INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Université d'Ottawa · University of Ottawa

CD1 RESTRICTED RECOGNITION BY MURINE T CELLS

Theresa A. Podrebarac M.D.

In partial fulfilment of the requirement for the degree of
Master's of Science
Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine

School of Graduate Studies
University of Ottawa
Ottawa, Ontario, Canada
May 6, 1999

ABSTRACT

CD1 molecules are non-polymorphic glycoproteins distantly related to major histocompatiblity complex (MHC) molecules and represent a third lineage of antigen-presenting molecules in the immune system. Uniquely, CD1 molecules have the ability to bind and to present lipid antigens to T cells. An unusual subset of CD1d restricted T cells in mice called NK T cells, express an invariant Vα14-Jα281 T cell receptor (TCR)α rearrangement. NK T cells have been shown to play a pivotal role in the regulation of the immune response, the development of autoimmunity and in tumour rejection. However, CD1d reactivity is not limited to NK T cells alone. By immunizing mice with CD1d expressing transfectant cells, a panel of CD1d restricted, directly reactive T cell clones was generated. Functionally similar to NK T cells, these T cell clones secreted both Type1 (IFN-γ) and Type 2 (IL-4, IL-10) cytokines upon stimulation. In contrast, detailed TCR analysis revealed that a diverse repertoire of T cells could recognize CD1d. T cells expressing Vα10, -11, -15 and –17 and having non-germline-encoded nucleotides resulting in diverse V-J junctions were identified. Three clones expressed the invariant Vα14-Jα281 TCR but were functionally indistinguishable from the clones expressing diverse TCRs. Murine γδ T cells were examined for similar reactivity to CD1d, but specific recognition of CD1d could not be demonstrated. These data establish that the universe of TCRs capable of direct recognition of CD1d is more diverse than was previously appreciated, indicating the flexibility of the adaptive immune system in response to CD1d mediated processes. Given the conservation of NK T cells between humans and mice, and

the important immunological roles attributed to NK T cells, further insights into this complementary population of CD1d restricted T cells will have a significant impact on our understanding of how dysregulation of these cells could potentially lead to autoimmunity and tumour spread.

ACKNOWLEDGEMENTS: I would like to thank my collaborator Dr. Samuel Behar for his invaluable intellectual input, technical assistance and tutelage without whom, the completion of this project would not have been possible. I wish to also express my thanks to my supervisor, Dr. Michael Brenner for allowing me the opportunity of working on this challenging project and for his excellent guidance. My sincere gratitude is extended to my mentor and co-supervisor Dr. Rose Goldstein, for her unfailing support and advice. I would like to acknowledge the tremendous technical assistance of Christopher Roy. To the other members of my thesis advisory committee, Dr. Jack Karsh, Dr. Christina Parker and Dr. Jo-Anne Dillon, I offer my heartfelt thanks for their thoughtful criticism and guidance.

TABLE OF CONTENTS:

LIST OF TABLES:

LIST OF FIGURES:

INTRODUCTION

T lymphocytes are the principle specific effector cells in the adaptive immune response and are responsible for the development and maintenance of immunological memory. Tremendous excitement has arisen with the recent description of an unusual lineage of T cells called natural killer T (NK T) cells. NK T cells present in both mice and humans are defined as T cells bearing the NK cell marker NK1.1 or its homologue NKR-P1A in humans and are distinguished from conventional T cells by their expression of an extremely restricted αβ T cell receptor (TCR) (1). Strikingly, NK T cells have been shown to protect against the development of Type I diabetes in NOD mice and to play a profound role in the rejection of certain tumours, suggesting that NK T cells are strategic in the regulation of the immune response (2-4). NK T cells are particularly interesting since they are restricted by CD1d1 rather than by major histocompatibility complex (MHC) molecules.

CD1 molecules are non-MHC encoded surface glycoproteins that have the unique ability to present foreign mycobacterial lipid antigens to T cells. Given the notable diversity of human TCRs directed against CD1a, -b, and –c proteins and the antigens they present (5), we hypothesized that murine T cells able to recognize CD1d1 were not restricted to the NK T cell subsets. This research was undertaken to investigate the diversity of the T cell populations that could recognize CD1d1 and to fully characterize the functions of these T cells using an *in vivo* mouse model.

BACKGROUND

Critical to our understanding of T cell biology was the discovery that T cells could recognize specific peptide antigens presented by MHC molecules on the cell surface of antigen presenting cells (APCs). The antigen specific (TCR), MHC molecule and peptide antigen form a trimolecular complex that activates the T cell (6). The derivation of a human $CD4^-CD8^-$ double negative (DN) αβ T cell line (DN1) challenged this traditional paradigm (7). Specifically, DN1 was shown to recognize mycolic acid, a mycobacterial glycolipid antigen, presented by the CD1b molecule expressed on the surface of human monocytes. Subsequently, additional non-peptide antigens present in the cell wall of mycobacteria, including glucose monomycolate and lipoarabinomannan (LAM), were identified as ligands for CD1 restricted human T cells, clearly demonstrating that certain TCRs can recognize antigens chemically distinct from peptides (8,9).

CD1 genomic classification

The CD1 genes, *CD1A,-B,-C,-D* and *E* in humans and *CD1D1* and *CD1D2* in mice, encode novel, nonpolymorphic antigen presenting molecules. In humans, the CD1 gene family is located on chromosome 1 and is unlinked to the MHC on chromosome 6 (10,11). The murine *CD1D* genes are located on chromosome 3. Based mainly on their amino acid sequences, the CD1 proteins are divided into two groups. Group I is comprised of CD1a, CD1b and CD1c proteins while CD1d, which shows close homology to the protein products of the two murine *CD1D* genes (CD1d1 and –d2), defines group II. No protein product has been identified for *CD1E*, but the gene is transcribed (10,11). In every

mammalian species thus far studied, CD1 genes and proteins have been identified but differences exist with respect to the complexity and number of CD1 genes (11).

CD1 tissue distribution

Group I CD1 proteins initially identified on immature human thymocytes are expressed on the surface of several APCs, especially dendritic cells. CD1a is highly expressed on Langerhans cells in the epidermis, whereas dendritic cells in the spleen, liver, lung, skin and kidney express CD1b and –c (11). A subset of B lymphocytes within tonsil, spleen and blood also express CD1c. Group I proteins are inducible *in vitro* by incubating circulating monocytes with granulocyte macrophage-colony stimulating factor (GM-CSF) suggesting that these proteins are upregulated by tissue macrophages during inflammation (12). The group II protein CD1d1 has distinct differences in its distribution. It is found on thymocytes but the level generally decreases with maturation. In mice however, a residual population of thymocytes expresses high levels of CD1d1 (13). An unusual form of CD1d1 has been reported on gut epithelium, which lacks glycosylation and is not associated with β2-microglobulin (14). In mice, cells of hematopoietic origin including B cells, in particular splenic marginal zone B cells and T cells express CD1d1 in addition to splenic dendritic cells (13,15). Human CD1d has been detected at low levels on circulating monocytes and subsets of T and B cells (SA Porcelli, M Exley, SP Balk unpublished data). In contrast to the group I proteins, CD1d is not inducible by GM-CSF treatment (SA Porcelli, F Spada unpublished data).

MHC-like characteristics of CD1

CD1 molecules are related to both MHC class I and MHC class II glycoproteins in structure and evolutionary origin. CD1 and MHC class I proteins have a similar intron/exon organization with three major exons encoding three extracellular domains of approximately 90 amino acids in size designated α1, α2, and α3 (11). CD1 protein is expressed on the cell surface as a polypeptide of 45kDa noncovalently associated with β2-microglobulin, similar to MHC class I molecules. The recent X-ray crystallographic structure of murine CD1d1 confirms that the molecule is similar to MHC class I in its overall topography (16,17). Most notably, it was found that the CD1d1 ligand binding groove is much deeper than peptide binding grooves for either MHC class I or II (Fig.1). The MHC molecules have six to nine small binding pockets to accommodate amino acid side chains in contrast to CD1d1, where only two large pockets, designated A' and F' exist. Hydrophobic, non-polar amino acid residues form the interior of CD1d1, which has little potential for hydrogen binding. In this light, it would be unlikely that the CD1d1 groove would bind ligands in the same way as MHC molecules and confirms that the CD1d1 cavity would be capable of binding hydrophobic lipid antigens (11,16).

Direct recognition of CD1d1

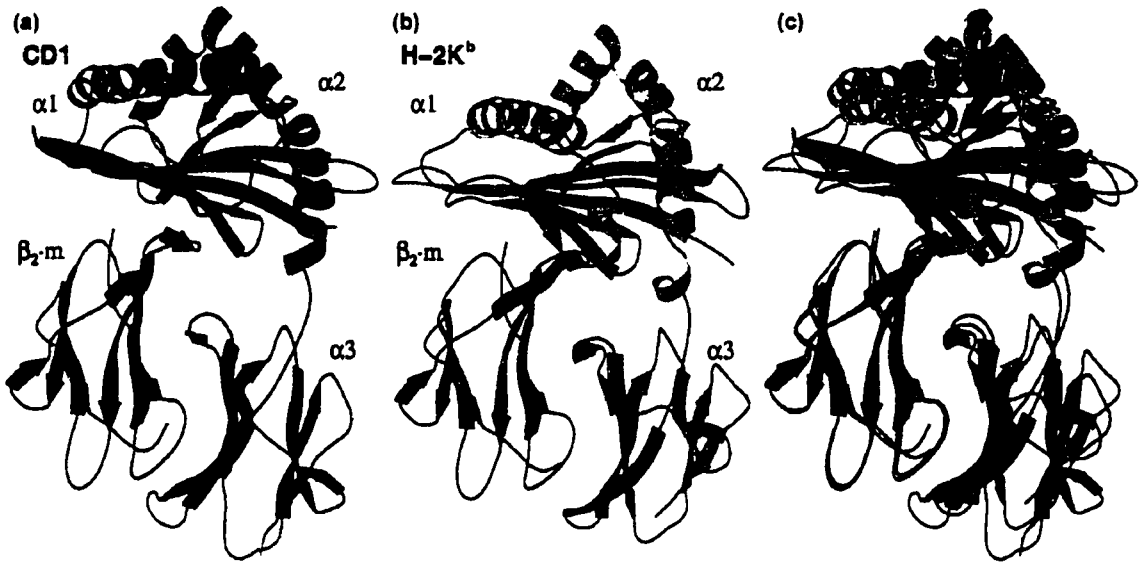
Prior to the discovery of mycolic acid as a lipid antigen presented by the CD1b molecule to human DN T cells, the first description of CD1 reactivity came from DN αβ and γδ T cell clones in humans that could lyse tumor cell lines expressing various CD1 isoforms (18). In the years following, studies in mice

FIGURE 1: Comparison of the crystal structure of CD1 to MHC class I.

A) Backbone ribbon diagram of mouse CD1d1: α-helices (red); β-strands (blue).

B) Ribbon diagram of MHC class I molecule H-2K^b: α-helices (turquoise); β-strands (green).

C) Superposition of the two molecules using alignment of β2m domains illustrates structural differences. Note that CD1d1 has a deeper, more voluminous antigen-binding pocket compared to H-2K^b and a more narrow entrance. (Figure reproduced with permission from Reference 17).


(a)
CD1
α1
α2
β2·m
α3
(b)
H-2K^b
α1
α2
β2·m
α3
(c)

demonstrated CD1d1 T cell reactivity in the $NK1.1^{+}$T cell population of normal mice as well as in the residual $CD4^{+}$ population of MHC class II deficient mice (19,20). These studies were conducted using T cell hybridomas derived from thymus. In the absence of added foreign antigen, these unusual DN T cells were found to recognize CD1d1 expressed on cell lines transfected with *CD1D* as well as CD1d1 on thymocytes. T cell recognition was specifically blocked with monoclonal antibodies directed against murine CD1d1. This phenomenon is referred to as direct reactivity or "autoreactivity" and suggests that this form of T cell autoreactivity is inherent. This unusual lineage of T cells became known as natural killer (NK) T cells (1).

CD1-restricted NK T cells

Numerically, NK T cells constitute up to 20% of the mature thymus, 20-30% of the T cells in the liver and bone marrow and 1% of splenocytes, representing the largest T cell clones in mouse (21). NK T cells characteristically express most known natural killer (NK) cell lineage markers including the NK1.1 receptor and coexpress an invariant TCRα chain encoded by the precise joining of Vα14 and Jα281 gene segments in association with a limited Vβ repertoire predominantly Vβ8, 7 or 2 (1). The limited variability of the TCR suggests that these cells will respond to only a restricted number of ligands. The NK T cell homologue in humans similarly expresses an invariant Vα24, JαQ with Vβ11 and NKR-P1A (22). These T cells have been found to be either $CD4^{+}$ or $CD4^{-}CD8^{-}$.

Upon activation, NK T cells release cytokines of both T-helper (Th)1 and (Th)2 origin. However, within an hour of TCR engagement, NK T cells secrete

large amounts of IL-4. It has been proposed that this potent biological effect can polarize the immune response to that of the Th2 phenotype leading to antibody production and immunity to extracellular parasites (23,24). In contrast to the concept that all T cells are selected through the interaction between the TCR and MHC molecule, NK T cells are selected by CD1. As evidence, mice with either a targeted mutation of β2-microglobulin or *CD1D* lack NK T cells (25-27). Similarly, mice with a deletion of the Jα281 gene segment have an absence of NK T cells (3).

$CD1D^{-/-}$ mice phenotype

Since this project was begun, several important discoveries with regard to CD1 have occurred. Three separate groups have reported on the generation of $CD1D^{-/-}$ mice, 2 on the C57BL/6 background and one on the BALB/c haplotype (25-27). Together, these animals confirmed the absolute dependence on CD1d1 for the selection and development of the NK T^{+} Vα14 Jα281 population. Immediate production of IL-4 after TCR cross-linking was impaired in these animals. In contrast to β2-microglobulin-deficient mice, CD1-deficient thymocytes produced comparable levels of IFNγ to their control littermates suggesting that the immediate production of IL-4 is a CD1-dependent process, whereas IFNγ production is β2-microglobulin-dependent but CD1-independent. However, IgE class switching in response to stimulation with anti-IgD, an IL-4 dependent process, was unimpaired indicating that NK T cells are not the exclusive source of Th2 cytokines.

CD1d1-presented antigens

Initial reports of CD1d1 antigen reactivity suggested that peptide antigens could bind murine CD1d. These findings arose from a random synthetic peptide library screening technique that identified the synthetic hydrophobic peptide p99a as a potent stimulator of CD1d1 restricted, peptide-specific cytolytic T cells (28). This suggested that in contrast to the non-peptide ligands identified for the group I CD1 proteins, CD1d1 had maintained the potential to react with peptides. Subsequently, accumulative data has now made this hypothesis much less likely.

Since initiation of this project, a recent breakthrough was made with the discovery of specific antigens from a family of ceramide-like glycolipids, specifically α-glycosyl acylphytosphingosines and glycosylphosphatidylinositol (GPI), that could stimulate CD1d1 restricted murine NK T cells (15,16). These compounds consist of an α-anomeric hexose sugar linked to an acylphytospingosine moiety. At present these ceramides are known to naturally exist in marine sponges but ongoing efforts are to identify structural analogues of these lipid antigens in pathogens and inflamed or injured tissues. Intriguingly, mammalian GPI is similar to the MTb antigens LAM and PIM, GPIs identified earlier as CD1b presented antigens (8). These data support the hypothesis that the group II CD1 proteins, like the group I CD1 molecules in humans, evolved to present lipid antigens to T cells.

