Published in final edited form as:

J Immunol Methods. 2008 June 1; 335(1-2): 106–115.

Rapid identification of MHC class I-restricted antigens relevant to autoimmune diabetes using retrogenic T cells

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Abstract

The method described herein provides a novel strategy for the rapid identification of CD8⁺ T cell epitopes relevant to type 1 diabetes in the context of the nonobese diabetic (NOD) mouse model of disease. Obtaining the large number of antigen-sensitive monospecific T cells required for conventional antigen discovery methods has historically been problematic due to (1) difficulties in culturing autoreactive CD8⁺ T cells from NOD mice and/or (2) the large time and resource investments required for the generation of transgenic NOD mice. We circumvented these problems by exploiting the rapid generation time of retrogenic (Rg) mice, relative to transgenic mice, as a novel source of sensitive monospecific CD8⁺ T cells, using the diabetogenic AI4 T cell receptor on NOD.SCID and NOD.Rag1^{-/-} backgrounds as a model. Rg AI4 T cells are diabetogenic *in vivo*, demonstrating for the first time that Rg mice are a means for assessing the pathogenic potential of CD8⁺ T cell receptor specificities. In order to obtain a sufficient number of Rg CD8⁺ T cells for antigen screens, we optimized a method for their *in vitro* culture that resulted in a ~500 fold expansion. We demonstrate the high sensitivity and specificity of expanded Rg AI4 T cells in the contexts of (1) specific peptide challenge, (2) islet cytotoxicity, and (3) their ability to resolve previously defined mimotope candidates from a positional scanning peptide library. Our method is the first to combine the speed of Rg technology with an optimized *in vitro* Rg T cell expansion protocol to enable the rapid discovery of T cell antigens.

Keywords

antigen; CD8⁺ T cells; diabetes; epitope; mimotope; NOD; retrogenic; peptide library

1. Introduction

The development of type 1 diabetes (T1D) in humans and in the nonobese diabetic (NOD) mouse model of disease depends on the recognition of β cell epitopes by autoreactive T cells

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(Tisch and McDevitt, 1996; Walter and Santamaria, 2005). CD8⁺ T cells, which recognize MHC class I-associated peptides, have recently come into focus as important contributors to diabetes initiation and development (Liblau et al., 2002; DiLorenzo and Serreze, 2005). Because pancreatic β cells express MHC class I but not class II molecules, only the CD8⁺ T cell subset appears capable of directly recognizing and killing β cells. NOD mice rendered MHC class I-deficient by deletion of the $\beta 2m$ gene, which is required for MHC class I expression, do not develop CD8⁺ T cells, and do not develop diabetes or insulinitis (Serreze et al., 1994; Wicker et al., 1994). As with MHC class II-restricted CD4⁺ T cells, several examples now exist of particular CD8⁺ specificities which are sufficient to cause disease when transferred or expressed transgenically in NOD mice (Haskins, 2005). Despite their suggested importance, the number of human and mouse CD8⁺ T cell epitopes described to date remains small (Di Lorenzo et al., 2007). Indeed, MHC tetramers for all three known pathogenic CD8⁺ T cell specificities in NOD mice were able to stain approximately 25% of islet-infiltrating CD8⁺ T cells on average (Lieberman et al., 2004), suggesting that a significant number of disease-relevant CD8⁺ T cell epitopes remain to be discovered.

To date, attempts to identify the epitopes recognized by an established diabetogenic CD8⁺ T cell specificity have relied on a sensitive biological readout of T cell function (*e.g.* cytolytic activity, interleukin-2 (IL-2) secretion) to differentiate between antigen pools derived from biochemical fractions (Lieberman et al., 2003), cDNA libraries (Wong et al., 1999) or peptide libraries (Lieberman et al., 2004). The agonist peptide(s) could then be isolated by the iterative screening of progressively lower-complexity antigen pools. Although identification of the natural antigen recognized by an autoreactive T cell is desirable, direct approaches such as screening cDNA expression libraries or screening HPLC-fractionated peptides eluted from antigenic peptide-MHC complexes are labor-intensive and technically demanding. For example, the antigen may be at so low an abundance as to not be represented in a cDNA expression library or not detectable in a complex peptide fraction. Alternatively, labile antigens may be lost during biochemical purification. A more robust and convenient, albeit indirect, approach to antigen discovery in the context of T1D has been the identification of mimotope peptides, which differ in sequence from the natural antigen, but are nonetheless recognized by T cells in the context of the appropriate MHC restriction (Anderson et al., 1999; Lieberman et al., 2004). The identification of mimotope peptides provides reagents (*e.g.* agonists and MHC tetramers) useful for studying and functionally manipulating particular autoreactive T cell specificities, and can lead to the identification of the natural autoantigen based on sequence or structural similarities. Indeed, a natural antigen recognized by the AI4 CD8⁺ T cell specificity was identified by sequence similarity to a mimotope specificity, previously deduced from a positional scanning peptide library (PSPL) screen (Lieberman et al., 2004).

In their most straightforward construction, PSPL consist of peptide pools synthesized by randomly incorporating amino acids at all but one peptide position (Houghten et al., 1991). A single amino acid is incorporated at the nonrandom position, but the identity and positional incorporation of the nonrandom amino acid are systematically varied from pool to pool such that all 20 amino acids are “scanned” at each position along the 9mer. One can measure the relative biological response of T cells to each peptide pool (*e.g.* proliferation, cytolytic activity, etc.) and derive a set of preferred amino acids at each position (Gundlach et al., 1996). Candidate mimotopes based on all possible permutations of the preferred amino acids may then be synthesized and tested. Although PSPL pools are very complex (typically $>10^7$ different peptide species), activated CD8⁺ T cells are highly sensitive to antigen (Purbhoo et al., 2004), and in the context of ^{51}Cr release assays, have proven capable of resolving T1D relevant mimotopes from PSPL (Anderson et al., 1999; Lieberman et al., 2004). On the order of 10^8 – 10^9 activated CD8⁺ CTL are typically required to thoroughly screen a PSPL.

