Modification Form for Permit BIO-RR1-0020

Permit Holder: Joaquin Madrenas

Approved Personnel
(Please stroke out any personnel to be removed)

Panagiotis Mitsopoulos

Luan Chau

Darah Christie

Thu Chau

Additional Personnel
(Please list additional personnel here)

Cynthia Tang
Holly Lemmon
Teresa Fernandes
Kumool Raghavendra

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. Give the full name - do not abbreviate.
Approved Microorganisms	Superantigen, S. aureus cell wall components, peptidoglycan, PAM2CSK4, PAM3CSK4.	
Approved Primary and Established Cells	Human (established) - E. 6.1 JurKats, HEK 293 E, Rodent (primary, established):mouse IAg7 L-Cell line (non-biohazardous) [Primary] - (Human): blood, cell lines. (Rodent) - Mice	Human Cell Line CCL-247
Approved Use of Human Source Material	blood, h. CTLA4 pbig2i Plasmid. Peritoneal fluid, human SLP-2-gfp cDNA in pBIg2i plasmid., Human TLR1, TLR2, and TLR6 cDNAs ready for expression in mammalian cells and signalling studies with S. aureus	
Approved Genetic Modifications (Plasmids/Vectors)	E. Coli DH5 Alpha. (Plasmid) - pBIG2i	
Approved Use of Animals	2007-078-12 for MICE	
Approved Biological Toxin(s)	superantigen, SEE (Staphylococcal Enterotoxin E)	

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF..*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: Jmadison

Current Classification: 2 **Containment Level for Added Biohazards:** _____

Date of Last Biohazardous Agents Registry Form: Sep 17, 2010

Date of Last Modification (if applicable): _____

BioSafety Officer(s): Ronald Hozbor December 17, 2010

Chair, Biohazards Subcommittee: _____ **Date:** _____

We are interested to look at the functions & characterization of the SLP-2 protein in the tumour conditions & do the in vitro experiments. The cell lines will be useful for this study and will ~~xxx~~ allow us to evaluate the unknown functions of SLP-2, a novel and unsual stomatin homologue.

Cell Biology

ATCC® Number:	**CCL-247™** Order this Item	Price:	**$256.00**
Designations:	HCT 116		
Depositors:	MG Brattain		
Biosafety Level:	1		
Shipped:	frozen		
Medium & Serum:	See Propagation		
Growth Properties:	adherent		
Organism:	*Homo sapiens* (human)		
Morphology:	epithelial 		
Source:	**Organ:** colon **Disease:** colorectal carcinoma		
Cellular Products:	carcinoembryonic antigen (CEA) 1 ng per 10 exp6 cells per 10 days; keratin		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Applications:	transfection host (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents)		
Tumorigenic:	Yes		
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 7,10 D13S317: 10,12 D16S539: 11,13 D5S818: 10,11 D7S820: 11,12 THO1: 8,9 TPOX: 8,9 vWA: 17,22		

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Cell, microbial and molecular genomics products for the life sciences

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Bio-materials management; basic repository to complex partnership-level services

Cytogenetic Analysis:	The stemline chromosome number is near diploid with the modal number at 45 (62%) and polyploids occurring at 6.8%. The markers 10q+ and t(?8p;18q) are present in all metaphases and t(9q;?16p-), in 80% of the cells karyotyped. N16 is monosomic in the presence of, but disomic in the absence of t(9q;?16p-). N10 and N18 are monosomic and other chromosomes from those mentioned above are disomic. Q-band observations revealed the presence of the Y chromosome, but not in all cells (50% of cells lacked the Y in G-band karyotypes).
Isoenzymes:	AK-1, 1 ES-D, 1-2 G6PD, B GLO-I, 1 PGM1, 1 PGM3, 1
Age:	adult
Gender:	male
Comments:	The cells are positive for keratin by immunoperoxidase staining. HCT 116 cells are positive for transforming growth factor beta 1 (TGF beta 1) and beta 2 (TGF beta 2) expression. This line has a mutation in codon 13 of the ras protooncogene, and can be used as a positive control for PCR assays of mutation in this codon.
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated McCoy's 5a Medium Modified, Catalog No. 30-2007. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C **Growth Conditions:** Growth and plating efficiency are enhanced by using a feeder layer of murine fibroblasts.

Subculturing:	**Protocol:** 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. 6. Incubate cultures at 37°C.
Preservation:	**Subcultivation Ratio:** A subcultivation ratio of 1:3 to 1:8 is recommended **Medium Renewal:** Every 2 to 3 days **Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2007 recommended serum:ATCC 30-2020 feeder layer cells:ATCC 56-X

References:

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Modification Form for Permit BIO-RRI-0020

Permit Holder: Joaquin Madrenas

Approved Personnel
(Please stroke out any personnel to be removed)

Luan Chau

~~Samar Sayedyahossein~~

Darah Christie

Thu Chau

Additional Personnel
(Please list additional personnel here)

PETER (PANAGIOTIS) MITSOPOULOS

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. Give the full name - do not abbreviate.
Approved Microorganisms	Superantigen, S. aureus cell wall components, peptidoglycan, PAM2CSK4, PAM3CSK4.	
Approved Primary and Established Cells	Human (established) - E. 6.1 JurKats, HEK 293 E, Rodent (primary, established), [Primary] - (Human): blood, cell lines. (Rodent) - Mice	Mouse IAg7 L-Cell line (non-biohazardous)
Approved Use of Human Source Material	blood, h. CTLA4 pbig2i Plasmid. Peritoneal fluid, human SLP-2-gfp cDNA in pBIg2i plasmid., Human TLR1, TLR2, and TLR6 cDNAs ready for expression in mammalian cells and signalling studies with S. aureus	
Approved Genetic Modifications (Plasmids/Vectors)	E. Coli DH5 Alpha. (Plasmid) - pBIG2i	
Approved Use of Animals	2007-078-12 for MICE	
Approved Biological Toxin(s)	superantigen, SEE (Staphylococcal Enterotoxin E)	

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE STORED, USED AND DISPOSED OF..*

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Signature of Permit Holder: J Madrenas

Current Classification: 2 **Containment Level for Added Biohazards:**

Date of Last Biohazardous Agents Registry Form: Sep 23, 2009

Date of Last Modification (if applicable): Mar 16, 2010

BioSafety Officer(s): J Stanley Sept 16/10 Ronald Mosesorky

Chair, Biohazards Subcommittee: Date: Sept 17/10

This cell line is used for antigen-presenting studies in the context of the I-A^{97}, diabetes prone NHE molecule. See attached publication for details. No biosafety hazard.

Journal of Autoimmunity (1998) 11, 63–71

JAI

Development of an I-A^{g7}-expressing Antigen-presenting Cell Line: Intrinsic Molecular Defect in Compact I-A^{g7} Dimer Generation

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[1]*Department of Microbiology and Immunoloy, The University of Western Ontario, Canada*
[2]*Department of Medicine, The University of Western Ontario, Canada*
[3]*The John P. Robarts Research Institute, London, Ontario, Canada*
[4]*Department of Medical Microbiology and Immunology, The University of Alberta, Edmonton, Alberta, Canada*

Insulin-dependent diabetes mellitus (IDDM) results from chronic, T-cell dependent, autoimmune destruction of the insulin-producing β-cells in the Langerhans' islets of the pancreas. Non-obese diabetic (NOD) mice spontaneously develop IDDM that resembles human type I diabetes. The susceptibility to diabetes in the NOD strain is a complex polygenic trait that determines a phenotype of immune alterations. The unique MHC class II molecule expressed by NOD mice (I-A^{g7}) plays a major role in the development of disease. Recently, it has been reported that I-A^{g7} molecules generate a lower proportion of compact αβ heterodimers, compared to other haplotypes. However, it is not clear whether this reflects an intrinsic defect of this molecule to bind peptide stably or is the result of abnormal processing and/or peptide loading into the I-A^{g7} molecule. Our aim was to develop and characterize a suitable antigen-presenting cell (APC) that expressed I-A^{g7} in the context of a non-diabetes-prone antigen processing and presentation machinery. Here, we report the generation of a mouse DAP.3 fibroblast cell line (DAP.3A^{g7}) that constitutively expresses high levels of I-A^{g7}. Using DAP.3 cells transfected with I-A^{g7} or I-A^{k}, we show that the expression of compact dimers in the same cell type is proportionally less for I-A^{g7} molecules than for I-A^{k} molecules, implying an intrinsic defect of the I-A^{g7} molecule as the cause for the low generation of compact dimers. However, DAP.3A^{g7} cells are able to process and present antigen, as indicated by I-A^{g7}-dependent IL-2 production by a GAD67-specific NDO T-cell hybridoma after stimulation with GAD and live, but not fixed, DAP.3A^{g7} cells. The IL-2 response to GAD when presented by DAP.3A^{g7} was significantly higher than the response to GAD presented by NOD splenocytes. Based on these data, we conclude that the low generations of compact dimers is an intrinsic feature of I-A^{g7} molecules and not affected by other genes in the NOD background. The DAP.3A^{g7} cell line should be a valuable tool with which to dissect the role of the I-A^{g7} molecule in antigen presentation and T-cell activation in NOD mice, which clearly contributes to the development of IDDM.

Received 5 September 1997
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Key words: Diabetes, MHC, antigen-presenting cell line

Introduction

Human insulin-dependent diabetes mellitus (IDDM) and its counterpart in the non-obese diabetic (NOD) mouse is an autoimmune disease characterized by T-cell dependent destruction of the insulin-secreting β-cells in the pancreatic islets of Langerhans [1, 2]. β-cell destruction in IDDM represents the final stage of a progressive T-cell infiltration of the islets or insulitis [3, 4], followed by chronic inflammation of the islets leading to β-cell death and loss of insulin production with subsequent hyperglycaemia [5, 6].

The primary event leading to the autoimmune response observed in IDDM is still unknown. Specifically, it is not known whether autoreactive T cells in this model result from defective thymic development of T cells, abnormal antigen presentation in the thymus and/or in the periphery, or impaired T-cell activation. In addition, multiple molecules have been proposed as primary autoantigens in the autoimmune destruction of β-cells (e.g. glutamic acid decarboxylase (GAD), insulin, carboxypeptidase-H (CPH), etc.) but the initial antigen that triggers T-cell mediated β-cell destruction is still unclear [7–12].

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Abnormal antigen presentation in the context of class II NOD MHC molecules may play a role in the initiation and development of the autoimmune response. Multiple previous studies have demonstrated the importance of class II major histocompatibility complex (MHC) molecules in the development of diabetes [8, 13–16]. In humans, there is a strong association between susceptibility to diabetes and expression of HLA-DQ3.2 as well as DR-3 and DR-4 MHC molecules [17]. McDevitt *et al.* have shown that the HLA-DQ β-chain of non-diabetic individuals has an aspartic acid residue at position 57 which is not present in patients with IDDM [18–23]. These patients often have a valine, serine, or alanine residue at this position. It has been suggested that the aspartic acid at position 57 of the DQ β-chain might form a salt bridge to an arginine residue in the adjacent α-chain of the DQ molecule [24]. The change to an uncharged residue would disrupt this salt bridge, altering the stability of the MHC molecule and potentially accommodating the binding of peptides that may then be recognized as autoantigens. Interestingly, NOD mice share this alteration in the β-chain of their I-A^{g7} MHC molecule, the equivalent of DQ-β in the mouse [25]. Thus, although the contribution of antigen-presentation defects in the context of diabetes-prone animals has not yet been established, there are plausible molecular mechanisms by which this may occur.

Recently, two different groups have reported that I-A^{g7} molecules form a low level of compact dimers [26, 27]. The reduced level of compact forms of I-A^{g7} correlated with a shorter half-life of peptide–I-A^{g7} complexes on the cell surface and inability to demonstrate peptide binding to the MHC molecule [26]. Reizis *et al.* also reported a lower amount of compact dimers for I-A^{g7} than for other alleles [27]. However, they did not find any significant defect in the peptide-binding capacity of I-A^{g7} molecules, a result supported by Harrison *et al.* [28]. The compact forms represent stable class II MHC molecules following peptide binding [29–32]. A reduced level of compact dimers would correlate with reduced peptide presentation. It is therefore important to establish whether the generation of low amounts of compact forms of I-A^{g7} is due to a primary defect of this molecule or is the result of abnormal peptide-loading machinery in the NOD background. To address this issue, we have generated a cell model in which I-A^{g7} expression takes place in a non-NOD background.

It has previously been shown that DAP.3 fibroblasts transfected with the appropriate MHC class II coding genes express class II MHC molecules indistinguishable from the class II molecules expressed by B cells, and can present immunogenic peptides to T cells [33]. This system provides a readily available and non-expensive source of APC for functional T-cell studies. An additional advantage of this system is that fibroblasts do not express class II MHC molecules so that class II-dependent presentation occurs only in the context of the MHC molecules coded by the transfected class II MHC cDNAs. Therefore, we transfected normal mouse fibroblast cells with the I-A^{g7} class-II MHC α and β cDNA coding for the corresponding MHC I-A^{g7} chains. Using fluorescent activated cell sorting (FACS) analysis we confirmed the expression of high levels of the I-A^{g7} molecules on the surface of the L cells. In this system, we found that I-A^{g7} molecules also generated proportionally fewer compact forms than I-A^{k} molecules. However, the transfected DAP.3 cells did show a good capacity to process and present a diabetogenic antigen such as GAD67 to a specific T-cell hybridoma in an I-A^{g7}-dependent and fixation-sensitive manner.

Material and Methods

Cells

RT2.2.3 and RT7.3 cells are murine DAP.3 L cells transfected with the I-A^{d} and I-A^{k} coding cDNAs respectively [34] and were kindly provided by R. Germain (National Institutes of Health, Bethesda, MD). The cell line generated in this project, DAP.3A^{g7}, was generated by transfection of DAP.3 L cells with the I-A^{g7} α- and β-chain cDNAs as described below. All the L cells were cultured in Dulbecco's modified Eagle medium with 10% FCS, 2 mM l-glutamine, non-essential amino acids, HEPES buffer, sodium pyruvate, and 2-mercaptoethanol.

E3 is a $CD4^{+}$ T cell hybridoma specific for GAD67 presented by I-A^{g7} molecules, and was generated from T cells of NOD mice immunized with GAD67 (B. Singh *et al.*, unpublished data). E3 T cells were kept in culture in RPMI 1640 medium with 10% FCS and L-glutamine, non-essential amino acid, HEPES buffer, sodium pyruvate, and 2-mercaptoethanol supplements. A20 cells are a previously described BALB/c mouse B-cell lymphoma line [35].

Antigens and antigen-derived peptides

The following antigens were used in these experiments: ovalbumin (OVA) protein and its OVA peptide fragment (323–339), pigeon cytochrome c (PCC) and its derived peptide encompassing the fragment between residues 81 and 104. Peptides were commercially provided by Procyon Biopharma Inc, London. Recombinant GAD67 protein was purified from transfected *Escherichia coli*, as previously reported [36].

Monoclonal antibodies

The following monoclonal antibodies (mAbs) were produced in our laboratory from culture supernatants (ATCC, Rockville, MD) or purchased from Pharmingen (San Diego, CA) and used in these experiments: 10.2.16, a mouse antibody against the I-A^{k} molecule and cross-reactive with the I-A^{g7} molecule [37]; 14.4.4S, a mouse antibody against the I-E^{k} molecule; M5/114, a rat antibody against the I-A^{b} molecule; 34.2.12, a mouse antibody against the H-$2D^{d}$

molecule; 16.1.2N, a mouse antibody against epitopes in the D^k/K^k molecules; 1G10, a rat antibody against mouse B7-1; GL1, a rat antibody against mouse B7-2; YN1/1.7.4, a rat antibody against mouse ICAM-1; and In-1, a rat monoclonal antibody against mouse invariant chain (Ii) (provided by R. Germain, NIH, Bethesda, MD).