NK T cells and tumours

IL-12 is known to mediate antitumour effects and it was hypothesized that this effect was due to the activation of NK T cells. To clarify the role of IL-12 in

the FBL-3 erythroleukemia or B16 melanoma tumour models, mice that lacked the invariant Vα14Jα281 TCR were generated (3). The numbers of NK1.1^{+} were reduced but the overall number of hematopoietic cells was unaffected in these mice. With injection of IL-12 intraperitoneally, liver metastases were suppressed in the liver of wild type mice but not Jα281$^{-/-}$ mice. To confirm that this effect was due to Vα14^{+}NK T cells, the Vα14Vβ8.2 transgene was introduced into the recombination activating gene (RAG)$^{-/-}$ mouse which then preferentially generates Vα14 NK T cells but blocks the development of all other NK, B and T cells. In these transgenic mice, the tumour was rejected (3). The known primary effect of IL-12 on Vα14NK T cells is to induce proliferation of these cells and to increase their cell volume. These findings suggest that our understanding of NK and T cell functions may need to be reevaluated with our present knowledge of the importance of NK T cells.

NK T cells and autoimmunity

Accumulating data suggest that NK T cells play a pivotal role in the induction of autoimmune disease. A powerful analysis of identical twins/triplets and the development of Type I diabetes mellitus (DM) showed that prior to the onset of disease, the diabetic sibling had a lower frequency of Vα24JαQ+ T cells, the human NK T cell subset, than the non-diabetic sibling (2). Furthermore, the Vα24JαQ+ T cell clones isolated from the at-risk siblings that did not progress to DM, produced both IFNγ and IL-4. In contrast, the siblings that did develop disease, their Vα24JαQ+ T cell clones produced only the Th1 cytokine IFNγ. This suggests that NK T cells regulate the development of autoimmunity by initially

producing both Th1 and Th2 cytokines and that disease progression correlates with the cessation of IL-4 production. Pancreatic injury could be prevented in diabetes prone NOD mice by the adoptive transfer of NK T cells, further illustrating that these cells play a crucial role in regulation of the immune response (4).

Several mouse models of SLE have demonstrated that NK T cells are specifically reduced prior to the development of disease as seen in aging C57BL/6 or MRL/*lpr/lpr* mice, C3H *gld/gld* and (NZBXNZW)F1 mice, suggesting that this diminution of NK T cells is associated with disease induction (31). Injection of MRL *lpr/lpr* mice with an anti-Vα14 antibody accelerates the development of lymphosplenomegaly and causes increased titers of anti-ds DNA antibodies.

An analysis of patients with scleroderma demonstrated that compared to healthy individuals, there was a selective reduction in Vα24JαQ+ T cells. These patients had an expansion of the $V\alpha24^{+}$ T cells but paired with alternate Jα gene elements. Together these data suggest that the canonical receptors Vα14Jα281 in mice and Vα24JαQ in humans serve specialized immunoregulatory functions.

SPECIFIC AIMS

What remains unclear is whether additional subsets of CD1d1 restricted T cells exist. What role do these cells play in the host immune response? The function and location of these T cells, the cytokines they produce, their antigen specificity, and the signals that stimulate or anergize these T cells will have

tremendous biologic importance. Given the broad diversity of human TCRs able to recognize CD1a,-b, and -c molecules and the antigens they present, we hypothesized that diverse murine T cell populations have the ability to recognize the CD1d1 molecule as well as CD1d1 presented hydrophobic antigens. The research described in this thesis dissertation was designed to address the following questions:

AIM 1: What other T cells in normal mice recognize CD1d1?

Do peripheral T cells have the ability to recognize CD1d1?

AIM 2: What is the functional outcome of CD1d1 recognition?

What is the cytokine profile of CD1d1 restricted T cells?

Do these T cells proliferate or cause cytolysis in response to CD1d1 recognition?

AIM 3: What is the nature of the TCRs capable of recognizing CD1d1?

Can diverse TCRs recognize CD1d1?

Does TCR analysis reveal conserved sequences that are required to confer CD1d1 reactivity?

CHAPTER 2: MATERIALS AND METHODS

Cell culture

All T cells were grown in complete media consisting of RPMI 1640 media supplemented with 10 mM HEPES, 2 mM L-glutamine, 10 mM nonessential amino acids, 10 mM essential amino acids, 0.055 mM 2-ME (all from Life Technologies, Gaithersburg, MD), 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 1% penicillin/streptomycin and 1.5 nM recombinant IL-2 (gift of Ajinomoto, Kawasaki, Japan). RMA-S cells were grown in complete media without IL-2.

T Cell lines and clones

The RMA-S cell line, which is MHC class II negative, was derived by mutagenesis from a C57BL/6 T cell lymphoma cell line (RBL-5) and contains a mutant *TAP2* gene, thereby making these cells also defective in the MHC class I processing pathway (33). These cells were chosen as an ideal APC for studying CD1d restricted T cell responses. RMA-S cells were transfected with the expression vector pSRa-NEO containing the CD1D1 cDNA (kindly provided by Dr. Steve Balk, Beth Israel Hospital, Boston, MA) by electroporation. Stably transfected cells were first selected for G418 resistance and then for high surface expression of CD1d1 by sorting cells that stained brightly with the 3C11 and 1H1 Abs using the FACSort (Becton Dickinson, Raritan, NJ). $CD1d1^+$ RMA-S (RMAS.CD1d1) clones were subsequently established by limiting dilution. The $CD1d1^+$ L cell transfectant, a fibroblast derived cell line, was provided by Dr. Steve Balk (Beth Israel Hospital, Boston, MA).

C57BL/6 mice were immunized with either of two protocols. The first protocol was used to generate Line 14. RMAS.CD1d1 cells that had been incubated with 10 μM p99a peptide at 37°C for 4 h, were washed, treated with mitomycin C (Sigma, St. Louis, MO) and injected i.m. into the thigh. Simultaneously, these mice each received 0.1 μmol p99a peptide in CFA (initial immunization) or IFA (subsequent immunizations) s.c. in the contralateral thigh. The immunization was repeated X 2 each 10 days apart. The second protocol simply immunized the mice with 0.1 μmol p99a peptide in CFA then IFA s.c. in the absence of RMAS.CD1d1 cells to generate Line 24. Mice were euthanized by CO_2 asphyxiation and the spleen and draining lymph nodes were removed. T cells were enriched for by removing MHC class II bearing cells from a single-cell suspension by incubating the cells using the Ab M5/114.15.2 followed by immunomagnetic bead depletion (Dynal Inc., Great Neck, NY). T cells were cultured at a 2:1 ratio with mitomycin C-treated RMAS.CD1d1 cells in the presence of 10 μM p99a peptide. After two weeks, the bulk cultures were restimulated with the RMAS.CD1d1 APCs, 10 μM p99a and IL-2. Following two restimulations, the bulk T cell line was assayed for reactivity to CD1d1 in the presence and absence of p99a antigen.

T cell clones were isolated from T cell lines by limiting dilution on the third restimulation. T cells were plated at 1, 5, or 10 cells/well in 96-well round-bottom microtiter plates in the presence of IL-2, 10 μM p99a and 2 X 10^5 mitomycin C-treated RMAS.CD1d1 cells. Plates were fed after 7 days culture at 37°C and then every 3-4 days thereafter. Once T cell functional assays revealed CD1d1

reactivity in the absence of p99a antigen, clones were expanded by restimulation with RMA-S.CD1d1 mitomycin C-treated APCs alone every 2-3 weeks. The prefixes used to label the clones, 14 and 24, refer to original T cell line from which the clones were derived.

Antibodies and antigens

The Abs M1/42.3.9.8.HLK (anti-MHC I), M5/114.15.2 (anti-MHC II), GK1.5 (anti-CD4) and 53-6.72 (anti-CD8α) were obtained from the American Type Culture Collection (Rockville, MD). Abs 53-5.8 (anti-CD8β), H57-597 (anti-αβ TCR), GL3 (anti-γδ TCR), PK136 (anti-NK1.1), 5E6 (anti-Ly49C), 1B1 (anti-CD1d1) were obtained from PharMingen (San Diego, CA). The hamster Abs 38-4.5 (IgG control), 3H3.23.2 and 5C6.4 (both anti-CD1d1) were used as spent culture supernatants and were kindly provided by C.R. Wang (University of Chicago, Chicago, IL) (34). The Abs 1H1 and 2C11 (rat anti-CD1d1) were generously provided by Steve Balk (Beth Israel Hospital, Boston, MA) and 10A7 (anti-NKR-P1A/B) was a kind gift of James Ryan (University of California at San Francisco, San Francisco, CA). The CD1d restricted peptide, p99a (EHDFHHIREWGNHK) (28) was synthesized by the Brigham and Women's Hospital Biopolymer Laboratory.

Flow Cytometry

Cells (2 X 10^5) were stained with 50 μl of ascites (diluted 1:400) or purified Ab (5 μg/ml) in FACS buffer (PBS/5% bovine calf serum/0.01% azide) for 1 h at 4°C. The cells were washed and resuspended in 20 μg/ml FITC labeled $F(ab')_2$ rabbit anti-hamster Ig or PE-labeled donkey anti-rat Ig (Jackson

ImmunoResearch Laboratories, West Grove, PA) for 1h at 4°C. Cells were washed, resuspended in FACS buffer and analyzed on a FACSort (Becton Dickinson). Dead cells were excluded based on propidium iodide counterstaining.

T Cell proliferation assays

T cells (5×10^4/well) were cultured in triplicate in 96-well flat bottom microtiter plates in the presence of 10^5 mitomycin C treated APCs (RMAS.CD1d1 cells or untransfected RMA-S cells) in the presence or absence of p99a peptide in a final volume of 200 μl/well. To assess CD1-restriction, the indicated antibodies were used at a final concentration of 20 μg/ml or a final dilution of 1:4 for spent culture supernatants. Cells were cultured for a total of 48-72 h at 37°C in 5% CO_2, pulsed with ^{3}H-thymidine (1 μCi/well, 6.7 Ci/mmol, New England Nuclear, Boston, MA) and incubated for 6h. The plates were harvested on a Tomtec 96-well plate harvester (Wallac, Gaithersburg, MD) and thymidine incorporation was measured with a Betaplate liquid scintillation counter (Wallac). In general, the standard deviation (SD) of the triplicates was 5-10% of the mean.

Cytotoxicity assays

RMAS.CD1d1 or untransfected RMA-S cells were used as targets in a standard ^{51}Cr release assay. Targets were labeled with 200 μCi of ^{51}Cr for 2h. Two thousand target cells were incubated with the effector T cells at the indicated E:T ratios for 4h in triplicate. CD1d1 restricted responses were assessed by using Abs specific for CD1d1 at a final concentration of 20 μg/ml or supernatants at a final dilution of 1:3. Chromium release was evaluated by spotting 25 μl of the supernatant onto fiberglass filter mats that were counted in a

1205 Betaplate liquid scintillation counter (Wallac). Specific lysis was calculated as [sample cpm - spontaneous cpm)/(maximum cpm – spontaneous cpm)] X 100. The SD of the triplicates was in general 1-5% of the mean.

Cytokine assays

IL-2, -4, -10 and IFN-γ production by the T cell clones was assessed using a sandwich ELISA 48 h after stimulation. Specific stimulation of the T cells incubated with RMAS.CD1d1 was compared to T cells incubated with untransfected RMA-S. The Abs specific for the cytokines were obtained from PharMingen and used according to the manufacturer's directions. The limits of detection were 78 pg/ml.

PCR and sequencing

RNA was extracted from the T cell clones using RNAzol (Tel Test, Friendswood, TX) and first strand cDNA synthesis was conducted using random hexamers. Gene-specific oligonucleotide primers to the Vα14 and Cα gene segments and *Taq* polymerase were used initially (35,36). Expression of the invariant TCR Vα14-Jα281 was then assessed by using internal primers specific for Vα14 and Jα281 and the products were visualized on a 2% agarose gel using ethidium bromide.

The remaining TCRα chains were determined by using inverse PCR as described (37). Oligo(dT)-primed double-stranded cDNA was synthesized from total RNA, and incubated with T4 DNA polymerase to form blunt-ended cDNA. Circularization of the cDNA was done with DNA ligase and used as a template for PCR using Cα primers oriented in opposite directions and KlenTaq

(CloneTech) (38). The amplified genes were cloned into pBluescript II (Stratagene, La Jolla, CA) and sequenced at the Brigham and Women's Hospital automated DNA sequencing facility using a 3' Cα-specific primer (5'-CGAGGATCTTTTAACTGG TAC-3');Vα-specific primers (Vα10: GGAAGTCTCGTCAGCCTGTT; Vα11: CCTCCCATTCTCCTTTGT; Vα15: GAGAAGGTCGAGCAACATGAG (35,36).Vβ gene usage was done by RT-PCR using primers previously described (39). The additional primers used were Vβ5:AACAAGTTCAGCAGATTCTGG;Vβ5:GGGTTGTCCAAGTCTCCAAGA;Vβ8: GAAACAAGGTGGCAGTAACAGGAGG;Vβ14: CTGTTGGCCAGGTAGAGTCGG Cβ: CCCAGGCCTCTGCACTGATGT and Cβ:GATGGCTCAAACAAGGAGACC.

CHAPTER 3: FEATURES OF αβ T CELL RECOGNITION OF CD1d1

RESULTS

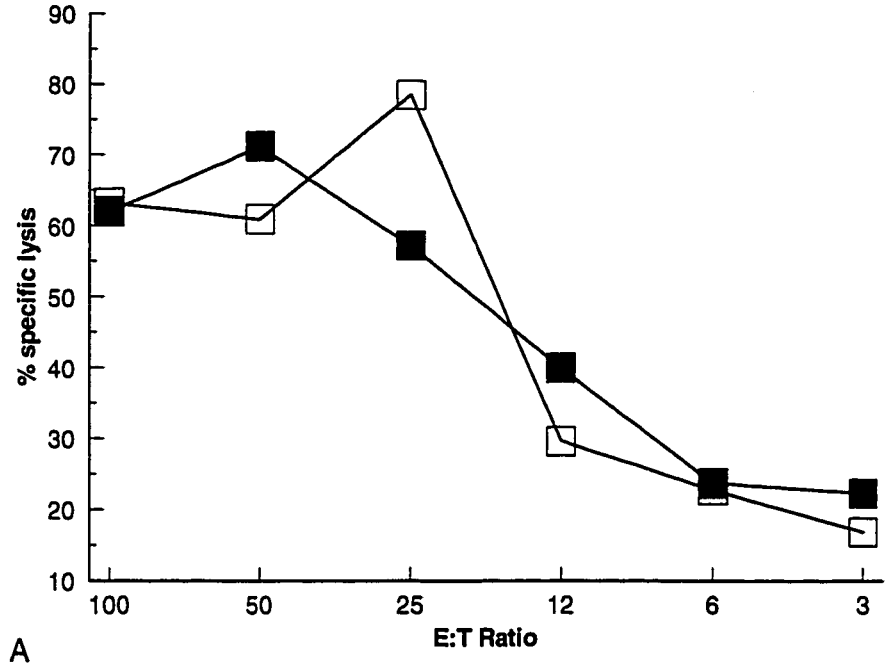
Derivation of T cell lines

T cells were isolated from the lymph nodes and spleens of normal C57BL/6 mice (an NK1.1 positive strain) that had been immunized with RMAS.CD1d1 mitomycin C-treated cells pulsed with p99a peptide or from mice that had been immunized with p99a peptide alone. The p99a peptide was included in the initial protocol since an earlier report indicated that this foreign peptide primed murine CD1d1 restricted antigen-specific cytolytic T cells (28). Despite the inclusion of p99a peptide in the immunization protocol and in the primary culture, none of the T cell lines derived showed specificity for the peptide. Instead, T cell lines recognized CD1d1 bearing APCs in the absence of exogenous antigen. In a representative experiment, Line 24 was able to specifically lyse RMAS.CD1d1 targets with an efficiency of 40-60% (Fig. 2A). Preincubation of the target cells with 10 μM p99a antigen did not affect the degree of lysis indicating that T cell recognition of the target cell was not antigen dependent. Antibody blocking experiments illustrated that T cell cytotoxicity of $CD1d1^+$ target cells could be inhibited from 60-70% specific lysis to 23-40% with monoclonal antibodies specific for CD1d1. In contrast, isotype control antibodies IgG2a (rat) and 38-4.5 (hamster) along with antibodies against MHC class I, MHC class II, CD4 and CD8 did not appreciably diminish cytolysis (Fig. 2B).

