Dr. S. Kim

[redacted]

[redacted]

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

~~Boram Ham~~

~~Sarah Spanton~~

Andrew Martins

Soon-Duck Ha

Sangwook Park

~~Anthony Bruni~~

Additional Personnel
(Please list additional personnel here)

	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	P. aeurogenosa, S. typhimurium, actobacillus rhamnosus, E. coli EC1000, E.Coli BL21	Vesicular ~~stomatitis virus~~ (Indiana strain) HSV type 1. Influenza A virus
[redacted]	RAW 264.7, HEK293, HepG2, CHO/CHO-K1, L929, Caco2, u937, NPC-1 deficient fibroblast (GMO3123) and wildtype	
Approved Use of Human Source Material	Blood (whole), PARF-CFP, mRFP-Rab7, mRFP-Rab5	
Approved Genetic Modifications (Plasmids/Vectors)	pTRK830, pOR128, pTRK669, pDisrup8.0-10x, pBABE-cre	PYPF2 (NLRP2) PYPF5 (NLRP6) PYPF3 (NLRP2) " 6 (" 11) " 4 (" 4)
Approved Use of Animals	mice	
Approved Biological Toxin(s)	[redacted] aeruginosa exotoxin A, Anthratoxin	

* *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.*

[redacted]

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: _____

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

Date of Last Biohazardous Agents Registry Form: Apr 28, 2008

Date of Last Modification (if applicable): Dec 21, 2009

BioSafety Officer(s): _____

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

Descriptions on researches using the reagents added:

Viruses
We recently found that the lysosomal protease cathepsin B plays important role in multi-vesicle body trafficking in cells. Previous studies showed that several viruses are packed and egress through multi-vesicle body in infected cells. We will examine whether cathepsin B inhibitor or cells deficient in cathepsin B prevent viral egress in vitro. No modification will be done in these reagents.

Plasmids
We found several PYPAF interacting proteins through yeast two hybrid screening. We will verify our findings through ectopically expressing these full or truncated proteins together with our interacting proteins identified, followed by immunoprecipitation. The plasmids are full length constructs of PYPAFs which will be used for the expression of full length proteins or for constructing LRR region truncated or PYRIN domain truncated forms.

Kim

Subject: Re: Containment Levels for 3 viruses
From: ImportZoopath <ImportZoopath@inspection.gc.ca>
Date: Mon, 23 Aug 2010 11:01:48 -0400
To: Jennifer Stanley <jstanle2@uwo.ca>

Good Morning,

the first two viruses (Rhabdovirus and Influenza A virus (H3N2)) are containment level 2 animal pathogens, the human herpes virus is a level 1. If you have any further questions, do not hesitate to contact our office.

Thank you,
Steven Burns

Please note our new contact information / Prière de noter nos nouvelles coordonnées

Office of Biohazard Containment & Safety, CFIA | Bureau du confinement des biorisques et de la sécurité, ACIA
Government of Canada | Gouvernement du Canada
1400 Merivale, Ottawa ON K1A0Y9
Phone/Tél.: (613) 773-6520
Fax/ Téléc.: (613) 773-6521
ImportZoopath@inspection.gc.ca

Please visit our website http://www.inspection.gc.ca/english/sci/bio/bioe.shtml / Veuillez visiter notre site internet http://www.inspection.gc.ca/francais/sci/bio/biof.shtml

Jennifer Stanley <jstanle2@uwo.ca> 2010/08/19 4:03 pm >>>
Hello there -
I am wondering if you agree with these three viruses as Level 2 (per ATCC website).
If there are any special Canadian requirements, please let me know.
Thanks
Jennifer

Animal Viruses and Antisera
ATCC^® Number: *VR-1238^(TM)* Price: $115.00
Classification: Rhabdovirus
Agent: Vesicular stomatitis virus deposited as Vesicular stomatitis,
Indiana
Strain: Indiana Lab [V-520-001-522]
Depositors: NIH/NIAID
Biosafety Level:
</CulturesandProducts/TechnicalSupport/BiosafetyLevels/tabid/660/Default.aspx>

2
Shipped: freeze-dried
Permits/Forms: In addition to the MTA
</ProductUsePolicy/MaterialTransferAgreement/tabid/613/Default.aspx>
mentioned above, other ATCC and/or regulatory permits
</CulturesandProducts/HowtoOrder/SpecialForms/tabid/696/Default.aspx>
may be required for the transfer of this ATCC material. Anyone

Animal Viruses and Antisera

ATCC® Number: **VR-1238™** Order this Item Price: **$115.00**

Classification:	Rhabdovirus
Agent:	Vesicular stomatitis virus deposited as Vesicular stomatitis, Indiana
Strain:	Indiana Lab [V-520-001-522]
Depositors:	NIH/NIAID
Biosafety Level:	2
Shipped:	freeze-dried
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.
Comments :	BSL II practices and facilities are recommended for activities utilizing laboratory-adapted strains of VSV (HHS Pub. No.(CDC)93-8395).
Related Products:	immune ascites fluid:ATCC VR-1238AF also distributed as:ATCC VR-158

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

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Biological Reference Material and Consensus Standards for the life science
- community

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Product Description

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Customers in Europe, Australia, Canada, China, Hong Kong, India, Israel, Japan, Korea, Macau, Mexico, New Zealand, Singapore, and Taiwan, R.O.C. should contact a local distributor for pricing information and to place an order for ATCC cultures and products.

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Animal Viruses and Antisera

ATCC® Number: VR-1493™ Order this Item **Price:** $360.00

Classification	Herpesviridae; Simplexvirus
Agent	Human herpesvirus 1
Strain	KOS
Original Source	derived from ATCC VR-1487 by passage in the presence of MRA to remove mycoplasma contaminants
Depositors	PA Schaffer
Biosafety Level	2
Shipped	frozen
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.
Host Organism	Vero cells (ATCC CCL-81), HEp-2 cells (ATCC CCL-23), other human and mammalian cell lines
Incubation	Duration: 1-3 days at 37.0°C in Vero cells (ATCC CCL-81)
Effect	Yes, in vitro effects: Cytopathic effects (syncytium formation and cell destruction)
Store at	-70.0°C or lower
Comments	antigenically cross-reactive with HSV-2 Host of choice: Vero cells (ATCC CCL-81)
Related Products	Virus DNA: ATCC VR-1493D
References	47027: Smith KO. Relationship between the envelope and the infectivity of herpes simplex virus. Proc Soc Exp Biol Med. 115: 814-816, 1964. PubMed: 14155835

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Animal Viruses and Antisera

ATCC® Number:	**VR-1680™** Order this Item	Price:	**$240.00**
Classification:	Orthomyxoviridae, Influenzavirus A		**Related Links ▸**
Agent:	Influenza A virus (H3N2)		NCBI Entrez Search
Strain:	~~A/Aichi/2/68 (TC-adapted)~~ unknown		Make a Deposit
Original Source:	Isolated from ~~sailor on Israeli ship docking at Aichi, Japan~~, 1968. patient in St Joseph's hospital [redacted]		Frequently Asked Questions
Depositors:	M.T. Coleman and ATCC		Agreement
Biosafety Level:	2		Technical Support
Shipped:	frozen		Related Products
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.		**BioProducts** Cell, microbial and molecular genomics products for the life sciences **BioServices** Bio-materials management; basic repository to complex partnership-level services **BioStandards** Biological Reference Material and Consensus Standards for the life science community
Host Organism :	**Production Host:** MDCK (ATCCCCL-34) **Alternate Host:** CE, MDCK and Monkey kidney cells (MkK)		
Incubation :	For best results, plate cells 24-48 hours in advance. Infect at 80%-90% confluent (not 100% confluent) using a multiplicity of infection (MOI) of 0.01 to 1.0. Incubate at 35.0°C in a humidified, 5% CO2 atmosphere for 2-4 days, until CPE are well advanced in 100% of the culture. In TC, ATCC® VR-1680 should be grown in serum free media that contains 1 ug/ml TPCK-treated trypsin, 0.125% BSA and 1% HEPES (1 molar stock).		
Effect :	CPE (cell rounding, degeneration, sloughing)		
Store at :	-70°C or colder		
Comments :	Derived by adaptation of egg-passage A/Aichi/2/68 (ATCCVR-547) to MDCK (ATCCCCL-34) cells. (Note: ATCCVR-547 and ATCCVR-1680 have not been compared with respect to sequence or infectivity in chick embryo and tissue culture).		
Related Products:	Virus: ATCC VR-547		
References :	33639: Coleman MT, et al. The Hong Kong-68 influenza A2 variant. Lancet 2: 1384-1386, 1968. PubMed: 4177941		

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-------- Original Message --------
Subject: Re: Fwd: Re: Permit for virus/plasmids - Kim lab
Date: Wed, 25 Aug 2010 10:15:42 -0400
From: Sung Kim <Sung.Kim@schulich.uwo.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>

Jennifer: The backbone of the plasmids is pCI (Promega) except PYPAF4 which is pCMV-4(Stratagene). Full length sequence were inserted into the multicloning site. The paper did not disclosed the exact insertion sites. Attached are the maps of the plasmids and you should be able to find the sequences in the commercial sites.

