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Development and Characterization of a Preclinical Model for the Evaluation of CD205-Mediated Antigen Delivery Therapeutics in Type 1 Diabetes

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ABSTRACT
Dendritic cells (DCs) are crucial for the production of adaptive immune responses to disease-causing microbes. However, in the steady state (i.e., in the absence of an infection or when Ags are experimentally delivered without a DC-activating adjuvant), DCs present Ags to T cells in a tolerogenic manner and are important for the establishment of peripheral tolerance. Delivery of islet Ags to DCs using Ag-linked Abs to the DC endocytic receptor CD205 has shown promise in the NOD mouse model of type 1 diabetes (T1D). It is important to note, however, that all myeloid DCs express CD205 in humans, whereas in mice, only one of the classical DC subsets does (classical DC1; CD8α− in spleen). Thus, the evaluation of CD205-targeted treatments in mice will likely not accurately predict the results observed in humans. To overcome this challenge, we have developed and characterized a novel NOD mouse model in which all myeloid DCs transgenically express human CD205 (hCD205). This NOD.hCD205 strain displays a similar T1D incidence profile to standard NOD mice. The presence of the transgene does not alter DC development, phenotype, or function. Importantly, the DCs are able to process and present Ags delivered via hCD205. Because Ags taken up via hCD205 can be presented on both class I and class II MHC, both CD4+ and CD8+ T cells can be modulated. As both T cell subsets are important for T1D pathogenesis, NOD.hCD205 mice represent a unique, patient-relevant tool for the development and optimization of DC-directed T1D therapies. ImmunoHorizons, 2019, 3: 236–253.
INTRODUCTION
Type 1 diabetes (TID) is characterized by T cell–mediated destruction of the pancreatic islet β cells (1). Because TID involves inappropriate lymphocyte targeting of the β cells, immune modulatory therapies represent an attractive means of treatment. This is in contrast to merely treating the disease with insulin replacement, which, although necessary, is unable to stop disease progression (2). Immune modulatory treatments that have been attempted include the targeting of CD3, CD20, IL-2, or CD80 and CD86 (3). These therapies have met with varying clinical success. At best, the response to these short-term treatments was not durable, despite initial positive outcomes, and patient disease returned (3). It is possible that, as TID is a chronic disease, long-term treatment with these reagents would be more successful, but this also exposes patients to the complications inherent in immune modulatory therapies. Targeting only T cells specific for β cell Ags is a more attractive option, as it represents a strategy to eliminate or suppress the disease-causing T cells while leaving immune responses to pathogens and cancers intact (4). Using β cell Ags themselves as treatments is predicated, in part, on the ability of dendritic cells (DCs) to present Ags in a tolerogenic manner in the absence of a DC-activating adjuvant, leading to T cell tolerance (5, 6). However, clinical trials where β cell Ags or epitopes were delivered to patients have also met with only moderate success (4). One reason for this may be that previous attempts have delivered these Ags nonspecifically rather than targeting them directly to DCs.

Delivery of Ags to DCs in murine models of autoimmunity has shown promise (7–12) and is feasible because of the presence of a number of DC-specific endocytic receptors, including CD205 (DEC-205; lymphocyte Ag 75) and DCIR2 (13). These markers, respectively, help define the murine classical DC (cDC) subsets cDC1 and cDC2 (14). Treatment of DCs with Ag-linked Abs against these receptors results in the uptake and degradation of the Ag (15).

We have previously shown that delivery of specific epitopes via murine CD205 (mCD205), expressed only by the mouse cDC1 subset, can lead to induction of CD8+ T cell tolerance toward those epitopes in the NOD mouse model of TID (8, 9). Others have shown that delivery of Ag via DCIR2, expressed by the mouse cDC2 subset, results in CD4+ T cell tolerance in NOD mice and delayed TID onset in an adoptive transfer model (11). Both DC subsets are, thus, capable of inducing T cell tolerance. This is important, as the different DC types are proposed to be somewhat specialized for presentation to different T cell subsets, with cDC1 (CD8α+ in spleen) optimally presenting to CD8+ T cells and cDC2 (CD8α−) to CD4+ T cells (15).

Although humans possess DCs with similar phenotypes to the murine subsets, they are not exactly parallel (16). In particular, in humans, CD205 is expressed by all myeloid DCs, not just the cDC1 subset (17). Because Ags taken up via human CD205 (hCD205) can be optimally presented on both class I and class II MHC (MHC II) (15), DCs treated in this manner can impact both CD4+ and CD8+ T cells, both of which are important for TID pathogenesis (1). Therefore, to efficiently modulate both T cell subsets, potentially only CD205 must be targeted in humans. An unmet need for progress in this area is a preclinical model for the development and optimization of such a strategy. To meet this need, we have developed and extensively characterized an NOD mouse model having transgenic expression of hCD205 under the control of the DC-specific CD11c promoter. As the hCD205 transgene is expressed by both cDC1 and cDC2, the expression of this endocytic receptor more closely mirrors the situation found in humans. We find that the transgenic mice have a similar TID disease profile to standard NOD mice. They retain typical APC subset levels and maturation status for NOD mice, and their DCs remain functional, in addition to being able to process and present Ag taken up via hCD205. NOD mice with transgenic expression of hCD205 thus represent a useful model for the preclinical testing of DC-directed therapies for TID.

MATERIALS AND METHODS
Production of Ag-linked anti-hCD205 Abs
The human anti-hCD205 Ab 3G9 was previously obtained by immunization of human Ig-transgenic mice with hCD205 (18), which bears 77% aa sequence identity to mCD205. To produce 3G9 linked to either OVA, murine proinsulin 2 (Ins2), human proinsulin (hIns), the mimotope peptide AL [YQLENYCAL; recognized by the NOD-derived CD8+ islet T cell clone AI4 (19)], or the HIV-derived peptide SL9 (SLYNTVATL), each was cloned in frame with the C terminus of the 3G9 H chain in a plasmid also encoding the 3G9 L chain. OVA, AL, and SL9 were preceded by a linker encoding DMAKKETWRLFFEGR, derived from the MHC II sequence, whereas Ins2 and hIns were preceded by the sequence for the flexible linker (GGGGS)3 to allow for proper proinsulin folding (20). Insertion of the proper Ag was assessed by restriction digest analysis and sequencing of the construct. The Abs were produced by transient transfection of 293T cells using a calcium phosphate transfection kit (Invitrogen). Briefly, 293T cells were seeded at 7.5 × 106 cells/150 mm tissue culture dish in 20 ml DMEM (Life Technologies) containing 10% low IgG FBS (Life Technologies), antibiotic/antimycotic (5 U/ml penicillin, 5 µg/ml streptomycin, and 0.25 µg/ml amphotericin B; Life Technologies), and 1 mM sodium pyruvate (Life Technologies) and incubated at 37°C overnight. The following day, the cells were transfected following the protocol included in the transfection kit with 60 µg of a 3G9 construct and 5 µg of pAdVantage vector (Promega) per plate. The pAdVantage vector enhances transient protein expression by inhibiting the activity of the dsRNA-activated inhibitor DAI. The transfection solution was added dropwise, and cells were incubated at 37°C overnight. Cells were then washed with 10 ml of serum-free DMEM and incubated for 4–6 d at 37°C in 30 ml of DMEM containing antibiotic/antimycotic and 1% Nutridoma (Roche). To remove debris, the supernatant was centrifuged at 486 × g for 5 min and sequentially passed through 0.45-, 0.22-, and 0.1-µm filters. The supernatant was passed through a Protein G column (GE Healthcare), and the Ab was eluted with 0.1 M glycine HCl (pH 2.7). Eluted Ab was concentrated using a 30-kDa Amicon...
Ultra centrifugal filter (EMD Millipore), and three buffer exchanges were performed with PBS (pH 7.2). Ab concentration was determined using a Bio-Plex assay (Bio-Rad) with bovine γ-globulin (Bio-Rad) as the standard, and purity was assessed by SDS-PAGE.

