11-1-1999

The CTL Memory Responses to Influenza A Viruses in Humans: a Dissertation

Julie Marie Jameson

University of Massachusetts Medical School

Follow this and additional works at: http://escholarship.umassmed.edu/gsbs_diss

Part of the Life Sciences Commons, and the Medicine and Health Sciences Commons

Recommended Citation


This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
THE CTL MEMORY RESPONSES TO INFLUENZA A VIRUSES IN HUMANS

A Dissertation Presented By

JULIE MARIE JAMESON

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester, in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY AND VIROLOGY

NOVEMBER 1999
THE CTL MEMORY RESPONSES TO INFLUENZA A VIRUSES IN HUMANS

A Dissertation Presented By

Julie Jameson

Approved as to style and content by:

________________________________________
Dr. Raymond M. Welsh, Chairperson of the Committee

________________________________________
Dr. John E. Herrmann, Member of the Committee

________________________________________
Dr. Dale Greiner, Member of the Committee

________________________________________
Dr. Kendall Knight, Member of the Committee

________________________________________
Dr. Jack F. Bukowski, Member of the Committee

________________________________________
Dr. Francis A. Ennis, Thesis Advisor

________________________________________
Dr. Thomas B. Miller, Jr., Dean of the
Graduate School of Biomedical Sciences

Program of Immunology and Virology,
November 2, 1999
COPYRIGHT NOTICE

Parts of this dissertation have appeared in the following publications:


ACKNOWLEDGMENTS

I would like to thank all of my parents for their encouragement and support; they have shown me how to work hard by example. I also want to thank my boyfriend Alan who has made my time in graduate school fun and exciting. I want to thank Dr. Ennis and Dr. Rothman for giving me so much of their time and showing me their enthusiasm for science. I would like to thank John Cruz for teaching me how to grow cells and how he has learned to experiment flawlessly.

There are several others I want to thank who have helped me in various ways along my graduate years; Drs. Ray Welsh, John Hermann, Dale Greiner, and Ken Knight for being on my committee, the other graduate students especially Neal Iwakoshi for his FACS expertise, and Marcia Woda along with the other members of the FACS facility.
ABSTRACT

Influenza A virus infections are a major cause of morbidity and mortality in the United States and throughout the world. The current vaccine elicits primarily a humoral response that is specific for the external glycoproteins hemagglutinin (HA) and neuraminidase (NA). However, these are the viral proteins that are most susceptible to antigenic shift and drift, and can evade the humoral response. Cytotoxic T lymphocytes (CTL) recognize and lyse virus-infected cells and are important in clearing influenza A virus infections. CTL can recognize epitopes on both the external glycoproteins and the more conserved internal viral proteins. This thesis investigates the hypothesis that there is a broad CTL memory response in humans, and, if boosted by vaccines, these CTL may help clear influenza A virus strains of different subtypes.

The CTL repertoire specific for influenza A viruses reported in inbred mice is extremely limited and has focused on a few immunodominant epitopes. We performed preliminary bulk culture chromium release assays using human peripheral blood mononuclear cells (PBMC) stimulated with influenza virus strain A/PR/8/34 (H1N1) in vitro. CTL activity was observed against autologous B-lymphoblastoid cell lines (B-LCL) infected with vaccinia constructs that expressed several influenza A viral proteins, including nucleoprotein (NP), matrix (M1), nonstructural 1 (NS1) and polymerase (PB1). This was more diverse than the limited response reported in inbred mice. To further characterize the CTL repertoire in humans, PBMC from healthy adult donors were stimulated and CTL were cloned by limiting dilution. Isolated cell lines were further
characterized by their CD4/CD8 surface expression, histocompatibility leukocyte antigen (HLA) restriction, cross-reactive or subtype-specific influenza A subtype recognition, and epitope recognition.

CTL lines isolated from three donors recognized epitopes on many different influenza virus proteins. The ELISPOT assay was used to identify the number of IFN-γ-secreting cells and determine the precursor frequency of the CTL specific for the epitopes that were mapped. The precursor frequency of IFN-γ producing CTL ranged from 1 in 4,156 PBMC to 1 in 31,250 PBMC. The precursor frequency for one epitope was below the level of detection of this assay, but most of the memory CTL were readily detected. The cross-reactive or subtype-specific recognition of various human influenza A subtypes by these T cell lines was determined by chromium release assays. Most of the CTL lines recognized B-LCL infected with any of the three influenza A subtypes that have caused epidemics in the last century (H1N1, H2N2, and H3N2) and recognized epitopes on conserved internal influenza viral proteins. Most of the subtype-specific cell lines recognized the surface HA or NA glycoproteins, which are not well conserved between influenza subtypes.

Although most of the T cell lines that were characterized were cross-reactive with influenza viruses of human origin, infection of humans with a divergent swine or avian derived strain could cause a global pandemic. To study the human CTL responses to non-human influenza viruses, B-LCL were infected with an Hsw1N1 influenza A virus of swine origin, and cell lines were tested for recognition of these targets in a chromium release assay. Most cell lines lysed the targets infected with the Hsw1N1 subtype to the
same degree as targets infected with the human H1N1 strain. Two influenza viruses of duck origin were also tested and were recognized by many of the cell lines. The subtypes of these duck strains were Hav1N1 and H5N2. The isolates of influenza A virus from the Hong Kong outbreak of 1997 were also used to infect targets and analyze recognition by these CTL. We found that approximately 50% of the human T cell lines tested recognized both of the Hong Kong isolates, 25% recognized at least one isolate, and 25% recognized neither isolate to the same degree as the A/PR/8/34 (H1N1) virus. We analyzed the amino acid (aa) changes in the epitopes of the T cells lines from the 25% of cell lines that did not recognize either Hong Kong virus isolate. Non-conservative mutations were found in all of the epitopes that lost recognition by the human CTL lines. Bulk cultures of PBMC from three donors that were stimulated with A/PR/8/34 (H1N1) influenza A virus of human origin recognized all of the non-human virus strains tested. Thus, humans have memory CTL that recognize influenza viruses of avian and swine species. This may provide a second line of defense against influenza infection in case of exposure to a novel influenza A virus derived from these species.

These results made it clear that humans have broad CTL memory to influenza A virus. In order to determine whether these T cells could be boosted in a vaccine, immune-stimulatory complexes (Iscom) incorporating inactivated influenza particles were tested in vitro. Iscoms containing inactivated influenza A vaccine (Flu-Iscom) were used to pulse autologous B-LCL overnight that were then used as targets in chromium release assays with human CTL lines as effectors. A CD8+ HA-specific CTL line lysed these targets, but not targets pulsed with Iscoms alone or with inactivated influenza A
vaccine alone. An NS1-specific cell line recognized targets pulsed with NS1 protein and Iscoms, but not targets pulsed with Iscoms or NS1 protein alone. Therefore, CTL could recognize in vitro target cells that were exposed to the Iscom vaccines containing their specific epitope.

Flu-Iscom and Iscom mixed with inactivated influenza virus particles (Flu-Iscomatrix) were then used as vaccines in a clinical trial to test CTL and neutralizing antibody induction against influenza. Fifty-five donors were bled pre-vaccination, and on days 14 and day 56 post-vaccination. Bulk culture chromium release assays were performed using targets infected with live vaccine strain viruses. There were significantly more increases in the influenza A specific CTL activity in the PBMC of donors that were vaccinated with the Flu-Iscom and Flu-Iscomatrix vaccines than in recipients of the standard vaccine. In order to determine whether these increases in cytotoxicity were due to an increase in the precursor frequency of influenza specific CTL, the PBMC were used in ELISPOT assays to assess the changes pre-and post-vaccination. When there was an increase in the level of cytotoxicity detected in bulk culture CTL, there was often also an increase in the precursor frequency of influenza-specific CTL. Peptide-specific increases in the number of CTL that recognize epitopes such as M1 aa 58-66 were detected in several donors confirming the increase in influenza-specific CTL post-vaccination.

Another type of T cell that may be involved in defense against viruses is the γδ T cell. T cells expressing the γδ T cell receptor (TCR) have been found extensively in mucosal tissues in mice and humans. Influenza A viruses enter via the airway tract,
infecting the epithelial cells at the mucosal surface. These epithelial cells have been shown in vitro to be targets for influenza-specific cytolytic recognition of αβ T cells. To analyze whether γδ T cells can respond to influenza A-infected APCs, PBMC were stimulated with influenza A virus. Intracellular IFN-γ staining was used to determine whether γδ T cells can secrete IFN-γ in response to the influenza A virus infection. We observed an increase in the percentage of γδ T cells secreting IFN-γ post-influenza A virus infection of PBMC compared to uninfected or allantoic fluid-stimulated cultures. These T cells also upregulated CD25 and CD69 in response to live influenza A virus. We focused on the responses in the CD8- population of γδ T cells, which are the majority of γδ T lymphocytes. Furthermore, the increases in IFN-γ production and activation marker expression were much more clear in the CD8- γδ+ T cells. The level of CD8- γδ T cell activation with inactivated influenza A virus was much less, and in some cases no higher than uninfected PBMC. The CD8+ αβ and γδ responses could be partially blocked by anti-class I antibodies, but the CD8- γδ responses could not. Vaccinia virus infection did not activate the CD8- γδ T cells to the same degree as influenza virus infection. γδ T cells are thought to have a regulatory role that includes the secretion of cytokines and epithelial growth factors to help restore tissue back to health.

Humans have broad multi-specific T lymphocyte responses by αβ T cells to influenza A viruses and those responses are cross-reactive with human, avian, and swine virus strains. These CTL can be activated in vitro and boosted in number in vivo by Iscom incorporating vaccines. There is also a population of γδ+ T lymphocytes in
humans that responds to influenza virus infection by producing cytokines and becoming activated. Increasing memory CTL as a second line of defense against influenza A viruses may be important in future vaccine development.
TABLE OF CONTENTS

COPYRIGHT NOTICE .......................................................... ii
ACKNOWLEDGEMENTS ............................................................. iv
ABSTRACT ........................................................................... v
TABLE OF CONTENTS ............................................................ x-xviii
LIST OF TABLES .................................................................. xviii-ix
LIST OF FIGURES ................................................................. xx-xxi
ABBREVIATIONS ............................................................... xxii-iii

CHAPTER I: INTRODUCTION .................................................. 1-42

A. BACKGROUND ................................................................. 1-21
   1. The Nature of Influenza A Viruses ................................. 1-2
   2. The Epidemiology of Influenza A Viruses ....................... 2-4
   3. Pandemics of the 20th Century .................................... 5-7
   4. Influenza A Virus Structure, Composition, and Pathogenesis .... 8-13
   5. The Role of Cytotoxic T Lymphocytes in Eradicating Virus Infection 13-17
6. The Role of CTL in Eradicating Influenza Virus Infection in mice ................................................................. 17-19

7. The Role of CTL in Eradicating Influenza A Infection in Humans ................................................................. 20-21

B. THE CYTOTOXIC T LYMPHOCYTE MEMORY REPERTOIRE SPECIFIC FOR INFLUENZA A ..................................................... 21-27

1. The CTL Repertoire Specific for Influenza A in the Mouse is Limited ................................................................. 22-24

2. The CTL Repertoire Specific for Influenza A Viruses in Humans ........................................................................... 25-27

C. HETEROTYPIC IMMUNITY SPECIFIC FOR INFLUENZA A ........... 28-32

1. Mice Can Recognize Influenza A Virus Strains of Differing Subtypes ................................................................. 28-29

2. Heterotypic Immunity Specific for Influenza A Viruses in Humans ........................................................................... 29-31

3. H5N1 Subtype Influenza A Virus Infection of Humans .......... 31-32

D. INFLUENZA VACCINES HAVE BEEN DEVELOPED TO BOOST CTL ................................................................. 33-38

1. Getting Antigen into the Class I Pathway to Activate CTL ...... 33-34

2. Vaccine Strategies Previously Developed to Induce CTL ...... 34-36

3. Immune Responses to the Flu-Iscom Vaccine in Mice .......... 36-37

4. Immune Responses to the Flu-Iscom Vaccine in Humans ...... 37-38
E. THE ROLE OF γδ T LYMPHOCYTES IN INFLUENZA A VIRUS INFECTION ........................................................... 38-41

1. γδ T Lymphocyte Responses Specific for Influenza
   in the Mouse ......................................................... 39-40

2. γδ T Lymphocyte Responses in Humans ....................... 40-41

F. THESIS OBJECTIVES .................................................. 42

CHAPTER II: MATERIALS AND METHODS ........................... 43-54

A. VIRUSES ............................................................... 43-45

B. HUMAN PBMC FOR DERIVATION OF CELL LINES .......... 46-47

C. VOLUNTEER PBMC FOR ISCOM STUDY ..................... 48-49

D. BULK CULTURES OF PBMC ........................................ 50

E. DERIVATION OF CTL LINES ..................................... 50-51

F. PREPARATION OF TARGET CELLS ............................. 51-52

G. CHROMIUM RELEASE ASSAYS .................................. 52-53

H. SINGLE CELL ELISPOT ASSAY FOR DETECTION
   OF IFN-γ-SECRETING CELLS .................................... 53

I. INTRACELLULAR IFN-γ STAINING ................................. 53-54

J. STATISTICAL ANALYSIS ........................................... 54

CHAPTER III: ANALYSIS OF THE T CELL REPERTOIRE
IN HUMANS ........................................................................ 55-77
A. BULK CULTURE RESPONSES TO INFLUENZA A VIRUS AND
CONSTRUCTS EXPRESSING INFLUENZA A VIRAL
PROTEINS IN DONOR 1 ...................................................... 55-57
B. BULK CULTURE T CELL RECOGNITION OF INFLUENZA VIRUS
AND VACCINIA CONSTRUCTS EXPRESSING INFLUENZA VIRUS
PROTEINS ................................................................. 58-60
C. PROTEIN SPECIFICITY OF CTL LINES ISOLATED
FROM DONOR 1 .............................................................. 61-62
D. HLA RESTRICTION OF DONOR 1 CTL LINES ...................... 63-66
E. CHARACTERIZATION OF THE EPITOPES RECOGNIZED BY
CTL LINES FROM DONOR 1 .............................................. 67-71
F. DONORS 2 AND 3 ALSO HAVE BROAD CTL REPERTOIRES FOR
INFLUENZA A VIRAL PROTEINS ........................................ 72-74
G. CROSS-REACTIVE LYSIS BY CTL LINES ISOLATED FROM
DONORS 1, 2, AND 3 OF INFLUENZA VIRUS SUBTYPES
H1N1, H2N2, H3N2 ............................................................. 75-77

CHAPTER IV: HUMAN CD8+ AND CD4+ T LYMOPHOCYTE
MEMORY TO INFLUENZA A VIRUSES OF SWINE AND
AVIAN SPECIES ................................................................. 78-92
A. RECOGNITION OF SWINE AND AVIAN INFLUENZA A SPECIES BY T CELL LINES ................................................................. 78-81

B. RECOGNITION OF THE 1997 HONG KONG STRAINS BY HUMAN T CELL LINES ................................................................................................................. 82-84

C. RECOGNITION OF TARGETS INFECTED WITH AVIAN, SWINE, AND HUMAN-SPECIES OF INFLUENZA A VIRUSES BY BULK CULTURE PBMC .......................................................................................................... 85-86

D. HUMAN T CELL LINE RECOGNITION OF MUTATED PEPTIDES FROM THE A/HK/97 STRAINS ............................................................... 87-88

E. PRECURSOR FREQUENCY DETECTION BY ELISPOT .......... 89-92

CHAPTER V: IN VITRO AND IN VIVO T CELL RESPONSES TO FLU-ISCOM VACCINATION ................................................................. 93-111

A. IN VITRO CTL LYSIS OF TARGET CELLS PULSED WITH FLU-ISCOM .................................................................................. 93-96

B. HUMAN CTL RESPONSES TO FLU-ISCOM VACCINES .......... 97-99

C. PEPTIDE SPECIFIC RESPONSES WERE DETECTED IN FLU-ISCOM VACCINE RECIPIENTS .............................................................. 100-102

D. INCREASES IN THE VIRUS-SPECIFIC IFN-γ SECRETION AND IN...
VIRUS-SPECIFIC CTL ACTIVITY ........................................ 103-108

E. FLUZONE CONTROL VACCINE RECIPIENTS DID NOT HAVE
INCREASES IN THE PRECURSOR FREQUENCY OF
INFLUENZA A SPECIFIC CTL ........................................ 109-111

CHAPTER VI: \( \gamma^\delta \) T CELL ACTIVATION IN RESPONSE TO
INFLUENZA A INFECTION IN HUMANS ....................... 112-130

A. \( \gamma^\delta \) T CELLS ARE ACTIVATED TO SECRETE IFN-\( \gamma \) IN
RESPONSE TO INFLUENZA INFECTION OF HUMAN PBMC..... 112-117

B. TIME COURSE OF INF-\( \gamma \) PRODUCTION BY \( \gamma^\delta \) T CELLS AFTER
EXPOSURE TO INFLUENZA VIRUS AND DOSE RESPONSE OF
VIRUS DILUTIONS ....................................................... 118-120

C. EXPRESSION OF ACTIVATION MARKERS ON \( \gamma^\delta \) T CELLS IN
RESPONSE TO INFLUENZA INFECTION .......................... 121-126

D. DETERMINATION OF WHETHER IFN-\( \gamma \) SECRETION BY \( \gamma^\delta \) T
CELLS CAN BE INHIBITED BY ANTI-CLASS I MONOCLONAL
ANTIBODIES ............................................................. 127-128

E. VACCINIA VIRUS DOES NOT ACTIVATE \( \gamma^\delta^+ \) CELLS IN PBMC AS
WELL AS INFLUENZA .................................................. 129-130

CHAPTER VII: DISCUSSION ............................................. 131-
A. THE HUMAN CYTOTOXIC T CELL REPERTOIRE SPECIFIC FOR INFLUENZA A VIRUS ............................................. 131-139
B. HETEROSUBTYPIC IMMUNITY TO INFLUENZA A VIRUSES IN HUMANS ............................................................... 139-143
C. FLU-ISCOM VACCINES INCREASE CTL MEMORY IN VACCINATED HUMANS ............................................................... 143-149
D. THE ROLE OF γδ T CELLS IN INFLUENZA A VIRUS INFECTION IN HUMANS ............................................................... 150-154
E. CONCLUSIONS ............................................................... 155-156