Thanks
SK

1052
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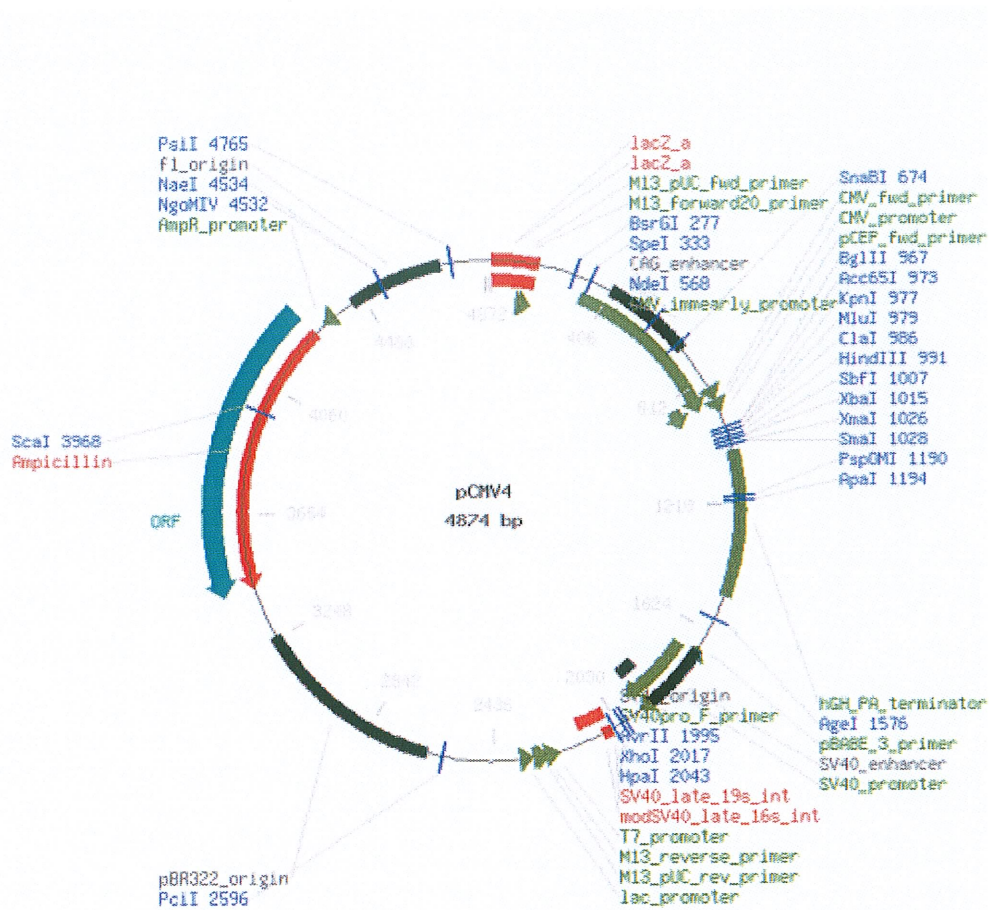
Xho I
EcoR I
Mlu I
Kpn I
Xba I
Sal I
Acc I
Sma I
BstZ I
Not I
Bgl II
CMV I. E.
Enhancer/Promoter
Asp 9 II
Pst I
BsrG I
intron
pCI
Vector
(4006bp)
SV40 Late
poly(A)
BamH I
f1 ori
Amp'
ori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NaeI 4534
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AmpR_promoter
lacZ_a
lacZ_a
M13_pUC_fwd_primer
M13_forward20_primer
BsrGI 277
SpeI 333
CAG_enhancer
NdeI 568
CMV_immearly_promoter
SnaBI 674
CMV_fwd_primer
CMV_promoter
pCEF_fwd_primer
BglII 967
Acc65I 973
KpnI 977
MluI 979
ClaI 986
HindIII 991
SbfI 1007
XbaI 1015
XmaI 1026
SmaI 1028
PspOMI 1190
ApaI 1194
ScaI 3968
Ampicillin
ORF
pCMV4
4874 bp
hGH_PA_terminator
AgeI 1576
pBABE_3_primer
SV40_enhancer
SV40_promoter
SV40pro_F_primer
NcoI 1995
XhoI 2017
HpaI 2043
SV40_late_19s_int
modSV40_late_16s_int
T7_promoter
M13_reverse_primer
M13_pUC_rev_primer
lac_promoter
pBR322_origin
PciI 2596

FEBS 26602 FEBS Letters 530 (2002) 73–78

Functional screening of five PYPAF family members identifies PYPAF5 as a novel regulator of NF-κB and caspase-1

Jill M. Grenier[1], Lin Wang[1], Gulam A. Manji[2], Waan-Jeng Huang, Amal Al-Garawi, Roxanne Kelly, Adam Carlson, Sarah Merriam, Jose M. Lora, Michael Briskin, Peter S. DiStefano[3], John Bertin*

Millennium Pharmaceuticals Inc., 75 Sidney Street, Cambridge, MA 02139, USA

Received 22 August 2002, accepted 28 August 2002

First published online 26 September 2002

Edited by Veli-Pekka Lehto

Abstract PYRIN-containing Apaf-1-like proteins (PYPAFs) are a recently identified family of proteins thought to function in apoptotic and inflammatory signaling pathways. PYPAF1 and PYPAF7 proteins have been found to assemble with the PYRIN–CARD protein ASC and coordinate the activation of NF-κB and pro-caspase-1. To determine if other PYPAF family members function in pro-inflammatory signaling pathways, we screened five other PYPAF proteins (PYPAF2, PYPAF3, PYPAF4, PYPAF5 and PYPAF6) for their ability to activate NF-κB and pro-caspase-1. Co-expression of PYPAF5 with ASC results in a synergistic activation of NF-κB and the recruitment of PYPAF5 to punctate structures in the cytoplasm. The expression of PYPAF5 is highly restricted to granulocytes and failed to colocalize with ASC and activate NF-κB. PYPAF5 also synergistically activated caspase-1-dependent cytokine processing when co-expressed with ASC. These findings suggest that PYPAF5 functions in immune cells to coordinate the transduction of pro-inflammatory signals to the activation of NF-κB and pro-caspase-1.

Key words: PYPAF5; PYRIN-containing Apaf-1-like protein; Caspase-1; NF-κB; ASC; Inflammation

1. Introduction

Apaf-1 plays a central role in apoptosis by transducing pro-apoptotic signals to the activation of pro-caspase-9 [1,2]. During apoptosis, cytochrome *c* is released from mitochondria into the cytoplasm, where it induces the oligomerization of Apaf-1 in the presence of dATP. Oligomerization of Apaf-1 results in the formation of an apoptosome that recruits and activates pro-caspase-9. Apaf-1 has a tripartite domain structure consisting of an N-terminal caspase-recruitment domain (CARD) that mediates recruitment of pro-caspase-9 to the apoptosome, a central nucleotide-binding site (NBS) domain, and a C-terminal domain comprised of WD-40 repeats. The NBS domain mediates Apaf-1 oligomerization in the presence of dATP, whereas the WD-40 repeats function as binding sites for cytochrome *c*. Thus, Apaf-1 functions as a sensor-like molecule that signals apoptosis in response to the release of cytochrome *c* from mitochondria.

A search for Apaf-1-like molecules has resulted in the identification of numerous proteins that belong to the NBS/leu-

[illegible] CARD) each contain an N-terminal CARD domain(s) that mediates assembly with a downstream signaling partner and a central NBS domain that likely coordinates nucleotide-dependent oligomerization [3–8]. In addition, each protein contains a C-terminal LRR domain that has been proposed to function as a binding site for specific upstream regulators. CARD4 and CARD15 signal the activation of NF-κB following assembly with RICK, a CARD-containing kinase that mediates inflammatory signaling for both the innate and adaptive immune systems [9,10]. Intriguingly, CARD15 variants confer susceptibility to Crohn's disease and Blau syndrome, two chronic inflammatory disorders [11–13]. In addition to coordinating the activation of NF-κB, Apaf-1-like proteins have also been found to regulate the activation of pro-inflammatory caspases. CARD12 is thought to play a critical role in inflammatory signaling by binding to and activating pro-caspase-1, a CARD-containing caspase that processes pro-interleukin-1β (IL-1β) and pro-interleukin-18 into active cytokines. The inflammatory signals that regulate the assembly and activation of these protein complexes are not presently understood.

A second group of Apaf-1-like proteins that contain N-terminal PYRIN domains instead of CARD domains has been identified recently [14–17]. We have named these proteins PYPAFs for PYRIN-containing Apaf1-like proteins [18,19]. The PYRIN domain is a novel protein–protein interaction domain that shares homology with pyrin, a protein that is associated with a rare inflammatory disorder called familial Mediterranean fever [20]. This domain is a new member of the death domain fold superfamily that functions to mediate homotypic interactions between PYRIN-containing proteins [14,15,18,19]. PYPAF1 (cryopyrin) variants cause several autoinflammatory diseases, including Muckle–Wells syn-

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E-mail address: bertin@mpi.com (J. Bertin).

