Modification Form for Permit BIO-UWO-0256

Permit Holder: Lakshman Gunaratnam

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

Ola Ismail

Xinghong Xhang

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	E.coli, Lentivirus	
Approved Primary and Established Cells	Mouse[primary] kidney, spleen, blood, bone marrow. Human[established]786-0, 769-P, HEK293, HEK293T/17, Jurkat, HK-2 Rodent[established] JAWSII, mIMCD-3, CMT-93 Porcine [established]LLC-PK1, canine	DC, 2, 4 (mouse)
Approved Use of Human Source Material		
Approved Genetic Modifications (Plasmids/Vectors)	[plasmids]: pcDNA3. [vectors]: pLUX-puro proprietory	
Approved Use of Animals	Musculus	
Approved Biological Toxin(s)	C3 Exotoxin, Phalloidin	SB202190 (S)-(+)-Camptothecin GM6001 DMSO

* *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: _____

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

Date of Last Biohazardous Agents Registry Form: Jun 29, 2010

Date of Last Modification (if applicable): Feb 08, 2011

BioSafety Officer(s): _____

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

1) Captothecin: Used to induce apoptosis in cells. Apoptotic cells are used as targets for phagocytosis in our experiments.[1]
2) SB 202190: Used to block MEK kinase in our cells. MEK kinase is believed to involved in metalloproease-mediated cleavage of KIM-1.[2]
3) GM6001: Used to block metalloproteases involved in KIM-1 shedding.[2] GM6001 will be injected into mice after ischemia-reperfusion injury surgery. This protocol will be submitted as a modification to our animal protocol.
4) DC 2.4 mouse dendritic cells were acquired from Dr. Kenneth Rock at Dana Farber Cancer Institute (via MTA). They will be used to study the interrelationship of antigen presenting cells with our kidney epithelial cells.

1. Ichimura T, Asseldonk EJ, Humphreys BD, Gunaratnam L, Duffield JS, Bonventre JV. Kidney injury molecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. *J Clin Invest.* May 2008;118(5):1657-1668.

2. Zhang Z, Humphreys BD, Bonventre JV. Shedding of the urinary biomarker kidney injury molecule-1 (KIM-1) is regulated by MAP kinases and juxtamembrane region. *J Am Soc Nephrol.* Oct 2007;18(10):2704-2714.

For the Captothecin:
How much (in ug) do you handle at once? 5mM in 15 uL
How much (in ug) do you store? 100mg
Please provide an LD50. Acute oral toxicity (LD50): 50.1 mg/kg [Mouse]

per email March 23, 2011

For the SB202190:
How much (in ug) do you handle at once? 0.05mg
How much (in ug) do you store? 5mg
Please provide an LD50. Acute dermal toxicity (LD50): 40000 mg/kg [Rat].

For the GM6001:
How much (in ug) do you handle at once? 0.010.05 mg
How much (in ug) do you store? 1mg-1g
Please provide an LD50. LD50: Not available.

0.01-.05 mg per e-mail March 23, 2011

-------- Original Message --------
Subject: Re: Biohazards Modification: Gunaratnam
Date: Mon, 14 Feb 2011 13:56:38 -0500
From: Lakshman Gunaratnam <Lakshman.Gunaratnam@lhsc.on.ca>
To:jstanle2@uwo.ca

Here is the MSDS for DMSO

Use: as solvent for chemicals in lab and for GM6001 injections into mice (10% solution)
Max single use: 200 microlitres
Max Lab Stock: 0.5L
LD50:ral, mouse: LD50 = 7920 mg/kg;
Oral, rat: LD50 = 14500 mg/kg;
Skin, rat: LD50 = 40 gm/kg;

Thanks for adding this to my Protocol.

Lakshman Gunaratnam, MD.,M.Sc.
Assistant Professor of Medicine and
Microbiology & Immunology
Transplant Nephrologist
London Health Sciences Centre-UH

Material Safety Data Sheet

Version 4.1
Revision Date 10/22/2010
Print Date 02/07/2011

1. PRODUCT AND COMPANY IDENTIFICATION

Product name : (*S*)-(+)-Camptothecin

Product Number : C9911
Brand : Sigma
Product Use : For laboratory research purposes.

Supplier : Sigma-Aldrich Canada, Ltd
2149 Winston Park Drive
OAKVILLE ON L6H 6J8
CANADA

Manufacturer : Sigma-Aldrich Corporation
3050 Spruce St.
St. Louis, Missouri 63103
USA

Telephone : +19058299500
Fax : +19058299292
Emergency Phone # (For both supplier and manufacturer) : 1-800-424-9300
Preparation Information : Sigma-Aldrich Corporation
Product Safety - Americas Region
1-800-521-8956

2. HAZARDS IDENTIFICATION

Emergency Overview

WHMIS Classification

D1B Toxic Material Causing Immediate and Serious Toxic Effects — Toxic by ingestion

GHS Classification

Acute toxicity, Oral (Category 3)

GHS Label elements, including precautionary statements

Pictogram

Signal word: Danger

Hazard statement(s)

H301	Toxic if swallowed.

Precautionary statement(s)

P264	Wash skin thoroughly after handling.
P270	Do not eat, drink or smoke when using this product.
P301 + P310	IF SWALLOWED: Immediately call a POISON CENTER or doctor/ physician.
P321	Specific treatment (see supplemental first aid instructions on this label).
P330	Rinse mouth.
P405	Store locked up.
P501	Dispose of contents/ container to an approved waste disposal plant.

HMIS Classification

Health hazard:	2
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	Toxic if swallowed.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Formula : $C_{20}H_{16}N_2O_4$
Molecular Weight : 348.35 g/mol

CAS-No.	EC-No.	Index-No.	Concentration
(S)-(+)-Camptothecin			
7689-03-4	-	-	-

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Take victim immediately to hospital. Consult a physician.

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. Consult a physician.

5. FIRE-FIGHTING MEASURES

Conditions of flammability
Not flammable or combustible.

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.

Hazardous combustion products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx)

Explosion data - sensitivity to mechanical impact
no data available

Explosion data - sensitivity to static discharge
no data available

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Wear respiratory protection. Avoid dust formation. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas. Avoid breathing dust.

Environmental precautions
Prevent further leakage or spillage if safe to do so. Do not let product enter drains.

Methods and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

Recommended storage temperature: 2 - 8 °C

Keep in a dry place.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
Where risk assessment shows air-purifying respirators are appropriate use a full-face particle respirator type N99 (US) or type P2 (EN 143) respirator cartridges as a backup to engineering controls. If the respirator is the sole means of protection, use a full-face supplied air respirator. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Face shield and safety glasses Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
Complete suit protecting against chemicals, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
Avoid contact with skin, eyes and clothing. Wash hands before breaks and immediately after handling the product.

Specific engineering controls
Use mechanical exhaust or laboratory fumehood to avoid exposure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	powder
Colour	no data available

Safety data

pH	no data available
Melting/freezing point	Melting point/range: 260 °C (500 °F) - dec.
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available

Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	no data available
Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
no data available

Materials to avoid
Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx)

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
LD50 Oral - rat - 153 mg/kg

Inhalation LC50
no data available

Dermal LD50
no data available

Other information on acute toxicity
no data available

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitization
no data available

Germ cell mutagenicity

Genotoxicity in vitro - mouse - lymphocyte
Other mutation test systems

Genotoxicity in vitro - Hamster - ovary
Sister chromatid exchange

Genotoxicity in vitro - Human - leukocyte
DNA inhibition

Genotoxicity in vitro - Hamster - Lungs
Mutation in mammalian somatic cells.

Genotoxicity in vitro - Hamster - Lungs

Sister chromatid exchange

Genotoxicity in vitro - mouse - leukocyte
DNA inhibition

Genotoxicity in vitro - Hamster - ovary
DNA damage

Genotoxicity in vitro - Human - HeLa cell
DNA inhibition

Genotoxicity in vitro - Chicken - Embryo
DNA inhibition

Genotoxicity in vitro - Human - HeLa cell
Other mutation test systems

Genotoxicity in vitro - Human - leukocyte
Other mutation test systems

Genotoxicity in vitro - Human - lymphocyte
Sister chromatid exchange

Genotoxicity in vitro - Hamster - Lungs
Cytogenetic analysis

Genotoxicity in vitro - Hamster - ovary
Cytogenetic analysis

Genotoxicity in vitro - Hamster - Lungs
DNA damage

Genotoxicity in vitro - mouse - lymphocyte
DNA inhibition

Genotoxicity in vitro - mouse - leukocyte
DNA damage

Genotoxicity in vitro - Monkey - Kidney
DNA damage

Genotoxicity in vitro - Human - lymphocyte
Cytogenetic analysis

Genotoxicity in vitro - Human - Other cell types
Cytogenetic analysis

Genotoxicity in vitro - Human - fibroblast
DNA damage

Genotoxicity in vitro - Human - HeLa cell
DNA damage

Genotoxicity in vitro - Human - lymphocyte
DNA damage

Genotoxicity in vitro - Human - Other cell types
DNA damage

Genotoxicity in vivo - mouse - Intraperitoneal
Cytogenetic analysis

Carcinogenicity

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.

ACGIH: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.

Reproductive toxicity

no data available

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
no data available

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

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

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

Synergistic effects
no data available

Additional Information
RTECS: UQ0492000

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product
Offer surplus and non-recyclable solutions to a licensed disposal company. Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.

Contaminated packaging
Dispose of as unused product.

14. TRANSPORT INFORMATION

DOT (US)
UN-Number: 1544 Class: 6.1 Packing group: III
Proper shipping name: Alkaloids, solid, n.o.s. ((S)-(+)-Camptothecin)
Marine pollutant: No

Poison Inhalation Hazard: No

IMDG
UN-Number: 1544 Class: 6.1 Packing group: III EMS-No: F-A, S-A
Proper shipping name: ALKALOIDS, SOLID, N.O.S. ((S)-(+)-Camptothecin)
Marine pollutant: No

IATA
UN-Number: 1544 Class: 6.1 Packing group: III
Proper shipping name: Alkaloids, solid, n.o.s. ((S)-(+)-Camptothecin)

15. REGULATORY INFORMATION

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

	CAS-No.
(S)-(+)-Camptothecin	7689-03-4

WHMIS Classification

D1B	Toxic Material Causing Immediate and Serious Toxic Effects	Toxic by ingestion

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

16. OTHER INFORMATION

Further information
Copyright 2010 Sigma-Aldrich Co. License granted to make unlimited paper copies for internal use only.
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.

Conforms to 93/112/EC and ISO 11014-1

SAFETY DATA SHEET

CALBIOCHEM®

Order Number **Customer Number**

1. Identification of the substance/preparation and of the company/undertaking

Product name : GM6001 in Solution

Chemical formula : $C_{20}H_{28}N_4O_4$

Synonym : Not available.

Catalog # : 364206

Supplier : EMD Biosciences, Inc.
10394 Pacific Center Court
San Diego, CA 92121
(858)450-5558/(800)854-3417
FAX: (858)453-3552

Emergency telephone number : Call Chemtrec®
(800)424-9300 (within U.S.A.)
(703)527-3887 (outside U.S.A.)

2. Composition / information on ingredients

Substance/Preparation : Substance

Chemical name*	CAS No.	EC Number	Symbol	R-Phrases
GM6001 in Solution		Not available.	Xi	R36/38

3. Hazards identification

Physical/chemical hazards : Not applicable.

Human health hazards : CAUTION!
MAY CAUSE EYE AND SKIN IRRITATION.

4. First-aid measures

First-Aid measures

Inhalation : If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Get medical attention.

Ingestion : Do NOT induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If large quantities of this material are swallowed, call a physician immediately. Loosen tight clothing such as a collar, tie, belt or waistband.

Skin Contact : In case of contact, immediately flush skin with plenty of water. Cover the irritated skin with an emollient. Remove contaminated clothing and shoes. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention.

Eye Contact : Check for and remove any contact lenses. In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical attention.

Effects and symptoms

Skin Contact : Hazardous in case of skin contact (irritant). Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering.

Eye Contact : Hazardous in case of eye contact (irritant).

Aggravating conditions : Repeated or prolonged exposure is not known to aggravate medical condition.

5. Fire-fighting measures

Flammability of the Product : May be combustible at high temperature.