To identify CD8⁺ T cell specificities relevant to T1D, several groups including ours have isolated clones of CD8⁺ T cells from the islets of NOD mice (Haskins, 2005). However, for reasons which are not well understood, we (unpublished observations) and others (Santamaria et al., 1995) have found that islet-reactive CD8⁺ T cell clones from NOD mice often fail to grow or lose antigen reactivity with repeated passage. NOD mice transgenic (Tg) for a β cell reactive CD8⁺ T cell receptor (TCR) can reliably provide responsive monoclonal T cells (Anderson et al., 1999; Takaki et al., 2004). However, TCR Tg mice may take 6 months to produce under optimal conditions. In addition, transgenesis in NOD mice is notoriously inefficient in most hands (Coligan, 1996), and therefore typically carried out in other strains, requiring a lengthy subsequent backcross period. Hence, the requirement for large numbers of sensitive monoclonal T cells has made the identification of diabetes-relevant CD8⁺ T cell antigens in NOD mice particularly difficult.

Retrogenic (Rg) mice have previously been shown to be a viable and rapid alternative to Tg mice for studying T cell biology (Szymczak et al., 2004; Holst et al., 2006a, 2006b), including the *in vivo* evaluation of MHC class II-restricted T cell specificities with respect to pathogenic potential in NOD mice (Arnold et al., 2004). Rg mice are produced by the *in vitro* transduction of donor hematopoietic stem cells (HSC) with a retroviral construct containing the TCR α and β chains encoding a TCR of interest. When bone marrow donor and recipient mice are used that bear the SCID mutation or Rag1 knockout, the resultant transferred hematopoietic compartment is monoclonal for the desired TCR. Importantly, Rg mice are ready for experimentation 6–8 weeks after HSC transplant.

Although Rg mice provide a rapid source of sensitive monoclonal T cells, the yield from an individual spleen is on the order of 10⁵ T cells per mouse (Holst et al., 2006b), far short of the number of T cells required for traditional antigen discovery methods (10⁸–10⁹). We therefore developed a simple and robust means of expanding cytolytic T cells from precursors in the spleens of Rg mice without the requirement for specific antigen, which would likely be unavailable or poorly characterized in the case of a novel TCR specificity. Using Rg AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} mice as a model, we present a method for the *in vitro* expansion of Rg CTL and demonstrate the utility of these cells for the identification of MHC class I-restricted antigens relevant to T1D.

2. Materials and Methods

2.1 Mice

Rg NOD.Rag1^{-/-} (Shultz et al., 2000) and NOD.SCID (Christianson et al., 1993) mice expressing the diabetogenic AI4 TCR specificity (Graser et al., 2000) were derived from strain-matched donor HSC transduced with an AI4 TCR α and β construct as previously described (Arnold et al., 2004; Holst et al., 2006a). Briefly, the AI4 TCR was cloned from separate pCR2.1 plasmids containing the corresponding AI4 V α 8 or V β 2 TCR cDNAs (DiLorenzo et al., 1998) into a single pMIG II vector (AI4-2A.pMIG II). Within the vector, TCR α and β genes were separated by a picornavirus P2A “cleavage” sequence (Szymczak et al., 2004). An IRES-driven eGFP gene, also contained within the construct, served as a transfection reporter. This construct was transiently cotransfected with a helper plasmid encoding gag, pol, and env proteins into 293T cells, which produced retrovirus capable of transducing GP+E86 ecotropic producer cell lines for the generation of high titer retrovirus. Bone marrow was harvested from 8–10 week old NOD.SCID or NOD.Rag1^{-/-} mice pretreated with 150 mg/kg 5-fluorouracil (Pharmacia & UpJohn, Kalamazoo, MI) and allowed to proliferate for 48 hours in a culture containing complete DMEM, 20% fetal calf serum (FCS), 20 ng/ml IL-3, 50 ng/ml IL-6, and 50 ng/ml stem cell factor (R&D Systems, Minneapolis, MN) before being added to irradiated (1200 rad) retroviral producers for transduction in the presence of 6 μ g/ml polybrene. Sublethally irradiated NOD.SCID (300 rad) or NOD.Rag1^{-/-} (450 rad) mice received 4 $\times$

10^6 transduced bone marrow cells in phosphate buffered saline (PBS)/2% FBS with 2 U/ml heparin via tail vein injection, and were used for experiments 8–10 weeks post transplant. Retrogenic mice were generated and maintained at the St. Jude Animal Resources Center (Memphis, TN) in *Helicobacter*-free, specific pathogen-free conditions, following national, state, and institutional guidelines. The St. Jude Animal Resources Center is accredited by the American Association for the Accreditation of Laboratory Animal Care. All animal experiments followed animal protocols approved by the St. Jude Institutional Animal Care and Use Committee. NOD. $\beta 2m^{-/-}$ (Serreze et al., 1994), Tg AI4(TCR α)-NOD.Rag1 $^{-/-}$, and Tg AI4-NOD.Rag1 $^{-/-}$ mice (DiLorenzo et al., 2002) were maintained by brother-sister mating at Albert Einstein College of Medicine under specific pathogen-free conditions and used in accordance with institutional guidelines for animal welfare.

2.2 Peptide libraries and synthetic peptides

A positional scanning combinatorial 9mer peptide library with H-2D^b-preferred anchor residues fixed at position 5 (asparagine) and position 9 (leucine), peptide MimA2 (YAIENYLEL) (Takaki et al., 2004), and peptide FNL9 (FQDENYLYL), derived from dystrophin myotonic kinase (Lieberman et al., 2004), were purchased from Mimotopes (Clayton, Victoria, Australia). Within the library, all 20 standard amino acids were incorporated into the peptide position being scanned, though only a single amino acid species was allowed at the scanned position within an individual mixture. Cysteine was excluded from incorporation at the randomized peptide positions. The negative control H-2D^b-binding peptide TRL9 (TSPRNSTVL) was synthesized by standard solid-phase methods using fluorenylmethoxycarbonyl chemistry in an automated peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA) and confirmed by mass spectrometry.