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[2] Present address: Elan Pharmaceuticals, South San Francisco, CA, USA.
[3] Present address: Elixir Pharmaceuticals, Cambridge, MA, USA.

0014-5793/02/$22.00

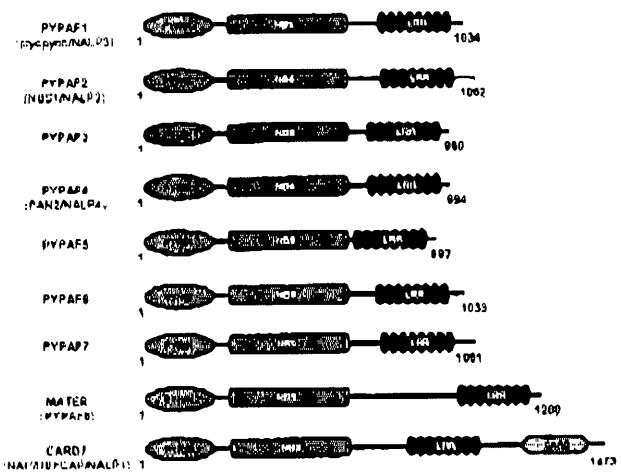


Fig. 1. Domain structure of PYPAF family members. Shown are PYPAF1, PYPAF2, PYPAF3, PYPAF4, PYPAF5, PYPAF6, PYPAF7, MATER (PYPAF8) and CARD7. PYRIN domains (red shading), NBS domains (blue shading) and LRR domains (black shading) are indicated.

ical cutaneous and articular syndrome [21–23]. We recently identified PYPAF1 and PYPAF7 as PYPAF family members that assemble together with ASC and signal the activation of NF-κB and pro-caspase-1 [18,19]. The recruitment and activation of pro-caspase-1 by these PYPAF proteins is mediated by the C-terminal CARD domain of ASC [19,24]. To determine if other PYPAF proteins regulate inflammatory signaling pathways, we screened five other family members for their

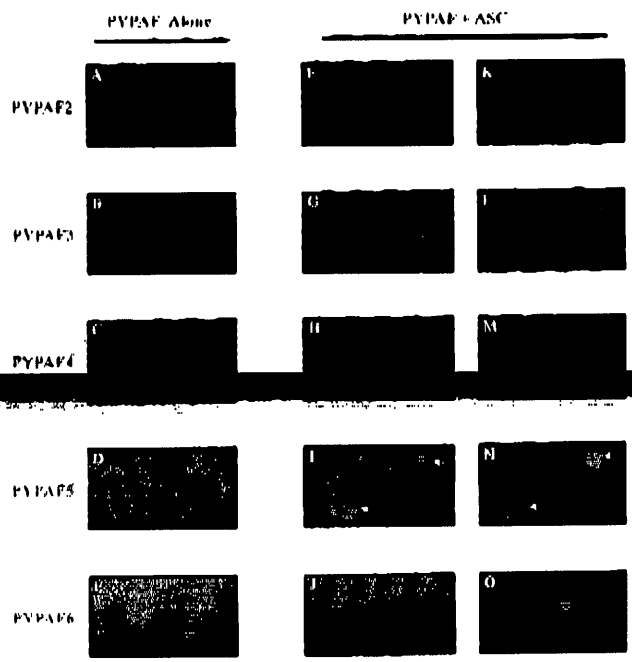


Fig. 2. PYPAF5 is recruited to ASC punctate structures. HA-tagged ASC (blue staining) and FLAG-tagged PYPAF proteins (red staining) were expressed in 293T cells. A–E show localization of PYPAF proteins when expressed alone. F–J and K–O show the immunostaining patterns observed when each protein was co-expressed with ASC. Note the co-localization of PYPAF5 (J and N) to the ASC punctate structures (arrows).

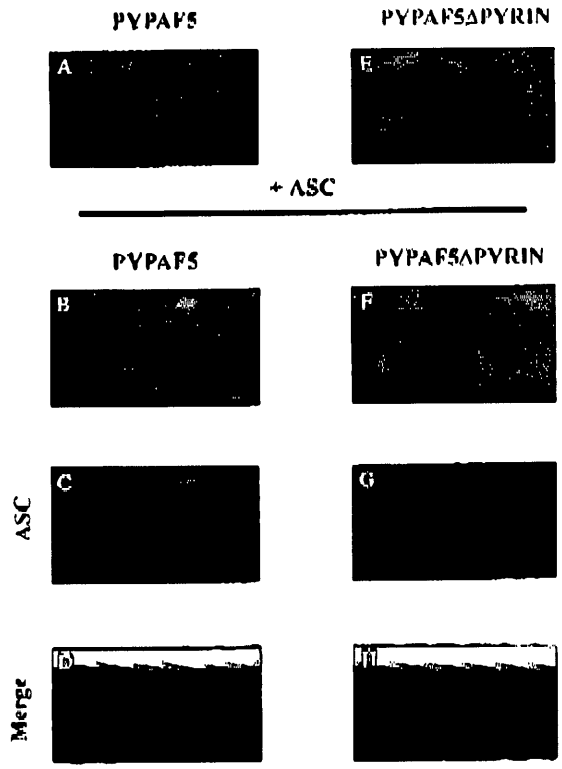


Fig. 3. The PYRIN domain of PYPAF5 is needed for recruitment to ASC punctate structures. HA-tagged ASC (blue staining) and FLAG-tagged PYPAF5 proteins (red staining) were expressed in 293T cells. A and E show PYPAF5 and PYPAF5ΔPYRIN, respectively, when expressed alone. B–D and F–H show the immunostaining patterns observed when each protein was co-expressed with ASC. Note the co-localization of PYPAF5 (B–D) and the lack of co-localization of PYPAF5ΔPYRIN (F–H) to the ASC punctate structures (arrows).

ability to activate NF-κB and pro-caspase-1. We report here that ASC recruits PYPAF5 to distinct cytoplasmic loci. In addition, PYPAF5 shows restricted expression to immune cells and synergistically activates NF-κB and caspase-1 when co-expressed with ASC. These findings identify PYPAF5 as a cell-type-specific upstream regulator of pro-inflammatory signaling pathways.

2. Materials and methods

2.1. Expression plasmids

Plasmids expressing full-length PYPAF2 (accession number AF464764), PYPAF3 (accession number AF464765), PYPAF4 (accession number AF479747), PYPAF5 (accession number AF479748) and PYPAF6 (accession number AY095145) with a C-terminal FLAG epitope were constructed using either pCI (Promega) or pCMV-4 (PYPAF4, Stratagene). The plasmid expressing a PYPAF5 truncation mutant lacking the PYRIN domain (PYPAF5ΔPYRIN, residues 81–892) with a C-terminal FLAG epitope was also constructed using pCI (Promega). Plasmids expressing mouse pro-IL-1β, pro-caspase-1, dominant negative IKKγ and inactive pro-caspase-1 (C285) were described previously [6,18].

2.2. PYPAF5 expression analysis

Total RNA from cells in culture was extracted (Qiagen) and expression profiles were determined by real time quantitative PCR analysis (Taqman®). In brief, an oligonucleotide probe (5′-ACGGAGCGGGCGTTCCTCTCCT-3′) was designed to anneal to PYPAF5 between two PCR primers (forward: 5′-CCGTGTCCGAG-

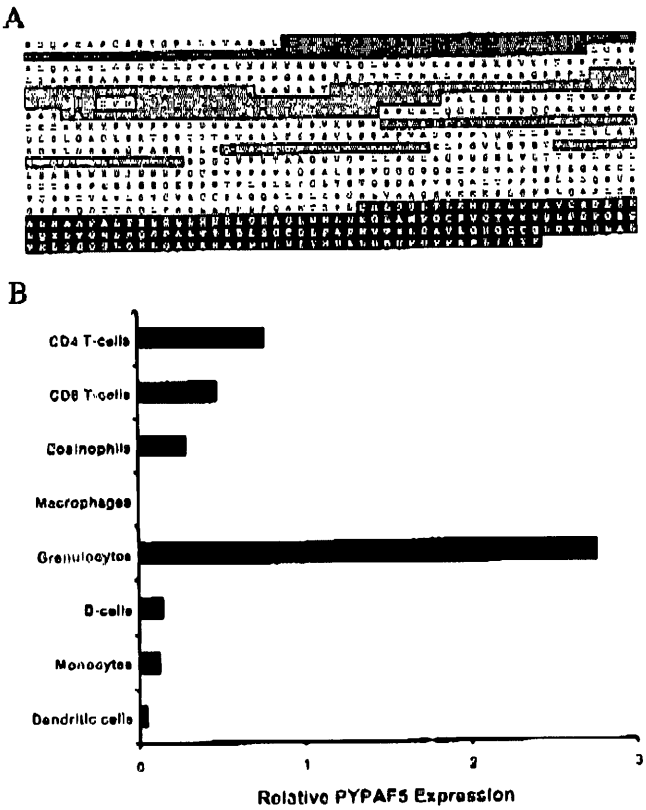


Fig. 4. Sequence and expression of PYPAF5. A: Amino acid sequence of PYPAF5. N-terminal PYRIN domain (residues 22–96, red shading), NBS domain (residues 194–513) with seven consensus motifs that are found in the NACHT subfamily of NTPases (blue shading), and C-terminal domain (residues 729–892) comprised of at least four LRRs. B: PYPAF5 expression in various immune cells by real time quantitative PCR. Level of PYPAF5 expression in various cell types was normalized to the expression of β2 microglobulin.