Extinguishing Media

Suitable : SMALL FIRE: Use DRY chemical powder.
LARGE FIRE: Use water spray, fog or foam. Do not use water jet.

Hazardous thermal (de)composition products : These products are carbon oxides (CO, CO2), nitrogen oxides (NO, NO2...).

Special fire-fighting procedures : Fire fighters should wear positive pressure self-contained breathing apparatus (SCBA) and full turnout gear.

Protection of fire-fighters : Be sure to use an approved/certified respirator or equivalent.

6. Accidental release measures

Personal precautions : Splash goggles. Full suit. Boots. Gloves. Suggested protective clothing might not be sufficient; consult a specialist BEFORE handling this product.

Small Spill and Leak : Absorb with an inert material and put the spilled material in an appropriate waste disposal

Large Spill and Leak : Absorb with an inert material and put the spilled material in an appropriate waste disposal.

7. Handling and storage

Handling : Keep away from heat. Keep away from sources of ignition. Empty containers pose a fire risk, evaporate the residue under a fume hood. Ground all equipment containing material. Do not breathe gas/fumes/ vapor/spray. Wear suitable protective clothing. If you feel unwell, seek medical attention and show the label when possible. Avoid contact with skin and eyes.

Storage : Keep container tightly closed. Keep container in a cool, well-ventilated area. Do not store above 4°C (39.2°F).

Packaging materials

Recommended use : Use original container.

8. Exposure controls/personal protection

Engineering measures : Provide exhaust ventilation or other engineering controls to keep the airborne concentrations of vapors below their respective threshold limit value. Ensure that eyewash stations and safety showers are proximal to the work-station location.

Hygiene measures : Wash hands after handling compounds and before eating, smoking, using lavatory, and at the end of day.

Ingredient Name	Occupational Exposure Limits
GM6001 in Solution	Not available.

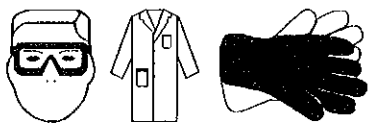
Personal protective equipment

Skin and body : Lab coat.

Hands : Gloves.

Eyes : Splash goggles.

Protective Clothing (Pictograms) :



9. Physical and chemical properties

Physical state : Liquid. (Supplied in DMSO)

Color : Not available.

Molecular Weight : 388.5 g/mole

Solubility : Not available.

Flash point : Not available.

Explosive properties : Risks of explosion of the product in presence of mechanical impact: Not available.
Risks of explosion of the product in presence of static discharge: Not available.

10. Stability and reactivity

Stability : The product is stable.

Conditions to avoid : Hygroscopic; keep container tightly closed.

Hazardous Decomposition Products : These products are carbon oxides (CO, CO2), nitrogen oxides (NO, NO2...).

11. Toxicological information

RTECS # : Not available.

Local effects

Skin irritation : Hazardous in case of skin contact (irritant).

Acute toxicity : LD50: Not available.
LC50: Not available.

Chronic toxicity : Repeated or prolonged exposure is not known to aggravate medical condition.

Other Toxic Effects on Humans : Not available.
Hazardous in case of skin contact (irritant), of eye contact (irritant).

Not available.

To the best of our knowledge, the toxicological properties of this product have not been thoroughly investigated

Carcinogenic effects	:	Not available
Mutagenic effects	:	Not available
Reproduction toxicity	:	Not available
Teratogenic effects	:	Not available.

12. Ecological information

Ecotoxicity	:	Not available.
Toxicity of the Products of Biodegradation	:	The product itself and its products of degradation are not toxic.

13. Disposal considerations

Methods of disposal: Waste of residues: Contaminated packaging	:	Waste must be disposed of in accordance with federal, state and local environmental control regulations.

14. Transport information

International transport regulations

Land - Road/Railway

ADR/RID Class	:	Not controlled under ADR (Europe).

Sea

IMDG Class	:	Not controlled under IMDG.

Air

IATA-DGR Class	:	Not controlled under IATA.
Special Provisions for Transport		Not applicable.

15. Regulatory information

EU Regulations

Hazard symbol(s) :

Classification	:	Irritant
Risk Phrases	:	R36/38- Irritating to eyes and skin.
Safety Phrases	:	S26- In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

U.S. Federal Regulations :

TSCA: No products were found.
SARA 302/304/311/312 extremely hazardous substances: No products were found.
SARA 302/304 emergency planning and notification: No products were found.
SARA 302/304/311/312 hazardous chemicals: No products were found.
SARA 311/312 MSDS distribution - chemical inventory - hazard identification: No products were found.
SARA 313 toxic chemical notification and release reporting: No products were found.
Clean Water Act (CWA) 307: No products were found.
Clean Water Act (CWA) 311: No products were found.
Clean air act (CAA) 112 accidental release prevention: No products were found.
Clean air act (CAA) 112 regulated flammable substances: No products were found.
Clean air act (CAA) 112 regulated toxic substances: No products were found.

HCS Classification	:	CLASS: Irritating substance.
State Regulations	:	
WHMIS (Canada)	:	Not controlled under WHMIS (Canada).

No products were found.

16. Other information

Hazardous Material Information System (U.S.A.)

[illegible]	2
[illegible]	1
Reactivity	0
Personal Protection	J

National Fire Protection Association (U.S.A.)

Notice to Reader

To the best of our knowledge, the information contained herein is accurate. However, neither the above named supplier nor any of its subsidiaries assumes any liability whatsoever for the accuracy or completeness of the information contained herein.
*Final determination of suitability of any material is the sole responsibility of the user. All materials may present unknown hazards and should be used with caution. Although certain hazards are described herein, we cannot guarantee that these are the only hazards that exist. **This product is intended for research use only.***

Material Safety Data Sheet

Version 4.1
Revision Date 10/21/2010
Print Date 02/07/2011

1. PRODUCT AND COMPANY IDENTIFICATION

Product name	: SB 202190			
Product Number	: S7067			
Brand	: Sigma			
Product Use	: For laboratory research purposes.			
Supplier	: Sigma-Aldrich Canada, Ltd 2149 Winston Park Drive OAKVILLE ON L6H 6J8 CANADA	Manufacturer	:	Sigma-Aldrich Corporation 3050 Spruce St. St. Louis, Missouri 63103 USA
Telephone	: +19058299500			
Fax	: +19058299292			
Emergency Phone # (For both supplier and manufacturer)	: 1-800-424-9300			
Preparation Information	: Sigma-Aldrich Corporation Product Safety - Americas Region 1-800-521-8956			

2. HAZARDS IDENTIFICATION

Emergency Overview

WHMIS Classification

D2B	Toxic Material Causing Other Toxic Effects	Moderate skin irritant Moderate respiratory irritant Moderate eye irritant

GHS Classification

Skin irritation (Category 2)
Eye irritation (Category 2A)
Specific target organ toxicity - single exposure (Category 3)

GHS Label elements, including precautionary statements

Pictogram

Signal word: Warning

Hazard statement(s)

H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.

Precautionary statement(s)

P261	Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray.
P264	Wash skin thoroughly after handling.
P271	Use only outdoors or in a well-ventilated area.
P280	Wear protective gloves/ eye protection/ face protection.
P302 + P352	IF ON SKIN: Wash with plenty of soap and water.
P304 + P340	IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.
P305 + P351 + P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

P312	Call a POISON CENTER or doctor/ physician if you feel unwell.
P321	Specific treatment (see supplemental first aid instructions on this label).
P332 + P313	If skin irritation occurs: Get medical advice/ attention.
P337 + P313	If eye irritation persists: Get medical advice/ attention.
P362	Take off contaminated clothing and wash before reuse.
P403 + P233	Store in a well-ventilated place. Keep container tightly closed.
P405	Store locked up.
P501	Dispose of contents/ container to an approved waste disposal plant.

HMIS Classification

Health hazard:	2
Flammability:	0
Physical hazards:	0

Potential Health Effects

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

3. COMPOSITION/INFORMATION ON INGREDIENTS

Synonyms : 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole

Formula : $C_{20}H_{14}FN_3O$

Molecular Weight : 331.34 g/mol

CAS-No.	EC-No.	Index-No.	Concentration
SB 202190			
152121-30-7	-	-	-

4. FIRST AID MEASURES

General advice
Consult a physician. Show this safety data sheet to the doctor in attendance.Move out of dangerous area.

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration. Consult a physician.

In case of skin contact
Wash off with soap and plenty of water. Consult a physician.

In case of eye contact
Rinse thoroughly with plenty of water for at least 15 minutes and consult a physician.

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

5. FIRE-FIGHTING MEASURES

Conditions of flammability
Not flammable or combustible.

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.

Hazardous combustion products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Hydrogen fluoride

Explosion data - sensitivity to mechanical impact
no data available

Explosion data - sensitivity to static discharge
no data available

6. ACCIDENTAL RELEASE MEASURES

Personal precautions
Use personal protective equipment. Avoid dust formation. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas. Avoid breathing dust.

Environmental precautions
Do not let product enter drains.

Methods and materials for containment and cleaning up
Pick up and arrange disposal without creating dust. Sweep up and shovel. Keep in suitable, closed containers for disposal.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes. Avoid formation of dust and aerosols.
Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection.

Conditions for safe storage
Keep container tightly closed in a dry and well-ventilated place.

Recommended storage temperature: 2 - 8 °C

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Contains no substances with occupational exposure limit values.

Personal protective equipment

Respiratory protection
For nuisance exposures use type P95 (US) or type P1 (EU EN 143) particle respirator.For higher level protection use type OV/AG/P99 (US) or type ABEK-P2 (EU EN 143) respirator cartridges. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Hand protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

Eye protection
Safety glasses with side-shields conforming to EN166 Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin and body protection
impervious clothing, The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Hygiene measures
Handle in accordance with good industrial hygiene and safety practice. Wash hands before breaks and at the end of workday.

Specific engineering controls
Use mechanical exhaust or laboratory fumehood to avoid exposure.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance

Form	solid
Colour	no data available

Safety data

pH	no data available

Melting/freezing point	no data available
Boiling point	no data available
Flash point	no data available
Ignition temperature	no data available
Autoignition temperature	no data available
Lower explosion limit	no data available
Upper explosion limit	no data available
Vapour pressure	no data available
Density	no data available
Water solubility	no data available
Partition coefficient: n-octanol/water	no data available
Relative vapour density	no data available
Odour	no data available
Odour Threshold	no data available
Evaporation rate	no data available

10. STABILITY AND REACTIVITY

Chemical stability
Stable under recommended storage conditions.

Possibility of hazardous reactions
no data available

Conditions to avoid
no data available

Materials to avoid
Strong oxidizing agents

Hazardous decomposition products
Hazardous decomposition products formed under fire conditions. - Carbon oxides, nitrogen oxides (NOx), Hydrogen fluoride

11. TOXICOLOGICAL INFORMATION

Acute toxicity

Oral LD50
no data available

Inhalation LC50
Dermal LD50
no data available

Other information on acute toxicity
no data available

Skin corrosion/irritation
no data available

Serious eye damage/eye irritation
no data available

Respiratory or skin sensitization

no data available

Germ cell mutagenicity
no data available

Carcinogenicity

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.

ACGIH: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH.

Reproductive toxicity

no data available

Teratogenicity

no data available

Specific target organ toxicity - single exposure (Globally Harmonized System)
Inhalation - May cause respiratory irritation.

Specific target organ toxicity - repeated exposure (Globally Harmonized System)
no data available

Aspiration hazard
no data available

Potential health effects

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

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

Synergistic effects
no data available

Additional Information
RTECS: Not available

12. ECOLOGICAL INFORMATION

Toxicity

no data available

Persistence and degradability
no data available

Bioaccumulative potential
no data available

Mobility in soil
no data available

PBT and vPvB assessment
no data available

Other adverse effects

no data available

13. DISPOSAL CONSIDERATIONS

Product
Offer surplus and non-recyclable solutions to a licensed disposal company. Contact a licensed professional waste disposal service to dispose of this material.

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.
SB 202190	152121-30-7

WHMIS Classification

D2B	Toxic Material Causing Other Toxic Effects	Moderate skin irritant Moderate respiratory irritant Moderate eye irritant

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations and the MSDS contains all the information required by the Controlled Products Regulations.

