Structural and functional characterization of a single-chain peptide–MHC molecule that modulates both naive and activated CD8+ T cells

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Peptide–MHC (pMHC) multimers, in addition to being tools for tracking and quantifying antigen-specific T cells, can mediate downstream signaling after T-cell receptor engagement. In the absence of costimulation, this can lead to anergy or apoptosis of cognate T cells, a property that could be exploited in the setting of autoimmune disease. Most studies with class I pMHC multimers used noncovalently linked peptides, which can allow unwanted CD8+ T-cell activation as a result of peptide transfer to cellular MHC molecules. To circumvent this problem, and given the role of self-reactive CD8+ T cells in the development of type 1 diabetes, we designed a single-chain pMHC complex (scKd.IGRP) by using the class I MHC molecule H-2Kd and a covalently linked peptide derived from islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP206–214), a well-established autoantigen in NOD mice. X-ray diffraction studies revealed that the peptide is presented in the groove of the MHC molecule in canonical fashion, and it was also demonstrated that scKd.IGRP tetramers bound specifically to cognate CD8+ T cells. Tetramer binding induced death of naive T cells and in vitro- and in vivo-differentiated cytotoxic T lymphocytes, and tetramer-treated cytotoxic T lymphocytes showed a diminished IFN-γ response to antigen stimulation. Tetramer accessibility to disease-relevant T cells in vivo was also demonstrated. Our study suggests the potential of single-chain pMHC tetramers as possible therapeutic agents in autoimmune disease. Their ability to affect the fate of naive and activated CD8+ T cells makes them a potential intervention strategy in early and late stages of disease.

CD8+ cytotoxic T lymphocytes (CTLs) use their T-cell receptors (TCRs) to recognize peptides presented by class I MHC molecules, and this recognition can lead to the demise of the cell displaying the cognate peptide–MHC (pMHC) complex. As a result, CD8+ T cells are important pathogenic effectors in a number of autoimmune diseases, including type 1 diabetes (1). The development of strategies to interfere with their function offers new therapeutic opportunities. Treatment of CTLs with multimers of pMHC complexes has shown promise in inhibiting CTL-mediated cytotoxicity (2–5). For example, pMHC multimers constructed with short flexible linkers cause rapid death of peptide-specific CTLs (3), whereas those with long rigid linkers inhibit CTL-mediated cytotoxicity by interfering with intermedium-mediated CTL adhesion (2). In addition, dimeric Ig fusions of pMHC complexes have been shown to inhibit lysis of target cells by alloreactive CTLs (4, 5).

We reasoned that, in addition to their inhibition of already differentiated CTLs (2–5), pMHC multimers should also be effective against naive T cells, as they would present antigen in the absence of a second costimulatory signal and would be predicted to drive the T cells to apoptosis or anergy (6–9). This is a profoundly unexplored area, perhaps because of the early unexpected finding that pMHC tetramers could instead activate naive CD8+ T cells (10). This behavior was subsequently found to result from the release of the peptide from the tetramers and its transfer to MHC molecules on T cells, which then acted as antigen-presenting cells capable of activating their naive counterparts (11, 12). Thus, the activity of pMHC multimers against CD8+ T cells, both naive and antigen-experienced, requires reevaluation with the use of pMHC complexes in which the peptide is rendered nonexchangeable by virtue of covalent linkage to the complex (13, 14).

To this end, we used a disease-relevant model system consisting of autoreactive CD8+ 8.3 T cells. The 8.3 T-cell clone was originally isolated from the pancreatic islets of a nonobese diabetic (NOD) mouse (15), a model system for type 1 diabetes in which CD8+ T cells have an important pathogenic role (16). The 8.3 T-cell clone is specific for the peptide composed of residues 206 to 214 of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP206–214) presented by H-2Kd (17), and its pathogenicity has been demonstrated by adoptive transfer studies (15) and the accelerated disease that occurs in NOD mice that transgenically express the 8.3 TCR (18). T cells specific for IGRP206–214 represent a prevalent population in the islets of NOD mice (17, 19, 20), and the monitoring of their numbers in the blood can be used to predict disease (20). IGRP epitopes have also been found to be targeted by CD8+ T cells in human type 1 diabetes (21–23).