2.3 Splenocyte isolation

TCR Rg spleens were cleaned of extraneous tissue and dissociated by mashing between the frosted ends of two glass slides (Fisher) into complete RPMI media (Gibco) including 10% FCS (Hyclone). Dissociated splenocytes were subjected to two rounds of hypotonic lysis with ammonium chloride potassium (ACK) buffer (Biowhittaker), washed twice with RPMI/10% FCS, and counted by hemocytometer.

2.4 In vitro expansion of CD8⁺ cytotoxic T cells from whole splenocytes

A 75 cm² tissue culture flask (BD Falcon) was coated with 32 μ g/ml anti-CD3 ϵ (BD Pharmingen, clone 145-2C11), 32 μ g/ml anti-CD28 (BD Pharmingen, clone 37.51) in PBS, pH 7.2 (Gibco) overnight at 4°C with the cap sealed. Following coating, flasks were rinsed at room temperature 3 times with 10 ml PBS, pH 7.2, followed by 1 rinse with 10 ml complete RPMI/10% FCS. Rg AI4-NOD.Rag1 $^{-/-}$ splenocytes were resuspended at 1×10^5 cells/ml in RPMI/10% FCS containing 1000 U/ml recombinant human IL-2 (Peprotech) and seeded in antibody-coated tissue culture flasks laid on their 75 cm² surface (25 ml/flask). Tg AI4-NOD.Rag1 $^{-/-}$ splenocytes were seeded similarly but at a cell density of 1×10^4 /ml. After 4 days (96 hours) of expansion, cells were harvested by collecting the supernatant. Adherent cells were also harvested by gently washing the culture flask 4 times with 5 ml calcium and magnesium-free Hanks' Balanced Salt Solution (CMF HBSS, Gibco), followed by two 5 minute room temperature incubations and washes with 5 ml cell dissociation buffer (CDB, Gibco). The supernatant and washes were consolidated and cells were subsequently washed twice with 10 ml complete RPMI/10% FCS. Expanded cells were then resuspended at 1×10^4 /ml in complete RPMI/10% FCS containing 1000 U/ml IL-2 and seeded in no more than 50 ml per T175 tissue culture flask (BD Falcon #353028). T175 flasks were laid on their 175 cm² surface. On day 6, the supernatant was centrifuged and pelleted cells were resuspended in three times the original volume, typically 150 ml, in the same flask. On day 8, cells were

harvested and split as on day 4, but using 10 ml per wash for CMF HBSS and CDB. On day 10, the media was exchanged as on day 6. On day 12, cells were harvested as on day 8. Harvested cells were consolidated and washed 3 times with 10 ml complete RPMI/10% FCS, counted, and kept on ice until needed for the assay.

2.5 Flow cytometry and determination of total fold expansion and T cell fold expansion

Rg splenocytes were assessed for TCR (PE-anti-TCR β , BD Pharmingen, clone H57-597, 1:30 dilution) or CD3 ϵ (PE-anti-CD3 ϵ , BD Pharmingen, clone 145-2C11, 1:30 dilution), and CD8 α (APC-anti-CD8 α , BD Pharmingen, clone 53-6.7, 1:30 dilution) expression. A PE-coupled hamster IgG2 λ 1 antibody specific for keyhole limpet cyanin (BD Pharmingen, clone Ha4/8) served as the isotype control for PE-anti-TCR β , and an APC-coupled rat IgG2ak antibody served as the isotype control for APC-anti-CD8 α (BD Pharmingen, clone R35-95). Gross fold expansion (FE) was calculated as total live cells (7AAD $^{-}$) on day 12 versus day 0. T cell FE was calculated as specific minus isotype control antibody-stained CD8 $^{+}$ T cells on day 12 versus day 0. *i.e.*

$$\text{T cell FE} = \frac{\left[(\text{TCR}\beta^{+}\text{CD8}^{+}\text{cells})_{\text{specific antibody}} - (\text{PE}^{+}\text{APC}^{+}\text{cells})_{\text{isotype control}} \right]_{\text{day 12}}}{\left[(\text{TCR}\beta^{+}\text{CD8}^{+}\text{cells})_{\text{specific antibody}} - (\text{PE}^{+}\text{APC}^{+}\text{cells})_{\text{isotype control}} \right]_{\text{day 0}}}$$

Absolute numbers of live and CD8 $^{+}$ TCR β^{+} cells were derived by reference to a known number of TruCOUNT (BD Pharmingen) fluorescent beads added to each FACS sample according to manufacturer's instructions.