16. OTHER INFORMATION

Further information
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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.

MATERIAL SAFETY DATA SHEET MSDS#GCC1-10
DIMETHYL SULFOXIDE (DMSO) *September 28, 2010*

1. CHEMICAL PRODUCT AND COMPANY IDENTIFICATION

PRODUCT NAME: Dimethyl Sulfoxide (DMSO) **CHEMICAL NAME:** Dimethyl Sulfoxide

SYNONYMS/TRADE NAMES: Enviro S®, dimethyl sulphoxide, methyl sulfoxide, sulfinylbis [methane]
MOLECULAR FORMULA: C_2H_6OS **MOLECULAR WEIGHT:** 78.13
USE of the substance: *Solvent for manufacture of pharmaceuticals, fine chemicals and polymers.*

MANUFACTURER/SUPPLIER:
GAYLORD CHEMICAL COMPANY, LLC
P. O. BOX 1209
SLIDELL, LA 70459-1209

EMERGENCY HEALTH, SAFETY & ENVIRONMENTAL INFORMATION, CALL:
Gaylord Chemical Company, LLC (985) 649-5464 (8:00 am - 5:00 pm CST)
(205) 342-0652 (Nights and Weekends)
EMERGENCY TRANSPORTATION INFORMATION, CALL:
CHEMTREC (800) 424-9300
OTHER INFORMATION, CALL: Gaylord Chemical Company, LLC (985) 649-5464

2. COMPOSITION/INFORMATION ON INGREDIENTS

Hazardous Ingredients	CAS No	EINECS	Weight %	OSHA PEL	ACGIH TLV®
Dimethyl Sulfoxide	67-68-5	200-664-3	≥99%	Not established	Not established

NTP/IARC/OSHA CARCINOGEN: No
EC Classifications *(R or S phrases)*: None Required

3. HAZARDS IDENTIFICATION

WARNING! COMBUSTIBLE LIQUID AND VAPOR
PHYSICAL APPEARANCE: Clear liquid
ODOR: Essentially odorless
EFFECTS OF OVEREXPOSURE:

General: DMSO has shown very few toxic symptoms in humans. The most common are nausea, skin rashes and an unusual garlic-onion-oyster smell on body and breath.
Inhalation: High vapor concentrations may cause headache, dizziness, and sedation.
Eyes: Low hazard for usual industrial/ commercial handling by trained personnel.
Skin: Stinging and burning of the skin as well as rashes and vesicles have been seen. A heat reaction may occur if applied to wet skin. Avoid contact with DMSO solutions containing toxic material or materials whose toxicological properties are not known. DMSO easily penetrates the skin and may enhance the rate of skin absorption of skin-permeable substances. But because of DMSO's low toxicity and its inability to carry less-permeable substances with it through the skin, it can be concluded that DMSO does not pose a significant threat by skin absorption.
Ingestion: A low ingestion hazard.

HMIS AND NFPA HAZARD RATINGS: Health - 1, Flammability - 1, Chemical Reactivity - 0
Note: HMIS and NFPA ratings involve data and interpretations that may vary from company to company. In both cases the lower the number, the less the hazard. They are intended only for rapid, general identification of the magnitude of the specific hazard. To deal adequately with the safe handling of this material, all the information contained in this MSDS must be considered.

4. FIRST AID MEASURES

NEVER GIVE FLUIDS OR INDUCE VOMITING IF PATIENT IS UNCONSCIOUS OR IS HAVING CONVULSIONS.

General advice:
Remove contaminated clothing promptly (launder before reuse).

Eye Contact:
Flush thoroughly with running water (including under eyelids) for at least 15 minutes. If irritation persists after flushing, seek medical attention.

Skin Contact:
Wash contaminated skin with water. Seek medical attention if irritation persists.

Ingestion:
Seek immediate medical care. Do not induce vomiting.

Inhalation:
Remove to fresh air. If breathing has stopped, provide artificial respiration, keep the victim warm and seek medical attention.

Special advice:
In general, DMSO is not dangerous to people, but like any other chemical, it should be treated with care, respect and common sense.

5. FIRE-FIGHTING MEASURES

Combustible liquid and vapor

Extinguishing Media:
Foam, carbon dioxide, dry powder, and water spray.

Special protective equipment for fire-fighters:
Wear a self-contained Breathing Apparatus (SCBA).

Special Exposure Hazards:
Burning dimethyl sulfoxide produces poisonous gases (sulfur oxides). Wear rubber gloves, SCBA, and rubber suit.

Flashpoint and method:
89°C (192°F) closed cup
95°C (203°F) open cup

Flammable Limits (% in air):
LEL: 3.0 - 3.5% by volume
UEL: 42 - 63% by volume

Autoignition Temperature:
300-302°C (572-575°F)

Spill, Leak, or Release:
Note: *Review sections 3, 4, and 5 of this MSDS before proceeding with clean up. Use appropriate PERSONAL PROTECTIVE EQUIPMENT during clean up.*

6. ACCIDENTAL RELEASE MEASURES

Personal Precautions:
In case of mist formation use a respirator or Self-Contained Breathing Apparatus (SCBA).

Environmental Precautions:
If a spill or leak occurs, immediately consult your environmental supervisor. Remove ignition sources. Ventilate the area. Do not breathe the vapor or get liquid in eyes or on skin/clothing.

Spill Clean-up Methods:
Dilute and flush to wastewater treatment or absorb with inert material. Do not allow the material to enter streams or waterways.

Recommended Decontamination Facilities:
Eye bath, water washing facilities

7. HANDLING AND STORAGE

Usage/Handling Precautions:

Keep away from sources of ignition. No Smoking. Do not breathe vapor or mist. Avoid contact with skin, eyes, or clothing.

Storage Precautions:

Keep container tightly closed, in a well-ventilated place. Freezes (solidifies) at 18°C (64°F)

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Exposure Limits:

ACGIH Threshold Limit Value (TLV): not established

OSHA (USA) Permissible Exposure Limit (PEL, 1989 Table Z-1-A values or section-specific standards): not established

AIHA Workplace Environmental Exposure Level "WEEL" guideline for airborne concentrations in the workplace: 250 ppm (8-hr TWA)

Ventilation:

Good general ventilation (typically 10 air changes per hour) should be used. Ventilation rates should be matched to conditions. Use process enclosures, local exhaust ventilation, or other engineering controls to maintain ventilation.

Respiratory Protection:

In case of mist formation use a respirator. Respirator type: organic vapor cartridge, SCBA or SAR. If respirators are used, a program should be instituted to assure compliance with OSHA standard 29 CFR § 1910.134.

Hand Protection/Protective clothing:

Butyl rubber or nitrile (NBR) rubber gloves. Rubber apron and boots if splash hazard.

Eye Protection:

Safety glasses with side shield, tight-fitting goggles or face shield.

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance:	colorless liquid
Odor:	essentially odorless
Odor Threshold:	not available
pH:	8.5 (50/50 in water)
Boiling Point:	189°C (372°F)
Flashpoint and method:	89°C (192°F) closed cup 95°C (203°F) open cup
Flammable Limits (% in air):	LEL: 3.0 - 3.5% by volume UEL: 42-63% by volume
Autoignition Temperature:	300-302°C (572-575°F)
Vapor Pressure:	0.55 mbar (0.46 mmHg) @ 20°C (68°F)
Specific Gravity:	1.1 at 20°C (68oF) (water = 1)
Solubility in water at 20°C:	miscible
Octanol/Water Partition Coefficient:	$\log P_{ow}$ = -2.03
Viscosity at 25°C (77°F):	2.0 mPas or cP
Vapor Density (Air = 1):	2.7
Evaporation rate (n-butyl acetate = 1):	0.026
Melting Point:	18°C (64°F)
Sensitivity to Static Discharge:	Material is unlikely to accumulate a static charge, which could act as an ignition source.

10. STABILITY AND REACTIVITY

Stability: Stable

Conditions to avoid: Prolonged heating above 150°C (302°F) can cause rapid, *exothermic* decomposition

Materials to avoid: Organic and inorganic acid chlorides, strong oxidizing agents, alkali metals, hydrobromic acid, acidic solutions of alkali bromides

Hazardous decomposition products: Sulfur dioxide, formaldehyde, methyl mercaptan, dimethyl sulfide, dimethyl disulfide, and bis (methylthio) methane

Hazardous polymerization: Will not occur. *No stabilizers are needed or present.*

11. TOXICOLOGICAL INFORMATION

Data for Dimethyl Sulfoxide:

Acute Toxicity Data:

Oral LD-50 (male rat): 14,500-28,300 mg/kg

Inhalation (rat): No mortality rate @ 2,900 mg/m^3 (900 ppm)/ 24 hrs.

Dermal LD-50 (rat): 40,000 mg/kg

Skin irritation (human): Mild

Repeated skin application (human): Slight irritation

Skin sensitization (human): None by EC protocols

Eye irritation (human): None by EC protocols

Subchronic Toxicity Data:**

Oral study (13 weeks, rat): LOEL = 8800 mg/kg/day (minor target organ effects: liver) (reduced body weight gain): NOEL = 1100 mg/kg/day

Inhalation study (13 weeks, rat): NOAEL = 0.964 mg/L (302 ppm)

** **Note - definitions for data:**
LOEL = lowest observed effect level **NOAEL** = no observed adverse effect level **NOEL** = no observed effect level.

Developmental Toxicity Data:

DMSO is not considered to be directly embryotoxic and has been shown to be a successful cryoprotectant for mammalian semen and embryos.

A mouse teratology NOEL of 12 g/kg/day has been established based on research with a 50% DMSO solution administered orally. Teratology data suggests that:

1. DMSO is not a teratogen to mammals when administered via oral and dermal routes at dose level that do not produce overt maternal toxicity.
2. DMSO is not a teratogen at low dose levels regardless of the route of administration.
3. The teratogenic potential of DMSO is dependent on route of administration, the dose level and the gestational time of exposure, but in all cases is extremely low or non-existent.

Mutagenicity/Genotoxicity Data:

Salmonella typhimurium assay (Ames test): negative (+/- activation). DMSO is used as a neutral solvent in the Ames mutagen test.

12. ECOLOGICAL INFORMATION

Introduction:

This environmental effects summary is written to assist in addressing emergencies created by an accidental spill which might occur during shipment or handling of this material. It is not meant to address discharges to sanitary sewers or publicly owned treatment works.

Aquatic Toxicity:

The LC_{50} (96 hrs.) for ten species of fish range from 32,500 to 43,000 ppm. The LC_{50} for two species of protozoans are 32,000 and 38,000 ppm. The concentration required to inhibit growth (EC_{50}) for five species of blue-green algae and one green algae species ranged from 0.4 to 4.0%. DMSO is non-bio-accumulating since the log of the octanol/water partition coefficient is -2.03.

Phytotoxicity:

Soaking tomato, cucumber, and bean seeds for 18hrs in up to 8% DMSO solutions had no effect on germination rate. DMSO has no effect on the growth rate of corn when sprayed on at rates up to 30 gallon/acre. When diluted with a large amount of water, release of DMSO, directly or indirectly, to the environment is not expected to have significant effect.

Biological Oxygen Demand:

Theoretical Oxygen Demand at 10 ppm:	**123 mg oxygen**
Chemical Oxygen Demand at 10 ppm:	**107 mg/l**
Biological Oxygen Demand-5 at 10 ppm:	**≤1.0 mg/l**

13. DISPOSAL CONSIDERATIONS

Disposal Methods

Waste Disposal:

Dilute and flush to an approved wastewater treatment system. Bacterial decomposition of dimethyl sulfoxide during wastewater treatment can result in the release of dimethyl sulfide (a volatile substance with a strong disagreeable odor). Waste DMSO can also be incinerated in an approved furnace where permitted. Consult federal, state or local authorities for proper disposal procedures.

Empty Containers:

Should be transported/delivered using a registered waste carrier for recycling or waste disposal in accordance with local regulations.