REFERENCES ............................................................... 157
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>Subtypes of Influenza A Viruses Isolated from Various Species</td>
</tr>
<tr>
<td>I-2</td>
<td>Influenza A Virus Epidemics or Pandemics Occurring in Humans in the Last Century</td>
</tr>
<tr>
<td>I-3</td>
<td>Previously Characterized Human CD8+ CTL Epitopes on Influenza A Viral Proteins</td>
</tr>
<tr>
<td>II-1</td>
<td>Recombinant Vaccinia Viruses Used in the Thesis</td>
</tr>
<tr>
<td>II-2</td>
<td>Adult Healthy Donors Used for T Cell Line Isolation and γδ T Cell Analysis</td>
</tr>
<tr>
<td>II-3</td>
<td>Volunteers from Flu-Iscom Vaccine Study that Were Analyzed by ELISPOT Assay</td>
</tr>
<tr>
<td>III-1</td>
<td>Identification of Influenza Virus Proteins Recognized by CTL Lines Generated from the PBMC of Donor 1</td>
</tr>
<tr>
<td>III-2</td>
<td>Peptide Specific CTL Frequencies in Donor 1 PBMC as Determined by ELISPOT</td>
</tr>
<tr>
<td>III-3</td>
<td>Recognition of Influenza Virus Proteins by CTL Lines Generated from the PBMC of Donors 2 and 3</td>
</tr>
<tr>
<td>III-4A</td>
<td>Subtype Cross-Reactive Recognition by Influenza Virus-Specific CTL from Donors 1, 2, and 3</td>
</tr>
<tr>
<td>III-4B</td>
<td>Subtype-Specific Recognition by Influenza Virus-Specific CTL from Donors 1, 2, and 3</td>
</tr>
<tr>
<td>IV-1</td>
<td>Recognition of Swine/Avian Influenza A Virus-Infected Targets by Human CTL Lines</td>
</tr>
<tr>
<td>IV-2</td>
<td>Recognition of the Hong Kong Avian Virus Strains by Human CTL Lines</td>
</tr>
</tbody>
</table>
IV-3 Effect of Mutations on the HK/156/97 Virus on CTL Recognition
by Human CD8+ and CD4+ CTL Lines to H1N1 Epitopes... 88

IV-4 Precursor Frequency Analysis of Mutations in HK/156/97 and
HK/483/97 Using ELISPOT Assay ................................. 91

IV-5 Amino Acid Sequence of CTL Epitopes on Different Influenza
Virus Strains ......................................................... 92

V-1 Recognition by HLA-B18 Restricted HA-Specific Human CD8+
CTL Cell line of A/Texas H1N1 Flu-Iscom Vaccine Pulsed
B-LCL .............................................................. 95

V-2 Recognition by Human HLA-A2.1 Restricted NS1 aa 122-130
Specific CD8+ CTL Cell line of Recombinant NS1 Protein and
Iscomatrix Pulsed B-LCL ........................................ 96

V-3 CTL Responders in the Flu-Iscom Phase 1 Trial .............. 99

V-4 Precursor Frequency of Influenza Virus (A/Johan/97, H3N2)-
specific T Cells in Recipients of Adjuvanted Vaccines Pre
and Post Vaccination .............................................. 107

V-5 Precursor Frequency of Influenza A Virus (A/Texas/97, H1N1)-
specific T Cells in Recipients of Adjuvanted Vaccines Pre
and Post Vaccination .............................................. 108

V-6 Precursor Frequency of Influenza-A Virus Specific T Cells of
Three Fluzone Vaccine Recipients Pre and Post Vaccination. 110

VII-1 Summary of CTL Lines Isolated from All Three Donors...... 135
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>RNA Polymerase and Surface Glycoproteins of Influenza A.</td>
<td>12</td>
</tr>
<tr>
<td>III-1</td>
<td>Donor 1 Bulk Culture CTL Responses Specific for Influenza A Viruses of Multiple Subtypes</td>
<td>56</td>
</tr>
<tr>
<td>III-2</td>
<td>Donor 1 Bulk Culture Responses Specific for Vaccinia Constructs Expressing Influenza Viral Proteins</td>
<td>57</td>
</tr>
<tr>
<td>III-3</td>
<td>Donor 2 Bulk Culture Responses Specific for Influenza and Vaccinia Constructs Expressing Influenza Proteins</td>
<td>59</td>
</tr>
<tr>
<td>III-4A</td>
<td>Donor 4 Bulk Culture Responses Specific for Influenza and NP</td>
<td>60</td>
</tr>
<tr>
<td>III-4B</td>
<td>Donor 5 Bulk Culture Responses Specific for Influenza and NP</td>
<td>60</td>
</tr>
<tr>
<td>III-5</td>
<td>HLA Restriction of CTL Lines</td>
<td>65</td>
</tr>
<tr>
<td>III-6</td>
<td>Donor 1 Bulk Culture Recognition of NP 174-184</td>
<td>68</td>
</tr>
<tr>
<td>III-7</td>
<td>Fine Epitope Mapping of T Cell Lines</td>
<td>69</td>
</tr>
<tr>
<td>III-8</td>
<td>Dose Response Recognition of Peptide Pulsed Targets</td>
<td>70</td>
</tr>
<tr>
<td>IV-1</td>
<td>Bulk Culture Recognition of Avian, Swine, Human Viruses.</td>
<td>86</td>
</tr>
<tr>
<td>V-1</td>
<td>Peptide-specific Responses of Two Donors Who Received the Flu-Iscom Vaccine</td>
<td>102</td>
</tr>
<tr>
<td>V-2</td>
<td>CTL Responses Specific for A/Johan/33/94</td>
<td>105</td>
</tr>
<tr>
<td>V-3</td>
<td>CTL responses specific for A/Texas/36/91</td>
<td>106</td>
</tr>
<tr>
<td>V-4</td>
<td>Number of A/Johan/94-specific SFC in Fluzone Vaccine Recipients</td>
<td>112</td>
</tr>
<tr>
<td>VI-1</td>
<td>Donor 1 γδ+ T Cells Respond to Influenza Infection</td>
<td>115</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>VI-2</td>
<td>Donor 2 γδ T Cells Respond to Influenza Infection</td>
<td>116</td>
</tr>
<tr>
<td>VI-3</td>
<td>Donor 5 γδ T Cells Respond to Influenza Infection</td>
<td>117</td>
</tr>
<tr>
<td>VI-4</td>
<td>Time Course of IFN-γ Production by CD8-γδ+ Cells in the PBMC of Donor 1</td>
<td>119</td>
</tr>
<tr>
<td>VI-5</td>
<td>Dose Response of Influenza Virus Infection of PBMC</td>
<td>120</td>
</tr>
<tr>
<td>VI-6</td>
<td>CD69 Expression on γδ T Cells Infected with PR/8/34 (H1N1) in Donor 1</td>
<td>123</td>
</tr>
<tr>
<td>VI-7</td>
<td>Surface Expression of CD69 on CD8+ or CD8- γδ+ T Cells from Donor 1</td>
<td>124</td>
</tr>
<tr>
<td>VI-8</td>
<td>CD25 Surface Expression on the γδ T Cell Population from Donor 5</td>
<td>126</td>
</tr>
<tr>
<td>VI-9</td>
<td>Anti-Class I Antibody Inhibits CD8+ γδ+ T Cell Secretion, but Not CD8-γδ+ T Cells in Donor 1</td>
<td>128</td>
</tr>
<tr>
<td>VI-10</td>
<td>Donor 1 γδ T Cells Do Not Respond to Vaccinia Virus</td>
<td>130</td>
</tr>
<tr>
<td>VII-1</td>
<td>Potential Role of Flu-Iscoms: Increasing CTLp to Respond to Influenza Infection by a Novel Subtype</td>
<td>149</td>
</tr>
<tr>
<td>VII-2</td>
<td>Models for γδ T Cell Activation</td>
<td>154</td>
</tr>
</tbody>
</table>
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>B-LCL</td>
<td>B lymphoblastoid cell line</td>
</tr>
<tr>
<td>CDR</td>
<td>complementary determining region</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLp</td>
<td>cytotoxic T lymphocyte precursors</td>
</tr>
<tr>
<td>E:T</td>
<td>effector to target ratio</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr Virus</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Flu-Iscom</td>
<td>Iscom with inactivated influenza vaccine incorporated in</td>
</tr>
<tr>
<td>Flu-Iscomatrix</td>
<td>Iscom particles mixed with inactivated influenza vaccine</td>
</tr>
<tr>
<td>Fluzone</td>
<td>standard influenza A vaccine</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>i.n.</td>
<td>intranasal</td>
</tr>
<tr>
<td>i.m.</td>
<td>intramuscular</td>
</tr>
<tr>
<td>Iscom</td>
<td>immune-stimulatory complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>M1, M2</td>
<td>matrix proteins</td>
</tr>
<tr>
<td>MAL</td>
<td>mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLN</td>
<td>mediastinal lymph node</td>
</tr>
<tr>
<td>moi</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>NA</td>
<td>neuraminidase</td>
</tr>
<tr>
<td>NP</td>
<td>nucleoprotein</td>
</tr>
<tr>
<td>NS1, NS2</td>
<td>non-structural proteins</td>
</tr>
<tr>
<td>PB1, PB2, PA</td>
<td>polymerase proteins</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>SFC</td>
<td>spot forming cell</td>
</tr>
<tr>
<td>SIL</td>
<td>specific immune lysis</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TDM</td>
<td>trehalose 6,6’-dimycolate</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Vac</td>
<td>vaccinia</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

A. BACKGROUND

Influenza A virus infections cause morbidity and mortality on an annual basis (Stuart-Harris and Schild, 1976; Langmuir and Schoenbaum, 1977). This excess mortality is usually observed in the elderly and in individuals with chronic illness. Older children and adults have generally had previous infections with influenza A viruses, but mutations accrue at the major antibody combining sites allowing reinfections to occur (Webster et al., 1992). Therefore, even in the healthy adult population, influenza is a major cause of morbidity.

1. The Nature of Influenza A Viruses.

Influenza is a negative sense, single-stranded, segmented RNA virus from the Orthomyxoviridae family. There are three types of influenza in the Orthomyxo family, designated type A, B, and C. Only types A and B have caused serious disease in humans; and type A has caused the most epidemics due to its amino acid sequence variability. Influenza A virus has eight RNA strands that encode ten proteins including an RNA polymerase. The error prone RNA polymerase-dependent replication creates point mutations that can cause antigenic drift on the external glycoproteins, hemagglutinin (HA) and neuraminidase (NA) (Laver et al., 1979; Moss et al., 1980). Changes in these
surface glycoproteins leads to virus escape from the humoral response, variation in host species range, and changes in disease severity.

In addition, periodically a drastic antigenic change occurs in which a reassortment of genes from one virus subtype with the genes from another virus subtype occurs, and can result in antigenic shift. Antigenic shift may be dangerous if the new genes come from a subtype to which humans have not been previously exposed (Webster and Laver, 1975). Influenza subtypes are classified into different categories based on the antigenic differences between the viral proteins. The nomenclature of subtypes is indicated by the hemagglutinin (HA) and neuraminidase (NA) in parenthesis. Thus far, 15 different HA and 9 different NA antigens have been identified (Table 1-1). Within each subtype of influenza virus are numerous viral strains; indicated by the influenza type A or B, location of isolation, strain number, and year of isolation, for example, A/Puerto Rico/8/34.

2. The Epidemiology of Influenza A Viruses.

The aquatic bird reservoir is the primary source of influenza A virus strains because all of the influenza subtypes have been isolated from these animals (Table I-1) (Webster et al., 1992). Several influenza epidemics in modern times were caused by viruses that underwent antigenic shift, picking up genes from avian viruses (Table I-2). For example, the 1957 “Asian Flu” was caused by a H2N2 subtype influenza A virus that acquired three genes (PB1, HA, and NA) from avian viruses. Another example is the 1968 “Hong
Kong Flu" which was caused by a H3N2 subtype of influenza A virus that acquired two avian genes (PB1 and HA) (Murphy and Webster, 1996).

The origin of influenza A virus pandemics is a matter of intense debate. Since the mid-eighteen-hundreds most pandemics have appeared to originate in southern China. Inter-species transmission is possible there due to the close living conditions between individuals and their pigs/ducks (Webster et al., 1992). The H3, H4, and H6 subtypes of influenza A viruses have been isolated from the domestic avian population in southern China (Shortridge, 1982), and recently the H5 subtype was transmitted from domestic chicken to humans in Hong Kong (Subbarao et al., 1998). The 1918 pandemic may be unique in origin, possibly starting in the U.S. and being spread to Europe by U.S. troops (Crosby, 1989), but it is also possible that the 1918 pandemic originated in Europe.
Table I-1. Subtypes of Influenza A Viruses Isolated from Various Species\textsuperscript{a,b}.

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>Hemagglutinin</th>
<th>Species of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Humans</td>
<td>Swine</td>
</tr>
<tr>
<td>H1 (H0, H1, Hsw1)</td>
<td>PR/8/34</td>
<td>Sw/la/15/30</td>
</tr>
<tr>
<td>H2</td>
<td>Japan/305/57</td>
<td>-</td>
</tr>
<tr>
<td>H3 (Hav7 Heq2)</td>
<td>Hong Kong/1/68</td>
<td>Sw/Taiwan/70</td>
</tr>
<tr>
<td>H4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H5</td>
<td>HK/156/97</td>
<td>-</td>
</tr>
<tr>
<td>H6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N1</td>
<td>PR/8/34</td>
<td>Sw/la/15/30</td>
</tr>
<tr>
<td>N2</td>
<td>Japan/305/57</td>
<td>Sw/Taiwan/70</td>
</tr>
<tr>
<td>N3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N9</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The table was updated from (Murphy and Webster, 1996). A dash indicates that no strains have been isolated from that species.

The influenza A pandemic of 1918-1920 was the most severe in modern times with over 20,000,000 deaths (Collins, 1957). Most mortality occurred in young adults, unlike most influenza epidemics which cause mortality in the elderly and young children (Collins, 1957). There were no major influenza pandemics in the two decades prior to 1918, suggesting that a lack of immune memory may have contributed to the significant mortality among the young adults. The hemagglutinin of the virus which caused the 1918 pandemic has recently been partially sequenced from autopsy tissue and has swine-like H1N1 sequences (Taubenberger et al., 1997; Reid et al., 1999). Serologic studies of individuals who were alive during that pandemic also strongly suggest that the virus had a swine-like hemagglutinin antigen (Masurel and Marine, 1973).

It is not known why the 1918 influenza A virus caused such a high mortality rate. Several groups speculate that acceleration of HA cleavage by a number of possible mechanisms may have allowed the virus to spread systemically (Goto and Kawaoka, 1998). Influenza is usually limited to the respiratory tract because local trypsin-like enzymes, found only in the lung and tracheal tissue, can cleave the HA into two subunits to allow for the infection of the virus (Steinhauer, 1999). A more virulent influenza A virus has been hypothesized to use a more ubiquitous subtilisin-like protease, such as furin or PC6, to accelerate cleavage, while avirulent viruses utilize enzymes only found in the respiratory tract (Steinhauer, 1999). One way that other influenza A viruses have been able to use these more ubiquitous enzymes is if the virus has many basic amino acids at the cleavage site. It has been shown that influenza viruses with between 3 and 5
basic aa at the cleavage site can be cleaved by these subtilisin-like proteases and be more virulent (Kawaoka and Webster, 1988; Barr, 1991; Vey et al., 1992; Horimoto and Kawaoka, 1994). Interestingly, the 1918 strain HA sequence does not have the numerous basic aa at the cleavage site (Reid et al., 1999), and there is no evidence that the virus was spread systemically so the mystery of its virulence remains.

Other major influenza A virus pandemics in the last century include the H2N2 subtype 1957 “Asian” flu. This subtype only circulated for approximately 10 years. The H2N2 subtype may have been crowded out by the 1968 H3N2 subtype (Hong Kong) which rapidly caused a pandemic and has remained to circulate in the population with antigenic drift and to reinfect humans for over three decades. There was evidence that the immunity from the 1957 H2N2 virus provided some protection from the 1968 H3N2 strain, since areas hit heavily by the H2N2 virus suffered far less when the H3N2 virus arrived (Gill et al., 1971). The mortality during each of these pandemics occurred primarily in the elderly and small children, unlike the 1918 pandemic.
Table I-2. Influenza A Virus Epidemics or Pandemics Occurring in Humans in the Last Century \(^a\).

<table>
<thead>
<tr>
<th>Year</th>
<th>Virus Subtype</th>
<th>Excess Mortality Caused by Influenza A Viruses in U.S.</th>
<th>Avian or Swine Derived Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1918</td>
<td>Hsw1N1</td>
<td>500,000</td>
<td>All 10 genes? (Avian/Swine)</td>
</tr>
<tr>
<td>1957</td>
<td>H2N2</td>
<td>70,000</td>
<td>HA, NA, PB1 (Avian)</td>
</tr>
<tr>
<td>1968</td>
<td>H3N2</td>
<td>40,000</td>
<td>HA, PB1 (Avian)</td>
</tr>
<tr>
<td>1977</td>
<td>H1N1</td>
<td>50,000</td>
<td>Similar to H1N1 strains of 1950s</td>
</tr>
</tbody>
</table>

\(^a\)The sources: (Langmuir and Schoenbaum, 1977) and (Murphy and Webster, 1996).
4. Influenza A Virus Structure, Composition, and Pathogenesis.

Influenza A virus particles have been visualized by electron microscope as 120 nm in diameter with irregular sphere shapes that have spikes protruding from the surface (HA, NA) (Choppin et al., 1960). Immunogold electron microscopy has proven that the M2 protein is associated with the surface of the virus (Jackson et al., 1991). The eight RNA segments inside the virus exist as a ribonucleoprotein associated with nucleoprotein (NP) and the three polymerase proteins (PB1, PB2, and PA) in a helical structure. The terminal ends of each segment create a panhandle structure that is involved in the replication process.

The HA viral protein is found on the surface of the viral particle, and accounts for up to 25% of the total viral protein (Murti and Webster, 1986). This protein is important in attachment to sialyloglycolipids/sialyloglycoproteins and penetration into the cell. The RNA segment encoding HA is translated as a single polypeptide chain. Posttranslationally the molecule has an N-terminal signal sequence cleaved off, and then the polypeptide is cleaved again removing several intervening residues to create the HA1 and HA2 molecules which are linked by a disulfide bond (Murphy and Webster, 1990). This HA cleavage into the HA1 and HA2 subunits allows the virus to be infectious. At the N-terminus of the HA2 molecule there is a fusion peptide, very conserved between subtypes, which undergoes a conformational change (pH = 5) to allow fusion of the viral envelope to the endosome and release the virus particles into the cytoplasm (Murphy and Webster, 1990). Homology between different HA subtypes is variable, the two most homologous subtypes are the H2 and H5 (80% homology), and the two most divergent
are the H1 and H3 subtypes (25% homology)(Air, 1981). Certain cysteine residues and other amino acids are conserved between all of the HA subtypes (Air, 1981). The three-dimensional structure of the H3 molecule has been determined using X-ray crystallography (Wilson et al., 1981; Wiley and Skehel, 1987). The HA protein is a trimer composed of two regions, a triple-stranded coiled-coil of alpha-helices and a globular region composed of antiparallel beta-sheets (Wilson et al., 1981; Wiley and Skehel, 1987). The globular region contains the receptor binding site in a conserved canyon inaccessible to antibody, the surface area outside of this canyon is where the antigenic variability in each HA subtype occurs (Wiley and Skehel, 1987).