We used 8.3 T cells to investigate whether a single multimeric pMHC reagent could be developed that would inactivate or eradicate both CTLs and naive CD8+ T cells. We designed a single-chain pMHC complex in which IGRP206–214 is covalently attached to β2-microglobulin (β2m), which itself is covalently linked to the heavy chain of H-2Kd. X-ray diffraction analysis of the single-chain H-2Kd/IGRP206–214 (scKd.IGRP) demonstrated that the covalently linked peptide is presented in the canonical binding groove of the MHC molecule in a fashion that would support productive TCR engagement. Tetramers of scKd.IGRP exhibit high-specificity binding for the cognate 8.3 TCR. Most importantly, scKd.IGRP tetramers specifically induce apoptosis of naive CD8+ 8.3 T cells, as well as of in vitro-generated CTLs and islet-infiltrating CTLs naturally differentiated in vivo. The tetramers also gain access to splenic and pancreatic T cells when administered in vivo. These characteristics support further exploration of the therapeutic potential of single-chain pMHC tetramers for type 1 diabetes and other conditions in which CD8+ T cells contribute to the pathogenic process.


The authors declare no conflict of interest.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3NWM).

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Results

Design and Biochemical Characterization of scKd.IGRP. To circumvent the complications associated with peptide transfer to cellular MHC molecules (11, 12), we designed the single-chain construct scKd.IGRP, which contained the heavy chain of H-2Kd, β2m, and the target peptide, following a strategy similar to that reported for a single-chain construct of H-2Kβ presenting a peptide derived from ovalbumin (13, 14). Specifically, the C terminus of IGRP206–214 (VYLKTNVFL) was fused to the N terminus of β2m with a GGGAS(G4S)2 linker and the C terminus of β2m was fused to the N terminus of the H-2Kd heavy chain with a (G4S)4 linker (Fig. 1A).

The scKd.IGRP construct was expressed in Escherichia coli as inclusion bodies that were refolded and purified in milligram quantities (Fig. S1A). The refolded material exhibited excellent solution properties and was monodisperse as demonstrated by analytical size-exclusion chromatography (Fig. S1C), and exhibited a well resolved single tight band on a native polyacrylamide gel (Fig. S1B). A control single-chain H-2Kd molecule presenting the tumor-derived peptide KYQAVTTTL (24), which is not recognized by 8.3 T cells, was prepared in an identical fashion and designated scKd.TUM (Fig. S2).

Anchoring of IGRP206–214 in H-2Kd Groove and Implications for TCR Recognition. The bacterially expressed and refolded scKd.IGRP protein crystallized in the monoclinic space group P21 with one molecule per asymmetric unit. Initial phase estimates were obtained by molecular replacement using a conventional H-2Kd complex (Protein Data Bank ID 2FWO) (25) incorporating the influenza virus-derived peptide TYQRTALV (FLU). After initial refinement, interpretable electron density was observed for the IGRP206–214 peptide and the two linker glycine residues immediately C-terminal to the peptide (Fig. 1B). The final electron density map was of excellent quality, with no ambiguities observed for the main-chain or side-chain atoms of IGRP206–214 except for the phenylalanine present at position 8 of the peptide (Fig. 1C and Fig. S3). Although the electron density of that phenylalanine side chain was weak, electrospray ionization Fourier transform MS revealed a mass of 49,463.6 Da, which is consistent with the predicted molecular weight of 49,463.1 Da, including the phenylalanine at position 8 (Fig. S4). The phenylalanine side chain was modeled on the basis of weak density and occupies one of the most favored rotamer positions. Diffraction data to 2.7 Å resolution were used for refinement,
resulting in a final atomic model with \( R_{\text{work}} \) and \( R_{\text{free}} \) of 20.8% and 28.4%, respectively, and good stereochemistry (Table S1).

