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T-cell epitope analysis using subtracted expression libraries (TEASEL): Application to a 38-kDa autoantigen recognized by T cells from an insulin-dependent diabetic patient

(autoimmunity/pancreas/β-cell/islets of Langerhans)

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ABSTRACT **Studies on circulating T cells and antibodies in newly diagnosed type 1 diabetic patients and rodent models of autoimmune diabetes suggest that β-cell membrane proteins of 38 kDa may be important molecular targets of autoimmune attack. Biochemical approaches to the isolation and identification of the 38-kDa autoantigen have been hampered by the restricted availability of islet tissue and the low abundance of the protein. A procedure of epitope analysis for CD4+ T cells using subtracted expression libraries (TEASEL) was developed and used to clone a 70-amino acid pancreatic β-cell peptide incorporating an epitope recognized by a 38-kDa-reactive CD4+ T-cell clone (1C6) isolated from a human diabetic patient. The minimal epitope was mapped to a 10-amino acid synthetic peptide containing a DR1 consensus binding motif. Data base searches did not reveal the identity of the protein, though a weak homology to the bacterial superantigens SEA (*Streptococcus pyogenes* exotoxin A) and SEB (*Staphylococcus aureus* enterotoxin B) (23% identity) was evident. The TEASEL procedure might be used to identify epitopes of other autoantigens recognized by CD4+ T cells in diabetes as well as be more generally applicable to the study low-abundance autoantigens in other tissue-specific autoimmune diseases.**

Insulin-dependent diabetes mellitus results from selective cell-mediated autoimmune destruction of the β cell of the pancreatic islet. A number of diabetes-associated autoantigens have been defined at the molecular level as the result of investigations of circulating autoantibodies (1) and include insulin (2), glutamate decarboxylase (3), carboxypeptidase H (4), peripherin (5), sulfatides (6), ICA512 (7), and ICA69 (8). The molecular targets of islet-reactive T cells, on the other hand, are largely uncharacterized, yet these probably play a more important role in the pathogenesis of the disease as indicated by transfer of disease to neonatal (9) and irradiated nonobese diabetic (NOD) mice by CD4+ T-cell clones and by the success of immunotherapy directed against individual T-cell subsets or their functions (10–12).

We have shown previously that the majority of newly diagnosed type 1 diabetics have circulating T cells which proliferate in response to membrane proteins of rat insulinoma tissue (13, 14). A series of T-cell lines and clones have been obtained using this antigen source including a CD4+ cytotoxic T-cell clone (1C6) which recognizes a 38-kDa membrane protein present in insulinoma subcellular fractions enriched in secretory granules (15). Proliferative responses to insulinoma proteins in the region of 36–40 kDa can be elicited in a large proportion of diabetic patients in assays involving two or three rounds of antigen-specific restimulation (13). The presence of circulating antibodies to proteins of 38 kDa in human (16, 17) and experimental diabetes (18) and the isolation of a 38-kDa responsive diabetogenic T-cell line from NOD mice (19) further suggest that an antigen of this size is of pathological importance in the disease.

We report here the cloning of the epitope of the 38-kDa protein which is recognized by the human T-cell clone 1C6 using an approach based upon the screening of a prokaryotic expression library constructed from β-cell cDNA fragments which were enriched by subtraction of cDNAs shared by pancreatic α cells. The technique should be generally applicable to other autoantigens like the 38-kDa protein which are of low abundance or prove intractable to biochemical purification based on T-cell proliferation assay screening procedures.

MATERIALS AND METHODS

Epitope Library Construction. Molecular cloning techniques were performed by standard procedures unless otherwise specified (20). Poly(A)+ cDNA prepared from mouse insulinoma (βTC3) and glucagonoma (αTC2) cell lines was subjected to a PCR-based subtractive hybridization procedure (21) to generate a library of β-cell-enriched cDNA fragments of ≈200–500 bp in length. The procedure involves digestion of both cDNA preparations with *Alu* I and a mixture of *Alu* I and *Rsa* I, size selection (0.2–2 kb), and blunt-end ligation of the fragments with a 24-mer *Eco*RI adaptor/primer followed by six successive rounds of PCR amplification and subtractive hybridization using 10 μg of target cDNA (βTC3-derived) and 100 μg of *Eco*RI-digested and biotinylated driver cDNA (αTC2). A further short hybridization (20 min at 69°C) was included at the end of the procedure to deplete abundant β-cell-enriched cDNAs. The subtracted cDNA was separated from hybrids and nonhybridized driver cDNA by the addition of streptavidin and repeated phenol/chloroform extraction. The final products were digested with *Eco*RI, blunted using T4 polymerase, phosphorylated with T4 polynucleotide kinase, and blunt-end ligated into the *Sma* I-cut dephosphorylated

