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The Cross-Priming Capacity and Direct Presentation Potential of an Autoantigen Are Separable and Inversely Related Properties

Jinguo Wang,*,†,1 Roopa Hebbandi Nanjundappa,*,†,1 Afshin Shameli,*,† Xavier Clemente-Casares,*,† Jun Yamanouchi,*, John F. Elliott,‡,§,*, Robyn Slattery,‖ Pau Serra,‡ and Pere Santamaria*†,‡,#

We investigated whether a prevalent epitope of the β-cell–specific autoantigen islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP206–214) reaches regional Ag-presentation pathways via unprocessed polypeptide chains, as free IGRP206–214 Peptide or via preformed IGRP206–214/Kd complexes. This was accomplished by expressing bacterial artificial chromosome transgenes encoding wild-type (stable) or ubiquitinated (unstable) forms of IGRP in IGRP-deficient NOD mice carrying MHC class I–deficient β-cells, dendritic cells, or B cells. We investigated the ability of the pancreatic lymph nodes of these mice to prime naïve IGRP206–214–reactive CD8+ T cells in vivo, either in response to spontaneous Ag shedding, or to synchronized forms of β-cell necrosis or apoptosis. When IGRP was made unstable by targeting it for proteasomal degradation within β-cells, the cross-priming, autoimmune-initiating potential of this autoantigen (designated autoantigenicity) was impaired. Yet at the same time, the direct presentation, CTL-targeting potential of IGRP (designated pathogenicity) was enhanced. The appearance of IGRP206–214 in regional Ag-presentation pathways was dissociated from transfer of IGRP206–214 or IGRP206–214/Kd from β cells to dendritic cells. These results indicate that autoantigenicity and pathogenicity are separable and inversely related properties and suggest that pathogenic autoantigens, capable of efficiently priming CTLs while marking target cells for CTL-induced killing, may have a critical balance of these two properties. The Journal of Immunology, 2014, 193: 3296–3307.

Autoimmune diseases such as type 1 diabetes (T1D) result from tissue-specific or systemic autoimmune responses against self-antigens. The autoimmune response leading to T1D in both humans and NOD mice is effected by T cells recognizing many autoantigens (1, 2).

CD8+ T cells are recognized as important effectors of diabetogenic autoimmunity (3). A population of CD8+ T cells recognizing an insulin-derived epitope (B:15–23) appears in the islets of NOD mice as early as at 3 wk (4), albeit in small numbers and accompanied by a much larger contingent of CD8+ T cells of unknown specificity (5). The size of this population declines quickly with age and is replaced by other specificities, primarily a subset of CD8+ T cells that use CDR3-invariant Vα17/Jα42+ TCRs (6–9). We have shown that the latter are highly diabetogenic (6, 7) and target IGRP206–214 (10). Remarkably, these T cells are prevalent in the peripheral repertoire, particularly as clinical disease nears (11). Nevertheless, islet-associated CD8+ T cells in T1D target numerous other islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP) epitopes (12), indicating that IGRP is a prevalent source of diabetogenic epitopes.

Understanding what makes certain β-cell proteins, like IGRP, preferential targets of autoreactive CD8+ T cells remains a fundamental gap in our knowledge. We reasoned that the dominance of IGRP in T1D might be related to its molecular nature, intra-cellular location, or both. IGRP is a highly hydrophobic transmembrane protein that contains an endoplasmic reticulum (ER) retention sequence and is buried in the ER membrane (13). Because: 1) cross-presentation of viral Ags to naïve CD8+ T cells is primarily driven by capture of intact viral proteins (as opposed to processed peptides) by dendritic cells (DCs) (14–16); 2) initiation
of T1D is preceded by a neonatal wave of β-cell apoptosis (17); and 3) capture of apoptotic cells by DCs facilitates cross-presentation of cellular Ags (2), we reasoned that IGRP’s autoantigenicity might be associated with its ER location, which would both protect it from proteolytic cleavage and promote its delivery to DCs via apoptotic bodies. In contrast, >30% of proteins are degraded by proteasomes shortly after synthesis, presumably because they do not fold properly, and a large fraction of peptide–MHC (pMHC) class I complexes in most cells originate from this pool (referred to as defective ribosomal initiation products [DRiPs] or ribosomal degradation products [RDPs]) (18–21). This implies that antigenic stability, although promoting access to DCs (and cross-presentation), would impair the efficiency of pMHC formation in β-cells and thus reduce their susceptibility to killing by the primed CTLs (direct presentation). Thus, it remains unclear to what extent the rules that govern the presentation (direct and indirect) of viral Ags apply to the presentation of cellular autoantigens. Recently, we have used a mathematical model to analyze the contribution of antigenic stability to autoantigenicity and pathogenicity and hypothesized that autoantigens that are too stable would efficiently cross-prime T cells but would yield too few target pMHCs in β-cells, whereas those that are too unstable would be inefficient at priming naïve T cells, but would yield high levels of target pMHCs in β-cells (22). This line of thinking predicts that IGRP’s molecular nature may be responsible for defining an optimal ratio of stable conformers (contributing to the pool of pMHCs that are cross-presented by DCs to naïve T cells in the pancreatic draining lymph nodes [PLNs]) and DRiPs/RDPs (contributing to the pool of pMHCs that are directly presented by the target β-cells to CTLs). Unfortunately, experimental evidence for this hypothesis is lacking. In fact, recent evidence suggests that the prevalent insulin epitope targeted by autoreactive CD4+ T cells in NOD mice is generated directly in β-cells and presented to cognate autoreactive CD4+ T cells in situ (23, 24), suggesting that protein stability is necessary for autoantigenicity of MHC class II–restricted epitopes. Whether IGRP predominance in T1D is associated with unusual stability, an unusually high degree of DRiP/RDP formation, an appropriate balance between the two, or completely dissociated from these parameters is unknown. Likewise, it is unclear to what extent β-cell–derived IGRP206–214 peptide and/or β-cell–assembled IGRP206–214/Kd class I complexes contribute to productive activation and recruitment of autoreactive CD8+ T cells in T1D.