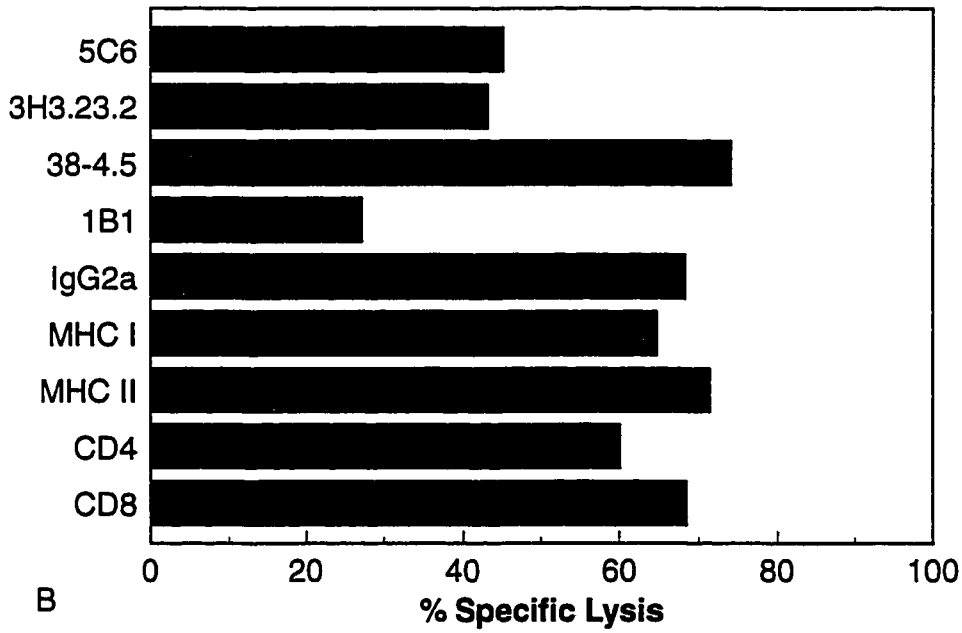
Proliferation as measured by $[^3H]$ thymidine incorporation yielded similar results. For example, T cells from lines 14DND8, 24.CD8.1 and 24.CD8.2

FIGURE 2: Cytolysis of CD1$^+$ APCs by αβ T cell lines

A) RMA-S cells stably transfected with murine CD1 cDNAs were labeled with ^{51}Cr for 2h either with 10 μM p99a peptide □; or without p99a peptide ■; and were used as targets in a standard 4h cytotoxicity assay. Line 24 T cells were added to 2000 ^{51}Cr-labeled RMAS.CD1d1 target cells at the indicated E:T ratios. B) The 14S T cell line was incubated with 2000 ^{51}Cr-labeled RMAS.CD1d1 target cells at E:T=50:1 in the presence of the indicated antibodies at 20 μg/ml in a standard 4h ^{51}Cr release assay. Only the CD1d1 specific antibodies, 5C6, 3H3.23.2 (both hamster) and 1B1 (rat), inhibited specific lysis. Neither isotype control antibody [IgG2a (rat), 38-4.5 (hamster)] nor antibodies specific for murine MHC class I, MHC class II, CD4 or CD8α inhibited cell lysis. Similar results were obtained with the other CD1-restricted T cell lines. Results are representative of at least three experiments.


90
80
70
60
50
40
30
20
10
% specific lysis
100
50
25
12
6
3
E:T Ratio

A


5C6
3H3.23.2
38-4.5
1B1
IgG2a
MHC I
MHC II
CD4
CD8
0
20
40
60
80
100
% Specific Lysis

B

proliferated vigorously in response to RMAS.CD1d1 APCs. When this response was compared to the proliferative response to the non-transfected RMA-S cells, a 2-3 log difference was seen (Fig. 3A). In order to rule out that the T cells were recognizing a clonal variation in the target RMA-S population unrelated to CD1, a second set of CD1d1 transfectants was used. T cells from line 14DND8 proliferated only when cultured with L cell fibroblasts transfected with CD1d1 (L.CD1d1) confirming CD1d1 specificity (Fig. 3B).

By the third restimulation, the T cell lines were found to be heterogeneous containing predominantly $CD8^+$ and DN T cells with some $CD4^+$ T cells. This heterogeneity may in part have explained the incomplete blockade of cytolysis by monoclonal antibodies. Hence, further functional experiments were conducted on T cell clones derived from these lines to determine if reactivity to CD1d1 was dependent on co-receptor expression and to analyze the TCR repertoire.

Functional assessment of murine T cell clones

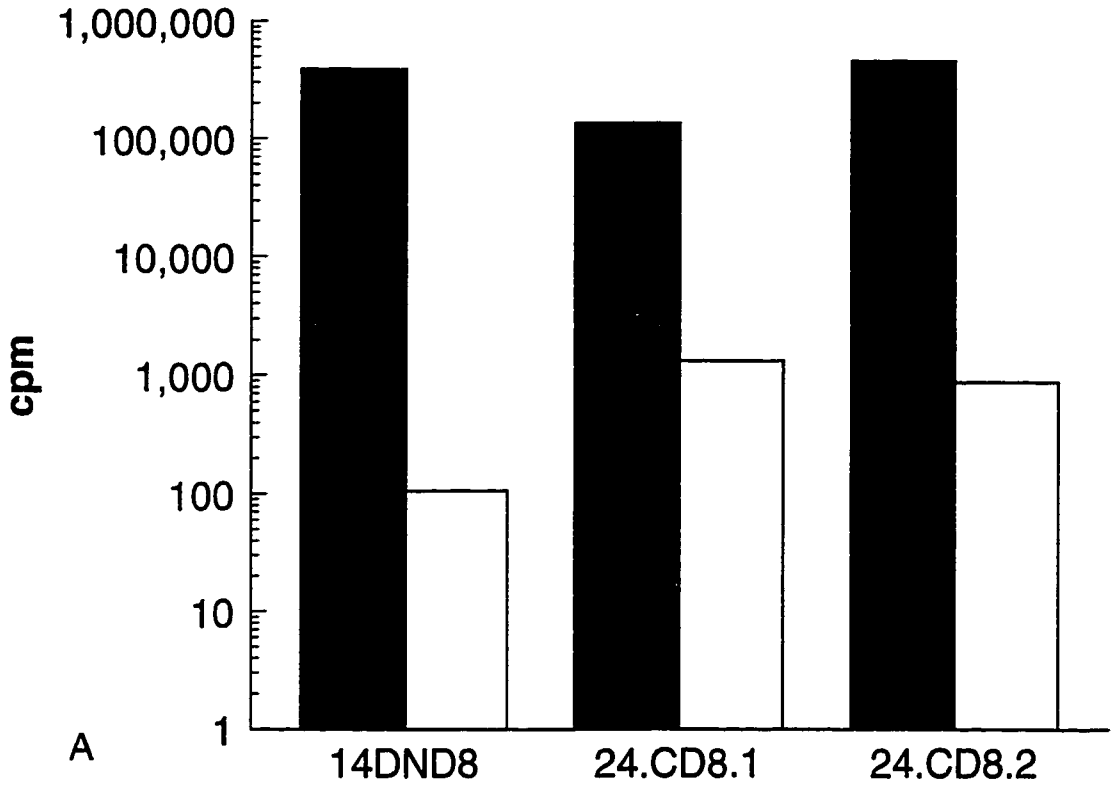
T cell clones were derived by limiting dilution and a panel of 10 clones was analyzed in depth. All of the clones expressed an αβ TCR, CD28 and were either double negative (7/10) or expressed CD8αα homodimer (3/10). Interestingly, although C57BL/6 mice are an NK1.1 positive mouse strain, neither the T cell lines nor clones expressed NK1.1 lineage markers as detected by specific monoclonal antibodies to NK1.1 (PK136), NKR-1PA or NKR-1Pb (recognized by 10A7 mAb), nor Ly-49C. This suggests that the T cell clones derived from these normal mice, were distinct from the NK T cell subset previously described.

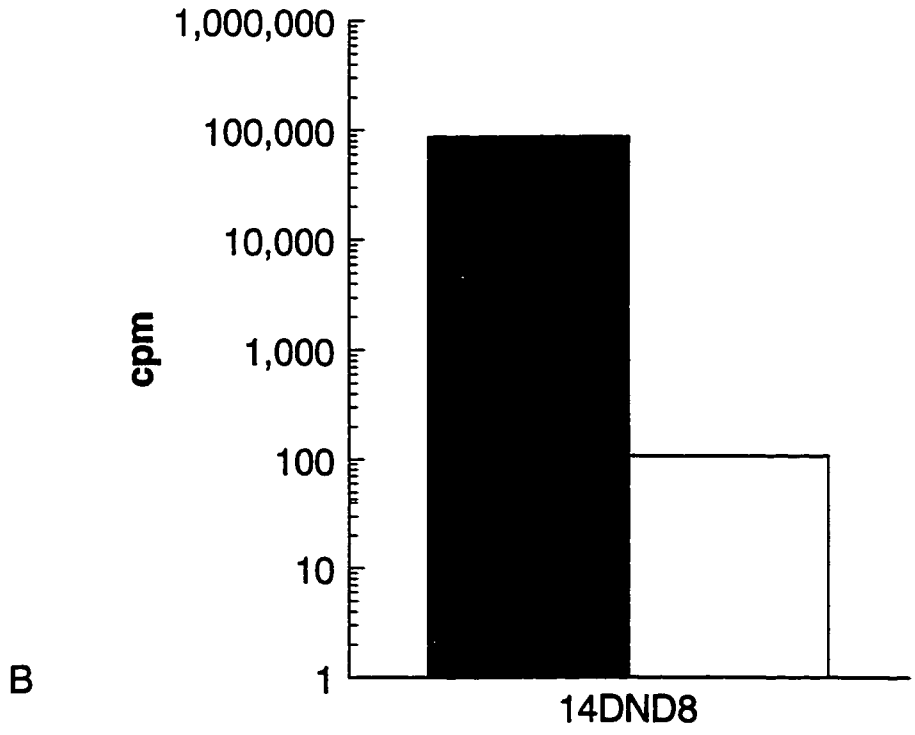
Like the parent lines, the T cell clones were efficient cytolytic cells able to

FIGURE 3: Recognition of CD1d1 by T cell lines

A) RMAS.CD1d1 ■; and non-transfected RMA-S cells □; cells were treated with mitomycin C and plated in triplicate at 10^5/well. T cells from lines 14DND8, 24.CD8.1 and 24.CD8.2 were added to the wells at $5X10^4$/well. No antigen was used in the assay. [^{3}H] thymidine was added to the cultures for the final 6h. The T cells proliferated only in the presence to the $CD1^+$ APCs.

B) In a similar assay, L.CD1d1 ■; and non-transfected L cells □; were used as another pair of APCs in the proliferation assay. T cells from 14DND8 proliferated with L.CD1d1 cells but not when cultured with the non-transfected L cells. Results are representative of three experiments.


1,000,000
100,000
10,000
1,000
100
10
1
cpm
A
14DND8
24.CD8.1
24.CD8.2


1,000,000
100,000
10,000
1,000
100
10
1
cpm
B
14DND8

recognize $CD1^+$ targets in the absence of added antigen but did not lyse the untransfected targets (Fig.4). The clones varied in their lytic efficiency, but consistently the RMAS.CD1d1 target cell was killed without the addition of antigen. This effect was titratable with varying E:T ratios. For example, the T cell clones 24.7 and 24.8 at E:T 40:1 lysed 25-35% of RMAS.CD1d but only 5% of the untransfected RMA-S targets (Fig. 4A and 4B). Clone 14S3, at the same E:T ratio 40:1, lysed 90% of the RMAS.CD1d target cells and <20% of the untransfected RMA-S cells (Fig. 4C). To confirm the specificity of the interaction, monoclonal antibody blocking was used demonstrating that only CD1d1 specific antibodies could inhibit cell lysis (Fig. 5). The rat anti-CD1d1 antibody 1B1 virtually abolished lysis by clone 14S10 and the hamster 5C6 and 3H3.23.2 anti-CD1d1 antibodies inhibited lysis by 60%.

Proliferation assays were also used as a measure of CD1d1 restricted activity of the T cell clones. T cells proliferated only when cultured with $CD1^+$APCs in the absence of antigen. This proliferative response was striking in some experiments with stimulation indices of up to 1000 comparing the ratio of T cells proliferating in the presence of RMAS.CD1d1 to T cells cultured with untransfected RMA-S. Antibody blocking studies confirmed that this reactivity was CD1d1 specific. Only monoclonal antibodies 1B1 (rat), 3H3.23.2 and 5C6.4 (both hamster), all specific for CD1d1 blocked proliferation whereas the antibody controls to MHC class I, MHC class II, CD4 and CD8 did not inhibit recognition (Fig. 6). The effect was dramatic and nearly complete.

FIGURE 4: T cell clones specifically lyse CD1$^+$APCs.

Untransfected RMA-S □; or RMAS.CD1d1 ■; cells were labeled with ^{51}Cr for 2h and used as target cells in a standard 4h cytotoxicity assay. T cell clones 24.7 (A), 24.8 (B) and 14S3 (C) were incubated with 2000 target cells at the indicated E:T ratios in the absence of added antigen. The lysis profile is similar for the other T cell clones. These results are representative of two to four experiments.

