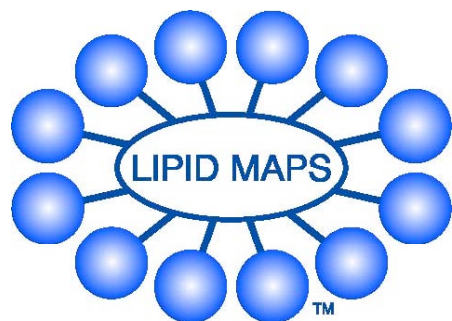
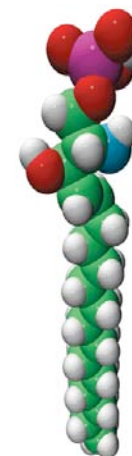




www.lipidmaps.org

LIPID MAPS Lipidomics Workshop

April 18, 2009



Analysis of Protein-Lipid Complexes

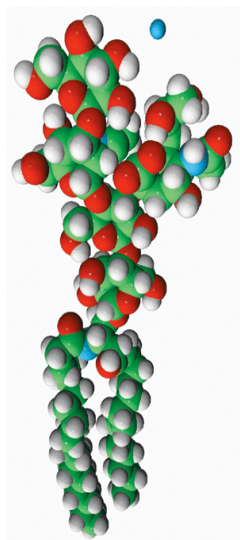
Ziqiang Guan

Department of Biochemistry
Duke University Medical Center

Other Lipid Maps Core K members:

Christian R. H. Raetz

Greg Laird, David Six



Outline

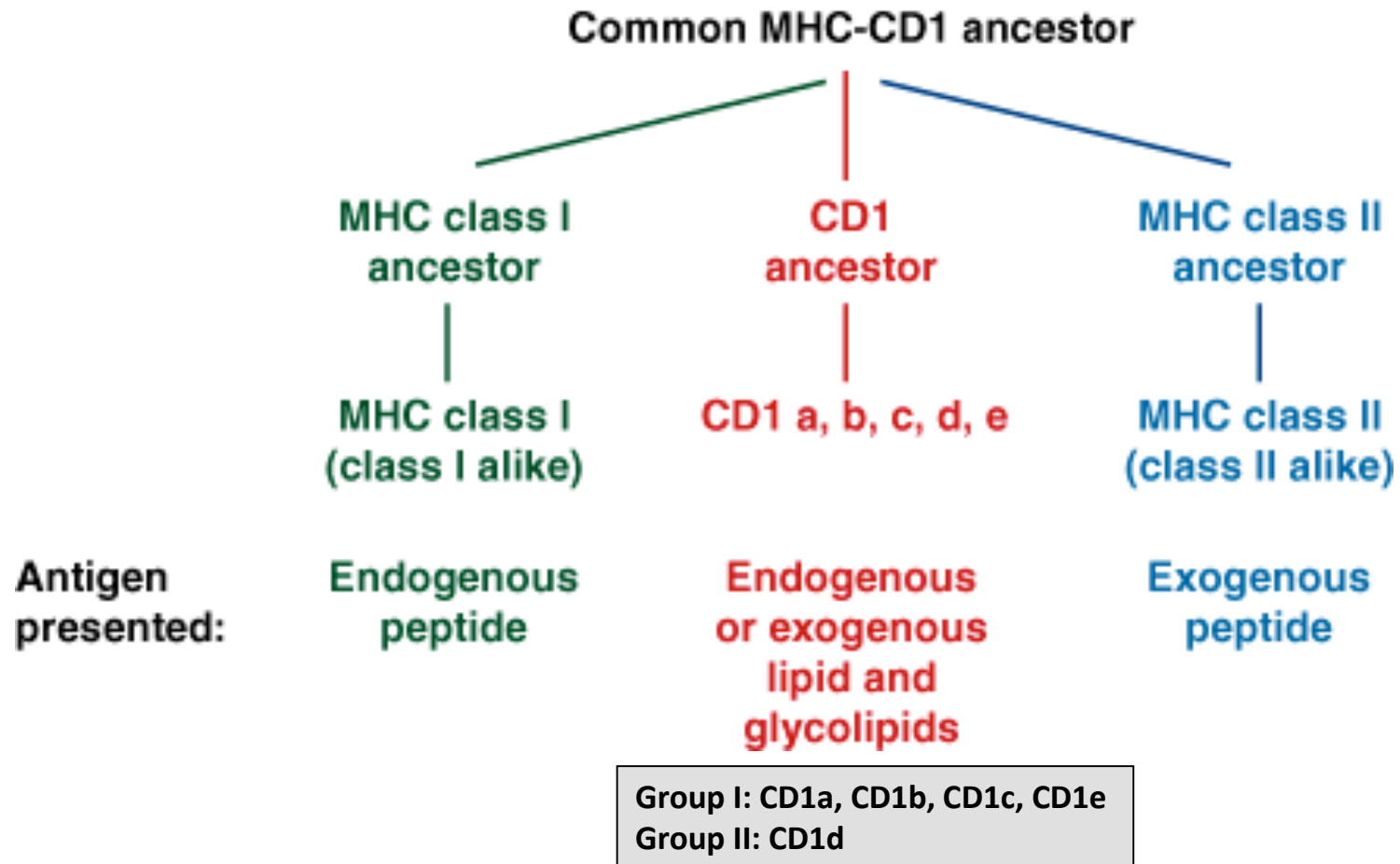
1. Identification of CD1d-binding endogenous lipids

- CD1 and lipid antigens: background
- CD1d protein expression and isolation (Haig, Xu and McMichael)
- Bligh-Dyer lipid extraction of purified CD1d
- Lipid identification by LC/MS/MS

2. Identification of small, lipid-like membrane proteins

- ^{15}N labeling
- Bligh-Dyer extraction
- Identification by LC/MS/MS

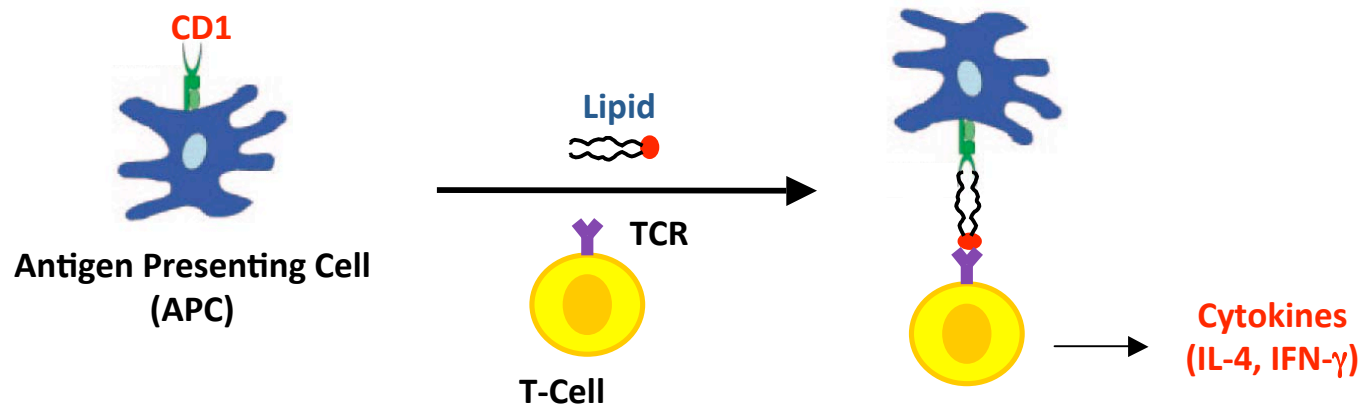
Antigen Presentation Molecules



M. Brenner and S. Porcelli, *Science*, **277**, 332 (1997)

CD1d and Natural Killer T (NKT) Cells

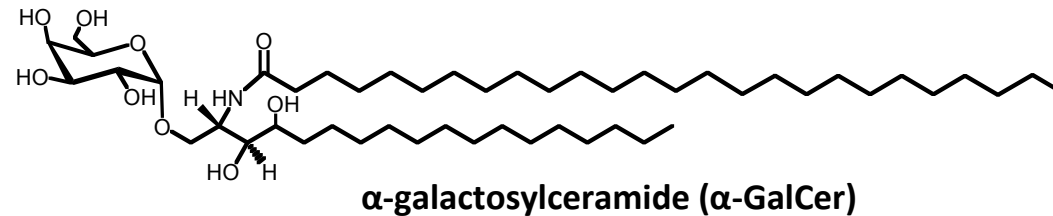
- CD1d (belonging to group II CD1) is highly conserved in all mammalian species.
- CD1d-restricted T cells are NKT cells (expressing both conserved T cell receptor and natural killer cell receptors).
- Upon stimulation, NKT cells rapidly produce a variety of cytokines and influence diverse immune responses and pathogenic processes, ranging from autoimmunity to tumor and anti-viral immunity.



Science (1997) 278, 1626.