Generation of the DAP.3A^{g7} cell line by cDNA transfection

The following quantities of three different plasmid DNAs were used for transfection of DAP.3 fibroblast cells: 200 µg of the I-Aα^{NOD} (i.e. I-Aα^d) cDNA in pSRaSD7, 200 µg of the I-Aβ^{NOD} (i.e. I-Aβ^{g7}) cDNA in pSRaSD7, and 40 µg of pSSDneo. Prior to electroporation plasmids were digested separately with Sal I, pooled, precipitated with ethanol and sodium acetate, and resuspended in 400 µl of HBSE (20 mM Hepes pH 7.0, 0.75 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 138 mM NaCl, 5 mM KCl, 5.5 mM dextrose). Cells (1×10^7) were harvested from confluent monolayers, washed once in HBSE, resuspended in the 400 µl HBSE plus DNA, and electroporated in a 0.4 cm cuvette (260 V, 960 µF) using a Genepulser apparatus (BIORAD). Following electroporation cells were cultured in complete DMEM medium plus 100 µg/ml gentamicin (GIBCO) as a prophylaxis against the possibility of bacterial contamination during the transformation. After 24 h, geneticin (400 µg/ml; GIBCO) was also added in order to select for G418-resistant cells. After 14 days the bulk population of G418-resistant cells were stained with FITC-conjugated 10.2.16 monoclonal antibody and analysed for IA^{g7} expression by flow cytometry. Ten thousand of the brightest-staining cells were selected by FACS sorting and propagated under continued geneticin selection. Two further rounds were performed.

Surface staining and flow cytometric analysis (FACS) of transfected L cells

Transfected L cells (1×10^6) were washed in 1×PBS buffer and used for staining. FACS analysis for expression of different MHC-class II molecules and other surface receptors was performed on a Becton-Dickinson® FACScan, after staining with primary antibody followed by secondary detection with FITC-labelled goat anti-mouse or goat anti-rat. As negative controls, we used samples directly stained with labelled secondary antibody in the absence of the primary antibody.

For intracellular staining of Ii, A20 and DAP.3A^{g7} cells were fixed in 10% formalin/PBS buffer for 10 min, washed twice and resuspended in PBS/0.1% saponin. Cells were then incubated with In-1 monoclonal antibody at 4°C for 30 min, followed by two washes in PBS/0.1% saponin. FITC-conjugated goat anti-mouse monoclonal antibody was added at 4°C for 30 min followed by three washes in PBS/0.1% saponin. Cells were then analysed by flow cytometry.

Analysis of I-A^{g7} molecule forms

I-A^{g7} or I-A^k-expressing DAP.3 cells (20×10^6 cells per group) were lysed in 1× lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, 10 µg/ml leupeptin, 0.02% sodium azide, 100 µg/ml PMSF, and 1 µg/ml aprotinin) in ice for 30 min. Lysates were then spun at 14,000 rpm for 10 min, and the supernatant divided in two equal aliquots and SDS-PAGE run in Laemmli's

← Methodology used to create IAg7 cell line

with the 10.2.16 antibody. Membranes were then analysed by chemiluminescence (Boehringer-Mannheim Chemiluminescence Blotting Substrate (POD)) and signals were quantified by densitometry (Bio-Rad, model GS 700, Hercules, CA) and the Molecular Analyst® Software (version 1.0, 1994, Bio-Rad laboratories).

T-cell functional assays

Interleukin-2 (IL-2) production by E3 T-hybridoma cells in response to peptide presented by the different DAP.3 cell transfectants was measured following standard procedures [38]. Briefly, 5×10^4 T-hybridoma cells were incubated (37°C, 5% CO_2) with 5×10^4 transfected DAP.3 cells in triplicate in 96-well flat-bottom culture plates, at a total volume of 200 µl/well, with or without increasing concentrations of peptide. After 24 h, supernatants were collected and assayed for IL-2 content following standard ELISA protocol [38]. The results were expressed as mean±SD.

In experiments testing the need for GAD processing, DAP.3A^{g7} cells were fixed with paraformaldehyde following a standard protocol. Briefly, DAP.3A^{g7} (3×10^6/ml) were washed twice with 1×PBS, and resuspended in 0.93% paraformaldehyde in 1×PBS for 10 min at room temperature. Next, the same volume of 0.2 M glycine in PBS was added to the cell suspension for another 10 min, after which the cells were washed with complete medium five times (1,000 rpm, 10 min, 4°C).

Mixed lymphocyte reactions

Proliferation of red blood cell-depleted, NOD, BALB/c, and B10.A splenocytes to DAP.3A^{g7} cells was measured by ^{3}H-thymidine uptake after culturing a suspension of splenocytes (4×10^5 cells/well) as responders, with mitomycin-treated DAP.3A^{g7} cells (4×10^5 cells/well) as stimulators, in triplicate in a 96-well round-bottom culture plate. The final volume per well was 200 µl. After 5 days, the cells were pulsed with ^{3}H-thymidine and, following incubation for 20–24 h, ^{3}H-thymidine uptake was determined with a micro-beta counter.

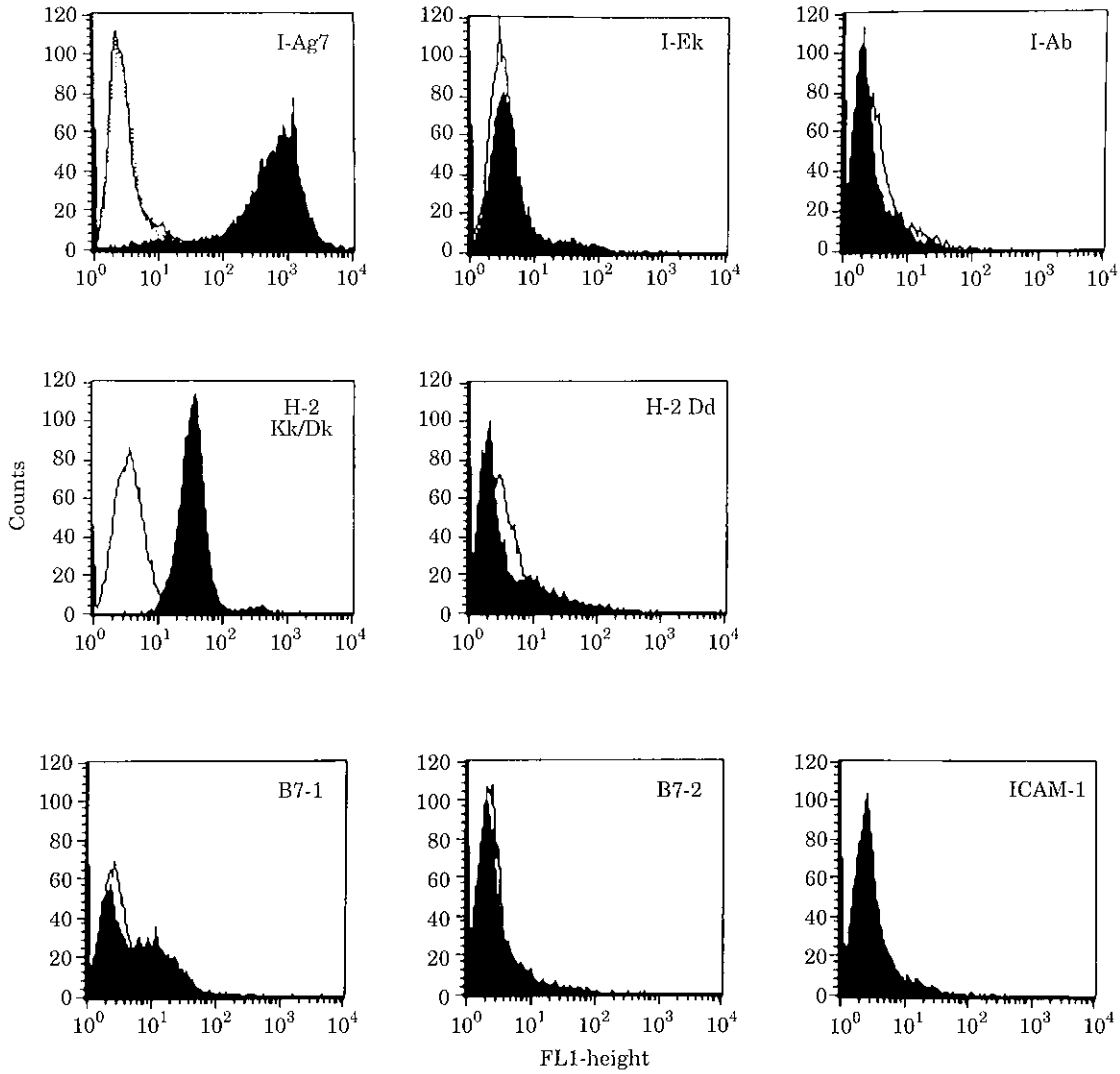

Figure 1. Phenotype of DAP.3A^{g7} cells. Flow cytometric analysis of the expression of various cell surface molecules on I-A^{g7}-transfected L cells (DAP.3A^{g7}). Cells (1×10^6 per group) were stained with specific monoclonal antibodies (black profiles) or PBS (white profiles) followed by the appropriate, FITC-labelled secondary antibodies, and staining was analysed by flow cytometry. The broken line on the top-left profile represents staining with primary (10.2.16) and secondary antibody of non-transfected DAP.3 cells.

Results

Phenotypic characterization of the I-A^{g7}-transfected (DAP.3A^{g7}) cells

The first set of experiments was designed to examine the level of surface expression of I-A^{g7} on the transfected cells as well as the level of surface expression of other molecules involved in Ag presentation. After transfection and selection, confluent G418-resistant cells were examined for I-A^{g7} expression by flow cytometry. DAP.3A^{g7} cells were harvested and stained with the 10.2.16 monoclonal antibody. This antibody, initially derived against the I-A^k molecule, was shown previously to cross-react with the I-A^{g7} molecule [37]. As shown in Figure 1, DAP.3A^{g7} cells express high levels of I-A^{g7}. The 10.2.16 staining is not due to cross-reactivity with the endogenous class II MHC molecules (I-A^k) expressed by the DAP.3 cells for two reasons. First, it has been shown previously that DAP.3 cells do not express any detectable levels of their endogenous class II MHC genes [33]. Second, we did not detect any staining with 10.2.16 antibodies in non-transfected DAP.3 cells or when DAP.3 cells were transfected with irrelevant MHC cDNAs, indicating that staining with 10.2.16 requires transfection of the I-A^{g7} cDNAs (Figure 1). As expected, DAP.3A^{g7} cells do not express I-E^k, or I-A^b MHC class II molecules (Figure 1). DAP.3A^{g7} cells do, however, express the MHC class I molecules from the original DAP.3 cells (K^k/D^k) (Figure 1). This is of potential interest given the NOD MHC class I gene background (H-2K^d and D^b), because the difference in class I MHC molecules may determine alloreactivity of NOD-generated T-cell clones or hybridomas to DAP.3A^{g7} cells.

Full activation of fresh and cloned T cells requires costimulation, provided by different molecules on the surface of the APC [39]. Among these, the B7 family of

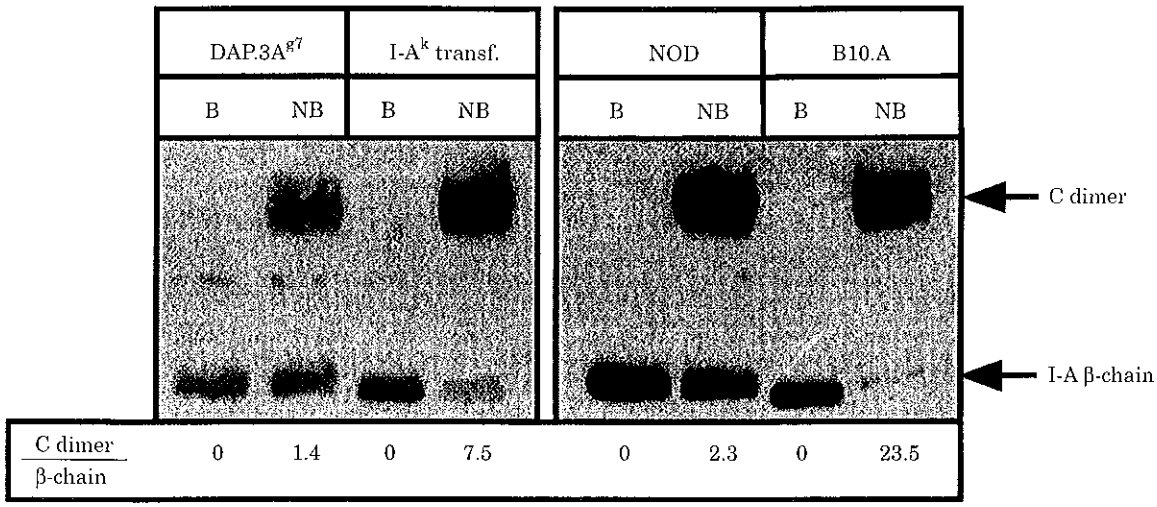

Figure 2. Low proportion of I-A^{g7} compact dimers in transfected DAP.3 cells and NOD splenocytes. Cell lysates from DAP.3A^{g7} or I-A^{k}-transfected DAP.3 cells (460,000 cell equivalent per lane) or from NOD or B10.A splenocytes (1.15×10^{6} cell equivalent per lane) were run in SDS-PAGE in Laemmli's sample buffer directly or after boiling for 5 min. The gel was transferred to a PVDF membrane and immunoblotted with 10.2.16 monoclonal antibody and signals developed by chemiluminescence. Signal intensity was measured by densitometry and expressed as a compact dimer to β-chain arbitrary unit ratio for each group.

molecules is the most extensively characterized [40, 41]. It was of interest, therefore, to determine the expression of several costimulatory molecules on the surface of the DAP.3A^{g7} cells. DAP.3A^{g7} cells express very low levels of B7-1, and do not express detectable levels of B7-2, or ICAM-1 accessory molecules (Figure 1). Based on this result, we predicted that DAP.3A^{g7} cells may not be good stimulators of naive or cloned T cells, given the requirement of these cells for co-stimulation. However, they could still be good stimulators of T-cell hybridomas, since these are not costimulation-dependent.

Low proportion of compact I-A^{g7} molecules in non-NOD background

It has been recently suggested that I-A^{g7} molecules are poor at binding peptides [26], which is consistent with the low percentage of compact class II MHC molecules in NOD APC. This finding could be due to an intrinsic property of the I-A^{g7} molecule, or to a defect in the processing and presenting of immunogenic peptides by the NOD APC. DAP.3A^{g7} cells provide a useful tool for addressing this issue given that, in these cells, I-A^{g7} expression takes place in a non-NOD background. Thus, we examined the formation of compact dimers in DAP.3 cells transfected with I-A^{g7}- and I-A^{k} coding cDNAs. Representative results from three different experiments are shown in Figure 2. In all these experiments, we found that the proportion of compact dimers to β-chains was significantly reduced for I-A^{g7} molecules compared with I-A^{k} molecules. This was the case for both the splenocytes and the transfected DAP.3 cells, although the proportion of compact dimers to β-chains was lower for both haplotypes in transfected DAP.3 cells than in splenocytes. A possible explanation for this is that the expression of class II MHC molecules in I-A^{g7}- and I-A^{k}-transfected DAP.3 cells takes place in cells expressing very low levels of Ii (Figure 3). However, in contrast to reports on bone-marrow derived APC [42], addition of CLIP did not cause any significant changes in the level of expression of I-A^{g7} II MHC molecules on DAP.3-transfected cells (data not shown). The proportion of compact forms did not significantly increase after incubation of the cells with an I-A^{g7}-binding peptide (data not shown).

DAP.3A^{g7} cells can present GAD67 to GAD-specific T cells

Given that DAP.3A^{g7} showed a decreased formation of compact I-A^{g7} dimers, it was of interest to examine the ability of these cells to process and present antigen. This was done by examining the response of an NOD-derived, GAD-specific T-cell hybridoma (E3) to GAD67. As shown in Figure 4A, E3 T cells produced significant amounts of IL-2 in response to GAD presented by DAP.3A^{g7} cells. There was no IL-2 production in cultures of E3 T cells with DAP.3A^{g7} cells in the absence of GAD67, excluding the possibility of E3 T-cell alloreactivity to DAP.3A^{g7} cells. The E3 response to GAD presented by DAP.3A^{g7} cells was significantly stronger than the response to GAD presented by NOD splenocytes as previously seen with other cloned T cells of different specificity responding to other MHC class II-transfected L cells (data not shown).