14. TRANSPORT INFORMATION

DOT (USA) Status:

Bulk (>119 gallons per container)
Proper shipping name: Combustible liquid, N.O.S. (Dimethyl Sulfoxide)
Hazard Class: Combustible liquid
I.D. Number: NA 1993
Packing Group: III
Reportable Quantity: N/A
Placards: 1993 (Combustible)
Quantity limitations: None

Drum (<119 gallons per container)
Proper shipping name: Dimethyl Sulfoxide
Hazard Class: Not regulated
I.D. Number: None
Packing Group: None
Label(s): None

TDG (Canada) Status: unregulated.
ICAO - International Civil Aviation Organization status: unregulated
IATA - International Air Transport Agency status: unregulated
ADR and IMDG - International Dangerous Goods status: unregulated

15. REGULATORY INFORMATION

This document has been prepared in accordance with the MSDS requirements of the OSHA Hazard Communication Standard 29 CFR 1910.1200.
Occupational Safety and Health Administration "OSHA" hazardous chemical(s): Dimethyl sulfoxide
Material(s) known to the State of California to cause cancer: None
Material(s) known to the State of California to cause adverse reproductive effects: None
Massachusetts Substance List: None

New Jersey Workplace Hazardous Substance List: None
Pennsylvania Hazardous Substance List: None
This document has been prepared in accordance with the MSDS requirements of the WHMIS Controlled Products Regulation.
WHMIS (Canada) Ingredient Disclosure List: Listed
WHMIS9 (Canada) Status: Regulated
WHMIS (Canada) Hazard Classification: B/3 – Combustible Liquid
IARC - International Agency for Research on Cancer Carcinogenicity Classification (components present at 0.1% or more): Not Listed
ACGIH - American Conference of Governmental Industrial Hygienists: Not Listed
NTP - National Toxicology Program: Not listed
Reporting requirements of Section 313 or Title III of the superfund Amendments and Reauthorization Act (SARA) of 1986 and 40 CFR Part 372: None
SARA (U.S.A.) Sections 311 and 312 hazard classification(s): fire hazard
TSCA - US Toxic Substances Control Act: This product is listed on the TSCA inventory.
CEPA/DSL - Canadian Environmental Protection Act/ Domestic Substances List: Listed.
EINECS - European Inventory of Existing Commercial Chemical Substances: No. 200-664-3
AICS/NICNAS - Australian Inventory of Chemical Substances/National Industrial Chemical notification and Assessment Scheme: This product is listed on AICS.
Japanese Handbook of Existing and New Chemical Substances: Listed.
EC Classification and User Label Information (Council Directive 67/548/EEC and 1999/45/EC): Hazard Symbols and Risk Phrases – None Required
ICH (International Council on Harmonization): Class III – Solvent with low toxic potential

16. OTHER INFORMATION

US/CANADIAN Label Statements:
WARNING!
COMBUSTIBLE LIQUID AND VAPOR
HIGH VAPOR CONCENTRATIONS MAY CAUSE DROWSINESS
Store away from heat and light.
Distill with caution.
Keep away from heat and flame.
Avoid breathing high vapor concentrations.
Keep container tightly closed.
Use with adequate ventilation and proper protective equipment given elsewhere in this MSDS.
FIRST AID:
If inhaled, move to fresh air. Treat symptomatically. Get medical attention if symptoms persist.
IN CASE OF FIRE:
Eliminate all ignition sources. Flush spill area with water spray. Prevent runoff from entering drains, sewers, and streams.
Since emptied containers retain product residue, follow label warnings even after container is emptied.
CAUTION:
FOR MANUFACTURING, PROCESSING OR REPACKAGING BY TRAINED PERSONNEL

MSDS Issue Date: September 28, 2010 **Compiled by: John Ferguson**
Supersedes MSDS Dated: September 12, 2007
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DC 2.4

Cloned Dendritic Cells Can Present Exogenous Antigens on Both MHC Class I and Class II Molecules[1]

Zhenhai Shen,*‡ Glen Reznikoff,† Glenn Dranoff,† and Kenneth L. Rock[2]*‡

Pathways for presenting proteins from the extracellular fluids on MHC class I molecules have been described in macrophages. However, it is uncertain whether similar mechanisms exist in dendritic cells, because conventional preparations of these cells can be contaminated with macrophages. We addressed this issue by transducing granulocyte-macrophage CSF into bone marrow cultures followed by supertransfection with *myc* and *raf* oncogenes. These immortalized clones displayed dendritic morphology, and many expressed the dendritic cell-specific markers DEC-205 and 33D1 as well as high levels of MHC molecules and costimulatory molecules. Using these cloned dendritic cells, we found that exogenous OVA could be presented on both their MHC class I and class II molecules. This presentation was markedly enhanced when the Ag was particulate and internalized by phagocytosis. Presentation of particulate OVA on MHC class I molecules was insensitive to the weak base chloroquine, but was blocked by peptide aldehyde inhibitors of the proteasome, indicating that the class I-presented peptides were generated in the cytosol. Brefeldin A, which inhibits the exocytosis of newly synthesized proteins from the endoplasmic reticulum, also inhibited Ag presentation. These results establish that dendritic cells can present exogenous Ags on MHC class I molecules and appear to use a similar phagosome to cytosol pathway as macrophages. Therefore, dendritic cells are likely to play an important role in generating immune responses to tissue transplants and tumors in vivo. Furthermore, these findings provide an approach for targeting vaccine Ags into these cells to prime immune responses in vivo. *The Journal of Immunology,* **1997, 158: 2723–2730.**

MHC class I molecules play an important role in immune surveillance by displaying antigenic peptides on the cell surface (1). The majority of these presented peptides are generated by a multicatalytic proteolytic particle, the proteasome, which is present in the cytosol and nucleus of all eukaryotic cells (2). These antigenic fragments are then translocated into the endoplasmic reticulum (ER)[3] by the transporter associated with Ag presentation (TAP), where they bind to newly assembled MHC class I molecules and then are transported to the plasma membrane (1). As a consequence of these mechanisms, class I-presented peptides are derived in most situations exclusively from cellular and viral proteins synthesized by the APCs. Ags in the extracellular fluids do not gain access to this pathway in most cells and fail to be presented (3).

However, a subset of APCs can present exogenous Ags on class I molecules (4, 5), and this process is markedly enhanced when the Ag is internalized by phagocytosis (6, 7) or macropinocytosis (8). In these cells the exogenous Ag is transferred from the endocytic compartment into the cytosol where it is degraded and presented by the classical MHC class I pathway (8–10). Alternatively, in some cases peptides from the exogenous Ag appear to be generated in the endocytic compartment and to bind to MHC class I molecules on the cell surface (7, 11–13).

The APCs that can present exogenous Ags on MHC class I are quantitatively recovered from lymphoid tissues in a low density fraction that is enriched in macrophages and dendritic cells (DC) (5). That macrophages can mediate this form of Ag presentation has been shown in assays with highly purified macrophages and cloned macrophage cell lines (14). It has been more difficult to address whether DC possess a similar Ag-presenting capability because purified preparations of these cells are often contaminated with macrophages. Therefore, this question can only be resolved with absolutely pure DC. This is an important issue because DC are extremely potent APCs that are believed to play a key role in the initiation of T cell responses (15). This was the rationale in the present study for isolating cloned dendritic cell lines.

The exogenous pathway is active in vivo (6, 16) and probably plays an important role in generating CTL responses in several situations. A major pathway for stimulating CTL to tumors and transplanted tissue involves the representation of tumor or alloantigens on host bone marrow-derived APCs (17–19). Moreover, a similar pathway may be involved in generating anti-viral responses (3). Finally, this pathway can be exploited for stimulating T cell responses to Ags in vaccines (20). Therefore, it is important to elucidate the underlying cellular and molecular mechanisms underlying this pathway of presentation.

Materials and Methods

Cell lines and Abs

NIH J2 Leuk was provided by Dr. U. Rapp (Wurzburg, Germany) (21). FDCP-1 is a GM-CSF-dependent cell line used to measure GM-CSF production (22). The retrovirus-producing cell line Crip MFG-murine GM-CSF (23), the macrophage cell line A3.1A7 (14), and T-T hybridomas RF33.70 (anti-OVA, K^b) (24) and MF2.2D9 (anti-OVA $^+$ IA^b) (6) were

Divisions of *Lymphocyte Biology and †Divisions of Hematologic Malignancies and Human Cancer Genetics, Dana Farber Cancer Institute, and ‡Department of Pathology, Harvard Medical School, Boston, MA 02115

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[1] This work was supported by Grants from the National Institutes of Health (to K.L.R.), a postdoctoral training grant from the National Institutes of Health (to Z.S.), and a Markey Young Scientist Award, the Claudia-Adams Barr Foundation, and the Cancer Research Institute (to G.D.).

[2] Address correspondence and reprint requests to Dr. Kenneth L. Rock, Division of Lymphocyte Biology, Dana Farber Cancer Institute, 44 Binney St., Boston, MA 02115.

[3] Abbreviations used in this paper: ER, endoplasmic reticulum; TAP, transporter associated with antigen presentation; DC, dendritic cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; RT-PCR, reverse transcription-polymerase chain reaction; LLnL, *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal; LLM, *N*-acetyl-L-leucinyl-L-leucinyl-methional.

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previously described mAbs Y3 (anti-H2-K^b) (25), Y3P (anti-IA) (26), 2.4G2 (anti-FcγRII) (27), 16-10A1 (anti-B7-1) (28), GL-1 (anti-B7-2) (29), YN1/1.7.4 (anti-ICAM-1) (30), D7 (anti-Ly6A/E) (31), M5/49 (anti-Thy-1) (32), MI/70 (anti-Mac-1) (33), NLDC145 (anti-DEC-205) (34), 3ID1 (35), and Moma-2 (36) were provided by the laboratories of origin and/or obtained from the American Type Culture Collection (Rockville, MD).

Immortalization of DC

Bone marrow cells flushed from the femurs and tibias of C57BL/6 mice (The Jackson Laboratories, Bar Harbor, ME) were depleted of RBC by ammonium chloride treatment. Nucleated cells (5×10^6) were then infected with an amphotropic, replication defective, retrovirus-expressing murine GM-CSF (CRIP MFG-murine GM-CSF) by cocultivation for 48 h in the presence of polybrene as previously described (37). Nonadherent cells were displaced by gentle pipetting and then placed into culture in 24-well dishes in the presence of RPMI, 10% FCS, and 10 ng/ml murine GM-CSF. Cells were refed every 2 days. Cultures developed an adherent monolayer and clusters of DC colonies. Cultures were dispersed when confluent and placed into medium lacking GM-CSF. After an additional 2 wk in culture in the absence of GM-CSF to expand the cell population, the floating cells were collected and infected with a retrovirus encoding *myc* and *raf* using supernatant (50%) from NIH J2 Leuk cells. After 36 h at 37°C, floating cells were collected, washed, and resuspended in DMEM high glucose supplemented with 10% FCS, L-glutamine, and antibiotics and passaged in tissue culture flasks. From these cell lines DC were subcloned by limiting dilution. For the subcloning and initial passage, conditioned media (50%) from the uncloned DC lines was added to the culture medium.

Immunofluorescence

Immunofluorescence staining was performed as previously described (38). Briefly, dendritic clones were incubated with mAb-containing supernatants for 45 min at 4°C, followed by FITC-conjugated goat anti-rat IgG or FITC-conjugated goat anti-mouse IgG (1/40; Cappel, Organon Teknika Corp., West Chester, PA). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson).

RNA extraction and RT-PCR

Total RNA was extracted from cells and purified using the RNeasy Total RNA kit (Qiagen, Chatsworth, CA), and cDNA was prepared using reverse transcriptase. The oligonucleotide primers CCTTTGTGCCCAGCCT TATA (complementary to the positive strand of DEC-205 sequence, position 478) and CATTCTTTTCCCAGTTACCT (complementary to the negative strand of DEC-205 sequence, position 685) were synthesized by the Molecular Biology Core Facility of Dana-Farber Cancer Institute. A plasmid containing 5′ 2-kb DEC-205 cDNA (kindly provided by Drs. Wanping Jiang and Michel Nussenzweig, Rockefeller University, New York, NY) and cDNA from various clones were used as templates in PCR reaction. PCR-amplified products were analyzed on a 1.2% agarose gel.