In this study, we investigate these issues by comparing the autoantigenicity and pathogenicity of wild-type and unstable forms of IGRP encoded in bacterial artificial chromosomes (BACS) expressed in G6pc2 and b2m gene-targeted (IGRP206–214 and b2m–microglobulin [b2m] double-deficient) NOD mice coexpressing a loxP-flanked b2m transgene and various cell-specific Cre transgenes. We demonstrate that IGRP206–214 peptides arising from the stable (wild-type) form of IGRP are significantly more effective at priming naïve autoreactive CD8+ T cells in the pancreas-draining lymph nodes, as compared with those arising from a form of IGRP that is fused to ubiquitin and is therefore targeted for proteasomal degradation. This phenomenon holds, regardless of the form of β-cell death. Conversely, we find that the destabilized IGRP is significantly more effective than the wild-type protein at promoting both pMHC class I formation in target cells and at marking β-cells for CTL-induced β-cell killing. As a result, mice selectively expressing the unstable form of IGRP experience higher rates of CTL-induced β-cell death and Ag shedding than those expressing wild-type IGRP, despite being less efficient at driving the activation and recruitment of cognate autoreactive CD8+ T cells. In addition, we demonstrate that cross-priming of IGRP206–214-reactive CD8+ T cells in the PLNs is dissociated from β-cell–derived MHC class I molecules, is selectively driven by CD11c+ cells with a negligible role for B cells or CD11b+ cells, and requires the expression of MHC class I molecules by the CD11c+ cells. Taken together, these data indicate that the cross-priming capacity and pathogenic potential of IGRP206–214 are separable and inversely related properties and suggest that the pathogenicity of an autoantigen may be a function of the balance between its ability to feed the cross-presentation pathway and mark target cells for CTL-mediated lysis.

Materials and Methods

**Mice**

NOD.LtJ, NOD.LtJ-TgIns2-TAg (28) (NOD.CD11c-DTR), and NOD.FVB-Tg(Igax-DTR/EGFP) 34Lan/Jdk/J (NOD.CD11c-DTR), and NOD.FVB-Tg(Igax-DTR/EGFP) 57Lan/Jdk/J (NOD.CD11c-DTR) mice were from The Jackson Laboratory (Bar Harbor, ME). 8.3–NOD (7), NOD.G6pc2KdKd (25), human insulin promoter (HIP)-Cre+ and HIP-Cre− NOD.BGmEPro/Pdkh2m−/− (26, 27), NOD.CD19-Cre, and NOD.CD11c-Cre mice (28) have been described. BAC-transgenic mice were produced by direct microinjection into NOD zygotes. To produce H2-Kd− negative 129/NOD.G6pc2KdKd mice, we intercrossed Ins2-Tag−transgenic, G6pc2KdKd.H2Kd−/− mice to generate Ins2−Tag−transgenic, 129/NOD.G6pc2KdKd.H2Kd−/− homozygotes. NOD. RIP-DTR mice were produced by backcrossing a rat insulin promoter (RIP)-HB-EGF receptor transgene (29) onto the NOD background for >10 generations. All other strains described in this study were produced by intercrossing and selecting progeny for the desired genotypes by allele-specific PCR. These studies were approved by the Faculty of Medicine’s Animal Care Committee and followed the guidelines of the Canadian Council of Animal Care.

**Proteosomal cleavage site prediction**

Prediction of proteosomal cleavage sites was done using NetChop3.1, setting the threshold at 0.9699 to identify preferential target sites (http://www.cbs.dtu.dk/services/NetChop/).

**Islet isolation and collection of islet-associated CD8+ T cells**

Pancreatic islets were isolated by hand-picking after collagenase P digestion of pancreas, cultured overnight in RPMI 1640 media supplemented with 10% FBS and 0.5 U/ml recombinant human IL-2 (Takeda), and disrupted into single cells.

**Bone marrow–derived DCs and splenic B cells**

Bone marrow–derived DCs were prepared by culturing hind leg bone marrow cell clumps in complete media supplemented with recombinant murine GM-CSF (BD Biosciences, San Jose, CA) and recombinant murine IL-4 (R&D Systems, Minneapolis, MN) (5 ng/ml each) for 6 to 7 d. DCs and B cells were purified with anti-CD11c– and anti-B220–coated beads, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany).

**Insuloma cell lines**

Insuloma cell lines were established from H-2Kd−negative 129/NOD.G6pc2KdKd.Jns2-TAg mice essentially as described previously (30).

**H-2Kd transfection**

HEK cells were transfected with 2 μg pcK-H2-Kd using Lipofectamine following the manufacturer’s protocol (Life Technologies). The transfected cells were stained with anti-Kd–PE mAb (BD Biosciences), FACS sorted, and used for downstream experimentation.

**Lentivirus packaging and transduction**

IGRP-EGFP or ubiquitin (Ub)-IGRP-EGFP genes were cloned into pLV-CMV. The third-generation packaging plasmids pRRE, pCMV-VSV-G, and pR8/SREV were cotransfected into 293T cells using JetPrime (Polyplus Transfection). Supernatants were collected at 16, 40, and 64 h of posttransfection, pooled, centrifuged to remove cells and debris, and subjected to ultracentrifugation at 25,000 rpm for 2 h at 18°C. The pellet was resuspended in PBS and centrifuged at 10,000 rpm for 1 min to remove residual debris. HEK and insuloma cells (104) were transduced with plv-IGRP-EGFP or pLV-Ub-IGRP-EGFP in the presence of 4 μg/ml polybrene and cultured at 37°C, 5% CO2. Enhanced GFP (eGFP)-expressing cells were FACS sorted and expanded.
Flow cytometry

Cells were stained in FACS buffer (0.1% sodium azide and 1% FBS in PBS) at 4°C for 30 min in the dark, washed, and analyzed immediately or fixed with 1% paraformaldehyde until analysis. We used the following mAbs: anti-CD8a–PerCP, PE, or FITC (53.6.7), anti-CD4–PE or FITC (GK1.5), and anti-H-2Kd–PE (SF1-1-1.1) (BD Biosciences). In other experiments, lentivirally transduced HEK or insulinoma cell lines or endogenous islet cells from transgenic mice were examined for eGFP fluorescence after a 4-h incubation with the proteasome inhibitor MG132 (8 μM) dissolved in DMSO or with DMSO alone.