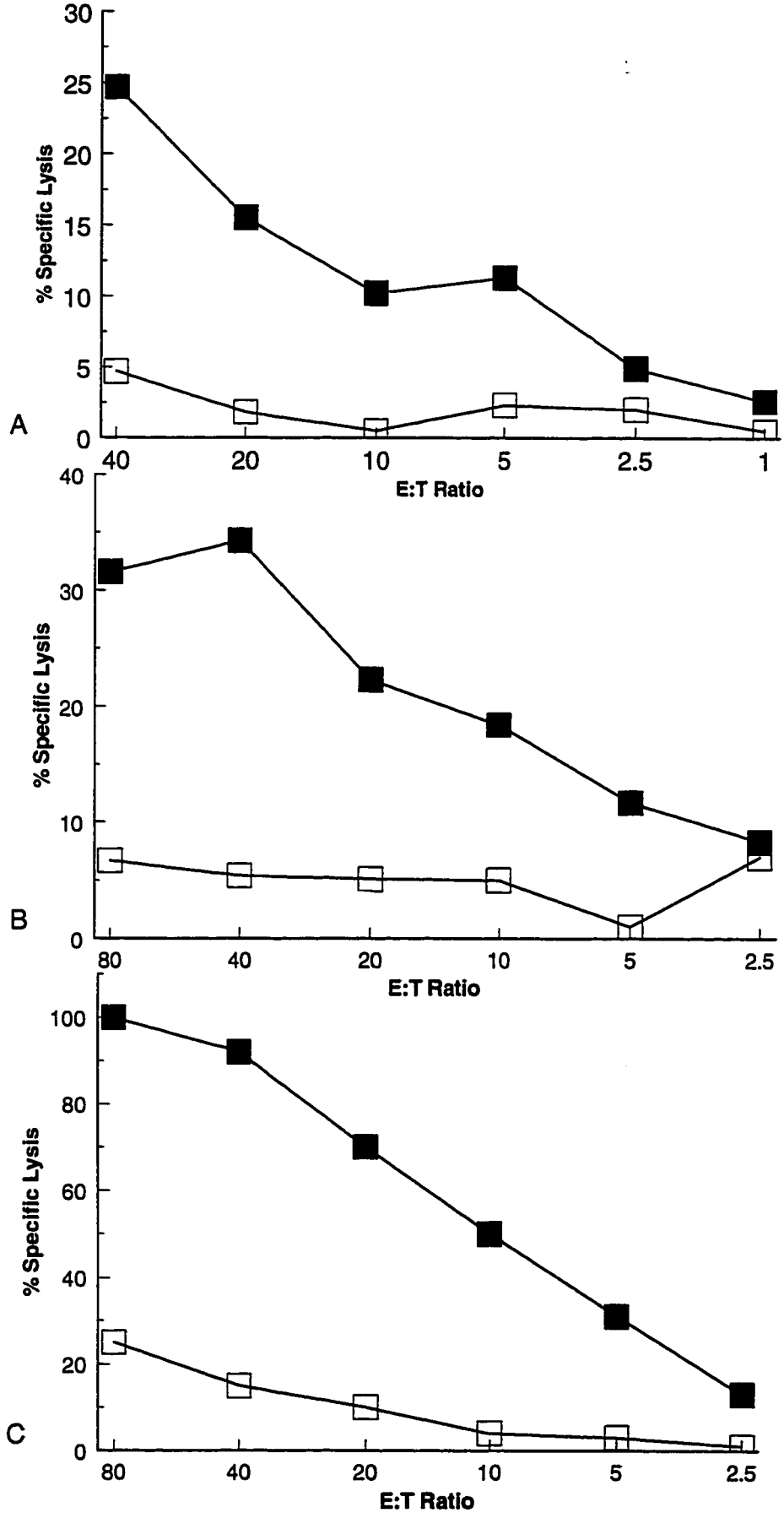

A
% Specific Lysis
30
25
20
15
10
5
0
40
20
10
5
2.5
1
E:T Ratio
B
% Specific Lysis
40
30
20
10
0
80
40
20
10
5
2.5
E:T Ratio
C
% Specific Lysis
100
80
60
40
20
0
80
40
20
10
5
2.5
E:T Ratio

FIGURE 5: Cytolysis of $CD1^+$ APCs is inhibited by CD1 antibodies

CD1d1 restricted cytolysis by T cell clones is inhibited in the presence of monoclonal antibodies specific for CD1. In a typical experiment, 14S10 T cells were incubated for 4 hours with 2000 ^{51}Cr-labeled RMAS.CD1d1 targets at E:T=10:1 in the presence of the antibodies indicated in the figure at 20 μg/ml. Only the CD1d1 specific antibodies, 5C6, 3H3.23.2 (both hamster) and 1B1 (rat), inhibited specific lysis. Isotype control antibodies IgG2a (rat), 38-4.5 (hamster) and antibodies to MHC class I, MHC class II, CD4 and CD8α did not alter the degree of lysis by the T cells.

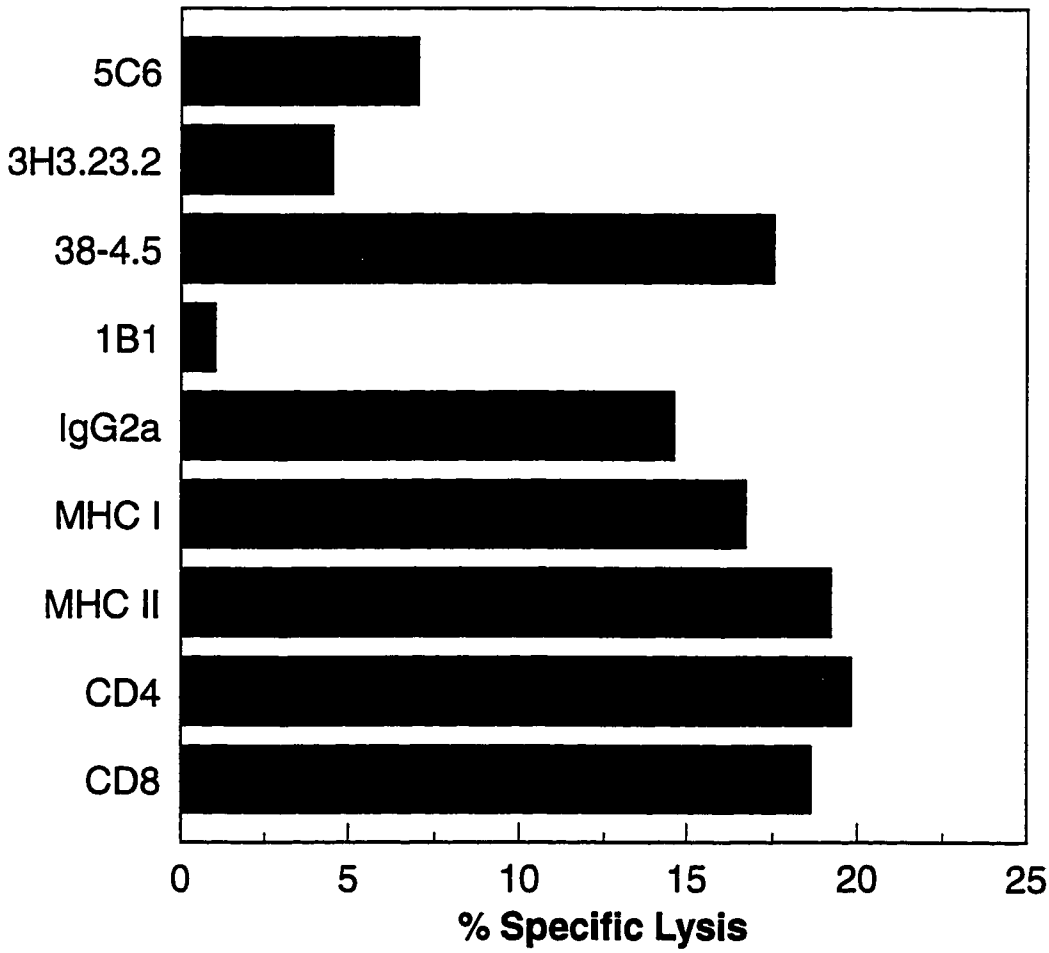
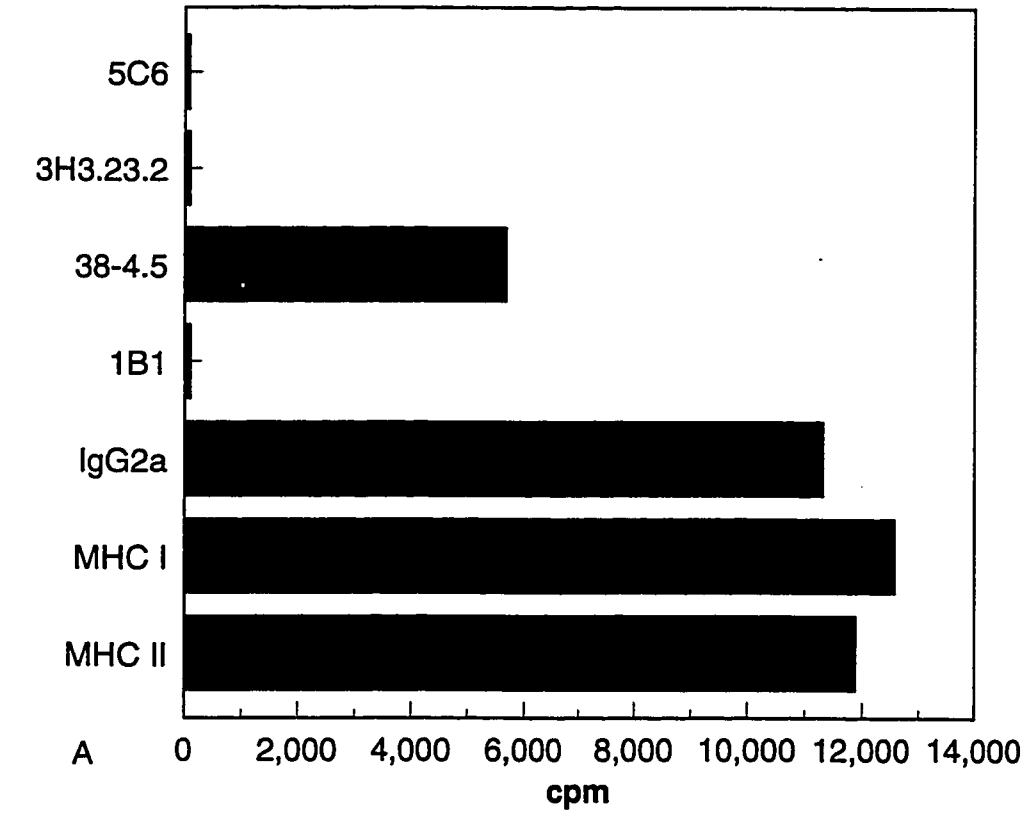
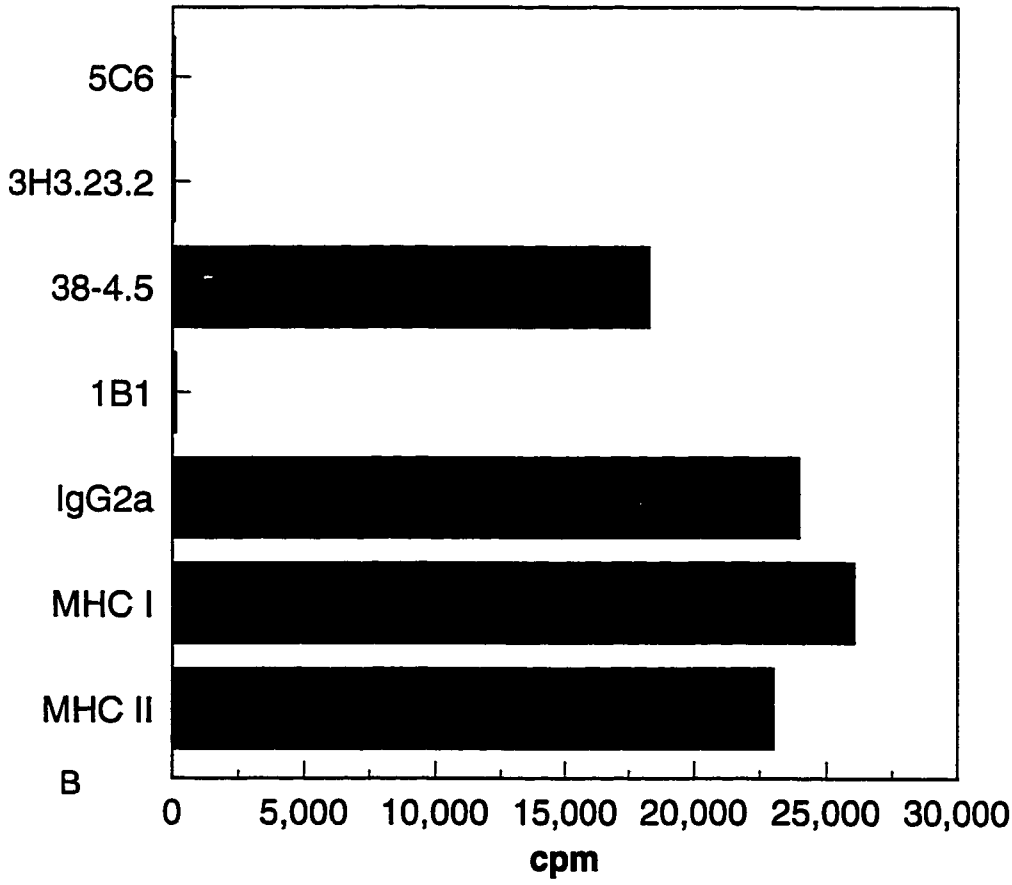

5C6
3H3.23.2
38-4.5
1B1
IgG2a
MHC I
MHC II
CD4
CD8
0
5
10
15
20
25
% Specific Lysis

FIGURE 6: CD1d1 recognition by T cell clones through proliferation.

Clones 24.7 (A) and 24.8 (B) were co-cultured with mitomycin C-treated RMAS.CD1d1 APCs at T:APC=1:2 in the presence of the indicated antibodies at a final concentration of 20 μg/ml. The assay was harvested on day 3 following a 6h pulse with 1μCi/well of [^{3}H] thymidine. The presence of anti-CD1d1 monoclonal antibodies 1B1 (rat), 5C6 and 3H2.23.2 (hamster) dramatically reduced the proliferative responses of the clones.


5C6
3H3.23.2
38-4.5
1B1
IgG2a
MHC I
MHC II
A
0
2,000
4,000
6,000
8,000
10,000
12,000
14,000
cpm


5C6
3H3.23.2
38-4.5
1B1
IgG2a
MHC I
MHC II
B
0
5,000
10,000
15,000
20,000
25,000
30,000
cpm

Cytokine analysis

Since NK T cells are capable of producing Th1 and Th2 type cytokines, the cytokine profile of these directly reactive CD1d1 T cell clones was assessed for comparison. Upon stimulation with $CD1^+$APCs, all of the T cell clones tested produced significant amounts of IFNγ, >80,000 pg/10^6 T cells with the exception of clone 24.8. Similarly, IL-10 production ranged from 8400-22,000 pg/10^6 T cells (Table I). Of note, neither the RMA-S cells nor the RMA-S transfectant RMAS.CD1d1 cells produced any detectable cytokine in the absence of T cells.

TABLE I **Cytokine profile of CD1d1 restricted αβ T cell clones**

T cell clones	IL-2		IL-4		IFNγ		IL-10	
	CD1	NT	CD1	NT	CD1	NT	CD1	NT
14S3	ND	ND	510	ND	>80000	ND	14,235	ND
14S4	ND	ND	2838	ND	>80000	ND	11,877	ND
14S7	ND	ND	2267	ND	>80000	702	21,347	ND
14S10	7321	ND	1931	ND	>80000	95	21,846	ND
24.7	16,927	ND	ND	ND	>80000	ND	8482	ND
24.8	3881	ND	ND	ND	13,539	4860	166	ND

T cells were co-cultured with RMAS.CD1d1 **[CD1]** or with non-transfected **[NT]** RMA-S cells at T:APC=1:2 without antigen. Supernatants were sampled at 48h following stimulation and frozen at -20°C until cytokine determination by ELISA. Results are shown as pg/10^6 T cells/ml. ND= not detected.

IL-4 production by the T cell lines initially exceeded 10,000 pg/10^6 T cells but with further propagation in culture, the production of IL-4 diminished. Analysis of the clones confirmed this variability in IL-4 production. Clones 24.7 and 24.8 did not produce any detectable IL-4. In contrast, only clones 14S.10, 24.7 and 24.8 produced IL-2. For comparison, clone AE7 (40), a $CD4^+$, pigeon cytochrome *c*

specific T cell clone, produced only IL-2 and IFNγ when stimulated with irradiated splenocytes and pigeon cytochrome *c*, a pattern typical of Th1 polarized T cells. In summary, the cytokine phenotype of these directly reactive CD1d1 T cell clones is unusual and suggests that these T cells may play a critical role in the regulation of the immune response.

