2O_2$ , saturated with normal goat or rabbit sera, and then incubated with anti-CD11c (N418; Serotec, Raleigh, NC) and the anti-gp40 antibody (murine homologue of human EpCAM) G8.8 (DSHB, University of Iowa, Iowa City, IA) antibodies for 30 min. After three successive washings in PBS (3  $\times$  5 min), the sections

were incubated with affinity-purified biotin-conjugated secondary antibodies goat anti-hamster IgG antibody (Vector Laboratories, Burlingame, CA) and rabbit anti-rat IgG (Vector Laboratories), respectively. Negative control sections were incubated only with secondary antibodies. Slides were then incubated with Vectastain Elite ABC reagent for 30 min (Vector Laboratories) using the horseradish peroxidase substrate diaminobenzidine tetrahydrochloride (Vector).

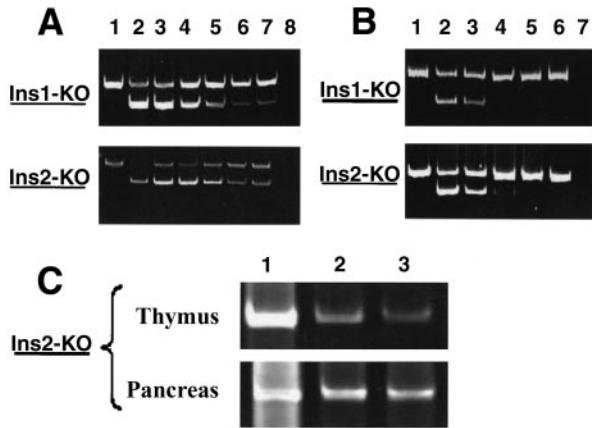
**Isolation of thymic cells.** Thymic DCs were isolated as described by Crowley et al. (29). In the first digestion, individual thymic lobes were perfused with 100 units/ml collagenase D (Sigma, Oakville, Ontario, Canada) and then incubated for 15 min at 37°C with 400 U/ml collagenase D. The glands were then disrupted, and all released cells were centrifuged at 1,400 rpm for 10 min. The cell pellet was resuspended in BSA (35%,  $\rho = 1.080$ ; Sigma), overlaid with 2 ml of RPMI medium and centrifuged for 20 min at 7,500g. In an alternative protocol, thymi were minced and digested with two 15-min incubations at 37°C with collagenase IV (Sigma), pelleted, and resuspended in BSA. The low-density floating fraction was collected, washed, and incubated for 90 min in a Petri dish. The nonadherent cells were removed by pipetting. The monolayer was incubated for an additional hour to remove additional small round lymphocytes. Then the monolayer was cultured overnight, at which time most DCs became nonadherent and were harvested. For further purification and enrichment for DCs, the cells were sorted in a Vantage FACS (fluorescent-activated cell sorter) (Becton Dickinson, Franklin Lakes, NJ) using anti-CD11c antibodies. For examining freshly isolated DCs without the phenotypic changes that might occur during overnight culture, the low-density floating fraction was incubated in 25 mmol/l EDTA/PBS at 37°C for 5 min and DCs were then directly FACS sorted with CD11c alone, DEC205 (Serotech, NLDC-145) alone, or CD11c and I-A<sup>b</sup> (MHC II; Pharmingen, Mississauga, Ontario, Canada) with essentially identical results.

Thymic epithelial cells (TECs) were isolated according to Klein et al. (30). Briefly, 4–5 thymi of 2- to 4-week-old mice were digested for 30–40 min with Collagenase/Dispase (Sigma; 0.2 mg/ml each) and DNase (Roche, Mannheim, Germany; 25  $\mu$ g/ml) in PBS-1% FCS. EDTA was added to a final concentration of 10 mmol/l for the last 5 min. Cells were then separated on a discontinuous Percoll (Amersham Biosciences, Uppsala, Sweden) density gradient. The TEC-enriched fraction (the two upper phases) were harvested, washed, and stained with G8.8 followed by phycoerythrin-conjugated rabbit anti-rat IgG antibody (Serotech). After 30 min at 4°C, cells were stained with anti-CD45-FITC (Pharmingen) to distinguish epithelial cells from G8.8-positive marrow-derived cells. TECs were sorted according to the phenotype of CD45<sup>low</sup> and G8.8<sup>+</sup>. Blocking with the anti-FcR mAb 2.4G2 (Pharmingen) preceded the stainings for DCs, whereas blocking with 1% horse serum was done for the TEC isolations. The average cell yield per thymus was 30,000 TECs.