In vitro direct presentation assays

Splenocytes from 8.3-NOD mice (10^5/well) were cultured in the presence of 10 μg/ml NRP-V7 for 3 d. CD8+ T cells were then purified from the 3-d cultures using anti-CD8-coated magnetic beads or BD IMag anti-mouse CD8a particles-DM (BD Biosciences). The CD8+ T cells (2 × 10^5) were then cultured in U-bottom 96-well plates in the presence of 1 μg/ml TUM or NRP-V7 (as negative and positive controls, respectively) or with irradiated HEK cells expressing or lacking H-2Kd and Ub-IGRP-eGFP or IGRP-eGFP fusions (2 × 10^5 cells/well) for 30 at 37°C, in the absence or presence of the proteasome inhibitor MG132 (8 μM). The supernatants were assayed for IFN-γ by ELISA using the DuoSet ELISA development system (R&D Systems).

In vivo indirect presentation assays

Cell cultures were trypsinized, subjected to three rounds of freeze/thawing, and injected into NOD.G6pc<sup>-<sup>+</sup>ki</sup> hosts prior to (Figs. 1F, 2D, 2E, left panel) and/or after a 4-h incubation with MG132 (10^5 cells into the right footpad, Fig. 2E, right panel), or 10–15 × 10^6 cells s.c. between the scapulae, Figs. 1F, 2D). The hosts were transfused with 10^7 CFSE-labeled 8.3 CD8+ T cells 24 h later as described in the section below. The mice were sacrificed 7 d later to measure the extent of CFSE dilution in the indicated lymphoid organs via flow cytometry.

In vivo studies of Ag-induced autoreactive CD8+ T cell proliferation

Splenic 8.3-CD8+ T cells were purified from 8.3-NOD.G6pc<sup>-<sup>+</sup>ki</sup> donor mice using BD IMag anti-mouse CD8 particles-DM (BD Biosciences), labeled with 2.5 μM CFSE (Molecular Probes, Eugene, OR), and 10^7 cells were injected i.v. into different host types. In Figs. 4A–C and 7B, the hosts were killed 7 or 14 d after T cell transfer, and their spleens, PLNs, mesenteric draining lymph nodes (MLNs), and islets were examined for presence of donor CD8+ T cells and for dilution of CFSE in the CD8+ gate. In other experiments (Figs. 2E, 4D, 4E, 6B, 6C, 7B), the hosts were treated, with alloxan (Sigma-Aldrich, St. Louis, MO) at the day of T cell transfer, with alloxan (Sigma-Aldrich, St. Louis, MO) at the day of T cell transfer, and then cultured in U-bottom 96-well plates in the presence of 1 μg/ml TUM or NRP-V7 (as negative and positive controls, respectively) or with irradiated HEK cells expressing or lacking H-2Kd and Ub-IGRP-eGFP or IGRP-eGFP fusions (2 × 10^5 cells/well) for 30 at 37°C, in the absence or presence of the proteasome inhibitor MG132 (8 μM). The supernatants were assayed for IFN-γ by ELISA using the DuoSet ELISA development system (R&D Systems).

Real-time RT-PCR analysis

RNA was extracted from 50–150 islets from transgenic lines using RNeasy kit (Qiagen). DNase I–treated RNA was used in the quantitative RT-PCR reaction with TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA) using eGFP probe (6FAM-5′-CGGATCA-CATGCTGCTGTCT-3′ TAMRA), eGFP forward primer (5′-AGCAGAACGA-CCTCGACAGAA-3′), eGFP reverse primer (5′-GGGCGCGGCGAAGC-3′), and GAPDH TaqMan Rodent GAPDH Control Reagents (VIC probe) (Applied Biosystems) (1 cycle: 48°C, 30 min; 1 cycle: 95°C, 10 min; 40 cycles: 95°C, 15 s and 60°C, 1 min).

Confocal microscopy

Wild-type and transduced HEK and insulinoma cells were fixed with PermFix (BD Biosciences), stained with rabbit anti-calnexin Abs (Abcam; 1:300), followed by donkey Cy3-conjugated anti-rabbit Abs (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:500), mounted with ProlongGold/DAPI (Life Technologies), and examined under a confocal microscope.

Fluorescence microscopy

To examine pancreatic islets for GFP expression, pancreata were embedded in OCT medium and frozen in a dry ice/acetone bath. Frozen pancreatic sections (5 μm) were fixed with 2% paraformaldehyde in PBS, washed twice with PBS, mounted in Aqua-Mount (Lerner Laboratories, Pittsburgh, PA), and analyzed with a Delanyvision microscope system (Applied Precision, Issaquah, WA). Analysis of GFP mean fluorescence intensities was performed with Velocity software.

Western blotting

A total of 400 isolated islets from transgenic lines was lysed using 2% SDS-loading buffer and electrophoresed in 12% SDS-PAGE, incubated overnight at 4°C with mouse anti-GFP mAb (1/1000) (clone JL-8; Clontech Laboratories, Mountain View, CA), washed, incubated with HRP-labeled donkey anti-mouse secondary Ab (1/10,000; Jackson ImmunoResearch Laboratories) for 1 h at room temperature, and developed using Super Signal West Pico chemiluminescent substrate kit (Thermo Scientific, Rockford, IL).

Insulitis scores

Pancreata were fixed in formalin, embedded in paraffin, sectioned at 5 μm, stained with H&E, and examined for insulitis as described (7).