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Abbreviations: SEA, *Streptococcus pyogenes* exotoxin A; SEB, *Staphylococcus aureus* enterotoxin B; APC, antigen-presenting cell; PHA, phytohemagglutinin; IL-2, interleukin 2; CMF, crude membrane fraction from rat insulinoma; ISG, insulin secretory granule; DR1, MHC DR A1, DR B1*0101; NOD, nonobese diabetic; TCR, T-cell receptor; LDAO, lauryldimethylamine oxide; PBMC, peripheral blood mononuclear cell.

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plasmid expression vectors pUEX1, pUEX2, and pUEX3 (22) to obtain three separate libraries representing each reading frame. A fourth library with the same reading frame as pUEX3 was prepared by direct ligation of *Eco*RI-digested subtracted cDNA into *Eco*RI-cut dephosphorylated pUEX2. *Escherichia coli* DH10B cells (Max efficiency cells; GIBCO/BRL) were transformed by electroporation to obtain libraries of $>5 \times 10^5$ independent clones. Each library had >50% insert content with >80% of inserts being 200–500 bp as expected. The pUEX1 version of the library was shown to contain β-cell-specific mRNAs in high frequency and a low frequency of housekeeping gene transcripts (23). The particular clone in question (31G) was enriched >50-fold by the subtraction procedure.

Screening and Cloning Procedures. Titered glycerol stocks of the libraries were diluted and sampled in duplicate batches of 100,000 clones for initial assay. A 10-fold serial dilution of the positive pUEX2 library was then made to ascertain the clonal frequency, and limiting dilution analyses were then carried out to isolate reactive clones. The latter procedure was performed by a matrix analysis initially with groups of 250 clones in a 96-well microtiter plate which were grown to A_{600} of ≈1.0 in 2xTY Amp broth at 30°C and preserved by the addition of glycerol and storage at −70°C. Samples of bacteria representing each row and column of the plate were pooled, their recombinant proteins were expressed and purified, and T-cell proliferation assays were performed. The points of intersection of positive rows and positive columns were reanalyzed and positive wells were used to generate a second matrix of 96 × 5 clones. The matrix analysis was repeated as above, the positive wells were plated on 2xTY Amp plates, and individual colonies were assayed.

Expression and Purification of Recombinant Proteins. Overnight cultures of bacteria in 2xTY Amp broth at 30°C were diluted 1:200 into 10 ml of fresh medium and grown at 30°C to A_{600} = 0.25–0.4; then protein expression was induced by incubation at 42°C for 2 h. Inclusion bodies were then prepared by a modification of the method of Bohmann and Tjian (24) as follows. Bacterial pellets were sonified [30 s at 4°C in 1 ml of 25 mM Hepes, pH 7/1 mM $MgSO_4$/0.5 M LiCl/0.1% lauryldimethylamine oxide (LDAO)] and centrifuged in a Microfuge (5 min at 22,000 × *g* at 4°C), and the pellet was resuspended in 10 mM Tris/1 mM EDTA/0.1 mg of lysozyme per ml, incubated for 30 min on ice, and centrifuged. The pellet was extracted twice with 10 mM Tris, pH 7.8/1 mM EDTA/0.5 M LiCl/0.5% LDAO/1 mM dithiothreitol and once with 10 mM Tris, pH 7.8/1 mM EDTA/0.5% LDAO. The resultant inclusion bodies were washed three times in phosphate-buffered saline (PBS) for use in T-cell proliferation assays or resuspended in 10 mM Tris, pH 6.8, prior to SDS/PAGE on 10% (wt/vol) acrylamide/0.13% *N,N'*-bisacrylamide gels. Gels were rinsed in distilled water for 1 min and the fusion protein bands were visualized by staining in ice-cold 1 M potassium acetate for 10 min. The prominent fusion protein band was excised and electroeluted (LKB), and the proteins were recovered by precipitation in 7 vol of acetone containing 1 M HCl, washed twice with acetone/H_2O, 7:1 (vol/vol), air dried, and resuspended by sonication in PBS.