**$\beta$ -Gal activity assay/FACS.** Approximately  $10^6$  cells from the Percoll gradient isolation (epithelial cells) or from the DC isolation (low-density fraction) were labeled with G8.8 or CD11c antibodies and resuspended in 1 ml of RPMI (10% FCS) with 10  $\mu$ l of endogenous  $\beta$ -Gal-like activity inhibitor from the C<sub>12</sub>FDG kit (ImaGene Green Kit; Molecular Probes, Eugene, OR) for 30 min at 37°C. After this, 0.5 ml of prewarmed C<sub>12</sub>FDG (15  $\mu$ mol/l) substrate was added to the cells and incubated for 20 min at 4°C, room temperature, or 37°C. Results from the incubations at all three temperatures were very similar, but the background was significantly lower at 4°C and the results shown were obtained at this temperature. This method was used to compare the percentage of cells positive for both G8.8 (or CD11c) and C<sub>12</sub>FDG between *Ins2*-KO mice ( $\beta$ -Gal expressed under the *Ins2* promoter) and *Ins1*-KO mice (no  $\beta$ -Gal transgene). The difference in double-positive cells between the two genotypes was taken to represent cells that truly express transgenic  $\beta$ -Gal. The number, as a percentage of all G8.8<sup>+</sup> cells, was much higher (10- to 30-fold higher) in *Ins2*-KO mice than in *Ins1*-KO in all four experiments regardless of whether the background, in the absence of C<sub>12</sub>FDG, was subtracted from each. In CD11c<sup>+</sup> cells, no significant difference from background was seen.

## RESULTS

**Proinsulin/ $\beta$ -Gal expression in the thymus and pancreas.** In mice, two unlinked, nonallelic, *Ins1* and *Ins2* genes encode insulin. *Ins1* is expressed at two to three times lower than *Ins2* in the pancreas and thymus (8,31,32). Figure 1A shows proinsulin expression in the thymus of 2- to 4-week-old mice. Proinsulin is expressed two to three times less in the *Ins2*-KO mice when compared with the *Ins1*-KO mice, confirming previous results (8). For both mice, the concentration of proinsulin was normalized with cyclophilin (Fig. 1B). As expected,  $\beta$ -Gal



**FIG. 1.** Proinsulin/ $\beta$ -Gal expression in the thymus and the pancreas. *A* and *B*: Quantitative RT-PCR of proinsulin in the thymus of *Ins1-KO* and *Ins2-KO* mice normalized with cyclophilin. Lower band, competitor sequence; upper band, endogenous insulin; 1–8 are serial dilutions of competitor. *C*: Semiquantitative RT-PCR of  $\beta$ -Gal in thymus and pancreas using serial dilutions of cDNA (1, 2, 3 = 1:1, 1:10, 1:100, normalized with cyclophilin).

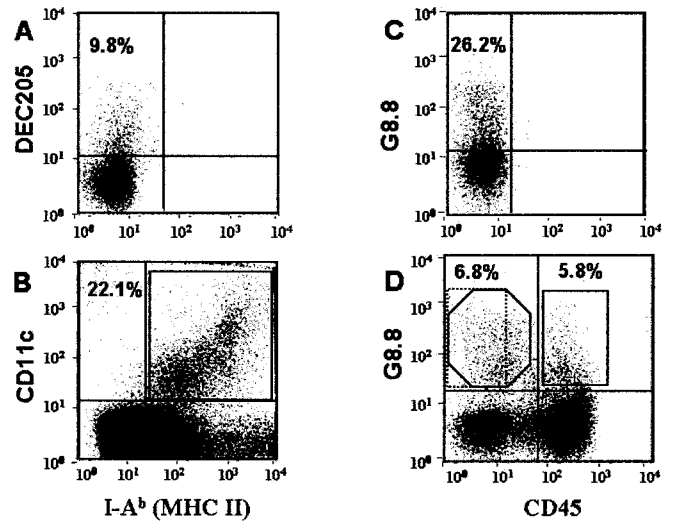
expression was detected in thymus and pancreas of *Ins2-KO* but not *Ins1-KO* mice (Fig. 1*C*). These results show that proinsulin is abundantly expressed in the thymus of *Ins1-KO* mice and bioactive  $\beta$ -Gal in the thymus of *Ins2-KO* mice. These mice were used for the analyses of proinsulin/ $\beta$ -Gal expression in fractionated thymic stromal cells.