2.6 Pancreatic islet isolation and culture

Islets from 6–10 week old female NOD. β 2m $^{-/-}$ or AI4(TCR α)-NOD.Rag1 $^{-/-}$ (T cell-deficient, β 2m $^{+/+}$) mice were isolated by cannulation of the common bile duct followed by pressurized distention of the pancreas with 2.5 ml ice cold 1 mg/ml collagenase P (Roche) in HBSS (Gibco). Distended pancreata were digested for 12 minutes at 37°C, followed immediately by trituration on ice with 10 ml cold HBSS containing 2% FCS (Hyclone) and centrifugation for 2 minutes at 240 $\times$ g and 8°C. The supernatant was gently decanted, the pellet was resuspended by flicking in 10 ml HBSS/2% FCS, and centrifuged at 240 $\times$ g and 8°C for 2 minutes. A third 10 ml wash was followed by a 1 minute centrifugation at 39 $\times$ g and 8°C. The pellet was resuspended in 10 ml cold HBSS/2% FCS and dispersed in a non-tissue culture treated Petri dish (Falcon #1029) from which islets were individually picked under microscope using a suction micromanipulator. High purity and dilution into the target culture media were achieved by two serial pickings into CMRL-1066 (Gibco) media supplemented with 2 mM Glutamax (Gibco), 50 U/ml penicillin + 50 μ g/ml streptomycin (Gibco), 1.9 mM leucine (Sigma), 2 mM nicotinamide (Sigma), 10% heat inactivated FCS, and 28.6 μ M β -mercaptoethanol (Sigma). Ten purified islets were individually seeded per round bottom tissue culture well (Corning) in a final volume of 200 μ l CMRL/10% FCS plus supplements. Culture plates were centrifuged to collect the islets at the bottom of each well and placed in a 37°C/5% CO $_2$ incubator. After 24 hours of culture, serum cycling of islet cultures was performed to encourage spreading and adherence of islets to the culture wells. Islet plates were centrifuged at 590 $\times$ g, supernatant was carefully aspirated and islets were washed three times with 200 μ l serum-free (SF) CMRL, otherwise supplemented as for the initial islet culture, before being centrifuged and returned to culture in 200 μ l SF CMRL. After an additional 24 hours of culture, 22 μ l FCS was added per well to return the media to ~10% FCS, and cells were returned to culture. After an additional 24 hours in culture, islets were cycled off of serum again, and back onto serum the next day, for a total of 2 serum cycles. Cultured islets were used for cytotoxicity assays on days 7–10.

2.7 Islet cytotoxicity assay

After 7–10 days in culture, islets were washed twice with 200 μ l SF CMRL, and overlaid with 50 μ l SF CMRL containing 0.1 μ Ci/ μ l 51 Cr (Amersham). Islets were labeled for 3 hours in a 37°C/5% CO₂ incubator, followed by 5 washes with 200 μ l complete SF RPMI. Islets were then overlaid with 100 μ l SF RPMI to which 100 μ l of the appropriate number of cytolytic effectors were added. Wells containing labeled islets alone (*i.e.* no effectors) were assayed in parallel for background subtraction (see below). Assay plates were then centrifuged at 590 $\times$ g for 5 minutes and placed in a 37°C/5% CO₂ incubator for 6 hours. The assay plates were then centrifuged again and 50 μ l of supernatant was collected and added to 150 μ l scintillation fluid (Perkin Elmer). 50 μ l 8% triton X-100 (Sigma) was then added to each well and mixed by pipetting to lyse any remaining 51 Cr labeled cells. Plates were centrifuged, and 50 μ l of triton lysate was mixed with 150 μ l scintillation fluid. Sample activities in cpm were determined using a Microbeta Trilux counter (Wallac) for 1 minute per sample.

The % lysis was calculated as:

$$\% \text{ lysis} = \frac{(\text{supernatant activity})}{(\text{total activity})} = \frac{(\text{supernatant})}{[(\text{triton lysate}) + (1/4) (\text{supernatant})]}$$

The % specific lysis = (% lysis of sample) – (% lysis of “islets alone” sample)

2.8 Cytotoxicity assays for screening PSPL and for challenges with specific peptides

In vitro-expanded Rg AI4-NOD.Rag1^{-/-} CTL were used in 16-hour 51 Cr-release cytotoxicity assays to test for recognition of peptide-pulsed target cells as previously described (Takaki et al., 2004). Briefly, TAP-deficient RMA-S cells were labeled for 1 hour with 51 Cr at 50 μ Ci/ (2 $\times$ 10⁶) cells in 200 μ l SF RPMI, washed 5 times with 2 ml SF RPMI, and used as CTL targets via coinubation with antigenic peptide and CTL. Effector:target ratios, synthetic peptide concentrations, and peptide library mix concentrations were as indicated in the figures. Percent specific lysis was calculated as:

$$\% \text{ specific lysis} = \frac{[(\text{sample activity}) - (\text{background activity})]}{[(100\% \text{ lysis activity}) - (\text{background activity})]}$$

where “sample”, “background”, and “100% lysis” activities refer to 50 μ l of supernatant from wells containing an experimental sample (*i.e.* peptide antigen or phytohemagglutinin, 3 $\times$ 10³ labelled RMA-S cells and the appropriate number of effectors), labeled RMA-S cells alone, or triton-lysed labeled RMA-S cells, respectively.

2.9 Determination of diabetes in AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} Rg mice

Rg AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} mice were monitored weekly and considered diabetic following a positive glucosuria reading by Clinistix (Bayer, Elkhart, IN) and a blood glucose reading above 200 mg/dL as measured by glucometer (One Touch Profile; Lifescan, Milpitas, CA).

2.10 Histological assessment of the pancreatic islets of Rg AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} mice

Newly diabetic AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} Rg mice were sacrificed and their pancreata placed in 10% buffered Formalin for paraffin embedding. Four μ m-thick sections were cut 150 μ m apart to ensure the examination of unique islets. Sections were stained by hematoxylin and eosin before being examined for the presence of inflammatory infiltration.

3. Results

3.1 Generation of Rg mice expressing the AI4 TCR

We examined CD8⁺ T cell yields and TCR expression levels from the spleens of AI4 TCR Rg mice generated on the NOD.SCID and NOD.Rag1^{-/-} backgrounds. In both AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} Rg mice, CD8⁺ T cells could be detected by staining for CD8 α and either CD3 ϵ or TCR β (Fig. 1). As expected, nearly all CD8⁺ T cells were GFP⁺, confirming expression of the retroviral construct, which contains a GFP reporter in addition to the TCR α and β transgenes (Fig. 1A). Average TCR expression levels for AI4 Rg mice were also comparable to strain-matched AI4 Tg mice albeit with a broader distribution (Fig. 1B). Rg mice yielded on the order of 5×10^6 splenocytes per mouse with CD8⁺ T cells comprising approximately 1% (AI4-NOD.Rag1^{-/-}) or 4% (AI4-NOD.SCID) of all splenocytes (Table 1, Fig. 1). CD8⁺ T cell percentages from Rg AI4-NOD.Rag1^{-/-} mice are consistent with a previous report in which non-autoreactive $\alpha\beta$ TCRs were expressed in Rg Rag1^{-/-} mice (Holst et al., 2006b). Therefore, CD8⁺ T cell yields for AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} mice were approximately 10^5 per spleen (Table 2).