Production of Ag-linked anti-mCD205 Abs
Ins2 was cloned in frame at the C terminus of the H chain of anti-mCD205 (NLDC-145), which had been previously modified to contain murine Ig κ and IgGI constant regions with mutations that interfere with Fc receptor binding (21). The flexible linker (GGGGS)₃ was inserted in between the Ab and proinsulin to allow for proper proinsulin folding (20); this Ab was designated anti-mCD205/Ins2. Anti-mCD205/OVA (15) and anti-mCD205/NRP-V7 (8) have been described. The Ag-linked anti-mCD205 Abs were produced by transient transfection of 293T cells with calcium phosphate and purified as described above for the Ag-linked 3G9 Abs, except that for each 150-mm plate to be transfected, 30 μg of DNA encoding the Ab H chain and 30 μg of DNA encoding the Ab L chain were used along with 5 μg of pAdvantage vector (Promega).

Assessment of Ag-linked 3G9 binding to hCD205
Chinese hamster ovary (CHO), CHO/mCD205 (18), and CHO/hCD205 cells (18) were incubated with 3G9/Al (for the experiments depicted in Supplemental Fig. 1), 3G9/OVA, 3G9/Ins2, or 3G9/hIns, washed once with PBS containing 1% FBS (HyClone) and 0.1% (w/v) sodium azide, and then incubated with a 1:100 dilution of PE-labeled donkey anti-human IgG (H+L) F(ab’)₂ (Jackson ImmunoResearch). Samples were washed twice, incubated in 1 μg/ml DAPI (Sigma-Aldrich) for 15 min on ice, and filtered through a 35-μm cell strainer before proceeding to data acquisition. Flow cytometry data were acquired using a BD FACScan or LSRII and analyzed using FlowJo software (version 8.8.6).

Assessment of Ag-linked anti-mCD205 binding to mCD205
CHO/mCD205 cells were incubated with anti-mCD205/OVA or anti-mCD205/Ins2, followed by the aliphophycocyanin-labeled secondary Ab anti-mouse IgG1 (BD Biosciences). For specific detection of the proinsulin moiety of anti-mCD205/Ins2, the detection reagents used were 5 μg/ml biotinylated anti-proinsulin (R&D Systems) followed by 5 μg/ml streptavidin–allophycocyanin (BD Biosciences). After washing, cells were resuspended in 5 μg/ml DAPI (Sigma-Aldrich) before addition of the fluorescent secondary Ab and data acquisition. Flow cytometry data were acquired using a BD FACSscan or LSRII and analyzed using FlowJo software (Tree Star).

Mice
C57BL/6 mice transgenically expressing hCD205 (C57BL/6.hCD205) (18) were obtained from R. Steinman (The Rockefeller University). The hCD205 transgene is under the control of a synthetic CD11c promoter (GenBank accession number: DG688851) containing both C57BL/6 and 129 sequences. To generate NOD mice with transgenic expression of hCD205, C57BL/6.hCD205 were backcrossed for 12 generations with NOD mice and fixed to homozygosity for markers of NOD origin delineating known Idd loci (22). The presence of the hCD205 transgene was determined by PCR of tail-tip DNA using the following primer pair: 5’-TTGAA GACATCGAGAAACCT-3’ and 5’-TCTCACGCCCAGTCCA GAAGTA-3’. The transgene was maintained in a hemizygous state. Ragl-deficient OT-I TCR-transgenic C57BL/6 mice were provided by F. Macian (Albert Einstein College of Medicine). C57BL/6 mice transgenically expressing the OT-II TCR were obtained from The Jackson Laboratory (B6.Cg-Tg[TcraTcrtb] 425Chn/J; stock no. 004194). NOD mice with transgenic expression of the TCRs from the NOD-derived islet CD8⁺ T cell clones 8.3 and A14, denoted NOD.8.3 and NOD.A14, respectively, were maintained in-house and have been described (23, 24).

Preparation of bone marrow–derived DCs
Bone marrow cells were harvested from the femur and tibia of NOD and NOD.hCD205 mice and incubated for 3 min in ACK lysing buffer (Lonza). Cells were cultured in RPMI 1640 (Life Technologies) containing 10% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin (Life Technologies), 1 mM sodium pyruvate (Life Technologies), and 1× nonessential amino acids (Life Technologies) (RPMI-10) supplemented with 10 ng/ml GM-CSF (PeproTech) and 10 ng/ml IL-4 (PeproTech) for 7 d.

Splenocyte preparation
Mice were sacrificed using CO₂ asphyxiation, followed by cervical dislocation. Splenocytes were harvested and placed in ice-cold Ca²⁺/Mg²⁺-free HBSS (Life Technologies) supplemented with 40 U/ml collagenase D (Roche). Collagenase-containing medium was injected into each spleen; spleens were then teased into small pieces and digested for 25 min at 37°C. The collagenase was inactivated by the addition of 10 μM EDTA (Sigma-Aldrich) for 5 min at 37°C. Samples were then washed in 40–μm cell strainer and remaining pieces of spleen were crushed. Samples were washed with 5 ml of RPMI-10 and centrifuged at 486 × g for 5 min. RBCs were lysed in ACK lysing buffer for 4 min at room temperature and washed with RPMI-10. The resultant splenocytes were centrifuged and washed twice with PBS. Prior to the final wash, splenocytes were passed through a 40-μm cell strainer. The splenocytes were then counted and suspended in PBS.

CD11c⁺ DC isolation
Splenocytes were washed once with autoMACS buffer (Miltenyi Biotec), counted, and suspended in 40 μl of autoMACS buffer/10³ cells and 5 μl of anti-CD11c microbeads (Miltenyi Biotec). Cells were incubated for 30 min at 4°C, washed with 10 μl of autoMACS buffer, and passed through an MS column (Miltenyi Biotec). Following removal from the magnet, the CD11c⁺ DCs were eluted from the column, suspended in RPMI-10, and counted.