**Proinsulin/ $\beta$ -Gal expression in thymic stromal cells.**

To examine proinsulin/ $\beta$ -Gal gene expression in thymic stromal cells, we enriched distinct cell populations to high purity by a combination of density fractionation and cell sorting. DCs were isolated from thymi of 2- to 4-week-old mice using the standard protocol (29) that includes enrichment by adherence to culture dishes after overnight incubation before cell sorting with anti-CD11c antibody. Alternatively, for avoiding possible phenotypic changes during the overnight incubation, possibly comparable to DC maturation in vivo (33), the overnight incubation step was omitted, and the carefully removed low-density cell fraction from the BSA gradient was sorted immediately using an anti-CD11c or anti-DEC205 antibody (Fig. 2*A*). Both techniques yielded CD11c-positive cells within the expected range of 30,000–70,000 DCs per mouse thymus (29). Without overnight incubation, sorting by CD11c and I-A<sup>b</sup> (MHC II) antibodies yielded similar results (Fig. 2*B*). Virtually the entire population of CD11c<sup>+</sup> cells were also positive for I-A<sup>b</sup>.

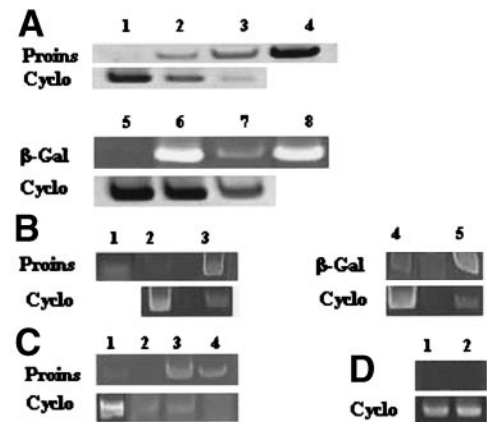
Epithelial cells were isolated as described by Klein et al. (30) and enriched by cell sorting using G8.8 and CD45 antibodies. The G8.8<sup>+</sup> population consists of 40–60% TECs, with the remainder of cells being mostly thymocytes (30,34). Marrow-derived cells were eliminated by selecting for G8.8<sup>+</sup>/CD45<sup>-lo</sup> providing exclusively medullary and cortical epithelial cells (19,30) (Fig. 2*C* and *D*). Such isolations yielded very similar results to Derbinski et al. (19), as we consistently isolated ~30,000 epithelial cells/thymus. G8.8<sup>+</sup>/CD45<sup>-</sup> and G8.8<sup>+</sup>/CD45<sup>hi</sup> cell fractions were also isolated (Fig. 2*D*).

By RT-PCR, strong proinsulin and  $\beta$ -Gal bands were detected in G8.8<sup>+</sup>/CD45<sup>-lo</sup> TECs from *Ins1-KO* and *Ins2-KO* mice, respectively, whereas only a very faint band

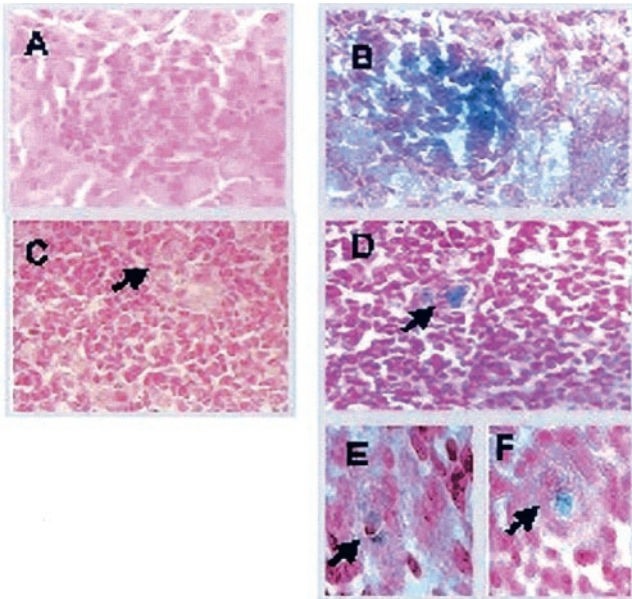


**FIG. 2.** FACS sorting of thymic dendritic and epithelial cell populations. Sort regions for DC without overnight incubation: with DEC205 (*A*) and with CD11c and I-A<sup>b</sup> (major histocompatibility complex [MHC] II; *B*). Epithelial sorts: G8.8<sup>+</sup> (mainly TEC; *C*) and G8.8<sup>+</sup>/CD45<sup>-lo</sup> (TEC; *D*). Dotted square, G8.8<sup>+</sup>/CD45<sup>-</sup> cells.

was detected in the CD11c<sup>+</sup>, DEC205<sup>+</sup>, or CD11c<sup>+</sup>/I-A<sup>b</sup> DCs from the same mice, isolated without overnight incubation (Fig. 3*B*, lanes 1–5, and *C*, lanes 1–4). Even with a two- to threefold greater number of DCs, as compared with TECs, and with a substantially larger amount of RNA used for RT-PCR, controlled for by cyclophilin, only very weak proinsulin and  $\beta$ -Gal bands could be detected within the DC extracts (Fig. 3*B*, lanes 1, 2, and 4, and *C*, lanes 1 and 2). Reducing the cDNA quantity in the DCs only reduced the intensity of the proinsulin and  $\beta$ -Gal