CD1d-Restricted and TCR-Mediated Activation of V $_{\alpha}$ 14 NKT Cells by Glycosylceramides

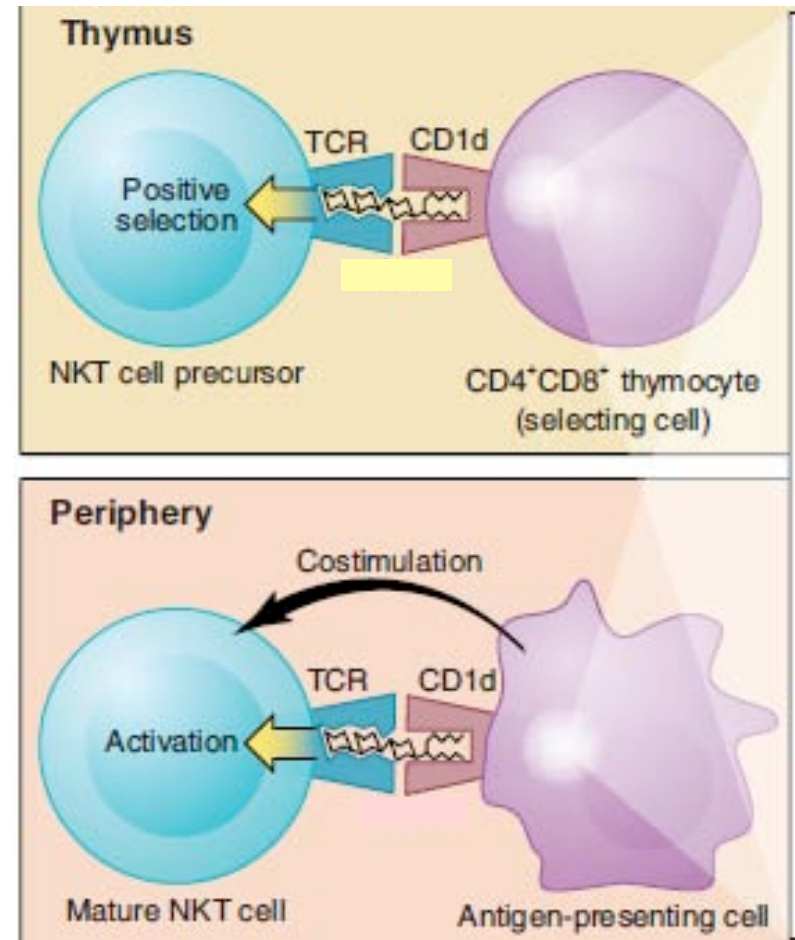
Tetsu Kawano, Junqing Cui, Yasuhiko Koezuka, Isao Toura, Yoshikatsu Kaneko, Kazuhiro Motoki, Hitomi Ueno, Ryusuke Nakagawa, Hiroshi Sato, Eisuke Kondo, Haruhiko Koseki, Masaru Taniguchi*



- Isolated from marine sponge
- Potently stimulates NKT cells in a CD1d-dependent manner
- Being tested as a cancer therapy

Endogenous Lipid Antigen(s) for NKT Cells?

- **Responsible for positive selection/development of NKT cells in the thymus**
- **Stimulation of NKT cells in the periphery**



Searching for CD1d-Binding Endogenous Lipids

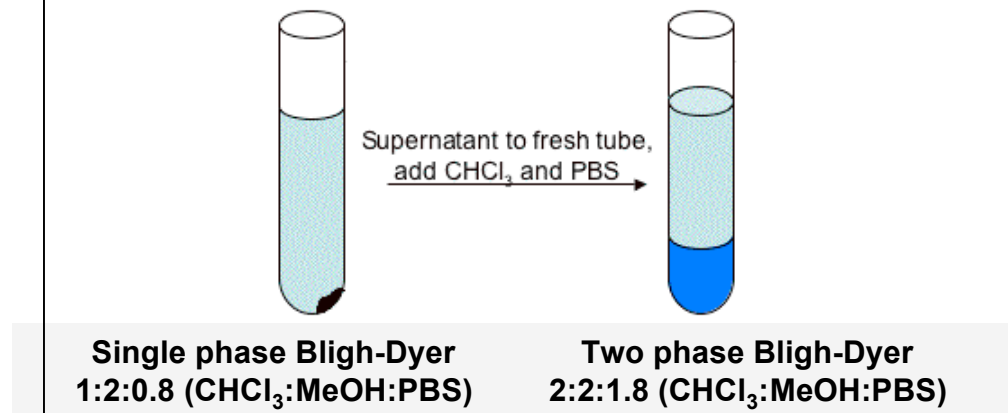
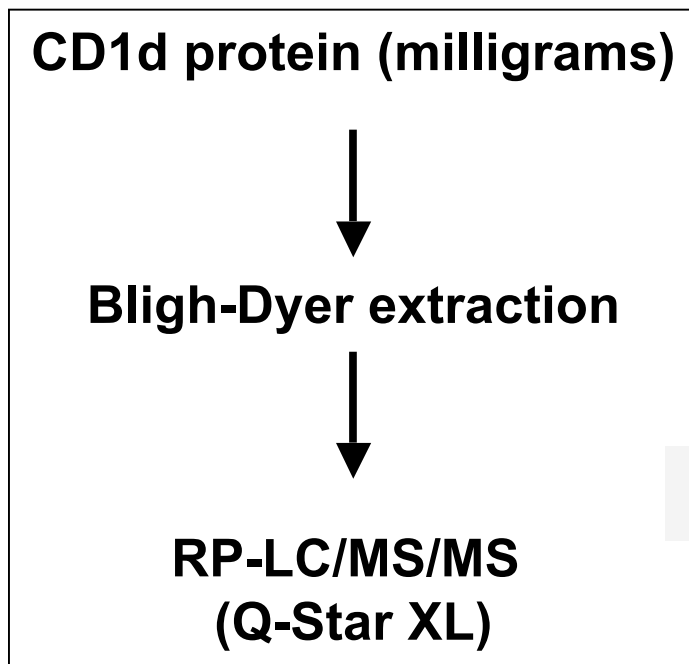
Approach:

Identifying lipids that co-purify with CD1d

Collaborators:

**Neil Haig, Dr. Xiaoning Xu, Sir Andrew McMichael
(Univ. of Oxford)**

Extraction and Identification of CD1d-Binding Endogenous Lipids



Reverse-Phase LC/MS

Column:

Zorbax SB-C8 (2.1 mm x 50 mm, 5 μ m)

Mobile phases:

Solvent A = 60/20/20 (methanol/acetonitrile/aqueous 1 mM ammonium acetate)

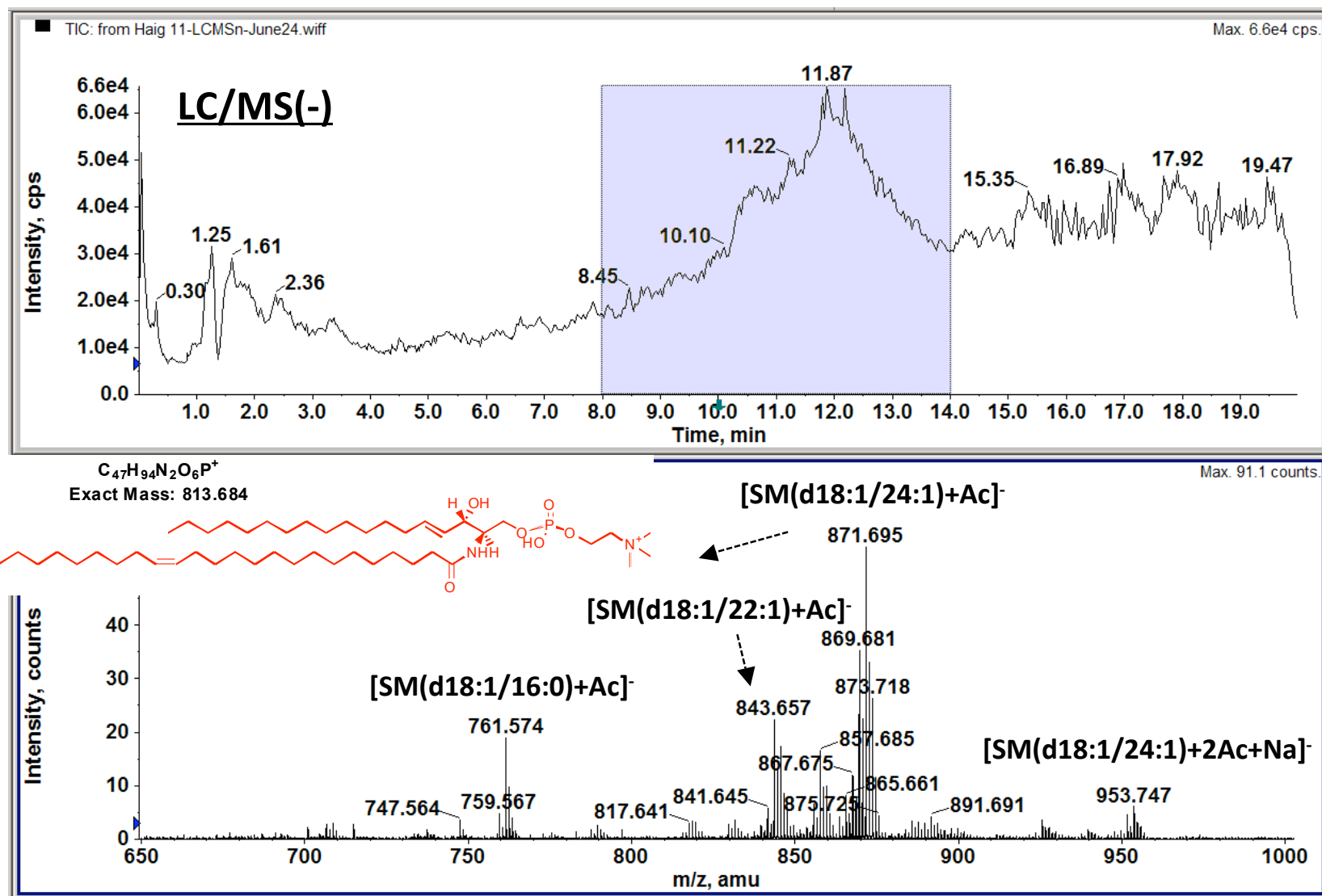
Solvent B= 100% ethanol containing 1 mM ammonium acetate.