CD8α⁺ and CD8α⁻ DC isolation by magnetic separation
Splenocytes were washed once with autoMACS buffer (Miltenyi Biotec) and counted. CD8α⁺ DCs were obtained using a CD8α⁺ DC isolation kit (Miltenyi Biotec). Briefly, splenocytes were depleted of T cells, B cells, and NK cells. The remaining cells were labeled
with anti-CD8α+ conjugated to magnetic beads, and the cells were passed through an MS column. Following removal from the magnet, the CD8α+ DCs were eluted from the column. The flow-through fraction contained cells that had been depleted of T cells, B cells, NK cells, and CD8α+ DCs. These cells were labeled with anti-CD11c microbeads (Miltenyi Biotec) and passed through an MS column (Miltenyi Biotec). Following removal from the magnet, the CD8α+ DCs were eluted from the column, suspended in RPMI-10, and counted.

### CD4+ and CD8+ T cell isolation

Splenocytes were washed once with autoMACS buffer (Miltenyi Biotec), counted, and suspended in 40 µl/10^7 cells autoMACS buffer and 5 µl of CD8 or CD4 T cell biotin Ab mixture (Miltenyi Biotec)/10^7 cells. Cells were incubated for 10 min at 4°C, at which point 30 µl of autoMACS buffer/10^7 cells and 10 µl of anti-biotin microbeads (Miltenyi Biotec)/10^7 cells were added. Cells were incubated for 15 min at 4°C, washed with 10 ml of autoMACS buffer, and passed through an LS column (Miltenyi Biotec). T cells were eluted from the column, suspended in RPMI-10, and counted.

### T cell proliferation assay based on BrdU incorporation

CD11c+ DCs (or CD8α+ DCs, CD8α- DCs, or bone marrow-derived DCs [BMDCs], as indicated) were treated with the indicated Abs or Abs in a flat-bottom 96-well plate and incubated at 37°C. After 2 h, DCs were matured with 0.5 µg/ml LPS. The following day, T cells were added to the DCs. After an additional 72 h, BrdU labeling reagent (BD Biosciences) was added to each well at a concentration of 10 µM. The next day, a BrdU detection ELISA (BD Biosciences) was performed. Absorbance of each well at 450 nm was detected using an EMax precision microplate reader (Molecular Devices), and the results were analyzed using GraphPad Prism 7 software.

### Assessment of T1D

Female mice were monitored weekly from 4 to 30 wk for glucosuria using Diastix reagent strips (Bayer). Mice were considered diabetic following two consecutive positive tests. The first positive test was recorded as the date of diabetes onset. Based on our prior studies with NOD mice, this corresponds to a blood glucose value at onset of 467 ± 121 mg/dl (n = 11).

### IFN-γ ELISPOT assay

Islets were isolated from pancreata of NOD.hCD205 mice as described (25) and cultured with IL-2 for 7 d. After 6 d, CD11c+ DCs were isolated from NOD mice, treated with peptides of interest, and matured with 0.5 µg/ml LPS. DCs were incubated overnight at 37°C. An ELISPOT plate (EMD Millipore) was coated overnight with anti–IFN-γ (R4-6A2; BD Biosciences). The following day, 2 × 10^4 islet-infiltrating cells were added to each well containing treated DCs. The mixed cell population was added to the Ab-coated ELISPOT plate and incubated at 37°C for 48 h. After this time, an IFN-γ ELISPOT assay was performed as described (25). Spots were counted using an automated ELISPOT reader (Autoimmun Diagnostika), and the results were analyzed using GraphPad Prism 7 software. The stimulation index was determined by dividing the number of spots per well by the number of spots in the negative control wells.

### Analysis of hCD205 expression by APC populations

Splenocytes were added to a 96-well V-bottom plate, with 2 × 10^6 cells per well. The plate was centrifuged at 486 × g for 5 min, and cells were washed one time in PBS (pH 7.2) containing 2% FBS. This buffer was used for all subsequent washing and dilution steps. Cells were blocked with 100 µl of Fc block (20 µg/ml; BD Biosciences) on ice for 15 min. Cells were then washed once. Samples were incubated on ice for 30 min with 12.5 µg/ml 3G9/OVA and other appropriate Abs in 100 µl (see Supplemental Table I, top) and mixed at 15 min into the incubation (14). Samples were washed twice. Biotinylated Abs were then detected with 2 µg/ml streptavidin/Brilliant Violet 510 (BD Biosciences), and 3G9/OVA was detected with 1:100 donkey anti-human IgG (H+L) F(ab′)2/PE (Jackson ImmunoResearch). Samples were washed twice and incubated in 1 µg/ml DAPI for 15 min on ice. Samples were filtered through a 35-µm cell strainer prior to data collection. Data were collected on a BD LSRII flow cytometer with five lasers (355, 405, 488, 561, and 640 nm) and analyzed using FlowJo software (version 8.8.6).

### Analysis of maturation markers on APC populations

Splenocytes were added to a 96-well V-bottom plate, with 2 × 10^6 cells per well. The plate was centrifuged at 486 × g for 5 min, and cells were washed one time in PBS (pH 7.2) containing 2% FBS. This buffer was used for all subsequent washing and dilution steps. Cells were blocked with 100 µl of Fc block (20 µg/ml; BD Biosciences) on ice for 15 min. Cells were then washed once. Samples were incubated on ice for 30 min with appropriate Abs in 100 µl (Supplemental Table I, top) and mixed at 15 min into the incubation (14). Samples were washed twice. The appropriate Abs (Supplemental Table I, middle) were added, and biotinylated Abs were detected with 2 µg/ml streptavidin/Brilliant Violet 510 (BD Biosciences). Samples were washed twice, incubated in 1 µg/ml DAPI for 15 min on ice, and filtered through a 35-µm cell strainer prior to data collection. Data were collected on a BD LSRII flow cytometer with five lasers (355, 405, 488, 561, and 640 nm) and analyzed using FlowJo software (version 8.8.6).