3.2 Rg T cells in AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} mice are pathogenic in vivo

After confirming expression of the AI4 TCR in our Rg mice, we wished to determine whether Rg AI4 T cells were pathogenic *in vivo* as previously observed for Tg AI4 mice (Graser et al., 2000). Rg AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} mice develop diabetes as soon as 10 weeks post transplant (Fig. 2A) with kinetics similar to that observed in AI4-NOD Tg mice (Graser et al., 2000). Examination of histological sections of the islets of recent-onset diabetic AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} Rg mice revealed a lymphocytic infiltration consistent with autoimmune diabetes (Fig. 2B).

3.3 In vitro expansion of Rg T cells

We wished to evaluate the use of Rg NOD mice as a future source of CD8⁺ T cells for defining the specificities of potentially diabetogenic TCRs. However, the relatively low yields of CD8⁺ T cells ($\sim 10^5$) from individual Rg mice made them unsuitable for antigen discovery assays directly *ex vivo*. We therefore explored several methods of expanding CTL without cognate antigen, including the use of *in vitro*-generated dendritic cells in combination with soluble anti-CD3 antibodies, the use of soluble anti-CD3 ϵ and anti-CD28 antibodies, and the use of platebound anti-CD3 ϵ and anti-CD28 antibodies in combination with IL-2 (data not shown). The greatest CD8⁺ T cell expansion was achieved by stimulating splenocytes with platebound anti-CD3 ϵ and anti-CD28 in the presence of 1000 U/ml IL-2 for 4 days, after which the cells were removed from CD3/28 stimulation and growth was maintained in IL-2 supplemented media for a further 8 days. Superior expansion with 1000 U/ml IL-2 versus 100 U/ml or 10 U/ml was observed using control retrogenic splenocytes from mice bearing an alternative TCR (not AI4), which were otherwise generated by the same method as AI4-expressing retrogenic mice (data not shown). This approach for expansion takes into consideration (i) the detrimental effects on growth attributable to activation induced non-responsiveness of CD8⁺ T cells when stimulating with anti-CD3 ϵ and anti-CD28 for longer than approximately 4 days (Tham and Mescher, 2002; Hedfors and Brinckmann, 2003), and (ii) the significant T cell growth enhancement seen with high doses of exogenous IL-2 in culture (Montes et al., 2005). Our method was capable of generating T cell expansions (#TCR β ⁺CD8 α ⁺ cells harvested)/(#TCR β ⁺CD8 α ⁺ cells seeded) on the order of 500 fold (Table 2). TCR β ⁺CD8 α ⁺ T cells comprise greater than 50% of the total recovered cells on average, compared to $\sim 2\%$ of cells on day 0 (Fig. 1B, Table 2). The extent of T cell expansion using our method was sufficient to boost Rg T cell numbers into a range useful for antigen screens (*i.e.* $\sim 10^5 \times \sim 500$ fold expansion = 5×10^7 CTL per mouse).

In theory, such cultures could be expanded for a longer period of time in order to produce a greater yield of CTL per mouse; however, we observed slower growth at around 2 weeks of culture (data not shown), consistent with a previous report for extended CD8⁺ T cell cultures (Hedfors and Brinchmann, 2003). Also, early attempts to expand Rg splenocytes for 4 weeks yielded T cells which were not responsive in antigen challenges (data not shown), suggesting that the culture problems previously observed for NOD autoreactive CD8⁺ clones may also manifest in Rg T cells during extended cultures. Therefore, 12 days of culture provided an adequate amount of expansion in a reasonable amount of time without compromising antigen sensitivity.

3.4 In vitro-expanded Rg AI4-NOD.Rag1^{-/-} T cells are sensitive and specific cytolytic effectors

We next examined the ability of *in vitro*-expanded AI4-NOD.Rag1^{-/-} Rg T cells to recognize agonist peptides in the context of the appropriate MHC class I restriction. To do this, we tested the ability of expanded Rg AI4-NOD.Rag1^{-/-} T cells to lyse ⁵¹Cr-labeled RMA-S target cells bearing either the superagonist peptide MimA2 (YAIENYLEL), the antigenic self peptide FNL9 (FQDENYLYL), or the H-2D^b-binding irrelevant peptide TRL9 (TSPRNSTVL). Expanded Rg AI4 CTL efficiently killed relevant but not irrelevant peptide-pulsed targets (Fig. 3A and 3B). Percent specific lysis plateaued at an effector:target ratio of approximately 40:1. However, expanded Rg T cells were able to kill agonist-bearing targets even at E:T ratios less than 1 (Fig. 3A). Expanded Rg AI4 CTL were also able to specifically kill FNL9- and MimA2-pulsed targets at peptide concentrations as low as 10⁻¹⁰ M and 10⁻¹¹ M, respectively, while not killing TRL9-pulsed targets incubated with up to 10⁻⁷ M peptide (Fig. 3B).

Future efforts to evaluate the function of Rg T cells expressing an uncharacterized TCR specificity will not have the benefit of known agonist peptides. Hence, we also evaluated phytohemagglutinin (PHA) as a potential positive control for expanded Rg cells in our ⁵¹Cr release assays. PHA is a plant lectin which can activate T cells and direct cytolysis of target cells by CTL. Although the mechanism of action of PHA is poorly understood, it likely acts by both conjugating CTL with target cells (*i.e.* agglutination) and by direct triggering of antigen receptors and other activating molecules on the surface of T cells (Green et al., 1978). Expanded Rg CTL efficiently killed PHA-pulsed targets with maximal % specific lysis at 10 µg/ml PHA and plateauing near E:T=40:1 (Fig. 3A and Fig. 3B). Notably, PHA and the superagonist peptide MimA2 had similar maximal responses and agonist dose response slopes, suggesting that in the absence of a known peptide agonist, PHA can serve as an index of the intrinsic lytic potential of expanded Rg T cells. Thus, PHA should prove useful as a positive control for the lytic activity of expanded Rg T cells of known or unknown specificity.