The IL-2 response of E3 to GAD presented by DAP.3A^{g7} could be the result of GAD67 processing and classical presentation of GAD67-derived peptides bound to the groove of the I-A^{g7} molecules, or could be secondary to T-cell stimulation by peptides bound

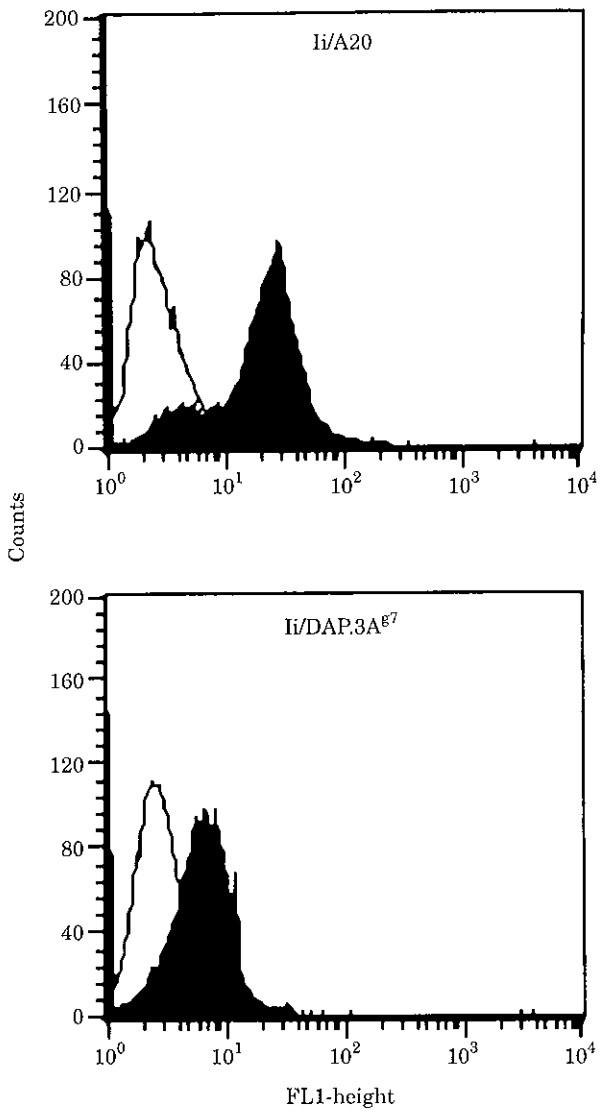

Figure 3. Flow cytometric analysis of the expression of invariant chain by DAP.3A^{g7} cells. DAP.3A^{g7} or A20 cells were made permeable with saponin, and stained for invariant chain using the In-1 antibody.

in alternative sites as previously reported for superantigens or certain peptides [43]. This is unlikely given that the response of E3 was not seen with all the alleles tested. E3 T cells were cultured with DAP.3A^{g7} cells, I-A^{d}-transfected cells (RT2.2.3), or I-A^{k}-transfected cells (RT7.3), in the absence or presence of GAD67. As shown in Figure 4B, E3 T cells produced IL-2 in response to GAD67 presented by DAP.3A^{g7}. A similar response was observed for E3 T cells cultured with GAD67 and RT2.2.3, but not with GAD67 and RT7.3 cells. This response was GAD67-specific and was not observed in response to other proteins, including human GAD65 (Figure 4C). In addition, the E3 response to GAD67 and DAP.3A^{g7} cells, but not to GAD67 and RT2.2.3. cells, was I-A^{g7}-dependent because it was completely blocked with monoclonal antibodies against I-A^{g7} (10.2.16) (Figure 4D). From these experiments, we concluded that the I-A^{g7} molecules were critical for the presentation of GAD67 to a GAD-specific, I-A^{g7}-restricted T-cell hybridoma. However, the E3 TCR recognition event showed some degeneracy, as indicated by the response of E3 to GAD67-derived peptides when presented by I-A^{d}-transfected DAP.3 cells. This degeneracy was applicable to GAD67 recognition, but not to peptides derived from other proteins (data not shown).

Next, we investigated whether stimulation of IL-2 production by E3 T cells required GAD67 processing. The IL-2 response by E3 T cells in response to GAD67 and DAP.3A^{g7} was completely abolished when GAD67 was incubated with paraformaldehyde-fixed DAP.3A^{g7} (Figure 5A). However, DAP.3A^{g7} cells pulsed with GAD67 and subsequently fixed were still able to present GAD67 to E3 T cells and stimulate IL-2 production by these cells. Thus, stimulation of T cells by GAD67 presented by DAP.3A^{g7} requires an intact processing machinery.

Finally, we examined the allostimulatory potential of DAP.3A^{g7} cells to fresh T cells. As mentioned before, DAP.3A^{g7} cells express very little B7-1 compared to splenocytes. Therefore, it was unlikely that they would have allostimulatory potential for primary T cells. This prediction was confirmed by using DAP.3A^{g7} cells as stimulators in mixed lymphocyte reactions with splenocytes from NOD, BALB/c, and B10.A mice as responders. In all three cases, there was no proliferative response (Figure 5B).

Discussion

The experiments described here were carried out to develop and characterize an antigen-presenting cell suitable for studying different biochemical and immunological aspects of IDDM in NOD mice. In particular, we were interested in addressing the biology of antigen presentation in the context of the I-A^{g7} class II MHC molecule. As we show in this report, the phenotype and function of the DAP.3A^{g7} cell line indicate that this cell line is a valuable tool for addressing this issue, given the high level of expression of the I-A^{g7} molecule and the ability of this cell line to process and present to T cells one potential autoantigen, GAD67, involved in the development of IDDM in NOD mice.

It has been recently reported that the proportion of compact dimers generated by I-A^{g7} molecules is lower than the proportion generated by MHC class II molecules from other haplotypes [26, 27]. This finding raises two questions: what is the cause for such low generation of compact dimers, and what is the relevance of low compact-form generation to peptide binding and the pathogenesis of IDDM. The cause is not known. Compact dimers are formed by peptide loading into class II MHC molecules, stabilizing the class II molecules [29, 32]. Thus, low generation of compact dimers may be secondary either to an intrinsic defect of the class II MHC molecule, causing poor stability of the molecule, or to a defect in the peptide-loading step, either in the machinery involved in the

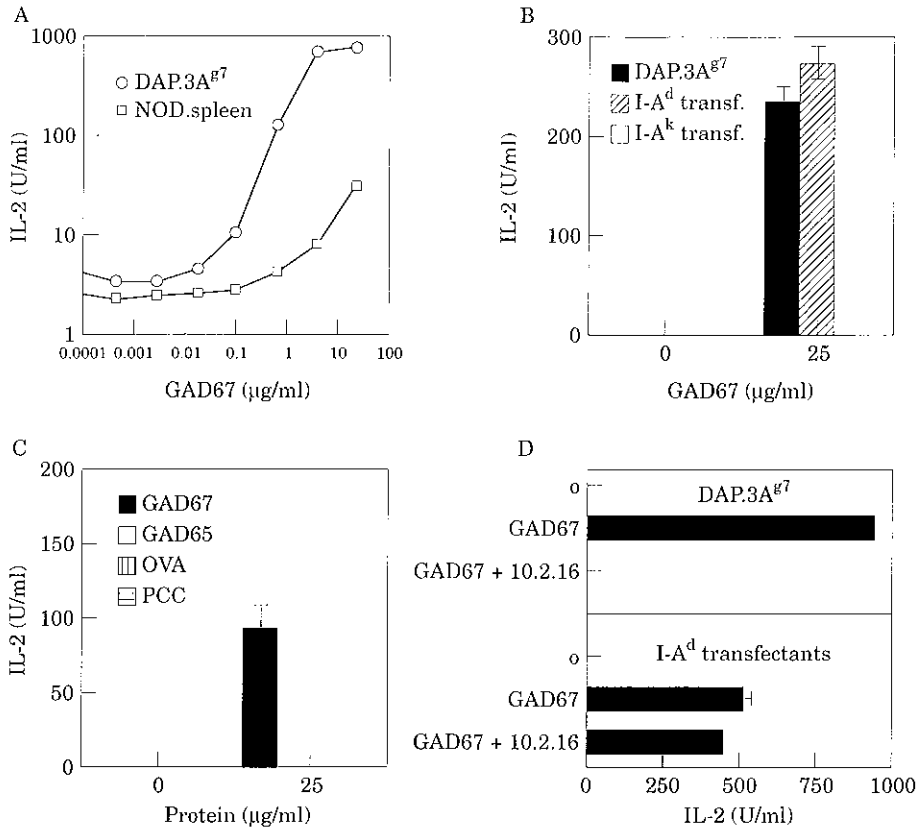

Figure 4. Functional characterization of DAP.3A^{g7} cells. (A) DAP.3A^{g7} can present GAD67 to a GAD67:I-A^{g7} specific T-cell hybridoma. E3 T cells (50,000/well) were incubated with DAP.3A^{g7} cells (50,000/well) or NOD splenocytes (1×10^6 cells/well) in the absence or presence of increasing concentrations of GAD67 protein, at 37°C for 24 h. Supernatants were then collected and the concentration of IL-2 in them was measured by ELISA. (B) Production of IL-2 by E3 T cells in response to GAD67 presented by I-A^{g7}-, I-A^{d}-, or I-A^{k}-transfected DAP.3 cells. Set-up similar to that in A. (C) The IL-2 response of E3 T cells is specific to GAD67 presented by DAP.3A^{g7} cells. (D) The E3 IL-2 response to GAD67:DAP.3A^{g7} is I-A^{g7}-dependent. E3 T cells (50,000/well) were incubated with DAP.3A^{g7} cells (50,000/well) or I-A^{d}-transfected DAP.3 cells (50,000/well) and GAD67 (25 μg/ml) in the absence or presence of a 1:40 dilution of a supernatant from a 10.2.16 monoclonal antibody-producing hybridoma culture. IL-2 in 24 h supernatants was measured by ELISA.

loading or in the peptide species being loaded. Our results favour the first interpretation: the low proportion of I-A^{g7} compact dimers is a consequence of an intrinsic feature of the I-A^{g7} molecule. This result was suggested by Carrasco-Marin *et al.* based on the observation that the low generation of compact dimers is still observed in (NOD×B6) F_1 splenocytes [26]. However, this type of experiment would not take into account the possibility of a dominant defect in Ag processing in NOD splenocytes. The structural basis for the low generation of compact dimers remains to be established. Obvious candidates are the histidine and serine residues at positions 56 and 57 of the I-A^{g7} β-chain, which are associated with an increased susceptibility to IDDM [16]. However, Carrasco-Marin *et al.* demonstrated that changing these residues for the residues present in other haplotypes did not enhance the generation of I-A^{g7} compact forms [26].

We have confirmed that the generation of compact dimers of I-A^{g7} molecules is low compared to other haplotypes and that this is an intrinsic feature of the I-A^{g7} molecules, as it occurs upon expression of I-A^{g7} molecules in non-NOD cells. However, it is not clear whether the low generation of compact dimers indicates an intrinsically poor peptide-binding capacity. In our system, we consider this not to be the case, because if peptide binding to I-A^{g7} molecules is abnormal, then one would expect the expression of I-A^{g7} molecules to be more dependent on Ii expression, given that Ii plays a critical role in the stabilization of class II MHC molecules in their transport to the loading compartment [44–46]. Different MHC class II haplotypes show different dependencies on Ii, H-2^{k} and H-2^{d} being less dependent on Ii expression than H-2^{b} haplotypes [47]. However, we did not see any disproportionate reduction in the percentage of I-A^{g7} compact dimers, compared to I-A^{k}, in conditions of limited expression of Ii. On the contrary, the reduction in compact dimer generation in conditions of limited Ii expression was proportionally greater for I-A^{k} (a low Ii-dependent haplotype) than for I-A^{g7}. This could indicate that the I-A^{g7} molecule has a low requirement for Ii in DAP.3 transfectants, the expression of Ii is low, and Ii may not be essential for class II MHC surface expression, as already reported for Ltk fibroblasts [48].

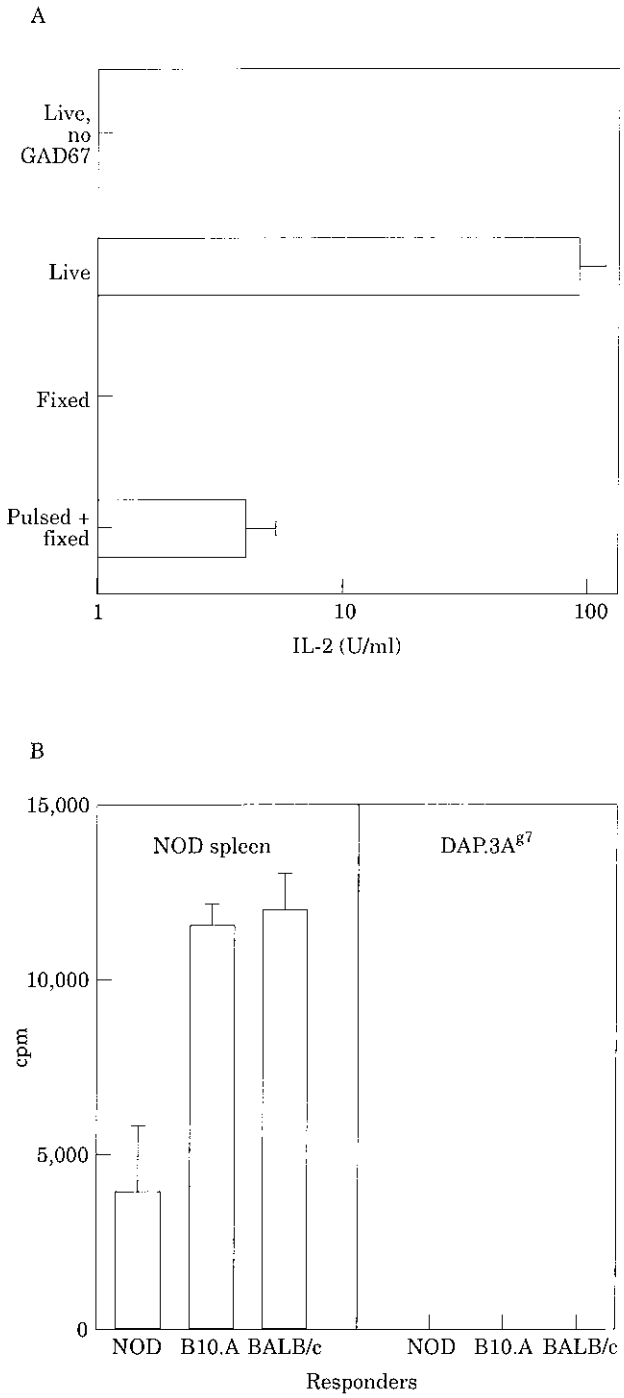

Figure 5. Functional characterization of DAP.3A^{g7} cells. (A) The IL-2 response to GAD67 presented by DAP.3A^{g7} requires processing. E3 T cells (50,000/well) were incubated for 24 h with GAD67 (25 μg/ml) and live or fixed (0.9% paraformaldehyde-treated) DAP.3A^{g7} cells, or fixed DAP.3A^{g7} cells having been pulsed with 25 μg/ml of GAD67 at 37°C for 2 h. IL-2 in 24 h-supernatants was measured by ELISA. (B) DAP.3A^{g7} are not good stimulators of MLR. Proliferation of red blood cell-depleted NOD, BALB/c, and B10.A splenocytes to splenocyte preparations or to DAP.3A^{g7} cells was measured by ^{3}H-thymidine uptake after culturing a suspension of splenocytes (4×10^{5} cells/well) as responders with mitomycin-treated DAP.3A^{g7} cells (4×10^{5} cells/well) as stimulators, or with irradiated splenocytes (4×10^{5} cells/well), in triplicate, after a 5-day culture.

Our results show that the low generation of compact dimers is not associated with lack of T-cell response to Ag presented by DAP.3A^{g7} cells, implying that the functional repercussion of low formation of compact forms may not be limiting for T-cell responses. This is still a controversial issue. On one hand, Carrasco-Marin *et al.* failed to show a biochemical interaction between peptides and I-A^{g7} molecules, and suggested that this may cause a defect in T-cell selection contributing to autoreactivity [26]. On the other hand, Harrison *et al.* [28] using purified I-A^{g7} molecules, and Reizis *et al.* [27], using activated peritoneal macrophages, showed good peptide binding. This would be consistent with preserved Ag presentation and induction of T-cell responses. The preservation of T-cell responsiveness despite low compact dimer formation would be further supported by the fact that only 100–200 peptide–MHC complexes are required for T-cell stimulation [49, 50]. The proportion of compact dimers required for activation is not known.