**FIG. 3.** Proinsulin/ $\beta$ -Gal expression in TECs versus DCs. *A*: Proinsulin expression in *Ins1-KO* mice: 1, CD11c<sup>+</sup> (DC; with overnight incubation [O/N]; *n* = 4); 2, G8.8<sup>+</sup> cells (mainly TEC; *n* = 4); and 3, G8.8<sup>+</sup>/CD45<sup>-lo</sup> cells (*n* = 8; TEC). Cyclophilin-loading control: 4, pancreatic proinsulin.  $\beta$ -Gal expression in *Ins2-KO* mice: 5, CD11c<sup>+</sup> (DC; O/N; *n* = 4); 6, G8.8<sup>+</sup> cells (mainly TEC; *n* = 4); and 7, G8.8<sup>+</sup>/CD45<sup>-lo</sup> cells (*n* = 8; TEC). 8, Pancreatic  $\beta$ -Gal. *B*: Proinsulin RT-PCR from FACS sorted CD11c<sup>+</sup> cells (DC, *Ins1-KO* mice; 1, half as much cDNA as one used for the PCR (2), and G8.8<sup>+</sup>/CD45<sup>-lo</sup> cells (3). Corresponding cyclophilin levels for 2 and 3 are shown.  $\beta$ -Gal RT-PCR from *Ins2-KO* mice FACS sorted 4 CD11c<sup>+</sup> (DC) and 5 G8.8<sup>+</sup>/CD45<sup>-lo</sup> cells with the corresponding cyclophilin levels. *C*: Proinsulin RT-PCR in CD11c<sup>+</sup> and DEC205<sup>+</sup> sorts with the corresponding cyclophilin levels (1 and 2). Proinsulin RT-PCR in G8.8<sup>+</sup>/CD45<sup>-lo</sup> and G8.8<sup>+</sup>/CD45<sup>hi</sup> sorts (3 and 4, isolated as per Fig. 2*D*) along with the corresponding cyclophilin levels. *D*: RT-PCR for proinsulin (1) and  $\beta$ -Gal (2) in G8.8<sup>+</sup>/CD45<sup>hi</sup> extractions along with the corresponding cyclophilin levels (*n* = 2).



**FIG. 4.** Histochemistry of  $\beta$ -Gal activity on pancreatic and thymic frozen sections. *A* and *B*: Pancreatic  $\beta$ -Gal activity detected in  $\beta$ -cells of Ins2-KO mice but not in Ins1-KO. *C* and *D*: Thymic  $\beta$ -Gal activity detected in mTECs (arrows) of HC of Ins2-KO mice but not in Ins1-KO mice. *E* and *F*: Zoom of HC cells positive for  $\beta$ -Gal activity. Magnification  $\times 40$ .

bands, ruling out saturation of cDNA as a possible inhibitor of the PCR (Fig. 3*B*, lanes 1 and 2). Similarly and as expected, the intensity of the proinsulin band increased with epithelial enrichment of the G8.8<sup>+</sup> fraction by double sorting (G8.8<sup>+</sup>/CD45<sup>-lo</sup>; Fig. 3*A*, lanes 2 and 3).  $\beta$ -Gal was also detectable in G8.8<sup>+</sup> and G8.8<sup>+</sup>/CD45<sup>-lo</sup> cells from Ins2-KO mice (Fig. 3, lanes 6 and 7; note that loading was not normalized for this result). No proinsulin/ $\beta$ -Gal expression was detected in G8.8<sup>+</sup>/CD45<sup>hi</sup> cells (Fig. 3*D*) and from DCs with overnight incubation (Fig. 3*A*, lanes 1 and 5). Proinsulin was also strongly detected in G8.8<sup>+</sup>CD45<sup>-</sup> epithelial cells even when poor RNA yields did not allow for the detection of cyclophilin (Fig. 3*C*, lane 4). All of these data demonstrate that in the thymus, proinsulin mRNA transcripts are predominantly detected in TEC.

