Dendritic Cells in Human Thymus and Periphery Display a Proinsulin Epitope in a Transcription-Dependent, Capture-Independent Fashion


*J Immunol* 2005; 175:2111-2122; doi: 10.4049/jimmunol.175.4.2111

http://www.jimmunol.org/content/175/4/2111

**References**

This article cites 57 articles, 30 of which you can access for free at: http://www.jimmunol.org/content/175/4/2111.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Dendritic Cells in Human Thymus and Periphery Display a Proinsulin Epitope in a Transcription-Dependent, Capture-Independent Fashion

Carlos A. Garcia,2∗ Kamalaveni R. Prabakar,2∗ Juan Diez,2∗ Zhu Alexander Cao,‖ Gloria Allende,* Markus Zeller,* Rajpreet Dogra,∗ Armando Mendez,∗† Eliot Rosenkranz,§‖ Ulf Dahl,* Camillo Ricordi,∗†† Douglas Hanahan,‖ and Alberto Pugliese3∗†‡

The natural expression of tissue-specific genes in the thymus, e.g., insulin, is critical for self-tolerance. The transcription of tissue-specific genes is ascribed to peripheral Ag-expressing (PAE) cells, which discordant studies identified as thymic epithelial cells (TEC) or CD11c+ dendritic cells (DC). We hypothesized that, consistent with APC function, PAE-DC should constitutively display multiple self-epitopes on their surface. If recognized by Abs, such epitopes could help identify PAE cells to further define their distribution, nature, and function. We report that selected Abs reacted with self-epitopes, including a proinsulin epitope, on the surface of CD11c+ cells. We find that Proins+ CD11c+ PAE cells exist in human thymus, spleen, and also circulate in blood.

Human thymic Proins+ cells appear as mature DC but express CD8α, CD20, CD123, and CD14; peripheral Proins+ cells appear as immature DC. However, DC derived in vitro from human peripheral blood monocytes include Proins+ cells that uniquely differentiate and mature into thymic-like PAE-DC. Critically, we demonstrate that human Proins+ CD11c+ cells transcribe the insulin gene in thymus, spleen, and blood. Likewise, we show that mouse thymic and peripheral CD11c+ cells transcribe the insulin gene and display the proinsulin epitope; moreover, by using knockout mice, we show that the display of this epitope depends upon insulin gene transcription and is independent of Ag capturing. Thus, we propose that PAE cells include functionally distinct DC displaying self-epitopes through a novel, transcription-dependent mechanism. These cells might play a role in promoting self-tolerance, not only in the thymus but also in the periphery.

Self-tolerance is established and maintained through central (thymic) and peripheral mechanisms. Thymic selection is crucial for shaping a self-tolerant T cell repertoire in early life during the maturation of the immune system (1). Peripheral tolerance mechanisms (deletion, anergy, ignorance, and regulatory cells) are active in peripheral lymphoid tissues (PLT) through life and are considered critical for self-molecules with tissue-restricted expression or peripheral proteins that are not expressed in the thymus. However, many peripheral proteins are “ectopically” expressed in the thymus (2–7), and their thymic expression contributes to the induction of self-tolerance (8–11). Proinsulin/insulin is a prototypical peripheral protein almost exclusively produced by pancreatic β cells (12). In humans, allelic variation and parent of origin effects correlate with levels of insulin gene (INS) transcription in the thymus and with the risk of type 1 diabetes (T1D), a disease in which autoimmunity to proinsulin/insulin plays an important role (3, 4). Mouse models in which proinsulin/insulin expression was genetically perturbed demonstrate that thymic proinsulin/insulin expression dramatically affects the development of self-tolerance (13–16). Mice have two nonallelic insulin genes, Ins1 and Ins2 (17), encoding slightly different proteins that are both functional and secreted by β cells. However, only Ins2 is expressed in the thymus of wild-type mice (14). The lack of proinsulin II expression resulting from the disruption of Ins2 (18) causes accelerated diabetes development, heightened disease incidence, and more severe autoimmune responses to proinsulin/insulin in NOD mice (15, 16), a well-known model of autoimmune diabetes (19).

A subset of cells in the thymus expresses ostensible tissue-specific proteins. However, data about their distribution, phenotype, and function are limited and discordant (20). Such cells were first described in the mouse and named peripheral Ag-expressing (PAE) cells (9). Initial studies in human thymus could not assign self-molecule expression to a single cell type but found evidence for ectopic transcription of self-molecule genes in cell fractions enriched for thymic epithelial cells (TEC) and bone marrow (BM)-derived APC (5). Later studies identified PAE cells as dendritic cells (DC)/macrophages (21, 22) or, alternatively, as medullary TEC (mTEC) in both mouse (6, 21, 23) and human thymus (7, 22) by demonstrating peripheral protein gene transcripts in sorted cell populations. In some studies, these transcripts were not found in
sorted DC populations. In our earlier studies, we used immuno-histochemistry to demonstrate cells stained for proinsulin, glu-tamic acid decarboxylase (GAD) and IA-2, islet cell molecules with tissue-restricted expression that can become target autoanti-gens in TID (22), in the human thymus. These cells expressed markers of BM-derived APC by double immunofluorescence, as defined by the expression of CD11c, CD14, CD40, CD80, CD86, CD83, CD86a, and HLA class II (22). Moreover, we found similar cells in human spleen and lymph nodes, tissues that also transcribe genes encoding for tissue-specific proteins, including INS (22, 24). These findings suggest but do not prove that human DC directly transcribe peripheral protein genes.

We hypothesized that, consistent with APC function, PAE cells should constitutively display multiple self-epitopes on their surface. If recognized by Abs, such epitopes could help identify PAE cells and allow further studies to more precisely define their nature, distribution, and function. We report that selected Abs react with self-epitopes, including a proinsulin epitope, on the surface of CD11c+ cells. By using this proinsulin epitope as a selecting ele-ment, we demonstrate that PAE cells include DC characterized by a distinct phenotype. These cells display multiple self-epitopes on their surface and, critically, such display is achieved through au-tonomous gene transcription.

Materials and Methods

Human subjects and tissue specimens

Blood samples were obtained from healthy subjects (age range, 23–53 years; six males, two females). Spleen specimens were obtained from ca-daver organ transplant donors (22–57 years old; four females, two males). Thymus was discarded material from children undergoing heart surgery (1 wk to 3 mo old; three females, three males). Specimens were obtained through informed consent and/or institutional approval.