Since DAP.3 cells do not normally act as APC, we examined antigen processing and presentation by these cells. We used GAD67, and made use of a GAD67-specific T-cell hybridoma. GAD67 was used as antigen since it appears to be the predominant GAD isoform expressed in mouse islets [36, 51] and previous work by Tisch *et al.* [52] has supported the assumption that GAD is a major target autoantigen of the autoimmune response in NOD mice. Furthermore, T-cell hybridomas were used because, in contrast to T-cell clones, hybridomas do not need costimulation for their activation. This was advantageous in this study since B7-1 was the only costimulatory molecule expressed (at low levels) on these L cells. In fact, the effect of a lack of the costimulatory signal molecule was seen with MLR. DAP.3A^{g7} cells used as stimulators were unable to induce proliferation of splenocytes of BALB/c, NOD, or B10.A mice. Our data indicate that DAP.3A^{g7} cells are capable of processing and presenting GAD67 protein to specific T cells in a conventional way.

In summary, we have generated an I-A^{g7} transfected DAP.3 cell line that can be used as antigen-presenting cell line and is useful in analysing antigen processing and presentation and T-cell activation in IDDM. Using this cell line, we have shown that the low generation of compact forms of the I-A^{g7} molecule is the result of an intrinsic defect of this class II MHC molecule, and is not dependent on the NOD cellular environment. This cell line can be further used to dissect the molecular basis of this abnormality.

Acknowledgements

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DAP cells were derived from NIH 3T3 cells.

Designations:	NIH/3T3
Biosafety Level:	1
Shipped:	frozen
Medium & Serum:	See Propagation
Growth Properties:	adherent
Organism:	*Mus musculus* (mouse)
Morphology:	fibroblast

Source:	**Organ:** embryo **Strain:** NIH/Swiss **Cell Type:** fibroblast fibroblast;
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.
Applications:	transfection host (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents)
Virus Susceptibility:	Murine leukemia virus
Age:	embryo
Comments:	The NIH/3T3 is highly sensitive to sarcoma virus focus formation and leukemia virus propagation and has proven to be very useful in DNA transfection studies [PubMed ID: 222457]. Tested and found negative for ectromelia virus (mousepox).
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Dulbecco's Modified Eagle's Medium, Catalog No. 30-2002. To make the complete growth medium, add the following components to the base medium: bovine calf serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C **Growth Conditions:** The serum used is important in culturing this line. Calf serum is recommended and not fetal bovine serum. The calf serum initially employed and found to be satisfactory was from the Colorado Serum Co. Denver.
Subculturing:	**Protocol:** 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. 6. Incubate cultures at 37C. DO NOT ALLOW THE CELLS TO BECOME CONFLUENT! Subculture at least twice per week at 80% confluence or less. **Subcultivation Ratio:** Inoculate 3 to 5 X 10(3) cells/cm2 **Medium Renewal:** Twice per week
Preservation:	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2002
References:	22370: Jainchill JL, et al. Murine sarcoma and leukemia viruses: assay using clonal lines of contact inhibited mouse cells. J Virol. 4: 549-553

Related Links

NCBI Entrez Search

Cell Micrograph

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Frequently Asked Questions

Material Transfer Agreement

Technical Support

Related Cell Culture Products

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Product Information Sheet

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Notices and Disclaimers

ATCC products are intended for laboratory research purposes only unless noted otherwise They are not intended for use in humans

While ATCC uses reasonable efforts to include accurate and up-to-date information on this site ATCC makes no warranties or representations as to its accuracy Citations from scientific literature and patents are provided for informational purposes only ATCC does not warrant that such information has been confirmed to be accurate

All prices are listed in U S dollars and are subject to change without notice A discount off the current list price will be applied to most cultures for nonprofit institutions in the United States Cultures that are ordered as test tubes or flasks will carry an additional laboratory fee Fees for permits shipping and handling may apply

Back to my Search

Modification Form for Permit BIO-RRI-0020

Permit Holder: Joaquin Madrenas

Approved Personnel
(Please stroke out any personnel to be removed)

Thu Chau

Luan Chau

Samar Sayedyahossein

Darah Christie

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	Superantigen	Commercially available S. aureus cell wall components Peptidoglycan (Sigma, cat# 77140) PAM2CSK4 (TLRL-PAM2, InVIVOGEN) PAM3CSK4 (TLRL-PMS, InVIVOGEN)
Approved Cells	Human (established) - E. 6.1 JurKats, HEK 293 E, Rodent (primary, established), [Primary] - (Human): blood, cell lines. (Rodent) - Mice	
Approved Use of Human Source Material	blood, h. CTLA4 pbig2i Plasmid. Peritoneal fluid, human SLP-2-gfp cDNA in pBIg2i plasmid.	Human TLR1, TLR2, and TLR6 cDNAs ready for expression in mammalian cells and signalling studies with S. aureus cell wall components.
Approved GMO	E. Coli DH5 Alpha. (Plasmid) - pBIG2i	
Approved use of Animals	2007-078-12 for MICE	
Approved Toxin(s)	superantigen, SEE (Staphylococcal Enterotoxin E)	

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form. found at http://www.wph.uwo.ca.

Signature of Permit Holder: Adriani

Classification: 2

Date of Last Biohazardous Agents Registry Form: Sep 23, 2009

Date of Last Modification (if applicable): Feb 4, 2010

BioSafety Officer(s): Stanley Mar 16/10 Ronald Mar 04, 2010

Chair, Biohazards Subcommittee: G. Kidd

We are planning to study whether signalling from TLR2/1 and TLR2/6 complexes are different, both quantitatively and qualitatively, in response to ligands of TLR2 from the cell wall of the microbe Staphylococcus aureus. To do these experiments, we will transfect these constructs into fibroblasts and H293 cells, develop stable clones expressing these cDNAs, and examine their signalling response to these ligands, following standard protocols already available in my laboratory.

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Peptidoglycans (PGN)

Cat No.	
77145	Peptidoglycan dyed with Remazol Brilliant Blue R from Staphylococcus aureus
69554	Peptidoglycan from Bacillus subtilis
78721	Peptidoglycan from Methanobacterium sp.
53243	Peptidoglycan from Micrococcus luteus
72789	Peptidoglycan from Saccharomyces cerevisiae
77140	Peptidoglycan from Staphylococcus aureus
79682	Peptidoglycan from Streptomyces sp.

Product Description:
Most bacteria have a cell wall containing a special polymer called peptidoglycan. Over the cell membrane is a shift of peptidoglycan and other polymers including teichoic and teichuronic acids. This peptidoglycan gives a certain rigidity to the cell wall and gives the cell mechanical strength.
The bacterial cell wall is a unique biopolymer, it contains both D- and L-amino acids. Its basic structure is a carbohydrate backbone of alternating units of N-acetyl glucosamine and N-acetyl muramic acid. The N-acetyl muramic acid residues are cross-linked with oligopeptides. The terminal peptide is D-alanine although other amino acids are present as D- isomers. This is the only known biological molecule that contains D-amino acids and it is the target of numerous antibacterial antibiotics e.g. pencillin. Penicicllin inhibits the enzymes transpeptidase and carboxypeptidase, which are responsible for the building of peptidoglycan. Lysozyme, present in the tears liquid, is able to split the peptidoglycan between the N-acetyl glucosamine and N-acetyl muramic. The cell wall of Gram-positive bacteria is largely made up of pepidoglycan. There may be up to 40 layers of this polymer, conferring enormous mechanical strength on the cell wall. [3]
The primary immune recognition is based on structures common among invading pathogens. Surface molecules, such as lipopolysaccharide (LPS), peptidoglycan and peptidoglycan recognition protein (PGRP), are known to elicit immune reactions ranging from cytokine release to fever. [4-6]

Applications:
Used for the activity estimation of lytical enzymes (e.g. Lyticase). All peptidoglycan can be used for this purpose but Fluka 77145 can be used as chromogenic substrate for lytical enzymes. [1] It is recommended to use a peptidoglycan concentration of 0.15 – 3mg/l in water or buffer and measuring at 450 nm. The peptidoglycan can not be solubilized but it is possible to make a suspension. For the peptidoglycan dyed with remazol brilliant blue R (Fluka 77145) a possible working suspension is 3 mg/l glycin-buffer (0.2M, pH 10) which cam be measured at 595 nm. [7]
For the stimulation of lymphocytes: Peptidoglycan activates the Toll-like receptor 2 (TLR2), present in mammalian cells. Work as an antagonist of Poly (I:C).[2]

References:
1. R. Zhou, et al., Substrate for the determination of lysostaphin activity, Anal. Biochem., 171, 141 (1988)
2. L. Alexopoulou, et al. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3, Nature, Vol 413, 732 (2001)
3. D.E. Stewart-Tull, Major component of the cell wall in gram positive organisms. Consists of a glycan backbone with alternating β 1-4 linked residues of N-acetyl-D-glucosamine and muramic acid. The immunological activities of bacterial peptidoglycans, Ann. Rev. Microbiol. 34, 311 (1980)
4. K.H. Schleifer, O. Kandler, Peptidoglycan types of bacterial cell walls and their taxonomic implications, Bact. Rev. 36, 407 (1972)
5. L.J. Wheat, et al., Antibody response to peptidoglycan during staphylococcal infections, J. Inf. Dis. 147, 16 (1983)
6. Doyle R.J., Dziarski R., in Molecular Medical Microbiology (Susmsman M., ed.) p137-153, Academic Press (2001).
7. Fluka quality control or production data.

SIGMA-ALDRICH

Material Safety Data Sheet

Version 3.0
Revision Date 01/02/2009
Print Date 12/08/2009

1. PRODUCT AND COMPANY IDENTIFICATION

Product name	:	Peptidoglycan, from *Staphylococcus aureus*
Product Number	:	77140
Brand	:	Sigma
Company	:	Sigma-Aldrich Canada, Ltd 2149 Winston Park Drive OAKVILLE ON L6H 6J8 CANADA
Telephone	:	+19058299500
Fax	:	+19058299292
Emergency Phone #	:	800-424-9300

2. COMPOSITION/INFORMATION ON INGREDIENTS

CAS-No.	EC-No.	Index-No.	Concentration
Peptidoglycan			
-	-	-	-

3. HAZARDS IDENTIFICATION

WHMIS Classification

Not WHMIS controlled.

Not WHMIS controlled.

HMIS Classification

Health Hazard: 0
Flammability: 0
Physical hazards: 0

Potential Health Effects

Inhalation May be harmful if inhaled. May cause respiratory tract irritation.
Skin May be harmful if absorbed through skin. May cause skin irritation.
Eyes May cause eye irritation.
Ingestion May be harmful if swallowed.

4. FIRST AID MEASURES

If inhaled

If breathed in, move person into fresh air. If not breathing give artificial respiration

In case of skin contact

Wash off with soap and plenty of water.

In case of eye contact

Flush eyes with water as a precaution.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water.

5. FIRE-FIGHTING MEASURES

Flammable properties

Flash point	no data available
Ignition temperature	no data available

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Special protective equipment for fire-fighters
Wear self contained breathing apparatus for fire fighting if necessary.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Avoid dust formation.

Environmental precautions
Do not let product enter drains.

Methods for cleaning up
Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Handling
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Storage
Keep container tightly closed in a dry and well-ventilated place.

Recommended storage temperature: -20 °C

Store under inert gas. Moisture sensitive.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
Respiratory protection is not required. Where protection from nuisance levels of dusts are desired, use type N95 (US) or type P1 (EN 143) dust masks. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
For prolonged or repeated contact use protective gloves.

Eye protection
Safety glasses

Hygiene measures
General industrial hygiene practice.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	solid

Safety data

pH	no data available
Melting point	no data available
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Water solubility	no data available

10. STABILITY AND REACTIVITY

Storage stability
Stable under recommended storage conditions.

Conditions to avoid
Avoid moisture.

Materials to avoid
Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Nature of decomposition products not known.

11. TOXICOLOGICAL INFORMATION

Acute toxicity

no data available

Irritation and corrosion

no data available

Sensitisation

no data available

Chronic exposure

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as probable, possible or confirmed human carcinogen by IARC.

Signs and Symptoms of Exposure

To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

Potential Health Effects

Inhalation	May be harmful if inhaled. May cause respiratory tract irritation.
Skin	May be harmful if absorbed through skin. May cause skin irritation.

Eyes	May cause eye irritation.
Ingestion	May be harmful if swallowed.

12. ECOLOGICAL INFORMATION

Elimination information (persistence and degradability)

no data available

Ecotoxicity effects

no data available

Further information on ecology

no data available

13. DISPOSAL CONSIDERATIONS

Product
Observe all federal, state, and local environmental regulations.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

DSL Status
This product contains the following components that are not on the Canadian DSL nor NDSL lists.

	CAS-No.
Peptidoglycan	-

WHMIS Classification

Not WHMIS controlled.

Not WHMIS controlled.

16. OTHER INFORMATION

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Co., shall not be held liable for any damage resulting from handling or from contact with the above product. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Pam2CSK4

Synthetic bacterial lipoprotein - TLR2-TLR6 ligand

Catalog # tlrl-pam2

For research use only

Version # 05G12-MT

PRODUCT INFORMATION

Content:

- 100 µg Pam2CSK4 x 3TFA
- 2 ml endotoxin-free water

Storage and stability:

- Pam2CSK4 is provided as a lyophilized powder and shipped at room temperature. Store at -20°C.
- Upon resuspension, Pam2CSK4 should be aliquoted and stored at 4°C for short term storage or -20°C for long storage.
- Product is stable 1 month at 4°C and 6 months at -20°C when properly stored.

DESCRIPTION

Pam2CSK4 is a synthetic diacylayed lipopeptide (LP). Bacterial lipoproteins are strong immune modulators that activate early innate host responses after infection. LP analogues of these lipoproteins signal either through TLR2/1 or TLR2/6 heterodimers. According to the current model, triacylated LP like Pam3CSK4, are recognized by TLR2/1, whereas diacylated LP, such as FSL1, induce signaling through TLR2/6. However, it was recently reported that diacylated LP, such as Pam2CSK4, induce signaling in a TLR6-independent manner[1]. This finding suggests that both the lipid and peptide part of lipoproteins take part in the specificity of recognition by TLR2 heterodimers.

1. Buwitt-Beckmann U. *et al.*, 2005. Toll-like receptor 6-independent signaling by diacylated lipopeptides. Eur J Immunol. 35(1):282-9.
2. Schindler U. & Baichwal VR., 1994. Three NF-kB binding sites in the human E-selectin gene required for maximal tumor necrosis factor alpha-induced expression. Mol Cell Biol, 14(9):5820-5831

CHEMICAL PROPERTIES

Chemical name: S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine x 3 CF3COOH

Formula: $C_{65}H_{126}N_{10}O_{12}S$, 3TFA

Molecular weight: 1271.85 - 342.1

Solubility: soluble in water, saline and aqueous buffers at pH<7.5

Endotoxin level: <0.125 EU/mg

METHODS

Preparation of stock solution (1 mg/ml)

Stimulation of TLR2-TLR6 can be achieved with 1-100 ng/ml Pam2CSK4.

- Add 100 µl endotoxin-free water (provided) and vortex for 30 seconds or until complete solubilization.
- Prepare further dilutions by adding the appropriate amount of endotoxin-free water .

Pam2CSK4 stimulation

- Transfect your cell line with an NF-κB-inducible reporter plasmid, i.e. a plasmid carrying a reporter gene, such as SEAP or luciferase, under the control of an NF-κB-inducible ELAM-1 (E-selectin) promoter[2].

Note: InvivoGen provides pNiFty, a family of NF-κB-inducible reporter plasmids that can be transfected transiently (pNiFty) or stably (pNiFty2). pNiFty plasmids are available either with the SEAP or luciferase reporter genes (see Related Products).

If your cell line does not naturally express TLR2 (and TLR6), cotransfect with a plasmid expressing TLR2 such as pUNO-TLR2 (or a plasmid co-expressing both TLRs, such as pDUO-TLR6/TLR2).

- Twenty-four to forty-eight hours after transfection, stimulate cells with 1 to 100 ng/ml Pam2CSK4 for 6 hours to 24 hours.
- Determine Pam2CSK4 stimulation on TLR2-TLR6 by assessing reporter gene expression using the appropriate detection system.