### Anti-insulin Western blot

Anti-mCD205 Abs were run on a 4–15% Tris–HCl gel (Bio-Rad) and then either stained with Biosafe Coomassie (Bio-Rad) or transferred to a PVDF membrane (Bio-Rad). The membrane was blocked with 5% nonfat dry milk (Santa Cruz Biotechnology) in 0.05% Tween 20/PBS. The membrane was then incubated with guinea pig anti-insulin (Abcam) diluted 1:200 in blocking buffer. After a 1-h wash with 0.05% Tween 20/PBS, the membrane was incubated with rabbit anti–guinea pig IgG–alkaline phosphatase (Abcam) diluted 1:1000 in blocking buffer. The membrane was then washed for 40 min with 0.05% Tween 20/PBS and 20 min with PBS. Detection reagent was prepared by dissolving one tablet.
of SigmaFast BCIP/NBT (Sigma-Aldrich) in 10 ml of H2O and added to the membrane. After sufficient color development, the reaction was stopped by rinsing the membrane with H2O. Alternatively, to verify the presence of the entire proinsulin protein, an Ab specific for the C-terminal region of the insulin A chain was used. The membrane was incubated with goat anti-insulin A (C-12; Santa Cruz Biotechnology) diluted 1:500 in blocking bu-
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II-2 ELISA
Splenic CD11c+ DCs from NOD mice were plated at 2.5 × 10⁴ cells well per well in 100 μl of RPMI-10 in a 96-well flat-bottom plate and treated with 0.1 or 1 μM peptides InsB9,23 (SHLYEALYLC GREG; Mimotopes) or the BDC2.5 mimotope AHHIPWARMDA (26) (Mimotopes) or the indicated concentrations of Ag-linked anti-mCD205 Abs. The cells were incubated for 2 h at 37°C, at which point the DCs were treated with 1 μg/ml LPS to induce maturation. Cells were incubated overnight at 37°C. The following day, the InsB9,23-specific cell line PCR1-10.18 (27) was added to the DCs at 2.5 × 10⁴ cells per well in 100 μl of RPMI-10. Cocultures were incubated overnight at 37°C. IL-2 secretion was measured by ELISA (eBioscience). Briefly, an ELISA plate was coated overnight with anti-IL-2 capture Ab. After blocking and washing, cell supernatants were added to the plate at 100 μl/well. Biotin–anti-IL-2 was used as a detection Ab, followed by incubation with avidin–HRP. 3,3’,5,5’-Tetramethylbenzidine substrate was added, and after color development, the reaction was stopped by addition of 1 N H2SO4. ELISA plates were read at 450 nm, and IL-2 concentrations were calculated from an IL-2 standard curve.

RESULTS
hCD205-transgenic DCs process and present Ags delivered via hCD205 to both CD4+ and CD8+ T cells
In hCD205-transgenic C57BL/6 mice, the transgene is under the control of a CD11c promoter, allowing expression on both the CD8α− and CD8α+ classic myeloid DC subsets (18). As previously reported (18), the anti-hCD205 Ab 3G9 binds to CHO cells stably expressing hCD205 (CHO/hCD205), with no binding to non-transfected CHO cells or to CHO/mCD205 cells (Supplemental Fig. 1). To verify the functionality of the hCD205 transgene, we developed anti-hCD205 linked to OVA (3G9/OVA). 3G9/OVA was produced in 293T cells (Fig. 1A), and its binding to hCD205 but not mCD205 was verified (Fig. 1B). CD11c+ DCs were isolated from C57BL/6 or C57BL/6.hCD205 mice and treated with 3G9/OVA or 3G9 linked to the HIV-derived peptide SLYNTVAATL (3G9/SL9) followed by LPS. To determine whether Ag could be internalized, processed, and presented, we added OVA-specific CD8+ OT-I T cells to these cultures. We found that C57BL/6 DCs could induce OT-I proliferation in response to SIINFEKL, the peptide recognized by OT-I, but could not induce proliferation in response to 3G9/OVA (Fig. 1C), as expected. In contrast, C57BL/6.hCD205 DCs induced proliferation in response to both the peptide and 3G9/OVA (Fig. 1D). This indicated that the transgenic hCD205 was functioning as expected. Furthermore, the proliferation seen was not due to an effect of 3G9 in the absence of OVA, as 3G9/SL9 induced no proliferation. We performed similar experiments with OVA-specific CD4+ OT-II T cells, replacing SIINFEKL with the peptide recognized by OT-II, ISQAVHAA HAEINEAGR. Once again, C57BL/6 DCs could only induce OT-II T cell proliferation by presenting the OT-II peptide (Fig. 1E), whereas C57BL/6.hCD205 DCs induced proliferation in response to 3G9/OVA, as well (Fig. 1F). Proliferation of OT-I (Fig. 1D) and OT-II cells (Fig. 1F) was observed even at the lowest concentration of 3G9/OVA tested (0.1 μg/ml, or 0.4 nM), suggesting that efficient presentation via hCD205 does not require a large amount of Ag to be available.

To verify the in vitro function of hCD205 on the cDC1 and cDC2 subsets individually, CD8α− (Fig. 1G) and CD8α+ DCs (Fig. 1H) were isolated by magnetic separation from the spleens of C57BL/6.hCD205 mice and treated with 3G9/OVA or with the indicated control reagents. OT-II CD4+ T cells were added, and T cell proliferation was assessed by BrdU incorporation. We found that both of the cDC subsets could process hCD205-targeted Ags and present them to T cells (Fig. 1G, 1H).

NOD mice with transgenic expression of hCD205 are susceptible to T1D
To generate hCD205-transgenic NOD mice (NOD.hCD205), we backcrossed the C57BL/6.hCD205 mice (18) with NOD mice for 12 generations. To verify that NOD.hCD205 mice remain susceptible to T1D, we performed an incidence study, comparing female NOD.hCD205 mice to their nontransgenic NOD littermates (Fig. 2A). Mice were monitored weekly for glucosuria and were considered diabetic following two consecutive positive tests. The two groups of mice were equally susceptible to T1D, indicating that the transgene does not interfere with the disease process. To ensure that the disease remained of an autoimmune cause, we examined the specificities of T cells cultured from the islets of NOD.hCD205 mice. NOD DCs were treated with the peptides NRP-V7 and YQLENYCAL, which are mimotopes of β cell peptides recognized by 8.3- and AI4-like CD8+ T cells, respectively (19, 28). We found that two out of three mice contained 8.3-like T cells, whereas one out of three contained AI4-like T cells, with one mouse recognizing neither peptide (Fig. 2B). Individual NOD mice display a range of islet T cell specificities (29). Thus, it was both unsurprising and reassuring to see that range reflected in the NOD.hCD205 mice, as well.

APCs from NOD.hCD205 mice develop as is typical for NOD mice
The next step was to ensure that incorporation of the hCD205 transgene had not impacted APC development. Relative amounts of APCs and their maturation status are known to be important in

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**FIGURE 1.** hCD205-transgenic DCs treated with 3G9/OVA can process and present Ag to both CD8⁺ and CD4⁺ T cells.