T-Cell Proliferation Assays. T-cell proliferation assays were performed using $1–2 \times 10^4$ T cells (clone 1C6) and $5–10 \times 10^4$ irradiated HLA DR-matched peripheral blood mononuclear cells (PBMCs) [antigen-presenting cells (APCs)] in flat-bottomed 96-well plates in complete Iscove's modified Dulbecco's medium (15). Phytohemagglutinin (PHA, 250 ng/ml) and interleukin 2 (IL-2; recombinant, 10% Lymphocult) were used as positive controls along with either 2 μg of crude insulinoma secretory granules (ISGs) per ml (15) or 10 μg of a crude membrane fraction (CMF) per ml prepared from a transplantable rat insulinoma (25). For the latter preparation, 5 g (wet weight) of tissue was homogenized with a Dounce homogenizer in 0.3 M sucrose/10 mM MES/2 mM EGTA/2 mM $MgSO_4$, pH 6.5 (26) and centrifuged at 1700 × *g* for 10 min to remove nuclei and debris; the CMF was recovered as a 45,000 × *g* × 10-min pellet. Protein assays were performed using the Pierce BCA reagent using bovine serum albumin as standard. Membrane protein samples were incubated for 10 min at 60°C in 10 μl of 1% SDS before applying the recommended procedure.

Peptide Synthesis. Peptides were synthesized on an ABIMED 422 instrument (ABIMED, Langenfeld, Germany) using the simultaneous multiple peptide synthesis method. Peptide purity was verified by reverse-phase chromatography on a Lichrospher C_{18} column (5-μm 60RO-select B; Merck).

RESULTS AND DISCUSSION

A PCR-based subtractive hybridization procedure was developed to generate a library of cDNA fragments representing mRNAs which are enriched in pancreatic β cells relative to α cells. These were expressed as fusion proteins with β-galactosidase in all three possible reading frames, partially purified as inclusion bodies, and subjected to further purification by SDS/PAGE and electroelution. The expression of large fusion proteins in a vector–host combination which consistently produces inclusion bodies proved a key component of this strategy. The large size of the fusion partner relative to the inserted sequences ensured that all expressed fusion proteins fell in to a narrow size range (125–155 kDa) and allowed them to be purified as a group by SDS/PAGE and electroelution. Since the fusion proteins were larger than the majority of *E. coli* proteins, contamination with mitogenic and inhibitory components of bacterial origin was avoided. In addition, the preparation of antigens in this form appeared to facilitate their presentation to T cells compared to soluble proteins and enhanced the sensitivity of T-cell proliferation assays by a factor of 50 or more (P.I.N., A. Cooke, B.O.R., and J.C.H., unpublished findings).

Initial screening of the recombinant proteins from groups of 100,000 clones of the subtracted library induced a T-cell (1C6) proliferative response only to cDNAs inserted in one of the

Table 1. 1C6 proliferative response to purified recombinant proteins from pancreatic β-cell cDNA libraries

Vector	Library		Control, 20 μg/ml	
	20 μg/ml	5 μg/ml	Control only	+ ISG, 2 μg/ml
pUEX1.Sma I	244 ± 145	116 ± 94	37 ± 15	3874 ± 1110
pUEX2.Sma I	39 ± 31	125 ± 42	32 ± 3	5859 ± 2107
pUEX3.Sma I	21,638 ± 830	11,156 ± 1150	39 ± 4	5045 ± 1079
pUEX2.EcoRI	13,940 ± 788	10,889 ± 1582	37 ± 10	ND

β-Galactosidase fusion proteins expressed from 100,000 clones of each of the four libraries were purified by inclusion body preparation and electroelution from SDS/PAGE gels. Control samples were prepared from individual randomly selected colonies. Results are expressed as the mean cpm ± SD of triplicate assays. ND, not determined. PHA, 18,075 ± 2744; background, 54 ± 3; IL-2, 5632 ± 426; ISG, 8784 ± 1672.

three possible reading frames (Table 1). Control incubations in which irrelevant recombinant proteins were mixed with crude insulinoma membrane fraction established that there was no major interference in the T-cell proliferation assay by the β-galactosidase portion of the recombinant proteins or from contaminating bacterial components. Insertion of the cDNA library into a different cloning site in the pUEX2 vector which maintained the same reading frame induced a similar proliferative response. This indicated that the T-cell reactivity was unlikely to have arisen from a low-frequency recombination event in the particular library or detection of an epitope generated from the linker sequence flanking the inserted cDNA.