Despite the importance of H-2K\(^{\beta}\)/IGRP\(_{206-214}\) as a target of pathogenic CD\(^{8^+}\) T cells in type 1 diabetes in NOD mice (17, 19, 20), the structure of this pMHC complex has not been reported previously to our knowledge. We found that IGRP\(_{206-214}\) (VYLKTNFL) is presented in the H-2K\(^{\beta}\) groove between the \( \alpha_1 \) and \( \alpha_2 \) helices and on top of the \( \beta \)-sheet platform in canonical fashion (Fig. 1B and C) (26). The overall conformation of the IGRP peptide is shown in Fig. 1D and Fig. S5. The binding of the IGRP peptide is associated with complete or partial burial of Tyr\(^{P2}\), Thr\(^{P5}\), and Leu\(^{P9}\) in the H-2K\(^{\beta}\) groove (Fig. 1D and Table S2). Tyr\(^{P5}\) and Leu\(^{P9}\) are inside the H-2K\(^{\beta}\)-binding nonameric peptides (25). Amino acids present at the C-terminal P9 position are also highly conserved, with a preference for Ile, Leu, and Val (25). Our structural data suggest that Tyr\(^{P2}\) and Leu\(^{P9}\) are indeed important residues for anchoring IGRP\(_{206-214}\) in the H-2K\(^{\beta}\) groove. Our structural data also suggest that Lys\(^{P4}\), Asn\(^{P6}\), Val\(^{P7}\), and Phe\(^{P8}\) are the most likely to be involved in contacting residues of the cognate TCRs, as the side chains of these residues protrude from the groove and are accessible for TCR recognition (Fig. 1D and Table S2). This is consistent with the identification of Lys\(^{P4}\) and Phe\(^{P8}\) as major 8.3 TCR contact residues (27), and the finding that alteration of P7 in the IGRP\(_{206-214}\) mimotope peptide NRP (KYKNKANWFL) to Ala or Val endows the resulting NRP-A7 and NRP-V7 peptides with superagonist activity (28).

Structural alignment of scK\(^{\alpha}\)IGRP with the native (i.e., non-covalently linked) H-2K\(^{\beta}\)-FLU (25) highlights a high degree of similarity between these two complexes (Fig. 1E), with an rmsd of 1.06 Å calculated over all C-\(\alpha\) atoms from the \( \alpha_2m \) domain, the heavy chain, and the peptide (0.47 Å between the \( \alpha_2m \) domains and 1.08 Å between the heavy chains). Overall, the structural organization of the complex and the canonical mode of peptide binding are conserved in the covalently linked pMHC complex. These results further suggest that the single-chain pMHC complex presents peptide and contacts its cognate TCR in a canonical fashion.

Tetramerization of scK\(^{\alpha}\)IGRP. Because of the relatively weak binding affinity between pMHC complexes and their cognate TCRs, we generated scK\(^{\alpha}\)IGRP tetramers with enhanced avidity to assess the functional activity of the single-chain pMHC complex. The tetramers were constructed by using standard procedures based on the high-affinity biotin–streptavidin interaction. The scK\(^{\alpha}\)IGRP tetramers and the control tetramers (scK\(^{\alpha}\)TUM) behaved as single highly homogeneous peaks on size-exclusion chromatography (Fig. 2A and Fig. S2B). A potential challenge associated with the production of such tetramers is the generation of heterogeneous mixtures containing ill-defined multimers, which complicates mechanistic interpretations (29). Our preparations consisted nearly exclusively of tetrameric complexes (Fig. 2A and Fig. S2B).

Specific Binding of scK\(^{\alpha}\)IGRP Tetramers to CD8\(^{+}\) T Cells Bearing a Cognate TCR. To assess the binding capacity of the scK\(^{\alpha}\)IGRP for the cognate 8.3 TCR, scK\(^{\alpha}\)IGRP was tetramerized by using phycoerythrin (PE)-labeled streptavidin, and its binding to splenocytes from 8.3 TCR-transgenic NOD mice was assessed by flow cytometry. Tetramers of scK\(^{\alpha}\)IGRP bound to nearly all the CD8\(^{+}\) T cells from these mice, whereas PE-conjugated tetramers of scK\(^{\alpha}\)TUM did not bind (Fig. 2B). This behavior indicates that the scK\(^{\alpha}\)IGRP complex adopts a conformation in which the covalently linked antigenic peptide is properly presented for specific recognition by the cognate 8.3 T cells. The specificity of the binding was further substantiated by the lack of interaction between scK\(^{\alpha}\)IGRP tetramers and A41 T cells (Fig. 2B), which recognize an autoantigenic peptide in the context of H-2D\(^{b}\) (19).