NOD-derived islet-infiltrating CD8⁺ T cells bearing the AI4 TCR and activated CTL from AI4-NOD Tg mice have previously been shown to exhibit MHC-dependent cytolytic activity against NOD islets, suggesting that AI4 CTL were recognizing an MHC restricted antigen(s) on NOD islets (DiLorenzo et al., 1998; Takaki et al., 2004). We therefore tested whether *in vitro*-expanded AI4-NOD.Rag1^{-/-} Rg mice exhibited cytolytic activity against ⁵¹Cr-labeled β2m-sufficient versus β2m-deficient islets *in vitro*. *In vitro*-expanded Rg AI4-NOD.Rag1^{-/-} CTL efficiently killed β2m^{+/+} relative to β2m^{-/-} NOD islets (Fig. 3C). Therefore, Rg AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} CTL are clearly pathogenic *in vivo* and capable of targeting NOD islets in a β2m-dependent fashion *in vitro*.

3.5 In vitro-expanded AI4-NOD.Rag1^{-/-} splenocytes can accurately resolve a mimotope specificity for the AI4 TCR from a PSPL screen

Given the degree of expansion of Rg T cells (~5 × 10⁷ T cells/mouse) and the sensitivity of these expanded cells to antigen, we next wished to assess the ability of expanded Rg cells to resolve a mimotope specificity from a PSPL screen. Expanded Rg AI4-NOD.Rag1^{-/-} CTL

clearly identified preferred residues at each position when used to screen an H-2D^b biased PSPL, in which the residues at positions 5 and 9 were fixed to asparagine and leucine (*i.e.* xxxNxxxL), the preferred MHC anchor residues for H-2D^b (Fig. 4A). In addition, the residues with the highest % specific lysis at each position in the 9mer (Fig. 4A and 4B) agreed with previously published PSPL results using AI4-NOD Tg splenocytes (Fig. 4C), allowing for inference of the previously described mimotope peptide YFIENYLEL from the set of top ranking residues at each position (Takaki et al., 2004).

4. Discussion

Our approach to antigen discovery relies on maximizing the yield of CD8⁺ Rg T cells via *in vitro* expansion followed by the determination of mimotope candidates using screens of PSPL. These methods circumvent both the poor growth and poor sensitivity to antigen typical of cultured NOD CD8⁺ T cell clones as well as the considerable time and resource expenditures associated with the development of TCR Tg mice. Specifically, our *in vitro* expansion protocol yields $\sim 5 \times 10^7$ T cells per Rg mouse. Preliminary studies using AI12.B1.2-NOD.Rag1^{-/-} Rg mice, which carry a CD8⁺ TCR of unknown specificity (DiLorenzo et al., 1998), have shown a similar degree of *in vitro* T cell expansion (data not shown), suggesting that the expansions observed for AI4-NOD.Rag1^{-/-} mice are indeed representative of Rg splenocyte expansions using our methods. An unbiased (*i.e.* where all 9 positions are variable) PSPL covering all 20 amino acids has $9 \times 20 = 180$ library mixtures for screening, and requires $\sim 10^5$ CTL to screen each mixture, or 5.4×10^7 total CTL (*i.e.* $180 \times 10^5 \times 3$) for the entire library in triplicate. This is approximately the number of CTL recovered from a typical *in vitro* expansion from one Rg spleen. An equivalent number of additional CTL would be required for each MHC class I allele screened (~ 1 mouse per MHC allele), to test candidate mimotopes (~ 1 mouse per MHC class I allele), and to verify islet cytotoxicity (~ 1 mouse). In the case of a direct search for natural antigens, a first pass analysis of HPLC fractions from MHC class I binding peptides would likely require a further 2–3 Rg mice. We therefore estimate a total of 10 mice or less will be needed to identify and validate mimotope ligands and to assess the feasibility of a direct antigen identification effort for novel NOD CD8⁺ TCRs in the future. Should greater numbers of T cells be required, groups of 10–50 Rg mice can readily be produced and T cell yields can be doubled by harvesting lymph nodes in addition to the spleen, making post-expansion T cell numbers on the order of 5×10^9 achievable (data not shown). The cost and time involved in generating such a Rg cohort would be far less than required for an equivalent number of Tg mice.

High dose IL-2 appears to be critical for optimal expansion of CD8⁺ T cells. We have also observed that initial cell density greatly affects CTL yields, with Rg T cells expanding best when initially seeded at $\sim 10^5$ splenocytes/ml, while Tg T cells expand best at lower initial densities (approximately 10^4 splenocytes/ml). In both cases, sparse initial seed densities and frequent splittings to low densities would argue against media exhaustion as a factor in the observed density effect. We did observe a weak correlation between T cell precursor frequency and the degree of expansion, but saw no relationship between pre- and post-expansion CD8⁺ T cell frequencies (data not shown). Since total splenocyte counts were used to determine initial culture densities, this suggests that T cell density rather than total splenocyte density may help determine the degree of expansion, with optimal T cell expansions seen at densities of $\sim 1\text{--}2 \times 10^3$ T cells/ml (Table 2 and data not shown). T cell precursor frequency may therefore contribute to the variability seen between expansions of Rg splenocytes from individual mice as well as the difference seen between average expansions of Tg versus Rg AI4-NOD.Rag1^{-/-} splenocytes, although other factors likely play a role as well.