RELATED PRODUCTS

Product	Catalog Code
pNiFty-Luc (Amp^R)	pnifty-luc
pNiFty-SEAP (Amp^R)	pnifty-seap
pNiFty2-Luc (Zeo^R)	pnifty2-luc
pNiFty2-SEAP (Zeo^R)	pnifty2-seap
pUNO-hTLR2 (human gene)	puno-htlr2
pUNO-mTLR2 (mouse gene)	puno-mtlr2
pDUO-hTLR6/2 (human genes)	pduo-htlr62
pDUO-mTLR6/2 (mouse genes)	pduo-mtlr62

TECHNICAL SUPPORT
Toll free (US): 888-457-5873
Outside US: (+1) 858-457-5873
Europe: +33 562-71-69-39
E-mail: info@invivogen.com
Website: www.invivogen.com

InvivoGen
3950 Sorrento Valley Blvd. Suite A
San Diego, CA 92121 - USA

Section 1 - Product and Company Information

Product Name: **Pam2CSK4**

Cat. Code: tlrl-pam2

Company identification:

InvivoGen, 3950 Sorrento Valley Blvd, Suite 100
San Diego, California 92121, USA
(+1) 858 457 5873

Cayla-InvivoGen, 5 rue Jean Rodier
31400 Toulouse, FRANCE
+33 (0) 5 62 71 69 39

Section 2 - Composition/Information on Ingredient

Substance Name: Synthetic diacylated lipoprotein;
S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine x 3 CF3COOH
Formula: $C_{65}H_{126}N_{10}O_{12}S$, 3TFA
CAS #: N/A **EC number:** N/A

Section 3 – Hazards Identification

Emergency Overview: OSHA Hazards: No known OSHA hazards.

HMIS Classification: Health Hazard **0,** Flammability Hazard **0,** Reactivity Hazard **0**
NFPA Rating: Health Hazard **0,** Fire **0,** Reactivity Hazard **0**

Potential Health Effects
- **Eye contact:** May cause eye irritation.
- **Skin contact:** May cause skin irritation.
- **Skin absorption:** May be harmful if absorbed through the skin.
- **Inhalation:** May be harmful if inhaled. May cause respiratory tract irritation.
- **Ingestion:** May be harmful if swallowed.

Section 4 – First Aid Measures

General advice: Consult a physician. Show this material safety data sheet to the doctor in attendance.
After skin contact: Immediately wash skin with soap and plenty of water. Consult a physician.
After swallowing: Never give anything by mouth to an unconscious person. Rinse mouth with water provided person is conscious. Consult a physician.
After inhalation: Remove to fresh air. If not breathing give artificial respiration. Consult a physician.
After eye contact: Immediately flush eyes with plenty of water for at least 15 minutes. Consult a physician.

Section 5 – Fire Fighting Measures

Extinguishing Media: Water spray, carbon dioxide, dry chemical powder or appropriate foam.
Special Firefighting Procedures: N/A
Flammability: N/A

Unusual Fire and Explosions Hazard (s): N/A
Flash point: N/A
Auto-ignition Temp: N/A

Section 6 – Accidental Release Measures

Personal precautions: Wear protective equipment. Keep unprotected persons away. Avoid dust formation.
Method for cleaning up: Absorb on sand or vermiculite and place in closed containers for disposal. Dispose contaminated material as waste according to section 13. Ventilate area and wash spill site after material clean-up is complete.

Section 7– Handling and Storage

Handling: Avoid contact with eyes, skin and clothing. Avoid prolonged or repeated exposure.
User Exposure: Avoid inhalation. Use personal protective equipment (i.e. impermeable gloves, lab coat or apron).
Storage: Keep tightly closed. Store at -20°C.

Section 8 – Exposure Controls / PPE

Engineering Measures: Ensure adequate ventilation, especially in confined areas.

Personal Protective Equipment
Hand: Protective gloves to prevent skin contact. **Eye:** Chemical safety goggles
General Hygiene Measures: Wash hands thoroughly after handling.

Section 9 – Physical / Chemical Properties

Appearance: White color.
Physical State: Solid (lyophilized powder).

Section 10 – Stability and Reactivity

Stability: Stable
Hazardous Polymerization: Will not occur
Materials to avoid: Strong oxidizing agents
Hazardous Decomposition Products: Decomposition products are not hazardous.

Section 11 – Toxicological Information

Acute toxicity:
LD50 Oral: N/A
LD50 Intraperitoneal: N/A
LD50 Intravenous: N/A

Primary irritant effect: N/A
Sensitization: N/A
Target Organ: N/A
Additional toxicological information: N/A

Section 12 – Ecological Information

Ecotoxicity: Data not yet available.
Mobility: Data not yet available.
Biodegradation: Data not yet available.
Bioaccumulation: Data not yet available.

Section 13 – Disposal Considerations

Product: Observe all federal, state and local environmental regulations. Contact a licensed professional waste disposal service to dispose of this material. Must not be disposed of together with household garbage.
Contaminated Packaging: Dispose of as unused product.

Section 14 – Transport Information

DOT (US): Non-hazardous for transport.
IATA: Non-hazardous for transport.
IMDG: Non-hazardous for transport.

Section 15 – Regulatory Information

OSHA HAZARDS (US): No known OSHA Hazards
DSL Status: This product contains the following components that are not on the Canadian DSL nor NDSL lists.
Pam2CSK4 CAS no.: N/A
SARA 302 Component: None of the ingredients are listed.
SARA 313 Component: None of the ingredients are listed.
SARA 311/312 Hazards: None of the ingredients are listed.

U.S. State Regulations

Chemical Name	Massachusetts RTK	New Jersey RTK	Pennsylvania RTK
Pam2CSK4	-	X	X

California Proposition 65: This product does not contain chemicals listed under Proposition 65.

Labeling and risk phrase according to EU Directives: The product does not need to be labeled in accordance with EC directives or respective national laws.

Section 16 – Other Information

Disclaimer: All InvivoGen products are supplied for research and laboratory use only. Not for drug, household or other uses.

The information contained in this MSDS relates only to the material(s) designated and does not relate to use(s) in combination with any other material, process(es) and/or chemical reaction(s). InvivoGen provides this information in good faith and is based on our present knowledge. This MSDS is provided without warranty of any kind. The recipient is responsible for ensuring that, where applicable, existing laws and guidelines are observed.

Pam3CSK4

Synthetic bacterial lipoprotein - TLR2-TLR1 ligand

Catalog # tlrl-pms

For research use only

Version # 06B24-MT

PRODUCT INFORMATION

Content:

- 1 mg Pam3CSK4 x 3HCl
- 2 ml endotoxin-free water

Storage and stability:

- Pam3CSK4 is provided lyophilized and shipped at room temperature. Store at 4°C.
- Upon resuspension, Pam3CSK4 should be aliquoted and stored at 4°C for short term storage or -20°C for long storage.
- Product is stable 1 month at 4°C and 6 months at -20°C when properly stored.

DESCRIPTION

Bacterial lipoproteins are a family of proinflammatory cell wall components found in both Gram positive and Gram negative bacteria. The stimulatory activity of bacterial lipoproteins resides in their acylated amino terminus. Pam3CSK4 is a synthetic tripalmitoylated lipopeptide that mimicks the acylated amino terminus of bacterial lipoproteins. Pam3CysSerLys4 (Pam3CSK4) is a potent activator of the proinflammatory transcription factor NF-κB[1]. Recognition of Pam3CSK4 is mediated by TLR2 which cooperates with TLR1 through their cytoplasmic domain to induce the signaling cascade leading to the activation of NF-κB[2].

1. Aliprantis AO *et al.*, 1999. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. Science.285(5428):736-9.
2. Ozinsky A. *et al.*, 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. PNAS. 97(25):13766-71.
3. Schindler U. & Baichwal VR., 1994. Three NF-kB binding sites in the human E-selectin gene required for maximal tumor necrosis factor alpha-induced expression. Mol Cell Biol, 14(9):5820-5831

CHEMICAL PROPERTIES

Chemical name: N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine x 3 HCl

Formula: $C_{81}H_{156}N_{10}O_{13}S$, 3HCl

Molecular weight: 1509.6 - 109.5

Solubility: soluble in water, saline and aqueous buffers at pH<7.5

Endotoxin level: <0.125 EU/mg

METHODS

Preparation of stock solution (1 mg/ml)

Stimulation of TLR2-TLR1 can be achieved with 0.1-1 µg/ml Pam3CSK4.

- Add 1 ml endotoxin-free water (provided) and vortex for 30 seconds or until complete solubilization.
- Prepare further dilutions by adding the appropriate amount of endotoxin-free water .

Pam3CSK4 stimulation

- Transfect your cell line with an NF-κB-inducible reporter plasmid, i.e. a plasmid carrying a reporter gene, such as SEAP or luciferase, under the control of an NF-κB-inducible ELAM-1 (E-selectin) promoter[3].

Note: InvivoGen provides pNiFty, a family of NF-κB-inducible reporter plasmids that can be transfected transiently (pNiFty) or stably (pNiFty2). pNiFty plasmids are available either with the SEAP or luciferase reporter genes (see Related Products).

If your cell line does not naturally express TLR2 and TLR1, cotransfect with a plasmid co-expressing both TLRs, such as pDUO-TLR1/TLR2 (see Related Products).

- Twenty-four to forty-eight hours after transfection, stimulate cells with 0.1 to 1 µg/ml Pam3CSK4 for 6 hours to 24 hours.
- Determine Pam3CSK4 stimulation on TLR2-TLR1 by assessing reporter gene expression using the appropriate detection system.

RELATED PRODUCTS

Product	Catalog Code
pNiFty-Luc (Amp^R)	pnifty-luc
pNiFty-SEAP (Amp^R)	pnifty-seap
pNiFty2-Luc (Zeo^R)	pnifty2-luc
pNiFty2-SEAP (Zeo^R)	pnifty2-seap
pDUO-hTLR1/2 (human genes)	pduo-htlr12
pDUO-mTLR1/2 (mouse genes)	pduo-mtlr12

TECHNICAL SUPPORT
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Europe: +33 562-71-69-39
E-mail: info@invivogen.com
Website: www.invivogen.com

InvivoGen
3950 Sorrento Valley Blvd. Suite A
San Diego, CA 92121 - USA

Section 1 - Product and Company Information

Product Name: **Pam3CSK4**

Cat. Code: tlrl-pms

Company identification:

InvivoGen, 3950 Sorrento Valley Blvd, Suite 100
San Diego, California 92121, USA
(+1) 858 457 5873

Cayla-InvivoGen, 5 rue Jean Rodier
31400 Toulouse, FRANCE
+33 (0) 5 62 71 69 39

Section 2 - Composition/Information on Ingredient

Substance Name: Synthetic triacylated lipoprotein;
N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-lysyl-[S]-lysyl-[S]-lysyl-[S]-lysine x 3 HCl
Formula: $C_{81}H_{156}N_{10}O_{13}S$, 3HCl
CAS #: N/A **EC number:** N/A

Section 3 – Hazards Identification

Emergency Overview: OSHA Hazards: No known OSHA hazards.

HMIS Classification: Health Hazard **0**, Flammability Hazard **0**, Reactivity Hazard **0**
NFPA Rating: Health Hazard **0**, Fire **0**, Reactivity Hazard **0**

Potential Health Effects
- **Eye contact:** May cause eye irritation.
- **Skin contact:** May cause skin irritation.
- **Skin absorption:** May be harmful if absorbed through the skin.
- **Inhalation:** May be harmful if inhaled. May cause respiratory tract irritation.
- **Ingestion:** May be harmful if swallowed.

Section 4 – First Aid Measures

General advice: Consult a physician. Show this material safety data sheet to the doctor in attendance.
After skin contact: Immediately wash skin with soap and plenty of water. Consult a physician.
After swallowing: Never give anything by mouth to an unconscious person. Rinse mouth with water provided person is conscious. Consult a physician.
After inhalation: Remove to fresh air. If not breathing give artificial respiration. Consult a physician.
After eye contact: Immediately flush eyes with plenty of water for at least 15 minutes. Consult a physician.

Section 5 – Fire Fighting Measures

Extinguishing Media: Water spray, carbon dioxide, dry chemical powder or appropriate foam.
Special Firefighting Procedures: N/A
Flammability: N/A

Unusual Fire and Explosions Hazard (s): N/A
Flash point: N/A
Auto-ignition Temp: N/A

Section 6 – Accidental Release Measures

Personal precautions: Wear protective equipment. Keep unprotected persons away. Avoid dust formation.
Method for cleaning up: Absorb on sand or vermiculite and place in closed containers for disposal. Dispose contaminated material as waste according to section 13. Ventilate area and wash spill site after material clean-up is complete.

Section 7– Handling and Storage

Handling: Avoid contact with eyes, skin and clothing. Avoid prolonged or repeated exposure.
User Exposure: Avoid inhalation. Use personal protective equipment (i.e. impermeable gloves, lab coat or apron).
Storage: Keep tightly closed. Store at 4°C.

Section 8 – Exposure Controls / PPE

Engineering Measures: Ensure adequate ventilation, especially in confined areas.

Personal Protective Equipment
Hand: Protective gloves to prevent skin contact. **Eye:** Chemical safety goggles
General Hygiene Measures: Wash hands thoroughly after handling.

Section 9 – Physical / Chemical Properties

Appearance: White color.
Physical State: Solid (lyophilized powder).

Section 10 – Stability and Reactivity

Stability: Stable
Hazardous Polymerization: Will not occur
Materials to avoid: Strong oxidizing agents
Hazardous Decomposition Products: Decomposition products are not hazardous.

Section 11 – Toxicological Information

Acute toxicity:
LD50 Oral: N/A
LD50 Intraperitoneal: N/A
LD50 Intravenous: N/A

Primary irritant effect: N/A
Sensitization: N/A
Target Organ: N/A
Additional toxicological information: N/A

Section 12 – Ecological Information

Ecotoxicity: Data not yet available.
Mobility: Data not yet available.
Biodegradation: Data not yet available.
Bioaccumulation: Data not yet available.

Section 13 – Disposal Considerations

Product: Observe all federal, state and local environmental regulations. Contact a licensed professional waste disposal service to dispose of this material. Must not be disposed of together with household garbage.
Contaminated Packaging: Dispose of as unused product.

Section 14 – Transport Information

DOT (US): Non-hazardous for transport.
IATA: Non-hazardous for transport.
IMDG: Non-hazardous for transport.

Section 15 – Regulatory Information

OSHA HAZARDS (US): No known OSHA Hazards
DSL Status: This product contains the following components that are not on the Canadian DSL nor NDSL lists.
Pam3CSK4 CAS no.: N/A
SARA 302 Component: None of the ingredients are listed.
SARA 313 Component: None of the ingredients are listed.
SARA 311/312 Hazards: None of the ingredients are listed.

U.S. State Regulations

Chemical Name	Massachusetts RTK	New Jersey RTK	Pennsylvania RTK
Pam3CSK4	-	X	X

California Proposition 65: This product does not contain chemicals listed under Proposition 65.

Labeling and risk phrase according to EU Directives: The product does not need to be labeled in accordance with EC directives or respective national laws.

Section 16 – Other Information

Disclaimer: All InvivoGen products are supplied for research and laboratory use only. Not for drug, household or other uses.

The information contained in this MSDS relates only to the material(s) designated and does not relate to use(s) in combination with any other material, process(es) and/or chemical reaction(s). InvivoGen provides this information in good faith and is based on our present knowledge. This MSDS is provided without warranty of any kind. The recipient is responsible for ensuring that, where applicable, existing laws and guidelines are observed.

Modification Form for Permit BIO-RRI-0020

Permit Holder: Joaquin Madrenas

Approved Personnel
(Please stroke out any personnel to be removed)

~~Gaitlin Lemke~~

Thu Chau

~~Mark Kirchhof~~

Luan Chau

~~Brianne Davis~~

~~Sara Ramos~~

Samar Sayedyahossein

Darah Christie

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	Superantigen	
Approved Cells	Human (established) - E. 6.1 JurKats, HEK 293 E, Rodent (primary, established), (Primary] - (Human): blood, cell lines. (Rodent) - Mice	
Approved Use of Human Source Material	blood, h. CTLA4 pbig2i Plasmid. Peritoneal fluid	human SLP-2-gfp cDNA in pBIG2i plasmid
Approved GMO	E. Coli DH5 Alpha. (Plasmid) - pBIG2i	
Approved use of Animals	2007-078-12 for MICE	
Approved Toxin(s)	superantigen, SEE (Staphylococcal Enterotoxin E) - 1 mg maximum Feb 4, 2010 e-mail	

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

As the principal investigator, I have ensured that all of the personnel named on the form have been trained. I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca.