Preparation of human thymus light density cells (T-LDC)

Thymus cells were processed to T-LDC, enriched in DC, according to the protocol of Vandenabeele et al. (25). Thymus tissue was minced into pieces, digested for 20 min at room temperature, with 1 mg/ml grade II bovine pancreatic DNase and 1 mg/ml type II collagenase in HBSS. EDTA (10 mM) was added to the cell suspension, which was filtered through a nylon mesh and washed with HBSS, 10 mM EDTA, and 2% FBS. Cells were centrifuged at 300 × g at 4°C, and the pellet was resuspended in 10 ml of cold Nycodenz medium (d-1.069 g/ml, Accudenz, A.G.; Accurate Chemical and Scientific). Cell suspensions (5 ml) were layered over 5 ml of cold Nycodenz medium (d-1.069 g/ml) and the LDC fraction recovered. Samples were centrifuged at 1700 × g at 4°C, and the LDC fraction recovered from the upper zones.

Preparation of human splenocytes

Spleen specimens were cut into small pieces that were forced through the mesh of a sterile sieve using the plunger of a syringe. Cell aggregates were dissociated by adding 10 mM EDTA and shaking. Mononuclear cells were isolated by centrifugation over Ficoll-Paque Plus, d-1.077 g/ml (Amersham Biosciences).

Abs to self-molecules

We used two mAbs against human proinsulin, clone M32337 and 3A1, both specific for the B-chain/C-peptide junction; mAb M32337 has no cross-reactivity with human insulin, mAb 3A1 has no cross-reactivity with human, bovine, porcine insulin, or human C-peptide. When used in flow cytometry, both Abs were initially tested using the indirect method in combi-nation with a secondary Ab against mouse IgG. FC-specific F(ab’)2, con-jugated with FITC (Sigma-Aldrich). Once staining was demonstrated, we conjugated mAb M32337 with PE to facilitate staining for multiple mark-ers. We also used mAbs reacting with both human insulin and proinsulin: clone K36aC10 (26) and clone D6C4. For GAD65, we used a mAb (GAD65-AS) and a rabbit serum (lot no. 18080605) reacting with GAD65 and GAD67. Abs to human IA-2 included mAbs 103/2C4, 97/4D9, 98/4H6, and rabbit sera against the intracellular (lot no. 8959) and extracel-lular (lot no. 9218H) domains, kindly provided by S. Gleason at Bayer (Pittsburgh, PA). Abs to human myelin basic protein (MBP) were mAb clone P12 and rabbit serum ab2404. Abs to human thyroid peroxidase (TPO) were mAb clone 6H7 and rabbit serum lot no. L3012864. Table I reports Abs species, available data on epitope specificity, cross-reactivity, and sources. The rat mAb DH21 (27) was used in the experiments involving transgenic mice. This Ab reacts with the MHC/Ag complex con-sisting of the I-Aγ molecule and the 116-129 hen egg lysozyme (HEL) peptide. It is reported to stain mouse spleen cells of CBA and B10.A(4R) mice (both expressing I-Aγ) when pulsed with this HEL peptide or whole HEL (27). Abs to human cluster differentiation (CD) surface markers

FITC-conjugates included mAbs to human CD3, CD8a, CD14, CD15, CD16, CD20, CD40, CD80, CD83, CD86, HLA-DR, CD95 (BD Pharm-ingen), and CD178 (FasL; Calbiochem). CyChrome 5.5 conjugates included Abs against CD11c, HLA-DR, and CD123 (BD Pharmingen).

### Table I. Abs to self-molecules tested for surface staining experiments

<table>
<thead>
<tr>
<th>Clone/Lot</th>
<th>Species</th>
<th>Specificity</th>
<th>Stained PAE Cells</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>M32337</td>
<td>Mouse</td>
<td>Human proinsulin, C-peptide/B-chain junction</td>
<td>Yes</td>
<td>Fitzgerald Industries</td>
</tr>
<tr>
<td>3A1</td>
<td>Mouse</td>
<td>Human proinsulin, C-peptide/B-chain junction</td>
<td>Yes</td>
<td>Research Diagnostics</td>
</tr>
<tr>
<td>K36aC10</td>
<td>Mouse</td>
<td>Human insulin/proinsulin</td>
<td>No</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>D6C4</td>
<td>Mouse</td>
<td>Human insulin/proinsulin (A19, B21)</td>
<td>No</td>
<td>Abcam Limited</td>
</tr>
<tr>
<td>GAD65-AS</td>
<td>Mouse</td>
<td>Rat GAD65 aa 4–25, N terminus</td>
<td>Yes</td>
<td>Alpha Diagnostics</td>
</tr>
<tr>
<td>AB1511</td>
<td>Rabbit</td>
<td>Rat GAD65/67 aa 572–685, C terminus</td>
<td>Yes</td>
<td>Chemicon International</td>
</tr>
<tr>
<td>103/2C4</td>
<td>Mouse</td>
<td>Human IA-2, aa 771–979, intracellular</td>
<td>No</td>
<td>Labor fur Autoimmundiagnostik</td>
</tr>
<tr>
<td>97/4D9</td>
<td>Mouse</td>
<td>Human IA-2, aa 605–772, intracellular</td>
<td>No</td>
<td>Labor fur Autoimmundiagnostik</td>
</tr>
<tr>
<td>98/4H6</td>
<td>Mouse</td>
<td>Human IA-2, aa 618–628, transmembrane</td>
<td>No</td>
<td>Labor fur Autoimmundiagnostik</td>
</tr>
<tr>
<td>8959</td>
<td>Mouse</td>
<td>Human IA-2, aa 601–979, intracellular</td>
<td>Yes</td>
<td>Gift from S. Gleason, Bayer</td>
</tr>
<tr>
<td>9218H</td>
<td>Rabbit</td>
<td>Human IA-2, aa 389–576, intracellular</td>
<td>Yes</td>
<td>Gift from S. Gleason, Bayer</td>
</tr>
<tr>
<td>P12</td>
<td>Mouse</td>
<td>Human MBP, aa 84–89</td>
<td>Yes</td>
<td>Research Diagnostics</td>
</tr>
<tr>
<td>2404</td>
<td>Rabbit</td>
<td>Human MBP</td>
<td>No</td>
<td>Abcam Limited</td>
</tr>
<tr>
<td>6H7</td>
<td>Mouse</td>
<td>Human TPO</td>
<td>Yes</td>
<td>Research Diagnostics</td>
</tr>
<tr>
<td>L3012864</td>
<td>Rabbit</td>
<td>Human TPO</td>
<td>No</td>
<td>United States Biologicals</td>
</tr>
</tbody>
</table>
**Immunophenotyping and sorting of human cells**