(A) 3G9/OVA was produced in 293T cells by transient transfection, purified by protein G affinity chromatography, and characterized by SDS-PAGE (lane 1, m.w. standards; lane 2, 3G9/OVA). The H and L chains of 3G9/OVA are indicated. (B) Binding of 3G9/OVA to hCD205 was verified by flow cytometric staining of the indicated cell lines. (C–F) To assess the in vitro function of hCD205, CD11c⁺ DCs from either C57BL/6 (C and E) or C57BL/6.hCD205 mice (D and F) were treated with 3G9/OVA or with the indicated control reagents in a 96-well plate. OT-I CD8⁺ T cells (C and D) (Continued)
TID pathogenesis (13, 30), so we examined the status of several major APC subsets by flow cytometry: monocytes, monocyte-derived DCs (MoDCs), plasmacytoid DCs (pDCs), and CD8α+ and CD8α− DCs (Fig. 3A). Following a previously described strategy (14), we first gated on splenocytes negative for CD3ε, CD19, NKp46, and Ly6G to exclude T cells, B cells, NK cells, and neutrophils, respectively. To examine the pDCs, we gated on CD11c+ Siglec-H+ cells. Next, Siglec-H+ cells were defined based on whether they were CD11chhi or CD11chi. CD11bhi cells were further distinguished based on Ly6C or MHC II expression; CD11bhi Ly6C+ MHC II+ cells were considered monocytes, whereas CD11bhi Ly6C− MHC II+ cells were considered MoDCs. Meanwhile, CD11c− cells were gated on high MHC II expression, and these cells were then separated based on expression of DCIR2 or CD8α. CD11chhi MHC II+CD8α− DCIR2− cells were considered CD8α− DCs, whereas CD11c− MHC IIhi CD8α+ DCIR2+ cells were considered CD8α+ DCs. To confirm the identity of each subset, we examined a number of other markers for each (Fig. 3B). For example, pDCs are known to express CD4 and MHC II (14), which we observe, as well. Additionally, although MoDCs and CD8α− DCs both express CD11c, CD11b, and MHC II, they can be differentiated from each other by the DCIR2 and CD4 expression of CD8α− DCs, which MoDCs lack (14). As expected, only CD8α+ DCs exhibited significant mCD205 expression (14). Interestingly, although the expression of mCD205 in CD8α+ DCs from transgenic mice did not differ from their nontransgenic littermates, NOD mice expressed approximately two times the amount found in the C57BL/6 background (Fig. 3C). Also, C57BL/6 mice had an mCD205− population among their CD8α+ DCs, whereas all NOD CD8α+ DCs expressed mCD205, suggesting that mice of different backgrounds may regulate mCD205 differently.

We used the gating strategy illustrated in Fig. 3A to determine whether the hCD205 transgene had an impact on any APC populations, either in the C57BL/6 or the NOD strains, in terms of the percentage of splenocytes or the absolute number of cells (Fig. 4). No significant differences were found in either percentages or absolute numbers of cells when comparing the transgenic and parent strains. Thus, the hCD205 transgene had no impact on APC development. These experiments did reveal some differences between the C57BL/6 and NOD backgrounds, consistent with previously reported findings (14). We found that there was a significant increase in both percentage and absolute number of pDCs in the NOD background (Fig. 4). However, despite previous reports (14), we did not find significant increases in the percentages and absolute numbers of NOD MoDCs or decreases in CD8α+ DCs, as compared with C57BL/6 mice (Fig. 4).

**APCs from NOD.hCD205 mice have typical maturation statuses for NOD mice**

Although the percentages and absolute numbers of the five APC subsets do not differ between transgenic mice and their parent strains (Fig. 4), it is possible that these strains differ functionally.
FIGURE 3. The presence of hCD205 does not alter APC phenotype.
Splenocytes from C57BL/6 and NOD mice with and without hCD205 (three female mice per group; 15–19 wk old) were analyzed by flow cytometry for expression of APC phenotypic markers including CD11c, CD11b, MHC II, Ly6C, CD8α, and DCIR2. (A) Gating strategy used to identify pDCs, MoDCs, monocytes (Mono), and CD8α+ and CD8α- DCs. (B) Representative histograms for expression of (from left to right) mCD205, CD4, CD11b, CD11c, H2-Aq, and H2-Ab by (top to bottom) CD8α+ DCs, CD8α- DCs, pDCs, MoDCs, and Mono. (C) Relative mCD205 expression on CD8α+ DCs from each mouse strain compared with C57BL/6 mice. Graph depicts mean ± SEM; p values are indicated (t test).

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Knowing that DC maturation can be important to T1D pathogenesis (13, 30), we examined the maturation status of each subset by measuring steady-state expression of several maturation markers, including CD80, CD86, CD40, PD-L1, and class I MHC and MHC II (31, 32). Expression for each strain was compared with C57BL/6 mice, indicating that they remain sensitive to maturation stimuli and can elicit a T cell response. Free peptides most likely displace peptides already present on surface MHC rather than being taken up and loaded onto new peptide–MHC complexes. We therefore next examined the ability of NOD.hCD205 DCs to process and present Ag taken up by endocytosis by using anti-mCD205 that was linked to LPS. CD8+ T cells from either 8.3 or AI4 TCR-transgenic mice were added to these DCs, and their proliferation was assessed (Fig. 6A, 6B). We found that NOD.hCD205 DCs were able to induce proliferation of these cells to the same extent as NOD DCs, whereas CD8+ DCs from NOD mice appear to be more mature.

**NOD.hCD205 mice show reduced expression of hCD205 compared with C57BL/6.hCD205 mice**

The hCD205 transgene is under the control of a synthetic CD11c promoter containing both C57BL/6 and 129 sequences (18). CD8α+ DCs have high CD11c expression, as shown in the above gating strategy (Fig. 3A). However, MoDCs and pDCs have intermediate levels of CD11c expression, as well. Thus, hCD205 may be expressed not only by the former subsets but by the latter two also. We therefore used 3G9/OVA to probe for expression of hCD205 (Fig. 7A). We found that monocytes are not stained with 3G9, an unsurprising result, as they do not express CD11c. Similarly, pDCs, although they do express CD11c, did not have detectable hCD205 expression, consistent with what has been previously found in C57BL/6.hCD205 mice (18). In contrast, MoDCs exhibit high expression of hCD205. As expected, both CD8α+ and CD8α− DCs express hCD205; however, hCD205 expression in NOD.hCD205 mice is only ~25% of that observed in the C57BL/6.hCD205 strain (Fig. 7B). Finally, whereas almost all

status in this study does not support this definition (13, 36, 37). Monocytes from NOD and C57BL/6 mice have equivalent expression of CD80, CD86, CD40, PD-L1, and class I MHC; the same is true for pDCs (Fig. 5B).

In sum, NOD CD8α− DCs, MoDCs, pDCs, and monocytes have a similar maturation status as their C57BL/6 counterparts, whereas CD8α− DCs from NOD mice appear to be more mature. However, it should be noted that each subset was examined at steady state without addition of exogenous maturation stimuli, so although these DCs may have increased expression of maturation markers, they are likely still immature or at least not fully differentiated. Finally, in addition to finding minimal differences between NOD and C57BL/6 APCs, there are no differences when comparing transgenic and nontransgenic mice. We therefore find that APCs from NOD.hCD205 mice develop as is typical for NOD APCs.