Diabetes

Diabetes was monitored by measuring urine glucose level using Diastix (Bayer Inc., Ontario, Canada). Animals were considered diabetic after 2 consecutive d of glucosuria.

Statistical analysis

Data were compared using log-rank, χ², or Mann–Whitney U tests. Statistical significance was assumed at p < 0.05.

Results

Cross-presented IGRP<sub>206–214</sub> arises from unprocessed IGRP in donor cells, with minimal contribution of preformed IGRP<sub>206–214</sub> or IGRP<sub>206–214/K<sup>d</sup></sub> complexes

We first sought to compare the ability of stable and unstable forms of IGRP to yield IGRP<sub>206–214/K<sup>d</sup></sub> complexes in H-2K<sup>d</sup>–transfected HEK cells. To this end, we transfected H-2K<sup>d</sup>–transfected HEK cells with lentiviruses encoding IGRP-eGFP (IGRP-eGFP) or a Ub-IGRP-eGFP that targets IGRP-eGFP for quick proteasomal degradation (Fig. 1A). The Ub-IGRP fusion design complies with the N-end rule for ubiquitin-dependent degradation of proteins, which requires the conjugation of Arg to their N termini (20, 31). Proteasome degradation of substrates generated by debiquitinating enzymes has been shown to play a key role in DRIP/RDP Ag processing (32), and ubiquitin-dependent degradation of ER-targeting proteins plays a major role in MHC class I–restricted Ag processing (33). Analysis of predicted proteasomal cleavage sites revealed a significant clustering of sites at the IGRP-eGFP junction (Fig. 1B), suggesting that cells expressing either form of IGRP should express eGFP fluorescence, either from the ER membrane-targeted form (IGRP-eGFP) or from eGFP released by proteasomal cleavage. Indeed, cells transfected with theUb-IGRP-eGFP fusion expressed significantly lower levels of eGFP fluorescence than cells expressing its IGRP-eGFP counterpart (Fig. 1C, 1D), and such differences disappeared after incubation of the cells in the presence of the proteasome inhibitor MG132 (Fig. 1C). Confocal microscopy analysis of MG132-treated cells stained with anti-calnexin Abs confirmed these results and excluded effects of the proteasome inhibitor on subcellular localization of either form of eGFP-tagged IGRP (data not shown; also see further below). Induction of apoptotic cell death led to the formation of apoptotic cell bodies in which nuclear material was enveloped by eGFP–containing membrane only in cells expressing IGRP-eGFP but not in cells expressing Ub-IGRP-eGFP (Fig. 1D and data not shown).

We next compared the ability of nontransfected and H-2K<sup>d</sup>–transfected HEK cells expressing IGRP-eGFP or Ub-IGRP-eGFP to process and present IGRP<sub>206–214/K<sup>d</sup></sub> complexes in vitro. Flow cytometric analyses of H-2K<sup>d</sup>–HEK cells expressing either fusion confirmed that they expressed similar levels of total H-2K<sup>d</sup> on the cell surface (Fig. 1E, top panel).
In vitro–differentiated 8.3-CTLs produced significantly higher levels of IFN-γ in response to H-2Kd+ HEK cells expressing either IGRP-eGFP fusion type than in response to cultures pulsed with the negative control (the Kd-binding peptide TUM) or unpulsed H-2Kd+ HEK cells (Fig. 1E, middle panel). Notably, H-2Kd+ HEK cells expressing the Ub-IGRP-eGFP fusion had significantly superior activity in these assays, reaching the levels of IFN-γ secretion induced by cultures of 8.3-CTLs pulsed with 1 μg/ml of the superagonist NRP-V7 peptide (negative and positive controls, respectively, both Kd binding). Bottom panel, Secretion of IFN-γ by 8.3-CTL challenged with Kd+ HEK cells, Kd+ HEK cells expressing Ub-IGRP-eGFP or IGRP-eGFP fusions, or 1 μg/ml of TUM or NRP-V7 peptides (negative and positive controls, respectively, both Kd binding). (F) Proliferation of naïve CFSE-labeled 8.3-CD8+ T cells in the draining (axillary [Ax]) versus nondraining (MLN) LNs of NOD mice injected s.c. between the scapulae with dead H-2Kd+ or H-Kd2 HEK cells expressing either IGRP fusion. Data correspond to the following number of mice/cell type: HEK, n = 11; HEK-IGRP-eGFP, n = 10; HEK-Ub-IGRP-eGFP, n = 5; HEK-Kd+-IGRP-eGFP, n = 7; and HEK-Kd+-Ub-IGRP-eGFP, n = 3. Data were collected in one to six experiments per condition and compared by Mann–Whitney U test.

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was the case for HEK transfectants, insulinoma cells expressing IGRP-eGFP expressed significantly higher eGFP fluorescence than insulinoma cells expressing Ub-IGRP-eGFP, despite expressing similar levels of transgenic mRNA as evaluated by quantitative PCR (Fig. 2B, 2C and data not shown). In addition, these differences in eGFP expression disappeared upon a 4-h incubation of the cells in the presence of MG132 (Fig. 2B). As was the case with HEK cells, confocal microscopy analysis of MG132-treated insulinoma cells stained with anti-calnexin Abs confirmed the flow cytometry results shown in Fig. 2B and excluded effects of the proteasome inhibitor on subcellular localization of either form of eGFP-tagged IGRP (Fig. 2C).