T-LDC and spleen mononuclear cells were adjusted to a 10^6 cells/ml concentration in PBS, 2% FBS, 10 mM EDTA, and 0.02% sodium azide (washing solution) before staining. We stained fixed, unpermeabilized, T-LDC, spleen cells, and whole blood samples (red cells were removed with 1× TRIS-buffered saline solution). Incubation with normal rat serum was used to block nonspecific binding. Cells were stained with 10–20 μl of direct conjugates and gently mixed at 4°C for 30 min. Samples were washed and centrifuged at 300 × g for 5 min at 4°C. The pellet was resuspended in 100 μl of washing solution. If indirect staining was used, this was performed first and revealed with FITC-conjugated goat anti-mouse IgG, F(ab')2. After a final wash, cells were fixed in PBS containing 1% paraformaldehyde. Monocyte-gated (whole blood and spleen cells), and ungated T-LDC were analyzed using a FACS Calibur (BD Biosciences). Triplet staining was analyzed by gating cells positive for one marker in histogram analysis followed by double staining dot plot analysis. Negative controls included unstained cells, omitting the primary Ab, and the appropriate isotype control Abs. CD4^+ and CD8^+ lymphocytes were used as a lineage control populations. For cell sorting, cells were incubated with lineage-positive Abs (CD3, CD7, CD15, CD19, and anti-glycophorin A) to eliminate T cells, B cells, granulocytes, and RBC. The remaining cells were stained with the proinsulin mAb M32337 and mAbs to several differentiation markers and then purified by FACS.

**In vitro differentiation and maturation of human monocyte-derived DC**

Peripheral blood samples or buffy coats were obtained from nine healthy donors (age range, 23–50 years; three males, four females, gender was unknown for two donors). Mononuclear cells were isolated through Ficoll-Paque gradient centrifugation, suspended in medium (RPMI 1640, 1% glutamine, 1% penicillin/streptomycin, 25 mM HEPES, and 10% heat-inactivated FBS), plated in 6-well plates (3 × 10^5 cells/ml), and left adhering for 3 h at 37°C. Nonadherent cells were removed and adherent cells cultured in medium alone, and medium was supplemented with recombinant human (rh)GM-CSF (50 ng/ml) and rIL-4 (25 ng/ml) for 7 days. Fresh cytokines were added on days 4 and 7 by removing half of the medium and adding an equal volume of fresh cytokines. To induce maturation, the medium was supplemented with rTNF-α (50 ng/ml) and PGE2 (2 μg/ml) on day 7. Cultured cells were harvested on day 8 and washed with PBS. Viability was assessed by trypan blue exclusion. On day 8, cells were harvested, stained with Abs, and analyzed by flow cytometry.

**C-peptide and proinsulin levels measurements**

Supernatants from the cell cultures were tested for the presence of C-peptide using a radioimmunoassay with a lower limit of detection of 0.10 ng/ml (Diagnostic Products). Proinsulin levels were measured by ELISA (Merodia); the limit of detection is <0.5 pmol/L and cross-reactivity with insulin and C-peptide is <0.03 and <0.06%, respectively.

**RNA isolation and RT-PCR from human cells**

We used the Microto-Midi Total RNA Purification kit (Invitrogen Life Technologies) or the MicroPoly(A) Purist mRNA Purification kit (Ambion) to purify total or mRNA from human tissues (whole thymus, spleen and pancreatic islets, positive controls); peripheral blood lymphocytes, and cells sorted from thymus, spleen, and blood. RNA integrity was assessed by gel electrophoresis. DNase-treated total RNA (1–4 μg) was used in RT-PCR experiments to assess the expression of the insulin genes, which were performed using nested primers. For forward and reverse primers were as follows: Ins2 first pair, 5'-CAC CCAGGCTTTTGTGCAAGACG-3', and 5'-TGGTGGTGTCTGATGTCGAC TAGT-3'; Ins2 second pair, 5'-GGGGCGGTGGTTTCTCTACAC-3', and 5'-GCTGTGGTGCAGCACTGATCA-3'; Ins1 first pair, 5'-GGGGCGGTGGTTTCTCTACAC-3', and 5'-GCTGTGGTGCAGCACTGATCA-3'; and Ins1 second pair, 5'-GGGGCGGTGGTTTCTCTACAC-3', and 5'-GCTGTGGTGCAGCACTGATCA-3'. The β2-microglobulin transcript was amplified as control.

**RT-PCR in human tissues and cells**

RNA was extracted using the Microto-Midi Total RNA Purification kit (Invitrogen Life Technologies). RNA samples from mouse BM cells, thymus, and spleen cells sorted and populations (CD41^+CD81^-, CD41^+CD81^+, CD41^+) were used in RT-PCR experiments to assess the expression of the Ins2, glucagon, and LacZ genes, which were performed using nested primers. Forward and reverse primers were as follows: forward, 5'-CCATGGGAACGCTGGGCTGCTTCTGGC-3', and reverse, 5'-GAGGGTGTTGGGCTTCTGGC-3'. Primers for the control transcript, GAPDH, were as follows: forward, 5'-CCATGGGAACGCTGGGCTGCTTCTGGC-3', and reverse, 5'-GAGGGTGTTGGGCTTCTGGC-3'. Primers for the control transcript, GAPDH, were as follows: forward, 5'-CCATGGGAACGCTGGGCTGCTTCTGGC-3', and reverse, 5'-GAGGGTGTTGGGCTTCTGGC-3'. Products were electrophoresed in 2% agarose gels.