scK\(^{\alpha}\)IGRP Tetramers Do Not Activate Cognate Naïve CD8\(^{+}\) T Cells and Instead Drive Them to Apoptosis. To examine the biological activity of scK\(^{\alpha}\)IGRP tetramers on naïve T cells, splenocytes isolated from nondiabetic 8.3 TCR-transgenic NOD mice were treated with scK\(^{\alpha}\)IGRP or scK\(^{\alpha}\)TUM tetramers for 3 h. Splenic CD8\(^{+}\) T cells from these mice were previously demonstrated to be largely naive (18). We confirmed this observation on the basis of high CD2DL expression, which was insensitive to tetramer treatment (Fig. 3A), showing that the tetramer binding did not activate the CD8\(^{+}\) T cells. However, staining with Annexin V–FITC demonstrated that the scK\(^{\alpha}\)IGRP tetramers induced phosphatidyserine externalization, a marker of apoptosis, in nearly 20% of cognate CD8\(^{+}\) 8.3 T cells, whereas the irrelevant scK\(^{\alpha}\)TUM tetramers did not have this effect (Fig. 3B). In contrast, the scK\(^{\alpha}\)IGRP tetramers were unable to induce apoptosis of noncognate CD8\(^{+}\) A41 T cells (Fig. 3C).

Death of in Vitro- and in Vivo-Generated CTLs upon Treatment with scK\(^{\alpha}\)IGRP Tetramers. Peptide–MHC class I multimers, which are widely used as cell-surface staining reagents, have been reported to inhibit CTL activity and induce apoptosis of differentiated CD8\(^{+}\) T cells (2–5). However, single-chain pMHC multimers were not examined in these earlier studies. To explore the effect of the scK\(^{\alpha}\)IGRP tetramers on CTLs, we first generated CTLs in vitro by using splenocytes from 8.3 TCR-transgenic NOD mice. CTLs were then treated with the single-chain tetramers for 3 h and stained with Annexin V–FITC to assess apoptosis induction. It was found that, as with naïve cells, the CTLs were also driven to death specifically by the scK\(^{\alpha}\)IGRP tetramers, whereas the noncognate scK\(^{\alpha}\)TUM tetramers had no effect (Fig. 4A).

In NOD mice, diabetes is accompanied by infiltration of the pancreatic islets by autoreactive CTLs. In the 8.3 TCR-transgenic mice, the vast majority of the islet-infiltrating CD8\(^{+}\) T-cell population is specific for H-2K\(^{\beta}\)/IGRP\(_{206-214}\) (30). These islet-infiltrating cells have previously encountered their antigen during priming in the pancreatic lymph node (31). To investigate the effect of single-chain tetramers on these in vivo-differentiated CTLs, islets from diabetic 8.3 TCR-transgenic mice were isolated and cultured with IL-2 for 6 d. The islet infiltrates that exited the islets were then treated with scK\(^{\alpha}\)IGRP or scK\(^{\alpha}\)TUM tetramers for 3 h and analyzed for apoptosis by Annexin V staining. The scK\(^{\alpha}\)IGRP tetramers induced an approximately threefold increase in apoptosis of these in vivo-differentiated CTLs compared with untreated cells or those treated with the scK\(^{\alpha}\)TUM tetramers (Fig. 4B).
It is of considerable interest to consider the possible fate of those 8.3 T cells that bound tetramer but did not undergo apoptosis. A second pathway associated with scKd.IGRP tetramer binding (i.e., TCR engagement in the absence of costimulatory interactions) could be the induction of anergy or unresponsiveness (7, 8). For this purpose, islet-infiltrating CD8+ T cells from the 8.3 TCR-transgenic mice were treated with the scKd.IGRP tetramers or the noncognate scKd.TUM tetramers, and used in an IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assay to determine their responsiveness. There was an approximately 80% reduction in the number of spot-forming cells when the CD8+ T cells were treated with the scKd.IGRP tetramers and presented with the superagonist mimotope peptide NRP-V7 (28), whereas those treated with the scKd.TUM tetramers retained their responsiveness to the mimotope (Fig. 4C). Therefore, the binding of the single-chain pMHC tetramers to cells that have already been activated in vivo, in addition to causing cell death (Fig. 4B), may also render them nonresponsive even when presented with a superagonist peptide.

scKd.IGRP Tetramers Can Access Splenic and Islet-Infiltrating T Cells in Vivo. Given the ability of the scKd.IGRP tetramers to induce apoptosis or unresponsiveness of the cognate CD8+ T cells, it was of particular interest to determine whether they could be delivered in vivo. For these studies, we used tetramers prepared with PE-labeled streptavidin for the purpose of visualizing them in the different organs. The 8.3 TCR-transgenic mice were injected i.v. with the scKd.IGRP or the scKd.TUM PE-labeled tetramers. After 4 h of treatment, cells from the spleen and the pancreas were isolated and stained with anti-CD8 and analyzed by flow cytometry for PE-tetramer binding. CD8+ T cells in the spleen and the pancreas showed tetramer binding only in mice treated with the scKd.IGRP tetramers (Fig. 5), thus demonstrating the ability of this reagent to bind to cognate CD8+ T cells in vivo. These results also establish that the tetramers can traffic to the pancreas, the site of autoreactivity in type 1 diabetes, and bind the targeted cognate CD8+ T cells. Based on our in vitro results, this delivery would be expected to ultimately lead to apoptosis and/or the induction of unresponsiveness in this specific T-cell population.