Analyze the T cell receptor usage of CD1d1 directly reactive T cells

In order to gain more insight into the nature of the TCR diversity for CD1d1 directly reactive T cells, TCR analysis was performed (Table II).

TABLE II V and J gene usage of CD1d1 directly reactive T cell clones

T cell clones	Phenotype	Vα	Jα	Vβ
24.7	DN	AV14S1	281	BV6S1
24.8	DN	AV14S1	281	BV8S2A2
24.9	DN	AV14S1	281	BV8S3
14S3	DN	AV22S1	NEW.02	BV8S2A1
14S4	DN	AV22S1	NEW.02	BV8S2A1
14S7	DN	AV22S1	NEW.02	BV8S2A1
14S10	CD8αα	AV11S3	NEW.15	BV8S1
14S11	CD8αα	AV11S1	19	BV8S2A3
14S15	DN	AV10S[2/9]	TA65	BV5S1
14S6	CD8αα	AV17S1	TT11	BV14S1

Gene sequences were determined comparing the sequences to the NCBI database using the BLAST algorithm. Nomenclature for the Vα and Vβ family members is based on the WHO-IUIS recommendations.

Three of the ten clones analyzed 24.7, 24.8 and 24.9, all used the canonical Vα14, Jα281 rearrangement as found on most NK T cells. Inverse PCR followed by direct sequencing was required to determine the TCR Vα usage of the

remaining seven T cell clones (41). Three clones, 14S.3, 14S.4 and 14S.7 had identical Vα sequences encoded by Vα15 rearranged to JαNew.02. Clones 14S.10 and 14S.11 used two members of the Vα11 family with two separate joining sequences JαNew.15 and Jα19 respectively. Clone 14S.15 was encoded by Vα10 and used yet another joining segment JαTA65. PCR amplification of 14S.6 revealed two Vα products and upon further subcloning and sequencing, it was determined that Vα17 was paired with JαTT11.

With this unexpected diversity in the Vα gene usage of the TCR, we then examined the Vβ genes by RT-PCR followed by sequencing. Seventy percent of the clones analyzed including 14S.3, 14S.4, 14S.7, 14S.10, 24.8, 24.9 and 14S11 all used a member of the Vβ8 family (BV8S2A1, BV8S1, BV8S2A2, BV8S3 and BV8S2A3). The remaining three clones 24.7, 14S15 and 14S.6 used genes from Vβ5, Vβ6 and Vβ14 families (Table II). Thus, despite the diversity demonstrated at the Vα locus, there was relative consistency of Vβ gene usage suggesting that the recognition of CD1d1 may depend on interacting residues of the TCR Vβ8 chain.

CHAPTER 4: EVALUATION OF γδ T CELL RESPONSES TO CD1d1

INTRODUCTION

γδ T cells represent a separate lineage of T cells that do not transcribe the αβ TCR genes. Accumulating evidence suggests that γδ T cells can be divided into subsets that differ in respect to their origin, V gene usage, tissue localization and specialized function (42). Overlapping waves of γδ TCR^+ cells appear in fetal thymus. The initial wave of cells express Vγ3 and Vγ4 paired with Vδ1/Dδ2/Jδ2 genes. Surprisingly, there is no diversity in the junctions of these early γδ T cells. These cells are later replaced with Vγ2 and Vγ1 bearing TCRs using a variety of Vδ genes. In the adult mouse, Vγ3 expressing cells with invariant junctions are localized to the skin while the $V\gamma4^+$ T cells are restricted to the tongue and female reproductive tract. Vγ2 and $V\gamma1^+$ cells with diverse TCRs are found in lymphoid tissues and blood. Intestinal intraepithelial (IEL) γδ T cells appear to develop extrathymically and predominantly express Vγ5 with diverse Vδ genes (43).

γδ T cells in microbial infection

In the periphery, γδ T cells comprise <10% of the T cells. However, γδ T cells are highly concentrated in the intestinal epithelium of mice and are the major T cell lineage in the skin epithelia (42). Despite extensive study, γδ T cells have yet to be assigned any general functions. Striking clues as to the importance of these cells have arisen from investigations in mice lacking either αβ or γδ T cells. In several infection models tested, including *Listeria monocytogenes* (44-46), *Leishmania* (47), *Mycobacteria* (46), *Salmonella* (48)

and *Plasmodium* (49), γδ T cells clearly mediate a protective immune response. γδ T cells are often referred to as the "first line of defense" which in part relates to the qualitative roles observed for γδ T cells. In *Listeria* infection for example, γδ T cells are responsible for decreased bacterial growth in the early phase of infection. The absence of γδ T cells results in enlarged liver lesions with abscess formation, an effect not observed in mice lacking αβ T cells (44).

Antigen independent functions of γδ T cells

Several other functions have been ascribed to γδ T cells that appear to be independent of antigen recognition. γδ T cells can regulate αβ T cells, promote isotype switching in B cells (50), produce a tissue-specific growth factor (51) and regulate epithelial cell development (52). Anti-tumour effects have been observed for a TCRγ transgene. In a mouse T cell leukemia model, the presence of TCRVγ1.1Jγ4Cγ4 transgene protected the mice against the development of spontaneous leukemia (53).

Antigen recognition by γδ T cells

What is clear, is that γδ T cells have different requirements for antigen recognition. Unlike αβ T cells, antigen processing and MHC molecules are not required (54). Uniquely, human Vγ2/Vδ2$^+$ T cells can respond to phosphate-containing non-peptide ligands such as isopentenyl pyrophosphate in the absence of a presenting element or processing (55). These pyrophosphate components are present in normal mammalian cells as precursors in lipid metabolism and are present in the cell wall of mycobacteria. However, the biological significance of this reactivity remains to be determined.

CD1 recognition by human γδ T cells

Intriguing human data however, suggest that murine γδ T cells may have evolved the ability to recognize CD1d1 molecules. The major circulating pool of human γδ T cells express the Vγ2Vδ2 TCR and have been shown to recognize phosphate non-peptide ligands (55). In contrast, the majority of γδ T cells within tissues express Vγ1Vδ1 TCR. Recently, a major reactivity of this subset of cells was identified as the CD1 molecules. Specifically, Vγ1Vδ1 TCR^+ cells recognize both CD1a and CD1c directly in the absence of exogenous antigen. These cytolytic T cells utilize both Fas/Fas ligand interactions and perforin/granulysin mediated pathways to lyse their targets (18, F Spada, M Sugita, MB Brenner, unpublished data). An antigen present in culture media or a self-lipid antigen has not strictly been ruled out. Additional preliminary experiments also indicate that a human γδ T cell clone recognizes mycobacterial (MTb) derived antigen presented by the CD1c molecule (D Leslie, MB Brenner unpublished data).

Two well characterized murine γδ T cell clones, LBK5 and G8, have been shown to recognize the mouse class II MHC molecule $I\text{-}E^k$ and the non-classical MHC class I molecules T10 and T2 respectively (54,56). Both clones recognize these protein antigens directly in the absence of antigen processing.

SPECIFIC AIMS:

We hypothesized that given the human examples of both direct and antigen specific γδ T cell recognition of CD1 molecules, together with the known murine examples of γδ TCR recognition of both classical and non-classical

antigen presenting molecules, murine γδ T cells could also manifest recognition of a third antigen presenting molecule, CD1d1.

AIM 1: Is there a subset of γδ T cells that can recognize CD1d1?

Are γδ CD1d1 directly reactive T cells limited to certain anatomical locations?

What is the V gene usage of CD1d1 directly reactive γδ TCRs?

AIM 2: What is the phenotype of γδ T cells that recognize CD1d1?

AIM 3: Does CD1d1 present foreign antigen to γδ T cells?

MATERIALS AND METHODS

Derivation of γδ T cell lines

TCR β chain gene knockout ($TCR\beta^{-/-}$) mice, 4-10 weeks of age, on a C57BL/6 background (Jackson Laboratory, Bar Harbor, ME) were used as a pure source of γδ T cells. These mice have a targeted mutation of the TCR β chain rendering them deficient in αβ T cells.

To generate CD1d1 directly reactive γδ T cell lines, the approach described in chapter 2 was used. $TCR\beta^{-/-}$ mice were repeatedly immunized using mitomycin C-treated, RMAS.CD1d1 cells injected i.m. into the thigh. The p99a peptide was not used in this protocol. At the same time, emulsified CFA and subsequently IFA was injected s.c. into three sites. There were three immunizations in total each ten days apart. Complete media was supplemented with 5 ng/ml murine IL-7 (Genzyme, Cambridge, MA) as a growth factor for γδ T cells.

Lymphoid tissues were removed from the mice following euthanasia by CO_2 asphyxiation. Nylon wool columns (Polysciences Inc., Warrington, PA) were used as per the manufacturer's protocol to enrich for T cells that were then set up in primary culture with RMAS.CD1d1 cells as the APC to derive CD1d1 directly reactive γδ T cells. The T:APC ratio used for the initial and subsequent restimulations was 1:2. No antigen was added to the cultures. Reactivity to CD1d1 was assessed using proliferation assays as described in chapter 2 on either the second or third restimulation depending on the number of viable T cells.

A second protocol was used to generate antigen specific T cells. Crude mycobacterial tuberculosis sonicate (MTb-H37RA Difco, Detroit, MI), prepared by adding 5 ml distilled water to 1 vial of MTb-H37RA and sonicating for 5 minutes on ice, was pulsed overnight at 37°C onto RMAS.CD1d1 cells at a final concentration of 5 μg/ml. The RMAS.CD1d1 cells were then mitomycin C-treated and injected i.m. into the thigh of $TCR\beta^{-/-}$ mice. Simultaneously, crude MTb sonicate was emulsified in either CFA or IFA and injected s.c.. There were a total of three immunizations 10 days apart. Antigen specific CD1d1 reactivity was assessed in these mice by preparing splenic T cells and plating the cells directly in a proliferation assay using RMAS.CD1d1 and RMA-S as APCs with increasing amounts of MTb antigen.

To examine for CD1d1 direct reactivity in the gut, small intestine intraepithelial lymphocytes (IEL) were isolated as previously described (57) from unimmunized $TCR\beta^{-/-}$ mice. The IEL were directly plated in triplicate for a proliferation assay using RMAS.CD1d1 and RMA-S as APCs and increasing amounts of IL-2 in the absence of antigen.

Production of T cell hybridomas

Seven to ten days following stimulation of the T cell lines, growing blasts were fused to the BW5147 $TCR\alpha^{-}\beta^{-}$ thymoma fusion partner generously provided by Dr. Willi Born (58) at a 2:1 ratio in 0.5 ml of 50% polyethylene glycol as previously described (59). The cells were then distributed in 96-well flat-bottomed plates with HAT medium and screened by FACS with the anti-CD3 antibody (clone 500A2, PharMingen) for a productive rearrangement of the TCR.

RESULTS

γδ T cell lines

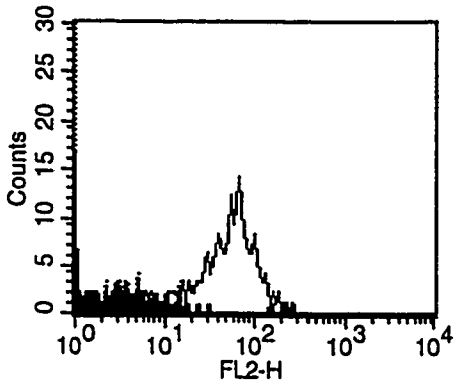
Several γδ T cell lines were generated by isolating T cells from TCRβ$^{-/-}$ mice that had been immunized with RMAS.CD1d1 cells and adjuvant. FACS analysis confirmed that the T cells uniformly expressed the γδ TCR (Fig. 7). To screen the T cell lines for CD1d1 restricted activity, a similar approach was used to the one that identified directly CD1d1 reactive αβ T cells. Prior to the second or third restimulation with RMAS.CD1d1 APCs, proliferation assays were performed with rested T cells comparing RMAS.CD1d1 as the APC to untransfected RMA-S cells. However, unlike the αβ T cells lines generated, the γδ T cells showed little discrimination between the APCs. In a representative experiment, γδ T cells from lines 1.1, 1.3M and 1.3S when cultured with RMAS.CD1d1 APCs, proliferated at a slightly higher rate than T cells cultured with the untransfected RMA-S cells (Fig. 8A). The proliferative response was weak and not significantly above the baseline proliferation of the APCs themselves.

The proliferation of line 1.3S could be augmented by adding exogenous IL-2, but this also increased the proliferative rate of the T cells assayed with the untransfected RMA-S cells (Fig. 8B). These experiments were repeated several times with similar results. In an attempt to isolate T cells that may have a higher reactivity to CD1d1 than the parent line, T cell cloning using limiting dilution in the presence of con A was performed. However, these attempts did not yield any CD1d1 specific T cell clones.

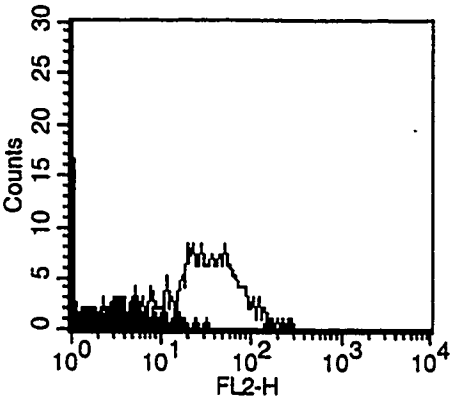
FIGURE 7: FACS profile of γδ T cell lines

FACS analysis was used to determine the TCR expression of the T cell lines derived from TCR $\beta^{-/-}$ mice. T cells from lines 1.1, 1.3S and 1.9S were stained with the GL3 monoclonal antibody (open histogram) and compared with the negative isotype control antibody (filled histogram). All of the positively staining cells were also CD3 positive. PI counterstaining was used to define the viable population within the lymphoid gate.