**Mice**

We studied tissues and cells from several mouse strains. These included C57BL6, C3H, BALB/c, FVB, CBA, and NOD mice. We also studied mice in which Ins1 and Ins2 were disrupted (18). In Ins2 knockout mice, the LacZ gene replaces Ins2, and thus, β-galactosidase is expressed where Ins2 would be whereas Ins2 is not expressed. Breeding pairs (Ins1^+/- Ins2^+/+) and (Ins1^+/- Ins2^-/-) were mated to obtain double knockout mice lacking the expression of both insulin genes (Ins1^-/- Ins2^-/-). Double homozygous mutant pups are smaller than heterozygous littermates and die within 48 h in diabetic ketoacidosis (18). The genotype of the mice was confirmed using a PCR-based typing method developed by M. Nakayama (Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Aurora, CO). We studied mice lacking expression of the pancreas-duodenum homeobox gene PDX1 (or insulin promoter factor 1), a transcription factor critical for pancreatic development, which suffer from pancreatic agenesis (28). We also studied transgenic mice expressing membrane-bound HEL (mHEL) under the control of a tissue-specific promoter, the crystalline promoter (mHEL mice). These mice were originated in the FVB background and bred with B10.BR mice so that they express the I-A^b molecule (29, 30). Mice were maintained according to institutional guidelines.

**Preparation of cells from mouse BM, thymus, and spleen**

BM cells from Ins2 knockout mice were obtained from femurs and tibiae by flushing cells out of the diaphysis with RPMI 1640 using a 23-gauge needle. Cells were filtered through a nylon mesh and counted. Viability was assessed with trypan blue exclusion. BM cells were cultured in AIM-V complete medium (Invitrogen Life Technologies) in the presence of GM-CSF (1–5 ng/ml) for 5 days before RNA extraction to assess LacZ expression by RT-PCR. Thymus and spleen from various mouse strains were dissected and submerged in RPMI 1640 and 2% FBS. Depending on the experiments, tissues were used to extract RNA or to obtain cells. For cell preparation, tissues were pressed gently with the plunger of a syringe. Thymus tissues were digested with type II collagenase (1 mg/ml) and grade II bovine pancreatic DNase I (1 μg/ml) in HBSS without calcium chloride, magnesium chloride, and magnesium sulfate for 15–20 min at room temperature. EDTA was added to a final concentration of 0.01 M to disrupt DC-T cell rosettes. Both thymus and spleen cell suspensions were layered over Ficoll-Paque (1077g/cm^3), centrifuged, and then the low-density fraction was recovered.

**Immunophenotyping and sorting of mouse cells**

Cells were washed with FACS buffer (PBS, 2% FBS, 0.02% sodium azide, and 0.01 M EDTA), and 0.5–1 million cells were used for surface staining and analyzed by flow cytometry. Before staining, cells were incubated with anti-CD16/CD32 to block nonspecific binding. Cells were then stained by flushing cells out of the diaphysis with RPMI 1640 using a 23-gauge needle. Forward and reverse primers were as follows: Ins2 first pair, 5'-CAC CCAGGCTTTTGTGCAAGACG-3', and 5'-TGGTGGTGTCTGATGTCGAC TAGT-3'; Ins2 second pair, 5'-GGGGCGGTGGTTTCTCTACAC-3', and 5'-GCTGTGGTGCAGCACTGATCA-3'; Ins1 first pair, 5'-GGGGCGGTGGTTTCTCTACAC-3', and 5'-GCTGTGGTGCAGCACTGATCA-3'; and Ins1 second pair, 5'-GGGGCGGTGGTTTCTCTACAC-3', and 5'-GCTGTGGTGCAGCACTGATCA-3'. The β2-microglobulin transcript was amplified as control.
Results

An epitope encompassing the B-chain/C-peptide junction of proinsulin is detected on the surface of human CD11c⁺ cells in thymus, spleen, and blood

Our initial phenotypic analysis of PAE cells in human thymus and PLT demonstrated that these cells express markers of BM-derived APC (including CD11c, CD14, and HLA class II) (22). We hypothesized that PAE-CD11c⁺ cells could display self-epitopes on their surface, in keeping with their APC phenotype, and that self-epitopes might be accessible to Ab recognition. This approach could also provide a selecting tool to study PAE cells. Using proinsulin as a representative peripheral protein expressed by PAE cells, we tested two mAbs specific for human proinsulin (Table I), both recognizing the B-chain/C-peptide junction but not reacting with mature insulin. Both proinsulin mAbs demonstrated similar levels of surface reactivity in preliminary experiments. Two other mAbs (Table I) reacting with insulin and proinsulin did not stain the cells’ surface. This may reflect the absence of intact protein on the cell surface, as at least one of these Abs targets a conformational epitope (26, 31). We then selected the anti-proinsulin mAb M32337 for further studies. As shown in Fig. 1, we found cells stained by this Ab or Proins⁻ cells in live, unpermeabilized cell populations from the thymus (T-LDC enriched in DC), and spleen monocytes (SP-M); we also tested whole blood samples and demonstrated Proins⁺ cells in the circulation when gating on peripheral blood monocytes (PB-M). Variable proportions of T-LDC (mean = 3.18 ± SD 1.3%, n = 6), SP-M (mean = 66.2 ± 19.4%, n = 6), and PB-M (mean = 62.9 ± SD 11.4%, n = 8) were stained on their surface by the proinsulin mAb M32337, with spleen and blood harboring higher proportions of Proins⁺ cells than thymus (p = 0.0004, T-LDC vs SP-M; p = 0.000001, T-LDC vs PB-M). Importantly, the vast majority of the Proins⁺ cells coexpressed CD11c (Fig. 1), which was the single marker most consistently coexpressed by Proins⁺ cells in human T-LDC (69%), SP-M (93%), and PB-M (94%) populations. Proinsulin staining was not detected in CD4⁺ or CD8⁺ lymphocytes (data not shown).

To further test the specificity of mAb M32337 and gain insight into the nature of the Ags recognized on the surface of CD11c⁺ cells, we performed blocking experiments with overlapping peptides spanning the proinsulin B-chain/C-peptide junction (B24-C32, B24-C33, and B24-C36). All peptides prevented staining, e.g., preincubation with the B24-C32 peptide prevented staining (Fig. 2A), and staining inhibition with the proinsulin peptide was dose dependent (Fig. 2B). This demonstrates that the mAb M32337 reacts with peptide Ags. Staining for CD11c and HLA-DR was unaffected by the proinsulin peptide (data not shown). Staining with the proinsulin mAb was also unaffected by preincubation with a GAD65 peptide (aa residues 4–25) (Fig. 2C).