Assays for phagocytosis

For ultrastructural analysis, DC2.4 cells were incubated with latex beads (3 μm in diameter) in 10-cm diameter culture dishes at 37°C for 30 min, and then washed and fixed with 1% paraformaldehyde. Subsequent embedding, ultrathin sectioning, and electron microscopy were performed at the Core Facility of Harvard Medical School (Boston, MA). For immunofluorescence analysis, cells were plated on coverslips, incubated with FITC-conjugated latex beads (3 μm in diameter) at 37°C for 30 min, and washed. Cells were examined on a fluorescence microscope (with the help of Dr. Joel Swanson, Harvard Medical School), and trypan blue (Fisher Scientific, Pittsburgh, PA; 25%, pH 5.0) was added to quench extracellular FITC-conjugated beads.

Preparation of Ag beads

Iron oxide beads (from PerSeptive Diagnostics, Cambridge, MA) were covalently conjugated to chicken egg OVA according to manufacturer's instruction. FITC (Sigma Chemical Co., St. Louis, MO) was passively absorbed to Latex beads (Polyscience, Inc., Warrington, PA; 1 μm in diameter) according to the manufacturer's instructions.

Ag presentation assays

APC were incubated in serum-free OptiMEM (Life Technologies, Gaithersburg, MD) supplemented with Nutridoma (Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C with or without the inhibitors brefeldin A (5 μg/ml, Sigma Chemical Co.), chloroquine (100 μM; Sigma Chemical Co.), cytochalasin B (5 μg/ml; Sigma Chemical Co.), LLnL (40 μM), LLM (40 μM), and MG132 (10 μM), followed by the addition of Ag. Ag was added in soluble form or bound to iron oxide beads. In some cases, OVA was loaded onto the cytosol by osmotic lysis of pinosomes (39), or SIIN FEKL was expressed in the cytosol using a vaccinia recombinant (a gift from Drs. Jon Yewdell and Jack Bennink) (40). After 5-h incubation at 37°C, the cells were washed, fixed with 1% paraformaldehyde solution, and added to microtiter plates. In some assays, live APCs (10^5/well) were incubated with varying concentrations of Ags in 200 μl of culture medium in flat-bottom microtiter plates. The culture medium was RPMI 1640 prepared as previously described and containing 0.25 μM indomethacin (41). Specific T-T hybrids were added to the microtiter plates and incubated for 20 h at 37°C, after which an aliquot (100 μl) of supernatant was collected and freeze-thawed. The IL-2 content in culture supernatants was assayed using an IL-2-dependent cell line CTLL as previously described (42, 43).

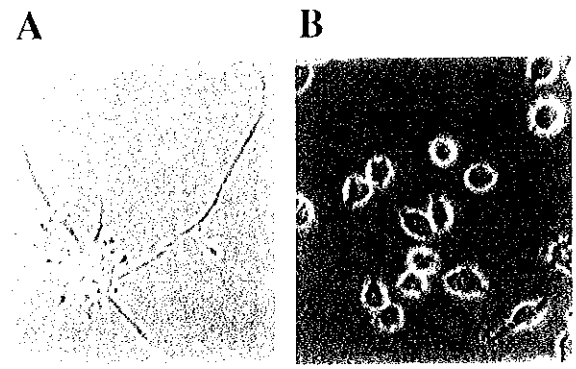

FIGURE 1. Photomicrographs of DC (*A*; DC2.4) and macrophage cells (*B*; A3.1A7). Magnification, ×630.

Results

Isolation of cell lines with DC morphology

It is well established that bone marrow-derived DC can be cultured in GM-CSF for short periods (44, 45). We attempted to develop long term cell lines by transducing bone marrow cells with GM-CSF. This resulted in the growth of cell populations that contained a subpopulation of cells with dendritic morphology. However, growth of these DC was not sustained beyond 6 wk, and we were unable to clone them.

We, therefore, modified this approach by superinfecting GM-CSF-transduced bone marrow cells with *myc* and *raf* oncogenes. The resulting cell populations contained cells with dendritic morphology that continued to grow in culture. By limiting dilution, 20 clones of DC were obtained in 4 to 5 wk. These cells had prominent dendritic processes and ruffled edges (Fig. 1*A*) that were not observed on macrophages (Fig. 1*B*); they attached to plastic and then detached over time, so that cultures contained both adherent and floating cells. Where examined, the DC clones do not continue to make measurable GM-CSF using a sensitive bioassay (data not shown). These DC clones have been in culture for >6 mo and can be frozen with cryopreservatives and thawed with good viability.

Characterization of cell surface molecules

We characterized the surface phenotype of these DC clones by immunofluorescence and flow cytometry. Individual fluorescence histograms for one clone are shown in Figure 2 (*A–K*), and the phenotypes of several different clones are summarized in Table I. All clones lacked T cell-specific (e.g., CD3) and B cell-specific (surface Ig) markers (data not shown). Most of these cells expressed high levels of MHC class I and class II molecules, the costimulatory molecules B7-1 and B7-2, as well as CD32 (FcγRII)

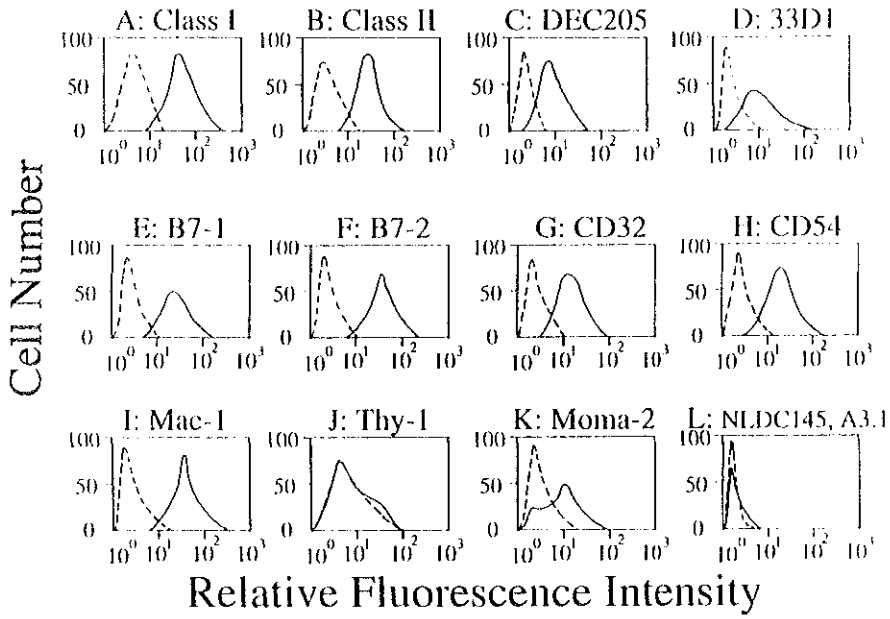

FIGURE 2. Immunofluorescence analysis of immortalized DC 2.4 cell line. DC 2.4 cells (*A–K*) and A3.1A7 macrophages (*L*) were stained by indirect immunofluorescence with mAb supernatants of the indicated specificities followed by appropriate FITC-conjugated secondary Abs as described in *Materials and Methods*. Dotted lines represent cell staining with FITC-conjugated secondary Abs only.

Table I. *Summary of surface phenotype of DC clones and A3.1A7*[a]

Marker	DC				
	DC1.2	DC 2.4	DC 2.5.1	DC4.1	A3.1A7
DEC205	+	+		+	
33D1	+/-	+		+	
B7-1	+ +	+	+	+	+/-
B7-2	+ +	+ +	+ +	+ +	+ +
Class I	+ +	+ +	+ +	+ +	+ +
Class II	+ +	+	+ +	+	+
CD32	+	+	+	+	+ +
Moma-2	+	+	+	+	+ +
Mac-1	+ +	+	+	+	+ + +
Thy-1					+/-
Ly 6A1		-			-
CD54	+ +	+ +	+ +	+ +	ND

[a] Summary of surface phenotype of representative clones of DC compared with that of macrophage cells, A3.1A7. Four representative DC clones and A3.1A7 were stained by indirect immunofluorescence with mAb supernatants of the indicated specificities followed by appropriate FITC-conjugated secondary Abs as described in *Materials and Methods*. Relative fluorescence intensity is indicated with plus and minus signs: one plus represents approximately one log scale.

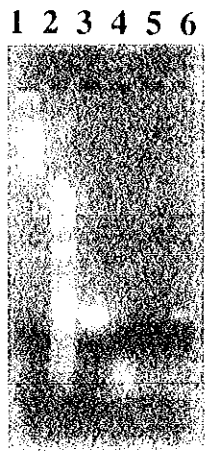

FIGURE 3. Analysis of RT-PCR products on 1.2% agarose gel. *Lane 1*, λ DNA *Hin*dIII digest; *lane 2*, PhiX174 RF DNA *Hae*III digest; *lane 3*, PCR product using plasmid containing DEC-205 cDNA as template; *lane 4*, RT-PCR product of mRNA from A3.1A7 macrophages; *lane 5*, RT-PCR product of mRNA from DC 2.5.1; *lane 6*, RT-PCR product of mRNA from DC 2.4.

and CD54 (intracellular adhesion molecule-1). These markers have all been reported on cultured DC (46–48), although they can also be expressed on other cell types.

The most specific markers for murine DC are the DEC-205 molecule recognized by the mAb NLDC145 (49) and the Ag detected by the mAb 33D1 (35). NLDC145 stained many of the DC clones, e.g., DC1.2, DC2.4, and DC4.1 (Fig. 2). The expression of DEC-205 in these cells was confirmed by RT-PCR (Fig. 3). The other dendritic marker, 33D1, also stained several clones. As expected, the macrophage cells A3.1A7 did not express DEC-205 (Fig. 2*L*) or 33D1 (data not shown). We concluded from these criteria that these cells represented cloned DC.

There were some lines with dendritic morphology that lacked the expression of both dendritic markers (Table I). For 33D1 this might simply reflect the sensitivity of the immunofluorescence assay because even on the DC where we detected expression, the intensity of staining was weak. However, these cells were also negative for the other dendritic marker, DEC-205, by both immunofluorescence and RT-PCR. The DEC-205-positive and -negative cells otherwise appeared to express the same surface molecules and were indistinguishable from one another on morphologic criteria. It is possible that the DEC-205-negative cells are of another lineage of cells (e.g., monocytes) or that there is heterogeneity in the expression of DEC-205 and 33D1 by primary isolated DC.

FIGURE 4. Ultrastructure of DC2.4 incubated with 3 μm-diameter Latex beads for 30 min at 37°C and then washed and handled as described in *Materials and Methods*. Magnification, ×8000.

DC clones are phagocytic

To determine whether the DC clones were phagocytic, they were incubated with latex beads. When viewed by phase microscopy, the DC appeared to rapidly internalize these particles (data not shown). To determine whether the beads were ingested and not simply bound to the cell surface, we initially performed ultrastructural studies. Figure 4 shows an electron micrograph of DC2.4 cells incubated with latex beads at 37°C for 30 min. Numerous beads were visualized in most cells and appeared to be in vesicles. However, it is possible that surface-bound beads could give a similar appearance, depending on the plane of sectioning. Therefore, to further verify that beads were internalized into cells, we performed an immunofluorescence analysis. The DC were incubated with FITC-labeled latex beads and then analyzed by fluorescence microscopy. Trypan blue was added to quench the fluorescence of beads outside the cells. Figure 5 showed both phase (Fig. 5*A*) and fluorescence (Fig. 5*B*) images of representative cells. Most DC contained beads whose fluorescence was not quenched with trypan blue. The fluorescence of surface-bound or free beads was quenched. These analyses indicated that the DC clones are phagocytic.