Discussion
For naive and antigen-experienced T cells, TCR engagement by pMHC in the absence of the costimulatory signal provided by binding of CD28 to its ligands (CD80 and CD86) can result in T-cell anergy or apoptosis (6–9). This so-called two-signal hypothesis of autoreactivity in type 1 diabetes, and bind the targeted cognate CD8+ T cells. Based on our in vitro results, this delivery would be expected to ultimately lead to apoptosis and/or the induction of unresponsiveness in this specific T-cell population.

Fig. 4. Induction of apoptosis in in vitro- and in vivo-generated CTLs and modulation of CTL activity by scKd.IGRP tetramers. (A) In vitro-generated 8.3 CTLs were incubated at 37 °C for 3 h with tetramers of scKd.IGRP or scKd.TUM at 25 nM. Cells were stained with anti-CD8, Annexin V, and 7-AAD and analyzed by flow cytometry. Samples were gated on CD8+ 7-AAD− cells. (B) As in A, except that islet-infiltrating cells were isolated from untreated (filled gray) and then stained with anti-CD8 and anti-CD62L and analyzed by flow cytometry. Samples were gated on CD8+ 7-AAD− cells. (C) As in B, except that splenocytes from 6- to 8-wk-old AI4 TCR-transgenic NOD mice were used. (A–C) Numbers denote the percentage of cells present in the indicated quadrants of the dot plots.
represents the rationale for a variety of therapeutic approaches that are currently being used or explored for the treatment of autoimmune disease (32). Although one such effective strategy is the use of CTLA4-Ig in the treatment of rheumatoid arthritis (33), antigen-specific approaches might be more desirable, as they would reduce the increased risk of infections and cancers that can accompany systemic immunosuppression. Single-chain pMHC class I molecules were originally designed to be used in the context of DNA vaccination to augment immune responses, as covalent linkage of the peptide would result in very stable cell surface expression of defined tumor- or pathogen-derived peptides (13, 14, 34). Their potential to manipulate autoreactive T cells in an antigen-specific manner when multimerized is underexplored. Here we demonstrate that these reagents possess considerable utility for this purpose, without the potential complication of peptide transfer to cells with costimulatory properties and the unintended activation of naïve T cells (11, 12). Such reagents should pose advantages over both peptide therapy, which suffers from short peptide half-life in vivo (35) and the risk of anaphylaxis (36), and administration of antigen-coupled fixed syngeneic cells (37, 38), which will require ex vivo manipulation of a patient’s cells.

The apoptosis, as marked by phosphatidylserine externalization, that we observed in naïve T cells treated with scKd.IGRP tetrasters is consistent with the requirement for both antigen exposure and costimulation to support the survival of naïve T cells (9). In contrast, the apoptosis observed upon treatment of CTLs with the cognate tetrasters may be more akin to activation-induced cell death, such as that observed by others when CD8+ CTLs with the cognate tetramers may be more akin to activation-tetramers is consistent with the requirement for both antigen targeting of CD4+ T cells (41) in autoimmune disease has focused almost exclusively on the expansion of low-avidity memory T cells that have autoregulatory functions, resulting in achievement of both disease prevention and reversal (45). Class II pMHC dimers have also been shown to have a beneficial effect, at least in part, by the fostering of T-cell populations that have regulatory or immunosuppressive properties (41, 42, 44). As we continue in vivo studies of our single-chain pMHC tetramers, the activities of induction of apoptosis and hyporeponsiveness we observed in vitro will be evaluated, as will the impact on regulatory T-cell populations. Although 8.3 TCR-transgenic mice will be used for some of this future work, even standard NOD mice, in which disease is caused by a variety of antigen specificities (46), will be invaluable to study, as they have a substantial population of CD8+ T cells reactive to the IGRP206–214 peptide (17, 19, 20). Regardless of the mechanisms at work, we have demonstrated that a single, readily produced reagent is capable of inducing apoptosis of naïve peptide-specific CD8+ T cells and differentiated CTLs, while at the same time modulating CTL activity. These findings suggest the potential of such reagents for both early and late intervention in the course of autoimmune disease progression.