The other surface glycoprotein involved in subtype characterization is the NA viral protein. NA is a mushroom shaped spike with a square head created by four subunits with a stalk that attaches it to the membrane (Laver and Valentine, 1969; Wrigley, 1979). NA removes sialic acid from glycoproteins and has been shown to be involved in the release of the virus particles from inside of the cell, and the spread of virus from cell to cell (Itzstein et al., 1993). Blocking NA using the drug zanamivir has been shown to stop the spread of influenza virus to other cells (Woods et al., 1992). The crystal structure of the protein demonstrates that the NA folds into six, four-stranded, antiparallel beta-sheets, arranged like propeller blades (Colman et al., 1983; Varghese et al., 1983). There is a four-fold axis of symmetry and a conserved region at the catalytic site that binds sialic acid.

Concerning the internal structure, the RNA of segment 7 codes for both matrix proteins, M1 and M2. The M1 viral protein is found under the envelope and has a role in
maintaining the structure of the virion. It is also found in the nucleus and cytoplasm of infected cells and is thought to have a role in downregulating the transcriptase (Murphy and Webster, 1990). The M2 protein is encoded by a spliced mRNA. M2 is an ion channel that is activated by low pH. It is found on the virion surface and in the cell membrane of virus infected cells. It is found in high copy number in the infected cell and is essential for virus uncoating.

NS1 and NS2 are the non-structural proteins found in infected cells. NS1 is an RNA-binding protein that is abundant in infected cells and has been implicated in a number of functions. The NS1 protein inhibits mRNA polyadenylation to shut off host protein synthesis (Numeroff et al., 1998), binds mRNA poly-A tails to inhibit mRNA export (Fortes et al., 1994; Qiu and Krug, 1994), and inhibits splicing of pre-mRNAs (Fortes et al., 1994). NS1 can also bind dsRNA in vitro to prevent activation of PKR (the kinase induced by IFN) (Lu et al., 1995). Influenza virus deficient in NS1 is not pathogenic in wild type mice, but is pathogenic in mice lacking an IFN response (STAT-/- mice) (Garcia-Sastre et al., 1998). The NS2 protein is encoded from a spliced mRNA, and the role of NS2 is to transport viral RNA into the cytoplasm from the nucleus (O'Neill et al., 1998). It has recently been renamed NEP (nuclear export protein) because the protein is found in the virion and is structural (O'Neill et al., 1998).

NP is a part of the ribonuclear complex that makes up the backbone of the helices, along with PB1, PB2, and PA. It is involved in transport of viral RNA into the nucleus during infection and may have a role in transcription and replication, since mutations in NP cause defects in RNA synthesis (Beaton and Krug, 1986; O'Neill et al., 1995). The
RNA polymerase is a multisubunit enzyme made up of PA, PB1, and PB2 viral proteins (Fig. III-1). The proteins each have a nuclear localization signal and they accumulate in the nucleus once the cell is infected. The stem of the panhandle formed by the complimentary ends of the vRNA is the polymerase complex binding site. Transcription is initiated by a "cap snatching" mechanism in which the proteins cleave capped RNA from host cell messages to start the transcription of viral mRNA (Plotch et al., 1981). An as yet undefined protein is necessary to make the complex switch from a transcription unit to a replication machinery.
Fig. III-1. RNA Polymerase and Surface Glycoproteins of Influenza A.

- **NH2 to COOH**
  - **PB1**
    - PB2 Contact
    - Nuclear Signal
    - PA Contact
  - **PB2**
    - Nuclear, Cap Binding
    - PB1 Contact
  - **PA**
    - PB1 Contact
    - Nuclear Signal
  - **HA**
    - Signal Peptide
    - Cleavage Action
    - Hydrophobic Membrane Attachment Sequence (Virulent viruses insert basic aa)
  - **NA**
    - Hydrophobic Membrane Attachment Sequence
Influenza virus is spread from person to person through aerosol. Replication occurs throughout the respiratory tract, peaking at about 48 hours. Viremia in humans is extremely rare, as generally the infection is limited to the respiratory tract. Onset of illness is marked by symptoms such as headache, chills, and cough. Fever usually begins soon after and does not decrease until day 2-3. The temperature goes back to normal by day 6. Birds generally have an asymptomatic infection and shed a lot of virus in the feces due to replication of virus in the intestinal tract as well as the lungs. Swine show symptoms such as cough, fever, nasal discharge, and labored breathing (Murphy and Webster, 1996). Pigs may be the “mixing vessel” where viruses reassort, since both avian and human viruses can infect them. Influenza can also naturally infect horses, seals, whales, and mink. Experimental infections have been studied in mice, ferrets, and primates (Murphy and Webster, 1996).

5. The Role of Cytotoxic T Lymphocytes in Eradicating Virus Infection.

There are two arms of the acquired immune system that work together to protect the host and to eradicate viruses. The humoral arm encompasses B lymphocytes and the antibodies that they secrete, and the cell-mediated response includes CTL that can lyse virus-infected antigen-presenting cells (APC) and T helper cells, which secrete cytokines. Neutralizing antibodies can protect cells from being infected by blocking entry of the extra-cellular virus particles or promoting virus particle clearance by Fc-bearing cells, and can eradicate infected cells using antibody-dependent cell-mediated cytotoxicity. Neutralizing IgA specific for HA or NA is produced in airway secretions of humans in
response to influenza A infection and is detectable for at least 3-5 months post-infection (Murphy and Webster, 1996). The levels of serum antibodies to HA and NA have correlated with protection from illness following either natural exposure (Couch and Kasal, 1983) or experimental infection (Clements et al., 1986). Both live attenuated influenza virus vaccines and inactivated influenza A vaccines induce HA-specific antibody responses (Ennis et al., 1982).

CTL are involved in destroying virus-infected cells and are important in decreasing virus titers. Recognition of cognate antigen occurs through the T cell receptor (TCR). The TCR is made up of two chains, either an α polypeptide chain pairs with a β, or a γ polypeptide chain pairs with a δ. The remainder of this section will focus on the αβ TCR expressing CTL. The αβ TCR has a variable region important for antigen recognition, and a constant region important in T cell activation and membrane association (Moss et al., 1992). CTL become activated to lyse virus-infected cells by recognition of viral peptides bound to class I or class II major histocompatibility antigens (MHC) with their TCR. MHC molecules are polymorphic proteins that bind peptides inside of the cell and present them on the surface of the cell. The N and C-terminal portions of the peptide bind grooves on the MHC molecule and the TCR generally recognizes the middle portion of the peptide with its complementary determining regions (CDR). Class I molecules are expressed on all nucleated cells, while class II molecules are expressed constitutively only on professional APCs such as dendritic cells, macrophages, and B cells. CTL can express either CD8 or CD4 molecules. CTL expressing CD8 molecules recognize class I bound viral peptides, while CTL expressing
CD4 molecules recognize class II bound viral peptides. The mechanisms of cytotoxicity for both subsets include perforin/granzyme, Fas/Fas Ligand interactions, and TNF.

Besides killing virus-infected cells, CTL are important mediators of cytokine secretion. IFN-γ is a type II IFN primarily secreted by T cells and NK cells. It can activate mononuclear phagocytes, increase MHC class I and II expression on APCs, promote T and B cell differentiation, activate neutrophils, stimulate NK cytolysis, and activate vascular endothelial cells. In the lymphocytic choriomeningitis virus (LCMV) system, CD8 T cells produce IFN-γ in response to infection and proliferate extensively (Gessner et al., 1989; Biron, 1994; Cousens et al., 1995). IFN-α is an important early mediator and promotes the conditions in the LCMV model that stimulate IFN-γ secretion (Cousens et al., 1999). During influenza infection, IFN-αR-/- mice undergo a systemic infection, suggesting that IFN-α has a role in the tissue restriction of influenza A virus (Garcia-Sastre et al., 1998). In the influenza system, mice were found to have the maximal number of IFN-γ secreting T cells in the mediastinal lymph nodes (MLN) 7 days post influenza infection (Sarawar and Doherty, 1994), and increases in IFN-γ mRNA have also been detected at this time point (Baumgarth et al., 1994). This correlates with experiments where human PBMC were stimulated with influenza in vitro. The increase in IFN-γ-secreting cells was found to be mostly CD8+ T cells, and the increase in IFN-γ spot-forming cells (SFC) on day 7 post stimulation correlated with increased cytotoxicity on that day (Fabio et al., 1994).

The balance between the activation and action of CTL to limit virus infections and the destruction of healthy cells is very important. In several systems, the over-
activation of the immune system is implicated in the pathogenesis of the disease. Thus homeostasis between the activation of CTL and destruction by CTL must be maintained. An example of a breakdown in this homeostasis is in dengue virus infection. There is a higher risk of getting dengue hemorrhagic fever if the patient has been previously exposed to a different dengue virus serotype (Halstead, 1988). There is evidence of antibody dependent enhancement of dengue infection. Individuals develop non-neutralizing antibodies to other dengue serotypes during primary infection and are more susceptible to severe disease during a second infection with another serotype (Halstead and O'Rourke, 1977; Halstead, 1979). Memory cross-reactive T cells also are demonstrable after primary infection. During a secondary dengue infection, T cells were shown to be activated in vivo and secrete IFN-γ, which can support infection of the monocytes by upregulating FcγR (Kurane et al., 1991; Mathew et al., 1998). By the time the virus is cleared, the CD8+ T cells are actively increasing in number (Green et al., 1999), and shock due to capillary leak syndrome occurs as virus is being cleared. This suggests that the immune system, with CTL, play a role in dengue shock syndrome (Halstead, 1988; Kurane and Ennis, 1992).

Another example is observed in the influenza system where nude mice actually live longer than control mice after a lethal dose of influenza virus (Sullivan et al., 1976; Wells et al., 1981). This suggests that although the virus infection itself eventually may destroy the host, host immune responses can play a role in the pathology of virus infections. To support this theory, F5-RAG-1/- TCR transgenic mice that only have T cells which recognize aa 366-374 on NP of influenza were given a low-dose influenza
infection, and even in the absence of protective antibodies the infection is eradicated
(Moskophidis and Kioussis, 1998). Virus titers decrease by day 8, lung pathology is
confined to several foci of inflammation, and the mice survive. However, if high doses
of influenza virus are administered, the illness is exacerbated, lung pathology is severe,
and the percent survival is lower than the Rag-/- mice, which lack all T and B cells
(Moskophidis and Kioussis, 1998).

6. The Role of CTL in Eradicating Influenza Virus Infection in Mice.
The importance of virus-specific CTL in immunity to influenza A has been well
documented in the murine system. Influenza virus-specific CTL have been shown to
limit influenza A virus replication and to protect against lethal influenza A virus
challenge (Yap et al., 1978; Wells et al., 1981; Taylor and Askonas, 1986; Kuwano et al.,
1988; Mackenzie et al., 1989; Kuwano et al., 1990). CTL have been recovered from the
lungs of influenza A virus-infected mice (Yap and Ada, 1978) and recovery from
influenza virus infection correlates with clearance by CD8+ CTL (Mackenzie et al.,
1989). After primary influenza infection there are substantial numbers of CTL that are
virus-specific in the bronchoalveolar lavage (BAL) fluid of mice. With a secondary
challenge, CTL precursors (CTLp) proliferate in the regional lymph nodes and are
recruited to the lung to clear the virus (Flynn et al., 1998).

Virus-specific CTL clones that recognize epitopes on NP, HA, or NS1 virus
proteins and influenza virus-stimulated immune splenocytes adoptively transferred into
naive recipients, reduced pulmonary virus titers after influenza virus challenge (Wells et
Furthermore, transfer of CTL clones into influenza A infected mice has been shown by histology to enhance lung tissue recovery (Mackenzie et al., 1989). Our laboratory has shown that active immunization with an HA fusion protein, that induced HA-specific CTL but not neutralizing antibody, resulted in a reduction in peak lung virus titers after virus challenge, and protection against a lethal challenge dose (Kuwano et al., 1989). In vitro, murine epithelial cells can be infected with influenza A virus and killed by CTL (Nguyen et al., 1998).

Much work has been done in transgenic or knock-out mice in order to discern which cell populations are important during influenza A infection in vivo. Transgenic mice that lack β-2 microglobulin, and thus have no class I-restricted CTL, have delays in viral clearance and increased mortality after infection with a virulent strain of influenza virus (Bender et al., 1992). However, these mice have defects other than a simple lack of CD8+ T lymphocytes. In mice depleted of CD4+ T cells, virus is still cleared and the CTLp frequency of influenza-specific CD8+ CTL in the lungs or lymph node is not altered significantly (Allan et al., 1990). However, CD4+ virus-specific T cells may help compensate for the absence of CD8+ CTL, because the virus is eventually cleared in CD8+ depleted mice. β-2 microglobulin deficient mice depleted of both CD4+ and CD8+ CTL do not clear virus or survive (Eichelberger et al., 1991). Surprisingly IFN-γ knock-out mice recover from an influenza H2N2 primary infection as well as control mice (Graham et al., 1993). However, when H3N2-immune IFN-γ knock-out mice were challenged with a H1N1 subtype influenza virus, they were not able to completely clear
virus by day 7 (Bot et al., 1998). Therefore, IFN-\(\gamma\) may play an important role in the memory recall response.

These CD8+ and CD4+ CTL may be killing virus-infected cells and secreting IFN-\(\gamma\) or other anti-viral cytokines to help clear the virus. The mechanism that CD8+ CTL use to kill influenza-infected lung epithelia has been studied extensively. Perforin or Fas-deficient mice can clear influenza A viruses with or without CD4+ T cells, although there is delayed kinetics (Topham et al., 1997). The perforin-deficient mice were shown to have enhanced antibody and IFN-\(\gamma\) production after influenza infection suggesting a role for perforin in down-regulating the immune response (Sambhara et al., 1998). Thus, CD8+ CTL must utilize both mechanisms (and possibly others) to clear virus.

Additionally, CD4 depleted mice with perforin -/- bone marrow (thus no perforin-forming CD8+ T cells) could not clear virus from a Fas -/- influenza-infected respiratory epithelium, but could from a Fas +/- influenza-infected respiratory epithelium (Topham et al., 1997). Therefore, mice lacking both the effectors that make perforin and the targets that express Fas can not clear virus, ruling out the use of another mechanism for killing influenza-infected epithelial cells. A CD4-depleted mouse with perforin +/- bone marrow (normal CD8+ effectors) could clear influenza virus from either the Fas/-/ or +/- infected respiratory epithelium (Topham et al., 1997). These results suggest that primarily the Fas and perforin pathways are utilized in the destruction of target cells infected with influenza virus.
7. The Role of CTL in Eradicating Influenza A Infection in Humans.

CTL responding to influenza A infection of humans has also been studied. Human CTL responses to influenza were analyzed in a study where 63 volunteers were experimentally inoculated; virus shedding, serum antibody, and CTL responses were measured. Volunteers with detectable levels of serum antibody did not shed more than a trace of virus; those without detectable antibody had low levels of viral shedding if they had CTL responses specific for influenza (McMichael et al., 1983). This suggests that in the absence of antibody, CTL can play a role in defense from virus infection. In another report the cytokine responses in the nasal lavage of experimentally infected volunteers were analyzed at various times post-infection (Hayden et al., 1998). IL-6 and IFN-α peaked at day 2, correlating with viral titers and symptom scores. TNF-α peaked later at day 4, and IL-8 increased by day 6 (Hayden et al., 1998).

In humans, CTL have roles in many other virus infections. For example, humans with T cell deficiencies often have uncontrolled EBV infections that can become disseminated and even lethal (Purtillo et al., 1992). CTL specific for EBV have been isolated from both patients with acute and chronic EBV-infections (Couedel et al., 1999). There is also literature on CTL isolated from HIV-infected patients that were found to recognize HIV-infected cells (Plata et al., 1987; Walker et al., 1987). A correlation between the increase in HIV-specific CD8+ T cells and the decrease in viral burden leading to the asymptomatic phase of HIV has been demonstrated (Borrow et al., 1994;
Koup et al., 1994). Additionally, progression towards AIDS has been correlated with a decrease in the HIV-specific CTL precursor frequency (Carmichael et al., 1993). It has also been shown that prior to prolonged viral suppression with anti-retroviral therapy, HIV infected individuals have CD4+ CTL specific for HIV (Pitcher et al., 1999). CD4+ and CD8+ T cells have been shown to recognize Env, Gag, and Pol HIV proteins (Kundu and Merigan, 1992). HSV is another virus that infects humans, and virus-specific CTL have been detected (Posavad et al., 1996). A correlation between CTL and clearance of HSV-2 from recurrent genital lesions has been demonstrated (Koelle et al., 1998).

Control of rhinovirus infections has been reported to involve CTL. Enhanced IL-2 and IFN-γ production were demonstrated in the PBMC of individuals experimentally infected with rhinovirus (Hsia et al., 1990). Experimental rhinovirus infection in humans was also shown to induce T cell responses in the PBMC (Hsia et al., 1990).

B. THE CYTOTOXIC T LYMPHOCYTE MEMORY REPERTOIRE SPECIFIC FOR INFLUENZA A.

In order for CTL to kill virus-infected APCs, they need to be either induced to become effector cells from naive cells or to be present in the memory population. Naïve T cells can survive long-term in the periphery if they receive TCR ligation through autologous MHC molecules (Freitas and Rocha, 1999). For naïve T cells to be induced to become virus-specific effectors, they need a signal by the autologous MHC-restricting allele containing antigenic peptide, and a second signal from co-stimulatory molecules such as
the B7 family members (Linsley et al., 1990). A longer period of stimulation is necessary for naïve cells to produce cytokines than for effector or memory cells (Bachmann et al., 1999). Once naïve cells are stimulated to become effector cells their role is to quickly destroy virus-infected cells. This may lead to recovery but also may result in immunopathology, as described earlier. Eventually these cells may enter the long-lived memory T cell pool.