TACAAGAAGAAG-3′ and reverse: 5′-CGCGATGAGCAGCTTG-GT-3′). The probe was then fluorescently labeled with FAM (reporter dye) on the 5′ end and TAMRA (quencher dye) on the 3′ end. Probe and PCR primers designed for β2 microglobulin were described previously [18]. PCR reactions were performed on cDNAs from various cell types using primers and probes for both PYPAF5 and β2 microglobulin genes. Fluorescent emissions generated during PCR reaction was measured by Sequence Detector 7700 (Perkin Elmer Life Sciences). The expression level of PYPAF5 was normalized to the expression of β2 microglobulin for each sample.

2.3. Immunostaining assays

293T cells were transfected in poly-D-lysine-coated glass chamber slides (BioCoat, Becton-Dickinson Labware) with plasmids expressing HA-tagged ASC and FLAG-tagged PYPAFs using SuperFect transfection reagent (Qiagen). Cells were fixed 24 h after transfection in 4% paraformaldehyde, permeabilized and blocked in a buffer containing 0.3% Triton X-100, and incubated with the following primary and secondary antibodies: rabbit anti-HA polyclonal Y-11 (Santa Cruz Biotechnology), mouse anti-FLAG monoclonal antibody M2 (Sigma), Alexa-350 goat anti-rabbit IgG (Molecular Probes), and Alexa-598 goat anti-mouse IgG (Molecular Probes).

2.4. Reporter gene assays

The NF-κB assay was described previously [25]. 293T cells were transfected with pNF-κB luciferase reporter (Stratagene), pRL-TK Renilla reporter (Promega), and indicated expression plasmids. Cells were harvested and firefly and Renilla luciferase activities were determined using the Dual-Luciferase reporter assay system (Promega).

2.5. IL-1β secretion assays

COS-7 cells (Gibco) were co-transfected in 12-well (22-mm) plates using LipofectAMINE 2000 transfection reagent (Invitrogen) with plasmids encoding mouse pro-IL-1β and indicated expression plasmids (total DNA – 1.04 μg). Supernatants were collected 24 h after transfection and subjected to ELISA for mouse IL-1β according to the manufacturer's protocol (R&D Systems). The caspase-1 inhibitor z-WEHD-FMK was added to cells following transfection at a final concentration of 100 μM (R&D Systems).

3. Results and discussion

3.1. PYPAF family members are putative regulators of NF-κB and caspase-1

The PYPAF family of signaling proteins is comprised of at least eight members (Fig. 1). In addition, six other genes that encode novel PYPAFs are also found in the HTG database of genomic sequences (J. Bertin, unpublished data). Each family member contains an N-terminal PYRIN domain, a central NBS domain, and a C-terminal domain comprised of multiple LRRs. In addition to these domains, CARD7 also contains a C-terminal CARD domain and is therefore a member of both the CARD and PYRIN protein families [14] (Fig. 1). We recently identified PYPAF1 and PYPAF7 as upstream activators of NF-κB signaling and pro-caspase-1 [18,19]. The PYRIN–CARD protein ASC plays a central role in PYPAF1 and PYPAF7 signaling by recruiting pro-caspase-1 to the PYPAF/ASC signaling complex. In this study, we screened five other PYPAF family members (PYPAF2, PYPAF3, PYPAF4, PYPAF5 and PYPAF6) for their ability to activate downstream inflammatory signaling pathways.

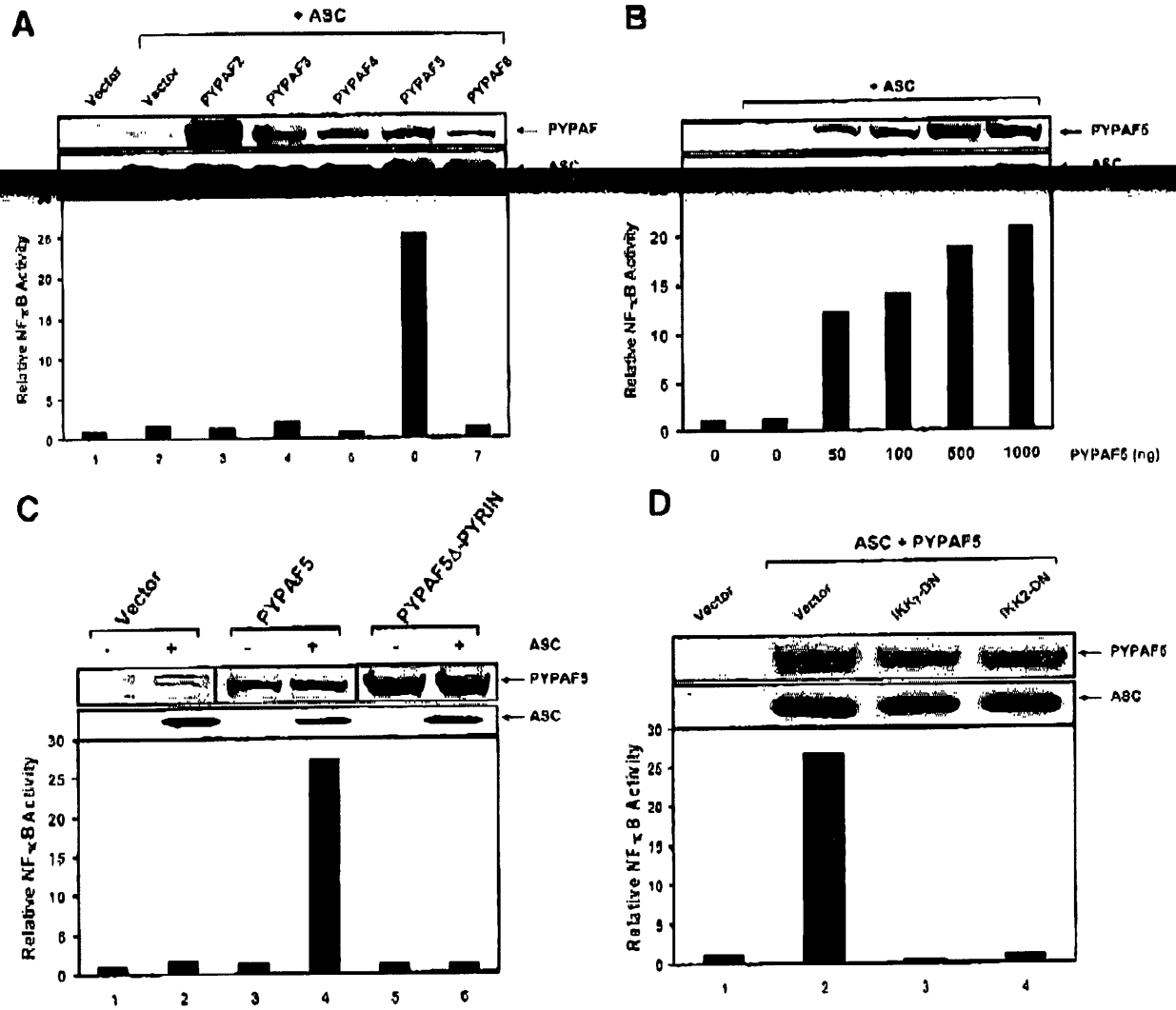
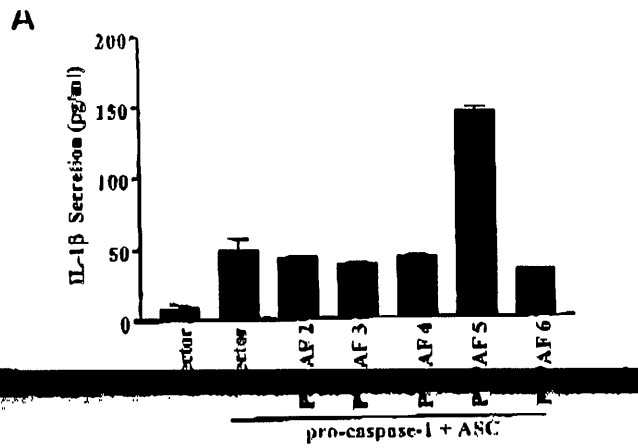
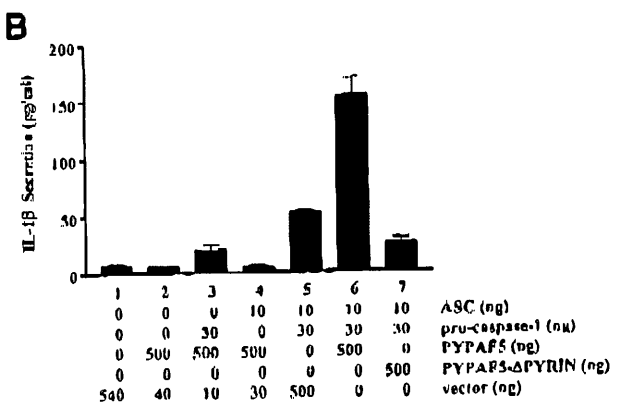