In general, we observed very good sensitivity and specificity when using expanded AI4-NOD.Rag1^{-/-} Rg T cells in antigen challenges. However, a significant amount of $\beta 2m^{-/-}$ islet

lysis was seen at the highest effector:target ratios. A similar level of background was observed when expanded AI4-NOD.Rag1^{-/-} Tg, rather than Rg, cells were used in islet cytotoxicity experiments (data not shown), suggesting the background cytolytic activity was not restricted to expanded Rg splenocytes. Also, similar background levels were not observed when RMA-S or other cells were used as antigen-presenting cells (Fig. 3, Fig. 4, and data not shown). We speculate that the elevated background lysis is related to an inherent sensitivity of cultured islets to non-specific killing by activated CTL. This is further supported by the short assay times required for the islet cytotoxicity assays (6 hours) versus antigen challenge assays with RMA-S cells (16 hours). Alternatively, Rg AI4 CTL may exhibit some minor reactivity to a non-MHC restricted antigen, expressed by NOD $\beta 2m^{+/+}$ and $\beta 2m^{-/-}$ islets alike, but not by antigen presenting cell lines such as RMA-S. It is also possible that NK cells co-expand in our culture conditions which are capable of killing $\beta 2m$ -deficient islets. Nonetheless, expanded Rg CD8⁺ T cells are highly sensitive and specific, and provide sufficient numbers for conventional antigen discovery screens, including screens of PSPL for mimotope identification.

It remains possible that differences in the fine specificity of the AI4 CTL used in the present versus previously published AI4 mimotope screen (Fig. 4B and Fig. 4C) exist as a result of intrinsic differences between Rg and Tg CD8⁺ T cells, or due to differences in the culture and activation of these cells prior to their use. For example, differences in the fine specificity of clonotypic CD8⁺ T cells have previously been attributed to TCR expression levels (Martin and Bevan, 1998) and surface glycosylation states (Kao et al., 2005) following activation. Also, the PSPL in the present study incorporated cysteine at the “scanned” position, though not at the randomized positions in a given mixture, as a precaution against the formation of spurious intrapeptide disulfide bonds, which might interfere with epitope recognition. The PSPL used to derive the original AI4 mimotope (Takaki et al., 2004) completely excluded cysteine during peptide synthesis, even at the “scanned” position. Nonetheless, the results of our PSPL screen using Rg AI4 splenocytes agree remarkably well with the previously published screen, and included the amino acid residues which define the original AI4 mimotope, YFIENYLEL.

Another approach to antigen discovery has recently been described which utilizes a fluorescent multimeric TCR reagent to stain cells expressing a random peptide-MHC library (Crawford et al., 2004; Wang et al., 2005). The library can then be enriched for optimal binding clones, containing candidate mimotopes, by flow cytometry. This approach to rapid antigen discovery does not require a bioassay, and as such, also circumvents the problem of culturing NOD CD8⁺ T cells for use in functional assays. While validation of candidate mimotopes using this display-based approach must still be done functionally, this is achievable with TCR hybridomas or transfectants and sufficiently high concentrations of specific antigen. However, the relatively low antigen sensitivity of TCR hybridomas and transfectants, as well as the specific homing and effector requirements for pathogenic CD8⁺ T cells, suggests that islet cytotoxicity assays and the assessment of diabetes and/or insulinitis would still require the generation of Tg or Rg mice.

In conclusion, we have developed a method for the *in vitro* expansion of TCR-Rg splenocytes for the purpose of identifying β cell antigens recognized by autoreactive CD8⁺ T cell specificities in NOD mice. This approach provides a large number of antigen-sensitive CTL, unlike long term cultures of islet-reactive NOD CD8⁺ T cell clones, which grow poorly and lose sensitivity to antigen. By allowing rapid antigen discovery and rapid *in vitro* and *in vivo* validation of candidate antigens, Rg NOD mice should accelerate the identification of diabetes-relevant CD8⁺ T cell antigens. It is also likely that the use of Rg mice could be similarly adapted for the discovery of diabetes-relevant CD4⁺ T cell antigens, and for the rapid identification of T cell antigens in other contexts, such as cancer or infectious disease. Identification of diabetes-relevant antigens is a necessary step towards determining which islet antigens are targeted in

autoimmune diabetes, how tolerance to these antigens is lost, and for the design of antigen-specific monitoring and interventions for T1D.

Acknowledgements

We are very grateful to the Vignali laboratory for assistance with bone marrow and spleen harvests, and to Toshiyuki Takaki and Irene Jarchum for technical advice. Work in the authors' laboratories was supported by National Institutes of Health (NIH) Grants DK64315 (T.P.D.), DK52956 (T.P.D.), DK51090 (D.V.S.), DK46266 (D.V.S.), and DK20541 (Albert Einstein College of Medicine's Diabetes Research and Training Center), and by grants from the Juvenile Diabetes Research Foundation (T.P.D., D.V.S., D.A.A.V. [1-2004-141 - The Robert and Janice Compton Research Grant, In Honor of Elizabeth S. Compton, and 1-2006-847]). D.A.A.V was also supported by the St. Jude Cancer Center Support CORE NIH Grant (CA21765) and the American Lebanese Syrian Associated Charities (ALSAC). The flow cytometry facility at Albert Einstein College of Medicine is supported by NIH Cancer Center Grant CA13330. R.J.C. was supported by NIH Molecular Neuropathology Training Grant NS07098.