Presentation of OVA on MHC class I and class II molecules

The availability of cloned DC allowed us to examine their Ag-presenting capabilities and particularly whether they could present exogenous Ags on MHC class I molecules. The presentation of Ag was determined by measuring the production of IL-2 from T-T hybrids specific for OVA peptides bound to MHC class I molecules. DC2.4 cells, a representative DC clone, presented on MHC class I molecules both soluble OVA and particulate OVA added to the culture medium (Fig. 6*A*). However, compared with soluble

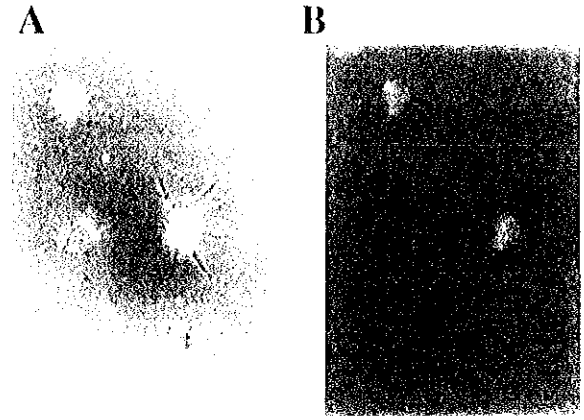

FIGURE 5. DC2.4 are phagocytic. Cells were incubated with 3-μm diameter Latex beads conjugated with FITC for 30 min at 37°C and then quenched with trypan blue (25%, pH 5). Both phase (*A*) and fluorescence (*B*) pictures of the same field were taken. Many intracellular fluorescent beads are of the plane of focus. Extracellular beads were not fluorescent in any plane of focus. Magnification, ×630.

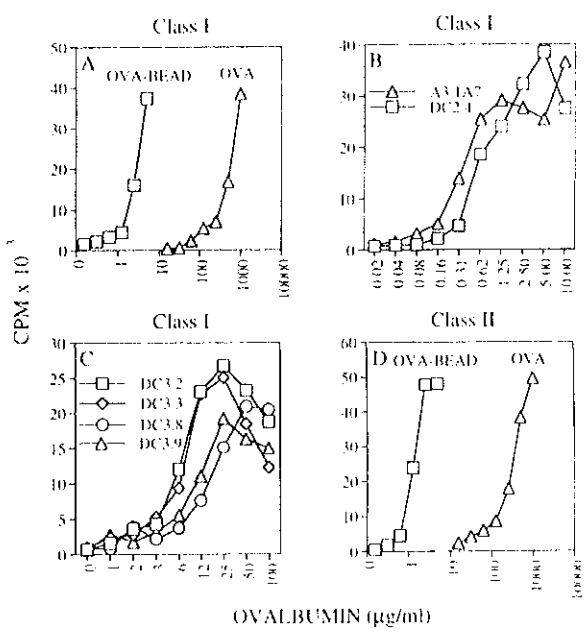

FIGURE 6. Presentation of exogenous OVA and soluble OVA on MHC class I and class II molecules. APCs (5×10^4/well) and RF33.70 (*A*, *B*, and *C*; 5×10^4/well) or ME.2D9 (*D*; 5×10^4/well) and the indicated amount of bead-conjugated OVA or soluble OVA were cultured in microtiter plates (200 μl). Cultures were then handled as described in *Materials and Methods*. *A*, Presentation of bead-conjugated OVA (open square) and soluble OVA (open triangle) by DC2.4 cells on MHC class I molecules; *B*, presentation of bead-conjugated OVA by DC2.4 (open square) and A3.1A7 (open triangle) on MHC class I molecules. *C*, Presentation of bead-conjugated OVA by various DC clones on MHC class I molecules. *D*, Presentation of bead-conjugated OVA (open square) and soluble OVA (open triangle) by DC2.4 cells on MHC class II molecules.

OVA, the presentation of bead-bound OVA was 100- to 1000-fold more efficient (Fig. 6*A*). Other DC clones were similarly capable of presenting OVA on MHC class I molecules (Fig. 6*C*). This

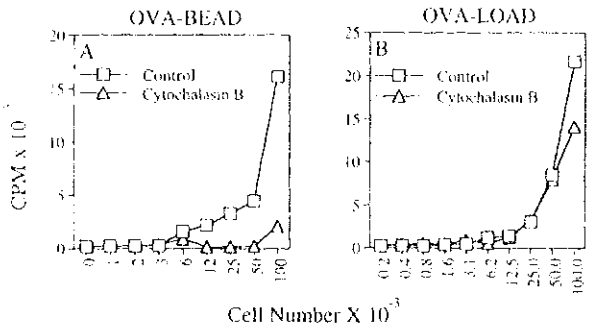

FIGURE 7. Effect of cytochalasin B on exogenous OVA presentation on MHC class I molecules. DC2.4 cells (2×10^5/ml) were pretreated with 5 μg/ml cytochalasin B for 30 min (open triangle) or medium alone (open square) followed by the addition of bead-conjugated OVA (50 μg/ml; *A*) or hypotonic loaded OVA by lysis of pinosome (2 mg/ml; *B*), further incubated for 6 h, and then fixed with 1% paraformaldehyde. Cells were then titrated in microtiter plates (100 μl), and the T-T hybrid cells (5×10^4/well), RF33.70 (*A* and *B*), were added to the cultures (200 μl total) and handled as described in *Materials and Methods*.

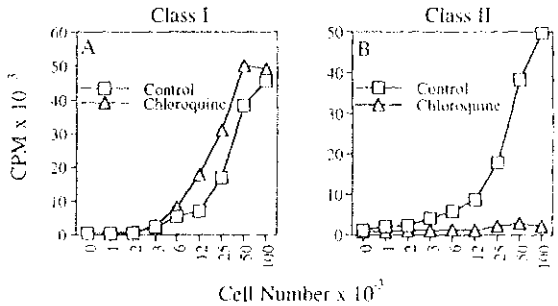

FIGURE 8. Effect of chloroquine on exogenous OVA presentation on MHC class I and class II molecules. DC2.4 cells (2×10^5/ml) were pretreated with 100 μM chloroquine for 30 min (open triangle) or with medium alone (open square) followed by the addition of bead-conjugated OVA (50 μg/ml), further incubated for 6 h, and fixed with 1% paraformaldehyde. Cells were then titrated in microtiter plates (100 μl), and the T-T hybrids (5×10^4/well), RF33.70 (*A*) and MF2.2D9 (*B*), were added to the cultures (200 μl total) and handled as described in *Materials and Methods*.

Ag-presenting capability was similar to that of A3.1A7, a macrophage cell line (Fig. 6*B*) (14). Since the DC were pure clones, we conclude that the pathway for presenting exogenous Ag on class I molecules is active in these APCs.

We also examined the ability of the DC to present Ag on MHC class II molecules. These cells presented soluble OVA to an OVA plus IA^b-specific T-T hybrid (Fig. 6*D*). Similar to the class I pathway, DC2.4 presented bead-bound OVA more efficiently than soluble OVA. Therefore, a DC can acquire exogenous Ag and present it simultaneously on both class I and class II molecules. Compared with similarly derived macrophage cell lines, the DC expressed higher levels of MHC class II molecules and were more potent at class II Ag presentation (data not shown). Without IFN-γ stimulation, the macrophage cell lines were either incapable or only weakly able to present Ag on class II (14).

Presentation of particulate OVA was inhibited by cytochalasin B

Phagocytosis was previously shown to be crucial for the presentation of particulate OVA by macrophages (11, 14). Here we examined the effect of cytochalasin B, an inhibitor of phagocytosis, on the presentation of OVA by DC. Treatment with cytochalasin B (5 μg/ml) inhibited the presentation of bead-bound OVA on MHC class I molecules (Fig. 7*A*). In contrast, this agent did not affect presentation when OVA was loaded directly into the cytosol (Fig. 7*B*), which indicates that it is interfering with an early event in the exogenous pathway and not at other steps in the class I pathway.

Presentation by DC of exogenous Ag on MHC class I molecules is chloroquine insensitive

The presentation of exogenous Ags on MHC class I molecules in some cases involves proteolysis of the Ag in the cytosol (8–10), while in other cases the proteolysis appears to occur in the endocytic compartment (7, 11–13). It was of interest to determine which of these mechanisms was operative in DC. To investigate whether presentation required proteolysis in acidic vesicles, we treated the DC with chloroquine during exposure to exogenous Ag. Chloroquine raises the pH in the endosomal and lysosomal compartments and thereby inhibits protein hydrolysis by cathepsins, which require an acidic environment for activity (50, 51). Treatment with chloroquine did not inhibit the presentation of particulate OVA on MHC class I molecules (Fig. 8*A*). In fact, this agent actually slightly enhanced this presentation, suggesting that proteolysis in vesicles might be limiting the availability of Ag for class I presentation. In contrast, chloroquine treatment completely inhibited the presentation of bead-bound OVA on class II molecules, as expected (Fig. 8*B*) (52). This latter finding serves as a positive control for the effectiveness of chloroquine in blocking proteolysis in vesicles. These results suggest that the class I-presented peptides are being generated outside of the endocytic compartments in DC.

Role of the proteosome in class I presentation by DC

The other major pathway for degrading proteins in cells is mediated by proteasome in the cytosol and nucleus (53). This pathway is responsible for generating the majority of class I-presented peptides from endogenous cellular and viral proteins (2) and has been implicated in the presentation of exogenous Ags by macrophages (9). Therefore, we next examined the effects of peptide aldehyde inhibitors of proteasome (2) on the presentation of exogenous OVA by DC. As shown in Figure 9*A*, two of these inhibitors, LLnL and MG132, inhibited the presentation of particulate OVA on MHC class I molecules. In contrast, a closely related peptide aldehyde, LLM, did not inhibit the presentation. This agent serves as a specificity control because it has activity similar to those of LLnL and MG132 on thiol proteases, but is much less potent against the proteasome (2). Furthermore, LLnL and MG132 did not inhibit the presentation of the OVA peptide SIINFEKL expressed in cytosol from a minigene in a vaccinia virus construct (40) (Fig. 9*B*). These results indicate that LLnL and MG132 inhibit the presentation of exogenous OVA by inhibiting peptide generation by the proteasome and not by affecting other steps in the class I pathway.

Brefeldin A inhibited bead-conjugated OVA on both MHC class I and class II molecules

Brefeldin A blocks the exocytosis of proteins from the endoplasmic reticulum and Golgi complex and thereby prevents newly assembled peptide-MHC molecules from reaching the cell surface (54). The presentation of exogenous Ag on MHC class I molecules by the vacuolar pathway has been reported to be insensitive to this inhibitor (7, 11, 13), while presentation by the cytosolic pathway is inhibited by brefeldin A (8–10). We therefore examined the

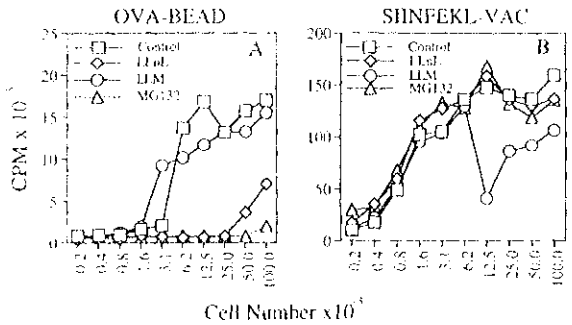

FIGURE 9. Exogenous OVA presentation was inhibited by proteasome inhibitors. DC2.4 cells were preincubated with medium alone (open square), 40 μM LLnL (open diamond), 40 μM LLM (open circle), or 10 μM MG132 (open triangle) for 30 min followed by the addition of bead-conjugated OVA (50 μg/ml) for 6 h (*A*) or infection with recombinant vaccinia virus encoding the SIINFEKL peptide (10 plaque-forming units/cell) for 3 h (*B*). Cells were then fixed with 1% paraformaldehyde and incubated with RF33.70 (10^5/well) for 20 h (200 μl). Cultures were then handled as described in *Materials and Methods*.

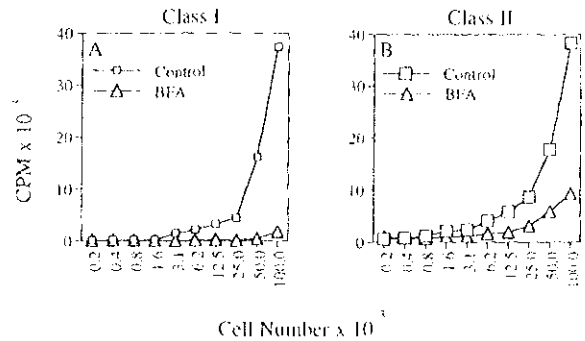

FIGURE 10. Effect of brefeldin A on exogenous OVA presentation on MHC class I and class II molecules. DC2.4 cells (2×10^6/ml) were pretreated with 5 μg/ml brefeldin A for 30 min (open triangle) or medium alone (open square) followed by addition of beads conjugated OVA (50 μg/ml), further incubated for 6 h, and fixed with 1% paraformaldehyde. Cells were then titrated in microtiter plates (100 μl), and the T-T hybrids (5×10^4/well) RF33.70 (*A*) and MF2.2D9 (*B*) were added to the cultures (200 μl total) and handled as described in *Materials and Methods*.

effect of this agent on the presentation of exogenous OVA by DC. Brefeldin A (5 μg/ml) completely inhibited the presentation by DC2.4 cells of particulate OVA on MHC class I molecules (Fig. 10*A*) and also inhibited the presentation of OVA on MHC class II molecules (Fig. 10*B*), suggesting a role for newly synthesized class II molecules in presentation of Ag by these DC.