1. The CTL Repertoire Specific for Influenza A in the Mouse is Limited.

After a primary infection with influenza virus, mice generate an increased influenza-specific CTLp frequency that peaks on day 7 and decreases by day 14. The memory T cells that persist can remain for the life of the mouse (Effros et al., 1977; Askonas et al., 1982; Owen et al., 1982; Ada and Jones, 1986; Flynn et al., 1999). There are different hypotheses on how the long-lived memory T cells are maintained. It has been proposed that antigen persists, possibly on follicular dendritic cells (DC), allowing constant re-stimulation of the memory T cells (Rooijen, 1992). However, no influenza virus RNA is detected by PCR two weeks post-infection (Eichelberger et al., 1991). Another group has shown in the mouse that the memory T cell population changes after subsequent infection with different viruses (Selin et al., 1998). Cross-reactivity with the low-affinity epitopes of other viruses may be a means of retaining influenza A-specific T cell memory, since memory T cells have been shown to be inducible with less signal (Beverly, 1990; Tanchot et al., 1997). There is also the theory that T cells recirculating through activated environments rich in cytokines will lead to bystander activation of T cells. This theory
relies on the fact that CD8+ T cells can be isolated from the lungs of influenza-infected mice that are not pathogen-specific, but have CD44^{hi} L-selectin^{low} activation markers (Hou and Doherty, 1993). On the other hand during acute infection of the HY-transgenic mouse with LCMV, the HY-specific cells do not enter blastogenesis and are diluted at the time of acute infection (Zarozinski and Welsh, 1997). Furthermore, recent techniques have been developed to enable a more accurate enumeration of virus-specific CTL during an acute LCMV infection and most of the activated T lymphocytes were virus-specific (Butz and Bevan, 1998; Murali-Krishna et al., 1998).

The CTL repertoire specific for influenza A viruses in inbred mice has been shown to be limited during primary and secondary infections. It has been reported that there are low or non-responder class I alleles for influenza A CTL responses (Bennink and Yewdell, 1988). For example, the L^d molecule was the restricting allele for only one of the influenza A viral protein expressing vaccinia constructs tested (NS1) (Bennink and Yewdell, 1988). Furthermore, D^d, D^k, and K^b were all found to be non-responder alleles for CTL responses to NP (Pala and Askonas, 1986). In both mice and humans the CTL responses detected in bulk culture were previously reported to be primarily directed at NP (Townsend et al., 1984; Townsend and Skehel, 1984; Yewdell et al., 1985; McMichael et al., 1986). The recognition of influenza virus by CTL in inbred mice was commonly found to be limited to immunodominant epitopes on one or two viral proteins (Townsend et al., 1986; Vitiello et al., 1996). During secondary exposure to influenza, CTL that recognize immunodominant epitopes again markedly expand (Flynn et al., 1999). Furthermore, nonimmunodominant epitopes on NP have also been reported, but
immunization with such epitopes did not protect mice from subsequent challenge as efficiently as immunization with the immunodominant epitope (Oukka et al., 1996).

The impact of a limited T cell repertoire specific for a virus (e.g. to one immunodominant epitope), has been studied in mouse models. When the potential repertoire was limited by using a mouse with a rearranged β-chain, influenza could still be cleared with only a slight delay; but the epitopes recognized by CTL were different than that of wild type mice (Daly et al., 1995). Theoretically, having CTL that recognize many different epitopes would be beneficial in case a mutation in the immunodominant epitope occurred. Oukka et al. found that immunization with both the immunodominant and subdominant epitopes of influenza was more protective than immunization with the immunodominant epitope alone (Oukka et al., 1996). This would argue that a broad response may be beneficial.

Acute LCMV infection of BALB/c mice generates T cells specific for NP 118-126 which is restricted by L^d. However, spleen cells which recognize a subdominant epitope GP 283-291 can clear a chronic LCMV virus infection from H-2^dm2 mice (L^d negative), suggesting that subdominant epitopes play a role in clearance also (Most et al., 1997). Oldstone et al. have shown that mice infected with LCMV variants which have changes in the immunodominant epitope, develop a CTL response to a different epitope on another protein (Oldstone et al., 1995). This suggests that although mice have a limited repertoire to some viruses, there is flexibility in the immune system allowing the generation of a response to a once silent epitope.

2. The CTL Repertoire Specific for Influenza A Viruses in Humans.
Our laboratory has previously shown that long-lived memory T cells exist in humans. Vaccinia virus-specific T cells can remain in the PBMC many years post-vaccination for smallpox (Demkowicz, 1993). In another system, the live attenuated measles vaccine provides long-term protection (Dhib-Jalbut and Jacobson, 1994). Also, volunteers who received experimental dengue vaccination exhibited CTL memory several years post vaccination (Green et al., 1993; Zeng et al., 1996; Green et al., 1997). In several donors we have also observed influenza-specific memory T cells that recognize the same viral proteins up to a decade later. But these donors may have been exposed to influenza A virus in the intervening years (unpublished data by Julie Jameson).

An early study using influenza stimulated bulk cultures of PBMC of six humans demonstrated that 6 of 6 donors’ PBMC recognized NP, 4 of 6 recognized PB2, 6 of 6 recognized M1, 1 of 6 recognized M2, and there was no recognition of the other six viral proteins (Gotch et al., 1987). After this study was published, many groups focused on NP or M1, often referring to them as the immunodominant influenza viral proteins. Previous groups had also identified several CTL clones in humans that recognize influenza A virus epitopes, (see Table 1-3). Most of these CTL clones were isolated from different individuals and were specific for epitopes on NP. The M1 aa 58-66 epitope which is HLA-A2.1 restricted was recognized by the PBMC of humans in several studies (Gotch et al., 1987). Since the A2.1 allele is a common HLA allele in the Caucasian population, this epitope has been studied extensively and been referred to as an immunodominant epitope by many groups. It is important to define influenza viral protein and epitope recognition at the clonal level in order to determine if the human CTL
memory response is restricted to a small number of immunodominant epitopes on a few proteins, or to a wider number of epitopes on many different viral proteins. It remained to be determined whether a single individual has a multispecific memory response, and whether many different HLA alleles are used by the APCs of each individual to present influenza A peptides. Furthermore, once the CTL become established as a part of the host’s repertoire, it is important to determine whether they continue to persist over time.
Table I-3. Previously Characterized Human CD8+ CTL Epitopes on Influenza A Viral Proteins.

<table>
<thead>
<tr>
<th>HLA</th>
<th>Viral Protein</th>
<th>Restricting Allele</th>
<th>Amino Acids</th>
<th>Amino Acid Sequence</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>B37</td>
<td>NP</td>
<td>342-351</td>
<td>FEDLRVLSFI</td>
<td>J. Exp. Med. (1986) 164:1397</td>
<td></td>
</tr>
</tbody>
</table>
C. HETEROTYPIC IMMUNITY SPECIFIC FOR INFLUENZA A.

If an influenza virus variant evades neutralizing antibody, other components of the immune system, such as CTL, can decrease morbidity and mortality (Schulman and Kilbourne, 1965; Anker et al., 1978; Couch and Kasal, 1983; Nguyen et al., 1999). This decrease in illness associated with challenge by influenza viruses of a different subtype is characterized as heterotypic immunity. Heterotypic immunity to influenza A has been observed in mice and humans (Schulman and Kilbourne, 1965; Anker et al., 1978; Couch and Kasal, 1983).

1. Mice Can Recognize Influenza A Virus Strains of Differing Subtypes.

Our laboratory has previously shown that adoptive transfer of a subtype cross-reactive NS1-specific CTL clone reduced virus titers in mice infected with H1N1, H2N2, and H3N2 influenza viruses (Kuwano et al., 1990). Mice immunized with an H3N2-subtype of influenza virus were protected during challenge with a virulent H3N8 virus (Riberdy et al., 1999). When T cells were depleted during the H3N8 infection, there was a delay in viral clearance. Furthermore, Ig-/-μMT H3N2-immune mice could clear low doses of the virulent H3N8 strain, suggesting that the cross-reactive CTL could decrease illness if there was enough time to expand to large enough numbers (Riberdy et al., 1999).

These and other findings revive the question regarding the effector mechanisms of heterotypic immunity. Heterotypic immunity occurs in the absence of neutralizing antibodies and it is thought to be mediated by CD8+ CTL. Nguyen et al. found that protection from lethal heterotypic influenza virus challenge by the mucosal route
correlated with heterotypic CTL in the mucosa-associated lymphoid (MAL) tissue (Nguyen et al., 1999). Long-lived CTL memory was induced at this site after mucosal immunization (Nguyen et al., 1999). The memory CTL can be activated, since secondary heterotypic influenza virus challenge has been shown to lead to a massive proliferation of NP 366-374 specific CTL in C57BL/6J mice during acute infection (Flynn et al., 1999). However, β2-m -/- mice have heterotypic immunity (Eichelberger et al., 1991; Bender et al., 1994; Epstein et al., 1997), and depletion of CD8+ T cells in the nose, trachea, or lung by mAb led to only a partial abrogation of heterotypic immunity (Liang et al., 1994). There may be an important balance between CD4+ and CD8+ T cells in protection from heterotypic influenza challenge. Furthermore, their location (ex. in lungs or MAL), precursor frequency, and virus dose all seem to play a role in protecting against subsequent challenge. Another factor involved may be the Th1 or Th2 nature of the cytokine environment. It was recently observed that stimulation with inactivated virus vaccine initiated a Th2 response that could not clear heterotypic influenza virus infection, however if a Th1 environment was established (+IL-12,-IL-4) then a heterotypic infection could be cleared (Moran et al., 1999).

2. Heterotypic Immunity Specific for Influenza A Viruses in Humans.

There have been few reports concerning cross-subtype protection of humans later infected with an influenza A virus of another subtype. One study observed Japanese students during the 1977-78 influenza season. When an H1N1 virus strain infected primary and middle school students in December of 1977, significantly fewer students
were infected and sick from the H3N2 virus subtype that caused another epidemic a month later as assessed by HAI antibodies and clinical features. This suggests that previous infection with the H1N1 virus conferred cross-reactive immunity (Sonoguchi et al., 1985). It is likely that the H3N2 virus escaped the neutralizing antibody response to the H1N1 virus so this protection was likely to have been mediated by cross-reactive CTL. Couch et al. followed individuals who had a documented H3N2 infection over a four year time span. He found that resistance to a homologous virus strain lasted at least 4 years (Couch, 1975), and this immunity also extended to a different strain of H3N2 (Couch and Kasal, 1983). In other studies he observed the heterologous protection for up to 7 years (Couch and Kasal, 1983), but this did not occur with all strains. The history of influenza epidemics provides another example. In the early H3N2 era, persons with a previous H2N2 infection had a significantly lower attack rate upon first exposure to the new H3N2 subtype of influenza virus infection (Hope-Simpson, 1971; Hope-Simpson, 1972; Gill and Murphy, 1977). This immunity may have been due to NA2 specific antibodies as well as T cell memory responses.

With the possibility of point mutations and reassortment, influenza A viruses of swine and avian origin have the potential to evade neutralizing antibody just as the H1N1, H2N2, and H3N2 human influenza viruses have. There have already been several examples of direct transmission of non-human influenza A subtypes into humans. During the winter of 1976 a soldier at Fort Dix died from an influenza A virus infection (A/New Jersey/76) that was later characterized as having HA and NA glycoproteins (Hsw1N1) similar to swine species. A serological survey found that several hundred
infections had occurred and at least 12 clinical cases were observed with person-to-person transmission suspected (Langmuir and Schoenbaum, 1977). This ignited the fear that this virus would cause a global pandemic like the 1918 swine-like virus had caused. Therefore, health officials ordered the development of an influenza A vaccine. Once the vaccine was finally administered, it was found to increase the chances of developing Guillain-Barre syndrome, and the program was terminated. Fortunately, there was no further spread of the swine virus outside of Ft. Dix.

3. H5N1 Subtype Influenza A Virus Infection of Humans in 1997.

The threat of an epidemic by a novel subtype of influenza occurred again in 1997 when an H5N1 influenza A virus was isolated from a 3 year old boy in Hong Kong. This child did not survive the infection and his was the first of six deaths in Hong Kong due to H5N1 influenza A virus infection (Doepel, 1998). The virus isolated was found to be similar to avian H5N1 viruses and those infected were known to have had contact with chickens (Suarez et al., 1998; Subbarao et al., 1998). All eight segments of the 1997 H5N1 influenza A virus were derived from an avian virus and had varying degrees of conservation with human strains (Subbarao et al., 1998). The internal viral proteins had the highest percent of conservation ranging from 83.3% homology for PB2 to 91.2% homology for M1 (Subbarao et al., 1998).

This was the first documented instance of an H5 subtype of influenza A virus natural human infection and of direct transmission of this virus from birds to humans. Avian viruses usually utilize the sialic acid α2,3 galactose determinants, whereas human
strains generally use sialic acid α2,6 galactose determinants (Webster, 1999). The isolate from this child (A/HK/156/97) was found to be virulent in chickens causing a systemic infection (Subbarao et al., 1998). Viral antigen was recovered from blood vessels, pulmonary capillary endothelium, and macrophages of chickens infected with the H5N1 isolate (A/HK/156/97)(Subbarao et al., 1998). Other H5 virus isolates have been previously detected in chickens and waterfowl in Mexico, and in 1983-84 there was an outbreak of virulent H5N2 virus in U.S. poultry farms (Webster et al., 1992). The viremia caused by these H5 viruses has been observed in naïve mice challenged with another virulent subtype of influenza virus (H3N8); virus was isolated from the brain, liver, and spleen (Riberdy et al., 1999).

It was important to determine whether human cross-reactive CD8+ and CD4+ CTL would recognize targets infected with influenza A virus strains of swine and avian species including the H5N1 virus strains recently isolated from patients in Hong Kong. Older children and adults living in the United States of America and elsewhere in the world in 1997 possess influenza A virus cross-reactive memory T cells that recognize the human subtypes, but some of these T lymphocytes may also recognize epitopes on the H5N1 avian-derived virus strains which caused human illness. If such heterotypic CTL existed in high enough precursor frequency they may restrict virus replication and prevent severe illness and morbidity. Since 2/3 of the people infected with the H5N1 avian influenza virus survived, it is possible that some humans have long-lived cross-reactive immunity to this virus.
D. INFLUENZA VACCINES HAVE BEEN DEVELOPED TO BOOST CTL.

The currently licensed influenza vaccine is composed of formalin-inactivated, detergent-disrupted virus particles. This vaccine is designed to induce antibodies that recognize surface HA and NA of the homologous and closely related influenza A virus strains. However, if a variant strain emerges, neutralizing antibodies could not provide protection because the major antigenic epitopes are on the variable surface glycoproteins. This can be demonstrated by the yearly outbreaks of diverse influenza strains that mutate and evade neutralizing antibodies, causing morbidity and approximately 10,000 deaths a year in the U.S. (Murphy and Webster, 1996). As a strategy to deal with this antigenic variation, our laboratory has focused on the development of influenza A vaccines that also augment virus cross-reactive T lymphocyte memory. CTL memory may be a second line of defense against serologically distinct influenza viruses by clearing infected cells and thereby decreasing the severity of illness (Kuwano et al., 1989; Kuwano et al., 1990). It is important to learn how to induce and augment cross-reactive CTL responses with killed or subunit vaccines in general and with the influenza vaccine in particular because of the high degree of antigenic variation between virus strains.

1. Getting Antigen into the Class I Pathway to Activate CTL.

The key to inducing a strong CTL response is to get the antigen presented by the class I pathway of the APC. The class I pathway usually only presents antigens synthesized in the cytoplasm, such as endogenous self antigens and viral proteins produced by live virus (reviewed in (Rock, 1996)). Proteins found in the cytoplasm are degraded by
proteasomes and proteases. The resulting oligopeptides can then be transported to the endoplasmic reticulum (ER) by means of the transporter associated with antigen processing (TAP), where the peptides can be further modified. Next, peptides bind to MHC molecules, stabilizing the MHC and the complex traffics to the surface of the cell. If an adjuvanted particle or liposome can help protein antigens enter into the cytoplasm, a peptide epitope may be able to be presented by the class I pathway. Some groups have also targeted peptides to the ER in order to skip proteolysis and transport (Restifo et al., 1994). Another way that an exogenous antigen is capable of entering the class I pathway is by penetrating into the cytosol on its own. Examples of such antigens are fusogenic proteins on viruses that allow membrane entry (Yewdell et al., 1988). Lastly, there are some cell types that have been implicated in exogenous antigen presentation by MHC class I molecules. These include certain types of macrophages and dendritic cells. Although the inactivated influenza vaccine has not been shown to induce CTL, it can elicit CD8+ CTL responses if presented by dendritic cells (Bender et al., 1995).

2. Vaccine Strategies Previously Developed to Induce CTL.

There have been several vaccination strategies developed that attempt to induce CTL activation by vaccination. Liposomes were created to mimic the natural virus infection using a lipid bilayer containing the viral glycoproteins. The liposomes were reconstituted with influenza glycoproteins HA and NA and delivered intranasally (i.n.) or intramuscularly (i.m.) to mice (Guink et al., 1989). Both routes of vaccine administration elicited antibody responses and provided protection (Guink et al., 1989). CTL are also
induced and activated by the presentation of the liposomal antigens on both macrophages and subsequently dendritic cells (possibly through the transfer of antigen) (Nair et al., 1994).

Another vaccination strategy utilizes plasmid DNA encoding antigenic proteins to induce both humoral and cell-mediated responses. The DNA vaccine is either dissolved in saline and delivered by injection or attached to gold beads and delivered by the gene gun. An i.m. injection of a plasmid encoding NP induced a strong T cell response to the NP 147-155 epitope in Balb/c mice (Fu et al., 1999). The mice were also protected against a lethal infection with an influenza virus of another subtype (H3N2) (Fu et al., 1999). This method allows for the presentation of antigen by both the class I and class II pathways, since both endogenous and exogenous antigen are made. DNA vaccination with NP was found to prime CD8+ CTL and CD4+ T helper cells (Ulmer et al., 1998). If these NP DNA vaccinated mice were depleted of either CD4+ or CD8+ T cells by mAb there was still protection against subsequent heterotypic challenge (Ulmer et al., 1998).