Proliferation assays performed with recombinant proteins isolated from a serial dilution of the positive library indicated that the clonal frequency was between 1:5000 and 1:20,000 (data not shown), a value consistent with the low abundance of the protein antigen in insulinoma membranes. A single bacterial clone, designated 31G, was subsequently isolated by two rounds of limiting dilution analysis starting from 25,000 bacteria. Nucleotide sequencing (Fig. 1) showed that the clone contained a 211-bp insert which was flanked by the PCR linker-primer sequence terminating in sites from a *Rsa* I to *Alu* I fragment of the original βTC3 cDNA. The single open reading frame of 70 amino acids (aa) was in the expected orientation and register. Retrieval of the insert as an *Eco*RI fragment followed by ligation into pGEX1 (27) resulted in the generation of a glutathione *S*-transferase fusion protein with antigenicity similar to that of the original β-galactosidase fusion (Fig. 2). The 211-bp insert used as a probe in Northern blot analyses of poly(A)$^+$ mRNA from a series of normal mouse tissues and βTC3, αTC2, and NIH 3T3 cell cultures showed the presence of a single 1500-base transcript which was expressed to a variable extent in a wide range of tissues. The highest level of expression was in βTC3 cells. Subsequent analysis has shown that the cloned sequence is related to an endogenous insulinoma 38-kDa protein (38).

The proliferative response of the T-cell clone 1C6 [T-cell receptor (TCR) Vβ19] to the recombinant β-galactosidase protein was observed with DR1/DR1 APCs but not with PBMCs of DR2/DR3 origin, consistent with the previously observed DR1 restriction for 1C6 reactivity with insulinoma membranes (data not shown). A control DR1-restricted T-cell clone, 3D7, which responds to a plasma membrane component of RIN m5F cells, but not insulinoma membranes, failed to respond to the recombinant antigen, consistent with the impression that the cloned sequence was presented to the TCR as a major histocompatibility complex (MHC)-bound processed peptide (data not shown).

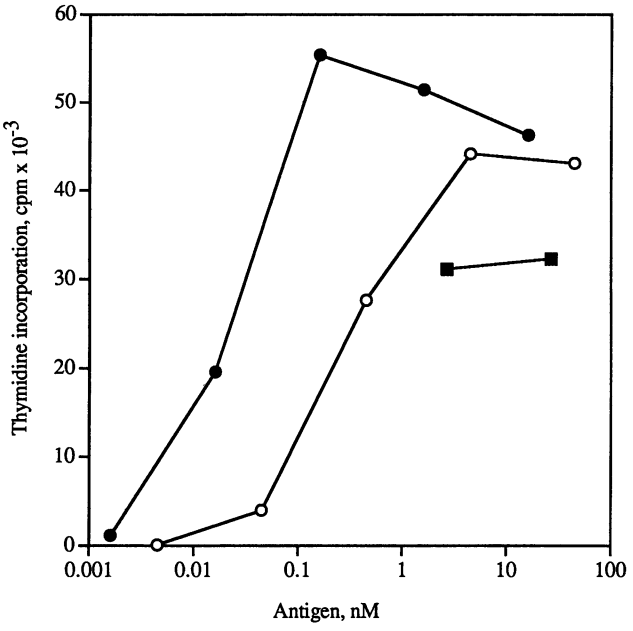

FIG. 2. Antigen concentration dependency of the 1C6 proliferative response. Results are shown for the β-galactosidase fusion protein (●), glutathione *S*-transferase fusion protein (■), and a synthetic 20-aa peptide (○) (peptide F; Table 2) bearing the epitope.