**DCs from NOD.hCD205 mice process and present Ags**

As the hCD205 transgene had no detectable impact on APC development or maturation in NOD mice, we next tested the function of NOD.hCD205 CD11c+ DCs. DCs were treated with either NRP-V7 or YQLENVC, peptides recognized, respectively, by the 8.3 and AI4 CD8+ T cell clones (19, 28), and matured with LPS. CD8+ T cells from either 8.3 or AI4 TCR-transgenic mice were added to these DCs, and their proliferation was assessed (Fig. 6A, 6B). We found that NOD.hCD205 DCs were able to induce proliferation of these cells to the same extent as NOD DCs, whereas CD8+ DCs from NOD mice display significantly higher PD-L1 and class I MHC expression than that seen in the C57BL/6 background (Fig. 5B). It would therefore appear that CD8α− DCs from NOD mice are more mature than those of C57BL/6 mice. Given their expression of PD-L1, they may be particularly useful in the generation of T cell tolerance.

Similar to the CD8α+ DC subset, NOD MoDCs do not significantly differ in their expression of any examined maturation marker when compared with C57BL/6 mice (Fig. 5B). Thus, despite a trend toward somewhat higher numbers of this subset in the NOD background (Fig. 4B), these cells have the same maturation status as those found in C57BL/6 mice. This is of interest, as this subset is often defined as inflammatory DCs, whereas we find that their maturation
FIGURE 5. APCs from NOD.hCD205 mice have a similar maturation status as NOD mice.
Expression of maturation markers by splenocytes from C57BL/6 and NOD mice with and without hCD205 (three female mice per group; 15–19 wk old) was investigated by flow cytometry. (A) Representative histograms for expression of (from left to right) CD80, CD86, CD40, PD-L1, and H2-Db by (top to bottom) CD8α+ DCs, CD8α- DCs, pDCs, MoDCs, and monocytes (Mono). (B) Relative expression of the indicated markers by each cell type in the different mouse strains. Expression for each subset was compared with the C57BL/6 strain. Graphs depict mean ± SEM; p values are indicated (t test).
FIGURE 6. CD11c+ DCs from NOD.hCD205 mice are able to process and present Ag to diabetogenic CD8+ T cells.

(A–C) To assess the function of DCs from NOD.hCD205 mice, NOD or NOD.hCD205 DCs were treated with 1 μM NRP-V7, 1 μM YQLENyCAL (AL), or appropriate controls in a 96-well plate. Either 8.3 (A and C) or AI4 (B) CD8+ T cells were added, and T cell proliferation was assessed by BrdU incorporation, which was quantified by ELISA. Graphs display mean absorbance ± SEM at the indicated DC:T cell ratios; each point indicates a mean of three replicates. (A) NOD or NOD.hCD205 DCs and 10,000 8.3 CD8+ T cells per well. (B) NOD or NOD.hCD205 DCs and 10,000 AI4 CD8+ T cells per well. (C) NOD.hCD205 DCs and 20,000 8.3 CD8+ T cells per well.

C57BL/6.hCD205 MoDCs are positive for hCD205, only ~75% of NOD.hCD205 MoDCs express the transgene (Fig. 7A).

Immune cells other than DCs can also express CD11c, including some activated T cells (38–41). For this reason, we examined CD11c+ splenocytes also positive for CD3ε, CD19, NKp46, or Ly6G (designated Lineage+ CD11c+ cells) for expression of the hCD205 transgene in our newly developed NOD.hCD205 mice. No evidence for expression of the transgene was observed (Supplemental Fig. 2A, 2B). When CD8+ and CD4+ T cells were specifically examined, we did not observe any population of cells expressing hCD205, regardless of their activation status (based on CD62L and CD44 expression) or the presence of CD11c (Supplemental Fig. 2C, 2D). Thus, as previously suggested for the C57BL/6.hCD205 mice (18), expression of hCD205 is limited to DCs in NOD.hCD205 mice.

DCs from NOD.hCD205 mice process and present Ag delivered via hCD205 to T1D-relevant T cells

We had thus far established that NOD.hCD205 mice behaved as typical NOD mice in their T1D susceptibility, APC development and maturation, and Ag-presenting abilities. The final step was to ensure that hCD205 functioned in these mice as already shown in the C57BL/6 background (Fig. 1). This was of particular importance, given our finding that hCD205 expression in the NOD.hCD205 strain was reduced relative to that observed in C57BL/6.hCD205 mice (Fig. 7B). For these experiments, 3G9 was linked to YQLENyCAL (3G9/AL), a mimotope peptide recognized by the islet-reactive CD8+ T cell clone AI4 (19) (Fig. 8A). CD11c+ splenic DCs from NOD and NOD.hCD205 mice were treated with 3G9/AL or 3G9/OVA followed by LPS. CD8+ AI4 T cells were then added to the cultures, and T cell proliferation was measured by BrdU incorporation (Fig. 8B). Robust proliferation was observed in the presence of splenic DCs isolated from NOD.hCD205 mice and treated with 3G9/AL. Similar findings were observed when BMDCs were used (Fig. 8C). Thus, despite lower transgene expression in the NOD background, hCD205 expressed by NOD mice is functional. The NOD.hCD205 strain can thus serve as a preclinical model for the evaluation of hCD205-mediated Ag delivery therapeutics.

Development of reagents to modulate the insulin-specific autoimmune response in T1D

Because proinsulin is a critical β cell Ag in T1D (42–45), we developed and characterized proinsulin-linked anti-mCD205 in a proof-of-principle study to determine whether Ab-linked proinsulin could be processed and presented by DCs. Proinsulin 2 (rather than proinsulin 1) was used for these studies because of its closer similarity to hIns. To produce anti-mCD205/Ins2, we cloned the Ins2 cDNA in frame with the C terminus of the H chain of an anti-mCD205 Ab (derived from NLDC-145) in which the mouse IgG1 C region had been mutated to interfere with Fc receptor binding (9). The flexible linker (GGGGS)3 (20) was inserted between the H chain and proinsulin to facilitate proinsulin folding, which we determined was necessary for the expression of the Ab when produced in 293T cells. Anti-mCD205/Ins2 was characterized by SDS-PAGE and showed an increase in the m.w. of the H chain compared to that of bovine γ-globulin due to the attached proinsulin (Fig. 9A). Western blotting with a polyclonal Ab to insulin revealed binding to the H chain of anti-mCD205/Ins2 but not anti-mCD205/OVA (Fig. 9B). Similar results were obtained using an Ab against the C terminus of the insulin A chain (Fig. 9B). Anti-mCD205/Ins2 bound similarly to CHO/mCD205 cells, as did the well-characterized anti-mCD205/OVA (15) (Fig. 9C, left). The presence of proinsulin on the bound Ab was verified using a secondary Ab specific for proinsulin (Fig. 9C, right).