Materials and Methods

Mice. Male 8.3 TCR-transgenic NOD mice (18) were obtained from The Jackson Laboratory and crossed with NOD mice bred in house to obtain female 8.3 TCR-transgenic NOD mice for our experiments. A14 TCR-transgenic NOD mice (47) were bred in house. All animals were bred and maintained under specific pathogen-free conditions at the Albert Einstein College of Medicine in accordance with protocols approved by the institutional animal care and use committee.

Cloning, Expression, and Purification of Single-Chain pMHC Monomers. The single-chain constructs of scKd.IGRP was solved and analyzed as described in SI Materials and Methods.

Crystallization and Structure Determination. The crystal structure of scKd.IGRP was solved and analyzed as described in SI Materials and Methods.

Tetramerization and Structure Determination. The crystal structure of scKd.IGRP was solved and analyzed as described in SI Materials and Methods.

Fig. 5. scKd.IGRP tetramers access splenic and islet-infiltrating T cells in vivo. 8.3 TCR-transgenic NOD mice were injected i.v. with PE-labeled tetramers of scKd.IGRP or scKd.TUM. After 4 h, cell suspensions of the spleen and pancreas were stained with anti-CD8 and analyzed by flow cytometry for PE-tetramer binding. Numbers denote the percentage of cells present in the indicated quadrants of the dot plots.

To generate 8.3 CTLs in vitro, splenocytes from 8.3 TCR-transgenic NOD mice were cultured in the presence of mitomycin C-treated splenic T cells (5) and a ratio of four molecules of biotinylated scKd.IGRP to one molecule of streptavidin. The formation of tetramers was analyzed by size-exclusion chromatography using a Superdex 200 10/30 prepacked column (Amersham Biosciences). Tetramers of scKd.TUM were prepared and characterized in an identical fashion.

Flow Cytometry. Flow cytometric studies were performed with a FACSCalibur or LSR II device (BD Biosciences) and analyzed by using Flowjo software (Treestar). Labeled monoclonal antibodies to murine CD8a (53-6.7) and CD62L (MEL-14) were purchased from BD Biosciences.

In Vitro 8.3 CTL Generation. To generate 8.3 CTLs in vitro, splenocytes from 8.3 TCR-transgenic NOD mice were cultured in the presence of mitomycin C-treated NOD splenocytes and 10 nM NRP-A7 at a ratio of 1:4. After 6 d of culture, live 8.3 CTLs were purified with Ficol and used for experiments.

Pancreatic islet Isolation and Culture of Inlet-Infiltrating CTLs. Islets were isolated after perfusion of the pancreas with collagenase P and cultured for 6 d in RPMI medium (RPMI 1640 supplemented with 1 mM sodium pyruvate, 28 μM 2-mercaptoethanol, and nonessential amino acids) supplemented with 10% FBS and 50 U/mL recombinant human IL-2 as described previously (48).

Cell Death Assay. Splenocytes or CTLs (1 × 10^6 cells/mL) were resuspended in RPMI medium containing 10% FBS and incubated in 100-μL aliquots at 37 °C for 3 h with 25 nM tetramers of scKd.IGRP or scKd.TUM or left untreated. Cells were washed and stained with FITC-labeled Annexin V and 7-aminoactinomycin D (7-AAD) according to the manufacturer’s protocol (BD Biosciences) and analyzed by flow cytometry. Dead (i.e., 7-AAD-positive) cells were excluded from analysis.
IFN-γ ELISPOT. Islet infiltrates from 8.3 TCR-transgenic NOD mice were collected after 6 d of culture, resuspended in RPMI medium containing 10% FBS, and incubated in 100-μL aliquots at 37 °C for 4 h. The mixture of scFV, scFv, or scFv2TUM or left untreated. ELISPOT plates (MAHA S45:10; Millipore) were precoated with anti-mouse IFN-γ mAb R4-6A2 (BD Pharmingen) and blocked with 1% BSA (Fraction V; Sigma-Aldrich). RMA-S cells engineered to express the MHC class I molecule H-2Kd (RMA-SKd; originally obtained as a gift from M. Bevan, University of Washington, Seattle, WA) were plated at a density of 2 × 10^5 cells per well and pulsed with 1 μM NRP-V7 peptide for 1 h at 26 °C. Islet-infiltrating T cells were cocultured with the peptide-pulsed antigen-presenting cells at 10^5 cells per well for 40 h at 37 °C. IFN-γ secretion was detected with a second, biotinylated anti-mouse IFN-γ mAb XMG1.2 (BD Pharmingen) and spots were developed by using streptavidin–alkaline phosphatase (Zymed Laboratories) and 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate (Sigma-Aldrich). Spots were counted using an automated ELISPOT reader system (Autoimmun Diagnostica).