Flow rate: 200 μ L/min; split ~10:1 to MS (Q-Star XL)

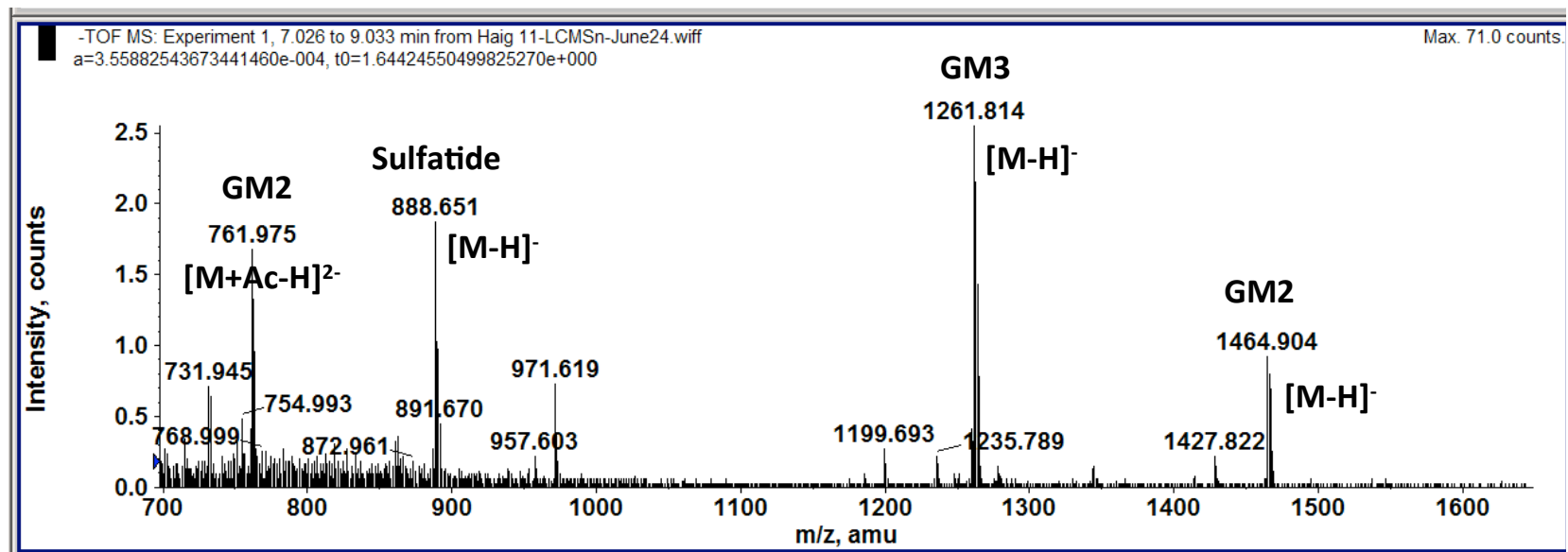
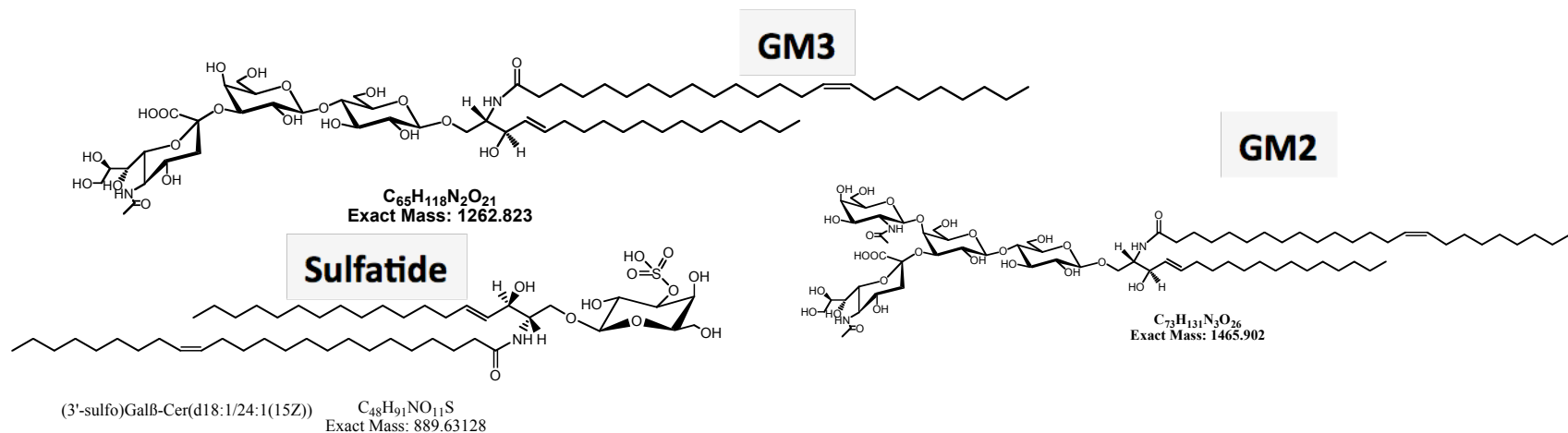
HPLC gradient:

Time (min)	A%	B%
0	100	0
2	100	0
10	0	100
13.5	0	100
14	100	0
20	100	0

Major Lipids Bound to CD1d: Sphingomyelins(SM)



Minor Lipids Bound to CD1d: Sulfatide, GM2 and GM3



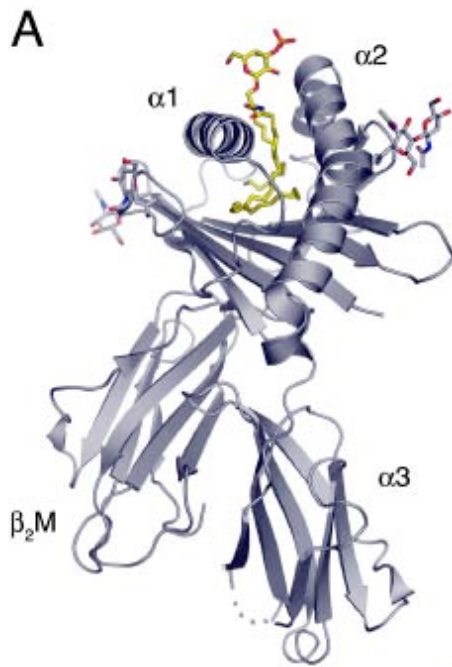
J. Exp. Med. 2005, 202, 1517

Structural basis for CD1d presentation of a sulfatide derived from myelin and its implications for autoimmunity

Dirk M. Zajonc,¹ Igor Maricic,⁴ Douglass Wu,^{2,3} Ramesh Halder,⁴ Keshab Roy,⁴ Chi-Huey Wong,^{2,3} Vipin Kumar,⁴ and Ian A. Wilson^{1,3}

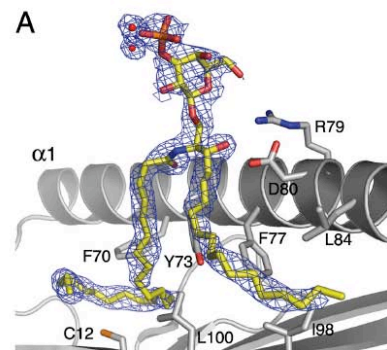
¹Department of Molecular Biology, ²Department of Chemistry, and the ³Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037

⁴Torrey Pines Institute for Molecular Studies, San Diego, CA 92121

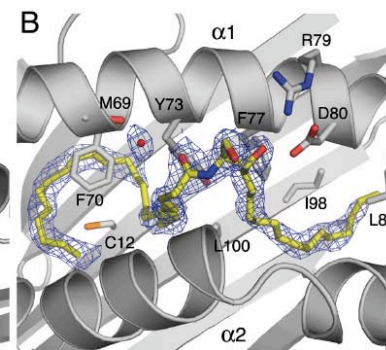


- **Sulfatide is implicated in T cell-mediated autoimmune disease in the CNS (inflammation and demyelination)**