Mapping of the epitope sequence was performed initially with overlapping 20-mer synthetic peptides spanning the entire sequence (Table 2). Only one peptide (peptide F) produced a response and did so over a similar concentration range to the recombinant antigen 31G (Fig. 2). The fact that the T-cell clone 1C6 responsed to the antigen in the subnanomolar range contributed to the facility with which the antigen could be detected in mixtures of several thousand proteins in the initial library screen. Assays of electroeluted proteins (10 μg/ml) prepared from mixtures of the positive clone 31G with increasing proportions with a negative clone (P3c) showed that the positive clone would be detectable at a frequency of 1/50,000 (3009 ± 395 cpm vs. background of 117 ± 68 cpm with PHA = 11,324 ± 647 cpm). This was well below the clonal frequency of 31G in the subtracted library (1/5000 by Southern dot blot analysis; P.I.N. and J.C.H., unpublished findings)

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                10                  20                  30                  40                  50
ACA AAG CCA CAG CAC CAG ATT CAG TTT GAC GAA GAC ATG GAC AGT TCT CTC
 T   K   P   Q   H   Q   I   Q   F   D   E   D   M   D   S   S   L
                                     10

        60                  70                  80                  90                  100
AAG CAG GAG AAG CCA ACT GAT TTC AGT AAA AGG AAA TAT TTA TTC AAG GGG
 K   Q   E   K   P   T   D   F   S   K   R   K   Y   L   F   K   G
        20                                          30

      110                 120                 130                 140                 150
AAA AGA CTT TCA ACT TTT GCT GAT AAG GCG TTT GCT GAT GAA CCA CCT GAA
 K   R   L   S   T   F   A   D   K   A   F   A   D   E   P   P   E
                        40                                      50

      160                 170                 180                 190                 200
CCA GAA GCA TCA CCT TCT CTC TGG GAG ATA GAG TTT GCT AAG CAG TTA GCC
 P   E   A   S   P   S   L   W   E   I   E   F   A   K   Q   L   A
                                    60