Overall, the reactivity pattern observed with the Abs to insulin/proinsulin and proinsulin is consistent with the surface display of a peptide epitope spanning the proinsulin B-chain/C-peptide junction with no evidence for the presence of conformation-dependent epitopes that would require the presence of intact proinsulin or larger fragments.

**Human Proins⁺CD11c⁺ cells correspond to PAE cells of the DC class**

Fig. 3 shows an example of Proins⁺ cells in T-LDC, SP-M, and PB-M populations coexpressing various differentiation markers together with CD11c. Fig. 4A compares the expression of these markers in these populations. Proins⁺CD11c⁺ cells often coexpress CD14 and HLA-DR in all three populations. Other APC markers were differentially expressed: Proins⁺CD11c⁺ cells more frequently express CD1a, CD20, CD40, CD80, CD83, and CD123 compared with Proins⁺CD11c⁻ populations. Proinsulin staining was often coexpressed CD14⁺ and HLA-DR⁺ cells in the periphery. Moreover, CD11c⁺CD8α⁺ T-LDC are enriched in Proins⁺ cells compared with CD11c⁺CD8α⁻ cells, on average 4-fold (mean = 49.3 ± SD
and thymic Proins \( ^+ \)CD11c \(^- \) cells (~30%) appear to include cells at various stages of differentiation and maturation, as indicated by the heterogeneous expression of phenotypic markers in the different compartments. Approximately 50% of the Proins \( ^+ \)CD11c \(^- \) cells in the thymus expressed and HLA-DR, with populations being CD123\(^{high} \) and HLA-DR\(^{high} \) (Fig. 3); 23% of the thymic Proins \( ^+ \)CD11c \(^- \) were CD14\(^+ \). Based on the purification of light density cells and gate selection, it is plausible that only a few of the Proins \( ^+ \)CD11c \(^- \) cells may be of epithelial origin.

In vitro-derived DC include Proins \( ^+ \) cells, which mature into a thymic-like PAE-DC phenotype

To further explore self-epitope expression by DC, also in relation to the maturation state, we derived DC from human PB-M by inducing their differentiation with GM-CSF/IL-4 and their maturation with TNF-\( \alpha \)/PGE\(_2\). Remarkably, ~30% of the cultured cells were Proins \( ^+ \). Although the proportion of Proins \( ^+ \) cells remained stable in response to the cytokines in the culture medium, we observed phenotypic changes typical of DC differentiation and maturation in the cultured cells. This was also confirmed by analyzing Proins \( ^+ \) and Proins \( ^- \) DC separately. Both populations responded to differentiation and maturation stimuli by expressing typical DC markers (HLA-DR, CD83, CD1a, CD40, CD80, and CD86), as shown in Fig. 5 for CD83 and CD80. However, unlike Proins \( ^+ \) cells, Proins \( ^- \) DC maintained CD14 expression and up-regulated CD86, CD20, and CD123, both in terms of percentage of positive cells (Fig. 5) and mean fluorescence intensity (data not shown). Thus, Proins \( ^+ \) cells have unique functional characteristics because they mature in vitro into cells having similar phenotype to thymic Proins \( ^+ \) PAE cells in their native microenvironment. C-peptide and proinsulin were not detectable in the supernatants of these cultures, indicating that the protein was not secreted (data not shown).

Human Proins \( ^+ \)CD11c \(^- \) cells display multiple self-epitopes

We then investigated whether other self-epitopes are detectable on the surface of CD11c \(^- \) cells and Proins \( ^+ \) cells. Using the Abs listed in Table I, we detected surface staining for selected GAD and IA-2 epitopes in T-LDC, SP-M, and PB-M CD11c \(^- \) populations. This was noted with both a mAb and a polyclonal to GAD and two polyclonal sera to IA-2, one reacting with the intracellular domain. Three other mAbs to IA-2 did not stain (Table I). Significant proportions of cells costained for GAD, IA-2, and proinsulin (Fig. 6A). In further blocking experiments, staining with the GAD65 mAb was virtually abolished by preincubating the Ab with the peptide used for its generation (aa 4–25) (Fig. 6B). Staining inhibition was related to the amount of peptide used; 60 \( \mu \)g of peptide resulted in almost complete blocking (from 53 to 5%) when analyzing the monocytes-gated cells from peripheral blood. The GAD peptide did not affect proinsulin staining (Fig. 2B). 14.1 vs 14.1 \( \pm \) 12.4, \( n = 6, p = 0.004; \) Fig. 4B). The results are consistent with our earlier characterization of proinsulin-positive PAE cells in human thymus and spleen tissue sections, where we also detected the coexpression of DC markers including CD8\( \alpha \) (22). Therefore, the surface expression of a proinsulin epitope identifies PAE-DC. The smaller proportions of peripheral (~5%),
gene, suggesting that an epitope of Golli-MBP is expressed on the surface of CD11c+ PAE cells. Overall, these data show that reactivity for multiple self-epitopes is detected with selected Abs on the surface of Proins+CD11c+ cells.

**Human Proins+CD11c+ cells transcribe INS**

Transcription of self-molecule genes has been reported previously in human thymus, spleen, and lymph nodes (in which PAE-DC were shown by immunohistochemistry/double immunofluorescence) (22, 24), in gradient-purified mouse (9) and human (5) thymic cells, and in cells sorted for the expression of DC (mouse) (21) or mTEC markers (mouse and human) (6, 7, 23). With the exception of one study in the mouse (21), these transcripts were not detected in thymic DC (6, 7, 23). Such discrepancies might be explained by the inability to enrich sufficiently for DC expressing self-Ags. Thus, we used proinsulin as a prototype Ag to test the hypothesis that the observed expression of self-epitopes is associated with gene transcription in PAE cells of the DC class. We sorted CD11c+ cells from human thymus, spleen, and peripheral blood based on the surface expression of the proinsulin epitope. We extracted RNA from sorted Proins+ and Proins− cells and assessed the presence/absence of full-length INS transcripts using a well-validated RT-PCR assay (3). INS mRNA was detected in Proins+ but not in Proins− cells (Fig. 7). The identity of the RT-PCR product was confirmed by sequencing or restriction enzyme digestion (data not shown). Thus, the surface display of the proinsulin epitope cosegregates with INS transcription.