In Vivo Delivery of scFv3IgR Tetramp. Tetramp of scFv3IgR or scFv2TUM were prepared with PE-labeled streptavidin for the purpose of visualization by flow cytometry after in vivo administration. The 8.3 TCR-transgenic NOD mice were injected i.v. with 200 μL of a 1-μM solution of PE-labeled tetramers of scFv3IgR or scFv2TUM for an immunogenic tumor specific. Int Immunol 4:1085–1090.


Supporting Information

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SI Materials and Methods

Cloning, Expression, and Purification of Single-Chain pMHC Monomers. The single-chain constructs of scK\textsuperscript{d}.IGRP and scK\textsuperscript{d}.TUM were cloned into pET3a (Novagen). Protein was expressed in E. coli strain BL21 (DE3) pLysS as insoluble inclusion bodies. Protein expression was induced at OD\textsubscript{600} of 0.5 with 1.0 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested and suspended in buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 20% (wt/vol) sucrose, 1 mM EDTA, and 10 mM DTT. DNase I (10 μg/mL) was added to the suspension, the cells lysed, and insoluble protein was pelleted by centrifugation. The inclusion bodies were washed three times with buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, and 10 mM DTT. The detergent was removed by washing the inclusion bodies twice with this buffer but omitting the Triton X-100. Protein purity was confirmed by SDS/PAGE.

The purified, detergent-free inclusion bodies were solubilized in buffer containing 6 M guanidine hydrochloride, 10 mM Na-acetate (pH 4.5), 5 mM EDTA, and 1 mM DTT. The solubilized material was refolded by rapid dilution (1, 2) and purified by size-exclusion chromatography with a buffer composed of 20 mM Hepes (pH 7.0), 150 mM NaCl, and 1 mM EDTA. Endotoxin was undetectable (<0.05 endotoxin units/μg), as determined using the ToxinSensor Gel Clot Endotoxin Assay Kit (GenScript).

Crystallization and Structure Determination. scK\textsuperscript{d}.IGRP (5.5 mg/mL in 20 mM Hepes, pH 7.0, 150 mM NaCl, and 1 mM EDTA) was crystallized by using the sitting drop vapor diffusion method at room temperature by mixing 0.5 μL of protein with 0.5 μL of precipitant composed of 15% PEG 8000 and 100 mM Tris-HCl buffer (pH 8.5) and equilibrating over 70 μL of precipitant. Thin plate-shaped crystals were obtained within 4 to 5 d. Crystals were cryoprotected in mother liquor supplemented with 15% ethylene glycol before flash-freezing in liquid nitrogen. Diffraction was consistent with the monoclinic space group P2\textsubscript{1} (a, 41.35 Å; b, 88.53 Å; c, 61.20 Å; and β, 102.92°; with one molecule per asymmetric unit). Data were collected at the X29A beam line (National Synchrotron Light Source) and integrated and scaled with HKL2000 (3). The structure was determined by molecular replacement with the program MOLREP (CCP4) using the model 2FWO (H-2K\textsuperscript{d}/FLU) (4). Initial placement of the H-2K\textsuperscript{d} heavy chain followed by placement of β2m and rigid body refinement with REFMAC5 (5) resulted in clear density for the covalently linked peptide. The model was further improved by alternative cycles of manual revision with COOT and refinement with REFMAC5. The final model was refined to 2.7 Å resolution, with R\textsubscript{work} and R\textsubscript{free} of 20.8% and 28.4%, respectively. Electron density for the β2m and the peptide is complete, including two glycine residues of the linker present just after the peptide. Residues 148 to 152 in the heavy chain are disordered; 87% of the residues are in most favored, 12% in additionally allowed, and 1% in generously allowed regions of the Ramachandran plot (Table S1).