Another vaccine approach is the use of a particulate adjuvant called immune-stimulating complexes (Iscoms). Iscoms are cage-like particles approximately 35 nm in diameter made up of cholesterol, phospholipid, the antigen of interest, and Quil A (Morein et al., 1984). Iscoms are taken up by APCs such as macrophages, monocytes, dendritic cells, and naïve B cells (Morein et al., 1994). They can enhance Th1 and Th2 cytokine production by antigen-specific T cells (Morein et al., 1995) and disseminate rapidly from the injection site to draining lymph nodes (Watson et al., 1989). Other groups have shown that Iscoms can upregulate MHC class II molecules on monocytes...
(Watson et al., 1989). Inside APCs, Iscoms associate with lipid membranes, thus localizing to the cytosolic or vesicular compartments. This allows for presentation by both class I and class II MHC molecules, but class I presentation is inhibited in TAP-deficient T2 cells, suggesting a role for TAP (Binnendijk et al., 1992). Iscoms have been used with various antigens and tested for antibody responses, CTL responses, and protection in animal models (Barr and Mitchell, 1996).

3. Immune Responses to the Flu-Iscom Vaccine in Mice.

Immunization of mice (i.n.) with Iscoms incorporating the HA and NA influenza surface glycoproteins elicited antibody (IgM, IgG, and IgA) responses that were comparable to antibody titers elicited during influenza virus infection (Jones et al., 1988; Lovgren and Morein, 1988). The influenza-specific antibody-secreting cells were found in the lung along with influenza-specific CTL precursors post-vaccination. After vaccination with the HA, NA Iscom vaccine, mice were protected from subsequent i.n. challenge with influenza virus (Sundquist et al., 1988; Lovgren et al., 1990). Influenza NP was integrated into Iscoms by LPS attachment and protected mice when delivered orally (Barr and Mitchell, 1996). Later, Iscom vaccines were formulated with inactivated, disrupted influenza virus (Flu-Iscom). Flu-Iscom vaccination was shown to induce antibody and CTL responses in mice and induce heterotypic immunity against influenza A virus challenge (Sambhara et al., 1998). Flu-Iscom vaccinated mice had a 10-fold higher hemagglutination inhibition titer than influenza monovalent subunit vaccinated mice and were protected from mortality by a different subtype of influenza A
virus challenge than the vaccinating strain (Sambhara et al., 1998). In another system, recently, an Iscom vaccine incorporating the surface glycoproteins of the A/Hong Kong/156/97 (H5N1) strain was demonstrated to provide protection in roosters challenged with the virulent H5N1 influenza virus (Rimmelzwann et al., 1999). An increase in the H5-specific antibody titer was observed, but CTL activity was not assessed (Rimmelzwann et al., 1999).

4. Immune Responses to the Flu-Iscom Vaccine in Humans.

Our laboratory has reported increases in influenza-specific bulk culture CTL memory in the PBMC of human vaccine recipients who received Flu-Iscom vaccines and vaccines with empty Iscomatrix particles mixed with inactivated influenza virus (Flu-Iscomatrix) (Ennis et al., 1999). In a double-blind study, only 5% of the donors who received a single dose of the standard influenza vaccine (Fluzone) had an increase in CTL bulk culture cytotoxicity, but 50% of the Flu-Iscom and 30% of the Flu-Iscomatrix recipients had increases in influenza A virus-specific CTL cytotoxicity. These increases in the killing of influenza-infected B-LCL by bulk culture PBMC could be due to increases in the number of CTL specific for influenza or possibly due to an increase in the avidity of memory cells to influenza A epitopes. It is important to discern if these increases in cytotoxic T cell activity were due to an increase in influenza virus-specific CTL post-vaccination, and if these CTL can be activated to secrete IFN-γ upon exposure to influenza virus.
E. THE ROLE OF γδ T LYMPHOCYTES IN INFLUENZA A VIRUS INFECTION.

The importance of T cells in protection from influenza A virus infection is well established, but the role of the γδ population of T cells has not been well described. Although αβ TCR expressing CTL make up most of the T lymphocytes in the blood, there is a population of γδ TCR expressing cells, usually 1-10% of the PBMC, whose function in viral infections is not well understood. γδ TCRs are CD3+, and the lengths of the γδ TCR CDR3 are closer to that of immunoglobulins, suggesting a different type of antigen recognition than by αβ TCRs (Rock et al., 1994). In mice, γδ T cells with distinct TCRs are localized to different tissues. For example, the invariant Vγ3/Vδ1 TCR is localized to the skin, while the Vγ4/Vδ1 TCR is found in the reproductive tract and tongue (Boismenu and Havran, 1998).

Extensive studies in humans have shown a more complicated distribution. In general the Vδ1 subset makes up 30-60% of the epithelial γδ T cells and only 1-20% of blood, while the Vδ2 subset makes up 10-30% of the epithelial γδ T cells and 50-90% of the blood γδ T cells (Wallace et al., 1995). Human blood γδ T cells are mostly Vγ9Vδ2 TCR+ with extensive diversity at the CDR3 region (Casorati et al., 1989). This population of γδ T cells has been shown to proliferate in response to mycobacteria and to be activated by nonpeptidic antigens such as alkyl phosphate molecules, a triphosphorylated complex, and prenyl phosphate antigens (Constant et al., 1994; Tanaka et al., 1994; Bukowski et al., 1995; Burk et al., 1995; Bukowski et al., 1999). Recently it
has been shown that this recognition relies on the junctional regions of the γ chain, along with Vδ2 pairing (Bukowski et al., 1998). Most γδ T cells are CD4-CD8-, but there are CD8+ and CD4+ subsets. Similar to αβ T cells, γδ T cells can secrete cytokines such as IL-4, IL-10, and IFN-γ, can upregulate activation markers such as CD25 and CD69, and can be cytotoxic to APCs infected with viruses or bacteria (Wallace et al., 1995).

1. γδ T Lymphocyte Responses Specific for Influenza in the Mouse.

γδ T lymphocytes expressing Vγ4 and Vγ7 are resident in the mucosal surfaces of the lung in C57BL6/J mice (Carding et al., 1990). When C57BL6/J mice are infected with influenza A H3N2 subtype virus, γδ T cells increase in number in the BAL after the infection has resolved (days 10-13) (Carding et al., 1990). After influenza virus infection with the X-31 strain (H3N2), an expansion of the resident Vγ4 population is seen up to day 13, and after day 13 an influx of Vγ2 cells take over (Carding et al., 1990). When mice were initially infected with a H3N2 influenza virus and then were given a H1N1 secondary influenza A infection one month later, γδ T cells were detected in the BAL by day 5, exhibiting a more rapid response than after challenge with a divergent influenza B virus (Allan et al., 1992). This suggests that there may be either memory γδ T cells or that the memory αβ T cells localize the γδ T cells to the site of infection faster after secondary challenge. Furthermore, mice infected with different strains of influenza have different Vγ expression, suggesting specificity (Carding, 1990). In vitro work on influenza virus X-31 (H3N2)-immune TCR β-/- mouse γδ T cell hybridomas has shown
that they produce IL-2 in response to influenza A and B infected cells, but not from cells infected with other viruses. No response could be detected to any of the influenza A virus proteins expressed in vaccinia constructs. These hybridomas seemed to recognize HSP-60 (Ponniah et al., 1996).

Trehalose 6,6'-dimycololate (TDM) is a component of the mycobacterial cell wall, and treatment with TDM leads to the accumulation of γδ-expressing cells in the lung. When mice are treated with TDM, they acquire resistance to influenza infection. These γδ cells are cytotoxic to targets transfected with either the H1 or H3 subtype of HA, but in a MHC non-restricted fashion (Hoq et al., 1997). Furthermore, TDM treatment of TCR δ -/- (no delta chain) mice prior to influenza infection did not provide protection in C57/BL/6J mice and provided only slight protection in Balb/c mice (Hoq et al., 1999). This suggests that the protection from influenza infection with TDM treatment is partially mediated by γδ T cells. γδ T cells have also been shown to play a major role during other pulmonary diseases; for example, Nocardia asteroides resulted in death within 14 days in TCR δ -/- mice, but control mice could clear the pathogen (King et al., 1999).

2. γδ T Lymphocytes Responses in Humans.

Several groups have reported increases in γδ T cells in humans after a viral infection or else isolated γδ T cell clones that recognize and kill virus infected APCs. The mechanism of these expansions or the recognition by the T cells has not been fully resolved. IL-12 has been shown to activate human γδ T cells in vitro, through a TNF-α dependent mechanism (Ueta et al., 1996). γδ T cells have been analyzed in several
human infections, such as Lyme disease (Vincent et al., 1998), HSV-1 infection (Maccario et al., 1993), EBV infection (Hacker et al., 1992), and HIV infection (Boullier et al., 1997). Lyme disease is caused by a spirochete *Borrelia Burgdorferi* transmitted by a tick that causes a nonspecific febrile illness that can become a chronic synovitis if not treated effectively. There are increased numbers of γδ T cells in the synovial fluid (Vincent et al., 1996), and γδ T cells expand rapidly when exposed to Borrelia, especially if dendritic cells and IL-2 are present (Vincent et al., 1998). The clones analyzed were Vδ1 paired with various Vγ chains and the antigen they recognized was thought to be a lipoprotein (Vincent et al., 1998).

In another system, the PBMC of HSV-1 immune donors were stimulated with autologous HSV-1 infected cells, and lysis of infected targets was reported and found to be partly due to γδ CD8+ T cells (Maccario et al., 1993). It was also reported that Vγ9/Vδ2 T cells from HSV-seropositive donors could grow out if cultured with HSV-infected cells (Bukowski et al., 1994). The γδ T cells were able to lyse HSV-infected targets in a non-MHC-restricted fashion (Bukowski et al., 1994). It is interesting that these T cells were also able to lyse vaccinia virus-infected targets, suggesting that a ligand may be induced by virus infection. Furthermore, this response was not blocked by anti-HSP antibodies. Murine γδ T cell clones that recognize glycoprotein 1 on HSV-1 were also isolated and found to be MHC-unrestricted (Johnson et al., 1992).

In acute EBV infections, the percentage of γδ T cells in the PBMC is increased, and even remains elevated during the convalescent phase (Paoli et al., 1990). There is a population of CD4- CD8- Vδ1 T cells that respond to either autologous or allogeneic
EBV-transformed B cell lines, but cell-cell interaction is necessary (Orsini et al., 1993). This response is independent of γ chain utilization. γδ T cells with another type of TCR, Vγ9/Vδ2, have been shown to be able to lyse and proliferate in response to the Daudi cell line (EBV-associated Burkitt’s lymphoma) (Fisch et al., 1990; Fisch et al., 1990; Fisch et al., 1992; Hacker et al., 1992; Bukowski et al., 1995). If antibodies were used to block the HSP 60 family, there was no outgrowth of this T cell subset which suggests a role for this molecule in γδ T cell stimulation (Fisch et al., 1990; Selin et al., 1992; Kaur et al., 1993).

The number of circulating γδ T cells is also increased in the PBMC of HIV-infected adults (Autran et al., 1989; Paoli et al., 1991; Maria et al., 1992). The expanded γδ T cell population in the periphery utilizes the Vδ1 gene, while the Vδ2 expressing γδ cells are underrepresented there (Boullier et al., 1997). These γδ T cells have been reported to express HLA-DR (Kabelitz et al., 1991), CD8 (Paoli et al., 1991), and CD38 (Boullier et al., 1995). More Vδ1+ cells from HIV-infected patients’ PBMC secreted TNF-α and IFN-γ upon stimulation than from control PBMC (Boullier et al., 1997). The role of γδ T cells in human diseases remains to be determined.

Many groups have speculated how the γδ T cells recognize the virus-infected cells. In the case of HSV-specific Vγ9/Vδ2 T cells, the lysis of infected targets could be blocked with an antibody to the TCR and also with an antibody to CD3 (Bukowski et al., 1994). This suggests that the γ and δ chains are important for the activation of γδ T cells. Recently, natural killer inhibitory receptors were found to be expressed on Vγ9/Vδ2 T
cells (Fisch et al., 1997; Halary et al., 1997; Bakker et al., 1998; Norris et al., 1999). The Vγ9/Vδ2 cells which were lytic expressed the natural killer inhibitory receptors while those without killing activity did not express the inhibitory receptor (Halar et al., 1997). The killer inhibitory receptors were found to inhibit lysis of MHC class I+ tumor B cell lines by the Vγ9/Vδ2 T cells. These receptors were not expressed on the Vδ1 subset of γδ T cells (Fisch et al., 1997). γδ T cells that express the natural killer inhibitory receptors may have a role in recognizing tumor cells that lack MHC class I expression.

F. THESIS OBJECTIVES.

1. The first objective of my thesis project was to determine the human CTL repertoire to influenza A virus by isolating and characterizing cytotoxic T lymphocytes from human donors.

2. Next, I used the CTL lines to determine whether human CTL lines recognize cells infected with non-human viruses, such as avian and swine species. PBMC were stimulated in bulk culture to look at recognition of these non-human viruses. I characterized the epitopes that these CTL recognized to analyze why they do or do not recognize the virus strain.

3. I wanted to determine whether these CTL could be boosted by an influenza vaccine comprised of Iscom particles and inactivated virus. I first used in vitro techniques to analyze cytotoxicity, and then I analyzed the PBMC from volunteers in a vaccine trial. The number of IFN-γ secreting cells in the PBMC were analyzed at several time points post-vaccination.
4. γδ T cells have a role in resolving influenza A infection or healing injured tissue. I determined whether they could recognize influenza-infected cells, upregulate activation markers, and secrete IFN-γ. I also analyzed whether these responses could be blocked by anti-class I mAbs or by inactivating the virus.
CHAPTER 2

MATERIALS AND METHODS

A. VIRUSES

Influenza A viruses, A/Puerto Rico/8/34 (H1N1) and A/Japan/305/57 (H2N2) were kindly provided from the Division of Virology (Bureau of Biologics, FDA, Bethesda, MD). Influenza A viruses A/Johannesberg/33/94 (H3N2) and A/Texas/36/91 (H1N1) were obtained from Paster Merieux Connaught (Toronto, Ontario). A/Hong Kong/156/97 (H5N1) and A/Hong Kong/483/97 (H5N1) were kindly provided by Nancy Cox PhD, World Health Organization Influenza Reference Laboratory at the Centers for Disease Control and Prevention (Atlanta, GA). A/Duck/Pennsylvania/10218/84 (H5N2), A/Duck/Alberta/35/76 (H1N1), and A/New Jersey/8/76 (Hsw1N1) were provided by the ATCC (Rockville, MD). Influenza A viruses were propagated in 10-day-old, embryonated chicken eggs. Infected allantoic fluids were harvested 2 days after infection with A/PR/8/34 or A/Japan/305/57, and 1 day after infection with A/HK/156/97 and A/HK/483/97, aliquoted, and stored at -80°C until use. Studies with the A/HK/97 virus strains were performed by John Cruz using biosafety laboratory level 3 conditions. Recombinant vaccinia viruses containing the genes coding for influenza A viral proteins HA, NA, M1, M2, PB1, PB2, PA, NS1, NS2, and NP were kindly provided by Dr. B. Moss. They are all derived from the various influenza A virus strains which are listed in Table II-1. They were constructed and propagated as previously described (Smith et al.,
1987). A recombinant vaccinia virus, which expressed segmented portions of the NP, was kindly provided by Drs. J. Bennink and L. Eisenlohr.
Table II-1. Recombinant Vaccinia Viruses Used in the Thesis.

<table>
<thead>
<tr>
<th>Vaccinia Virus</th>
<th>Amino Acids</th>
<th>Influenza Subtype</th>
<th>Construct Donated by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vac. HA</td>
<td>1-562</td>
<td>H1N1</td>
<td>Moss</td>
</tr>
<tr>
<td>Vac. NP</td>
<td>1-498</td>
<td>H1N1</td>
<td>Moss</td>
</tr>
<tr>
<td>Vac. NP1-3</td>
<td>1-168</td>
<td>H1N1</td>
<td>Bennink and Eisenlohr</td>
</tr>
<tr>
<td>Vac. NP2-3</td>
<td>147-315</td>
<td>H1N1</td>
<td>Bennink and Eisenlohr</td>
</tr>
<tr>
<td>Vac. NP3-3</td>
<td>296-498</td>
<td>H1N1</td>
<td>Bennink and Eisenlohr</td>
</tr>
<tr>
<td>Vac. NS1</td>
<td>1-237</td>
<td>H3N2</td>
<td>Moss</td>
</tr>
<tr>
<td>Vac. NS1A</td>
<td>1-167</td>
<td>H3N2</td>
<td>*</td>
</tr>
<tr>
<td>Vac. NS1B</td>
<td>1-139</td>
<td>H3N2</td>
<td>*</td>
</tr>
<tr>
<td>Vac. NS1C</td>
<td>1-81</td>
<td>H3N2</td>
<td>*</td>
</tr>
<tr>
<td>Vac. NS1D</td>
<td>1-52</td>
<td>H3N2</td>
<td>*</td>
</tr>
<tr>
<td>Vac. NS1E</td>
<td>1-40</td>
<td>H3N2</td>
<td>*</td>
</tr>
<tr>
<td>Vac. M2</td>
<td>1-97</td>
<td>H3N2</td>
<td>Moss</td>
</tr>
<tr>
<td>Vac. M1</td>
<td>1-252</td>
<td>H1N1</td>
<td>Moss</td>
</tr>
<tr>
<td>Vac. NA</td>
<td>1-454</td>
<td>H1N1</td>
<td>Moss</td>
</tr>
<tr>
<td>Vac. PB1</td>
<td>1-757</td>
<td>H1N1</td>
<td>Moss</td>
</tr>
<tr>
<td>Vac. PB2</td>
<td>1-759</td>
<td>H1N1</td>
<td>Moss</td>
</tr>
<tr>
<td>Vac. PA</td>
<td>1-716</td>
<td>H1N1</td>
<td>Moss</td>
</tr>
</tbody>
</table>

The NS1 clone was given to our laboratory by Robert Lamb. Kamal Saikh made the truncated NS1 vaccinia recombinants (Tamura et al., 1998).
B. HUMAN PBMC FOR DERIVATION OF CELL LINES.

PBMC specimens were obtained from normal, healthy, adult donors. All donors were Caucasian Americans. Most of the donor PBMC had convincing evidence of influenza A virus specific CTL activity in bulk culture. We concentrated our efforts on the PBMC of five donors who were available for obtaining repeat samples. PBMC were purified by Ficoll-Hypaque density gradient centrifugation (Boyam, 1968). Cells were resuspended at $2 \times 10^7$/ml in RPMI 1640 with 20% FBS (Sigma) and 10% dimethyl sulfoxide and cryopreserved until use. The HLA alleles of these donors are shown in Table II-2. HLA typing was performed in the HLA typing laboratory at the University of Massachusetts Medical Center.
Table II-2. Adult Healthy Donors Used for T Cell Line Isolation and γδ T Cell Analysis.