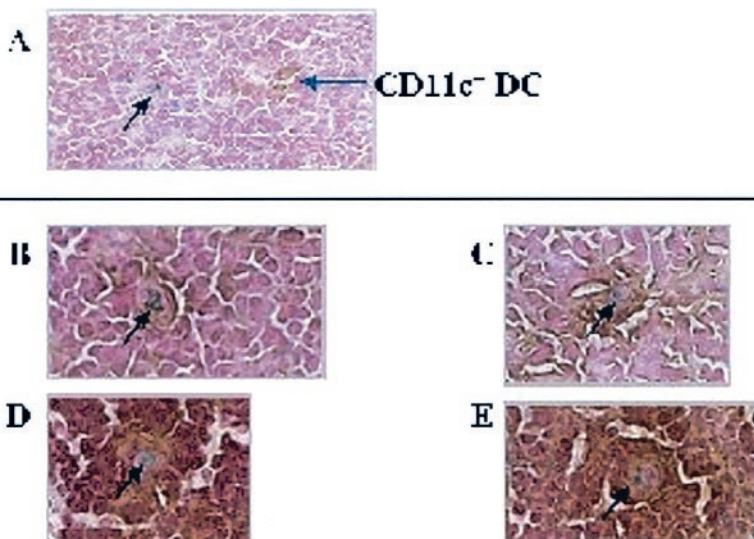
**Proinsulin/ $\beta$ -Gal is produced by rare TECs of the HC.** To be more specific about which population of cells is

responsible for insulin gene transcription in the thymus, we localized  $\beta$ -Gal activity in histologic sections. Figure 4 shows histochemistry results of the  $\beta$ -Gal activity on pancreatic and thymic frozen sections of Ins1-KO and Ins2-KO mice. These results show that  $\beta$ -Gal activity is specifically detected in pancreatic islets of Ins2-KO mice but not in Ins1-KO mice (Fig. 4*A* and *B*). In the thymus,  $\beta$ -Gal activity is detected in Ins2-KO mice but not in Ins1-KO and was localized in the mTECs forming the concentric structure that resembles HC. Approximately 1 in every 10 HC were positive for  $\beta$ -Gal activity, some of which contained as many as four X-gal-stained cells. (Fig. 4*C*–*F*). Because our sections represent only a slice of a three-dimensional HC, the true proportion must be higher, and it is indeed possible that every HC contains these cells. Immunohistochemistry co-localized all of the  $\beta$ -Gal-positive cells with G8.8 (Fig. 5*B*–*E*) but never with CD11c (DC; Fig. 5*A*). These data demonstrate that proinsulin-producing cells in the mouse thymus are mTECs of the HC.

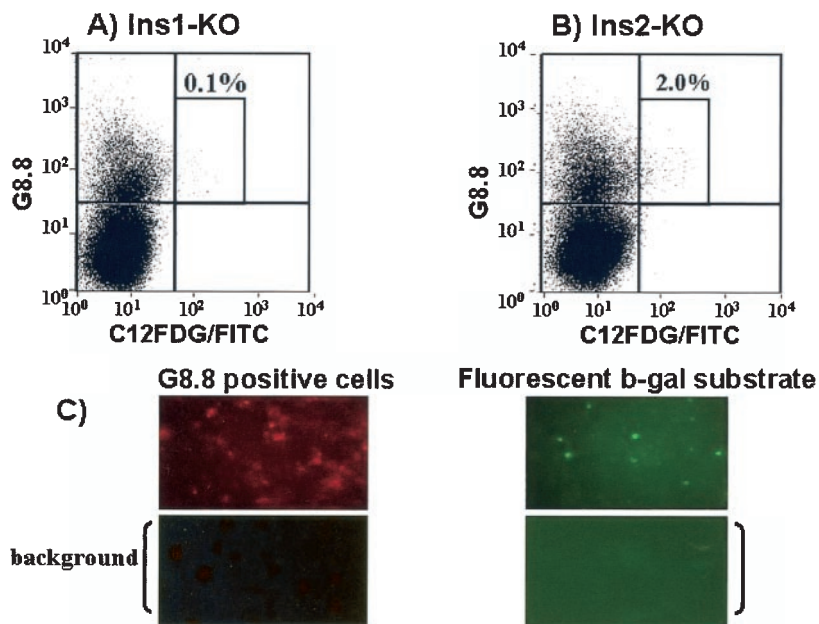
**Flow cytometry analysis of  $\beta$ -Gal/proinsulin-producing rare TEC.** We further localized  $\beta$ -Gal activity in purified populations of thymic stromal cells by flow cytometry using fluorescent  $\beta$ -Gal substrate (C<sub>12</sub>FDG) and cell type-specific antibodies. The results consistently revealed a population of between 1 and 3% G8.8<sup>+</sup>/ $\beta$ -Gal-positive cells within the purified G8.8<sup>+</sup> thymic epithelial cell extract (TEC) from Ins2-KO mice (Fig. 6*B*). We could not detect a similar  $\beta$ -Gal-positive population in G8.8<sup>+</sup> cells of control Ins1-KO mice (Fig. 6*A*). In a separate experiment, we incubated sorted G8.8<sup>+</sup> cells with C<sub>12</sub>FDG, under similar conditions as above and observed them by fluorescence microscopy. The results showed a similar proportion of  $\beta$ -Gal-positive cells in the Ins2-KO preparation, consistent with the FACS data (Fig. 6*C*). The same experiments were done for sorted CD11c<sup>+</sup> DCs, and no population of  $\beta$ -gal-positive DCs was observed in Ins2-KO mice that was above the background levels obtained with DCs from Ins1-KO mice (data not shown).