Signature of Permit Holder: J Madrenas

Classification: 2

Date of Last Biohazardous Agents Registry Form: Sep 23, 2009

Date of Last Modification (if applicable):

BioSafety Officer(s): J Stanley Feb 4, 2010 Ronald Rosenfeld

Chair, Biohazards Subcommittee: C.M. Kidder

USE OF THIS NEW REAGENT:
The SLP-2 protein was identified in a proteomics screening of lipid rafts of activated Jurkat T cells.

The cDNA was generated from these cells and subcloned in pBIG2i with a 3' gfp fusion.

See nucleotide and amino acid sequences attached.

We are currently using this construct to learn about the function of SLP-2.
Specific applications of this reagent are the transfection of the cDNA into the standard cell lines available in the laboratory, the induction of expression ofa chimeric SLP-2-gfp protein, and the biochemical characterization of molecular partners for this protein using standard protein biochemistry techniques.
These experiments are within the standard operating procedures of a level 2 lab.

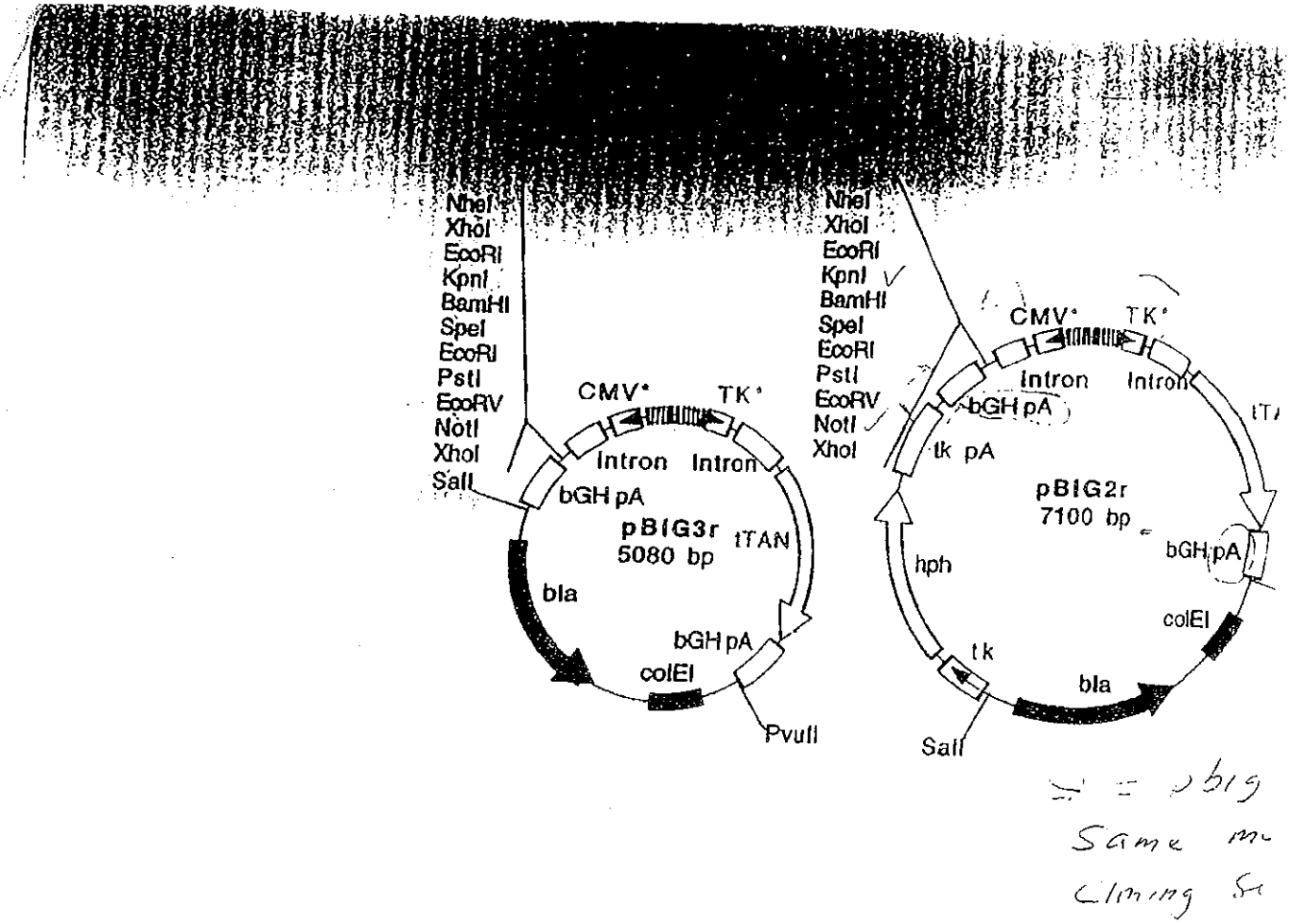


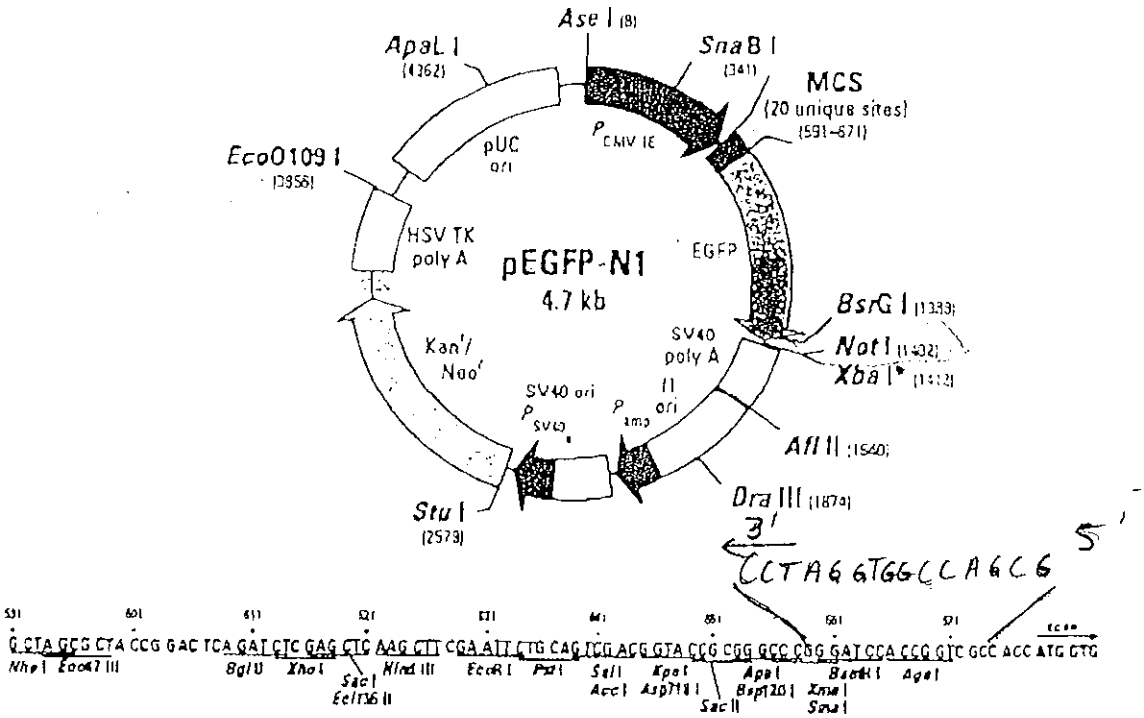
Fig.2: Autoregulated bi-directional tetracycline-responsive pBIG expression vectors. Each vector is based on a high copy number plasmid backbone containing the colE1 origin of replication and B'-lactamase gene that confers resistance to ampicillin. The bi-directional tetracycline-responsive promoter in each vector is comprised of a central tetO element, a stronger CMV* element to drive cDNA expression and a weaker TK* element to drive expression of the transactivator component.

The two vectors are essentially identical with the exception that pBIG2 contains a selectable marker conferring resistance to hygromycin B for the generation of stable cell lines. The "i" series of vectors ulilize the rtTAN transactivator such that cDNA expression is effectively induced by doxycycline.

VECTOR INFORMATION PT3027-5

pEGFP-N1 N-Terminal Protein Fusion Vector Catalog #6085-1

GenBank Accession #: U55762



Restriction Map and Multiple Cloning Site (MCS) of pEGFP-N1. (Unique restriction sites are in bold). The *Not* I site follows the EGFP stop codon. The *Xba* I site (*) is methylated in the DNA provided by CLONTECH. If you wish to digest the vector with this enzyme, you will need to transform the vector into a *dam*$^-$ host and make fresh DNA.

Description:

pEGFP-N1 encodes a red-shifted variant of wild-type GFP (1-3) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maxima = 488 nm; emission maxima = 507 nm.) pEGFP-N1 encodes the GFPmut1 variant (4) which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences (5). Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (6) to further increase the translation efficiency in eukaryotic cells. The MCS in pEGFP-N1 is between the immediate early promoter of CMV ($P_{CMV\,IE}$) and the EGFP coding sequences. Genes cloned into the MCS will be expressed as fusions to the N-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the EGFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen. A neomycin-resistance cassette (neo^r), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex thymidine kinase gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette (P_{amp}) expresses kanamycin resistance in *E. coli*. The pGFP-N1 backbone also provides a pUC19 origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