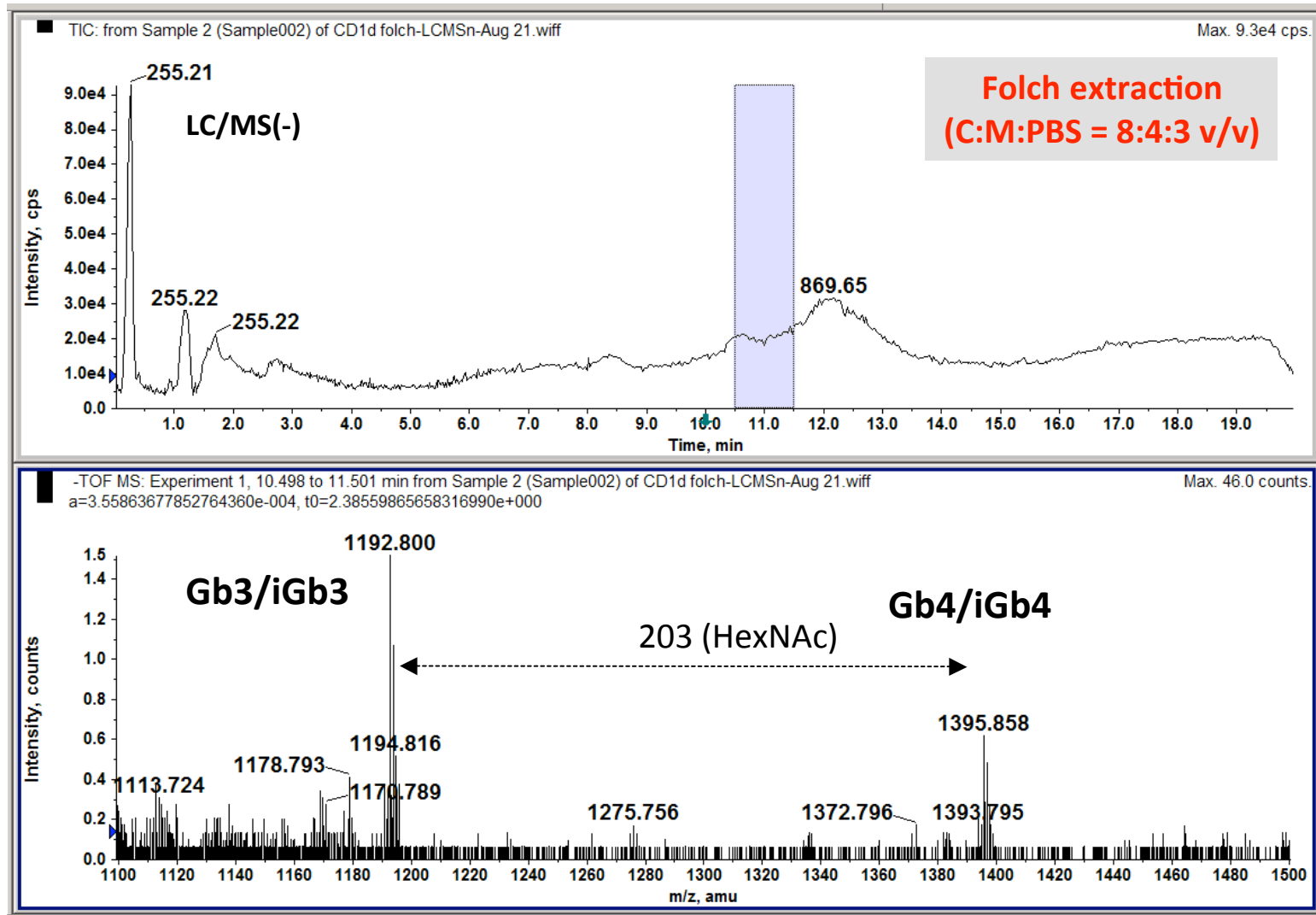
A. Side view



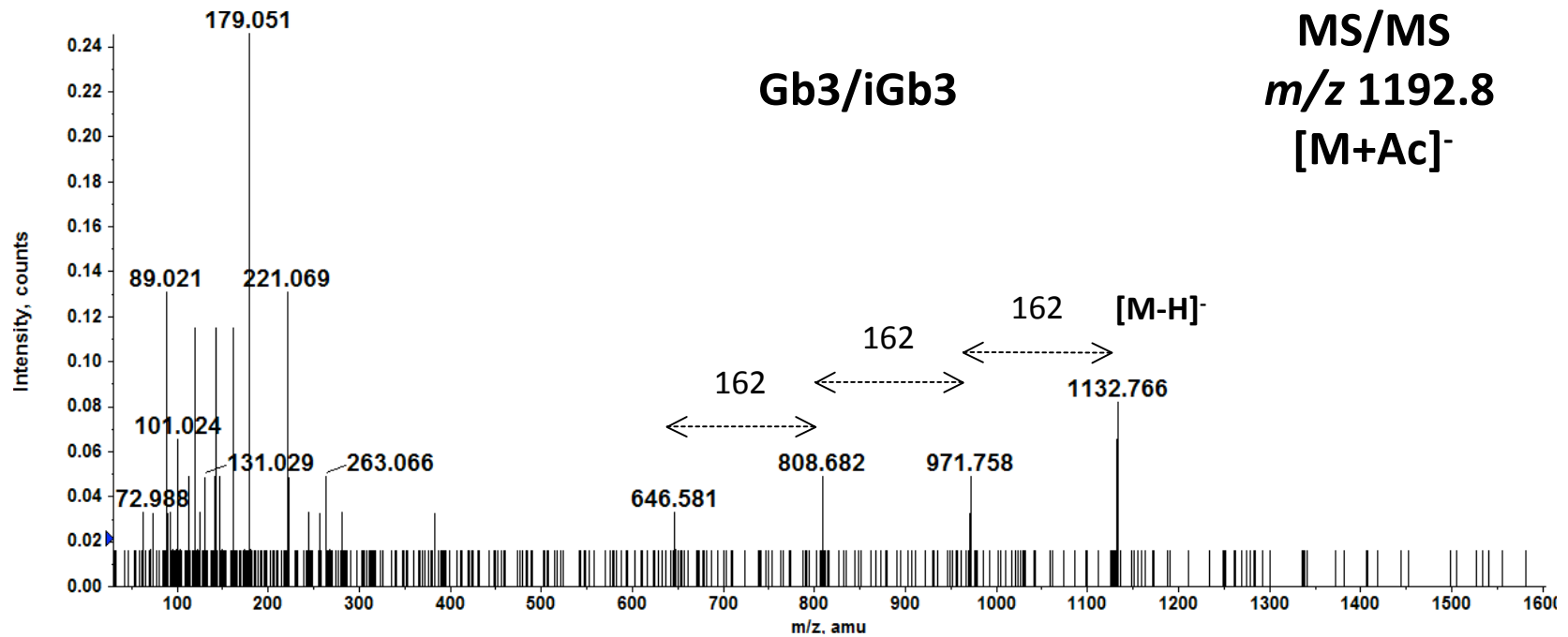
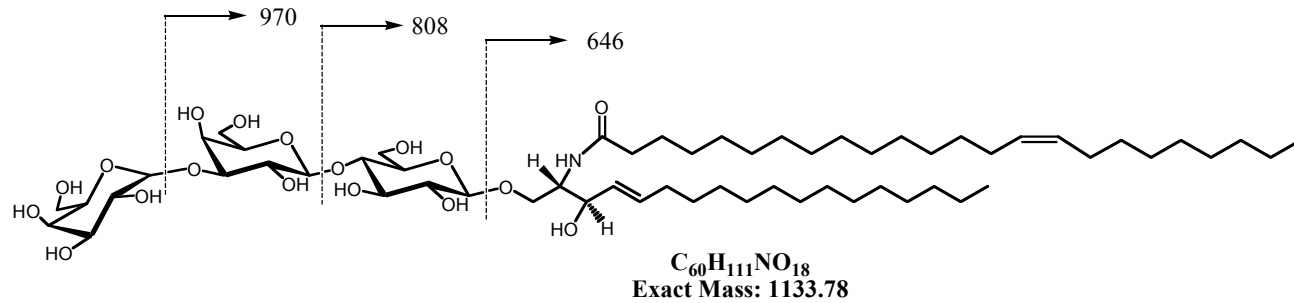
B. TCR view



Minor Lipids Bound to CD1d: Gb3/iGb3, Gb4/iGb4



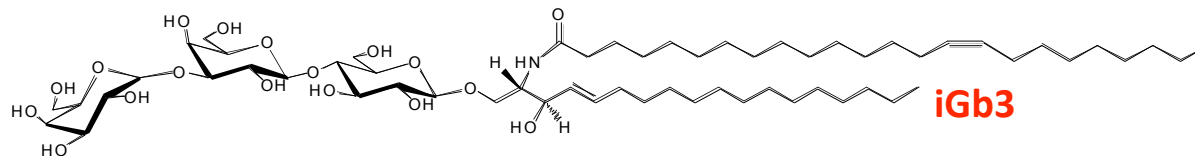
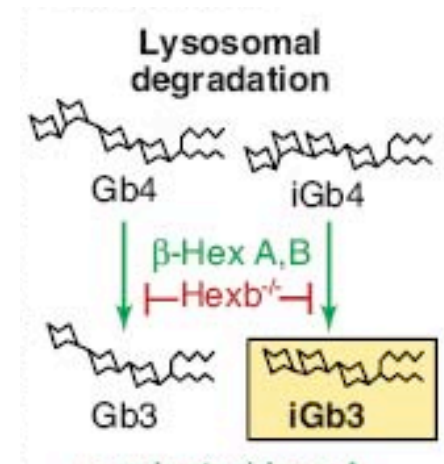
MS/MS Identification of Gb3 (or iGb3)



Science (2004) **306**, 1786-9.

Lysosomal Glycosphingolipid Recognition by NKT Cells

Dapeng Zhou,^{1*} Jochen Mattner,¹ Carlos Cantu III,²
Nicolas Schrantz,² Ning Yin,³ Ying Gao,³ Yuval Sagiv,¹
Kelly Hudspeth,¹ Yun-Ping Wu,⁴ Tadashi Yamashita,⁴
Susann Teneberg,⁵ Dacheng Wang,⁶ Richard L. Proia,⁴
Steven B Levery,⁷ Paul B. Savage,³ Luc Teyton,² Albert Bendelac^{1*}



- iGb3 is recognized both by mouse and human NKT cells.
- Impaired generation of lysosomal iGb3 in mice lacking β -hexosaminidase B results in severe NKT cell deficiency.

***Nature Review Immunol*, 7 May 2007**

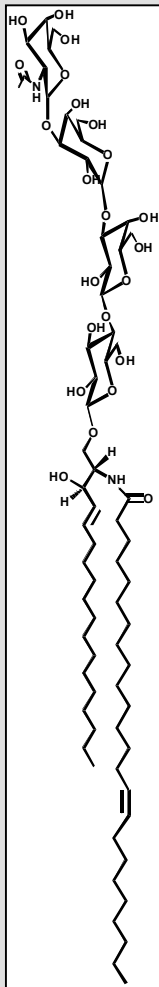
iGb3: to be or not to be?