## DISCUSSION

Our results show that in mouse thymus, proinsulin expression is limited to a very rare fraction of mTECs forming the HC. Previous studies that attempted to identify which cells



**FIG. 5.** Immunohistochemistry of  $\beta$ -Gal activity and thymic stromal cells. *A*: Co-localization of  $\beta$ -Gal activity and CD11c (DC). *B*–*E*: Co-localization of  $\beta$ -Gal activity and G8.8 (TEC). No staining was observed in Ins1-KO sections (data not shown). Magnification  $\times 40$ .



**FIG. 6.**  $\beta$ -Gal activity in sorted thymic G8.8<sup>+</sup> cells. **A** and **B**: Percoll-purified TECs were incubated with  $C_{12}$ FDG substrate (see RESEARCH DESIGN AND METHODS). A percentage of 1–3% more G8.8<sup>+</sup>/ $\beta$ -Gal-positive epithelial cells was consistently observed in the Ins2-KO sorts when compared with the Ins1-KO sorts. Percentages shown are normalized for the percentage of G8.8<sup>+</sup> cells in each extraction. No similar increase over the Ins1-KO control was seen in BSA-purified CD11c<sup>+</sup> cells (DC). **C**: G8.8<sup>+</sup> TEC cells were incubated with  $C_{12}$ FDG substrate and viewed by fluorescence microscopy. An increased number of  $\beta$ -Gal-positive cells in the Ins2-KO sample as compared with the Ins1-KO sample was observed (data not shown), confirming the FACS data. Magnification  $\times 10$ .

are responsible for this proinsulin expression, by RT-PCR, gave contradictory results with expression being detected in DCs (26) or TECs (19) but not both. To resolve this discrepancy and to best characterize the identity of these cells, we used  $\beta$ -Gal as a marker of transcriptional activity of the endogenous *Ins2* promoter. Semiquantitative RT-PCR shows that proinsulin/ $\beta$ -Gal is very strongly and easily detected in TECs but only very faintly detected in DCs that are sorted without overnight incubation. Throsby et al. (26) also detected a very faint band in DC extracts, and they required using a radiolabeled probe to see it adequately. This band may be the result of some epithelial cell contamination in the DC extracts, or it might represent expression at much lower levels or much fewer cells among the DCs.

Immunohistochemical experiments revealed that these thymic cells were confined to the thymic medulla and were part of the HC. The expression of insulin and other ectopic or promiscuous tissue-specific self-antigens by mTECs has been documented extensively by Derbinski et al. (19). The authors, however, did not identify the exact location of the mTECs responsible for the production of insulin or other antigens and their spatial relationship to the rest of the stroma. Our immunohistochemistry of  $\beta$ -Gal activity showed that proinsulin production is a unique property of rare mTECs of the HC.

It has been reported that self-antigen-expressing cells in the thymus, including proinsulin-producing cells, were frequently found in a cluster of two to four positive cells when analyzed ex vivo (35). This may reflect in vivo clustering, as suggested by our observation of clusters of more than one  $\beta$ -gal-positive cell. Rodewald et al. (36) demonstrated that the thymus medulla consists of epithelial cell islets, each derived from a single progenitor. It is not known how this clonality relates to the epigenetic differences that distinguish the rare cells involved in the thymic expression of tissue-restricted antigens from the rest of the TECs. It also is not known whether such expression is the property of a large number of cells, each specializing in a particular antigen, or of a small number of

cells that have developed the promiscuous transcriptional machinery to express many diversely regulated antigens. Our ability to isolate the insulin-producing cells by scaling up the flow cytometry depicted in Fig. 6B will enable us, in studies currently under way, to provide an answer to these questions.

Thymocyte-negative selection occurs mainly in the medulla (37,38), and the HC cells have been associated with apoptotic thymocytes (22). DCs and macrophages have been frequently observed around HC (23,38), and the DCs were shown to be responsible for phagocytosis of apoptotic thymocytes (23). In human thymus, Pugliese et al. (39) found that insulin can be detected in a number of thymic and secondary lymphoid DCs by double immunostaining. Our findings are compatible with the presence of a small number of proinsulin-expressing DCs, but it also is conceivable that these DCs do not express insulin at the transcriptional level but merely take it up for presentation. For tolerizing both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, presentation of the self-antigen by major histocompatibility complex class I and II molecules is necessary. It is known that mTECs can present endogenously produced self-antigens by both classes, whereas DCs do not seem to present endogenously produced antigen on class II (40).