NCBI Reference Sequence: NM_013442.1

Homo sapiens stomatin (EPB72)-like 2 (STOML2), mRNA

Comment Features Sequence

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LOCUS       NM_013442               1303 bp    mRNA    linear   PRI
01-NOV-2009
DEFINITION  Homo sapiens stomatin (EPB72)-like 2 (STOML2), mRNA.
ACCESSION   NM_013442
VERSION     NM_013442.1  GI:7305502
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SOURCE      Homo sapiens (human)
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            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;
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Haplorrhini;
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REFERENCE   1  (bases 1 to 1303)
  AUTHORS   Grass,S., Preuss,K.D., Ahlgrimm,M., Fadle,N., Regitz,E.,
            Pfoehler,C., Murawski,N. and Pfreundschuh,M.
  TITLE     Association of a dominantly inherited hyperphosphorylated
            paraprotein target with sporadic and familial multiple
myeloma and
            monoclonal gammopathy of undetermined significance: a
case-control
            study
  JOURNAL   Lancet Oncol. 10 (10), 950-956 (2009)
   PUBMED   19767238
  REMARK    GeneRIF: Familial MGUS and multiple myeloma were
associated with a
            dominant inheritance of hyperphosphorylated paratarg-7
REFERENCE   2  (bases 1 to 1303)
  AUTHORS   Kirchhof,M.G., Chau,L.A., Lemke,C.D., Vardhana,S.,
Darlington,P.J.,
            Marquez,M.E., Taylor,R., Rizkalla,K., Blanca,I.,
Dustin,M.L. and
            Madrenas,J.
  TITLE     Modulation of T cell activation by stomatin-like protein
2
  JOURNAL   J. Immunol. 181 (3), 1927-1936 (2008)
   PUBMED   18641330
  REMARK    GeneRIF: SLP-2 is an important player in T cell
activation by
            ensuring sustained TCR signaling
REFERENCE   3  (bases 1 to 1303)
  AUTHORS   Green,J.B. and Young,J.P.
  TITLE     Slipins: ancient origin, duplication and diversification
of the
            stomatin protein family
  JOURNAL   BMC Evol. Biol. 8, 44 (2008)
   PUBMED   18267007
  REMARK    GeneRIF: Endosymbiotic origin of paraslipin from an
            alphaprotoebacterial ancestor (SLP-2)
            Publication Status: Online-Only
REFERENCE   4  (bases 1 to 1303)
  AUTHORS   Cao,W., Zhang,B., Liu,Y., Li,H., Zhang,S., Fu,L., Niu,Y.,
```

Ning,L.,
Cao,X., Liu,Z. and Sun,B.
TITLE High-level SLP-2 expression and HER-2/neu protein expression are
associated with decreased breast cancer patient survival
JOURNAL Am. J. Clin. Pathol. 128 (3), 430-436 (2007)
PUBMED 17709317
REMARK GeneRIF: High-level SLP-2 expression was associated with decreased
overall survival (P = .011) and was more often found in patients
with tumors larger than 20 mm, lymph node metastasis, advanced
clinical stage, distant metastasis
REFERENCE 5 (bases 1 to 1303)
AUTHORS Cui,Z., Zhang,L., Hua,Z., Cao,W., Feng,W. and Liu,Z.
TITLE Stomatin-like protein 2 is overexpressed and related to cell growth
in human endometrial adenocarcinoma
JOURNAL Oncol. Rep. 17 (4), 829-833 (2007)
PUBMED 17342323
REMARK GeneRIF: SLP-2 was overexpressed in endometrial adenocarcinoma
compared with their normal counterparts.
REFERENCE 6 (bases 1 to 1303)
AUTHORS Guo,D., Han,J., Adam,B.L., Colburn,N.H., Wang,M.H., Dong,Z.,
Eizirik,D.L., She,J.X. and Wang,C.Y.
TITLE Proteomic analysis of SUMO4 substrates in HEK293 cells under serum
starvation-induced stress
JOURNAL Biochem. Biophys. Res. Commun. 337 (4), 1308-1318 (2005)
PUBMED 16236267
REFERENCE 7 (bases 1 to 1303)
AUTHORS Rush,J., Moritz,A., Lee,K.A., Guo,A., Goss,V.L., Spek,E.J.,
Zhang,H., Zha,X.M., Polakiewicz,R.D. and Comb,M.J.
TITLE Immunoaffinity profiling of tyrosine phosphorylation in cancer cells
JOURNAL Nat. Biotechnol. 23 (1), 94-101 (2005)
PUBMED 15592455
REFERENCE 8 (bases 1 to 1303)
AUTHORS Humphray,S.J., Oliver,K., Hunt,A.R., Plumb,R.W., Loveland,J.E.,
Howe,K.L., Andrews,T.D., Searle,S., Hunt,S.E., Scott,C.E.,
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Barker,D.J., Barlow,K.F., Bates,K., Beasley,H., Beasley,O.,
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Burrill,W., Burton,J., Carder,C., Carter,N.P., Chapman,J.C.,
Chen,Y., Clarke,G., Clark,S.Y., Clee,C.M., Clegg,S., Collier,R.E.,
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Frankish,A., Frankland,J.A., French,L., Fricker,D.G., Garner,P.,
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Kershaw,J.K., Kimberley,A.M., King,A., Knights,A., Laird,G.K.,
Langford,C., Lawlor,S., Leongamornlert,D.A., Leversha,M., Lloyd,C.,
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Nickerson,T., Nisbett,J., Nordsiek,G., Pearce,A.V., Peck,A.I.,
Porter,K.M., Pandian,R., Pelan,S., Phillimore,B., Povey,S.,
Ramsey,Y., Rand,V., Scharfe,M., Sehra,H.K., Shownkeen,R.,
Sims,S.K., Skuce,C.D., Smith,M., Steward,C.A., Swarbreck,D.,
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Thomas,D.W., Wall,M., Wallis,J.M., West,A.P., Whitehead,S.L.,
Willey,D.L., Williams,S.A., Wilming,L., Wray,P.W., Young,L.,

Related sequences
Full text in PMC
GEO profiles
Gene
Gene genotype
GeneView in dbSNP
Genome
HomoloGene
Map viewer
Master
OMIM
Order cDNA clone
Probe
Protein
PubMed
PubMed (RefSeq)
PubMed (weighted)
SNP
Taxonomy
UniGene
UniSTS
mRNA genome project
LinkOut

Ashurst,J.L., Coulson,A., Blocker,H., Durbin,R., Sulston,J.E., Hubbard,T., Jackson,M.J., Bentley,D.R., Beck,S., Rogers,J. and Dunham,I.
TITLE DNA sequence and analysis of human chromosome 9
JOURNAL Nature 429 (6990), 369-374 (2004)
PUBMED 15164053
REFERENCE 9 (bases 1 to 1303)
AUTHORS Owczarek,C.M., Treutlein,H.R., Portbury,K.J., Gulluyan,L.M., Kola,I. and Hertzog,P.J.
TITLE A novel member of the STOMATIN/EPB72/mec-2 family, stomatin-like 2 (STOML2), is ubiquitously expressed and localizes to HSA chromosome 9p13.1
JOURNAL Cytogenet. Cell Genet. 92 (3-4), 196-203 (2001)
PUBMED 11435687
REFERENCE 10 (bases 1 to 1303)
AUTHORS Wang,Y. and Morrow,J.S.
TITLE Identification and characterization of human SLP-2, a novel homologue of stomatin (band 7.2b) present in erythrocytes and other tissues
JOURNAL J. Biol. Chem. 275 (11), 8062-8071 (2000)
PUBMED 10713127
COMMENT PROVISIONAL REFSEQ: This record has not yet been subject to final NCBI review. The reference sequence was derived from AF190167.1.

Publication Note: This RefSeq record includes a subset of the publications that are available for this gene. Please see the Entrez Gene record to access additional publications.

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Subject: Re: Toxin Order
From: "Dr. J. (Quim) Madrenas" <madrenas@robarts.ca>
Date: Thu, 04 Feb 2010 10:58:49 -0500
To: Jennifer Stanley <jstanle2@uwo.ca>
CC: rsn@uwo.ca, Luan Chau <luan@robarts.ca>

Thank you very much Jennifer. Greatly appreciated.

J

On 04/02/10 10:24 AM, "Jennifer Stanley" <jstanle2@uwo.ca> wrote:

> Hi Dr. Madrenas:
> Your purchase order has been approved and that Ron is working on the
> PHAC and CFIA paperwork for Cedarlane.
> Please note that this amount (1 mg) should be the maximum that you keep
> on hand in the laboratory due to biosecurity requirements.
> Regards,
> Jennifer

--
J. (Quim) Madrenas, MD PhD
Canada Research Chair in Immunobiology
Professor, Microbiology & Immunology, and Medicine
The University of Western Ontario
Head of Immunology, Robarts Research Institute
Director, FOCIS Centre for Clinical Immunology and Immunotherapeutics

Robarts Research Institute
Room 2.05, P.O. Box 5015, 100 Perth Drive
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THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: June 26, 2009
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biohazardous agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biohazards being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA)

Completed forms are to be returned to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190) for distribution to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety

PRINCIPAL INVESTIGATOR	DR. J. MADRENAS
SIGNATURE	Jmadrenas
DEPARTMENT	ROBARTS RESEARCH INST / IMMUNOLOGY
ADDRESS	100 PERTH DRIVE, LONDON, ON
PHONE NUMBER	(519) 663-5777 ext 24211
EMERGENCY PHONE NUMBER(S)	(519) 679-6862
EMAIL	madrenas@robarts.ca

Location of experimental work to be carried out: Building(s) RRI Room(s) 2278 & 2276

*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 12.0, Approvals).

FUNDING AGENCY/AGENCIES: CIHR
GRANT TITLE(S): 1- The role of SLP-2 in TCR Signalling
2- Regulation of CTLA4 function

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED. A GRANT SUMMARY PAGE MAYBE ADEQUATE IF IT PROVIDES SUFFICIENT DETAIL ABOUT EACH BIOHAZARD USED.

Names of all personnel working under Principal Investigators supervision in this location:

LUAN A. CHAU
THU A. CHAU
DARAH CHRISTIE
SAMAR SAYEDYAHOSSEIN
ISAAC ELIAS
SARA RAMOS

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? ☑YES ○ NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)?
If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? ○ YES ☑NO
If YES, please give the name of the species. _____
What is the origin of the microorganism(s)? _____
Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

Please attach the CFIA permit.
Please describe any CFIA permit conditions:

_____
_____
_____

1.2 Please complete the table below:

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
SUPERANTIGEN	☑Yes ○ No	☑Yes ○ No	○ Yes ☑No	1 microliter	STAPH. AUREUS	○ 1 ☑2 ○ 3
	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No			○ 1 ○ 2 ○ 3
	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No			○ 1 ○ 2 ○ 3
	○ Yes ○ No	○ Yes ○ No	○ Yes ○ No			○ 1 ○ 2 ○ 3

*Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? ☑YES ○ NO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture:

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	☑Yes ○ No	Human blood + Human cell lines	Not applicable
Rodent	☑Yes ○ No	Mice	
Non-human primate	○ Yes ☑No		
Other (specify)	○ Yes ☑No		

2.3 Please indicate the type of established cells that will be grown in culture in:

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Supplier / Source
Human	☑ Yes ○ No	E6.1 Jurkats HEK 293	ATCC
Rodent	○ Yes ○ No		
Non-human primate	○ Yes ○ No		
Other (specify)	○ Yes ○ No		

*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required ○ 1 ☑ 2 ○ 3

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ☑ YES ○ NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used.

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Known to Be Infected With An Infectious Agent? YES/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid	NORMAL Volunteer Donors	○ Yes ☑ No		○ 1 ☑ 2 ○ 3
Human Blood (fraction) or other Body Fluid	Peritoneal Fluids from PD Patients	☑ Yes ○ No	S. Aureus	○ 1 ☑ 2 ○ 3
Human Organs or Tissues (unpreserved)		○ Yes ○ No		○ 1 ○ 2 ○ 3
Human Organs or Tissues (preserved)		○ Yes ○ No		○ 1 ○ 2 ○ 3

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? ☑ YES ○ NO If no, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? ☑ YES, complete table below ○ NO

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results
E. Coli DH-5α	pBIG2i	Internal Source	CTLA4 SLP 2	none

* Please attach a Material Data Sheet or equivalent if available.

4.3 Will genetic modification(s) involving viral vectors be made? O YES, complete table below ☒NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- HIV O YES, please specify _____ O NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens O YES, specify _____ O NO
- SV 40 Large T antigen O YES O NO
- E1A oncogene ☒YES O NO
- Known oncogenes O YES, please specify _____ O NO
- Other human or animal pathogen and or their toxins O YES, please specify _____ O NO

4.5 Will virus be replication defective? O YES O NO

4.6 Will virus be infectious to humans or animals? O YES O NO

4.7 Will this be expected to increase the containment level required? O YES O NO

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted involving a biological agent? O YES ☑NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

5.2 If YES, please specify which biological agent will be used: _____
Please attach a full description of the biological agent.

5.2 Will the biological agent be able to replicate in the host? O YES O NO

5.3 How will the biological agent be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted: _____

5.5 Has human ethics approval been obtained? O YES, number: _____ O NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? ☑YES O NO If no, please proceed to section 7.0

6.2 Name of animal species to be used MICE

6.3 AUS protocol # 2007-078-12

6.4 Will any of the agents listed be used in live animals O YES, specify: _____ ☑NO

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other body fluids including blood be used?

- Pound source dogs O YES ⊘NO
- Pound source cats O YES ⊘NO
- Cattle, sheep or goats O YES ⊘NO
- Non-human primates O YES, please specify species _____ ⊘NO
- Wild caught animals O YES, please specify species & colony # _____ ⊘NO
- Birds O YES ⊘NO
- Others (wild or domestic) O YES, please specify _____ ⊘NO

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? ⊘YES O NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s) SEE (Staphylococcal Enterotoxin E)
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin unknown for humans (please see attached note!)

8.4 How much of the toxin is handled at one time*? < 0.5ug

8.5 How much of the toxin is stored*? ~~50mg~~ 1 mg maximum
JF - Feb 1, 2010 (per email).

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

9.0 Insects Requiring CFIA Permits

9.1 Do you use insects that require a permit from the CFIA? O YES ⊘NO
If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. _____

9.3 What is the origin of the insect? _____

9.4 What is the lifestage of the insect? _____

9.5 What is your intention? O Initiate and maintain colony, give location: _____
O "One-off" use, give location: _____

9.6 Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

9.7 Please attach the CFIA permit.

9.8 Please describe any CFIA permit conditions:

_____
_____
_____

10.0 Plants Requiring CFIA Permits

10.1 Do you use plants that require a permit from the CFIA? O YES O NO
If no, please proceed to Section 11.0

10.2 If YES, please give the name of the species. _____

10.3 What is the origin of the plant? _____

10.4 What is the form of the plant (seed, seedling, plant, tree..)? _____

10.5 What is your intention? O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO
If yes, please describe: _____
_____

10.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:
_____
_____
_____

10.8 Is the CFIA permit attached? O YES O NO
If NO, please forward the permit to he Biosafety Officer when available.

10.9 Please describe any CFIA permit conditions:
_____
_____
_____

11.0 Import Requirements

11.1 Will any of the above agents be imported? O YES, please give country of origin_____
If no, please proceed to Section 12.0 O NO

11.2 Has an Import Permit been obtained from HC for human pathogens? O YES O NO

11.3 Has an import permit been obtained from CFIA for animal or plant pathogens? O YES O NO

11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ O NO

12.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

SIGNATURE _____

13.0 Containment Levels

11.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. O 1 ☑ 2 O 3

13.2 Has the facility been certified by OHS for this level of containment?

☑ YES, permit # if on-campus BIO-RRI-0020
O NO, please certify
O NOT REQUIRED for Level 1 containment

14.0 Procedures to be Followed

14.1 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca/

SIGNATURE _____ Date: August 31/2009

15.0 Approvals

UWO Biohazard Subcommittee: SIGNATURE: O.M. Calder
Date: 23 Sept. 2009

Safety Officer for Institution where experiments will take place: SIGNATURE: Ronald Wasserlauf
Date: September 01, 2009

Safety Officer for University of Western Ontario (if different from above): SIGNATURE: Stanley
Date: Sept 23/09

Approval Number: BIO-RRI-0020 Expiry Date (3 years from Approval): September 22, 12

Special Conditions of Approval:

1) Follow the "Biosecurity Requirements for Facilities Using Biological Agents" attached. See uwo.ca/humanresources/biosafety for information

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

Biosecurity Requirements for Facilities Using Biological Agents

(1) Biological agents protected by a lock. For example, biological agents in a freezer, fridge, laboratories or other type of container must be locked after-hours/if no one present.

(2) The supervisor must ensure that each person has the qualifications and training to do the work without supervision.

(3) Visitors must be accompanied.

(4) The supervisor must keep a current inventory and a list of the location(s) where the biological agent(s) are stored and handled.

(5) Labelling to identify samples and the container in which they are stored.

(6) Notify the biosafety officer if a sample is lost, stolen, or otherwise misused.

(7) Notify Campus Community Police Services of suspicious behaviour.

There are two additional requirements for Facilities Using or Storing Biological Toxins:

(8) Do not keep on hand more than the amounts regulated by the United States Select Agents regulation: www.selectagents.gov/index.htm/

(9) For best practices, it is recommended to use or handle less than one human dose at any given time.

8.3

The LD50 for staphylococcal superantigens for humans is not known. Preliminary studies using non-human primates suggested that it may be in the range of 0.02micrograms/Kg (1.4 micrograms for a normal 70Kg adult) when using inhalatory route. To further minimize the risk, we will aliquot our stocks to a maximum of 1 microgram per aliquot . Note that we never use more than 0.5 microgram per experiment. Also, all out stocks are stored under lock and key and under strict control of my chief technician Ms. Luan Chau.

Ron Noseworthy

From: madrenas [madrenas@robarts.ca]
Sent: September 22, 2009 6:30 AM
To: john McCormick; luan@robarts.ca; Ron Noseworthy
Cc: madrenas
Subject: SAgs

Thanks John, Luan and Ron,

The reference I got for the LD50 in monkeys by inhalatory route is the one from the US Army Manual. In the context of the tenuous and heterogenous data you reviewed in your mail, I believe that the LD50 in this paper is the closest we have as a proper reference. We should though qualify the claim indicating that the evidence for human LD50 is not available, that the evidence for non-human primates is very limited, and that there are very substantial species differences in terms of sensitivity to SAgs. Altogether, the LD50 may be not applicable as such to human beings.