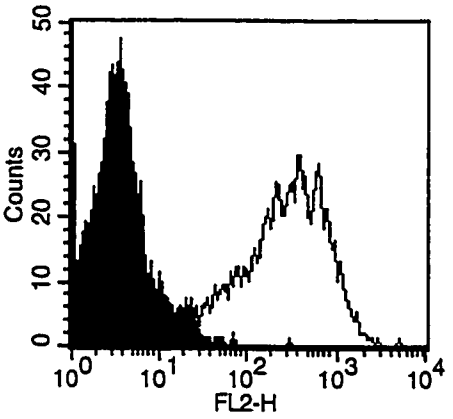
RELATIVE CELL NUMBER



Line 1.1



Line 1.3S

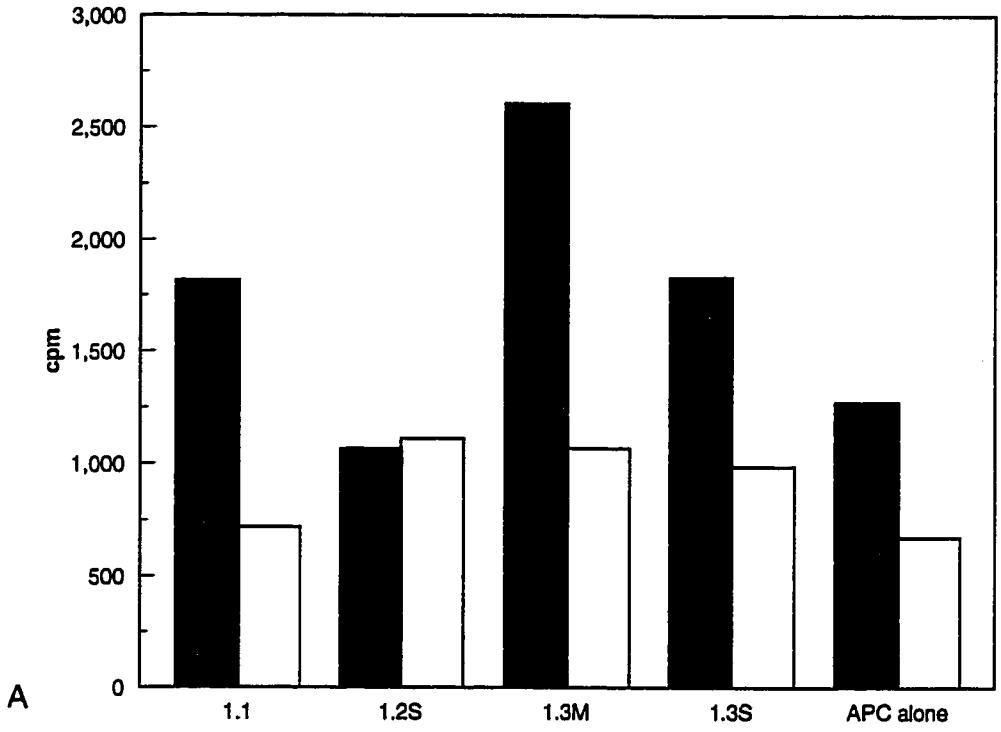


Line 1.9S

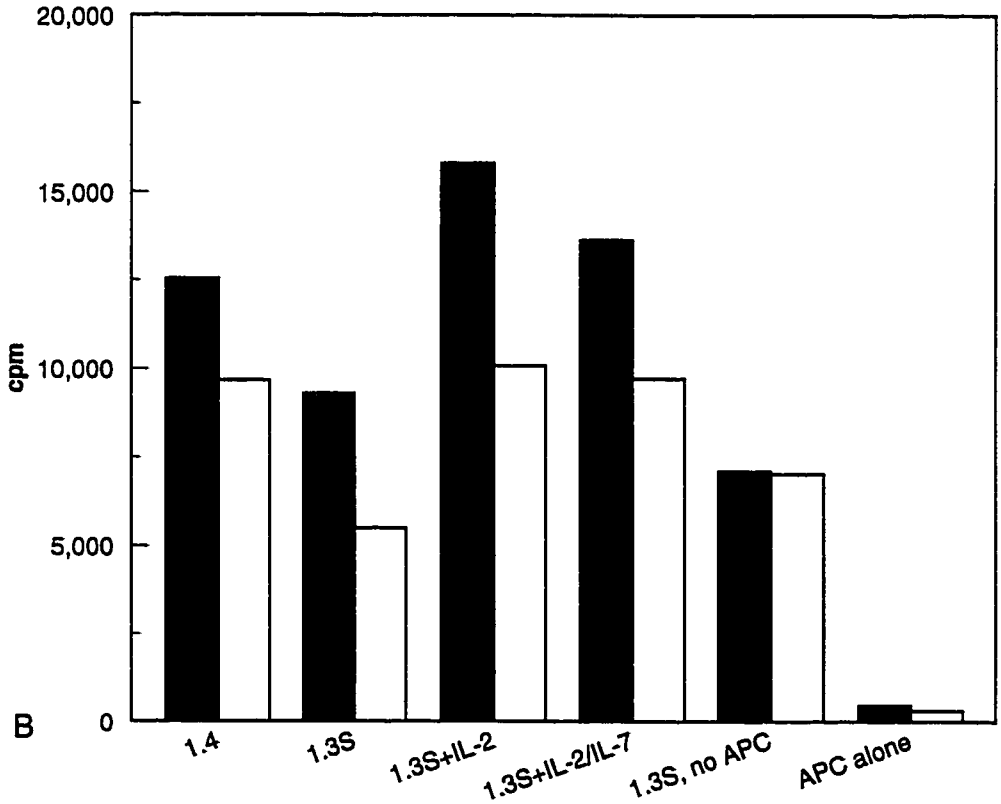
Fluorescence Intensity

FIGURE 8: Proliferation of γδ T cell lines in response to $CD1^+$ APCs.

A) Proliferation assays were done as previously described for figure 3. Lines 1.1, 1.2S, 1.3M and 1.3S were assessed for the ability of the T cells to proliferate in response to RMAS.CD1d1 ■; in comparison to RMA-S APCs □; in the absence of antigen. [^{3}H] thymidine was added for the final 6h of culture. The T cells react weakly above background. B) This proliferation assay compared the ability of lines 1.4 and 1.3S to proliferate in response to RMAS.CD1d1 ■; in contrast to RMA-S APCs □ in the absence of antigen. The effect of supplemental cytokines on γδ T cell proliferation in response to $CD1^+$ APCs was examined by adding 2nM IL-2 ± 5 ng/ml IL-7 to Line 1.3S. Line 1.3S shows an increase in proliferation with the addition of IL-2 to both RMAS.CD1d1 ■; and RMA-S APCs □.


3,000
2,500
2,000
1,500
1,000
500
0
cpm
1.1
1.2S
1.3M
1.3S
APC alone

A


20,000
15,000
10,000
5,000
0
cpm
1.4
1.3S
1.3S+IL-2
1.3S+IL-2/IL-7
1.3S, no APC
APC alone

B

γδ T cell responses to MTb sonicate and CD1

γδ T cells are difficult to grow and maintain so a number of other approaches were then attempted to elucidate CD1d1 recognition by the γδ T cell population. Instead of generating T cells lines, splenic T cells were purified from TCR $\beta^{-/-}$ mice immunized with RMAS.CD1d1 and MTb sonicate and set up directly in a proliferation assay to assess whether CD1d1 restricted responses were present (Fig. 9). MTb sonicate was selected since it had been used successfully as a source of lipid antigen in the human system to derive both αβ and γδ CD1 restricted T cells and could potentially prime CD1 restricted T cells *in vivo*. The T cells proliferated with increasing antigen titration however, the untransfected APCs presented the antigen equally well. In these limited experiments, MTb antigen did not appear to enhance CD1d1 recognition.

CD1d1 restricted responses in IEL

Unlike αβ T cells, γδ T cells have a skewed distribution in the organism dependent on anatomic site. We had geared the initial experiments to isolating peripheral T cells since they were the source of the CD1d1 restricted αβ T cells and were the most readily available. We hypothesized that the splenic γδ T cell population which expresses primarily Vγ2, Vγ1 and diverse Vδ genes has a low avidity for CD1d1. We decided to then examine the γδ T cells abundantly found in the intestine of mice which predominately express Vγ5 and various Vδ elements. IEL were freshly isolated from TCR$\beta^{-/-}$ mice and a proliferation assay was performed comparing the relative abilities of the RMAS.CD1d1 versus the untransfected RMA-S to stimulate CD1 restricted T cell responses (Fig. 10).

FIGURE 9: Proliferation of splenic T cells following immunization with MTb sonicate.

Freshly prepared nylon wool separated T cells, from the spleens of TCR $\beta^{-/-}$ mice that had been immunized with mitomycin C-treated RMAS.CD1d1 in combination with MTb crude sonicate, were plated in a proliferation assay with either RMAS.CD1d1 ■; or RMA-S APCs □ in the presence of increasing amounts of MTb antigen. [^{3}H] thymidine was added for the last 6 hours of this 72h assay.

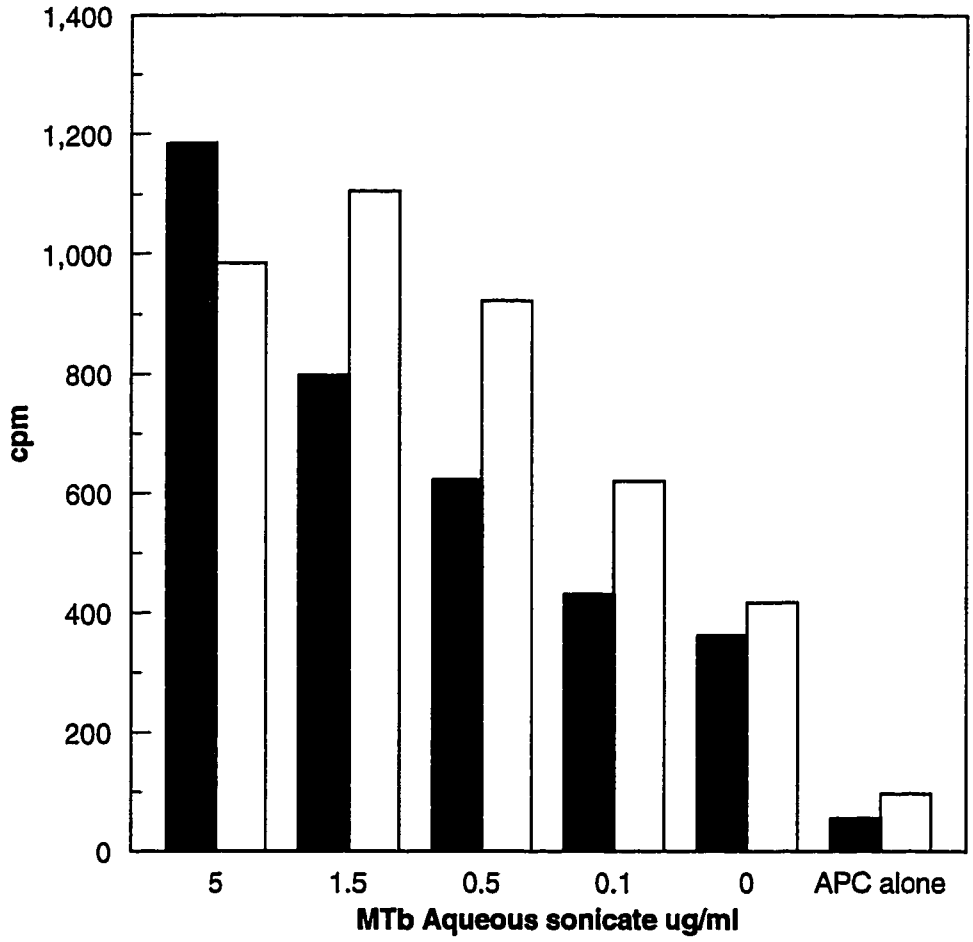
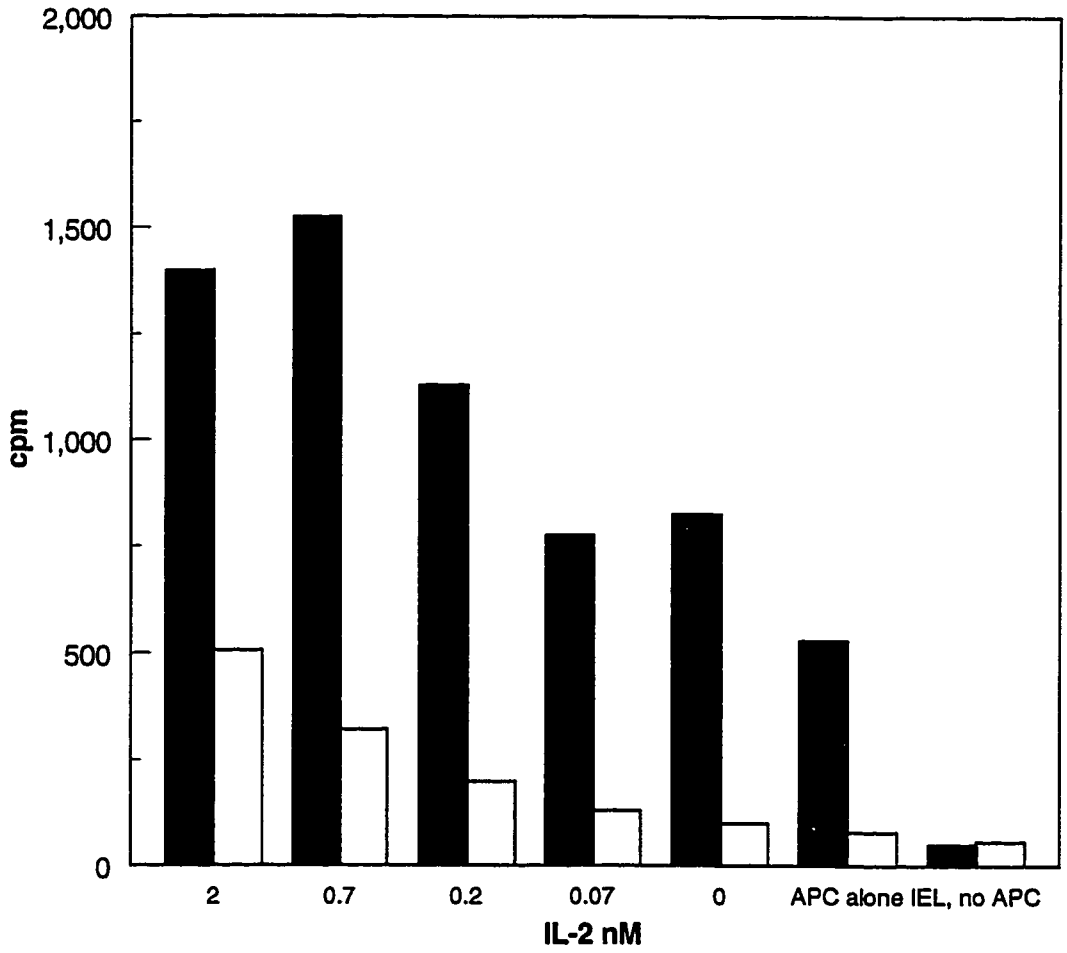

1,400
1,200
1,000
800
600
400
200
0
cpm
5
1.5
0.5
0.1
0
APC alone
MTb Aqueous sonicate ug/ml

FIGURE 10: IEL responses to $CD1^+$ APCs

Freshly isolated IEL from naive TCR $\beta^{-/-}$ mice were co-cultured with RMAS.CD1d1 ■; or RMA-S APCs □; at IEL:APC=1:2 with increasing concentrations of supplemental IL-2 in a proliferation assay. The assay was harvested after 72h of co-culture following a 6h pulse with 1μCi/well of [^{3}H] thymidine.


2,000
1,500
1,000
500
0
cpm
2
0.7
0.2
0.07
0
APC alone IEL, no APC
IL-2 nM

The results confirm that IEL, in general, do not proliferate well. There was a trend toward increasing proliferation with the addition of incremental amounts of IL-2 in response to RMAS.CD1d1 APCs. The IELs that tend to have a memory phenotype were able to distinguish subtle differences between the APCs. Further investigation is needed to determine if this reactivity can be optimized and reproduced.

CHAPTER 5: DISCUSSION

CD1 molecules represent a third distinct lineage of antigen presenting molecules and define a new paradigm for the recognition of non-peptide lipid and glycolipid antigens by T cells. The focus of CD1 mediated T cell recognition in mice has been dominated by NK T cells and their unique immunoregulatory functions. NK T cells co-express NK lineage markers and a canonical αβ TCR consisting of Vα14Jα281 germline encoded gene segments with a limited Vβ repertoire. The ability of NK T cells to produce large amounts of IL-4 upon activation in addition to IFNγ has prompted extensive investigation of these unusual lymphocytes.

The first evidence indicating that additional subsets of murine T cells could recognize CD1 was derived from a T cell hybridoma analysis of the residual $CD4^+$ T cells in MHC class II deficient mice (20). Splenic T cells were found to recognize CD1d1 independently of antigen and were termed "autoreactive". TCR analysis revealed that these cells were not derived from the NK T cell lineage. Subsequently, an analysis of foreign lipid antigen-specific human TCRs capable of recognizing CD1 demonstrated a surprising diversity of the TCR gene usage (5). Transfection of these TCRs into Jurkat cells (a TCR β chain mutant) reconstituted specificity for the lipid antigen along with the CD1 isoform recognized. Including the recent CD1d1 crystal structural analysis, these data suggest that lipids and CD1 molecules form complexes analogous to peptide/MHC complexes that are recognized by TCRs.