**Mouse BM-derived CD11c+ cells transcribe Ins2, exist in mouse PLT, and display multiple self-epitopes**

Earlier studies did not find peripheral protein gene transcripts in mouse peripheral DC (6, 21). In light of the above data demonstrating transcription of an organ-specific gene such as insulin in human DC (Fig. 7) and human PLT (22), we further investigated insulin gene expression in mouse DC and PLT. As expected, we detected Ins2, as well as glucagon transcripts, in mouse thymus but also in spleen and lymph nodes (Fig. 8A). Ins2 transcripts were found in CD11c+ cells sorted from mouse thymus and spleen (Fig. 8B) but not in spleen CD11c− cells and CD4+ T cells. These data are consistent with the expression of peripheral protein genes in human PLT (22, 24) and demonstrate it in mouse PLT and peripheral CD11c+ cells. To substantiate this observation, we examined mice in which the LacZ gene replaces one copy of the coding region in the Ins2 gene, serving as a marker of transcription (18). β-Galactosidase (LacZ) transcripts were detected in thymus and inguinal lymph nodes (Fig. 8C) and in both freshly isolated and cultured BM cells (Fig. 8D). This indicates that Ins2-expressing CD11c+ PAE cells are BM derived. We then investigated whether Ins2 transcription in mouse CD11c+ PAE cells correlates with cell surface reactivity for the proinsulin epitope, similar to human cells. We stained thymus and spleen cells from several mouse strains (C57BL6, BALB/c, and NOD) and detected surface staining using both the proinsulin and GAD Abs (Fig. 9). Proins− cells from ≥5 wk-old mice coexpressed CD11c, as well as other DC markers, and similar to human cells, they expressed Ins2 mRNA (data not shown).

**Proinsulin surface staining depends on insulin gene transcription and is independent of Ag capturing**

Having shown that a proinsulin epitope is detected on the surface of both human and mouse CD11c+ cells, which transcribe the insulin gene, we tested whether the display of this epitope depends on insulin gene transcription. Therefore, we asked whether Proins− cells could be detected in Ins2+/−Ins2−/− mice. We bred Ins2+/−Ins2−/− with Ins2+/−Ins2−/− mice to obtain Ins2−/−Ins2−/− mice. We evaluated newborn mice because these mice die shortly after birth with severe diabetic ketoacidosis (18). Although newborn mice have few CD11c+ cells and mature DC do not fully develop until 5 wk of age (35), thymus and spleen cells from newborn wild-type and Ins2−/−Ins2−/− mice stained with the polyclonal Ab to GAD. Thus, PAE cells were present at birth, although possibly not fully matured. Proins− cells were detected in the thymus and spleen of wild-type mice but were virtually absent in Ins2−/−Ins2−/− mice (Fig. 9). Thus, surface staining with the proinsulin Ab is dependent on the presence of intact insulin genes and inferentially on insulin gene transcription. This experiment definitively excludes any cross-reactivity of the proinsulin mAb.
M32337 with unknown molecules and provides a critical validation for our approach to identify PAE cells through the expression of this proinsulin epitope in both human and mouse cells.

DC are known to help maintain peripheral tolerance by capturing, processing, and presenting self-molecules to T cells (36, 37). Thus, we investigated whether the surface expression of the proinsulin epitope by PAE cells of the DC class is alternatively explained by the capture of proinsulin expressed in pancreatic β cells. We studied PDX1 knockout mice (28) because PDX1 is a transcription factor required for the normal development of the pancreas. PDX1−/− mice suffer from complete pancreatic agenesis, lack ectopic sites of insulin or amylase production, and die shortly after birth (28). Thus, PDX1−/− mice have no proinsulin/insulin in the circulation and lack pancreatic β cells that could provide pancreatic proinsulin for capturing by DC. We found that thymus and spleen of newborn PDX1−/− mice contained Proins (and GAD+) cells similarly to wild-type mice (Fig. 9). Consistent with the expectation that thymic and peripheral PAE cells autonomously produce proinsulin through Ins2 transcription, we detected Ins2 expression in the thymus and spleen of PDX1−/− mice (data not shown). Thus, Ins2 transcription in these tissues and by inference in thymic and splenic CD11c+ PAE cells is not affected by the lack of PDX1. Collectively, the results in the insulin and PDX1 knockout mice show that the expression of the proinsulin epitope on the surface of PAE cells is not dependent on the presence of the target organ as a source of captured Ag, clearly implicating gene transcription in PAE-DC with the surface display of cognate self-epitopes.

Proins+ cells from HEL transgenic mice display an HEL epitope in the context of MHC class II

To investigate whether self-epitopes expressed by PAE cells are associated with MHC molecules, we studied mice that express the HEL under the control of a tissue-specific promoter, the crystalline promoter. The pattern of expression of HEL in mHEL transgenic mice mimics the expression of Ins2, with expression in a specific tissue (the eye), as well as in the thymus. As with Ins2, thymic expression of the HEL gene has been shown to promote tolerance to the HEL protein (29, 30), which is a self-molecule in the transgenic mouse. Based on the finding that Proins+ PAE cells can express multiple self-epitopes, we tested the hypothesis that at least some Proins+ PAE cells from mHEL mice should also express HEL epitopes on their surface. Using HEL as surrogate example of natural self-epitopes expressed by Proins+ PAE cells, we tested the hypothesis that HEL epitopes could be associated with MHC molecules. As shown in Fig. 10, we demonstrate that Proins+ cells from mHEL transgenic mice, but not from nontransgenic controls, display both the proinsulin epitope and a HEL epitope on their surface. As expected, these cells expressed the transcripts for Ins2 and the HEL transgene (data not shown). Because the anti-HEL mAb used is known to recognize a MHC class II/HEL Ag complex when cells are pulsed with HEL (27), this experiment provides initial evidence that PAE cells can display self-epitopes in association with MHC molecules.