An s.c. injection (between the scapulae) of insulinoma cell lysates into NOD.G6pc2^{KIKI}/KI/KI mice transfused with naive CFSE-labeled 8.3-CD8^{+} T cells from 8.3-NOD.G6pc2^{KIKI} donors confirmed that cells expressing IGRP-eGFP were significantly more...
effective than those expressing Ub-IGRP-eGFP at delivering Ag for cross-presentation of IGRP$_{206-214}$Kd to 8.3-CD8$^+$ T cells in vivo (Fig. 2D). A similar outcome was seen when these insulinoma cells were killed by exposure to alloxan and then injected into the right footpad of NOD.G6pc2$^{KdKd}$ hosts transfused with CFSE-labeled 8.3-CD8$^+$ T cells (Fig. 2E, *top and bottom left panels*). In this study, too, incubation of these insulinoma cell lines with MGI32 for 4 h prior to alloxan exposure and injection into the footpad abrogated the above differences in their ability to trigger the proliferation of exogenous 8.3-CD8$^+$ T cells in vivo (Fig. 2E, *bottom right panel*).

Together, these data indicate that, unlike stable forms of IGRP, its proteolytic fragments (including IGRP$_{206-214}$) or pre-formed IGRP$_{206-214}$/Kd complexes cannot effectively access the cross-presentation pathway in vivo.

**Transgenic NOD mice expressing stable versus unstable forms of IGRP**

We next sought to compare the ability of stable and unstable forms of transgenic IGRP to trigger the activation, recruitment, and β-cell cytotoxic activity of IGRP$_{206-214}$reactive CD8$^+$ T cells in vivo. To this end, we created NOD transgenic lines using BACs encoding the IGRP-eGFP (BAC-IGRP-eGFP) or Ub-IGRP-eGFP (BAC-Ub-IGRP-eGFP) (Fig. 3A, Supplemental Table I). We note that transgene expression in these mice is driven by Ubiq, ubiquitin; WB, Western blot.

Islet RNA from progeny of four different transgene-expressing founder lines (two for each BAC transgene) was examined for levels of eGFP-containing mRNA by TaqMan RT-PCR. As shown in Supplemental Fig. 1A, these four lines expressed slightly different levels of mRNA, as expected, but these differences were not transgene type dependent; overall, NOD.BAC-IGRP-eGFP expressed full-length IGRP-eGFP (BAC-IGRP-eGFP) or Ub-IGRP-eGFP (BAC-Ub-IGRP-eGFP) using an anti-eGFP–specific mAb confirmed the presence of full-length IGRP-eGFP in NOD.BAC-IGRP-eGFP and its absence in NOD.BAC-Ub-IGRP-eGFP mice (Fig. 3D). In fact, the latter contained significantly higher levels of N-truncated forms of IGRP-eGFP (Fig. 3D). Thus, whereas NOD.BAC-IGRP-eGFP express full-length IGRP-eGFP, NOD.BAC-Ub-IGRP-eGFP do not, consistent with rapid ubiquitin-dependent proteasomal degradation in the latter.

**Cross-priming and recruitment of cognate CD8$^+$ T cells by IGRP$_{206-214}$ derived from endogenous stable versus unstable forms of β-cell IGRP**

To be able to compare the ability of the stable (BAC-IGRP-eGFP) and unstable (BAC-Ub-IGRP-eGFP) forms of IGRP to deliver IGRP$_{206-214}$ to pancreatic lymph node DCs for cross-presentation to cognate CD8$^+$ T cells, we introgressed the transgenics into NOD.G6pc2$^{KdKd}$ mice to produce NOD.G6pc2$^{KdKd}$ BAC-IGRP-eGFP and NOD.G6pc2$^{KdKd}$ BAC-Ub-IGRP-eGFP mice (Supplemental Table I). We then compared the proliferative activity of naive CFSE-labeled 8.3-CD8$^+$ T cells from 8.3-NOD.G6pc2$^{KdKd}$ donors in the PLNs, MLNs, and spleens of 10-wk-old NOD, NOD.G6pc2$^{KdKd}$, NOD.G6pc2$^{KdKd}$ BAC-IGRP-eGFP, and NOD.G6pc2$^{KdKd}$ BAC-Ub-IGRP-eGFP hosts and 14 d posttransfer (Fig. 4).

Naive 8.3-CD8$^+$ T cells were recruited to all of the secondary lymphoid organs examined without any obvious differences among host types (Supplemental Fig. 2), but proliferated primarily in the PLNs, as expected (Fig. 4A) (25). Remarkably, these T cells underwent similar degrees of proliferation in the PLNs of NOD.G6pc2$^{KdKd}$ BAC-IGRP-eGFP and NOD.G6pc2$^{KdKd}$ BAC-Ub-IGRP-eGFP hosts at both 7 (not shown) and 14 d posttransfer, regardless of transgenic protein stability (albeit to a significantly lower extent than in wild-type NOD hosts, expressing substantially higher levels of IGRP than these transgenic animals) (Fig. 4A). Proliferation in the PLNs was followed by recruitment of donor 8.3-CD8$^+$ T cells into host islets (Fig. 4B) and accompanied by additional rounds of proliferation in situ (Fig. 4C). Clearly, these responses were induced by the BAC-encoded IGRP$_{206-214}$ epitope, because no such responses were seen in nontransgenic NOD.G6pc2$^{KdKd}$ hosts (Fig. 4A–C). The magnitude of T cell recruitment into islets paralleled the magnitude of proliferation in the PLNs, and most, if not all, of the cells that were recruited into islets of mice expressing endogenous or transgenic IGRP were actively proliferating (Fig. 4A–C).
These data appeared to be at odds with the results obtained using lentiviral-transduced cells (Figs. 1, 2), suggesting that IGRP_{206-214} encoded in both types of transgenic IGRP-eGFP fusions (stable and unstable) are equally efficient at priming cognate CD8^+ T cells and promoting their recruitment into islets. However, this interpretation would only be valid if the rates of Ag shedding from endogenous β-cells (and subsequent uptake by DCs) were similar in both types of transgenic mice. Experiments aimed at measuring differences in eGFP fluorescence in the PLN-associated DCs of both types of mice were not informative, as the levels of eGFP fluorescence detected in these studies were below the limits of detection by flow cytometry (data not shown). To control for this variable and investigate the role of β-cell death type, we introduced an RIP-driven transgene encoding the human DT receptor (DTR) into both NOD.G6pc_{2KI/KI}.BAC-IGRP-eGFP and NOD.G6pc_{2KI/KI}.BAC-Ub-IGRP-eGFP mice (Supplemental Table I).