We then asked the question whether TCR diversity for CD1d1 recognition exists in the murine repertoire. Unlike previous investigators, we sought to examine the TCR diversity in mice with a normal immune system. By not pre-selecting for NK1.1 positive T cells, we identified a panel of αβ T cell clones that recognized CD1d1 directly in the absence of exogenous antigen. Functional characterization of these T cells revealed that they proliferated specifically in response to $CD1^+$APCs and lysed CD1 bearing target cells efficiently. Since none of the clones expressed the CD8αβ heterodimer, cytolysis was independent of CD8 co-receptor expression. Further analysis showed that these $NK1.1^-$ T cells were capable of producing both Th1 and Th2 type cytokines *in vitro* when stimulated by $CD1^+$APCs. This pattern is similar to NK T cells.

Remarkably, the majority of the clones derived expressed diverse TCRα gene elements. Seventy percent of the T cell clones did not use the Vα14Jα281 invariant receptor, suggesting that the repertoire capable of recognizing CD1d1 is more expansive than previously appreciated. This allows speculation that the universe of antigens presented and recognized by CD1d1 extends beyond the ceramide-like glycolipid antigens thus far identified. The overrepresentation of the Vβ8 TCR chains in this analysis suggests that amino acid residues within the conserved sequences of Vβ8 may be important for CD1d1 recognition. Recently, Chiu et al. have characterized non-Vα14-expressing CD1d autoreactive αβ T cell hybridomas derived from the spleens of MHC class II deficient mice (60). These $NK1.1^-$ T cells expressed a limited diversity in the TCR and secreted both IL-4 and IFN-γ. Interestingly, the $Vα14^+NK1.1^+$ T cells they studied required the

tyrosine based endosomal targeting motif in the cytoplasmic tail of CD1d1 for direct recognition while the Vα14$^-$NK1.1$^-$ T cell population could recognize tail deleted CD1d1. This suggests that the CD1d1 molecule has the ability to load antigen in two different cellular compartments that are sampled separately by MHC class I and MHC class II molecules (60).

None of the T cell lines or clones analyzed here, including clones 24.7, 24.8 and 24.9 which use the Vα14-Jα281 TCR, expressed the NK1.1 receptor. However, a recent report has shown that even limited short-term *in vitro* culture induces loss of expression of the NK1.1 receptor from the cell's surface (61). The mechanism is unknown but implies that the mechanism of CD1d1 recognition is independent of NK1.1 surface expression.

Although the T cell clones were shown to recognize CD1$^+$APCs in the absence of added antigen, it is highly likely that an endogenous self-lipid or an antigen present in the culture media may be being recognized. This hypothesis is supported by the crystal structure of murine CD1d1 that suggests that the putative antigen-binding groove is not empty but contains a single acyl chain (16). Furthermore, the discovery of non-peptide ceramide antigens as CD1d1 ligands strengthens this postulate that CD1d1 presents lipid antigen in a manner similar to the group I CD1 molecules (29,30). α-galactosylceramides closely resemble sphingolipids such as glucocerebroside and other gangliosides, the major structural components of the brain, and raise the possibility that these endogenous lipids may be among the naturally occurring CD1d1 ligands in humans. In combination with the other correlative data linking NK T cells to

autoimmune disease, this raises the exciting question of whether CD1 presentation of myelin derived lipids becomes altered or dysregulated in multiple sclerosis. Many tumour antigens contain glycolipid epitopes including gangliosides and could represent another instance of the importance of the cell mediated immune response to non-peptide antigen.

The findings presented here are significant for several reasons. Because these responses were generated in normal mice as opposed to MHC class II deficient animals that have an unusual residual CD4 population, this analysis is reflective of the T cells normally present in the lymphoid organs. Yet, little is known as to the function of this complementary population of CD1d1 restricted T cells. In the murine IL-12 tumour model described, the deletion of Vα14Jα281 T cells led to tumour dissemination (3). The diverse CD1d1 restricted population of cells would still be present in these mice suggesting that although the two populations of CD1d1 T cells produce similar cytokines, they are activated or anergized differently and serve distinct functions *in vivo*. One hypothesis is that the Vα14Jα281 T cells having pre-formed cytokines, in particular IL-4, may function as part of the early or innate host immune response and perform critical immunoregulatory functions. The diverse population of T cells that recognize CD1d1 may have evolved as part of the adaptive immune response with the ability to recognize diverse non-peptide antigens. The analysis performed by Chiu et al. supports this hypothesis that the invariant T cells and diverse T cells have different functions. Since T cells with the invariant TCR can only recognize full-length CD1d1, these T cells will sample antigen loaded in the endosome,

while T cells expressing diverse TCRs can recognize tail deleted CD1d1 and presumably, antigen loaded in other cellular compartments (60). By developing an integrated understanding of how these distinct populations of CD1d1 restricted T cells are activated and regulated, several therapeutic potentials may be realized.

Investigation of γδ T cell reactivity to CD1 suggests that there may be some modest recognition of CD1d1 but the data at present are unconvincing. In summary, immunization of TCR $\beta^{-/-}$ mice with CD1d1^{+} transfectants did not yield CD1d1 specific splenic γδ T cells. Further propagation of these peripheral T cells *in vitro* to generate lines did not enhance CD1d1 reactivity. The slight trend in the T cell proliferation in the gut suggests that this population of Vγ5 bearing T cells may represent a subset of γδ T cells on which to focus for CD1d1 reactivity. Given that the IEL are localized to the intestinal mucosa, CD1d1 reactivity in this population may have significant implications for mucosal immunity.

Most troublesome in the γδ T cell analysis was that the untransfected RMA-S APCs also supported proliferation. Subsequent analysis has shown that the parental line RMA-S expresses low levels of CD1d1 (MFI approximately 20). This may account for the low level of γδ T cell reactivity to the untransfected APC. Nevertheless, αβ T cells clones used in the same assays as positive controls, unequivocally proliferated only in response to RMAS.CD1d1 APCs, suggesting that this low level of CD1d1 expressed on RMA-S cells was not significant.

Due to the small numbers of γδ T cells available in the mutant mice and the difficulty maintaining these cells in culture, one potential solution is to

generate T-T cell hybridomas. By immortalizing the T cells in this way, it would then be possible to sample T cell populations in the mouse that are difficult to grow as bulk lines such as intestinal intraepithelial T cells and thymocytes. Secondly, normal C57BL/6 mice could be used as a source of γδ T cells. TCRβ$^{-/-}$ mice were chosen for these experiments so as to prevent the outgrowth of αβ T cells and to have a starting population that homogeneously expressed the γδ TCR. The characterization of the TCRβ$^{-/-}$ mice disclosed that these mice had very small thymuses but that the T cells had otherwise developed normally (62). γδ T cell populations have not yet been examined in the CD1d1$^{-/-}$ animals and it is unclear whether αβ T cells may be required for the ontogeny of CD1d1 reactive γδ T cells.

Initial T-T fusions using freshly isolated γδT cells from immunized animals, fresh thymocytes stimulated *in vitro* for 48 h with anti-CD3 and activated T cells from short-term lines have been done but, the experiments were technically flawed. This technique will likely be the most definitive method to assess whether CD1d1 reactivity exists in the γδ T cell population and will allow analysis of the TCRs. The main disadvantage is that functional experiments will be limited.

The analysis of NK T cells has focused on the invariant Vα14Jα281 TCR^{+} subset. However, there is a significant and largely ignored population of γδ T cells that do express the NK1.1 antigen (63). In contrast to NK TCR αβ^{+}T cells, NK1.1^{+}γδ T cells are actually increased in β2-microglobulin deficient mice suggesting that this population of T cells may be selected by a ligand other than CD1d1. More recently, a novel population of murine γδ thymocytes has been

described which appears to represent the γδ homologue to the αβ NK TCR Vα14-Jα281. Phenotypically, this subset of γδ T cells is capable of producing both IFNγ and IL-4 upon stimulation, is Thy-1^{dull} and expresses Vγ1Vδ6.4 with a highly restricted junctional region (64). However, only 50% of these T cells co-express the NK1.1 antigen. These γδ T cell hybridomas were not assessed for reactivity to CD1d1. Since only half of the cells are positive for NK1.1, it would be informative to look for the presence of $NK1.1^{-}$ Vγ1Vδ6.4 cells in both β2-microglobulin and CD1d1 deficient mice to determine whether CD1d1 is the selecting ligand for these cells.

Another possibility for the lack of CD1d1 reactivity in the γδ T cells studied, is that the CD1d1 restricted population may be limited to certain sublineages confined to specific anatomical locations. The invariant γδ T cell repertoires of the skin and the vaginal epithelia, which would be predicted to recognize a non-polymorphic ligand like CD1d1, have not yet been examined for CD1d1 specificity.

Despite the apparent homology of the γδ TCR to the αβ TCR, there is no data to support that γδ T cell recognition of antigen requires a presenting molecule. This has led others to hypothesize that γδ T cells behave more like immunoglobulin molecules that do not require antigen processing for recognition (65,66). CD1d1 protein is a group 2 protein and despite the direct recognition of CD1a and CD1c, both group 1 proteins by human γδ T cells, it is possible that γδ T cells cannot recognize group 2 proteins. Experiments to assess potential human γδ T recognition of CD1d are currently ongoing. In short, the T cells

expressing invariant TCRs and NK lineage markers differ between αβ and γδ T cells. Further studies are required to assess CD1d1 reactivity in these γδ subpopulations.

CONCLUSION

Until recently, it has been difficult to conceive of T lymphocyte biology outside the paradigm of the TCR recognition of MHC/peptide complexes. The emergence of an MHC-independent pathway of lipid antigen presentation mediated by CD1 molecules, is clearly an important mechanism through which T cells can respond to a ubiquitous class of molecules. This extended T cell immune repertoire likely evolved as a critical component of the host's defense against microbial pathogens and may have broad applicability with respect to autoimmunity and tumour surveillance.

The specialized subset of NK T cells is but one population of CD1d1 restricted T cells in mice and humans. These studies have clearly demonstrated the existence of a diverse repertoire of αβ T cells with the ability to recognize CD1d1 directly that share a similar NK T cell phenotype. Despite a concerted effort, a reciprocal reactivity in murine γδ T cells could not be demonstrated. Residues within the Vβ TCR chain may confer reactivity to CD1d1 as suggested by the relative conservation of the Vβ8 chain among T cell clones. The direct reactivity observed may be the result of self-lipid antigen recognition. Further investigation into the basic biology of CD1d1 restricted T cells is required. By understanding how dysregulation of these cells may lead to disease, one can begin to unravel how these potent cells may be activated *in vivo* to treat a number of inflammatory, neoplastic and autoimmune diseases.

REFERENCES

1. Bendelac A, Rivera MN, Park SH, Roark JH. 1997. Mouse CD1-specific NK1 T cells: development, specificity and function. *Annu Rev Immunol* 15:535-562.

2. Wilson SB, Kent SC, Patton KT, Orban T, Jackson RA, Exley M, Porcelli S, Schatz DA, Atkinson MA, Balk SP, Strominger JL, Hafler DA. 1998. Extreme Th1 bias of invariant Vα24JαQT T cells in type 1 diabetes. *Nature* 391:177-181.

3. Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M.1997. Requirement for Vα14 NK T cells in IL-12-mediated rejection of tumors. *Science* 278:1623-1626.

4. Hammond KL, Poulton LD, Palmisano LJ, Silveira PA, Godfrey DI, Baxter AG. 1998. αβ-T cell receptor $(TCR)^{+}CD4^{-}CD8^{-}$ (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. *J Exp Med* 187:1047.

5. Grant EP, Degano M, Rosat J-P, Stenger S, Modlin RL, Wilson IA, Porcelli SA, Brenner MB. 1999. Molecular recognition of lipid antigens by T cell receptors. *J Exp Med* 189:195-205.

6. Haskins K, Kappler J, Marrack P. 1984. The major histocompatibility complex-restricted antigen receptor on T cells. *Annu Rev Immunol* 2:51-66.

7. Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB. 1994. Recognition of a lipid antigen by CD1-restricted $\alpha\beta^{+}$ T cells. *Nature* 372:691-694.

8. Sieling PA, Chatterjee D, Porcelli SA, Prigozy TI, Mazzaccaro RJ, Soriano T, Bloom BR, Brenner MB, Kronenberg M, Brennan PJ. 1995. CD1-restricted T cell recognition of microbial lipoglycan antigens. *Science* 269:227-30.

9. Moody DB, Reinhold BB, Guy MR, Beckman EM, Frederique DE, Furlong ST, Ye S, Reinhold VN, Sieling PA, Modlin RL, Besra GS, Porcelli SA. 1997. Structural requirements for glycolipid antigen recognition by CD1b-restricted T cells. *Science* 278:283.

10. Porcelli SA. 1995. The CD1 family: a third lineage of antigen-presenting molecules. *Adv Immunol* 59:1-98.

11. Porcelli SA, Modlin RL. 1999. The CD1 System: Antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu Rev Immunol* 17:297-329.

12. Kasinrerk W, Baumruker T, Majdic O, Knapp W, Stockinger H. 1993. CD1 molecule expression on human monocytes induced by granulocyte-macrophage colony-stimulating factor. *J Immunol* 150:579-84.

13. Brossay L, Jullien D, Cardell S, Sydora BC, Burdin N, Modlin RL, Kronenberg M. 1997. Mouse CD1 is mainly expressed on hematopoietic-derived cells. *J Immunol* 159:1216-24.

14. Balk SP, Burke S, Polischuk JE, Frantz ME, Yang L, Porcelli SA, Colgan SP, Blumberg RS. 1994. β2-microglobulin-independent MHC class Ib molecule expressed by human intestinal epithelium. *Science* 265:259-62.

15. Roark JH, Park SH, Jayawardena J, Kavita U, Shannon M, Bendelac A. 1998. CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. *J Immunol* 160:3121-27.

16. Zeng Z-H, Castano AR, Segelke BW, Stura EA, Peterson PA, Wilson IA. 1997. Crystal structure of mouse CD1: An MHC-like fold with a large hydrophobic binding groove. *Science* 277:339-345.

17. Porcelli SA, Segelke BW, Sugita M, Wilson IA, Brenner MB. 1998. The CD1 family of lipid antigen-presenting molecules. *Immunol Today* 19:362-68.

18. Porcelli SA, Brenner MB, Greenstein JL, Balk SP, Terhorst C, Bleicher PA. 1989. Recognition of cluster of differentiation 1 antigens by human $CD4^-CD8^-$ cytolytic T lymphocytes. *Nature* 341:447-50.

19. Coles MC, Raulet DH. 1994. Class I dependence of the development of CD4+CD8- NK1.1+ thymocytes. *J Exp Med* 180:395-99.

20. Cardell S, Tangri S, Chan S, Kronenberg M, Benoist C, Mathis D. 1995. CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice. *J Exp Med* 182:993.

21. Ohteki T, MacDonald HR. 1994. Major histocompatibility complex class I related molecules control the development of CD4+8-and CD4-8- subsets of natural killer 1.1+ T cell receptor-α/β+ cells in the liver of mice. *J Exp Med* 180:699.

22. Exley M, Garcia J, Balk SP, Porcelli S. 1997. Requirements for CD1d recognition by human invariant Vα24$^+$CD4$^-$CD8$^-$ T cells. *J Exp Med* 186:109.

23. Arase H, Arase N, Nakagawa K, Good RA, Onoe K. 1993. NK1.1$^+$CD4$^+$CD8$^-$ thymocytes with specific lymphokine secretion. *Eur J Immunol* 23:307-310.