    210
TCG GTA G
 S   V
    70
```

FIG. 1. Nucleotide and deduced amino acid sequence of the cDNA insert in the positively reacting pUEX3 clone 31G.

Table 2. 1C6 proliferative response to synthetic peptides encoded by the cloned recombinant antigen

Peptide	Sequence	Residues	1C6 response	
			2.5 μg/ml	0.625 μg/ml
A	TKPQHQIQFDEDMDSSLKQE	1–20	128 ± 12	120 ± 28
B	EDMDSSLKQEKPTDFSKRKY	11–30	106 ± 29	142 ± 46
C	KPTDFSKRKYLFKGKRLSTF	21–40	64 ± 25	71 ± 32
D	LFKGKRLSTFADKAFADEPP	31–50	73 ± 28	113 ± 101
E	ADKAFADEPPEPEASPSLWE	41–60	184 ± 42	121 ± 38
F	EPEASPSLWEIEFAKQLASV	51–70	43,293 ± 9650	47,160 ± 1901

The overlapping peptides cover the deduced protein sequence shown in Fig. 1. Results are the mean ± SD of triplicate analyses. PHA, 18,608 ± 3969; background, 185 ± 48; IL-2, 6353 ± 882; CMF, 7071 ± 720.

but of the same order as the abundance of the mRNA in βTC3 cells.

Fine mapping of the epitope was achieved using a series of 11 synthetic 10-mer peptides spanning the sequence of peptide F (Table 3). The minimal epitope was defined as the sequence LWEIEFAKQL and a shift of 1 aa in either direction caused a marked drop in the proliferative response. The 10-mer peptides tested at a 10-fold higher dilution (100 ng/ml) did not induce a proliferative response, yet the 20-mer peptide F at 2 ng/ml produced a near-maximal response in the same assay. This difference may relate to the mode of presentation of the peptides or suggest that the naturally processed epitope is a more extended structure. To investigate this further, a series of candidate 13-aa epitopes within peptide F were modeled into the peptide binding groove of the HLA DR1 molecule using the three-dimensional structure of influenza hemagglutinin epitope aa 306–318 bound to DR1 as a reference (28) (PKYVKQNTLKLAT; the DR1 contact residues are underlined). The quality of fit of the candidate peptides was examined using the program WHAT IF (29), the energy of each model with MODELLER, and the coordinates visualized with RASMOL (30). The best fit was found to be an 11-aa sequence, SLWEIEFAKQL (major DR1 contact residues underlined). In the model, the tryptophan residue would be accommodated in a deep hydrophobic pocket and together with the leucine residue in a shallower pocket at the opposite end of the MHC binding groove would make a major contribution to the binding affinity (see ref. 28). This was in agreement with a consensus DR1 binding motif as defined by Hammer *et al.* (31) using a phage expression system. This deduced epitope is consistent with the observed reactivity of the 10-mer peptide series (Table 3) and further suggested that the greater response seen to the 20-mer compared to 10-mer peptide F8 peptide may relate to the additional interaction of the side chain of the NH_2-terminal serine residue with DR1 afforded by the former peptide.

Table 3. Fine mapping of the epitope encoded by the cloned recombinant antigen

Peptide	Sequence	Residues	1C6 response
F1	EPEASPSLWE	51–60	74 ± 6
F2	PEASPSLWEI	52–61	401 ± 140
F3	EASPSLWEIE	53–62	546 ± 154
F4	ASPSLWEIEF	54–63	257 ± 24
F5	SPSLWEIEFA	55–64	153 ± 75
F6	PSLWEIEFAK	56–65	370 ± 128
F7	SLWEIEFAKQ	57–66	353 ± 84
F8	LWEIEFAKQL	58–67	16,070 ± 1551
F9	WEIEFAKQLA	59–68	4,252 ± 1551
F10	EIEFAKQLAS	60–69	522 ± 349
F11	IEFAKQLASV	61–70	286 ± 45

The overlapping peptides which cover the sequence of the reactive 20-mer (peptide F; Table 2) were tested at 1 μg/ml in the 1C6 proliferation assay. Results are the mean ± SD of triplicate analyses. PHA, 35543 ± 8616; background, 185 ± 94; IL-2, 34,928 ± 6657; 20-mer peptide F (2 ng/ml), 26,171 ± 3711.

Nucleotide and amino acid data base alignments performed with either the cloned 210-nt sequence or the epitope did not reveal the identity of the protein. The 70-aa sequence showed a weak homology to the bacterial toxin superantigens SEA (*Streptococcus pyogenes* exotoxin A; ref. 32) (23.7% identity, 69.4% homology over 59 residues) and SEB (*Staphylococcus aureus* enterotoxin B; ref. 33) (22.8% identity, 70.1% homology over 57 residues). Comparison of the 31G sequence with the known three-dimensional structure of the SEB/DR1 complex (34) indicated that the residues involved in the interaction of SEB with DR1 and the putative contact sites of SEB and the TCR lie outside the region of homology of SEB with 31G. Furthermore, these superantigens, unlike 31G, are not active as short peptide fragments (35). Thus, although a linkage between diabetic autoimmunity and superantigen expression has been postulated (36), the observed homology is probably fortuitous. Alignment of the epitope sequence with the prolactin receptor AEWEIEFAGQQTE and bovine β-crystallin PSLWAHGFQDRVASV (identities underlined) was also evident and raised the possibility of mimicry between the 38-kDa protein and these proteins akin to that postulated between glutamic acid decarboxylase and Coxsackie P2 (37). However, the homology between these proteins and 31G did not extend to the rest of the 70-aa sequence and the conformational requirements for DR1 binding were not satisfied. It was therefore not surprising that synthetic peptides corresponding to the above sequences were inactive in the 1C6 proliferation assay (data not shown).

It is concluded that the subtractive expression cloning procedure which was developed correctly identified the T-cell epitope recognized by a DR1-restricted $CD4^+$ T-cell clone (1C6). The availability of a cloned cDNA fragment of the parent molecule will enable the primary structure of the antigen to be determined and facilitate studies of its importance to the immunology and genetics of type 1 diabetes. The TEASEL procedure can clearly be applied to other diabetic autoantigens. It may be especially useful to identify immunodominant low-abundance antigens such as the 38-kDa antigen or provide a shortcut to defining epitopes embedded in large proteins. Factors limiting application of the technique are the representation of the antigenic fragment in the subtracted library and the requirement that the reporter T-cell clone should exhibit a high sensitivity to the antigen and a high stimulation index.

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