Discussion

We tested the hypothesis that PAE cells of the DC class should function as APC and constitutively display multiple self-epitopes on their surface. We also hypothesized that the display of such epitopes could be demonstrated with Abs. Although Abs are not routinely used to detect self-epitopes on the cell’s surface, the TCR and Ig (the B cell receptor) share many structural features allowing Abs to mimic TCR. Moreover, some Abs have been shown to recognize HLA/peptide complexes (27, 38, 39). Our data show for the first time that a subset of CD11c+ cells constitutively display epitopes derived from self-molecules, in particular an epitope of proinsulin. We extrapolate the loss of proinsulin reactivity on the surface of thymus and spleen cells from insulin knockout mice to the human system as indicating that reactivity with the proinsulin
mAb M32337 is not spurious but rather depends on gene transcription. The blocking experiments demonstrate that the proinsulin Ab reacts with a peptide Ag, and the overall pattern of reactivity observed with Abs to proinsulin and insulin/proinsulin is consistent with the lack of conformational epitopes requiring the full molecule on the surface of the cells. The data from the mHEL transgenic mice further show that PAE cells displaying the proinsulin epitope also display an HEL epitope, the latter in the context of MHC class II using an Ab known to react with MHC class II (I-A^k) and HEL Ag complexes (27). Although extensive studies are needed to characterize the nature of the self-epitopes displayed by PAE cells and to fully determine their association with Ag-presenting molecules, our data suggest that the display of self-epitopes by PAE cells is relevant to Ag presentation.

Our data also show that the CD11c^+ cells expressing self-epitopes belong to the DC class of PAE cells, which have been associated with the expression of self-molecules in the thymus and self-tolerance. The data provide critical information on PAE cells because their nature, phenotype, and distribution has been controversial. In fact, studies have identified thymic PAE cells as TEC (6, 7, 23) or BM-derived APC (21, 22). Using double immunofluorescence, we previously identified CD11c^+ PAE-DC expressing proinsulin and other T1D autoantigens in human thymus and PLT (22), tissues that transcribe the genes coding for these molecules (22, 24). Other studies in mouse and humans found no evidence that PAE cells include BM-derived APC or that PAE cells exist outside the thymus (6, 7, 21). Our data show that PAE cells exist in human peripheral blood and provide definitive evidence that, in

**FIGURE 5.** In vitro-derived Proins^+ DC express CD8α, CD20, and CD123. Percentage of cells expressing CD8α, CD20, CD123, CD14, CD80, and CD83 by flow analysis of in vitro-derived DC, as a whole or as Proins^- and Proins^+ cell populations. The frequency of cells expressing these markers was compared in Proins^+ and Proins^- cells in both the immature (CD8α, p = 0.03; CD20, p = 0.02; CD123, p = 0.15; CD14, p = 0.03; CD83, p = 0.01) and mature states (CD8α, p = 0.006; CD20, p = 0.01; CD123, p = 0.01; CD14, p = 0.01); n = 9 except n = 4 for CD123. Asterisks denote values that are statistically different.

**FIGURE 6.** Proins^+ cells display multiple self-epitopes. A. Analysis of monocyte gated peripheral blood cells stained with Abs against proinsulin (M32337), GAD (GAD65-AS), and IA-2 (serum 8959, against the intracellular domain). Surface staining is demonstrated with Abs to GAD65 and IA-2 and is present on significant proportions of Proins^+ cells. B. Blocking experiment demonstrating the specificity of the GAD65 mAb. Staining with this mAb was virtually blocked by preincubation with the relevant GAD65 peptide. C. Analysis of monocyte-gated peripheral blood cells stained with Abs against proinsulin (M32337), MBP, and TPO. Surface staining is demonstrated with Abs to MBP and TPO and is present on a significant proportion of Proins^+ cells.
addition to mTEC, PAE cells do include BM-derived CD11c+ DC in both thymus and PLT in humans and mice. This is shown by the coexpression of CD11c and other APC markers in human Proins cells and by the selective presence of INS transcripts in sorted Proins CD11c+ cells from thymus, spleen, and blood. Mouse BM precursor cells also express Ins2 and CD11c+ cells maintain such expression in thymus and PLT.

The relative contribution to self-tolerance provided by mTEC and DC remains unclear (10). In NOD mice, diabetes develops despite the expression of Ins2 in the thymus, although this is lower than in control strains (40). NOD.Ins2−/− mice lacking Ins2 expression (in both mTEC and DC) develop diabetes at an accelerated rate with higher incidence in both sexes (15, 16). Conversely, the transgenic expression of Ins2 in MHC class II-positive cells, including in the thymus, prevents diabetes in NOD mice (13); moreover, syngeneic transplantation of transgenic BM expressing Ins2 in APC and the transfer of CD11c+ DC transgenically expressing Ins2 prevent diabetes in NOD mice (41, 42). Thus, there is evidence from genetically manipulated mouse models that the expression of self-Ags in the thymus and by adoptively transferred BM-derived APC can influence self-tolerance. In the AIRE knockout mouse (43, 44), a model of the autoimmune polyendocrinopathy (APE)-candidiasis (C)-ectodermal dystrophy (ED) syndrome, or APECED (45), the lack of the transcription factor AIRE (autoimmune regulator) causes mTEC to lose the ability to express self-molecule genes, including insulin (44). However, AIRE knockout mice do not develop autoimmune diabetes or insulinitis, even when AIRE knockout mice transgenically express HEL in pancreatic β cells and a HEL-specific TCR (46). Lack of NOD susceptibility genes, anergy, or ignorance of the target Ag could explain these findings (47). An additional explanation is the persistence of self-molecules expression by PAE-DC. It is unclear whether the lack of AIRE impairs self-molecules expression in these cells, but in our earlier studies, we did not observe colocalization of AIRE with proinsulin expression in human thymus (22). Our finding in humans and nontransgenic mouse strains that PAE cells of the DC class normally display self-epitopes in both thymus and periphery suggests that these cells might physiologically contribute to both thymic and peripheral tolerance.

Continuous exposure of the immune system to self is indeed thought to be critical for self-tolerance, but peripheral presentation of tissue-specific self-molecules is considered dependent on the capturing of self-molecules by APC, in particular immature DC (48). The analysis of DC isolated from PDX1 knockout mice demonstrates that the surface display of a proinsulin epitope does not require proinsulin expression from pancreatic β cells and thus is not readily explainable by Ag capture from peripheral sources. The combined data from the insulin gene double knockout mice (which lack Proins+ PAE cells) and the PDX1 knockout mice (which have such cells) show that the display of a proinsulin epitope by PAE-DC in both thymus and periphery depends on autonomous gene transcription. Thus, it appears that at least three mechanisms can explain the expression of self-molecules in the thymus and lymphoid tissues: expression by mTEC, expression by DC through capturing from peripheral tissues (48) or from thymic mTEC (29, 49), and expression by DC through the transcription-dependent mechanism described here. The existence of redundant and possibly complementary mechanisms may provide improved capacity to establish self-tolerance.