The authors of two reports published in the *Proceedings of the National Academy of Sciences of the USA* suggest that, contrary to popular belief, the glycosphingolipid iGb3 (isoglobotrihexosylceramide) is not the ligand involved in the positive

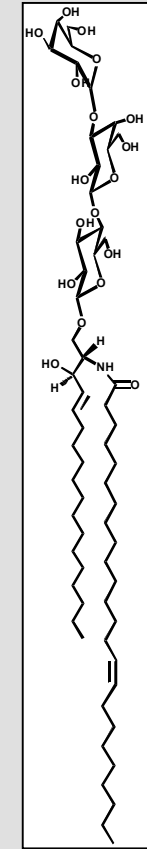
selection of invariant NKT cells that express an invariant T-cell receptor (TCR) α -chain and a restricted set of β -chains, and are selected in the thymus by the presentation of an endogenous lipid bound to CD1d on CD4⁺CD8⁺ cortical thymocytes. It is thought that this selectinoid lipid

CD1d-Binding Endogenous Lipids

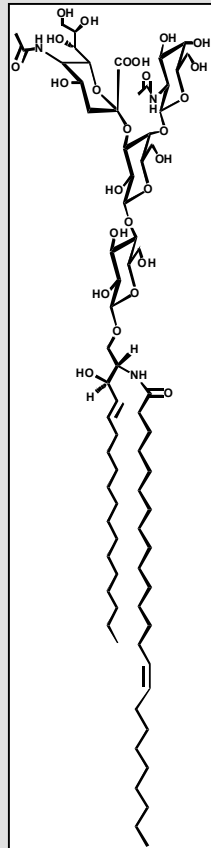
iGB4/GB4
(d18:1/24:1)



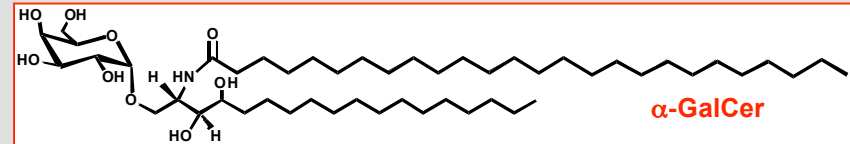
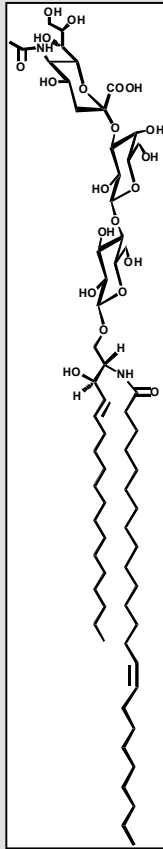
iGB3/Gb3
(d18:1/24:1)



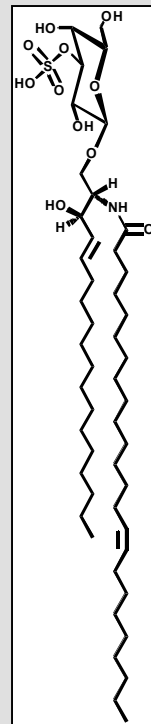
GM2
(d18:1/24:1)



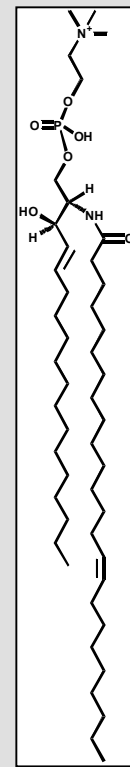
GM3
(d18:1/24:1)



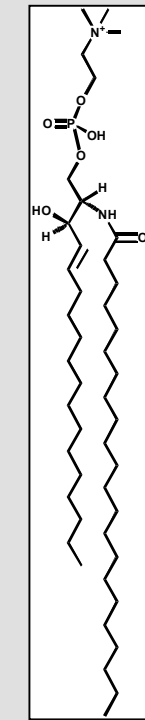
Sulfatide



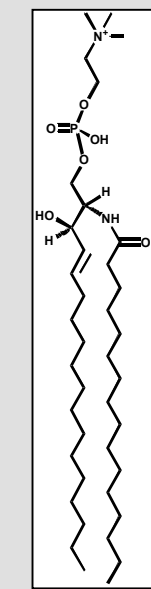
SM
(d18:1/24:1)



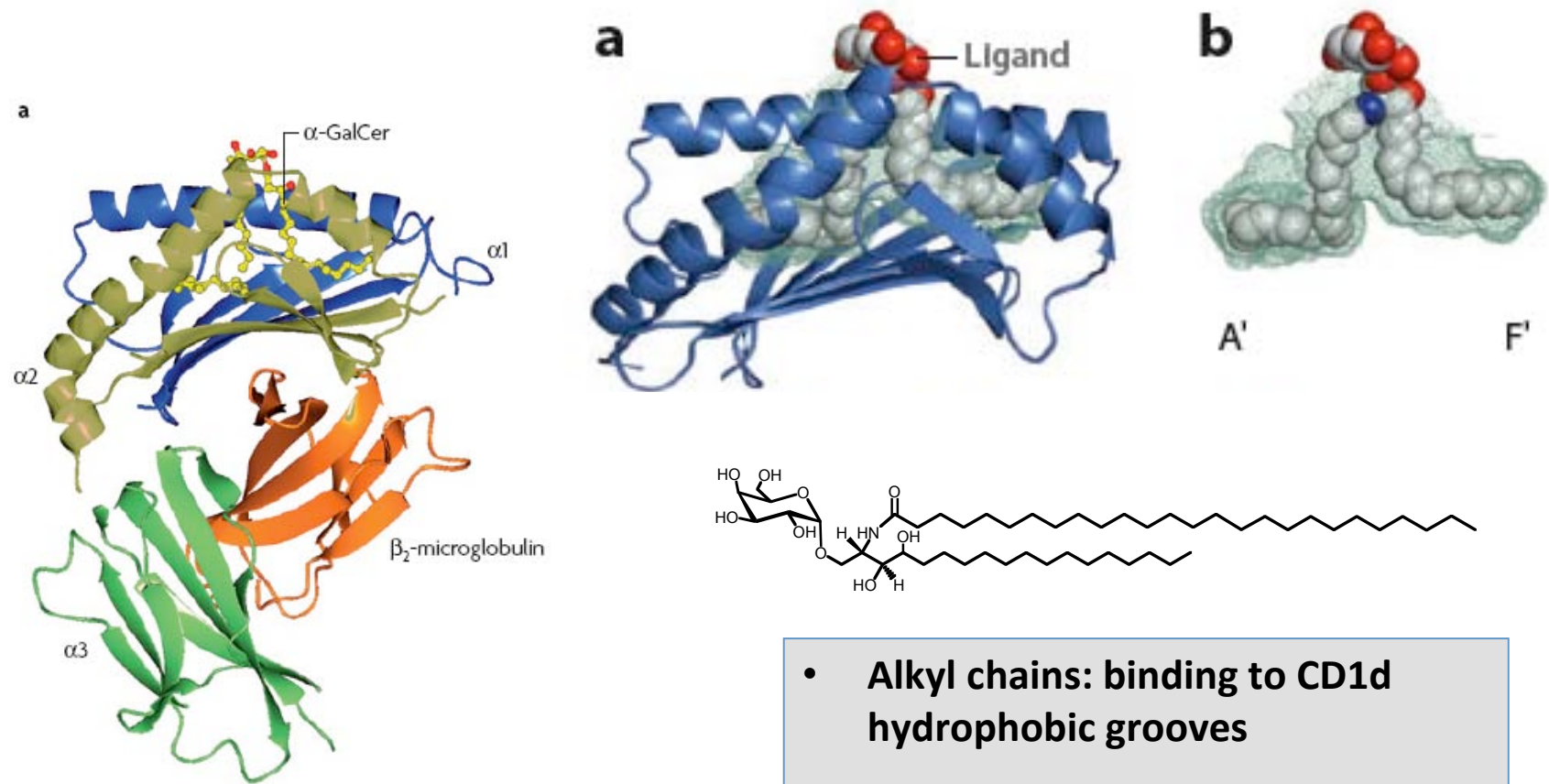
SM
(d18:1/22:0)



SM
(d18:1/16:0)



Complex of CD1d with α -GalCer



Zajonc, D. Wilson, I. *et al. Nature Immunol.* 6, 810 (2005)
Koch, K; Cerundolo, V *et al. et al. Nature Immunol.* 6, 819 (2005)

- Alkyl chains: binding to CD1d hydrophobic grooves
- Sugar configuration: recognition by the TCR of NKT cells

Outline

1. Identification of CD1-binding endogenous lipids

- CD1 and lipid antigens: background
- CD1d protein expression and isolation
- Bligh-Dyer lipid extraction of purified CD1d
- Lipid identification by LC/MS/MS

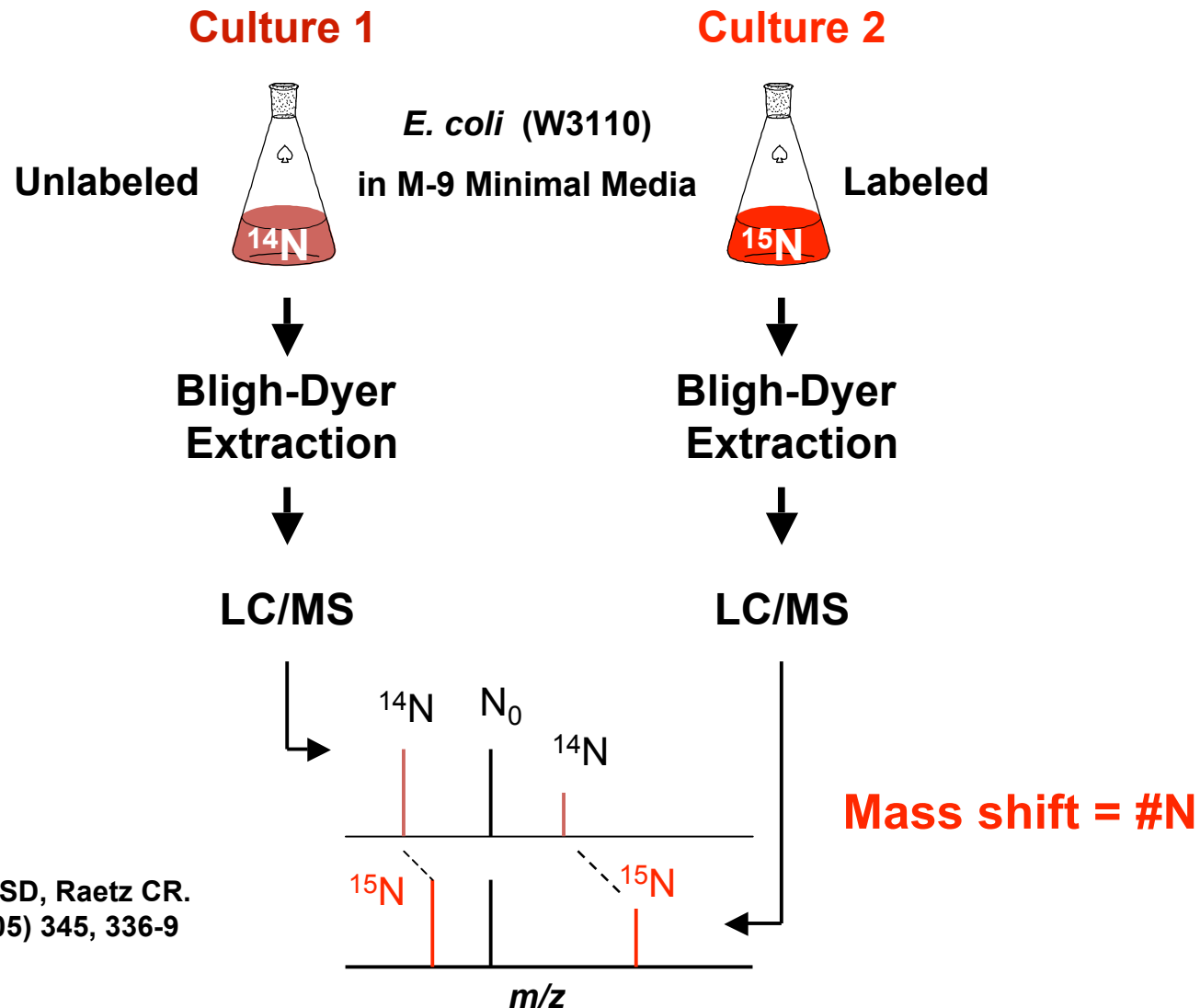
2. Identification of small, lipid-like membrane proteins