If proinsulin expression in thymus medulla is important in negative selection, then how can contact of all or most thymocytes with the few, scattered insulin-producing cells be ensured? This may be the result of chemoattractant properties (23) that drive each thymocyte to scan many HCs. Alternatively or in parallel, other antigen-presenting cells, particularly DCs, may also contribute by taking up secreted antigen for presentation.

We have previously shown that thymic insulin expression levels modulate insulin-specific autoreactive T-cell tolerance even in non-diabetes-prone mice (8). Against the NOD background, the *Ins2* knockout mice (with extremely low insulin expression in the thymus but normal levels in the pancreas) develop diabetes with higher incidence and earlier than the wild type (20, confirmed by our own

preliminary observations and those of the group of Dr. G. Eisenbarth; Moriyama et al., personal communication).

We believe that the isolation of proinsulin-producing cells, using the techniques that we have developed here, will allow detailed study of the cellular and molecular biology of proinsulin production, central tolerance mechanisms, and the possibility of designing novel forms of gene or cellular therapy.

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#### REFERENCES

- Eisenbarth GS: Type I diabetes mellitus: a chronic autoimmune disease. *N Engl J Med* 314:1360–1368, 1986
- Noble JA, Valdes AM, Cook M, Klitz W, Thomson G, Erlich HA: The role of HLA class II genes in insulin-dependent diabetes mellitus: molecular analysis of 180 Caucasian, multiplex families. *Am J Hum Genet* 59:1134–1148, 1996
- Robinson BH, Dosch HM, Martin JM, Akerblom HK: Savilahti E, Knip M, Ilonen J: A model for the involvement of MHC class II proteins in the development of type 1 (insulin-dependent) diabetes mellitus in response to bovine serum albumin peptides. *Diabetologia* 36:364–368, 1993
- Redondo MJ, Eisenbarth GS: Genetic control of autoimmunity in type I diabetes and associated disorders. *Diabetologia* 45:605–622, 2002
- Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, Wickramasinghe S, Colle E, Polychronakos C: Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. *Nat Genet* 15:289–292, 1997
- Pugliese A, Zeller M, Fernandez A Jr, Zalberg LJ, Bartlett RJ, Ricordi C, Pietropaolo M, Eisenbarth GS, Bennett ST, Patel DD: The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. *Nat Genet* 15:293–297, 1997
- Brimnes MK, Jensen T, Jorgensen TN, Michelsen BK, Troelsen J, Werdnlin O: Low expression of insulin in the thymus of non-obese diabetic mice. *J Autoimmun* 19:203–213, 2002
- Chentoufi AA, Polychronakos C: Insulin expression levels in the thymus modulate insulin-specific autoreactive T-cell tolerance: the mechanism by which the IDDM2 locus may predispose to diabetes. *Diabetes* 51:1383–1390, 2002
- Thebault-Baumont K, Dubois-Laforgue D, Krief P, Briand JP, Halbout P, Vallon-Geoffroy K, Morin J, Laloux V, Lehuen A, Carel JC, Jami J, Muller S, Boitard C: Acceleration of type 1 diabetes in proinsulin 2 deficient NOD mice. *J Clin Invest* 111:851–857, 2003
- Kappler JW, Roehm N, Marrack PT: T cell tolerance by clonal elimination in the thymus. *Cell* 49:273–80, 1987
- Sprent J, Lo D, Gao EK, Ron Y: T cell selection in the thymus. *Immunol Rev* 101:173–190, 1988
- Nossal GJ: Negative selection of lymphocytes. *Cell* 76:229–239, 1994
- Stockinger B: T lymphocyte tolerance: from thymic deletion to peripheral control mechanisms. *Adv Immunol* 71:229–265, 1999
- Mondino A, Khoruts A, Jenkins MK: The anatomy of T-cell activation and tolerance. *Proc Natl Acad Sci U S A* 93:2245–2252, 1996
- Miller JF, Heath WR: Self-ignorance in the peripheral T-cell pool. *Immunol Rev* 133:131–150, 1993
- Sprent J, Webb SR: Intrathymic and extrathymic clonal deletion of T cells. *Curr Opin Immunol* 7:196–205, 1995
- Hanahan D: Peripheral-antigen-expressing cells in thymic medulla: factors in self-tolerance and autoimmunity. *Curr Opin Immunol* 10:656–662, 1998
- Klein L, Kyewski B: Self-antigen presentation by thymic stromal cells: a subtle division of labor. *Curr Opin Immunol* 12:179–186, 2000