Fig. 5. PYPAF5 augments ASC-induced NF-κB activity. A: PYPAF5 synergizes with ASC to induce NF-κB activation. Plasmids expressing PYPAFs (1000 ng) and ASC (32 ng) were transfected into 293T cells, and relative luciferase activities were determined to measure the induction of NF-κB activity. B: PYPAF5 activates NF-κB in a concentration-dependent manner. C: The N-terminal PYRIN domain of PYPAF5 is required for synergy. 293T cells were transfected with plasmids expressing PYPAF5, and PYPAF5ΔPYRIN (1000 ng) with or without ASC (32 ng). The amount of DNAs in each transfection was kept constant by addition of empty vectors. Relative luciferase activities were then determined as a measure of NF-κB activity (lower panel). Immunoblot analysis shows expression of PYPAF5 and ASC (upper panels). D: PYPAF5 and ASC activate NF-κB through the IKK complex. 293T cells were transfected with plasmids expressing 32 ng of ASC and 500 ng of PYPAF5 with either 500 ng of empty vector or dominant negative mutants of IKKγ (IKKγ-DN) or IKK2 (IKK2-DN). Relative luciferase activities were then measured. Immunoblot analysis shows expression of PYPAF5 and ASC (upper panels).

3.2. Recruitment of PYPAF5 to ASC punctate structures

We first performed cellular co-localization studies to determine whether the PYPAF proteins interact with ASC. FLAG-tagged PYPAF2, PYPAF3, PYPAF4, PYPAF5 or PYPAF6 were co-expressed with HA-tagged ASC in cells and detected using a mixture of anti-HA and anti-FLAG antibodies. When expressed alone, the PYPAFs showed a broad cytoplasmic distribution that excluded the nucleus (Fig. 2A–E). As observed previously, ASC localized to cytoplasmic punctate structures when expressed alone ([26], and data not shown). However, PYPAF5 was found to co-localize with ASC when co-expressed (Fig. 2I and N). PYPAF2, PYPAF3, PYPAF4 and PYPAF6 failed to be recruited to the ASC punctate structures (Fig. 2, compare F and K, G and L, H and M, J and O). We also examined the ability of a PYPAF5 mutant lacking the N-terminal PYRIN domain (PYPAF5ΔPYRIN) to be recruited to the ASC punctate structures. When expressed alone, PYPAF5ΔPYRIN showed a broad cytoplasmic distribution similar to that of PYPAF5 (Fig. 3A and E). However, PYPAF5ΔPYRIN failed to associate with the ASC punctate structures, demonstrating that the PYRIN domain of PYPAF5 is necessary for recruitment (Fig. 3, compare B–D to F–H). We have been unable to detect an interaction between PYPAF5 and ASC by performing co-immunoprecipitation experiments when both proteins are overexpressed in cells. Our inability to detect an interaction is likely due to the transient nature of the interaction and/or the relative insolubility of PYPAF5/ASC complexes that form in the cell when these proteins are co-expressed. Nonetheless, the co-localization of PYPAF5 to the ASC punctate structures is required for the





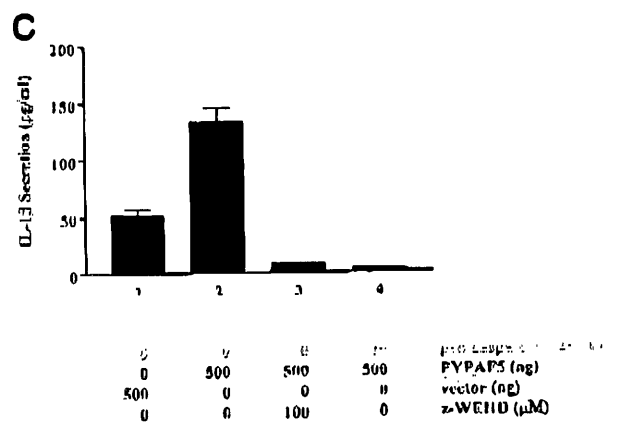


Fig. 6. PYPAF5 and ASC activate pro-caspase-1. COS-7L cells were co-transfected with plasmids encoding mouse pro-IL-1M in combination with various amounts of indicated plasmids encoding pro-caspase-1, ASC and PYPAFs. After 24 h, supernatants were collected and subjected to ELISA for IL-1M A: Activation of pro-caspase-1 by PYPAF5. B: Activation of pro-caspase-1 by PYPAF5 is dependent on its N-terminal PYRIN domain. C: PYPAF5 and ASC induction of IL-1M secretion is dependent on active caspase-1.

synergistic activation of NF-κB and caspase-1-dependent cytokine processing (see below).

3.3. PYPAF5 is expressed in immune cells

PYPAF5 has a tripartite structure consisting of an N-terminal PYRIN domain (residues 22–96), a central NBS domain (residues 194–513) and a C-terminal domain (residues NTPases and contains seven signature motifs, including the P-loop (residues 194–219, Fig. 4A) [27]. The PYRIN domain of PYPAF5 shares significant sequence similarity with the PYRIN domains of other PYRIN-containing proteins (data not shown). To determine if PYPAF5 is expressed in immune cells, peripheral blood leukocytes were fractionated into distinct cell populations. Quantitative real time PCR analysis showed PYPAF5 to be predominantly expressed in granulocytes with some expression in T-cells (Fig. 4B). Northern blot analysis using a multiple tissue expression array (CLONTECH) showed little or no expression in other tissues and cell lines (data not shown), suggesting a restricted expression of PYPAF5 to immune cells.

3.4. Activation of NF-κB by PYPAF5

We next examined whether PYPAF5 participates in NF-κB signaling pathways using a luciferase reporter plasmid. Expression of each PYPAF alone in cells failed to activate NF-κB at all protein levels examined (data not shown). In contrast, NF-κB was activated when high levels of ASC were expressed in cells (data not shown; [18,19]). However, when ASC was expressed at low protein levels that did not activate NF-κB, co-expression with PYPAF5 resulted in a potent activation of NF-κB (Fig. 5A, lane 6). Co-expression of ASC with PYPAF2, PYPAF3, PYPAF4 or PYPAF6 failed to activate NF-κB (Fig. 5A, lanes 3–5, 7). PYPAF5 activated ASC-dependent NF-κB signaling in a concentration-dependent manner (Fig. 5B). The N-terminal PYRIN domain of PYPAF5 was essential for NF-κB signaling, since deletion of this domain (PYPAF5ΔPYRIN) eliminated the synergistic induction of NF-κB activity (Fig. 5C, compare lanes 4 and 6). Immunoblot analysis revealed that ASC protein levels were not increased when co-expressed with PYPAF5, demonstrating that the activation of NF-κB was not due to increased levels of ASC (Fig. 5C, upper panels). NF-κB signaling occurred through the IKK complex because dominant-negative versions of IKK-γ and IKK-2 blocked the ability of PYPAF5 to synergistically activate NF-κB (Fig. 5D, lanes 3 and 4). Taken together, these data demonstrate that PYPAF5 is an activator of NF-κB signaling when co-expressed with ASC.

PYPAF5 and PYPAF6 play a role in caspase activation and cytokine processing. Active caspase-1 cleaves pro-IL-1β resulting in the release of IL-1β from cells. To measure the activation of pro-caspase-1, plasmids expressing pro-caspase-1 and mouse pro-IL-1β were transfected into COS-7L cells with plasmids encoding ASC and specific PYPAF family members. In this assay, the amount of murine IL-1β released into the culture medium 1 day after transfection correlates with the amount of intracellular caspase-1 activity [28]. When ASC was expressed at protein levels that resulted in low levels of caspase activity, co-expression with PYPAF5 resulted in a synergistic activation of pro-caspase-1 and a corresponding increase in IL-1β production (Fig. 6A). Co-expression of ASC with PYPAF2, PYPAF3, PYPAF4 or PYPAF6 failed to activate pro-caspase-1. The N-terminal PYRIN domain of PYPAF5 was essential for pro-caspase-1 activation, since deletion of this domain (PYPAF5ΔPYRIN) eliminated the syn-

analysis revealed that PYPAF5wPYRIN was expressed at levels similar to that of PYPAF5, indicating that loss of function was not due to reduced protein levels (data not shown). PYPAF5 and ASC were unable to synergistically induce cytokine expression when co-expressed with a caspase-1 active site mutant (C285A) (Fig. 6C, lane 4). Furthermore, addition of a caspase-1 inhibitor (z-WEHD) blocked the ability of PYPAF5 and ASC to induce the secretion of IL-1M(Fig. 6C, lane 3). Taken together, these data demonstrate that PYPAF5 and ASC synergistically activate pro-caspase-1.