InsB9-23 presented by H2-Aβ7 is a major target of CD4+ T cells in NOD mice, essential for diabetes development (45, 46), and also

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a target of CD4+ T cells in humans when presented by the structurally similar HLA-DQ8 (47–49). We evaluated processing and presentation of InsB9–23 by NOD mouse DCs from anti-mCD205/Ins2 using the cognate T cell hybridoma PCRI-10.18 (27). PCRI-10.18 cells released IL-2 in response to anti-mCD205/Ins2–treated DCs (Fig. 9D), demonstrating that Ab-linked proinsulin epitopes are functionally processed and presented to T cells.

Having found that proinsulin-linked anti-mCD205 could be produced in vitro and that proinsulin-derived T cell epitopes could be processed and presented when DCs were treated with anti-mCD205/Ins2 (Fig. 9D), the same strategy was used to link Ins2 to the C terminus of the H chain of the human anti-hCD205 Ab 3G9 (18). The resulting anti-hCD205/Ins2 was characterized by SDS-PAGE (Fig. 9E, left) and was found to bind similarly to CHO cells modified to express hCD205, as did anti-hCD205/OVA (Fig. 9E, right). hIns was similarly linked to the C terminus of the H chain of anti-hCD205, and anti-hCD205/hIns was successfully produced in 293T cells (Fig. 9F, left). We verified that, as expected, the presence of hIns did not disrupt the binding of the Ab to hCD205 (Fig. 9F, right). The availability of this reagent elevates the potential translational impact of this Ag delivery strategy.

**DISCUSSION**

Autoreactive T cells are responsible for the destruction of the insulin-producing β cells, leading to the development of T1D (1). One potential strategy for preventing disease is to inhibit the activation of, and/or induce tolerance in, these autoreactive T cells (5, 6). As both CD4+ and CD8+ T cells are required for T1D to occur (1, 50), it will likely be necessary to inhibit the function of both of these T cell subsets. Under steady-state conditions, DCs can present Ag to both CD4+ and CD8+ T cells in a tolerogenic manner, resulting in T cell deletion, anergy, and/or the induction and expansion of regulatory T cells (5, 6). This, therefore, represents an attractive method for preventing the pathogenicity of autoreactive T cells in T1D. Previous work in the NOD mouse has shown that Ags can be delivered to the CD8α+ or CD8α- DC subsets by linking an Ag to anti-mCD205 (8–10, 51) or anti-DCIR2 (11), respectively. In these cases, CD8α+ DCs induced tolerance in CD8+ T cells (8, 9), and CD8α- DCs did the same for CD4+ T cells (11). The ability of CD8α+ DCs to induce CD4+ T cell tolerance in this strain is less clear, as there are conflicting reports in the literature on this point (10, 51). Other studies, using Ag delivery without DC targeting, were also successful in impacting the disease process in NOD mice; however, such studies have failed to effectively translate to humans (4). By improving the approach, both by delivering Ag directly to DCs and by using a more clinically relevant model system, we can

**FIGURE 7.** CD8α+, CD8α−, and MoDCs from NOD.hCD205 mice express hCD205. 
(A) Expression of hCD205 by splenocytes from C57BL/6 and NOD mice with and without hCD205 (three female mice per group; 15–19 wk old) was analyzed by flow cytometry. (B) hCD205 expression by splenic DCs, CD8α+, CD8α−, and MoDCs from C57BL/6 (white) and NOD (black) hCD205-transgenic mice. Graph depicts mean MFI + SEM; p values are indicated (t test).

CD8α+, CD8α−, and MoDCs from C57BL6 (white) and NOD (black) hCD205-transgenic mice. Graph depicts mean MFI + SEM; p values are indicated (t test). Mono, monocytes.
improve the likelihood that the treatment will translate from mice to humans.

To that end, we have developed an NOD-based mouse model for T1D having transgenic expression of hCD205. Aside from the very fact that we focus on hCD205 rather than on murine endocytic receptors, there are several reasons to expect this model to have a higher translational potential than the DC-targeted treatments mentioned above. Whereas in mice, CD205 is expressed only by the CD8α+ subset, hCD205 is expressed by both the BDCA-1+ and the BDCA-3+ DC subsets in humans (13, 52). Although less studied than the murine DC subsets, BDCA-1+ DCs are thought to parallel the function of the murine CD8α2 subset, whereas BDCA-3+ DCs may serve the same function as the murine CD8α+ subset (13). The two DC subsets are believed to be specialized for presentation to either CD4+ or CD8+ T cells, respectively, although neither DC subset is limited to presentation to one type of T cell (15). CD205 can serve an important practical role in this context, as Ag endocytosed by human DCs via CD205 can be processed and presented on MHC II, as is typical for extracellular Ags; CD205 is also able to mediate cross-presentation, with the result that peptides can be presented on class I MHC, as well (52, 53). As a result, Ag delivered by anti-CD205 can impact both CD4+ and CD8+ T cells, both of which will likely need to be targeted for effective T1D therapy (1, 50). A drawback of using mCD205 targeting as a model system is that CD8α+ DCs may not be able to present to CD4+ T cells effectively, whereas the CD8α− subset lacks mCD205 (13, 15). This divide is not an issue in the case of human DCs, however, and CD205, therefore, is an ideal endocytic receptor for the targeting of both DC subsets. Furthermore, using one reagent to target multiple subsets is an improvement over needing to use a different reagent for each subset. In humans, BDCA-3+ DCs express ∼2-fold higher CD205 on their surface than do BDCA-1+ DCs (52). Interestingly, in the hCD205-transgenic C57BL/6 and NOD mice, the CD8α− DC subset trended toward enhanced surface expression of hCD205 compared with the CD8α+ subset (Fig. 7B; p = 0.206 and 0.131 for the C57BL/6 and NOD strains, respectively).

In developing the NOD.hCD205 model, we examined whether the transgene had any impact on T1D or on the status of APCs. We found that NOD.hCD205 mice had a T1D incidence indistinguishable from that of NOD littermates and contained AI4-like and 8.3-like T cells in their islet infiltrate. We further found that the presence of the transgene had no impact on the percentages or absolute numbers of any examined APC subset, including monocytes, MoDCs, pDCs, and CD8α+ and CD8α− DCs, nor was there any impact on maturation status. In all cases, APCs from NOD.hCD205 mice were indistinguishable from those from NOD mice; the same can be said when comparing C57BL/6.hCD205 mice to their nontransgenic counterparts. As expected, the amounts of several subsets differed when comparing the C57BL/6 and NOD backgrounds (14).

There was, however, one surprising result. We found that, in the NOD background, CD8α+ and CD8α− DCs had reduced surface hCD205 expression compared with transgenic C57BL/6 mice. This was despite a trend toward increased expression of endogenous CD11c in the NOD strain (Supplemental Fig. 3A).
FIGURE 9. Proinsulin delivered to DCs via CD205 can be processed and presented to T cells.