Upon treatment with DT, males and homozygous females develop massive β-cell loss (>99% of β-cells) (29). Male mice from both strains were treated with either DT or alloxan to induce massive (synchronous) β-cell apoptosis or necrosis, respectively, and then transfused with naive CFSE-labeled 8.3-CD8^+ T cells. As shown in Fig. 4D and 4E and Supplemental Fig. 3A and 3B, the transfused 8.3-CD8^+ T cells proliferated significantly better in the PLNs of male mice expressing the stable form of IGRP than in the PLNs of mice expressing its unstable, ubiquitinated form, regardless of whether the β-cell Ag shedding insult was initiated by necrosis or apoptosis. Similar differences were seen in RIP-DTR heterozygous females, in which the RIP-DTR transgene is expressed in approximately half of the pancreatic β-cells as compared with male mice (Supplemental Fig. 3C).

Taken together, these results confirmed that the stable form of IGRP is more efficient at delivering IGRP_{206-214} for cross-presentation than its unstable form and suggested that β-cells expressing Ub-IGRP somehow shed higher amounts of IGRP_{206-214}-bearing material than β-cells expressing wild-type IGRP.

Increased β-cell destruction by IGRP_{206-214}-reactive CD8^+ T cells in mice expressing the unstable form of IGRP, despite impaired rates of cross-presentation

Because pMHC class I molecules in target cells likely derive from DRiPs/RDPs and because K^b^ HEK cells expressing Ub-IGRP-eGFP were recognized by 8.3-CTLs significantly better than those...
expressing IGRP-eGFP (Fig. 1E), we reasoned that the islet cells of NOD.G6pc<sup>2K/KI</sup>.BAC-Ub-IGRP-eGFP mice might undergo higher rates of β-cell death (and consequently IGRP<sub>206-214</sub> shedding) in response to local 8.3-CTL than the islet cells of their NOD.G6pc<sup>2K/KI</sup>.BAC-IGRP-eGFP counterparts. In vitro cytotoxicity assays using in vitro–differentiated 8.3-CD8<sup>+</sup> CTL and islet β-cells from both types of mice were not informative (very low levels of specific versus spontaneous lysis), owing to the low levels of transgene expression by the β-cells of these mice relative to endogenous IGRP and the low levels of K<sup>d</sup> expression by murine β-cells in general (6–8).

To investigate this, we compared the incidence of spontaneous TID in 8.3-TCR–transgenic NOD.G6pc<sup>2K/KI</sup>.BAC-IGRP-eGFP versus 8.3-NOD.G6pc<sup>2K/KI</sup>Ub-IGRP-eGFP mice (Supplemental Table I). As shown in Fig. 5A, whereas 8.3-NOD.G6pc<sup>2K/KI</sup>.BAC-IGRP-eGFP were completely resistant to T1D (owing to the low levels of transgene-derived IGRP as compared with the levels of IGRP encoded in the endogenous wild-type locus of 8.3-NOD mice), one-third of 8.3-NOD.G6pc<sup>2K/KI</sup>.BAC-Ub-IGRP mice spontaneously developed TID. Although the incidence of TID in these mice is substantially lower than in wild-type 8.3-NOD mice (7), these differences in diabetes susceptibility between 8.3-NOD.G6pc<sup>2K/KI</sup>.BAC-IGRP and 8.3-NOD.G6pc<sup>2K/KI</sup>.BAC-Ub-IGRP are remarkable given that the latter express significantly lower levels of transgene-encoded IGRP-eGFP than the former (Supplemental Fig. 1). In agreement with this, nondiabetic (>22wk-old) 8.3-NOD.G6pc<sup>2K/KI</sup>.BAC-Ub-IGRP mice displayed significantly increased insulitis scores compared with age-matched 8.3-NOD.G6pc<sup>2K/KI</sup>.BAC-IGRP-eGFP mice (Fig. 5B).

Together, these observations suggest that: 1) the β-cells of NOD.G6pc<sup>2K/KI</sup>.BAC-Ub-IGRP mice have increased susceptibility to 8.3-CTL–induced lysis versus those from NOD.G6pc<sup>2K/KI</sup>.BAC-IGRP-eGFP mice; and 2) this enhances the shedding of IGRP and other autoantigens into the milieu, leading to increased rates of islet inflammation. Thus, ubiquitination of IGRP impairs its ability to be cross-presented in the PLNs, but enhances its ability to be directly presented on the β-cell surface.

IGRP<sub>206-214</sub> does not access the cross-presentation pathway via preformed IGRP<sub>206-214</sub>K<sup>d</sup> complexes (cross-dressing), even in mice expressing only ubiquitinated IGRP molecules.

The above results suggested that the IGRP<sub>206-214</sub> peptides that are cross-presented in the PLNs of NOD.G6pc<sup>2K/KI</sup>.BAC-Ub-IGRP mice originate from proteolytic fragments of IGRP or from preformed (in β-cells) IGRP<sub>206-214</sub>K<sup>d</sup> complexes. We thus took advantage of the availability of these mice to investigate the role of cross-dressing in the activation of naive autoantigenic CD8<sup>+</sup> T cells.