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Abbreviations

ACK, ammonium chloride potassium
CDB, cell dissociation buffer
CMF, calcium and magnesium-free
CMRL, Connaught Medical Research Laboratories
FCS, fetal calf serum
FE, fold expansion
HSC, hematopoietic stem cell
HBSS, Hanks' Balanced Salt Solution
IL-2, interleukin-2
NOD, nonobese diabetic
PBS, phosphate buffered saline
PHA, phytohemagglutinin
PSPL, positional scanning peptide library
Rg, retrogenic
RPMI, Roswell Park Memorial Institute
SF, serum free
TCR, T cell receptor
Tg, transgenic

FIGURE 1. Rg AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} splenocytes contain a population of CD8⁺ T cells which can be expanded *in vitro*

A. CD8 α ⁺CD3 ϵ ⁺ T cells can be detected in the spleens of Rg AI4-NOD.SCID mice (upper panel), and are comprised almost entirely of GFP⁺ cells (solid histogram, lower panel). CD8 α ⁻ and/or CD3 ϵ ⁻ cells are primarily negative for GFP (dashed histogram, lower panel). B. CD8 α ⁺TCR β ⁺ cells are present in both Tg and Rg AI4-NOD.Rag1^{-/-} splenocytes (upper panel) as compared to isotype controls (lower panel) at the start of *in vitro* expansion. CD8 α ⁺TCR β ⁺ cells typically comprise greater than half of all cells, versus isotype controls, following 12 days of *in vitro* expansion with anti-CD3 ϵ , anti-CD28 and high dose IL-2.

FIGURE 2. Rg AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} T cells cause diabetes *in vivo*
A. Rg mice were followed for diabetes as determined by weekly sampling for glucosuria plus verification of diabetes by blood glucose measurement (n=9).
B. Hematoxylin and eosin stains of representative pancreatic islets from diabetic AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} Rg mice showing insulitis.

FIGURE 3. Rg AI4-NOD.Rag1^{-/-} splenocytes contain a sensitive and specific cytotoxic effector population following *in vitro* expansion

A. *In vitro* expanded Rg AI4-NOD.Rag1^{-/-} CTL were incubated with RMA-S targets at various effector to target ratios with phytohemagglutinin at 10 µg/ml (diamonds with solid black line), peptide MimA2 at 1 × 10⁻⁸ M (squares with black medium dashed line), peptide FNL9 at 1 × 10⁻⁸ M (triangles with black short dashed line), or peptide TRL9 at 1 × 10⁻⁸ M (circles with grey line). The Y-axis indicates the % specific lysis ± SEM, n=4.

B. *In vitro*-expanded Rg AI4-NOD.Rag1^{-/-} CTL were incubated with RMA-S targets (E:T=20) at various peptide or phytohemagglutinin concentrations. Peptide designations are as in A.

C. % specific lysis (+SEM), n=4, was determined for Rg AI4-NOD.Rag1^{-/-} CTL incubated *in vitro* with β2m^{+/+} (closed bars) or β2m^{-/-} (open bars) NOD islets at the indicated E:T ratios.

FIGURE 4.

A. Rg AI4-NOD.Rag1^{-/-} CTL discriminate preferred amino acid residues from a PSPL. Specific lysis $\pm$ SEM was determined for CTL incubated with ⁵¹Cr-labeled RMA-S targets (E:T= 20) and H-2D^b anchored peptide library mixtures (0.5 μ g/ml peptide) corresponding to the indicated fixed amino acid positions (n=4).

B. The top six amino acid residues at each position from the PSPL screen using Rg AI4-NOD.Rag1^{-/-} CTL, ranked in descending order of % specific lysis from top to bottom. The amino acid residues comprising the original AI4 mimotope YFIENYLEL are in bold with heavy circles. Overlap in the top residues at each position between Figures 4B and 4C are denoted with hairline circles.

C. The top six amino acid residues at each position from the previously published PSPL screen using Tg AI4-NOD CTL (Takaki et al., 2004), ranked in descending order of % specific lysis from top to bottom.

TABLE 1Total splenocyte yields and CD8⁺ T cell yields for Rg AI4-NOD.SCID and AI4-NOD.Rag1^{-/-} mice.

Source of Splenocytes	Splenocyte yields	TCRβ ⁺ CD8α ⁺ Splenocytes	
		%	No.
AI4-NOD.SCID Rg $\frac{\circ}{+}$	7.00E+06	0.543%	3.80E+04
AI4-NOD.SCID Rg $\frac{\circ}{+}$	1.70E+06	10.9%	1.85E+05
AI4-NOD.SCID Rg $\frac{\circ}{+}$	5.00E+06	11.2%	5.61E+05
average	4.57E+06	7.55%	2.61E+05
geometric mean	3.90E+06	4.05%	1.58E+05
AI4-NOD.Rag1 ^{-/-} Rg $\frac{\circ}{+}$	5.94E+06	0.170%	1.01E+04
AI4-NOD.Rag1 ^{-/-} Rg $\frac{\circ}{+}$	8.20E+06	3.83%	3.14E+05
AI4-NOD.Rag1 ^{-/-} Rg $\frac{\circ}{+}$	7.00E+06	2.03%	1.42E+05
average	7.05E+06	2.01%	1.56E+05
geometric mean	6.99E+06	1.10%	7.67E+04

TABLE 2Relative *in vitro* expansion for AI4-NOD.Rag1^{-/-} CD8⁺ T cells from Rg versus Tg mice.

Source of Splenocytes	% TCRβ ⁺ CD8α ⁺ Splenocytes		Fold Expansion	
	day 0	day 12	Gross	TCRβ ⁺ CD8α ⁺
AI4-NOD.Rag1 ^{-/-} Rg ♀	0.170%	49.0%	2.07	597
AI4-NOD.Rag1 ^{-/-} Rg ♂	3.83%	91.6%	70.7	1687
AI4-NOD.Rag1 ^{-/-} Rg ♀	2.03%	45.7%	5.86	132
average	2.01%	62.1%	26.2	805
geometric mean	1.10%	59.0%	9.50	510
AI4-NOD.Rag1 ^{-/-} Tg ♀	16.7%	77.4%	513	2385
AI4-NOD.Rag1 ^{-/-} Tg ♂	19.7%	54.0%	1411	3868
AI4-NOD.Rag1 ^{-/-} Tg ♀	2.10%	54.5%	38.5	1000
average	12.8%	62.0%	654	2418
geometric mean	8.83%	61.1%	303	2097