Discussion

Properties of cloned DC

DC are present in both lymphoid and somatic tissues, but in relatively low abundance (55). Consequently, it is difficult to obtain large numbers of these cells with a high degree of purity. Many types of studies would be facilitated by the availability of reliable methods to isolate large numbers of homogeneous DC. Larger numbers of these cells can be obtained by culturing precursors from peripheral blood or bone marrow in GM-CSF and other cytokines (44, 45). This has been an important advance; however, these cultures typically contain other contaminating cell types, and this approach has not allowed the isolation of cloned DC lines. We also failed to grow clones of DC from bone marrow cultures transduced with GM-CSF. Presumably, GM-CSF stimulation is not sufficient for immortal growth of DC or their progenitors. This is consistent with previous studies examining the reconstitution of lethally irradiated mice with bone marrow that had been infected with a replication defective, retrovirus-expressing GM-CSF (56). Although a myeloproliferative disease occurred secondary to constitutive GM-CSF secretion in the primary recipients, this did not evolve into a clonal leukemia. Moreover, the myeloproliferative disorder could not be transplanted into secondary recipients (56). However, we found that supertransfection of *myc* and *raf* into GM-CSF-transduced cultures immortalizes these cells. In this case GM-CSF probably acts in a paracrine manner to expand infectable DC because immortal growth is maintained without the clones producing detectable levels of this cytokine. Similar approaches to immortalize DC using oncogenes (without GM-CSF) have been reported by others (57, 58), although whether the isolated cells are bona fide DC has not been firmly established in all cases.

The initial criteria we employed for identifying DC was morphologic. These clones had very obvious dendritic processes and veils, which was an appearance we had never observed in cells growing from fresh bone marrow infected with the same *myc*- and *raf*-expressing retrovirus (a procedure yielding macrophage and B cell clones) (14) (our unpublished observations). That at least some of these cells were indeed DC was confirmed by their expression of specific DC markers, DEC-205 and 33D1. These cells also expressed other molecules that are typical for DC, including high levels of B7 family members and MHC class I and class II molecules. They also expressed other receptors that are not present on freshly isolated DC, such as CD32 and Mac1, but which are found on cultured DC (47, 57, 59, 60).

The DC clones can avidly internalize micrometer-sized particles. This process requires microfiliments, and the particles are found in cells in large vacuoles. Therefore, the DC are phagocytic. This property has previously been observed for cultured DC and DC resident in tissues (61, 62). We show that this activity is important for the presentation on MHC molecules of peptides from particulate Ags (further discussed below).

We also isolated clones with similar morphology but that lacked expression of DEC-205 and 33D1. Given the similarity of their appearance and their expression of other cell surface molecules to the DEC-205-positive clones, we favor the possibility that these clones represent different subsets or different stages of maturation of DC. This interpretation would be consistent with other data demonstrating phenotypic heterogeneity in freshly isolated and cultured DC (63–65). However, in the absence of other objective criteria, it is difficult to rule out the possibility that these DEC-205-negative cells represent some other unrelated cell lineage or that their phenotype is abberent and an artifact of the immortalization conditions.

The immortalized DC lines are homogeneous and easily grown. These properties should be useful to studies exploring the cell biology and biochemistry of these cells. We have used them to analyze the Ag-presenting pathways that are operative in these cells.

Presentation of exogenous Ags by DC

The major question addressed by the present study is whether DC are capable of presenting Ags from the extracellular fluids on MHC class I molecules. Previously, cells with this capability were detected in fractions from both spleen and thymus enriched in DC

(5, 16). However, these same fractions contained some macrophages (5), and macrophages have been shown to have this Ag-presenting activity (11, 14). Our present results conclusively demonstrate that cloned DC that are free of macrophage contamination can present exogenous Ags on class I molecules. This pathway of presentation was detected in multiple different clones.

Soluble OVA added to culture medium was presented on class I molecules by DC, but required high concentrations of Ag. Approximately 1000-fold less Ag was required when the OVA was conjugated to a microsized particle that was internalized by phagocytosis. This enhanced presentation was blocked by cytochalasin B, which disrupts phagocytosis but not other endocytic processes. These results are similar to earlier findings with macrophages (14) and indicate that class I molecules can monitor the contents of phagosomes and potentially other endocytic compartments in DC. One such compartment might be the macropinosome, because this is a site in other cells that has been implicated in the delivery of exogenous proteins into the class I pathway (8), and macropinocytosis occurs in DC (66).

Two distinct pathways have been described for the presentation of internalized Ags on class I molecules of macrophages. One is independent of the proteasome and TAP and is resistant to brefeldin A (7, 11–13). In this case it is thought that the presented peptides are generated in the endocytic compartment and then bind to class I molecules on the plasma membrane. The other pathway requires proteasome and TAP and is sensitive to brefeldin A (8–10). In this case, Ags are transferred from phagosomes into the cytosol where they follow a common final pathway with endogenous Ags. The presentation of OVA particles by DC is sensitive to inhibitors of the proteasome and brefeldin A, but resistant to chloroquine. Therefore, it appears that in these APCs OVA is following the phagosome to cytosol pathway. Whether DC also have a vacuolar pathway for class I presentation remains to be determined.

Compared with macrophage lines, the cloned DC present exogenous Ag with similar efficiency to MHC class I-restricted T-T hybridomas. Nevertheless, it is likely that these cells will be more effective in stimulating responses to these Ags because of their potent immunostimulatory properties. T-T hybrids only require stimulation through their TCR, while normal T cells require additional signals, such as B7-1 and B7-2, which are expressed at high levels on DC. Moreover, the DC clones are constitutively better class II-presenting cells than macrophages, and this correlates with higher levels of expression of MHC class II molecules.

There is considerable evidence that the pathway for presenting exogenous Ags on class I molecules is active in vivo (6). Macrophages and DC isolated from immunized animals present the injected Ag on their class I molecules. Moreover, injection of antigenic particles primes CTL responses (6, 20). This pathway probably plays an important role in generating immunity in several pathologic situations, including responses against tissue transplants, tumors, and possibly even viruses (3, 17–19). In some of these situations, the exogenous pathway may be the major mechanism for initiating responses. This may be because the somatic cells that are producing the Ag are themselves poor stimulators of immunity, and professional APC, such as the DC, are needed to prime responses (15).

The exogenous class I pathway can potentially be exploited to generate CTL immunity to proteins in vaccines. Conventional vaccine preparations generally fail to elicit CTL responses, presumably because the Ags fail to be presented in vivo. However, particles can be used to target proteins into phagocytes, and this is effective in conferring protective CD8 T cell immunity (6, 20). The existence of the phagosome to cytosol pathway in DC makes this approach particularly attractive. Studying this pathway in the cloned DC should help to optimize approaches for targeting Ags into these key APCs.

Acknowledgments

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References

1. York, I., and K. L. Rock. 1996. Antigen processing and presentation by the class I major histocompatibility complex. *Annu. Rev. Immunol. 14:369.*
2. Rock, K. L., C. Gramm, L. Rothstein, K. Clark, R. Stein, L. Dick, D. Hwang, and A. L. Goldberg. 1994. Inhibitors of the proteasome block nonlysosomal protein degradation and the generation of peptides presented on MHC-class I molecules. *Cell 78:761.*
3. Rock, K. L. 1996. A new foreign policy: MHC class I molecules monitor the outside world. *Immunol. Today 17:131.*
4. Rock, K. L., S. Gamble, and L. Rothstein. 1990. Presentation of exogenous antigen with class I major histocompatibility complex molecules. *Science 249:918.*
5. Rock, K. L., L. Rothstein, S. Gamble, and C. Fleischacker. 1992. Characterization of APCs that present exogenous antigens in association with class I MHC molecules. *J. Immunol. 150:438.*
6. Kovacsovics-Bankowski, M., K. Clark, B. Benacerraf, and K. L. Rock. 1993. Efficient MHC class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc. Natl. Acad. Sci. USA 90:4942.*
7. Pfeifer, J. D., M. J. Wick, R. L. Roberts, K. Fidlay, S. J. Normark, and C. V. Harding. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature 361:359.*
8. Norbury, C. C., L. J. Hewlett, A. R. Prescott, N. Shastri, and C. Watts. 1995. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity 3:783.*
9. Kovacsovics-Bankowski, M., and K. L. Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science 267:243.*
10. Reis e Sousa, C., and R. N. Germain. 1995. Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. *J. Exp. Med. 182:841.*
11. Harding, C. V., and R. Song. 1994. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. *J. Immunol. 153:4925.*
12. Bachmann, M. F., A. Oxenius, H. Pircher, H. Hengartner, P. Ashton-Richardt, S. Tonegawa, and R. M. Zinkernagel. 1995. TAP1-independent loading of class I molecules by exogenous viral proteins. *Eur. J. Immunol. 25:1739.*
13. Song, R., and C. V. Harding. 1996. Roles of proteasomes, transporter for antigen presentation (TAP), and β-microglobulin in the processing of bacterial or particulate antigens via an alternate class I MHC processing pathway. *J. Immunol. 156:4182.*
14. Kovacsovics-Bankowski, M., and K. L. Rock. 1994. Presentation of exogenous antigens by macrophages: analysis of major histocompatibility complex class I and II presentation and regulation by cytokines. *Eur. J. Immunol. 24:2421.*
15. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol. 9:271.*
16. Grant, E. P., and K. L. Rock. 1992. MHC Class I-restricted presentation of exogenous Ag by thymic antigen-presenting cells in vitro and in vivo. *J. Immunol. 148:13.*
17. Bevan, M. J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross react in the cytotoxic assay. *J. Exp. Med. 143:1283.*
18. Gooding, L. R., and C. B. Edwards. 1980. H-2 antigen requirements in the in vitro induction of SV40-specific cytotoxic T lymphocytes. *J. Immunol. 124:1258.*
19. Huang, A., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, and H. Levitsky. 1994. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science 264:961.*
20. Falo, L. D., Jr., M. Kovacsovics-Bankowski, K. Thompson, and K. L. Rock. 1995. Vaccination with particulate antigen induces protective tumor immunity. *Nat. Med. 1:649.*
21. Blasi, E., B. Mathieson, L. Varesio, J. L. Cleveland, P. A. Borchert, and U. R. Rapp. 1985. Selective immortalization of murine macrophages from fresh bone marrow by a *raf/myc* recombinant murine retrovirus. *Nature 318:667.*
22. Dexter, T., J. Garland, D. Scott, E. Scolnick, and D. Metcalf. 1980. Growth of factor-dependent hemopoietic precursor cell lines. *J. Exp. Med. 152:1036.*
23. Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson, H. Hamada, D. Pardoll, and R. Mulligan. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA 90:3539.*