<table>
<thead>
<tr>
<th>Donor #</th>
<th>Class I HLA Type</th>
<th>Class II HLA Type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Donor 1</td>
<td>2.1, 11</td>
<td>18, 27</td>
</tr>
<tr>
<td>Donor 2</td>
<td>2, 24</td>
<td>7, 62</td>
</tr>
<tr>
<td>Donor 3</td>
<td>1</td>
<td>8, 44</td>
</tr>
<tr>
<td>Donor 4</td>
<td>26, 28</td>
<td>27, 39</td>
</tr>
<tr>
<td>Donor 5</td>
<td>2, 11</td>
<td>27, 60</td>
</tr>
</tbody>
</table>
C. VOLUNTEER PBMC FOR ISCOM STUDY.

Fifty-five healthy volunteers aged 18-45 years with no known allergy to eggs were recruited for the study as previously reported (Ennis et al., 1999). Volunteers received one of five study vaccines: (1) standard unadjuvanted influenza virus vaccine (Fluzone), (2) influenza virus vaccine incorporated into 50 μg of Iscoms (Flu-Iscom 50), or (3) into 75 mg of Iscoms (Flu-Iscom 75), (4) influenza virus vaccine mixed with 50 μg of Iscoms (Flu-Iscomatrix 50), or (5) mixed with 75 μg of Iscoms (Flu-Iscomatrix 75). The Iscom formulations were previously described (Ennis et al., 1999). PBMC were isolated on days 0 (prevaccination), 14, and 56 (Ennis et al., 1999) according to manufacturer’s instructions in the C.P.T.® tubes (Becton Dickinson, Franklin Lakes, NJ). Selection of the samples of PBMC for testing in ELISPOT assays was based on the volume of available cryopreserved PBMC, the HLA alleles of the individuals, and the results of bulk culture CTL assays performed previously by John Cruz.
Table II-3. Volunteers from Flu-Iscom Vaccine Study that Were Analyzed by ELISPOT Assay.

<table>
<thead>
<tr>
<th>Donor #</th>
<th>HLA Class I Alleles</th>
<th>Vaccine Received</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>3, 33</td>
<td>15, 58</td>
</tr>
<tr>
<td>8</td>
<td>1, 2</td>
<td>8, 15</td>
</tr>
<tr>
<td>16</td>
<td>3, 24</td>
<td>7, 35</td>
</tr>
<tr>
<td>23</td>
<td>2, 31</td>
<td>18, 57</td>
</tr>
<tr>
<td>26</td>
<td>2, 26</td>
<td>27, 51</td>
</tr>
<tr>
<td>29</td>
<td>2</td>
<td>44, 57</td>
</tr>
<tr>
<td>30</td>
<td>24, 30</td>
<td>13, 44</td>
</tr>
<tr>
<td>33</td>
<td>1, 2</td>
<td>7, 44</td>
</tr>
<tr>
<td>42</td>
<td>2, 24</td>
<td>13, 44</td>
</tr>
<tr>
<td>45</td>
<td>2, 3</td>
<td>49, 51</td>
</tr>
<tr>
<td>53</td>
<td>2, 29</td>
<td>44, 45</td>
</tr>
<tr>
<td>54</td>
<td>1, 2</td>
<td>27, 44</td>
</tr>
</tbody>
</table>
D. BULK CULTURES OF PBMC.

Responder PBMC were suspended at 10^6/ml in AIM-V medium (Gibco BRL, Grand Island, NY) containing 10% human AB serum (HuABS) (NABI, Boca Raton, FL), penicillin-streptomycin, glutamine, and HEPES in a 70ml flask (Falcon). Stimulators were infected with the influenza A virus strain A/PR/8/34 (H1N1) at an moi of 15 for 1.5 hours at 37°C in 1 ml PBS containing 0.1% BSA, and were then added to responders in a flask at a stimulator:responder ratio of 1:10. On day 7 of culture, cells were restimulated with gamma-irradiated (3,000 rad) autologous PBMC infected with A/PR/8/34 at an moi of 15 for 1.5 hours in 1 ml PBS containing 0.1% BSA. They were washed twice and added at a stimulator:responder ratio of 1:10 in fresh medium containing 10% Hu ABS and 20 U IL-2 (Collaborative Biomedical Products, Bedford MA). Bulk culture restimulated cells were assayed for cytolytic activity 7 days later.

E. DERIVATION OF CTL LINES.

Influenza specific CTL cell lines were established using a limiting dilution technique, as previously described (Kurane et al., 1989). PBMC which had been stimulated in bulk culture for 7 or 14 days were collected and plated at a concentration of 3, 10, or 30 cells per well in 96-well round-bottom microtiter plates in 100μl of AIM-V medium containing 10% FBS, 20 U IL-2, 1:1000 dilution of the anti-CD3 monoclonal antibody (12F6) kindly provided by Johnson Wong, and 10^5 gamma-irradiated allogeneic PBMC/well. On day 7, 50μl fresh medium with 10% fetal bovine serum (FBS) (Sigma Immunochemicals, St. Louis, MO) and IL-2 were added, and on day 14 fresh medium
with $10^4$ gamma-irradiated allogeneic PBMC/well and 1:1000 dilution of anti-CD3 monoclonal antibody were added. Growing cells were assayed for cytolytic activity on days 21 and 28. Cells from wells with influenza A specific cytolytic activity were expanded to 48-well plates. The two cell lines isolated from the PBMC of donor 3 were characterized by John Cruz. Some T cell epitopes have been previously defined by others: NP 383-391 (Huet et al., 1990), M1 58-66 (Gotch et al., 1987), NS1 122-130 (Man et al., 1995), and M1 17-31 (Rothbard et al., 1988). We have identified additional CD8+ and CD4+ human T cell epitopes (Jameson et al., 1998).

F. PREPARATION OF TARGET CELLS.

Autologous lymphoblastoid cell lines (BLCL) were established by culturing with Epstein Barr Virus (EBV) in 24-well plates as previously described (Green et al., 1993). For target cell usage, BLCL were infected with recombinant vaccinia viruses at an moi of 20:1 for 1.5 h at 37°C. The cells were then diluted in 1 ml of 10% FBS in RPMI 1640 and further incubated for 12-16 h. BLCL infected with influenza A viruses were infected at an moi of 15:1 in 1 ml of 10% FBS in RPMI 1640 for 12-16 h. These infected target cells were labeled with .25 mCi of $^{51}$Cr for 60 min at 37°C. After four washes, the target cells were counted and diluted to $2 \times 10^4$ cells/ml for use in the cytotoxicity assay. The HLA partially-matched allogeneic target cells used in chromium release assays were BLCL produced in our laboratory from the HLA-typed PBMC of unrelated donors, or were obtained from the National Institutes of General Medical Sciences (NIGMS).
Human Genetic Mutant Cell Repository or the American Society for Histocompatibility and Immunogenetics (ASHI) Cell Bank and Repository.

G. CHROMIUM RELEASE ASSAYS.

Cytotoxicity assays were performed in 96-well round-bottom plates, as previously reported (Bukowski et al., 1989). Briefly, effector cells in 100 μl of RPMI 1640 medium containing 10% FBS were added to 1.5x10^3 – 2x10^3 ⁵¹Cr-labeled target cells in 100 μl at various effector to target (E:T) ratios. In CTL assays using synthetic peptides, peptides were added to target cells at the indicated concentrations (.25μg/ml for screening) and incubated at 37°C for 30 min, after which the effector cells were added. Several of the M2, NS1, and NP peptides were kindly provided by Dr. Arthur Pedzcak and Dr. Pele Chong (Pasteur Merieux Connaught, Toronto, Canada), and all other peptides were synthesized at the Core Protein Chemistry Facility directed by Dr. R. Carraway (University of Massachusetts Medical Center, Worcester, MA). In assays testing the FluIscom bulk cultures, 1.5x10^4 K562 cells per well were added to the effectors for two hours prior and left in during the assay to reduce natural killer cell activity. Plates were centrifuged at 200g for 5 min and incubated for 4-5 h at 37°C. Supernatant fluids were harvested using the supernatant collection system (Skatron Instruments, Sterling, Virginia), and ⁵¹Cr content was measured in a gamma counter. Spontaneous release was less than 30% in all assays unless otherwise indicated. The percent-specific ⁵¹Cr release was calculated with the following formula: (cpm experimental release - cpm spontaneous release)/(cpm maximum release - cpm spontaneous release) x 100. All assays were
performed in triplicate, and the results were calculated from the average of the triplicate wells. Specific Immune Lysis (SIL) was calculated by subtracting the percent-specific lysis of peptide pulsed targets from lysis of uninfected targets.

**H. SINGLE CELL ELISPOT ASSAY FOR DETECTION OF IFN-γ-SECRETING CELLS.**

The ELISPOT assay was done as previously described (Lalvani et al., 1997). Briefly, 96-well filtration plates (Millipore, Bedford, MA) were coated with mouse anti-human IFN-γ antibody (clone NIB42, Pharmingen, San Diego, CA). Cryopreserved PBMC were thawed, washed and added to the plates at 1.5-5x10^5 cells per well in RPMI 1640 medium supplemented with 10% FBS, penicillin-streptomycin, glutamine, and HEPES. Cells were incubated for up to 15 hours with or without peptide (10μg peptide/ml) or virus stimulation (virus moi of 15). The plates were washed and then incubated with biotinylated mouse anti-human IFN-γ antibody (clone 4S.B3, Pharmingen). Spots were developed using fresh substrate buffer (.3 mg/ml of 3-amino-9-ethyl-carbazole and .015% H₂O₂ in .1M sodium acetate [pH 5]). The precursor frequency of specific CTL was calculated based on the number of spots counted out of the number of cells added to the wells. Spot forming cells (SFC) were plotted as the number of SFC per 1x10^6 PBMC.

**I. INTRACELLULAR IFN-γ STAINING.**

Two x10^6 PBMC were washed two times and stimulated for 4 hours with A/PR/8/34 influenza A virus at an moi of 5 in .5-1 ml 10% RPMI/FBS at 37°C. Four hours later
10μl of Golgi Plug (Pharmingen, San Diego, CA) or 10μg/ml Brefeldin A (Sigma) was added to each tube. The cells were washed once and fixed/permeabilized with 200μl Cytofix/Cytoperm (Pharmingen) for 20 minutes at 4°C. Cells were washed twice in Perm Wash buffer 1X (Pharmingen) and labeled with 20μl of anti-CD4 (SK3, Becton Dickinson), anti-CD8 (SK1, Becton Dickinson), or anti-CD56 (B159, Pharmingen) with 3μl of anti-IFN-γ (B27, Pharmingen) or control IgG1 (MOPC-21, Pharmingen) monoclonal antibodies in 50μl Perm Wash buffer for 1 hour at 4°C. Experiments with γδ T cells also utilized γδ-fc (#347903, Pharmingen) and Vδ2-fc (#33784A, Pharmingen). Cells were washed once and analyzed by a flow cytometer. Data was analyzed by the WinList program (Verity).

J. STATISTICAL ANALYSIS.

For the ELISPOT assay results the Student’s t-test was used to compare the significance of the difference between the various days assayed. Computer-assisted evaluation was used to calculate probability values. Values of p < 0.05 were considered significant.
CHAPTER III

ANALYSIS OF THE T CELL REPERTOIRE IN HUMANS

A. BULK CULTURE RESPONSES TO INFLUENZA A VIRUS AND CONSTRUCTS EXPRESSING INFLUENZA A VIRAL PROTEINS IN DONOR 1.

We began by analyzing the PBMC of several donors to detect influenza-specific lysis measured by chromium release assays. We wanted to determine whether individuals recognized more than one viral protein in bulk culture and to identify donors whose PBMC would be of interest for our studies. The PBMC from healthy, adult donors were isolated and stimulated with PR/8/34 (H1N1)-infected autologous stimulators twice (day 0 and 7). At day 14 the bulk culture was tested for lysis of autologous B-LCL infected with influenza. Uninfected B-LCL were used as a negative control. The bulk culture PBMC of donor 1 recognizes targets infected with each of the three subtypes of influenza A viruses tested (H1N1, H2N2, H3N2) (Fig. III-1). In order to determine which viral proteins were recognized by the bulk culture T cells, several vaccinia constructs were used to infect B-LCL targets. Specific lysis of target cells expressing the influenza viral proteins NP, HA, M1, and NA were detected (Fig. III-2). The highest lysis was detected against targets infected with the vaccinia construct that expresses NP. Similar results were observed in multiple experiments performed with this donor’s PBMC. These results suggest that donor 1 has influenza-specific memory CTL that recognize multiple proteins and is cross-reactive with the various influenza virus subtypes tested.
Fig. III-1. Donor 1 Bulk Culture CTL Responses Specific for Influenza A Viruses of Multiple Subtypes.

Targets were infected with influenza A viruses at an moi of 15:1. Bulk culture PBMC were stimulated with the PR/8/34 virus two times (at days 0 and 7) in vitro prior to the assay. The negative control was uninfected targets (media only).
Targets were infected with vaccinia virus constructs at an moi of 20. Bulk culture PBMC were stimulated with PR/8/34 (H1N1) influenza virus two times (at days 0 and 7) in vitro prior to the assay and then were tested for lysis of vaccinia virus construct infected B-LCLs. The negative control was targets infected with a vaccinia virus construct that does not express an influenza protein (vac control).
B. BULK CULTURE T CELL RECOGNITION OF INFLUENZA VIRUS AND VACCINIA CONSTRUCTS EXPRESSING INFLUENZA VIRUS PROTEINS.

To analyze the memory T cell responses to influenza virus by other healthy adult volunteers, PBMC were stimulated with autologous PR/8/34 infected stimulators on day 0 and 7. The bulk cultures were tested in chromium release assays to analyze the specific lysis of target cells infected with influenza virus or vaccinia constructs. The PBMC of donor 2 specifically recognized influenza PR/8/34 (H1N1) infected targets as compared to uninfected targets. This donor also had specific lysis of the Vac NP construct. In other experiments specific lysis of Vac M1 and PB1 targets were also observed (data not shown).

John Cruz stimulated bulk cultures from donor 3 and observed specific lysis of influenza infected targets and Vac NS1, Vac NP, and Vac M2 infected targets (data not shown). The bulk cultures from donors 4 and 5 both recognized targets infected with influenza PR/8/34 (H1N1) as well (Fig. III-4A,B). These donors both exhibited recognition of B-LCL infected with the Vac NP construct. Previously, in our laboratory the PBMC of other donors were tested as well. Overall, most donors had specific lysis at the bulk culture level of influenza infected targets (data not shown). This was expected, because most adults have been infected with influenza multiple times during their lifetime. We had identified several donors with memory T cell responses to influenza that could be used to characterize the CTL response further.
Fig. III-3. Donor 2 Bulk Culture Responses Specific for Influenza and Vaccinia Constructs Expressing Influenza Proteins.

Targets were infected with influenza virus at an moi of 15, and with vaccinia virus at an moi of 20. Bulk culture PBMC were stimulated with the PR/8/34 virus two times (at days 0 and 7) in vitro prior to the assay.
Targets were infected with influenza virus at an moi of 15, and with vaccinia virus at an moi of 20. Donor 4 and 5 PBMC were stimulated with PR/8/34 (H1N1) twice (at days 0 and 7) and tested for lysis of B-LCLs infected with either influenza virus or vaccinia virus constructs.
C. PROTEIN SPECIFICITY OF CTL LINES ISOLATED FROM DONOR 1.

The bulk culture of influenza A virus-specific CTL from donor 1 were put into 96-well plates by limiting dilution at 3, 10, and 30 cells/well, and wells positive for growth were screened for lysis of influenza A virus-infected targets. This was done on two more occasions also. All of the CTL lines were stained for CD4 or CD8 expression by flow cytometry (data not shown). Ten CTL lines generated from the PBMC of donor 1 were tested for lysis of targets infected with the recombinant vaccinia viruses and influenza A virus (PR/8/34, H1N1). The results obtained with 6 of the 10 lines are presented in Table III-1. These CTL have novel, previously unreported specificities. The other four cell lines characterized from this donor recognize epitopes that have been previously reported and will be summarized later.

Each of the T-cell lines characterized was specific for one influenza A virus protein (Table III-1). These cell lines and those with previously reported specificities recognized NP, PB2, M1, HA, PB1, NS1, and NA, making a total of seven different influenza A virus proteins recognized by the memory T lymphocytes of donor 1. These data indicate that the CTL responses of this donor to influenza virus are directed against a broad range of viral proteins and include both CD4+ and CD8+ components.
Table III-1. Identification of Influenza Virus Proteins Recognized by CTL Lines Generated from the PBMC of Donor 1.

<table>
<thead>
<tr>
<th>Virus Strain Used to Infect B-LCL*</th>
<th>% Specific $^{51}$Cr Release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD8+</td>
</tr>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>PR/8/34</td>
<td>10-1C4</td>
</tr>
<tr>
<td>Vac HA</td>
<td>-1</td>
</tr>
<tr>
<td>Vac M1</td>
<td>26</td>
</tr>
<tr>
<td>Vac M2</td>
<td>0</td>
</tr>
<tr>
<td>Vac NS1</td>
<td>0</td>
</tr>
<tr>
<td>Vac NA</td>
<td>-1</td>
</tr>
<tr>
<td>Vac NP</td>
<td>93</td>
</tr>
<tr>
<td>Vac PA</td>
<td>-1</td>
</tr>
<tr>
<td>Vac PB1</td>
<td>-1</td>
</tr>
<tr>
<td>Vac PB2</td>
<td>68</td>
</tr>
</tbody>
</table>

*The E:T ratio was 10:1. Lysis of targets infected with wild-type vaccinia virus was subtracted from lysis of recombinant vaccinia virus. Lysis of the target cell by the CTL that demonstrates specific protein recognition defined as greater than 25% specific lysis is underlined.
D. HLA RESTRICTION OF DONOR 1 CTL LINES.

MHC restriction of the cell lines was determined by using partially HLA-matched allogeneic B-LCL as targets for CTL lysis. Targets were infected with the recombinant vaccinia virus that had resulted in specific target cell lysis or with wild-type vaccinia virus as a negative control. Alternatively, partially HLA-matched target cells were pulsed with a peptide that contained the CTL epitope. The results in Fig. III-5A show that targets pulsed with NP peptide containing amino acids (aa) 173-193 are lysed by bulk culture effectors if they share HLA B27. When the cell line that recognized that epitope (10-1C4) was characterized it was also found to lyse only target cells with B27 in common when pulsed with the same NP peptide, and it is therefore also B27 restricted (data not shown). Cell lines 10-1B7 and 1-2F8 are also able to lyse only targets which share B27 and are therefore B27 restricted (Fig. III-5 B and C). Cell line 4-30E11 is either A11, Cw7, or Cw1 restricted, because it does not lyse targets expressing A2, B27, or B18 (Fig. III-5D). This cell line ceased growing and could not be tested further. HA-specific cell line 10-1G5 is B18 restricted because it lyases targets which have only B18 in common (Fig. III-5E).