- Derbinski J, Schulte A, Kyewski B, Klein L: Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol* 2:1032–1039, 2000
- Boyd RL, Tucek CL, Godfrey DI, Izon DJ, Wilson TJ, Davidson NJ, Bean AG, Ladyman HM, Ritter MA, Hugo P: The thymic microenvironment. *Immunol Today* 14:445–459, 1993
- Patel DD, Whichard LP, Radcliff G, Denning SM, Haynes BF: Characterization of human thymic epithelial cell surface antigens: phenotypic similarity of thymic epithelial cells to epidermal keratinocytes. *J Clin Immunol* 15:80–92, 1995
- Douek DC, Altmann DM: T-cell apoptosis and differential human leucocyte antigen class II expression in human thymus. *Immunology* 99:249–256, 2000
- Zaitseva M, Kawamura T, Loomis R, Goldstein H, Blauvelt A, Golding H: Stromal-derived factor 1 expression in the human thymus. *J Immunol* 168:2609–2617, 2002
- Romagnani P, Annunziato F, Manetti R, Mavilia C, Lasagni L, Manuelli C, Vannelli GB, Vanini V, Maggi E, Pupilli C, Romagnani S: High CD30 ligand expression by epithelial cells and Hassal's corpuscles in the medulla of human thymus. *Blood* 91:3323–3332, 1998
- Amakawa R, Hakem A, Kundig TM, Matsuyama T, Simard JJ, Timms E, Wakeham A, Mittrucker HW, Griesser H, Takimoto H, Schmits R, Shahinian A, Ohashi P, Penninger JM, Mak TW: Impaired negative selection of T cells in Hodgkin's disease antigen CD30-deficient mice. *Cell* 84:551–562, 1996
- Throsby M, Homo-Delarche F, Chevenne D, Goya R, Dardenne M, Pleau JM: Pancreatic hormone expression in the murine thymus: localization in dendritic cells and macrophages. *Endocrinology* 139:2399–2406, 1998
- Duvillie B, Cordonnier N, Deltour L, Dandoy-Dron F, Itier JM, Monthieux E, Jami J, Joshi RL, Bucchini D: Phenotypic alterations in insulin-deficient mutant mice. *Proc Natl Acad Sci U S A* 94:5137–5140, 1997
- Forster E: An improved general method to generate internal standards for competitive PCR. *Biotechniques* 1:18–20, 1994
- Crowley M, Inaba K, Witmer-Pack M, Steinman RM: The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell Immunol* 118:108–125, 1989
- Klein L, Klugmann M, Nave KA, Tuohy VK, Kyewski B: Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. *Nat Med* 6:56–61, 2000
- Deltour L, Leduque P, Blume N, Madsen O, Dubois P, Jami J, Bucchini D: Differential expression of the two nonallelic proinsulin genes in the developing mouse embryo. *Proc Natl Acad Sci U S A* 90:527–531, 1993
- Wentworth BM, Rhodes C, Schnetzler B, Gross DJ, Halban PA, Villakomaroff L: The ratio of mouse insulin I: insulin II does not reflect that of the corresponding preproinsulin mRNAs. *Mol Cell Endocrinol* 86:177–186, 1992
- Sornasse T, Flamand V, De Becker G, Bazin H, Tielemans F, Thielemans K, Urbain J, Leo O, Moser M: Antigen pulsed dendritic cells can efficiently induce an antibody response in vivo. *J Exp Med* 192:15–21, 1992
- Nelson AJ, Dunn RJ, Peach R, Aruffo A, Farr AG: The murine homologue of human Ep-CAM, a homotypic adhesion molecule, is expressed by thymocytes and thymic epithelial cells. *Eur J Immunol* 26:401–408, 1996
- Kyewski B, Derbinski J, Gotter J, Klein L: Promiscuous gene expression and central T-cell tolerance: more than meets the eye. *Trends Immunol* 23:364–371, 2002
- Rodewald HR, Paul S, Haller C, Bluethmann H, Blum C: Thymus medulla consisting of epithelial islets each derived from a single progenitor. *Nature* 414:763–768, 2001
- Surh CD, Sprent J: T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature* 372:100–103, 1994
- Bodey B, Bodey B Jr, Siegel SE, Kaiser HE: Novel insights into the function of the thymic Hassal's bodies. *In Vivo* 14:407–418, 2000
- Pugliese A, Brown D, Garza D, Murchison D, Zeller M, Redondo M, Diez J, Eisenbarth GS, Patel DD, Ricordi C: Diabetes-associated autoantigens exist in both thymus and peripheral lymphoid organs. *J Clin Invest* 107:555–564, 2001
- Oukka M, Colucci-Guyon E, Tran PL, Cohen-Tannoudji M, Babinet C, Lotteau V, Kosmatopoulos KCD: 4 T cell tolerance to nuclear proteins induced by medullary thymic epithelium. *Immunity* 4:545–553, 1996