- ^{15}N labeling
- Bligh-Dyer extraction
- Identification by LC/MS/MS

Small Membrane Proteins

- **Annotation of genes encoding the small proteins (16-50 aa) is challenging:**
 - **Numerous short open reading frames (ORFs)**
 - **Lacking sufficient sequence for domain and homology determination**
 - **Small size limits the frequency in which they are disrupted in random genetic screens.**
- **Few annotated short ORFs have been confirmed (to correspond to synthesized proteins).**
- **Hydrophobic polypeptides are difficult to analyze with common proteomics techniques**

Identification of Small Membrane Proteins: Experimental Approaches



Guan Z, Breazeale SD, Raetz CR.
Anal. Biochem.(2005) 345, 336-9

Normal Phase LC

HPLC: Agilent 1200 (with quaternary pumps)

Column: Ascentis silica column (5 μm , 25 cm x 2.1 mm)

Solvent A: CHCl_3 :MeOH: NH_4OH (800:195:5, v/v)

Solvent B: CHCl_3 :MeOH: H_2O : NH_4OH (600:340:50:5, v/v)

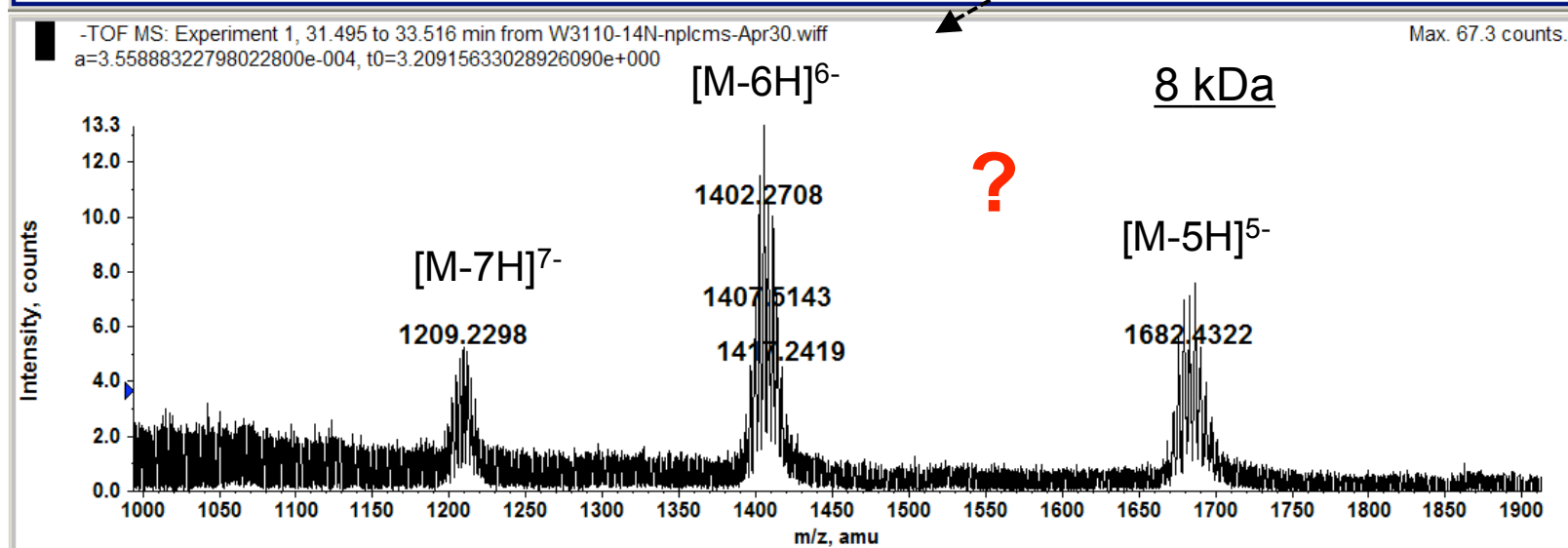
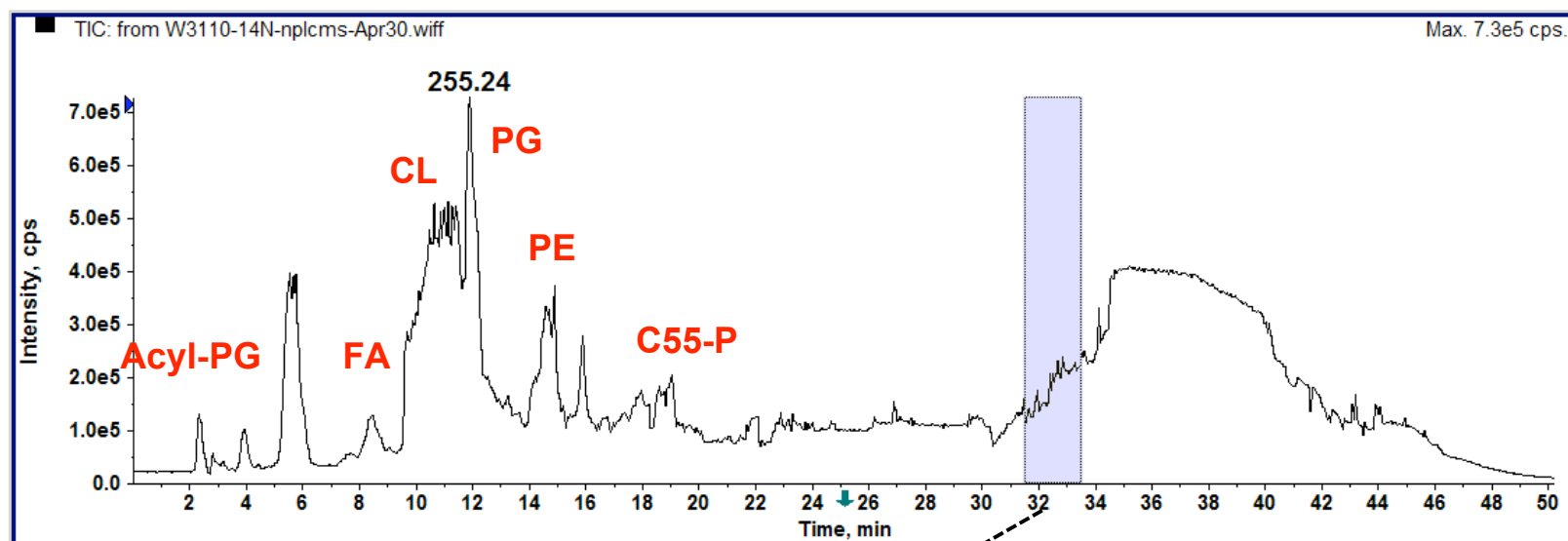
Solvent C: CHCl_3 :MeOH: H_2O : NH_4OH (450:450:95:5, v/v)

Flow rate: 300 $\mu\text{L}/\text{min}$ (1/10 split to Q-Star XL)