Fig. S1. Characterization of scK\textsuperscript{d}.IGRP monomers. (A) SDS/PAGE analysis shows refolded and purified scK\textsuperscript{d}.IGRP (right lane). Molecular weight standards are in the left lane. (B) Refolded and purified scK\textsuperscript{d}.IGRP exhibits a well resolved single tight band on native PAGE. (C) The refolded scK\textsuperscript{d}.IGRP exhibits excellent solution properties and is monodisperse as demonstrated by analytical size-exclusion chromatography on a Superdex 200 column. Elution volume is indicated.
Fig. S2. Characterization of scK<sup>d</sup>TUM monomers and tetramers. (A) SDS/PAGE analysis shows refolded and purified scK<sup>d</sup>TUM (right lane). Molecular weight standards are in the left lane. (B) scK<sup>d</sup>TUM monomers (blue) and tetramers (red) were analyzed by size-exclusion chromatography on a Superdex 200 column. Elution volumes are indicated.

Fig. S3. Omit-2Fo-Fc map shows electron density of the IGRP peptide. The membrane distal peptide-binding platform of H-2K<sup>d</sup> is depicted as a blue ribbon and the IGRP peptide is displayed as a ball-and-stick model. The N terminus of the peptide is at the top of the figure.
Electrospray ionization Fourier transform ion cyclotron resonance MS defined the experimentally determined molecular weight of the refolded and purified scKd.IGRP as 49,463.6 Da. The expected molecular weight of this protein with phenylalanine present at the eighth residue of the IGRP peptide and with the correct number of disulfide bonds is 49,463.1 Da. Spectra were acquired on an IonSpec FT-ICR mass spectrometer. The mass spectra were deconvoluted and the average mass was obtained with the Omega8 software package. For a protein in this mass range, the accuracy obtained from this instrument is ±0.5 Da.

The detailed conformation of IGRP_{206-214} (VYLKTNVFL) is shown on the membrane distal peptide-binding platform of H-2K^{d} (gray) in scK^{d}.IGRP. The IGRP peptide is rendered as a ball-and-stick model and the atoms are colored as follows: carbon, green; nitrogen, blue; and oxygen, red. The N terminus of the IGRP peptide is oriented on the left and the Gly residue (L1) on the right is the first amino acid of the linker between the peptide and β2m.
Table S1. Crystallographic data, phasing, and refinement statistics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<td>NSLS X29A</td>
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<tr>
<td>Wavelength (Å)</td>
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<tr>
<td>Resolution limits (Å)</td>
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<td>Unit cell a, b, c (Å), and β (°)</td>
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<td>No. of unique reflections</td>
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<td>Completeness (%)</td>
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<tr>
<td>Mean I/σI</td>
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<tr>
<td>Rmerge on I†</td>
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<td>Cutoff criteria I/σI</td>
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<td>Redundancy</td>
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Refinement

| Resolution limits (Å)              | 59.6–2.7                       |
| No. of reflections (work/test)    | 11289/564                      |
| Rwork‡                            | 0.208 (0.33)*                  |
| Rfree (4.8% of data)              | 0.284 (0.31)*                  |
| Protein/H2O                       | 3,160/14                       |
| Mean B values (Å^2)               | Main chain, 43.06; side chain, 43.02; H2O, 38.1 |
| Bonds (Å)/angles (°)              | 0.008/1.16                     |
| Ramachandran plot                 | 87.0% in most favored region, 12.0% in additionally allowed, and 1.0% in generously allowed |

PDB, Protein Data Bank.

*Values in parentheses correspond to the high resolution bin.

†$R_{\text{merge}} = \frac{\sum_{hkl} \sum_i I_i(hkl) - <I(hkl)>}{\sum_{hkl} \sum_i I(hkl)}$.

‡$R_{\text{work}} = \frac{\sum F_o - \sum F_c}{\sum F_o}$, where $F_o$ and $F_c$ are observed and calculated structure factors, respectively.

Table S2. Available surface area (Å^2) of each residue of IGRP206–214

<table>
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<tr>
<th>Location</th>
<th>1: Val</th>
<th>2: Tyr</th>
<th>3: Leu</th>
<th>4: Lys</th>
<th>5: Thr</th>
<th>6: Asn</th>
<th>7: Val</th>
<th>8: Phe</th>
<th>9: Leu</th>
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<tbody>
<tr>
<td>In peptide alone</td>
<td>199.4</td>
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<td>151.5</td>
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<tr>
<td>In pMHC complex</td>
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<td>2.0</td>
<td>6.5</td>
<td>96.9</td>
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<td>36.9</td>
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<td>16.7</td>
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