Q

Ron Noseworthy

From: Luan A. Chau [luan@robarts.ca]
Sent: September 21, 2009 11:06 AM
To: Ron Noseworthy
Cc: Quim Madrenas
Subject: Fwd: Re: Biosafety form

Dear Ron,

Please see below.

Thanks,
Luan

> From: John McCormick <john.mccormick@schulich.uwo.ca>
> To: "Luan A. Chau" <luan@robarts.ca>, Quim Madrenas <madrenas@robarts.ca>
> Subject: Re: Biosafety form
> Date: Mon, 21 Sep 2009 10:42:09 -0400
>
> Hi Luan and Quim,
>
> I haven't been able to find the data described below for the 1.4 microgram/70 kg adult by inhalation. But after some digging, this is what I did find:
>
> The LD50 for bacterial (staphylococcal enterotoxins) superantigens is not known. However, 3 historical events in the early 1960s from the now disbanded U.S. Offensive Biological Warfare Program indicates that accidental inhalation of staphylococcal enterotoxin B in the microgram range may result in symptoms of fever, cough, nausea and vomiting (among other symptoms), although there were no deaths (Rusnak et al., 2004 Emerg Infect Dis). There are no further documented reports of any additional aerosol exposures despite 5000 research publications on superantigens since 1970. In addition, aerosol models indicated that a "lethal dose" in monkeys was 190 micrograms/kg body weight (Tseng et al., 1993 Infect Immun). An LD50 by this route is probably closer to ~10-20 micrograms/kg although this was not made clear (Tseng et al, 1995 Infect Immun).
>
> In addition, anti-cancer clinical Phase I trials using wild-type staphylococcal enterotoxin A (fused with a monoclonal antibody to target cancer cells) infused over 3 hours as a single dose is safe when given at doses up to 4 nanograms/kg (total dose ~ 280 ng for a 70 kg adult) (Nielsen et al., 2000 J. Immunother). Infusion of a mutated version of the therapeutic indicated that the maximum tolerated dose (MTD) ranged from 103 ng/kg to 601 ng/kg infused per day for 4 days (~7 micrograms to 42 micrograms per day x 4 days) (Cheng et al., 2004 J. Clin Oncol).
>
> I think the evidence is clear we should be careful not to inhale these things, and that a dose to induce symptoms would probably be in the low microgram range, but I also think an actual lethal dose would be quite high.

Hope this helps.

John.

On 18-Sep-09, at 3:57 PM, Luan A. Chau wrote:

Thanks John. Have a great weekend!

Luan

Hi Luan,

I am working on getting you the references for human dose. I'm not sure I believe the inhalation doses are accurate however so please don't submit this information yet. Thanks,

John.

On 18-Sep-09, at 10:44 AM, Luan A. Chau wrote:

Dear John,

For our Biohazard Registry Form renewal, the reference for the LD50 as we claimed below needs to be included with the form.

Could you please send me the reference for this.

Thanks very much for your help,

Best regards,

Luan

Dear Luan,

For the biosafety form, John McC told me that he did answer just that it was not known for humans and that mouse strains are mostly resistant to superantigens. For the few that are susceptible, it was on the range of 100 to 500 micrograms.

My understanding is that we have to put it for humans. If so, please include the information below in the requested point (I believe it is 8.3). Show it to Ron Noseworthy before submission for approval.

The LD50 for staphylococcal superantigens for humans is not known. Preliminary studies using non-human primates suggested that it may be in the range of 0.02micrograms/Kg (1.4 micrograms for a normal 70Kg adult) when using inhalatory route. To further minimize the risk, we will aliquot our stocks to a maximum of 1 microgram per aliquot . Note that we never use more than 0.5 microgram per experiment. Also, all out stocks are stored under lock and key and under strict control of my chief technician Ms. Luan Chau.

Q

--
J. (Quim) Madrenas, MD PhD
Canada Research Chair in Immunobiology
Professor, Microbiology & Immunology, and Medicine
The University of Western Ontario
Head of Immunology, Robarts Research Institute
Director, FOCIS Centre for Clinical Immunology and Immunotherapeutics

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SYSTEMS IMMUNOLOGY OF *S. aureus* INFECTION

Overview: The goal of this research program is to develop a Systems Immunology of the human response to *Staphylococcus aureus* (*S. aureus*).

Background and preliminary data: During the previous grant term, we examined the regulation of human T cell activation by CTLA-4 using the response to *S. aureus* superantigens as a model. In the course of that work, we unraveled novel aspects of human T cell activation by these toxins such as the regulatory role of Lck (*J Immunol* 2004; 172: 222) and a new Gα11-PLCβ-dependent activation pathway (*Immunity* 2006; 25:67). More important, we identified a new mechanism used by *S. aureus* to down-regulate T cell activation by superantigens (*Nature Medicine* 2009; 15: 641). This mechanism is operational *in vivo* and may explain the balance between commensalism and pathogenicity by *S. aureus*. Preliminary data indicate that this mechanism involves binding of staphylococcal peptidoglycan (PGN)-embedded molecules to TLR2 complexes on antigen-presenting cells (APCs), and the induction of an NF-κB-dependent, interleukin-10 response leading to down-regulation of T cell response to superantigens. In addition, recent experiments suggest that the modulatory effects of staphylococcal PGN-embedded molecules is dependent on its selective binding to TLR2/6, but not TLR2/1, complexes on APCs. The specific focus of this grant is the comprehensive dissection of the molecular basis of such a mechanism.

Hypothesis: Selective binding of staphylococcal PGN-embedded molecules to TLR2/6 on APCs triggers unique genomic and proteomic profiles in these cells leading to modulation of the immune response to *S. aureus*.

Specific aims:

1. To identify the factors that control selective binding of PGN-embedded molecules to TLR2/6;
2. To build the signaling network involved in immunomodulation by staphylococcal PGN-embedded molecules;
3. To establish the genomic and proteomic profiles in APCs and in T cells during immunomodulation by *S. aureus* PGN preparations;
4. To generate a computational model of immunomodulation by the staphylococcal cell wall; and
5. To test the data network in a clinical setting of sepsis and shock by *S. aureus*.

Experimental approach: We will follow a systems approach to study the complex temporal and spatial interactions between APCs and T cells during the response to *S. aureus* superantigens in the presence or absence of staphylococcal PGN preparations. First, we will assess basal and ligand-induced TLR2/6 vs. TLR2/1 dimerization with varying amounts of each chain and in different monocyte subsets vs. monocyte-derived macrophages vs. monocyte-derived dendritic cells and correlate this with functional modulation of T cell responses to superantigens. Next, we will examine the activation of signaling pathways emanating from TLR2/6 and link their activation with modulation of T cell activation. Once the optimal cellular and biochemical conditions of immunomodulation have been identified, we will perform genomic and proteomic

analyses of the responding cells. The resulting body of data will undergo bioinformatic analysis and will be fed into a computer model that we have already started to build to predict the course of immune responses to staphylococcal superantigens. Predictions from this model will be tested in the clinic using peripheral blood cells from patients in ICU with *S. aureus* sepsis vs. shock, and iterative model refinements will be performed.

Relevance: *S. aureus* poses a paradox: on one hand, it is carried by up to 50% of healthy individuals but on the other hand, it is also one of the most common pathogens in the clinic. How *S. aureus* can act as a commensal or as a pathogen is not known. The proposed work will identify molecular profiles associated with commensalism and pathogenicity, and reveal potential therapeutic targets to act as alternatives to antibiotics.

Toxin Technology, Inc.

7165 Curtiss Ave
Sarasota, FL 34231
USA

941-925-2032 (ph)
941-925-2130 (fax)
Email "toxtech@atlas.net"

Certificate of Analysis

Product : Staphylococcal Enterotoxin E, partially purified

cat. no : EP404

lot no : 31301Pe

purity : approximately 50 % pure by SDS-PAGE, Coomassie Blue stain

serological : 10 ug / ml solution showed lines of identity with 10 ug SEE / ml standard when tested with anti SEE in double immunodiffusion assay. No cross reactivity was observed when tested at a 100ug/ml with anti SEA, SEB, SEC, SED and TSST (sensitivity approx. 5 ug / ml)

solubility : After lyophilization, the SEE was re-dissolved to a 1 mg / ml solution using deionized water. This solution was clear within several minutes.

storage : At - 20 °C, 1 mg / ml solution is stable for one year under serological freezer conditions.
At 4 °C, 1 mg / ml solution is stable for two weeks.
In lyophilized, desiccated form - stable for at least 5 years.

This product is for research purposes only and is not intended for in vivo or diagnostic use

Toxin Technology shall not be held responsible for any damages resulting from the use of this product

TOXIN TECHNOLOGY, INC.

7155 CURTISS AVENUE • SARASOTA, FLORIDA 34231
PHONE (941) 925-2032 • FAX (941) 925-2130 • Email: toxtech@worldnet.att.net

R.F. REISER, Ph.D. "Tox Tech" R.H. REISER, Ph.D.

MATERIAL SAFETY DATA SHEET

HEALTH HAZARD DATA

To the best of our knowledge, the chemical, physical and toxicological properties have not been thoroughly investigated.

OPEN AND REHYDRATE VIALS IN BIOSAFETY SHEET.

Acute effects
May be harmful if swallowed, inhaled, or absorbed through skin. Biomedical material. May cause human disease.
Causes emesis and diarrhea in experimental animals.
Associated with food poisoning and causes enteritis in humans. The dose of purified protein required to produce emesis or diarrhea in monkeys is 0.9ug/kg by oral feeding (Biochem. Vol. 4, 1965).

Aerosols may be harmful; 30ng/person (incapacitating), 1.7ug/person (may be lethal)[1]. Re-hydrate lyophilized toxins in bio-safety hood. Once rehydrated, handle liquid in chemical hood or bio-safety hood when mixing or agitating.

FIRST AID

In case of contact, flush with copious amounts of water. If swallowed, induce vomiting then wash mouth with water provided person is conscious. Call a physician.

In case of contact with eyes, flush with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers. Call a physician.

If inhaled, remove to fresh air. If breathing becomes difficult, call a physician.

FIRE AND EXPLOSION HAZARD

Use extinguishing media appropriate for surrounding fire.

Firefighters should wear proper protective equipment and self-contained breathing apparatus
with full facepiece.

1. Franz, DR et al Clinical Recognition and Management of Patients Exposed to Biological
Warfare Agents.1997. JAMA 278(5):399-411.

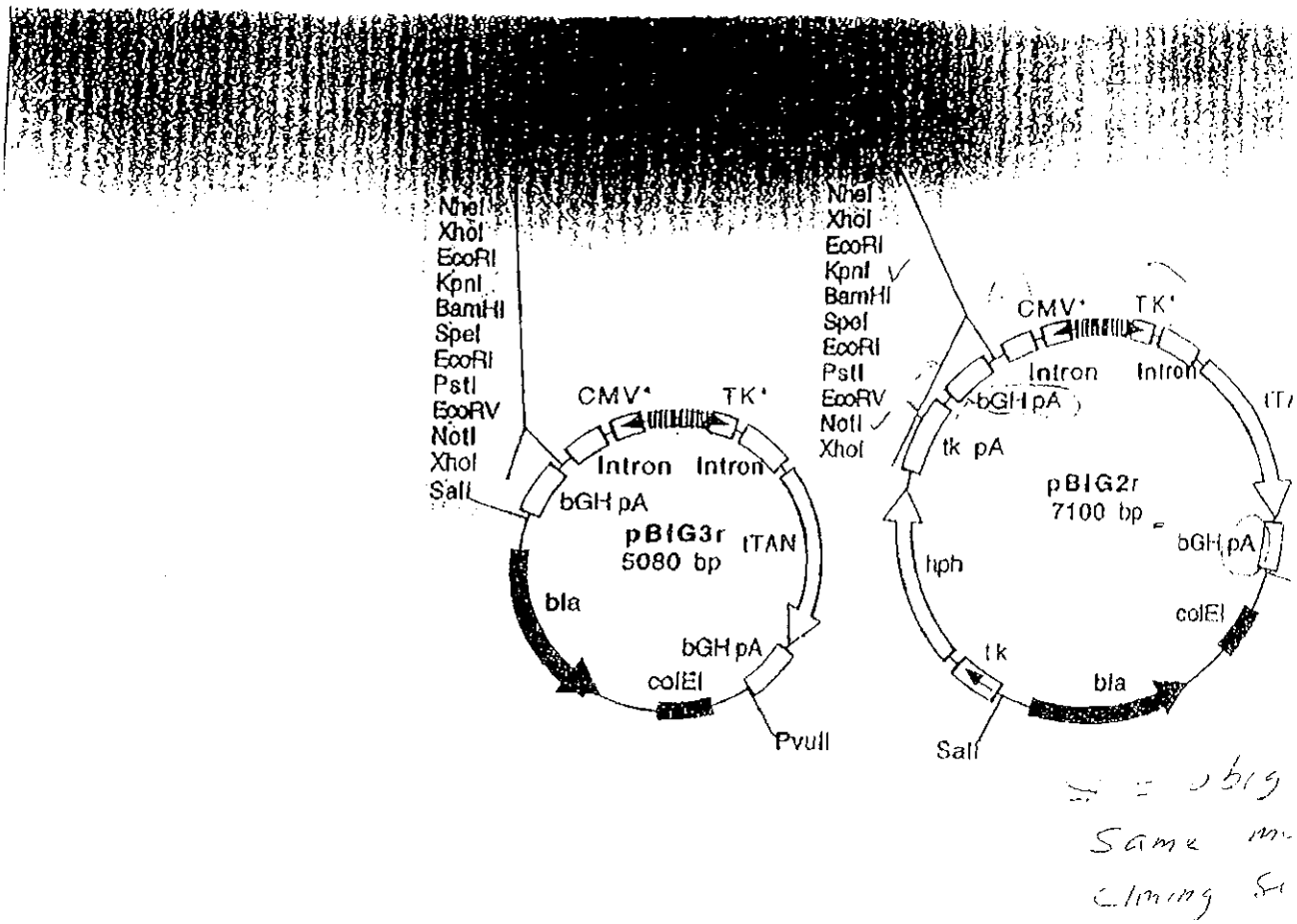

Fig.2: Autoregulated bi-directional tetracycline-responsive pBIG expression vectors. Each vector is based on a high copy number plasmid backbone containing the colE1 origin of replication and B'-lactamase gene that confers resistance to ampicillin. The bi-directional tetracycline-responsive promoter in each vector is comprised of a central tetO element, a stronger CMV* element to drive cDNA expression and a weaker TK* element to drive expression of the transactivator component.

The two vectors are essentially identical with the exception that pBIG2 contains a selectable marker conferring resistance to hygromycin B for the generation of stable cell lines. The "i" series of vectors ulilize the rtTAN transactivator such that cDNA expression is effectively induced by doxycycline.

Cell Biology

ATCC® Number:	**TIB-152™** Order this Item	Price:	**$264.00**
Designations:	Jurkat, Clone E6-1		
Depositors:	A Weiss		
Biosafety Level:	1		
Shipped:	frozen		
Medium & Serum:	See Propagation		
Growth Properties:	suspension		
Organism:	*Homo sapiens* (human)		
Morphology:	lymphoblast 		
Source:	**Disease:** acute T cell leukemia **Cell Type:** T lymphocyte;		
Cellular Products:	interleukin-2 (interleukin 2, IL-2) [1609]		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Applications:	transfection host (technology from amaxa Roche FuGENE® Transfection Reagents)		
Receptors:	T cell antigen receptor, expressed		
Antigen Expression:	CD3; Homo sapiens, expressed		
DNA Profile (STR):	Amelogenin: X,Y CSF1PO: 11,12 D13S317: 8,12 D16S539: 11 D5S818: 9 D7S820: 8,12 THO1: 6,9.3 TPOX: 8,10 vWA: 18		
Cytogenetic Analysis:	This is a pseudodiploid human cell line. The modal chromosome number is 46, occurring in 74% with polyploidy at 5.3%. The karyotype is 46,XY,-2,-18,del(2) (p21p23),del(18) (p11.2). Most cells had normal X and Y chromosomes.		

Related Links ▸

- NCBI Entrez Search
- Cell Micrograph
- Make a Deposit
- Frequently Asked Questions
- Material Transfer Agreement
- Technical Support
- Related Cell Culture Products

Gender:	male
Comments:	This is a clone of the Jurkat-FHCRC cell line, a derivative of the Jurkat cell line. [1609] The Jurkat cell line was established from the peripheral blood of a 14 year old boy by Schneider et al., and was originally designated JM. [50685] [112530]
Propagation:	Clone E6-1 cells produce large amounts of IL-2 after stimulation with phorbol esters and either lectins or monoclonal antibodies against the T3 antigen (both types of stimulants are needed to induce IL-2 production. [1609] The line was cloned from cells obtained from Dr. Kendall Smith and are mycoplasma free. [1609] **ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated RPMI-1640 Medium, Catalog No. 30-2001. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C
Subculturing:	**Protocol:** Cultures can be maintained by the addition of fresh medium or replacement of medium. Alternatively, cultures can be established by centrifugation with subsequent resuspension at 1 X 10(5) viable cells/ml. Do not allow the cell density to exceed 3 X 10(6) cells/ml. **Interval:** Maintain cultures at a cell concentraion between between 1 X 10(5) and 1 X 10(6) viable cells/ml. **Medium Renewal:** Add fresh medium every 2 to 3 days (depending on cell density)
Preservation:	**Freeze medium:** Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Doubling Time:	48 hrs
Related Products:	derivative:ATCC CRL-1990 derivative:ATCC CRL-2063 recommended serum:ATCC 30-2020 derivative:ATCC TIB-153 Recommended medium (without the additional supplements or serum described under ATCC Medium):ATCC 30-2001

Cell Biology

ATCC® Number:	CRL-1573™ Order this Item	Price:	$256.00
Designations:	293 [HEK-293]		
Depositors:	FL Graham		
Biosafety Leve...	2 [CELLS CONTAIN ADENOVIRUS]		
Shipped:	frozen		
Medium & Serum:	See Propagation		
Growth Properties:	adherent		
Organism:	*Homo sapiens* (human)		
Morphology:	epithelial 		
Source:	**Organ:** embryonic kidney **Cell Type:** transformed with adenovirus 5 DNA		
Permits/Forms:	In addition to the MTA mentioned above, other ATCC and or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.		
Restrictions:	These cells are distributed for research purposes only. 293 cells, their products, or their derivatives may not be distributed to third parties.		
Applications:	efficacy testing [92587] transfection host (Nucleofection technology from Lonza Roche FuGENE® Transfection Reagents) viruscide testing [92579]		
Receptors:	vitronectin, expressed		
Tumorigenic:	Yes		
DNA Profile (STR):	Amelogenin: X CSF1PO: 11,12 D13S317: 12,14 D16S539: 9,13 D5S818: 8,9 D7S820: 11,12 THO1: 7,9.3 TPOX: 11 vWA: 16,19		

Related Links ▸

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Cytogenetic Analysis:	This is a hypotriploid human cell line. The modal chromosome number was 64, occurring in 30% of cells. The rate of cells with higher ploidies was 4.2 %. The der(1)t(1;15) (q42;q13), der(19)t(3;19) (q12;q13), der(12)t(8;12) (q22;p13), and four other marker chromosomes were common to most cells. Five other markers occurred in some cells only. The marker der(1) and M8 (or Xq+) were often paired. There were four copies of N17 and N22. Noticeably in addition to three copies of X chromosomes, there were paired Xq+, and a single Xp+ in most cells.
Age:	fetus
Comments:	Although an earlier report suggested that the cells contained Adenovirus 5 DNA from both the right and left ends of the viral genome [RF32764], it is now clear that only left end sequences are present. [39768] The line is excellent for titrating human adenoviruses. The cells express an unusual cell surface receptor for vitronectin composed of the integrin beta-1 subunit and the vitronectin receptor alpha-v subunit. [23406] The Ad5 insert was cloned and sequenced, and it was determined that a colinear segment from nts 1 to 4344 is integrated into chromosome 19 (19q13.2). [39768]
Propagation:	**ATCC complete growth medium:** The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%. **Atmosphere:** air, 95%; carbon dioxide (CO2), 5% **Temperature:** 37.0°C The cell line does not adhere to the substrate when left at room temperature for any length of time, therefore, live cultures may be received with the cells detached.The cells will re-attach to the flask over a period of several days in culture at 37C.

Subculturing:

Protocol:

1. Remove and discard culture medium.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).
   Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.
4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.
5. Add appropriate aliquots of the cell suspension to new culture vessels.An inoculum of 2 X 10 (3) to 6 X 10 (3) viable cells/cm2 is recommended.
6. Incubate cultures at 37°C.6. Subculture when cell concentration is between 6 and 7 X 10(4) cells/cm2.

Preservation:

Subcultivation Ratio: 1:10 to 1:20 weekly.
Medium Renewal: Every 2 to 3 days

Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO
Storage temperature: liquid nitrogen vapor phase

Related Products:

derivative: ATCC CRL-12007
derivative: ATCC CRL-12013
derivative: ATCC CRL-12479
derivative: ATCC CRL-2029
derivative: ATCC CRL-2368
purified DNA:ATCC CRL-1573D
Recommended medium (without the additional supplements or serum described under ATCC Medium): ATCC 30-2003
derivative: ATCC CRL-10852
derivative: ATCC CRL-12006