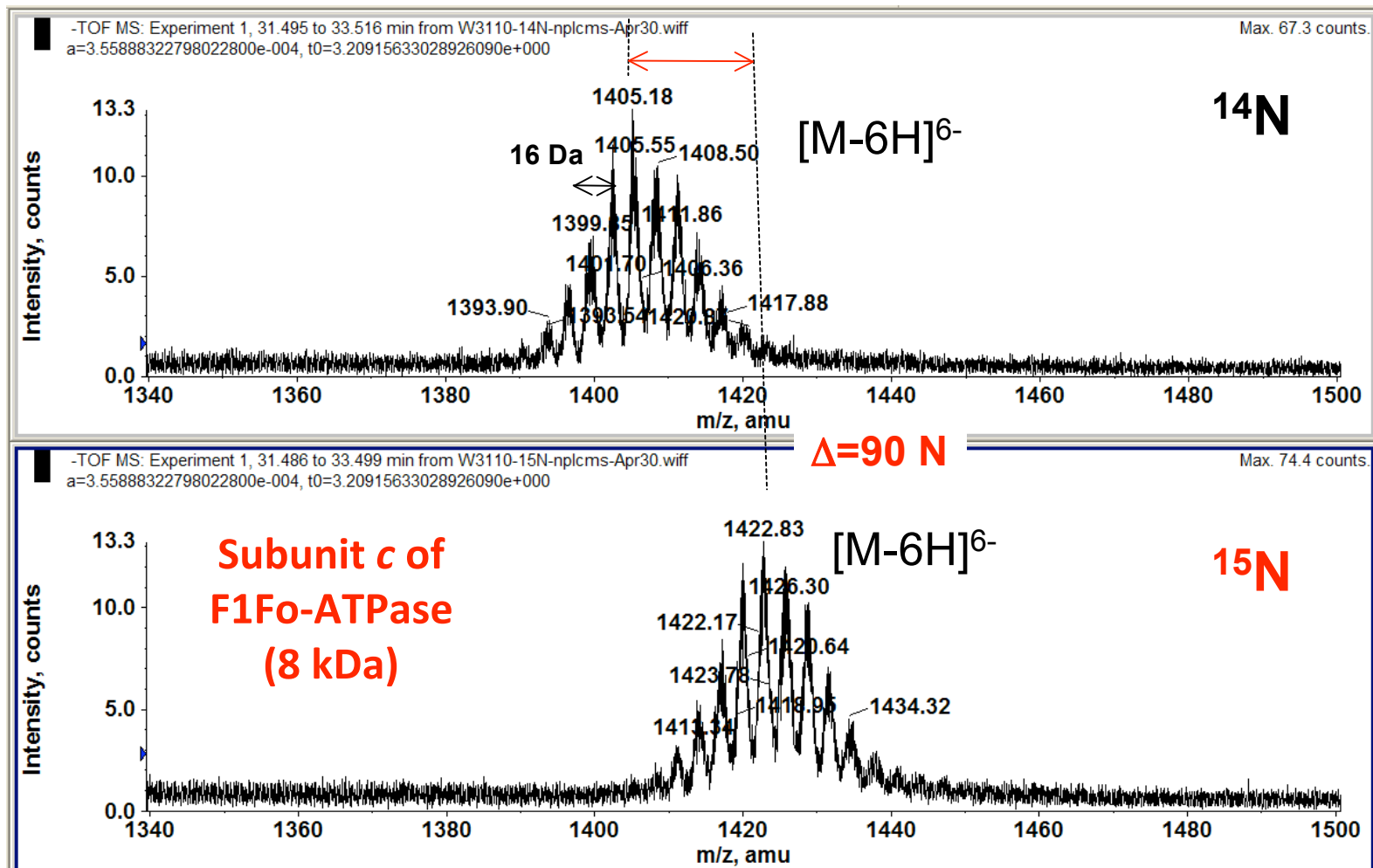
<u>Gradient:</u>	Time (min)	A (%)	B (%)	C (%)
	0	100	0	0
	2	100	0	0
	16	0	100	0
	27	0	100	0
	30	0	0	100
	33	0	0	100
	33.5	100	0	0
	40	100	0	0



NP-LC/MS of Bligh-Dyer Extract of *E. coli*

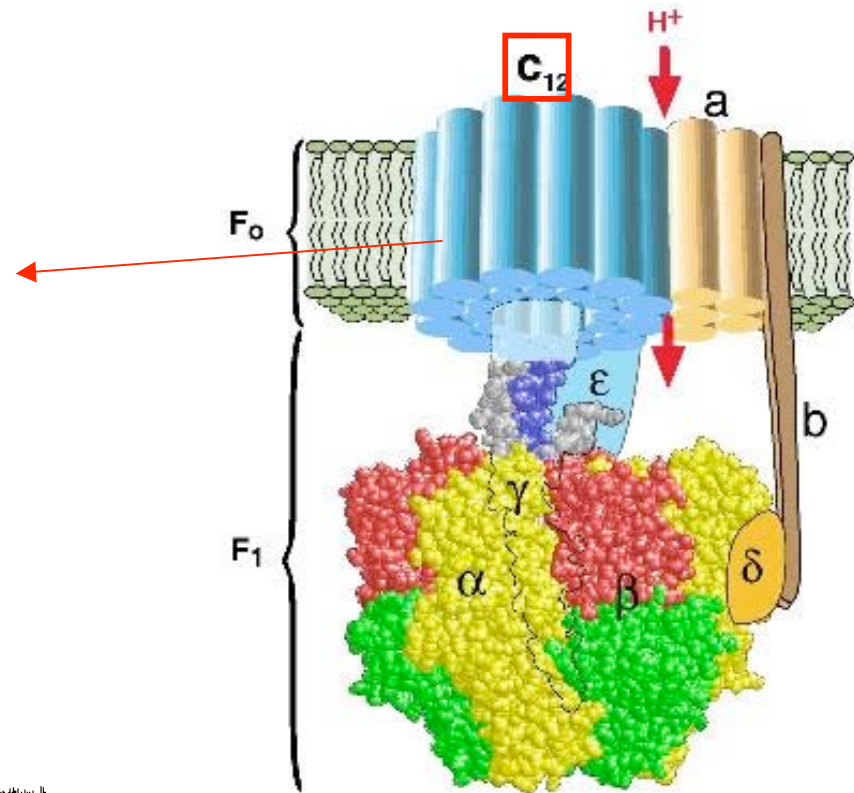
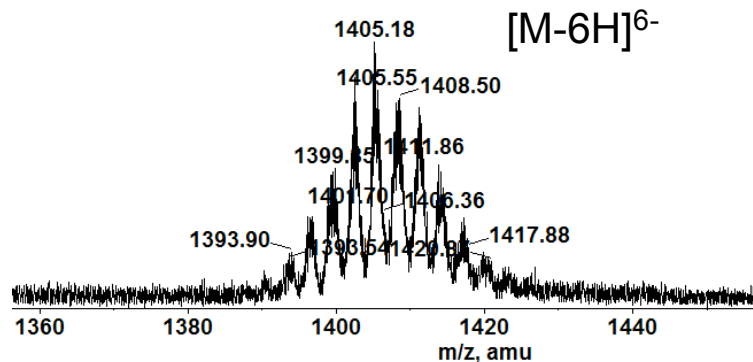


Detection of Subunit *c* of F1Fo-ATP Synthase from *E. coli*: Bligh-Dyer Extraction and NP-LC/MS



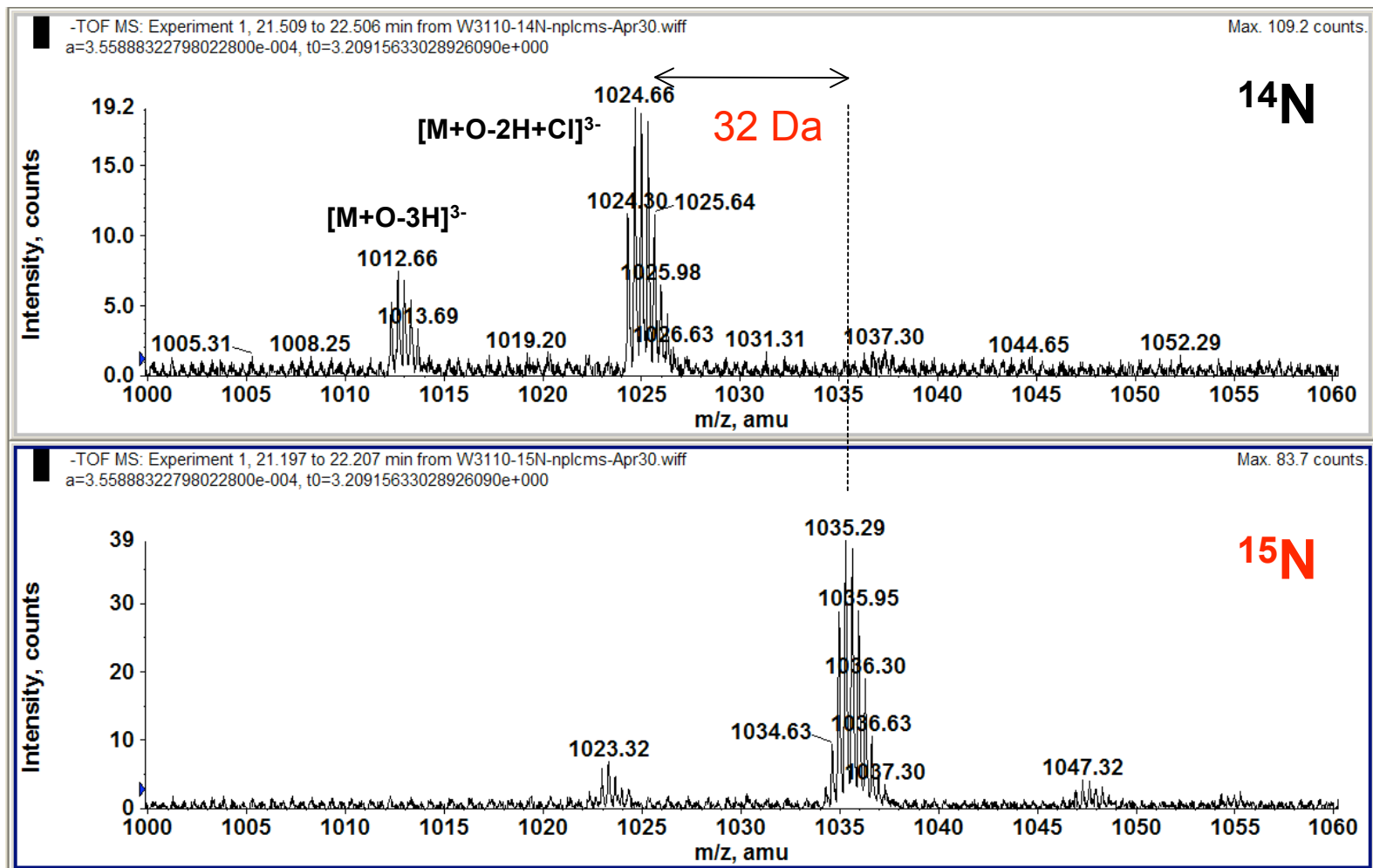
Subunit c of F1Fo-ATP Synthase (*E coli*): A “Proteolipid”