3.6. Conclusions

In conclusion, we have identified PYPAF5 as a novel activator of pro-inflammatory signaling pathways. Our finding that PYPAF5 is recruited to ASC punctate structures through its N-terminal PYRIN domain suggests that these two proteins assemble together into a complex that mediates signal transduction. The restricted expression of PYPAF5 to peripheral blood leukocytes indicates a role for this PYPAF family member in inflammatory signaling. Our data further suggest that PYPAF5 and ASC participate in the activation of both NF-κB and pro-caspase-1. PYPAF5 may function in a manner analogous to APAF-1 and activate ASC and pro-caspase-1 through an induced proximity mechanism [29]. Both pro-IL-1M and pro-IL-18 are processed by caspase-1, suggesting that PYPAF5 may play an important role in cytokine production and immune regulation [30]. PYPAF1 and PYPAF7 were also recently found to activate pro-caspase-1 when co-expressed with ASC, suggesting an analogous role for these proteins in the production of IL-1 and IL-18 [18,19]. In this study, PYPAF2, PYPAF3, PYPAF4 and PYPAF6 failed to co-localize with ASC and activate NF-κB and pro-caspase-1, suggesting that these PYPAF family members may regulate other signal transduction pathways. Alternatively, these PYPAFs could be folded in such a manner that renders them unable to associate with ASC. The findings presented here indicate the PYPAF5 belongs to a subgroup of PYPAF family members that function to regulate NF-κB and cytokine production.

References

[1] Zou, H., Henzel, W.J., Liu, X., Lutschg, A. and Wang, X. (1997) Cell 90, 405–413.
[2] Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cell 91, 479–489.
[3] Bertin, J., Nir, W.J., Fischer, C.M., Tayber, O.V., Errada, P.R., Grant, J.R., Keilty, J.J., Gosselin, M.L., Robison, K.E., Wong, G.H., Glucksmann, M.A. and DiStefano, P.S. (1999) J. Biol. Chem. 274, 12955–12958.
[4] Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J. and Nunez, G. (1999) J. Biol. Chem. 274, 14560–14567.
[5] Ogura, Y., Inohara, N., Benito, A., Chen, F.F., Yamaoka, S. and Nunez, G. (2001) J. Biol. Chem. 276, 4812–4818.
[6] Geddes, B.J., Wang, L., Huang, W.-J., Lavellee, M., Manji, G.A., Brown, M., Jurman, M., Cao, J., Morgenstern, J., Merriam, S., Glucksmann, M.A., DiStefano, P.S. and Bertin, J. (2001) Biochem. Biophys. Res. Commun. 284, 77–82.
[7] Poyet, J.L., Srinivasula, S.M., Tnani, M., Razmara, M., Fernandes-Alnemri, T. and Alnemri, E.S. (2001) J. Biol. Chem. 276, 28309–28313.
[8] Damiano, J.S., Stehlik, C., Pio, F., Godzik, A. and Reed, J.C. (2001) Genomics 75, 77–83.
[9] Kobayashi, K., Inohara, N., Hernandez, L.D., Galan, J.E., Nunez, G., Janeway, C.A., Medzhitov, R. and Flavell, R.A. (2002) Nature 416, 194–199.
[10] Chin, A.I., Dempsey, P.W., Bruhn, K., Miller, J.F., Xu, Y. and Cheng, G. (2002) Nature 416, 190–194.
[11] Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J.F., Sahbatou, M. and Thomas, G. (2001) Nature 411, 537–539.
[12] Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., Achkar, J.P., Brant, S.R., Bayless, T.M., Kirschner, B.S., Hanauer, S.B., Nunez, G. and Cho, J.H. (2001) Nature 411, 603–606.
[13] Miceli-Richard, C., Lesage, S., Rybojad, M., Prieur, A.M., Manouvrier-Hanu, S., Hafner, R., Chamaillard, M., Zouali, H., Thomas, G. and Hugot, J.P. (2001) Nat. Genet. 29, 19–20.
[14] Bertin, J. and DiStefano, P.S. (2000) Cell Death Differ. 7, 1273–1274.
[15] Martinon, F., Hofmann, K. and Tschopp, J. (2001) Curr. Biol. 11, 118–120.
[16] Staub, E., Dahl, E. and Rosenthal, A. (2000) Trends Biochem. Sci. 26, 83–85.
[17] Fairbrother, W.J., Gordon, N.C., Humke, E.W., O'Rourke, K.M., Starovasnik, M.A., Jian-Ping, Y. and Dixit, V.M. (2001) Protein Sci. 10, 1911–1918.
[18] Manji, G., Wang, L., Geddes, B., Brown, M., Merriam, S., Al-Garawi, A., Mak, S., Lora, J.M., Briskin, M., Jurman, M., Cao, J., DiStefano, P.S. and Bertin, J. (2002) J. Biol. Chem. 277, 11570–11575.
[19] Wang, L., Manji, G.A., Grenier, J.M., Al-Garawi, A., Merriam, S., Lora, J.M., Geddes, B.J., Briskin, M., DiStefano, P.S. and Bertin, J. (2002) J. Biol. Chem. 277, 29874–29880.
[20] The International FMF Consortium (1997) Cell 90, 797–807.
[21] Hoffman, H.M., Mueller, J.L., Broide, D.H., Wanderer, A.A. and Kolodner, R.D. (2001) Nat. Genet. 29, 301–305.
[22] Feldmann, J., Prieur, A.M., Quartier, P., Berquin, P., Certain, S., Cortis, E., Teillac-Hamel, D., Fischer, A. and de Saint Basile, G. (2002) Am. J. Hum. Genet. 71, 198–203.
[23] Dode, C., Le Du, N., Cuisset, L., Letourneur, F., Berthelot, J.M., Vaudour, G., Meyrier, A., Watts, R.A., Scott, D.G., Nicholls, A., Granel, B., Frances, C., Garcier, F., Edery, P., Boulinguez, S., Domergues, J.P., Delpech, M. and Grateau, G. (2002) Am. J. Hum. Genet. 70, 1498–1506.
[24] Srinivasula, S.M., Poyet, J.-L., Razmara, M., Datta, P., Zhang, Z.-J. and Alnemri, E.S. (2002) J. Biol. Chem. 277, 21119–21122.
[25] Wang, L., Guo, Y., Huang, W.J., Ke, X., Poyet, J.L., Manji, G.A., Merriam, S., Glucksmann, M.A., DiStefano, P.S., Alnemri, E.S. and Bertin, J. (2001) J. Biol. Chem. 276, 21405–21409.
[26] Masumoto, J., Taniguchi, S., Ayukawa, K., Sarvotham, H., Kishino, T., Niikawa, N., Hidaka, E., Katsuyama, T., Higuchi, T. and Sagara, J. (1999) J. Biol. Chem. 274, 33835–33838.
[27] Kounin, E.V. and Aravind, L. (2000) Trends Biochem. Sci. 25, 223–224.
[28] Lee, S.H., Stehlik, C. and Reed, J.C. (2001) J. Biol. Chem. 276, 34495–34500.
[29] Salvesen, G.S. and Dixit, V.M. (1999) Proc. Natl. Acad. Sci. USA 96, 10964–10967.
[30] Fantuzzi, G. and Dinarello, C.A. (1999) J. Clin. Immunol. 19, 1–11.

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gB and Virus Egress Require Functional Biogenesis of Multivesicular Bodies[V]

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Received 22 June 2007/Accepted 2 August 2007

The biogenesis of multivesicular bodies (MVBs) is topologically equivalent to virion budding. Hence, a number of viruses exploit the MVB pathway to build their envelope and exit from the cell. By expression of dominant negative forms of Vps4 and Vps24, two components of the MVB pathway, we observed an impairment in infectious herpes simplex virus (HSV) assembly/egress, in agreement with a recent report showing the involvement in HSV envelopment of Vps4, the MVB-specific ATPase (C. M. Crump, C. Yates, and T. Minson, J. Virol. 81:7380–7387). Furthermore, HSV infection resulted in morphological changes to MVBs. Glycoprotein B (gB), one of the most highly conserved glycoproteins across the *Herpesviridae* family, was sorted to MVB membranes. In cells expressing the dominant negative form of Vps4, the site of intracellular gB accumulation was altered; part of gB accumulated as an endoglycosidase H-sensitive immature form at a calreticulin-positive compartment, indicating that gB traffic was dependent on a functional MVB pathway. gB was ubiquitinated in both infected and transfected cells. Ubiquitination was in part dependent on ubiquitin lysine 63, a signal for cargo sorting to MVBs. Partial deletion of the gB cytoplasmic tail resulted in a dramatic reduction of ubiquitination, as well as of progeny virus assembly and release to the extracellular compartment. Thus, HSV envelopment/egress and gB intracellular trafficking are dependent on functional MVB biogenesis. Our data support the view that the sorting of gB to MVB membranes may represent a critical step in HSV envelopment and egress and that modified MVB membranes constitute a platform for HSV cytoplasmic envelopment or that MVB components are recruited to the site(s) of envelopment.