24. Chen H, Paul WE. 1997. Cultured NK1.1$^+$CD4$^+$ T cells produce large amounts of IL-4 and IFN-γ upon activation by anti-CD3 or CD1. *J Immunol* 159:2240-2249.

25. Chen Y-H, Chiu NM, Mandal M, Wang N, Wang C-R. 1997. Impaired NK1$^+$ T cell development and early IL-4 production in CD1-deficient mice. *Immunity* 6:459-467.

26. Mendiratta SK, Martin WD, Hong S, Boesteanu A, Joyce S, Van Kaer L. 1997. *CD1d1* mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* 6:469-477.

27. Smiley ST, Kaplan MH, Grusby MJ. 1997. Immunoglobulin E production in the absence of interleukin-4-secreting CD1-dependent cells. *Science* 275:977-979.

28. Castano AR, Tangri S, Miller JE, Holcombe HR, Jackson MR, Huse WD, Kronenberg M, Peterson PA. 1995. Peptide binding and presentation by mouse CD1. *Science* 269:223.

29. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M. 1997. CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science* 278:1626-1629.

30. Joyce S, Woods AS, Yewdell JW, Bennink JR, De Silva AD, Boesteanu A, Balk SP, Cotter RJ, Brutkiewicz RR. 1998. Natural ligand of mouse CD1d1: cellular glycosylphosphatidylinositol. *Science* 279:1541-1544.

31. Mieza MA, Itoh T, Cui JQ, Makino Y, Kawano T, Tsuchida K, Koike T, Shirai T Yagita H, Matsuzawa A, Koseki H, Taniguchi M. 1996. Selective reduction of Vα14$^+$ NK T cells associated with disease development in autoimmune-prone mice. *J Immunol* 156:4035-4040.

32. Sumida T, Sakamoto A, Murata H, Makino Y, Takahashi H, Yoshida S, Nishioka K, Iwamoto I, Taniguchi M. 1995. Selective reduction of T cells bearing invariant Vα24JαQ antigen receptor in patients with systemic sclerosis. *J Exp Med* 182:1163-68.

33. Ljunggren HG, Ohlen C, Hoglund P, Franksson L, Karre K. 1991. The RMA-S lymphoma mutant; consequences of a peptide loading defect on immunological recognition and graft rejection. *Int J Cancer Suppl* 6:38.

34. Mandal M, Chen X-R, Alegre M-L, Chiu NM, Chen Y-H, Castano AR, Wang C-R. 1988. Tissue distribution, regulation and intracellular localization of murine CD1 molecules. *Mol Immuno* 35:525.

35. Lantz O, Bendelac A. 1994. An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med* 180:1097.

36. Shimamura M, Ohteki T, Launois P, Garcia AM, MacDonald HR. 1997. Thymus-independent generation of NK1+ T cells in vitro from fetal liver precursors. *J Immunol* 158:3682.

37. DerSimonian H, Sugita M, Glass DN, Maier AL, Weinblatt ME, Reme T, Brenner MB. 1993. Clonal Vα12.1+ T cell expansions in the peripheral blood of rheumatoid arthritis patients. *J Exp Med* 177:1623.

38. Wang R, Taniguchi M. 1995. Limited T cell antigen receptor repertoire in tumor-infiltrating lymphocyte and inhibition of experimental lung metastasis of murine melanoma by anti-TCR antibody. *J Immunol* 154:1797.

39. Masuda K, Makino Y, Cui J, Ito T, Tokuhisa T, Takahama Y, Koseki H, Tsuchida K, Koike T, Moriya H, Amano M, Taniguchi M. 1997. Phenotypes and invariant αβ TCR expression of peripheral Vα14+ NKT cells. *J Immunol* 158:2076.

40. Carbone FR, Fox BS, Schwartz RH, Paterson Y.1987. The use of hydrophobic, α-helix defined peptides in delineating the T cell determinant for pigeon cytochrome c. *J Immunol* I138:1838.

41. Behar SM, Podrebarac TA, Roy CJ, Wang C-R, Brenner MB. 1999. Diverse T cell receptors recognize murine CD1. *J Immunol* 162:161-167.

42. Allison JP. 1993. γδ T cell development. *Curr Opin Immunol* 5:241-246.

43. Bandeira A, Itohara S, Bonneville M, Burlen-Defranoux O, MotaSantos T, Coutinho A, Tonegawa S. 1991. Extrathymic origin of intestinal

intraepithelial lymphocytes bearing T cell antigen receptor γδ. *Proc Natl Acad Sci* 88:43-7.

44. Hiromatsu K, Yoshikai Y, Matsuzaki G, Ohga S, Muramori K, Matsumoto K, Bluestone JA, Nomoto K. 1992. A protective role of γδ T cells in primary infection with *Listeria-monocytogenes* in mice. *J Exp Med* 175:49-56.

45. Mombaerts P, Arnoldi J, Russ F, Tonegawa S, Kaufmann SH. 1993. Different roles of αβ and γδ T cells in immunity against an intracellular bacterial pathogen. *Nature* 365:53-56.

46. Kaufmann SHE, Ladel CH. 1994. Role of T cell subsets in immunity against intracellular bacteria: experimental infections of knock-out mice with *Listeria monocytogenes* and *Mycobacterium bovis* BCG. *Immunobiology* 191:509-19.

47. Rosat JP, MacDonald HR, Louis JA. 1993. A role for γδ + T cells during experimental infection of mice with *Leishmania major. J Immunol* 150:550-55.

48. Mixter PF, Camerini V, Stone BJ, Miller VL, Kronenberg M. 1994. Mouse T lymphocytes that express a γδ T cell antigen receptor contribute to resistance to *Salmonella* infection in vivo. *Infect Immun* 62:4618-21.

49. Tsuji M, Mombaets P, Lefrancois L, Nussenzweig RS, Zavala F, Tonegawa S. 1994. γδ T cells contribute to immunity against the liver stages of malaria in αβ T cell deficient mice. *Proc Natl Acad Sci* 91:345-49.

50. Kaufmann SHE, Blum C, Yamamoto S. 1993. Crosstalk between αβ T cells and γδ T cells in vivo: activation of αβ T cell responses after γδ T cell modulation with the monoclonal antibody GL3. *Proc Natl Acad Sci* 90:9620-24.

51. Boismenu R, Havran W. 1994. Modulation of epithelial cell growth by intraepithelial γδ T cells. *Science* 266:1253-55.

52. Komano H, Fuijura Y, Kawaguchi M, Matsumoto S, Hashimoto Y, Obana S, Mombaerts P, Tonegawa S, Yamamoto H, Itohara S, Nanno M, Ishikawa H. 1995. Homeostatic regulation of intestinal epithelia by intraepithelial γδ T cells *Proc Natl Acad Sci* 90:6147-51.

53. Penninger J, Wen T, Timms E, Potter J, Wallaca V, Matsuyama T, Ferrick D, Sydora B, Kronenberg M, Mak T. 1995. Spontaneous resistance to acute T cell leukemias in TCRVγ1.1Jγ4Cγ4 transgenic mice. *Nature* 375:241-44.

54. Schild H, Mavaddat N, Litzenberger C, Ehrlich EW, Davis MM, Bluestone JA, Matis L, Draper RK, Chien Y-h. 1994. The nature of major histocompatibility complex recognition of γδ T cells. *Cell* 76:29-37.

55. Morita CT, Beckman EM, Bukaurki JF, Tanaka Y, Band H, Bloom BR, Golan DE, Brenner MB. 1995. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T cells. *Immunity* 3:495-507.

56. Weintraub BC, Jackson MR, Hedrick SM. 1994. γδ T cells can recognize non-classical MHC in the absence of conventional antigenic peptides. *J Immunol* 153:3051-58.

57. Nagler-Anderson C, McNair LA, Cradock A. 1992. Self-reactive, T cell receptor-$\gamma\delta^+$ lymphocytes from the intestinal epithelium of weanling mice. *J Immunol* 149:2315.

58. White J, Blackman M, Bill J, Kappler J, Marrack P, Gold DP, Born W. 1989. Two better cell lines for making hybridomas expressing specific T cell receptors. *J Immunol* 143:1822-1825.

59. Pereira P, Gerber D, Huang SY, Tonegawa S. 1995. Ontogenic development and tissue distribution of Vγ1-expressing γ/δ T lymphocytes in normal mice. *J Exp Med* 182:1921-1930.

60. Chiu Y-H, Jayawardena J, Weiss A, Lee D, Park S-H, Dautry-Varsat A. Bendelac A. 1999. Distinct subsets of CD1d-restricted T cells recognize self-antigens loaded in different cellular compartments. *J Exp Med* 189:103-110.

61. Chen H, Huang H, Paul WE. 1997. NK1.1+CD4+T cells lose NK1.1 expression upon in vitro activation *J Immunol* 158:5112.

62. Mombaerts P, Clarke AR, Rudnicki MA, Iacomini J, Itohara S, Lafaille JJ, Wang L, Ichikawa Y, Jaenisch R, Hooper ML, Tonegawa S. 1992. Mutations in T cell antigen receptor genes α and β block thymocyte development at different stages. *Nature* 360:225-231.

63. Vicari AP, Mocci S, Openshaw P, O'Garra A, Zlotnik. 1996. Mouse γδ TCR+NK1.1+ thymocytes specifically produce interleukin-4, are major histocompatibility complex class I independent, and are developmentally related to αβ TCR+NK1.1+ thymocytes. *Eur J Immunol* 26:1424-29.

64. Azuara V, Levraud J-P, Lembezat M-P, Pereira P. 1997. A novel subset of adult γδ thymocytes that secretes a distinct pattern of cytokines and expresses a very restricted T cell receptor repertoire. *Eur J Immunol* 27:544-553.

65. Chothia C, Boswell D, Lesk A. 1988. The outline structure of the T cell αβ receptor. *EMBO J7*:3745-55.

66. Rock EP, Sibbald PR, Davis MM, Chien Y-h. 1994. CDR3 length in antigen-specific immune receptors. *J Exp Med* 179:323-28.

Curriculum Vitae

Name: Theresa Anne Podrebarac MD

Office Address: Brigham and Women's Hospital
Smith Building, Room 518
One Jimmy Fund Way
Boston, MA
USA 02115

Home Address: 281 Canton Street
Westwood, MA
USA 02090

E:Mail: tpodrebarac@rics.bwh.harvard.edu **FAX:** 617-525-1010

Place of Birth: Sudbury, Ontario, Canada

Education:

Year	Degree	Institution
1996-1999	MSc - Immunology	University of Ottawa Ottawa, Ontario, Canada
1986-1990	M.D.	University of Western Ontario London, Ontario, Canada
1984-1986	Chemistry	University of Western Ontario London, Ontario, Canada

Post-Doctoral Training:

Year	Title	Specialty	Place of Training
1996-1999	Postdoctoral Research Fellow	Immunology/ Rheumatology	Brigham and Women's Hospital Department of Medicine Division of Rheumatology/ Immunology/Allergy
1995-1996	Postdoctoral Research Fellow	Rheumatology	University of Ottawa Department of Medicine Division of Rheumatology Ottawa, Canada
1993-1995	Rheumatology Fellow	Rheumatology	University of Ottawa Department of Medicine Division of Rheumatology Ottawa, Canada

Year	Title	Specialty	Place of Training
1993	Chief Medical Resident	Internal Medicine	St. Joseph's Hospital Department of Medicine University of Western Ontario London, Canada
1990-1993	Internal Medicine Resident	Internal Medicine	University of Western Ontario Department of Medicine London, Canada

Licensure and Certification:

Year	Type of License or Certification
1998-	Diplomate of the American Board of Internal Medicine Internal Medicine Specialty Certification
1996-	Medical Licensure, Commonwealth of Massachusetts
1995-	Rheumatology Sub-specialty Certification Royal College of Physicians and Surgeons of Canada I.D. No. 0455269
1994-	Internal Medicine Specialty Certification Royal College of Physicians and Surgeons of Canada I.D. No. 0455269
1991-	Medical Licensure No. 62158 College of Physicians and Surgeons of Ontario

Academic Appointments:

Year	Academic Title	Institution
1999-	Instructor	Harvard Medical School

Hospital or Affiliated Institution Appointments:

Year	Title	Hospital
1996-	Post-doctoral Research Fellow	Brigham and Women's Hospital Department of Medicine Division of Rheumatology/ Immunology/Allergy
1995-1996	Internal Medicine Clinical-Research Scholar	Ottawa General Hospital Department of Medicine Ottawa, Canada

Professional Society Involvement:

Year	Society	Role
1995-	American College of Rheumatology	Member
1995-1996	The Canadian Rheumatology Association	Member
1991-	The American College of Physicians	Member
1988-1996	The Ontario Medical Association Canadian Medical Association	Member

Awards and Honors:

Year	Name of Award
1997	Edmund Dubois Award Outstanding Young Investigator in Lupus American College of Rheumatology
1996-2003	Clinician Scientist Award Medical Research Council of Canada
1996-1999	University of Ottawa Excellence Scholarship
1994-1995	Arthritis Society Clinical Fellowship Award The Canadian Arthritis Society
1990	Hewlett Packard Top Medical Graduate Award University of Western Ontario Medical School
1989	Lange Medical Publications Prize University of Western Ontario Medical School
1990	Lange Medical Publications Prize University of Western Ontario Medical School
1984	University Entrance Scholarship University of Western Ontario

Part III: Bibliography

Original Articles:

1. Podrebarac TA, Jovaisas A, Karsh J. *Pneumocystis Carinii* pneumonia following discontinuation of hydroxychloroquine in two patients with SLE [letter]. J Rheumatol 1996;23:199-200.

2. Podrebarac TA, Boisvert D and Goldstein R. Clinical Correlates, Autoantibodies and the Role of the major histocompatibility complex comparing French Canadian and non-French Canadian Caucasians with systemic lupus erythematosus. Lupus 1998;7:183-191.

3. Behar SM, Podrebarac TA, Roy C, Wang C-R, Brenner MB. Diverse T cell receptors recognize murine CD1. J Immunol 1999;162:161-167.

Reviews and Educationally Relevant Publications:

1. Podrebarac TA, Mckendry R. Cellular Suicide: Critical concept or fleeting fad. Rheumatology, the year in review. Lancet 1995;346(suppl):22.

2. Podrebarac TA, Hebert P, Tugwell P. A reader's guide to the evaluation of causation. Postgrad Med J (BMJ) 1996;72(845):131-137.

3. Podrebarac TA, Goldstein R. What is the relationship between complement and systemic lupus erythematosus [editorial]? J Rheumatol 1998;25:2062-2064.

Book Chapters:

1. Podrebarac TA, Tugwell P. The Newer Second-Line Agents: Cyclosporin. In: van de Putte LBA, Furst DE, Williams HJ and van Riel PLCM, editors. Therapy of Systemic Rheumatic Disorders. Marcel Dekker, Inc., New York, USA; 1998: 207-220.

Abstracts:

1. Podrebarac TA, McPherson R, Goldstein R. Lipid profiles and lipoprotein(a) levels in Systemic Lupus Erythematosus and Primary Antiphospholipid Antibody Syndrome. Arthritis Rheum 38;9:S169,1995.

2. Podrebarac TA, McPherson R, Pham B, Goldstein R. A prospective follow-up of lipoprotein(a) and lipid levels in SLE and PAPS patients. Arthritis Rheum 04/97.

CONTRIBUTION OF COLLABORATORS

The scope of this work would not have been possible without the assistance of my collaborators. Specifically, I would like to thank Dr. Samuel Behar for his work on the sequencing and analysis of the TCRs. I wish to also acknowledge Christopher Roy's technical assistance in PCR and in sequencing of the TCRs.