We first measured the ability of naive CFSE-labeled 8.3-CD8<sup>+</sup> T cells from 8.3-NOD.G6pc<sup>2K/KI</sup> donors to proliferate in the PLNs of NOD.h2m<sub>null</sub> mice expressing endogenous IGRP and a loxP-flanked b2m<sup>+</sup> transgene in the presence or absence of a human insulin promoter-driven Cre (NOD.h2m<sub>null</sub>/b2ma<sup>loxP</sup>/HIP-Cre<sup>R</sup> or NOD.h2m<sub>null</sub>/b2ma<sup>loxP</sup>/HIP-Cre<sup>C</sup>, respectively) (Supplemental Table I). As reported earlier (26, 27), Cre expression in these mice renders their β-cells, but not professional APCs, MHC class I deficient; in this case, b2m in the circulation does not reconstitute MHC class I expression in β-cells (Fig. 6A). To be able to ascertain whether the form of β-cell death (necrosis versus apoptosis) played a role in defining the source of IGRP<sub>206-214</sub>– we also introgressed into these mice the RIP-DTR transgene (Supplemental Table I). The PLNs of both HIP-Cre<sup>R</sup> and HIP-Cre<sup>C</sup> mice induced the proliferation of naive 8.3-CD8<sup>+</sup> T cells equally well, regardless of whether β-cell death was induced by alloxan (necrosis) or DT treatment (apoptosis) (Fig. 6B). Similar results were obtained in HIP-Cre<sup>R</sup> versus HIP-Cre<sup>C</sup> NOD.G6pc<sup>2K/KI</sup>.BAC-Ub-IGRP-eGFP/h2m<sub>null</sub>/b2ma<sup>loxP</sup>/Ub-IGRP mice, in which all IGRP<sub>206-214</sub> originates from the unstable form of IGRP (Fig. 6C).

Thus, IGRP<sub>206-214</sub> does not access the cross-presentation pathway via preformed pMHC class I complexes (i.e., cross-dressing), even in mice expressing a highly unstable form of IGRP.

Cross-presentation of IGRP<sub>206-214</sub> requires MHC class I–expressing DCs

To confirm that the IGRP<sub>206-214</sub> that is cross-presented by PLN APCs requires the expression of MHC class I by the APC itself and that preformed pMHC class I complexes play a minimal role, we measured the proliferation of naive CFSE-labeled 8.3-CD8<sup>+</sup> T cells in the PLNs of NOD.h2m<sub>null</sub>/b2ma<sup>loxP</sup> mice expressing CD11c promoter– or CD19 promoter–driven Cre transgenes (Supplemental Table I). Expression of CD11c-Cre or CD19-Cre in these mice selectively abrogated K<sup>d</sup> expression in CD11c<sup>+</sup> or CD19<sup>+</sup> cells, respectively (Supplemental Fig. 7A). Interestingly, the PLNs of NOD.h2m<sub>null</sub>/b2ma<sup>loxP</sup>/CD11cP-Cre mice, carrying MHC class I–bald DCs, were significantly less able to induce the proliferation of 8.3-CD8<sup>+</sup> T cells than the PLNs of NOD.h2m<sub>null</sub>/b2ma<sup>loxP</sup>/CD19cP-Cre mice (Fig. 7B).

We confirmed these results using an alternative approach. We measured 8.3-CD8<sup>+</sup> T cell proliferation in the PLNs of NOD.G6pc<sup>2K/KI</sup>.BAC-Ub-IGRP mice coexpressing CD11c or CD11b promoter–driven DTR transgenes, after DT treatment, to kill CD11c<sup>+</sup> or CD11b<sup>+</sup> cells, respectively (Supplemental Table I). DT treatment significantly reduced the cross-presentation of IGRP<sub>206-214</sub> arising from ubiquitinated IGRP only in mice expressing the CD11c-DTR transgene (Fig. 7C).

Collectively, these data demonstrate that the IGRP<sub>206-214</sub> molecules that are responsible for activation of naive cognate CD8<sup>+</sup> T cells primarily arise from stable (nondegraded) forms of IGRP and require processing of IGRP by CD11c<sup>+</sup> DCs into the MHC.
class I-processing pathway, without any evidence for IGRP$_{206-214}$ peptide transfer or IGRP$_{206-214}/K^{d}$ cross-dressing. Furthermore, our data indicate that the autoantigenicity and pathogenicity of a self-antigen are distinct properties and that pathogenicity peaks at moderate levels of autoantigenicity.

**Discussion**

In this study, we explore the contribution of protein stability to the autoantigenicity and pathogenicity of a dominant CD8$^+$ T cell autoantigen in a polyclonal pathogenic autoimmune response as a step toward understanding what makes certain proteins preferred targets of autoreactive CD8$^+$ T cells. We provide direct experimental evidence that activation of naive IGRP$_{206-214}$-reactive CD8$^+$ T cells in the pancreatic lymph nodes is significantly compromised by an approach that reduces antigenic stability and promotes proteasomal degradation in target β-cells, arguing for protein stability as a property promoting autoantigenicity. Our data further indicate that access of IGRP$_{206-214}$ to regional Ag-protein stability as a property promoting autoantigenicity. Our presentation pathways is not chaperoned by molecules, regardless of the form of cell death. (A) Expression of Hip-Cre abrogates K$^{\beta}$ expression on the β-cells of Hip-Cre$^+$ versus Hip-Cre$^{-}$ NOD.b2m$^{loxP/loxP}$/b2m$^{-/-}$ mice (left panel). Islet cells were cultured in the presence of IFN-γ and IL-1β for 18 h and stained with an anti-K$^{\beta}$ mAb. Splenic B cells (right panel) were used as controls. (B) Proliferation of CFSE-labeled 8.3-CD8$^+$ T cells in the PLN, MLN, and spleen of alloxan-treated Hip-Cre$^+$ versus Hip-Cre$^{-}$ NOD.b2m$^{loxP/loxP}$/b2m$^{-/-}$ (left panel) or DT-treated Hip-Cre$^+$ versus Hip-Cre$^{-}$ NOD.b2m$^{loxP/loxP}$/b2m$^{-/-}$/RIP-DTR male mice (right panel). Data correspond to five mice per strain type. (C) Proliferation of CFSE-labeled 8.3-CD8$^+$ T cells in alloxan-treated BAC-Ubc-eGFP-transgenic NOD. G6pc$^{2K^{I}}$K/b2m$^{loxP/loxP}$/b2m$^{-/-}$ (Hip-Cre$^+$ versus Hip-Cre$^{-}$) mice (n = 6 and 5 mice, respectively). Data were compared using Mann–Whitney U test.