The possibility that Proins+ PAE-DC might contribute to self-tolerance is further supported by the phenotypic analysis of human Proins+ cells, which correspond to the PAE-DC we defined in the native state (22). Proins+ cells in T-LDC and monocyte populations from spleen and blood often coexpress CD11c, CD14, and

**FIGURE 7.** Proins+ cells transcribe INS. RT-PCR analysis to assess the expression of the INS transcript (437 bp, full length) in Proins+ and Proins- cells sorted from thymus, spleen, and peripheral blood. Data shown are representative of several experiments (thymus n = 3, spleen n = 2, peripheral blood n = 4). Pancreatic islets and negative controls are also shown (omitting the reverse transcriptase (−RT) or the template (NT)). RT-PCR products were loaded on 2% agarose gels, of which the negative image is shown. GAPDH was used as a housekeeping gene.

**FIGURE 8.** Ins2 is expressed in mouse PLT, thymic, and peripheral CD11c+ cells. A, Nested RT-PCR demonstrates Ins2 and glucagon transcripts in thymus, spleen, inguinal lymph nodes but not liver of C3H mice (+RT lanes, RT = reverse transcriptase). Control PCR in which the reverse transcriptase was omitted are also shown (−RT). B, β2-microglobulin was used as housekeeping gene. B, Thymic and splenic CD11c+ cells but not splenic CD11b+ and CD4+ T cells from C3H mice expressed Ins2 and glucagon transcripts. C, RNA from pancreas and thymus of Ins2−/−, LacZ+ mice were used in nested RT-PCR experiments to assess the expression of β-galactosidase. D, BM cells were isolated from Ins2−/−, LacZ−/+ mice. BM cells were also cultured in the presence of GM-CSF. Nested RT-PCR detected β-galactosidase gene expression in both freshly isolated and cultured BM cells.
other APC markers. PAE-DC from thymus more frequently express CD1a, CD83, CD40, and CD80 compared with their counterparts in the periphery, suggesting that these cells include mature DC. Thymic Proins⁻⁺ cells also expressed CD8α⁺, a marker of “lymphoid” DC, which have tolerogenic function in the mouse (48, 50) but are not well characterized in humans. We have previously presented evidence for the existence of CD8α⁺ DC expressing proinsulin in the human thymus using double immunofluorescence on tissue sections (22). We substantiate and extend those results by immunophenotyping and flow cytometry analysis of DC isolated from human thymus and produced in vitro from PB-M. Myeloid and lymphoid DC originate from a common myeloid progenitor, and the expression of CD8α⁺ likely marks the functional state rather than origin (51). In the mouse, CD8α⁺ DC induce T cell deletion through the FasR (52). Consistent with these findings, Proins⁺⁺ cells express FasL, and we previously demonstrated apoptosis in the lymphocytes surrounding Proins⁺⁺ cells in human thymus and spleen, suggesting that human PAE-DC can contribute to self-tolerance through deletion (22). Moreover, Proins⁺⁺ PAE-DC in the thymus frequently express CD20 and CD123, markers that are associated with regulatory function in some studies (53). In contrast, the predominant phenotype of peripheral PAE-DC resembled those of immature DC (infrquent expression of CD83, CD40, and CD80). Immature DC have also been reported to be tolerogenic (48). Thus, CD11c⁺ PAE-DC in thymus and periphery differ in their maturation state and express partially distinct phenotypic markers.

Remarkably, we discovered that ~30% of the DC derived in vitro from PB-M using a standard protocol are Proins⁺⁺ cells. These cells maintained CD14 expression, and their frequency was not affected by GM-CSF/IL-4, suggesting that these cells may be somewhat unresponsive to these cytokines. However, the Proins⁺⁺ cells in the cultures responded to the differentiation (GM-CSF/IL-4) and maturation (PGE₂/TNF-α) stimuli by up-regulating CD8α⁺, CD20, and CD123. Thus, Proins⁺⁺ cells acquired a phenotype similar to that of thymic PAE-DC in their native state. This observation suggests that the surface expression of a proinsulin epitope identifies a subset of DC with unique functional and maturation characteristics. The ability to generate DC in vitro that include Proins⁺⁺ cells uniquely maturing into a thymic DC-like and potentially tolerogenic phenotype may be relevant for developing improved DC-based strategies against autoimmunity. The epitopes expressed by PAE-DC may participate in thymic positive and negative selection processes and thus might be more likely to interact productively with the T cell repertoire, perhaps stimulating regulatory cells in the periphery, than those derived from pulsing DC with Ags (54). It should also be noted that the B-chain/C-peptide junction of proinsulin is a known target epitope in patients with T1D and NOD mice (55–57). Genetic factors are critical modifiers of the expression of self-molecules by PAE cells and can influence susceptibility to autoimmune responses against many of the self-Ags expressed by PAE cells (10); it remains to be determined whether PAE cells might activate autoreactive T cells against self-epitopes, perhaps in the presence of a predisposing genetic background and a proinflammatory microenvironment that modifies their functional state.

In conclusion, we demonstrate that PAE cells include a class of DC, which are present in the thymus and periphery. This subset of
DC displays self-epitopes through a novel mechanism based on autonomous gene transcription rather than Ag capturing. The presence of PAE-DC in the circulation and the ability to purify these cells by selective staining for self-epitopes should facilitate further studies to better characterize the self-epitopes expressed, the molecular mechanisms regulating such expression, the functional properties of these cells in both normal and autoimmune states, and, ultimately, their potential for therapeutic applications.

Acknowledgments

Dr. Ken Shortman kindly shared his protocol for T-LDC preparation. Dr. Len Harrison donated proinsulin peptides for the blocking experiments. We thank him and Dr. Michael Clare-Salzler for critical discussions of the data. Dr. Constantin Polychronakos provided breeding pairs to generate insulin double knockout mice. We thank Dr. Maki Nakayama for sharing her unpublished genotyping protocol and primer sequences. Ingela Berglund-Dahl helped with mouse husbandry, and Helena Edlund provided PDX1 knockout mice. Dr. Igal Gery provided mHEL mice and Dr. Ronald Germain the mAb D8H21. David Stenger gave many helpful suggestions for the manuscript. We acknowledge the support of Jim Phillips and the Flow Cytometry Core of the University of Miami.

Disclosures

The authors have no financial conflict of interest.

References