We also tested CD4+ T-cell line 4-10D9-1, which is DR1 restricted (data not shown) and another four T-cell lines which recognized previously reported epitopes and were found to be restricted by HLA A2.1, B27, and DR1 (data not shown) (Gotch et al., 1987; Rothbard et al., 1988; Huet et al., 1990; Man et al., 1995). The results shown in Fig. III-5 are a representation of the experiments done to confirm MHC restriction of the CTL lines. MHC restriction of each cell line was confirmed in multiple experiments.
using different allogeneic targets. A2.1-restricted lines were confirmed by lysis of infected Hmy C1R A2.1-transfected targets. Overall, these results indicate that at least four different class I HLA alleles (A2.1, B18, B27, and All, Cw1, or Cw7) and one class II HLA allele (DR1) restrict the CTL lines isolated from donor 1, and there is not one influenza A virus-specific dominant HLA allele.
Fig. III-5

A.

B27, Cw1
A2, B18, Cw1
A11.1, Cw1
A2, B27
Autologous

B.

A2, B18, Cw1
A2, B27
B18
A2, A11, B27, Cw1
Autologous

C.

A2, B18, Cw1
A2, B27
B18
A2, A11, B27, Cw1
Autologous

D.

A2, B27
B18
A2, A11, B27, Cw1
Autologous

E.

A2, B18, Cw1
A2, B27
B18
A2, A11, B27, Cw1
Autologous

% Specific Lysis
Legend for Fig. III-5

HLA alleles of partially HLA-matched targets in common with donor 1 are listed on the Y axis. % specific lysis of negative control vaccinia virus infected targets or uninfected targets were subtracted from lysis of infected targets. A) Bulk culture lysis of NP peptide 173-193 pulsed targets. B) Lysis by cell line 10-1B7 of vac PB2 infected targets. C) Lysis by cell line 1-2F8 of vac PB1 infected targets. D) Lysis by cell line 4-30E11 of vac M1 infected targets. E) Lysis by cell line 10-1G5 of vac HA infected targets.
E. CHARACTERIZATION OF THE EPITOPES RECOGNIZED BY CTL LINES FROM DONOR 1.

Recombinant vaccinia viruses that contain overlapping amino acid regions of the NP gene (see Table II-1) were used to localize the epitope recognized by NP-specific cell line 10-1C4. This cell line lysed targets infected with the vaccinia virus construct expressing NP aa 147 to 315. We synthesized 20-mer peptides that spanned aa 147 to 315, and this cell line lysed targets pulsed with a peptide containing aa 173 to 193. Target cells pulsed with this peptide were also recognized by effector cells from a 7-day bulk culture from this donor (Fig. III-6). Finer mapping with synthetic peptides indicated that the optimal epitope is contained within aa 174 to 184 (Fig. III-7A and Fig. III-8A). Precursor frequency analysis by ELISPOT single-cell IFN-γ secretion indicated that this CTL epitope is recognized by 1 in 4,156 PBMC from donor 1 (Table III-2).

Peptides containing epitopes that were previously reported were synthesized and tested for recognition by CTL lines if they shared MHC restriction and viral protein specificities with the cell lines we isolated. Cell line 10-2C2 lysed targets pulsed with a peptide representing aa 122-130 of NS1, line 1-7-K lysed targets pulsed with peptide aa 58 to 66 of M1, line 1-3 lysed targets pulsed with peptide aa 17 to 31 of M1, and line 1-lysed targets pulsed with peptide aa 383 to 391 of NP. Precursor analysis of these epitopes in this donor’s PBMC confirmed that these are not rare CTLs generated by the cloning process (Table III-2).
Fig. III-6. Donor 1 Bulk Culture Recognition of NP 174-184.

Donor 1 bulk culture was stimulated for 7 days with PR/8/34 and tested for recognition of virus infected targets. Peptides were pulsed at a concentration of 25µg/ml, vaccinia viruses were added at an moi of 20, and influenza virus was added at an moi of 15.
Fig. III-7. Fine Epitope Mapping of T Cell Lines.

Peptides were pulsed on targets at 25μg/ml for 30 minutes prior to the addition of the cell line. A) Cell lines 10-IC4, B) 3E5, C) 124, and D) 77 were added to the assay at an E:T ratio of 10:1.
Peptides were pulsed on targets at the designated concentrations for 30 minutes prior to the addition of cell lines A) 10-1C4, B) 3E5, C) 124, and D) 77 at an E:T of 10:1.
Table III-2. Peptide-Specific CTL Frequencies in Donor 1 PBMC as Determined by ELISPOT.

<table>
<thead>
<tr>
<th>Influenza Virus Protein</th>
<th>Amino Acids</th>
<th>MHC Class I Restriction</th>
<th>Precursor Frequency of Peptide-Specific IFN-γ spot-forming cells (SFC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>174-184</td>
<td>B27</td>
<td>1/4,156</td>
</tr>
<tr>
<td>M1</td>
<td>58-66</td>
<td>A2.1</td>
<td>1/31, 250</td>
</tr>
<tr>
<td>NP</td>
<td>383-391</td>
<td>B27</td>
<td>1/16,447</td>
</tr>
<tr>
<td>NS1</td>
<td>122-130</td>
<td>A2.1</td>
<td>&lt;1/500,000</td>
</tr>
<tr>
<td>M1</td>
<td>17-31</td>
<td>DR1</td>
<td>1/26,316</td>
</tr>
</tbody>
</table>

Negative controls included the addition of no peptide or an irrelevant peptide. Neither produced SFC in donor 1. The results of this experiment are representative of those observed in three experiments using this donor’s PBMC.
F. DONORS 2 AND 3 ALSO HAVE BROAD CTL REPERTOIRES FOR INFLUENZA A VIRAL PROTEINS.

After characterizing a broad repertoire of CTL lines that recognize influenza A virus in donor 1, we wanted to analyze the PBMC of other donors in a limited way to determine if they also had broad CTL responses. As before, a bulk culture was cloned by limiting dilution to characterize the CTL response at the clonal level. In donor 2, after one limiting dilution, five different cell lines were characterized. They were shown to have specific lytic activity against four different viral proteins (HA, NP, NA, and M1). Three CD4+ lines and two CD8+ lines were characterized (Table III-3). Two of these were NP-specific, CD8+ 3G11 and CD4+ 3E5. Recombinant vaccinia viruses expressing segmented portions of NP were used to determine the specific region that NP-specific lines recognize. Cell line 3G11 lysed targets infected with vaccinia virus NP aa 296 to 498, and 3E5 lysed targets infected with vaccinia virus NP aa 147-315. Fine epitope mapping using synthetic peptides demonstrated that cell line 3E5 recognizes NP aa 255 to 265 at 0.25μg/ml (Fig. III-6B and III-7B). CD8+ cell lines 3F4 and 3G11 were found to be B62 restricted (data not shown) by lysis of partially HLA-matched targets as described for donor 1. The restricting alleles of the three CD4+ CTL lines were not determined because they failed to lyse the available partially matched B-LCL targets.

T cells from donor 3 were also cloned by limiting dilution and two influenza virus-specific cell lines were established. Cell line 124 is CD8+ and recognizes M2, while cell line 77 is CD4+ and recognizes NS1 (Table III-3). A cell line that recognizes NP was also isolated but not further characterized. Synthetic peptides were used to
identify the epitopes. Cell line 124 recognizes aa 7 to 15 of M2 down to 0.25μg/ml (Fig. III-6C and III-7C), while cell line 77 recognizes aa 34 to 42 at 25μg/ml (Fig. III-6D and III-7D). HLA restriction analysis was performed by using allogeneic partially HLA-matched cell lines as done with the two previous donors; CTL line 124 is restricted by B44, and CTL line 77 is restricted by DR3 (data not shown).
Table III-3. Recognition of Influenza Virus Proteins by CTL Lines Generated from the PBMC of Donors 2 and 3.

<table>
<thead>
<tr>
<th>Virus Infecting</th>
<th>% Specific [^{31}\text{Cr} \text{ Release}</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Expt. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-LCL Targets*</td>
<td>CD8+ CD8+ CD4+ CD4+ CD8+ CD8+ CD4+ CD4+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3F4 3G11 3E5 3E9 10E7 124 77</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR/8/34</td>
<td>36 75 56 27 72</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac HA</td>
<td>52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac M1</td>
<td>-2</td>
<td>0</td>
<td>66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac M2</td>
<td>-10</td>
<td>69</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac NA</td>
<td>0</td>
<td>59</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Vac NP</td>
<td>65</td>
<td>65</td>
<td></td>
<td>-2</td>
<td>0</td>
</tr>
<tr>
<td>Vac NP (aa 1-168)</td>
<td>-2</td>
<td>-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac NP (aa 147-315)</td>
<td>1</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac NP (aa 296-498)</td>
<td>67</td>
<td>-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac PA</td>
<td></td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac PB1</td>
<td></td>
<td>-1</td>
<td>-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vac PB2</td>
<td></td>
<td>-3</td>
<td>-1</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>Vas NS1</td>
<td></td>
<td>3</td>
<td>74</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The E:T ratios were 10:1. The lysis of targets infected with wild-type vaccinia virus was subtracted from the lysis of targets infected with recombinant vaccinia viruses.

Lines 124 and 77 are isolated from donor 3, all others are isolated from donor 2. Lysis of the target cell demonstrating specific lysis of at least 25% by the cell line is underlined.
G. CROSS-REACTIVE LYsis BY CTL LINES ISOLATED FROM DONORS 1, 2, AND 3 OF INFLUENZA VIRUS SUBTYPES H1N1, H2N2, H3N2.

It has been previously shown that influenza virus-specific memory CTL in mice are either subtype specific or cross-reactive (Effros et al., 1977; Zweerink et al., 1977; Zweerink et al., 1977; Braciale et al., 1981; Kuwano et al., 1988). The cell lines were generated by stimulating PBMC with PR/8/34 (H1N1). We infected targets with virus strains of the three different subtypes (H1N1, H2N2, and H3N2) to analyze the subtype-cross-reactive nature of the T cell lines. In all donors, most of the cell lines that recognize epitopes on the internal viral proteins (10-1C4, 10-1B7, 4-30E11, 1-2F8, 3G11, 10E7, 77, and 3E5) were H1N1, H2N2, and H3N2 subtype cross-reactive (Table III-4A). Cell lines with specificity to the external glycoproteins (10-1G5, 4-10D9-1, 3F4, and 3E9) were either H1N1 and H2N2 subtype cross-reactive or H1N1 subtype specific (Table III-4B). In donor 1, HA-specific cell line 10-1G5 demonstrated H1N1 and H2N2-cross-reactive killing (Table III-4B). Cell line 124 is unique because it recognizes an epitope on the conserved internal protein M2 and is H1N1 and H2N2, but not H3N2 cross-reactive. These results indicate that these donors have both subtype-specific and cross-reactive CTLs. The cross-reactive CTL recognize targets infected with each of the influenza subtypes that have caused pandemics in humans this century.
Table III-4A. Subtype Cross-Reactive Recognition by Influenza Virus-Specific CTL from Donors 1, 2, and 3*.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Protein Specificity</th>
<th>Epitope</th>
<th>CD4/CD8</th>
<th>% Specific $^{31}$Cr Release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PR/8/34</td>
<td>H1N1</td>
</tr>
<tr>
<td>10-1C4</td>
<td>NP</td>
<td>174-184</td>
<td>CD8</td>
<td>18</td>
</tr>
<tr>
<td>10-1C4</td>
<td>NP</td>
<td>174-184</td>
<td>CD8</td>
<td>22</td>
</tr>
<tr>
<td>10-1C4</td>
<td>NP</td>
<td>174-184</td>
<td>CD8</td>
<td>15</td>
</tr>
<tr>
<td>10-1B7</td>
<td>PB2</td>
<td>174-184</td>
<td>CD8</td>
<td>25</td>
</tr>
<tr>
<td>10-1B7</td>
<td>PB2</td>
<td>174-184</td>
<td>CD8</td>
<td>46</td>
</tr>
<tr>
<td>4-30E11</td>
<td>M1</td>
<td>174-184</td>
<td>CD8</td>
<td>25</td>
</tr>
<tr>
<td>1-2F8</td>
<td>PB1</td>
<td>174-184</td>
<td>CD8</td>
<td>13</td>
</tr>
<tr>
<td>1-2F8</td>
<td>PB1</td>
<td>174-184</td>
<td>CD8</td>
<td>16</td>
</tr>
<tr>
<td>3G11</td>
<td>NP</td>
<td>315-496</td>
<td>CD8</td>
<td>56</td>
</tr>
<tr>
<td>10E7</td>
<td>M1</td>
<td>315-496</td>
<td>CD8</td>
<td>53</td>
</tr>
<tr>
<td>3E5</td>
<td>NP</td>
<td>315-496</td>
<td>CD8</td>
<td>97</td>
</tr>
<tr>
<td>77</td>
<td>NS1</td>
<td>315-496</td>
<td>CD8</td>
<td>82</td>
</tr>
<tr>
<td>77</td>
<td>NS1</td>
<td>315-496</td>
<td>CD4</td>
<td>ND</td>
</tr>
</tbody>
</table>
Table III-4B. Subtype-Specific Recognition by Influenza Virus-Specific

CTL from Donors 1, 2, and 3.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Protein Specificity</th>
<th>Epitope</th>
<th>CD4/CD8</th>
<th>% Specific $^{31}$Cr Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-1G5</td>
<td>HA</td>
<td>CD8</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>10-1G5</td>
<td>HA</td>
<td>CD8</td>
<td>40</td>
<td>48</td>
</tr>
<tr>
<td>4-10D9-1</td>
<td>NA</td>
<td>CD4</td>
<td>44</td>
<td>8</td>
</tr>
<tr>
<td>3F4</td>
<td>HA</td>
<td>CD8</td>
<td>55</td>
<td>2</td>
</tr>
<tr>
<td>3E9</td>
<td>NA</td>
<td>CD4</td>
<td>62</td>
<td>13</td>
</tr>
<tr>
<td>124</td>
<td>M2</td>
<td>7-15</td>
<td>69</td>
<td>55</td>
</tr>
</tbody>
</table>

*The E:T ratio was 10:1. Lysis of uninfected targets was subtracted from that of influenza virus-infected targets. Some cell lines were tested in multiple experiments. The same experiment may have included more than one cell line. ND, not tested in that experiment.
CHAPTER IV

HUMAN CD8+ AND CD4+ T LYMPHOCYTE MEMORY TO INFLUENZA A VIRUSES OF SWINE AND AVIAN SPECIES

A. RECOGNITION OF SWINE AND AVIAN INFLUENZA A VIRUS SPECIES BY T CELL LINES.

We wanted to determine whether humans have cross-reactive CTL that recognize APCs infected with influenza viruses that usually infect the avian or swine population. We tested the previously characterized cell lines for recognition of targets infected with avian or swine strains of influenza. The first influenza virus strain that we tested was A/NJ/76 (Hsw1N1), which is a swine-like virus isolated from a soldier who died at Fort Dix, NJ in 1976 (Webster et al., 1992). The human T cell lines characterized in chapter III were tested for recognition of targets infected with this swine-like virus. The results are shown in Table IV-1 (Expt. A), and they indicate that about two-thirds of the CD8+ human influenza A-specific, subtype cross-reactive CTL lines recognize and kill target cells infected with the swine-like A/NJ/76 virus at levels similar to those for lysis of target cells infected with the human PR/8/34 (H1N1) virus.

Avian strains that contained either the avian H5 HA (A/Duck/Pennsylvania/10218/84 [H5N2]) or the avian N1 (A/Duck/Alberta/35/76 [H1N1]) similar to the H5 and N1 proteins of the recently isolated Hong Kong H5N1 viruses were then tested. The results are shown in Table V-1 (Expt. B), and they...
demonstrate that several of the human serotype cross-reactive CTL lines recognized autologous targets infected with the avian viruses to a similar degree as the PR/8/34 human strain. Certain CD8+ and CD4+ T cell lines that recognized epitopes only on H1 or on H1 and H2 subtype viruses (e.g. 10D9 or 124) did not recognize the avian virus-infected cells. A HLA-B27-restricted CD8+ CTL line (1-1) from donor 1, which is known to recognize aa 383-391 on NP of the human subtypes H1, H2, and H3, recognized target cells infected with the 1976 H1N1 avian strain (26.5%) but did not recognize as well targets infected with the 1984 H5N2 avian virus (8%). There may be a mutation in this epitope that decreases the affinity, presentation, or TCR recognition. Similar results were obtained using another CD8+ CTL line (10-2C2) which is HLA-A2.1 restricted and recognizes aa 122-130 on the NS1 protein. However, another HLA-A2.1-restricted line (1-7-K) specific for M1 aa 58-66 recognized target cells infected with either of these avian viruses to a similar degree as A/PR/8/34-infected target cells. To extend these observations bulk culture PBMC from donor 1 was stimulated with the H1N1 avian strain, and there was specific lysis of targets pulsed with the NP 383-391 and M1 58-66 peptides (data not shown).