¹MENLNMDLLYMAAAVMMGLA
AIGAAIGIGILGGKFLEGAARQPD
IPLLRTQFFIVMGLVDAIPMIAVGL
GLYVMFAVA⁷⁹

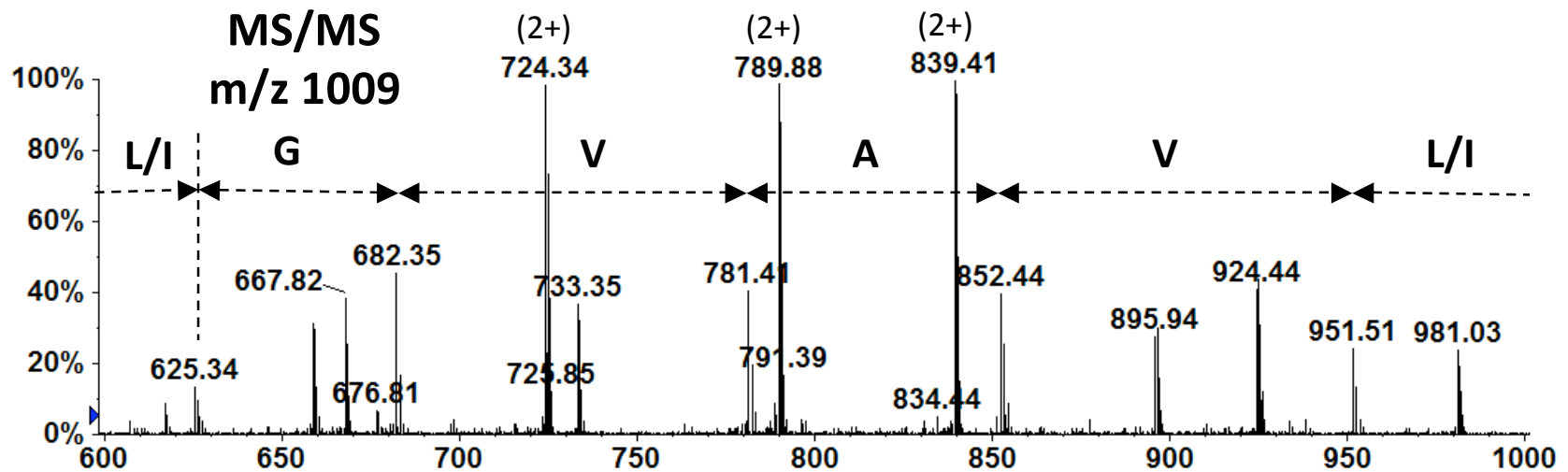
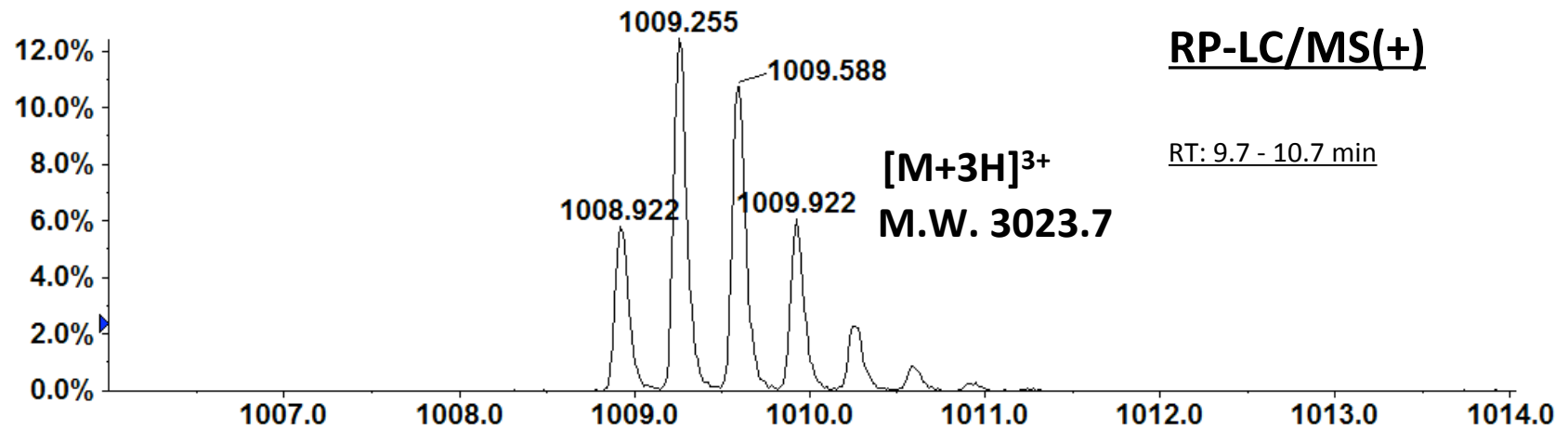


H. Wang and G. Oster (1998). Nature 396:279-282.

Unknown 3k Da species with 32 nitrogen atoms



It Is a Peptide: MS/MS



Sequence Determination of Unknown 3 kDa Hydrophobic Peptide (*E. coli*)

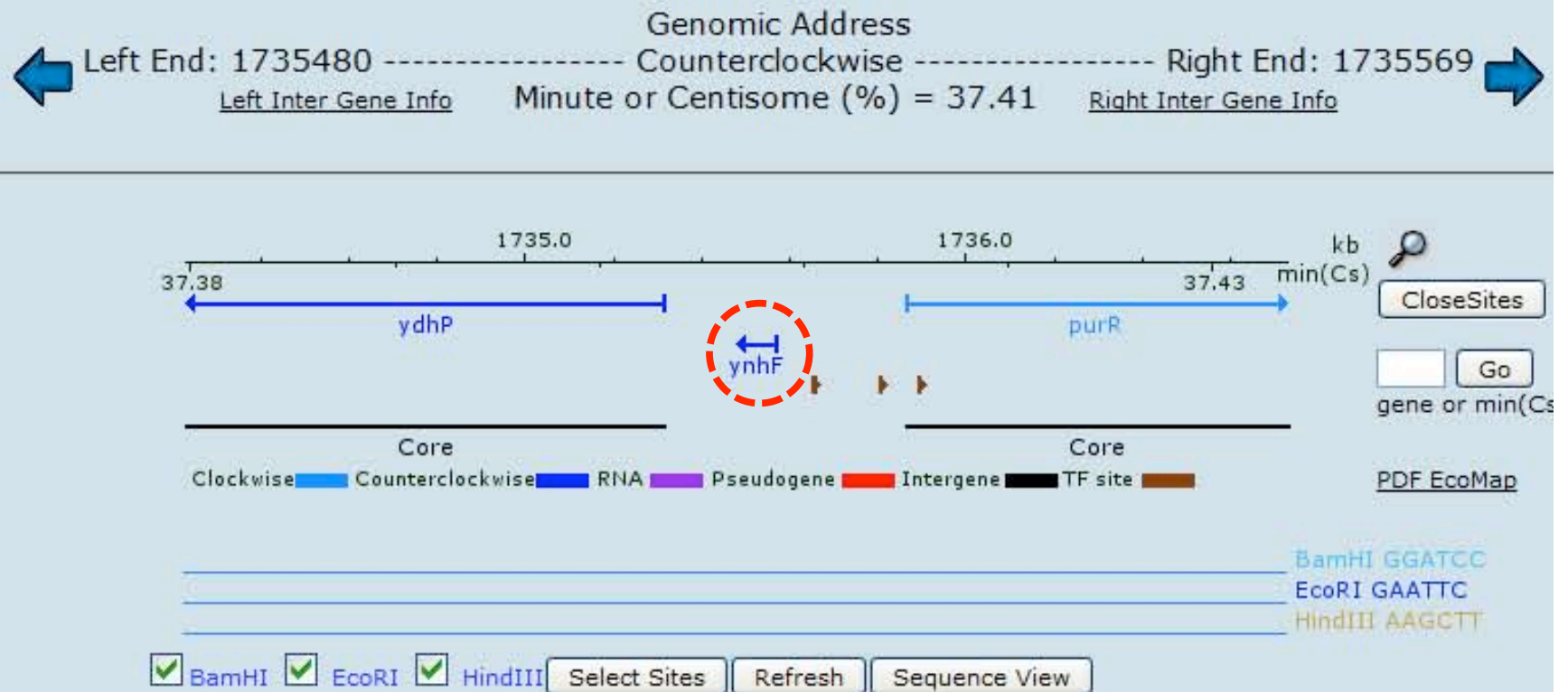
formyl-¹MSTDLKFSLVTTIIVLGLIVAVGLTAALH²⁹

Sequence determination by:

- MS/MS
- Accurate mass measurement
- tBLASTn search
- Comparison with a synthetic analog



Kenneth E. Rudd



Curator or user-submitted Comments:

YnhF has been identified as a chloroform-soluble peptide by partial peptide sequencing (Hydrophobic membrane mini-proteins in Escherichia coli and Salmonella, Z. Guan, X. Wang and C. R. H. Raetz, in preparation). Predicted TM segment.

Summary

1. Identification of CD1d-binding endogenous lipids

- SM, ST, GM2/GM3, iGb3/iGb4 (Gb3/Gb4)--sphingolipids with similar alkyl chains
- Different from those bound to CD1c (mainly PCs)
- GM2 and GM3 are shown to be recognized by CD1d-restricted T cells

2. Identification of small, lipid-like membrane proteins

- Several known and novel membrane mini-proteins are identified
- Their genomic locations hint that they are subunits of membrane structural complexes (such as transporters)