(A) Anti-mCD205/Ins2 was produced in 293T cells by transient transfection and purified from the culture supernatant. SDS-PAGE analysis was performed under reducing conditions (lane 1, m.w. standards; lane 2, bovine γ-globulin; lane 3, anti-mCD205/Ins2; lane 4, anti-mCD205/OVA). The H and L chains of anti-mCD205/Ins2 are indicated. (B) Western blot analysis using a polyclonal guinea pig anti-insulin Ab (lanes 1–3) or a polyclonal goat anti-insulin A chain Ab (lanes 4–6). Lanes 1 and 4, anti-mCD205/OVA; lanes 2 and 5, anti-mCD205/Ins2; lanes 3 and 6, bovine γ-globulin. (C) CHO/mCD205 cells were stained with the indicated Abs, followed by a labeled anti-IgG (left) or an anti-proinsulin (right) secondary Ab. (D) Splenic DCs from NOD mice were treated with the indicated peptides or Abs and then cocultured with PCR1-10.18 cells for 24 h. T cell response was quantified by IL-2 ELISA of culture supernatants. Graph depicts mean ± SD of two independent experiments; p values are indicated (t test). (E) 3G9/Ins2 was produced in 293T cells by transient transfection, purified using protein G, and analyzed by SDS-PAGE. Left, lane 1, m.w. standards; lane 2, 3G9/Ins2; lane 3, bovine γ-globulin. The H and L chains of 3G9/Ins2 are indicated. Right, CHO/hCD205 cells were stained with
The hCD205 transgene is under the control of a synthetic CD11c promoter (GenBank accession number: DQ6588851) containing both C57BL/6 and 129 sequences (18). When we compared the synthetic CD11c promoter sequence with the corresponding regions of the C57BL/6 and NOD genomes using Ensembl (54), only limited variation was observed for either strain, suggesting that the reduction in hCD205 expression in the NOD strain is unlikely to be due to reduced promoter activity. Additionally, to be sure that the hCD205 transgene had not undergone some alteration in the NOD mice that led to reduced surface expression of the protein, we crossed hemizygous transgene-positive C57BL/6 mice with transgene-negative NOD mice. Similar to hemizygous NOD mice, the transgene-positive F1 progeny showed a reduced level of surface hCD205 expression when compared with hemizygous C57BL/6 mice (Supplemental Fig. 3B). These results suggest that surface expression of hCD205 in hCD205 is under the control of one or more genes that differ between C57BL/6 and NOD mice and that the transgene in our NOD colony has not undergone an untoward alteration leading to reduced transcription. This finding could be related to the observations that APC populations from autoimmune-prone mice and humans can show alterations in phenotype and/or function when compared with control individuals (30). In any event, the critical point is that despite a decrease in expression, hCD205 still allows for processing and presentation of Ag delivered via 3G9 in the transgenic NOD strain, validating its potential as a preclinical model.

We envision a number of future uses for the NOD.hCD205 mouse model. Previous DC-targeted treatment studies have largely focused on the delivery of β cell mimotopes to either CD8α− or CD8α−DCs to specifically target CD8+ or CD4+ T cells (8, 9, 11). In our model, in which both subsets express hCD205, it would be ideal to deliver a hCD205-linked Ag that includes epitopes relevant to both CD4+ and CD8+ T cells. Proinsulin is the most appropriate Ag choice, as establishment of tolerance to insulin can lead to prevention of TID (42, 43), and remission of established disease (55), in NOD mice. Indeed, studies in mice suggest that it may be the long-sought-after initiating Ag in TID (42–45). Furthermore, in humans, the non-MHC locus that confers the strongest susceptibility to TID is the insulin gene regulatory region (56), and disease-associated alleles are correlated with reduced thymic expression (57, 58). Finally, CD4+ and CD8+ T cell responses to insulin are observed in TID patients (59–62), in whom the reduced thymic insulin expression correlates with impaired negative selection of high-avidity insulin-specific T cells (63). Inducing a tolerogenic response to insulin is, therefore, a primary goal of the DC-targeted therapies that can now be developed using the NOD.hCD205 mice.

We have shown that Ins2 and hIns can be linked to CD205-specific Abs and readily purified from 293T cell supernatants. 3G9/Ins2 will be of immediate use in investigations in which we will examine the impact of treatment with this Ab on TID incidence, T cell tolerance, and/or deletion of insulin-specific T cells. Of particular interest will be tracking the presence of particular T cell clones. The latter will be possible through the use of peptide–MHC tetramers; the epitopes of a number of insulin-specific T cell clones have been identified, including the CD8+ T cell clones A14 and G9C8 (19, 64). Also of interest is the impact the prevention of anti-insulin reactivity might have on non–insulin-specific T cells. If insulin is important as an initiating Ag and anti-insulin attack is prevented, we may also see a decrease in the population of T cells specific for other β cell Ags. Disease prevention and reversal studies with 3G9/Ins2 will require consideration and optimization of multiple parameters, including dose, timing, and frequency of administration. Finally, we have developed anti-hCD205 linked to hIns. By crossing our NOD.hCD205 mice with mice transgenically expressing human insulin (65) and treating them with 3G9/hIns, we can further improve the translational impact of our model.

In sum, we have generated and characterized a mouse model of TID that incorporates the transgenic expression of hCD205. These mice represent a unique, well-characterized, and useful tool for the preclinical development and optimization of DC-directed TID therapies. Of note, 3G9 linked to the tumor Ag NY-ESO-1 was recently tested in humans for its ability to elicit an immune response when administered along with an adjuvant to induce DC activation, and it was found to be effective and safe (66). This strategy was first tested for safety and efficacy using C57BL/6.hCD205 as the preclinical model (18). Related approaches to combat TID will use Ag-linked Abs without such an adjuvant because the desired outcome will be tolerance. Nonetheless, this precedent for the use of Ag-linked anti-CD205 in humans, coupled with the availability of our well-characterized NOD.hCD205 mouse strain, should facilitate the translation of 3G9/hIns and related reagents to clinical practice.

DISCLOSURES

L.-Z.H. and T.K. are employees and shareholders of Celldex Therapeutics. The other authors have no financial conflicts of interest.

3G9/Ins2 or 3G9/OVA followed by a labeled anti-human IgG secondary Ab. Binding to hCD205 was assessed by flow cytometry. 3G9/hIns was produced in 293T cells, and the purified Ab was analyzed by SDS-PAGE. Left, lane 1, m.w. standards; lane 2, 3G9/hIns; lane 3, bovine γ-globulin. The H and L chains of 3G9/hIns are indicated. Right, 3G9/hIns was added to CHO/hCD205 cells, and binding was assessed by flow cytometry using a labeled anti-human IgG secondary Ab.
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