Multivesicular bodies (MVBs) constitute a central station in the endocytic-lysosomal pathway. They are responsible for the biosynthetic delivery of hydrolases to lysosomes as well as for the sorting of a number of cell surface receptors, destined to degradation in the lysosome (69). At the ultrastructural level, early endosomes appear predominantly as tubulovesicular structures, whereas late endosomes, which are capable of fusing with lysosomes, exhibit a multivesicular aspect and, for this reason, are named MVBs. The transition between these two endosomal compartments occurs by involution of the limiting membrane to form intraluminal vesicles. When the MVBs fuse with lysosomes, the intraluminal vesicles and their contents are degraded. Both the lipid and protein compositions of the endosome change along the pathway to lysosomes (75). A major signal for sorting of cargoes along the MVB pathway is ubiquitination (41). In *Saccharomyces cerevisiae*, MVB biogenesis requires a total of 17 yeast class E Vps proteins (47). Vps23 and two other class E Vps proteins form a cytosolic complex termed ESCRT-I (endosomal *s*orting complex *r*equired for *t*ransport I), which recognizes ubiquitinated endosomal cargo (41). ESCRT-I activates another soluble class E Vps complex called ESCRT-II, which in turn is required to initiate the assembly of the ESCRT-III complex on endosomal membranes (3). ESCRT-III, the core of the apparatus that drives membrane curvature and MVB vesicle formation, is formed by four structurally related class E Vps proteins that exhibit homology to human *ch*romatin *m*odifying *p*roteins (CHMP) (3). Finally, to enable the recycling of MVB machinery, ESCRT-III recruits the AAA-type ATPase Vps4, a class E Vps protein that disassembles and thereby recycles the ESCRT machinery (3). Overexpression of ATPase-defective Vps4 proteins induces the formation of enlarged endosomes and dysfunctional MVBs that are defective in the sorting and recycling of endocytosed substrates ("class E" phenotypes) (3). The Vps4 mutants also prevent normal ESCRT protein trafficking, because these proteins are trapped on the surfaces of the aberrant MVBs (3). On the other hand, dominant negative forms of the ESCRT-III component Vps24/CHMP3, essential for vesicle invagination (47), like Vps24 fused to a bulky tag such as red fluorescent protein, induce class E-like phenotypes (81).

Numerous enveloped RNA viruses, including retroviruses

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Modification Form for Permit BIO-UWO-0147

Permit Holder: Sung Kim

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

Boram Ham

Anthony Bruni

Sarah Spanton

Andrew Martins

Soon-Duck Ha

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	P. aeurogenosa, S. typhimurium	Lactobacillus rhamnosus, E.coli EC1000, E.coli BL21
Approved Cells	Human (primary), rodent (primary), human (established), THP-1, rodent (established), RAW 2649	
Approved Use of Human Source Material	Blood (whole), PARF-CFP, mRFP-Rab7, mRFP-Rab5	
Approved GMO		pTRK830, pOR128, pTRK669. **
Approved use of Animals	mice	

* *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.*

Classification: 2

Date of last Biohazardous Agents Registry Form: Apr 28, 2008

Signature of Permit Holder

BioSafety Officer(s): Oct 5/09

Chair,Biohazards Subcommittee:

Modification Form for Permit BIO-UWO-0147

Permit Holder: Sung Kim

Approved Toxin(s)	cholera, diphtheria, CONT'D	anthrax toxin

† Researcher confirmed that the "Biosecurity Requirements Using Biological Agents" will be followed. See attached email (and requirements).

* *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.*

Classification: 2

Date of last Biohazardous Agents Registry Form: Apr 28, 2008

Signature of Permit Holder

BioSafety Officer(s): Oct 5/09

Chair,Biohazards Subcommittee:

Subject: [Fwd: Re: [Fwd: Re: [Fwd: [Fwd: Re: Anthrax toxin project]]]]
From: Sung Kim <Sung.Kim@schulich.uwo.ca>
Date: Fri, 02 Oct 2009 09:03:18 -0400
To: Jennifer Stanley <jstanle2@uwo.ca>

Hi Jennifer,
Yes, we will follow the requirements.
Thanks
Sk

Sung O. Kim Ph.D.
Assistant Professor
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University of Western Ontario

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http://publish.uwo.ca/~skim283/

>>> Jennifer Stanley <jstanle2@uwo.ca> 28/09/2009 12:47 pm >>>
Hi Dr. Kim
Please send me an e-mail confirming that you will follow the biosecurity requirements.
Thanks
Jennifer

Western

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.

Subject: Re: Use of lethal toxin
From: Sung Kim <Sung.Kim@schulich.uwo.ca>
Date: Fri, 06 Feb 2009 11:22:01 -0500
To: Jennifer Stanley <jstanle2@uwo.ca>

See below:

>>> Jennifer Stanley <jstanle2@uwo.ca> 2/6/2009 11:18 AM >>>
Thanks Dr. Kim

I understand from this that you store ~ 1 mg of each component. Total we have. We have in aliquots.
Do you have any LD50 information? about 100 micg/20g wt. in mice.
How do you dispose of the material when the experiments are complete? Toxin is inactivated in 3h at room temp. Bleach them, anyway.

Jennifer

Sung Kim wrote:
> Hi Jennifer:
>
> >>> Jennifer Stanley <jstanle2@uwo.ca> 2/5/2009 9:36 AM >>>
> Hi Dr. Kim
> Just a couple more questions:
> - how much of the toxins (PA and LF) do you keep on hand at once? ~1
> mg each
> - how much of each do you usually use at once? 5 micg What is the
> concentration of toxin? 50 micg/ml
> Thanks!
> Jennifer

The MTA is to obtain vectors to transform Lactobacillus rhamnosus for probiotic study. We will transform L. rhamnosus using these vectors to identify genes involved in macrophage activation in vitro.

pTRK830 (EmR vector for Lactobacillus rhamnosus) -
pORI28 (integration targeting plasmid, EmR)
pTRK669 (helper plasmid, CmR)
E. coli EC1000
/Purpose is for cloning and integration experiments in Lactobacillus rhamnosus.

We prepare recombinant lethal toxin and protective antigen from E. coli. After purification, we use them cell lines or primary peritoneal or bone marrow-derived macrophages in vitro. All toxins are kept in -80 in our lab. Our lab is locked all the time, unless some one is in site.

We are using E. coli-BL21 to express PA and Bacillus megaterium for LF. I believe they are commercial strains.
Mostly we use both toxins to treat cells.

We use toxins to treat mouse primary and immortalized macrophages, but sometimes use human or mouse fibroblasts.

------- Original Message --------
Subject: Re: Containment Question, lethal toxin
Date: Fri, 23 Jan 2009 11:10:08 -0500
From: Geneviève Lacroix <genevieve_lacroix@phac-aspc.gc.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>

Good morning Ms. Stanley,

The toxins you are describing me are 2 of the 3 components of anthrax toxin. There are too many variables for me to give you an answer. Although the toxins are produced separately in another host, the risk level is most probably the same, the toxins are probably as potent as if they were produced by B. anthracis. Once I receive the importation application, I will have to complete an in depth risk assessment, which will take some time.

However, I think this information will be useful to you.

Bacillus anthracis causes anthrax. B. anthracis requires 2 plasmids for its virulence. One plasmid contains the toxin genes (pX01) and the second plasmid contains the capsular genes (pX02). The exotoxins secreted by B. anthracis, encoded by pX01, are composed of three distinct components: protective antigen (PA), lethal factor (LF), and edema factor (EF). These proteins play a key role in the pathogenesis of anthrax. EF and LF have enzymatic functions but require PA, responsible for their transport into the host, to achieve their biological effects. These proteins individually cause no known physiological effects in animals but in pairs produce two toxic actions. Injection of PA with LF causes death of rats in 60 min, whereas PA with EF causes edema in the skin of rabbits and guinea pigs. S H Leppla, Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. PNAS May 1, 1982 vol. 79 no. 10 3162-3166.

This is as much as I can do for now. I hope this information will help you.