24. Rock, K. L., L. Rothstein, and S. Gamble. 1990. Generation of class I MHC restricted T-T hybridomas. *J. Immunol. 145:804.*
25. Jones, B., and C. A. J. Janeway. 1981. Cooperative interaction of B lymphocytes with antigen-specific helper T lymphocytes is MHC restricted. *Nature 292:547.*
26. Janeway, C. A., P. J. Conrad, E. A. Lerner, J. Babich, P. Wettstein, and D. B. Murphy. 1984. Monoclonal antibodies specific for Ia glycoproteins raised by immunization with activated T cells: possible role of T cell bound Ia antigens as targets of immunoregulatory T cells. *J. Immunol. 132:662.*
27. Unkeless, J. C. 1979. Characterization of a monoclonal antibody directed against mouse macrophages and lymphocyte Fc receptors. *J. Exp. Med. 150:580.*
28. Razi-Wolf, Z., G. J. Freeman, F. Galvin, B. Benacerraf, L. Nadler, and H. Reiser. 1992. Expression and function of the murine B7 antigen, the major costimulatory molecule expressed by peritoneal exudate cells. *Proc. Natl. Acad. Sci. USA 89:4210.*
29. Hathcock, K. S., G. Laszlo, H. B. Dickler, J. Bradshaw, P. Linsley, and R. J. Hodes. 1993. Identification of an alternative CTLA-4 ligand costimulatory for T-cell activation. *Science 262:905.*
30. Taket, F. 1985. Inhibition of mixed lymphocyte response by a rat monoclonal antibody to a novel murine lymphocyte activation antigen. *J. Immunol. 134:1403.*
31. Malek, T. R., G. Ortega, R. A. Chan, R. A. Kroczek, and E. M. Shevach. 1986. Role of Ly-6 in lymphocyte activation. II. Induction of T cell activation by monoclonal anti-Ly-6 antibodies. *J. Exp. Med. 164:709.*
32. Davignon, D., E. Martz, T. Reynolds, K. Kurzinger, and T. Springer. 1981. Lymphocyte function-associated antigen 1 (LFA-1): a surface antigen distinct from Lyt-2,3 that participates in T lymphocyte-mediated killing. *Proc. Natl. Acad. Sci. USA 78:4535.*
33. Springer, T., G. Galfre, D. S. Secher, and C. Milstein. 1979. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *Eur. J. Immunol. 9:301.*
34. Kraal, G., M. Breel, M. Janse, and G. Bruin. 1986. Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. *J. Exp. Med. 163:981.*
35. Nussenzweig, M. C., R. M. Steinman, M. D. Witmer, and B. Gutchinov. 1982. A monoclonal antibody specific for mouse dendritic cells. *Proc. Natl. Acad. Sci. USA 79:161.*
36. Kraal, G., M. Rep, and M. Janse. 1987. Macrophages in T and B cell compartments and other tissue macrophages recognized by a monoclonal antibody, MOMA-2. *Scand. J. Immunol. 26:653.*
37. Wilson, J. M., O. Danos, M. Grossman, D. H. Raulet, and R. C. Mulligan. 1990. Expression of human adenosine deaminase in mice reconstituted with retrovirus-transduced hematopoietic stem cells. *Proc. Natl. Acad. Sci. USA 87:439.*
38. Rock, K. L., E. T. H. Yeh, C. F. Gramm, S. I. Haber, H. Reiser, and B. Benacerraf. 1986. TAP, a novel T cell activating protein involved in the stimulation of MHC restricted T lymphocytes. *J. Exp. Med. 163:315.*
39. Moore, M. W., F. R. Carbone, and M. J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell 54:777.*
40. Cox, J. H., P. Galardy, J. R. Bennink, and J. W. Yewdell. 1995. Presentation of endogenous and exogenous antigens is not affected by inactivation of E1 ubiquitin-activating enzyme in temperature-sensitive cell lines. *J. Immunol. 154:511.*
41. Rock, K. L., and B. Benacerraf. 1983. Inhibition of antigen-specific T lymphocyte activation by structurally related *Ir* gene controlled polymers: evidence of specific competition for accessory cell antigen-presentation. *J. Exp. Med. 157:1618.*
42. Gillis, S., and K. A. Smith. 1977. Long term culture of tumor-specific cytotoxic T cells. *Nature 268:154.*
43. Gillis, S., K. A. Smith, and J. Watson. 1980. Biochemical characterization of lymphocyte regulatory molecules. II. Purification of a class of rat and human lymphokines. *J. Immunol. 124:1954.*
44. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med. 176:1693.*
45. Caux, C., C. Dezutter-Dumuyant, D. Schmitt, and J. Banchereau. 1992. GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells. *Nature 360:258.*
46. Larsen, C., S. Ritchie, T. Pearson, P. Linsley, and R. Lowry. 1992. Functional expression of the costimulatory molecule, B7/BB1, on murine dendritic cell populations. *J. Exp. Med. 176:1215.*
47. Umezu, H., M. Naito, K. Inaba, and K. Takahashi. 1995. Ultrastructural and immunophenotypic differentiation of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony stimulating factor (GM-CSF). *J. Submicrosc. Cytol. 27:227.*
48. Lu, L., W. A. Rudert, S. Qian, D. McCaslin, F. Fu, A. S. Rao, M. Trucco, J. J. Fung, T. E. Starzl, and A. W. Thomson. 1995. Growth of donor-derived dendritic cells from the bone marrow of murine liver allograft recipients in response to granulocyte/macrophage colony-stimulating factor. *J. Exp. Med. 182:379.*
49. Jiang, W., W. J. Swiggard, C. Heufler, M. Peng, A. Mirza, R. M. Steinman, and M. C. Nussenzweig. 1995. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature 375:151.*
50. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of intralysosomal pH in living cells and perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA 75:3327.*
51. Geisow, M. J., P. D'Arcy Hart, and M. R. Young. 1981. Temporal changes of lysosome and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy. *J. Cell. Biol. 89:645.*
52. Ziegler, H., and E. Unanue. 1982. Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. *Proc. Natl. Acad. Sci. USA 79:175.*
53. Goldberg, A., and K. L. Rock. 1992. Proteolysis, proteosomes and antigen presentation. *Nature 357:375.*
54. Yewdell, J. W., and J. R. Bennink. 1989. Brefeldin A specifically inhibits presentation of protein antigens to cytotoxic T lymphocytes. *Science 244:1072.*
55. Steinman, R. M., and Z. A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J. Exp. Med. 137:1142.*
56. Johnson, G. R., T. J. Gonda, D. Metcalf, I. K. Hariharan, and S. Cory. 1989. A lethal myeloproliferative syndrome in mice transplanted with bone marrow cells infected with a retrovirus expressing granulocyte-macrophage colony stimulating factor. *EMBO J. 8:441.*
57. Crowley, M., K. Inaba, M. D. Witmer-Pack, and R. M. Steinman. 1989. The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell. Immunol. 118:108.*
58. Paglia, P., G. Girolomoni, F. Robbiati, F. Granucci, and P. Ricciardi-Castagnoli. 1993. Immortalized dendritic cell line fully competent in antigen presentation initiates primary T cell responses in vivo. *J. Exp. Med. 178:1893.*
59. Ohnishi, K., M. Daigo, and T. Tokunaga. 1995. SV40-adenovirus immortalized cell line derived from mouse lymphoid dendritic cell preparations. *Immunol. Cell Biol. 3:205.*
60. Lu, L., D. McCaslin, T. E. Starzl, and A. W. Thomson. 1995. Bone marrow-derived dendritic cell progenitors induce alloantigen-specific hyporesponsiveness in murine T lymphocytes. *Transplantation 60:1539.*
61. Inaba, K., M. Inaba, M. Naito, and R. M. Steinman. 1993. Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. *J. Exp. Med. 178:479.*
62. Reis e Sousa, C., P. Stahl, and J. Austyn. 1993. Phagocytosis of antigens by Langerhans cells in vitro. *J. Exp. Med. 178:509.*
63. Koch, F., B. Trockenbacher, G. Schuler, and N. Romani. 1995. Antigen processing capacity of dendritic cells from mice of different MHC background: down-regulation upon culture and evidence for heterogeneity of dendritic cell populations. *Adv. Exp. Med. Biol. 378:203.*
64. Egner, W., and D. N. Hart. 1995. The phenotype of freshly isolated and cultured human bone marrow allostimulatory cells: possible heterogeneity in bone marrow dendritic cell populations. *Immunology 85:611.*
65. Xu, S., P. R. Bergstresser, and A. Takashima. 1995. Phenotypic and functional heterogeneity among murine epidermal-derived dendritic cell clones. *J. Invest. Dermatol. 105:831.*
66. Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: down-regulation by cytokines and bacterial products. *J. Exp. Med. 182:389.*

MATERIAL TRANSFER AGREEMENT

Dana-Farber Cancer Institute, Inc.

The University of Western Ontario at 1151 Richmond Street, London ON N6A 3K7 and its investigator(s) Dr Lakshman Gunaratnam (hereinafter collectively referred to as "Recipient"), in consideration of the receipt of biological materials (which material has been provided to Dr. Mansour Haeyrfar of The University of Western Ontario by way of Material Transfer Agreement dated September 29, 2006 and Dana-Farber Cancer Insitite (hereinafter "DFCI") herby consents to Dr. Haeyrfar providing said Material to Dr. Lakshman Gunaratnam) hereby agree to the following terms and conditions:

1. The biological materials to be provided to Recipient are: DC2.4. Material(s) shall mean the above referenced biological materials plus progeny, unmodified derivatives and any accompanying know-how or data.

2. The Materials shall be used exclusively for non-commercial research by Recipient to study Testing conditions that lead to DC maturation after renal transplantation.. The Material(s) shall be used solely by the named investigator and those under his or her direct supervision. Materials will not be used for *in vivo* testing in human subjects. Use will be in compliance with all applicable Federal, State and local laws and regulations, including, but not limited to animal welfare laws and regulations.

3. The Materials are the property of DFCI. Ownership of modifications and derivatives of Materials will be determined by the parties hereto depending upon (a) their relative contribution to the creation of said modifications and derivatives, which is to be considered but not required in said negotiation; and (b) any applicable laws and regulations relating to inventorship.

4. Recipient shall not sell or otherwise distribute Materials to a third party for any purpose. This Agreement and the resulting transfer of Material constitute a non-exclusive license to use the Material solely for the basic research or other not-for-profit purposes described herein. Recipient shall not use Materials for any products or processes for profit-making or commercial purposes.

5. This agreement is not assignable.

6. DFCI has, or may, make Materials available to others, both profit and non-profit.

7. To the extent supplies are available, DFCI agrees to make the Material available, under a separate agreement, to other scientists for teaching or not-for-profit research purposes only. Recipient will acknowledge DFCI as the source of the Material in all publications containing any data or information about the material unless DFCI indicates otherwise.

8. Recipient will arrange the return to DFCI or disposal of all unused Material whenever investigation for which it has been supplied discontinues or is terminated. In the event investigator(s) transfer to another institution, a new Material Transfer Agreement is to be executed.

9. The Material hereunder provided is experimental in nature, and it is provided WITHOUT ANY WARRANTIES, EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR USE. DFCI MAKES NO REPRESENTATION AND PROVIDES NO WARRANTY THAT THE USE OF THE MATERIAL

WILL NOT INFRINGE ANY PATENT OR OTHER PROPRIETARY RIGHT.

10. To the extent allowable under applicable laws, Recipient agrees to indemnify, defend, and hold harmless DFCI and its trustees, officers, staff, representatives and agents against all damages, expenses (including without limitation legal expenses), claims, demands, suits or other actions arising from Recipient's acceptance, use and disposal of the Materials and their progeny or derivatives, except insofar as such claims result directly from the gross negligence or willful misconduct of DFCI.

Accepted by: Institution: The University of Western Ontario

Authorized

Institutional Officer: _____ Investigator: Dr Lakshman Gunaratnam

Title: _____ Title: _____

Signature: _____ Signature: _____

Date: _____ Date: _____

Acknowledgement: Dr. Mansour Haeyrfar, in consideration of section 1 as provided, hereby acknowledges and consents to the transfer of DC2.4 cells to Dr. Lakshman Gunaratnam.

By: _____

Approved by: DANA-FARBER CANCER INSTITUTE, INC.

_____ Date: _____

Anthony A. del Campo, MBA

Vice President, Office of Research and Technology Ventures

Outbound MTA Agr [Agr ID], 18/05/2010

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	SB202190
Proposed Use Dose:	50 µg
Proposed Storage Dose:	500 µg
LD_{50} (species):	40000 µg

Calculation:

40000 µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg = 2000000

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = 200000

Comments/Recommendations:

Western

TOXIN USE RISK ASSESSMENT

Name of Toxin:	DMSO
Proposed Use Dose:	200000 µg
Proposed Storage Dose:	500000000 µg
LD_{50} (species):	14500000 µg

Calculation:

14500000 µg/kg x 50 kg/person

Dose per person based on LD_{50} in µg = 725000000

LD_{50} per person with safety factor of 10 based on LD_{50} in µg = *72500000*

Comments/Recommendations:

Calculation based on 1g = 1mL (density).

Store in two different locations.