IGRP is a major target of the diabetogenic CD8$^+$ T cell response, where it functions as the source of numerous epitopes (12). β-cells do not express costimulatory molecules or MHC class II molecules and thus cannot directly prime the diabetogenic autoimmune response. In addition, experimental evidence suggests that priming of some CD8$^+$ T cells occurs in the PLNs (34–36), although there is evidence that this may not be true for certain autoreactive CD4$^+$ T cell specificities targeting insulin-derived peptides that bind to IA$^\alpha$ molecules with low affinity (23, 24). How most β-cell Ags come to be presented by APCs in the PLNs and why certain Ags are preferred targets of the diabetogenic CD8$^+$ T cell response is not known. Presentation of β-cell Ags does not appear to require a preceding CD8$^+$ T cell attack on β-cells (37), but probably requires some sort of insult capable of triggering β-cell Ag shedding. The mode of shedding (as processed peptides, peptides bound to chaperones, preformed pMHCs, or stable full-length Ags) and the type of β-cell insult (ER stress, apoptosis, necrosis, or autophagy) may contribute to defining the autoantigenic hierarchy in T1D. For example, Ags concentrated in apoptotic bodies or β-cell secretory granules may be shielded from degradation and thus gain access to APCs more efficiently. A fundamental question arising from these considerations is whether the cognate epitopes responsible for productive activation and recruitment of autoreactive CD8$^+$ T cells in T1D are produced in β-cells and then acquired by APCs or produced by APCs that have captured unprocessed β-cell Ag shed-

Previous studies have proposed an important role for Ag stability in the cross-presentation of viral Ags to CD8$^+$ T cells (14, 15), but whether this is also true for an autoimmune disease-relevant endogenous tissue-specific autoantigen was unknown. We have shown in this study that cross-presentation of IGRP$_{206-214}$ to diabetogenic CD8$^+$ T cells is also regulated by antigenic stability. We find that the
magnitudes of the proliferative response of IGRP206–214-reactive CD8+ T cells in the PLNs decreases considerably in mice for which β-cells express a form of IGRP that is rapidly targeted for proteasomal degradation upon translation. Given that activation of IGRP206–214-reactive CD8+ T cells requires MHC class I–expressing DCs, our data support the hypothesis that autoreactive CD8+ T cell responses primarily target stable, cross-presentable autoantigens (14, 15) rather than unstable or rapidly degraded Ags, including peptides chaperoned by heat shock proteins or MHC class I molecules (38, 39).

Cross-dressing is a recently described pathway of Ag presentation in which preformed pMHC complexes are transferred from other cells to and presented by DCs without further processing (40). DCs can acquire pMHC complexes after direct contact with dead Ag-expressing cells (41), and DCs cross-dressed with pMHC complexes derived from monocytes that phagocytosed Ags from virus-infected dead cells could elicit antiviral CD8+ T cell responses (42). More recently, an elegant study demonstrated the in vivo significance of cross-dressed DCs in the activation of memory CD8+ T cells against a viral infection (43). The role and significance of cross-dressing in the activation of autoreactive CD8+ T cell responses against self-antigens has not been previously studied. Our experiments exclude cross-dressing as a major mechanism of IGRP-reactive CD8+ T cell activation.

Upon recognition of cognate pMHC on DCs, T cells acquire the ability to survey nonlymphoid tissues for the presence of their cognate target Ags. We have recently shown that recruitment of newly activated, differentiated (as CTL) and memory T cells to islets are exquisitely Ag-specific events (25, 44), such that pre-activated T cells fail to accumulate in the islets of Ag-deficient hosts. Because recruitment of autoreactive CD8+ T cells to islets is not significantly impaired in mice bearing b2m-deficient β-cells or β-cells that could not export pMHC class I to the surface (26, 37), we propose that a cross-presenting intraislet DC, perhaps the DC type that cross-primes these responses in the draining lymph nodes, is also responsible for local retention of autoreactive CD8+ T cells. In fact, there is extensive evidence supporting such a role for intraislet APCs in the recruitment, retention, and accumulation of CD4+ T cells targeting β-cell antigenic epitopes presented by self–MHC class II molecules, which are not expressed in β-cells (45). One attractive possibility, proposed by Calderon et al. (46) for autoreactive CD4+ T cells, is that cognate pMHCs class I complexes expressed on the transendothelial prolongations of dendrites from intraislet DCs into the vascular lumen are responsible for driving this process. Although the PLN is clearly a site of IGRP206–214 cross-priming, we do not rule out an additional role for intraislet DCs in the priming of naive CD8+ T cell responses.
Surprisingly, and despite its inferior cross-presentability, the unstable form of IGRP was found to be superior at driving the development of both insulin and diabetes in 8.3-TCR–transgenic mice as compared with its stable counterpart. Because the unstable form of IGRP results in enhanced pMHC class I expression in expressing cells (Fig. 1E), we speculate that these results are due to increased rates of DRiP/RDP formation owing to protein instability (19, 20), leading to increased expression of autoantigenic pMHC class I complexes on β-cells and more efficient β-cell targeting by CTLs.

Although it would seem logical to assume that the ability of an autotagonist to elicit the activation of cognate autoreactive T cells via DCs in the draining lymph nodes (autoantigenicity) is coupled to (or parallels) its pathogenicity (ability to elicit CTL-mediated lysis of β-cells), our data indicate that this is not true. This phenomenon suggests that differences in protein stability (or rates of proteosomal degradation) may regulate the pathogenicity of autoantigenic β-cell proteins and provide one explanation as to why certain T cells responses occur efficiently, yet exhibit low pathogenic potential (47). Accordingly, our data indicate that autoantigenicity does not imply pathogenicity and that pathogenicity peaks at intermediate levels of autoantigenicity.

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Disclosures
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References