Regards

Genevieve Lacroix
A/Head, Importation and Biosafety Program/
Chef intérimaire, Importation et Services de biosécurité
Office of Laboratory Security / Bureau de la sécurité des laboratoires
Public Health Agency of Canada/ Agence de la santé publique du Canada
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http://www.phac-aspc.gc.ca/bls-sbl/index.html

Modification Form for Permit BIO-UWO-0147

Permit Holder: Sung Kim

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

Sarah Spanton

Andrew Martins

Soon-Duck Ha

Additional Personnel
(Please list additional personnel here)

Anthony Bruni

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	P. aeurogenosa, S. typhimurium	
Approved Cells	Human (primary), rodent (primary), human (established), THP-1, rodent (established), RAW 2649	
Approved Use of Human Source Material	Blood (whole)	PARF - CFP mRFP - Rab7 mRFP - Rab5
Approved GMO		
Approved use of Animals	mice	

* *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.*

Date of last Biohazardous Agents Registry Form Apr 28, 2008

Signature of Permit Holder:

BioSafety Officer(s): Stanley Sept 30/08

Chair, Biohazards Subcommittee:

Modification Form for Permit BIO-UWO-0147

Permit Holder: Sung Kim

Approved Toxin(s)		

* *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.*

Date of last Biohazardous Agents Registry Form Apr 28, 2008

Signature of Permit Holder:

BioSafety Officer(s): V Hanley Sept 30/08

Chair, Biohazards Subcommittee: C.M. Childs

Bio - UWO - 0147

THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Revised Biohazards Subcommittee: September, 2007

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario where the use of biohazardous infectious agents are described in the experimental work proposed. The form must also be completed if animal work is proposed involving the use of biohazardous agents or animal carrying zoonotic agents infectious to humans. Containment Levels will be required in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Health Canada (HC) 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 (Stevenson-Lawson Building, Room 60) for forward to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Coordinator at extension 81135. If there are changes to the information on this form (excluding grant title and funding agencies) modifications must be completed and sent to Occupational Health and Safety. See website: www.uwo.ca/humanresources

PRINCIPAL INVESTIGATOR Kim, Sung Ouk
SIGNATURE
DEPARTMENT Micro & Immuno
ADDRESS SDRI Rm 119, 1400 Western Rd.
PHONE NUMBER 85961
EMAIL sung.kim@schulich.uwo.ca

Location of experimental work to be carried out: Building(s) SDRI Room(s) 119

*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 it being sent to Occupational Health and Safety (See Section 12.0, Approvals). For research being done at Lawson Health Research Institute, London Regional Cancer Centre, Child and Parent Research Institute or Robarts Research Institute, University Biosafety Committee members can also sign as the Safety Officer.

GRANT TITLE(S):
~~- Dissecting signaling mechanisms of TNF-induced necrotic cell death~~
- Investigating the mechanisms of Nod-like receptor-induced cytokine release and cell death in macrophages

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK, SUCH AS THE RESEARCH GRANT SUMMARY THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED.

FUNDING AGENCY/AGENCIES CIHR

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

Soon-Duck Ha
Andrew Martins
Anthony Bruni
Sarah Spanton

1.0 Microorganisms

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

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?	Source/ Supplier	Health Canada or CFIA Containment Level
P. aeruginosa	⊘Yes O No	⊘Yes O No	O Yes O No	2×10^9 cells		O 1 ⊘2 O 3
S. typhimurium	O~~Yes~~ O No	⊘Yes O No	O Yes O No	2×10^9 "		O 1 ⊘2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 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 O 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 in the table below

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue
Human	⊘Yes O No	primary peripheral blood, ~~THP-1~~
Rodent	⊘Yes O No	peritoneal, bone-marrow-derived macrophages
Non-human primate	O Yes O No	
Other (specify)		

2.3 Please indicate the type of established cells that will be grown in culture in the table below.

Cell Type	Is this cell type used in your work?	Specific cell line(s)	Supplier / Source
Human	⊘Yes O No	THP-1	ATCC
Rodent	⊘Yes O No	RAW 264.7	ATCC
Non-human primate	O Yes O No		
Other (specify)	O Yes O No		

2.4 For above named cell types(s) indicate HC or CFIA containment level required O 1 ⊘2 O 3

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

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

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? ✓ YES O 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)	HC or CFIA Containment Level (select one)
Human Blood (whole) or other Body Fluid	Volunteers	✓ Yes O No		O 1 ✓ 2 O 3
Human Blood (fraction) or other Body Fluid		O Yes O No		O 1 O 2 O 3
Human Organs (unpreserved)		O Yes O No		O 1 O 2 O 3
Human Tissues (unpreserved)		O Yes O No		O 1 O 2 O 3
Human Organs (preserved)		O Yes O No		O 1 O 2 O 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? O YES ✓ NO
If no, please proceed to Section 5.0

4.2 Will genetic sequences from the following be involved:

- HIV O YES O NO
  if YES specify_____
- HTLV 1 or 2 or genes from any CDC class 1 pathogens O YES O NO
  if YES specify_____
- Other human or animal pathogen and or their toxins O YES O NO
  if YES specify_____

4.3 Will intact genetic sequences be used from

- SV 40 Large T antigen O YES O NO If YES specify_____
- Known oncogenes O YES O NO If YES specify_____

4.4 Will a live viral vector(s) or bacterial plasmid be used for gene transduction O YES O NO
If YES name _____.
Please attach a Material Safety Data Sheet or equivalent.

4.5 List specific vector(s) to be used. _____

4.6 Will virus be replication defective O YES O NO

4.7 Will virus be infectious to humans or animals O YES O NO

4.8 Will this be expected to increase the Containment Level required O YES O NO

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

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials using the viral vector in 4.0 be conducted? O YES O NO
If no, please proceed to Section 6.0
If YES attach a full description of the make-up of the virus.

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

5.3 How will the virus 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 O NO O PENDING

6.0 Animal Experiments

6.1 Will any of the agents listed be used in live animals? ✓ YES O NO
If no, please proceed to section 7.0

6.2 Name of animal species to be used mouse, C57 BL/6

6.3 AUS protocol # pending

6.4 If using murine cell lines, have they been tested for murine pathogens? ✓ YES O 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 bodily 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 ✓ NO If YES specify species _____
- Wild caught animals O YES ✓ NO If YES specify species _____ colony # _____
- Birds O YES ✓ NO
- Others (wild or domestic) O YES ✓ NO

8.0 Biological Toxins

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

8.2 If YES, please name the toxin_____

8.3 What is the LD_{50} (specify species) of the toxin_____

8.4 Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

9.0 Import Requirements

9.1 Will the agent be imported? (✓) YES ( ) NO
If no, please proceed to Section 10.0
If yes, country of origin USA

9.2 Has an Import Permit been obtained from HC for human pathogens? (✓) YES ( ) NO

9.3 Has an import permit been obtained from CFIA for animal pathogens? (✓) YES ( ) NO

9.4 Has the import permit been sent to OHS? ( ) YES (✓) NO
If yes, Permit # _____

10.0 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
- Employee Health and Safety Orientatioin

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.

SIGNATURE _____

11.0 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 ( ) 1 (✓) 2 ( ) 3

11.2 Has the facility been certified by OHS for this level of containment? (✓) YES ( ) NO

11.3 If yes, please give the date and permit number. BIO-UWO-0147

12.0 Approvals

UWO Biohazard Subcommittee

Signature _____ Date 28 April '08

Safety Officer for institution where experiments will take place

Signature _____ Date Sept. 25/08

Safety Officer for University of Western Ontario (if different from above)

Signature _____ Date _____

Approval Expiration Date April 28, 2011

The University of Western Ontario
Biohazard Subcommittee Meeting

Minutes of February 9, 2009
10:00 – 11:30 am, SSB 5161

Present: Dr. G. Kidder, Dr. S. Siu, Dr. G. Dekaban, Dr. T. DeLangley, Dr. S. Barr, J. Stanley
Regrets: No regrets

1.0 Introductions

No introductions needed.

2.0 Approval of Minutes of January 30, 2009

Motion: Dr. S. Siu Dr. S. Barr

3.0 Biohazardous Agents Registry Forms

3.1 Dr. C. Y. Kang (Revisit)

Motion: Dr. S. Barr Dr. T. DeLangley

Approved: Given information from CFIA, this project remains Level 2.

3.2 Dr. S. O. Kim (Modification, revisit)

Motion: Dr. S. Siu Dr. S. Barr

Tabled: More information required on lethal dose of toxin.

3.3 Dr. R. Dekoter

Motion: Dr. S. Barr Dr. G. Dekaban

Tabled: More information required.

Western

BIOSECURITY SUBCOMMITTEE
February 5, 2009
2:00 – 3:00 P.M., SSB 5161

MINUTES

1. **Introductions**

   Present: G. Kidder, G. Thorn, G. Margaritis, M. Mics, T. Hammoud, J. Stanley, S. May (guest).

2. **Approval of minutes, June 10, 2008**
   Motion: G. Margaritis G. Thorn
   Minutes Approved

3. **Communication of Biosecurity Initiatives**

   We would like to use Western News to inform the community about Bill C-54 and that we have a biosecurity plan and a subcommittee who addresses these issues on campus. The revised biosecurity plan and requirements will be taken to the Biosafety Committee. This will be the reason / trigger for the story.

4. **Bill C-54**

   J. Stanley updated the Subcommittee on discussions with the Public Health Agency of Canada. Notable updates on policy include the removal of security clearances for Risk Group 2 Labs and inventories available only on request. Level 3 visitors would unlikely need security clearances.
   Level 3 users can get security clearances from Michael Mics in Campus Police Services. In ACVS, 5 years of consistent performance reviews with no biosecurity issues can put an employee in good standing, even if there is a history / event of concern.

5. **Kim Protocol**

   J. Stanley will get more info regarding LD_{50}, the disposal method and the US Select Agent regulating requirements. The goal is to dispose of all