Similar results were observed with CTL lines from donors 2 and 3. The CD8+ and CD4+ lines 3G11 and 3E5 recognized both virus strains well. However, 3E9 and 77 did not recognize the H5N2 subtype as well as the 1976 H1N1 virus. These results were obtained using PBMC of American donors; although the frequency of HLA alleles varies considerably between distinct populations groups, some, e.g., HLA A2, represent approx.
30% of both North American Caucasian and Southern Han, the dominant tribe in southern China (Charron, 1997).
Table IV-1. Recognition of Swine/Avian Influenza A Virus-Infected Targets by Human CTL Lines*

<table>
<thead>
<tr>
<th>Donor 1</th>
<th>Cell Lines</th>
<th>CD4/CD8</th>
<th>Protein</th>
<th>Epitopes</th>
<th>Subtype Specificity</th>
<th>HLA Restriction</th>
<th>% Specific (^{31})Cr Release of Targets Infected with Expt. A</th>
<th>Expt. B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>CD8</td>
<td>NP</td>
<td>383-391</td>
<td>H1,H2,H3</td>
<td>B27</td>
<td>17.0</td>
<td>13.2</td>
<td>8.0</td>
</tr>
<tr>
<td>1-7-K</td>
<td>CD8</td>
<td>M1</td>
<td>58-66</td>
<td>H1,H2,H3</td>
<td>A2.1</td>
<td>45.6</td>
<td>31.4</td>
<td>43.5</td>
</tr>
<tr>
<td>102C2</td>
<td>CD8</td>
<td>NS1</td>
<td>122-130</td>
<td>H1,H2,H3</td>
<td>A2.1</td>
<td>20.0</td>
<td>15.1</td>
<td>-9.9</td>
</tr>
<tr>
<td>101C4</td>
<td>CD8</td>
<td>NP</td>
<td>174-184</td>
<td>H1,H2,H3</td>
<td>B27</td>
<td>10.1</td>
<td>5.4</td>
<td>-4.0</td>
</tr>
<tr>
<td>101G5</td>
<td>CD8</td>
<td>HA</td>
<td>-</td>
<td>H1,H2</td>
<td>B18</td>
<td>26.8</td>
<td>7.2</td>
<td>3.3</td>
</tr>
<tr>
<td>101B7</td>
<td>CD8</td>
<td>PB2</td>
<td>-</td>
<td>H1,H2,H3</td>
<td>B27</td>
<td>43.8</td>
<td>8.7</td>
<td>14.3</td>
</tr>
<tr>
<td>1-2F8</td>
<td>CD8</td>
<td>PB1</td>
<td>-</td>
<td>H1,H2,H3</td>
<td>B27</td>
<td>21.1</td>
<td>10.1</td>
<td>12.4</td>
</tr>
<tr>
<td>1-3</td>
<td>CD4</td>
<td>M1</td>
<td>17-31</td>
<td>H1,H2,H3</td>
<td>DR1</td>
<td>65.6</td>
<td>65.2</td>
<td>19.1</td>
</tr>
<tr>
<td>10D9</td>
<td>CD4</td>
<td>NA</td>
<td>-</td>
<td>H1</td>
<td>DR1</td>
<td>37.5</td>
<td>-3.1</td>
<td>-10.5</td>
</tr>
<tr>
<td>Donor 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26.7</td>
<td>41.7</td>
<td>73.6</td>
</tr>
<tr>
<td>3G11</td>
<td>CD8</td>
<td>NP</td>
<td>315-496</td>
<td>H1,H2,H3</td>
<td>B62</td>
<td>76.9</td>
<td>77.9</td>
<td>80.8</td>
</tr>
<tr>
<td>3E5</td>
<td>CD4</td>
<td>NP</td>
<td>254-262</td>
<td>H1,H2,H3</td>
<td>-</td>
<td>14.1</td>
<td>3.2</td>
<td>-1.2</td>
</tr>
<tr>
<td>3E9</td>
<td>CD4</td>
<td>NA</td>
<td>-</td>
<td>H1</td>
<td>-</td>
<td>24.5</td>
<td>21.8</td>
<td>50.0</td>
</tr>
<tr>
<td>10E7</td>
<td>CD4</td>
<td>M1</td>
<td>-</td>
<td>H1,H2,H3</td>
<td>-</td>
<td>26.8</td>
<td>2.6</td>
<td>-0.8</td>
</tr>
<tr>
<td>Donor 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>79.1</td>
<td>71.3</td>
<td>3.8</td>
</tr>
<tr>
<td>124</td>
<td>CD8</td>
<td>M2</td>
<td>7-15</td>
<td>H1,H2</td>
<td>B44</td>
<td>26.8</td>
<td>2.6</td>
<td>-0.8</td>
</tr>
<tr>
<td>77</td>
<td>CD4</td>
<td>NS1</td>
<td>34-42</td>
<td>H1,H2,H3</td>
<td>DR3</td>
<td>70.3</td>
<td>71.3</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*The T cell lines from each donor were tested separately in three experiments. A dash indicates the HLA restriction or epitope are not yet defined. E:T ratio was 10:1 to 20:1 and uninfected targets were used as a negative control with lysis <1% by all of the T cell lines.
B. RECOGNITION OF THE 1997 HONG KONG VIRUS STRAINS BY HUMAN T CELL LINES.

The 1997 H5N1 Hong Kong strains were obtained and tested for recognition as virus-infected autologous targets by these human T cell lines (Table IV-2). Several of the donor 1 CD8+ cell lines recognized the H5N1 virus-infected target cells, e.g., cell line 1-1 and cell line 1-7-K. These cell lines recognize epitopes on NP and M1 respectively. Another interesting cell line, 10-1G5, which was previously shown to recognize H1 and H2 subtypes of influenza viruses, also recognized both strains of the H5N1 subtype. This cell line recognizes an epitope on the HA which is usually the least conserved viral protein, but H2 and H5 are the two most homologous HA molecules, thus explaining the cross-reactive recognition. Cell lines 1-2F8 and 10-1B7, both HLA-B27 restricted, recognize epitopes on the PB1 and PB2 proteins, and recognized target cells infected with at least one of the 1997 Hong Kong viruses to a similar degree as the earlier human H1 virus. Other CD8+ CTL lines recognized targets infected with H1N1 virus but not targets infected with the Hong Kong H5N1 strains, e.g., cell line 10-2C2 which recognizes aa 122-130 on NS1. Another example is the subtype-specific CD4+ CTL line 10D9-d10 which is HLA-DR1 restricted, NA-specific, and only recognized target cells infected with PR/8/34 (H1N1) virus.

Similar results were obtained using cell lines isolated from the PBMC of donor 2. Entirely cross-reactive killing of targets infected with the H5N1 Hong Kong strains was observed with one CD8+ CTL line, 3G11, which is HLA-B62 restricted and recognizes an epitope located within aa 315-496 on NP. Two CD4+ CTL lines that recognize
epitopes on NP (3E5) and M1 (10E7) also had cross-reactive recognition of the Hong Kong avian virus-infected targets. However, an NA-specific CD4+ line 3E9 recognizes only H1N1 virus-infected target cells. One cell line, 77, from the third donor exhibited a very cross-reactive pattern of lysis. This CD4+ CTL line is restricted by HLA-DR3 recognizes an epitope on aa 34-42 on the NS1 protein. On the other hand, the HLA-B44-restricted CD8+ CTL line 124, which recognizes aa 7-15 on the M2 protein, killed target cells infected with human H1N1 subtype virus but did not recognize target cells infected with either of the 1997 Hong Kong viruses.
**Table IV-2. Recognition of the Hong Kong Avian Virus Strains by Human CTL Lines*.**

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>CD4/C D8</th>
<th>Protein</th>
<th>Epitopes</th>
<th>Subtype Specificity</th>
<th>HLA Restriction</th>
<th>% Specific (^{31}\text{Cr} \text{ Release of Targets}</th>
<th>Expt. C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A/HK/156/97 (H5N1)</td>
<td>A/HK/483/97 (H5N1)</td>
<td></td>
</tr>
<tr>
<td>Donor 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-1</td>
<td>CD8</td>
<td>NP</td>
<td>383-391</td>
<td>H1,H2,H3</td>
<td>B27</td>
<td>48.5</td>
<td>20.4</td>
</tr>
<tr>
<td>1-7-K</td>
<td>CD8</td>
<td>M1</td>
<td>58-66</td>
<td>H1,H2,H3</td>
<td>A2.1</td>
<td>64.9</td>
<td>57.9</td>
</tr>
<tr>
<td>102C2</td>
<td>CD8</td>
<td>NS1</td>
<td>122-130</td>
<td>H1,H2,H3</td>
<td>A2.1</td>
<td>4.6</td>
<td>-1.3</td>
</tr>
<tr>
<td>101C4</td>
<td>CD8</td>
<td>NP</td>
<td>174-184</td>
<td>H1,H2,H3</td>
<td>B27</td>
<td>13.7</td>
<td>6.3</td>
</tr>
<tr>
<td>101G5</td>
<td>CD8</td>
<td>HA</td>
<td>-</td>
<td>H1,H2</td>
<td>B18</td>
<td>41.9</td>
<td>18.8</td>
</tr>
<tr>
<td>101B7</td>
<td>CD8</td>
<td>PB2</td>
<td>-</td>
<td>H1,H2,H3</td>
<td>B27</td>
<td>61.3</td>
<td>55.4</td>
</tr>
<tr>
<td>1-2F8</td>
<td>CD8</td>
<td>PB1</td>
<td>-</td>
<td>H1,H2,H3</td>
<td>B27</td>
<td>41.9</td>
<td>15.7</td>
</tr>
<tr>
<td>1-3</td>
<td>CD4</td>
<td>M1</td>
<td>17-31</td>
<td>H1,H2,H3</td>
<td>DR1</td>
<td>33.1</td>
<td>2.6</td>
</tr>
<tr>
<td>10D9</td>
<td>CD4</td>
<td>NA</td>
<td>-</td>
<td>H1</td>
<td>DR1</td>
<td>7.9</td>
<td>-2.5</td>
</tr>
<tr>
<td>Donor 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3G11</td>
<td>CD8</td>
<td>NP</td>
<td>315-496</td>
<td>H1,H2,H3</td>
<td>B62</td>
<td>79.7</td>
<td>84.0</td>
</tr>
<tr>
<td>3E5</td>
<td>CD4</td>
<td>NP</td>
<td>254-262</td>
<td>H1,H2,H3</td>
<td>-</td>
<td>82.2</td>
<td>75.1</td>
</tr>
<tr>
<td>3E9</td>
<td>CD4</td>
<td>NA</td>
<td>-</td>
<td>H1</td>
<td>-</td>
<td>14.4</td>
<td>9.7</td>
</tr>
<tr>
<td>10E7</td>
<td>CD4</td>
<td>M1</td>
<td>-</td>
<td>H1,H2,H3</td>
<td>-</td>
<td>83.3</td>
<td>79.6</td>
</tr>
<tr>
<td>Donor 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>CD8</td>
<td>M2</td>
<td>7-15</td>
<td>H1,H2</td>
<td>B44</td>
<td>-2.8</td>
<td>-0.7</td>
</tr>
<tr>
<td>77</td>
<td>CD4</td>
<td>NS1</td>
<td>34-42</td>
<td>H1,H2,H3</td>
<td>DR3</td>
<td>68.5</td>
<td>65.4</td>
</tr>
</tbody>
</table>

*The T cell lines from each donor were tested separately in three experiments. A dash indicates that the HLA restriction or epitope are not yet defined. E:T ratio was 10:1 to 20:1 and uninfected targets were used as a negative control with lysis <1% by all of the T cell lines.
C. RECOGNITION OF TARGETS INFECTED WITH AVIAN, SWINE, AND HUMAN-SPECIES OF INFLUENZA A VIRUSES BY BULK CULTURE PBMC.

Although the previous experiments demonstrate that influenza A-specific CTL isolated from humans can recognize avian and swine virus strains, we wanted to test the recognition at the short-term bulk culture level. PBMC were stimulated with human PR/8/34 (H1N1) and tested for recognition of targets infected with human H1N1 or H2N2 viruses, older avian viruses (H5N2 and H1N1), the recent avian-derived viruses isolated from humans in Hong Kong (H5N1), or the swine-like 1976 strain (Hsw1N1). The results in Fig. IV-1 demonstrate convincing killing by cells in these bulk cultures of targets infected with the recent Hong Kong viruses (A/Hong Kong/483/97 and A/Hong Kong/156/97) at least to a degree similar to that of target cells infected with the older human H1N1 and H2N2 virus strains (A/PR/8/34 and A/Japan/305/57). Killing was also observed of target cells infected with the older avian virus strains (A/Duck/Alb/76 and A/Duck/Penn/84) and the Hsw1N1 strain (A/NJ/1/76). This experiment demonstrates that humans have memory T lymphocytes that recognize epitopes on avian- and swine-derived viruses, including the recent Hong Kong-derived strains, in a highly cross-reactive fashion.
Fig. IV-1. Bulk Culture Recognition of Avian, Swine, Human Viruses.

A. A/NJ/1/76
   A/Duck/Alb/76
   A/Duck/Penn/84
   A/Hong Kong/483/97
   A/Hong Kong/156/97
   A/Japan/305/57
   A/PR/8/34

B. A/NJ/1/76
   A/Duck/Alb/76
   A/Duck/Penn/84
   A/Hong Kong/483/97
   A/Hong Kong/156/97
   A/Japan/305/57
   A/PR/8/34

C. A/NJ/1/76
   A/Duck/Alb/76
   A/Duck/Penn/84
   A/Hong Kong/483/97
   A/Hong Kong/156/97
   A/Japan/305/57
   A/PR/8/34

PBMC from A) donor 1, B) donor 2, and C) donor 3 were stimulated with PR/8/34 (H1N1) infected autologous stimulators. Specific lysis of uninfected control targets was subtracted from the infected targets.
D. HUMAN T CELL LINE RECOGNITION OF MUTATED PEPTIDES FROM THE A/HK/97 STRAINS.

The effect of mutations in the A/HK/156/97 (H5N1) virus sequence at epitopes recognized by T cells on the A/PR/8/34 (H1N1) epitope sequence were examined. The results in Table IV-3 show several mutations and recognition patterns. Cell line 10-2C2, is CD8+ and recognizes aa 122-130 on the NS1 protein of H1N1, H2N2, and H3N2 viruses, but it does not recognize target cells pulsed with the A/HK/156/97 peptide due to a single N→D mutation at aa 127. There is also a M→I change at aa 129, but this change in sequence is also found in the PR/8/34 (H1N1) strain that this cell line does recognize. Thus it is the N→D mutation that abolishes recognition by the 10-2C2 cell line. The CD4+ cell line 3E5, which recognizes NP aa 254-264 of the PR/8/34 (H1N1) strain, also recognized targets pulsed with the A/HK/156/97 peptide despite a T→I mutation at aa 258. A CD8+ CTL line 124, which recognizes an epitope on M2 aa 7-15 on the H1N1 subtype virus, does not recognize the H5N1 peptide which has changes at aa 10, 11, and 14. These results illustrate that epitopes of avian viruses with conservative aa changes can still be recognized by human CTL, while those with nonconservative changes cannot.
Table IV-3. Effect of Mutations on the HK/156/97 Virus on CTL Recognition by Human CD8+ and CD4+ CTL lines to H1N1 Epitopes.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Cell Line</th>
<th>CD4/CD8</th>
<th>Protein</th>
<th>Epitope</th>
<th>Virus Strain</th>
<th>% Specific Lysis of Peptide</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pulsed Targets</td>
<td>2.5μg/ml</td>
<td>.25μg/ml</td>
</tr>
<tr>
<td>1</td>
<td>102C2</td>
<td>CD8</td>
<td>NS1</td>
<td>122-130</td>
<td>Udorn/74</td>
<td>77.2</td>
<td>79.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HK/156/97</td>
<td>-5.1</td>
<td>-7.1</td>
</tr>
<tr>
<td>2</td>
<td>3E5</td>
<td>CD4</td>
<td>NP</td>
<td>254-264</td>
<td>PR/8/34</td>
<td>16.7</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HK/156/97</td>
<td>19.1</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>124</td>
<td>CD8</td>
<td>M2</td>
<td>7-15</td>
<td>PR/8/34</td>
<td>75</td>
<td>65.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HK/156/97</td>
<td>-1.9</td>
<td>-2.1</td>
</tr>
</tbody>
</table>

*The E:T ratio is 10:1. Changes in aa sequence are bold and underlined.
E. PRECURSOR FREQUENCY DETECTION BY ELISPOT.

Table IV-4 demonstrates the precursor frequency of CTL detected in the PBMC of donor 1 specific for two epitopes on the human H1N1 and Hong Kong H5N1 viruses determined using the ELISPOT assay for IFN-γ secretion. In donor 1, stimulation of PBMC with the peptide based on NP aa 174-184 of PR/8/34 (H1N1) had a precursor frequency of 1/4,348. Stimulation with the NP 174-184 peptide based on the HK/156/97 strain with a V→I change at aa 183 produced a similar number of IFN-γ-secreting cells (1/5,263). On the other hand, stimulation with a peptide that incorporates the E→Q change at aa 23 on the M1 protein of the avian Hong Kong 483 strain resulted in the loss of detectable IFN-γ secreting cells. Thus the quantitative detection of individual peptide-specific T cells showed either a similar frequency of IFN-γ secreting CTL or an absence of IFN-γ secreting CTL, depending on the specific epitope and the mutations present in the recently isolated Hong Kong viruses.

A summary of the effect of sequence conservation and mutation at CTL epitopes in the human isolates of avian-derived viruses (A/HK/156/97 and A/HK/483/97 (H5N1)) is presented in Table IV-5. Sequence data are available for comparison of seven human CTL epitopes. Three epitopes are entirely conserved and are cross-reactive. One CD4+ CTL epitope (M1 17-31) is conserved in A/HK/156/97, but not in A/HK/483/97. In this case the E→Q change at aa 23 is not recognized by the cell line. There are three other epitopes that are not conserved; a B27-restricted cell line recognizes a mutation on NP at aa 183 (V→I). The other two epitopes with mutations are not recognized by the cell lines (NS1 122-130 and M2 7-15). Therefore, there is cross-reactive recognition by the
human CTL lines of the avian virus strains that are conserved or have conservative aa changes.
Table IV-4. Precursor Frequency Analysis of Mutations in HK/156/97 and HK/483/97 Using ELISPOT Assay*.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Protein Specificity</th>
<th>Epitope</th>
<th>Virus Strain</th>
<th>Precursor Frequency</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NP</td>
<td>174-184</td>
<td>PR/8/34</td>
<td>1/4,348</td>
<td>RRSGAAGAAVK</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HK/156/97</td>
<td>1/5,263</td>
<td>RRSGAAGAAIK</td>
</tr>
<tr>
<td>1</td>
<td>M1</td>
<td>17-31</td>
<td>PR/8/34</td>
<td>1/50,000</td>
<td>SGPLKAEI&amp;QRLEDV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HK/483/97</td>
<td>&lt;1/500,000</td>
<td>SGPLKAI&amp;QRLEDV</td>
</tr>
</tbody>
</table>

*Changes in aa are bold and underlined.
Table IV-5. Amino Acid Sequence of CTL Epitopes on Different Influenza Virus Strains*.

<table>
<thead>
<tr>
<th>Protein Specificity</th>
<th>MHC</th>
<th>Epitope</th>
<th>Strain Sequence</th>
<th>Virus Strain</th>
<th>Relative SIL</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS1</td>
<td>A2.1</td>
<td>122-130</td>
<td>AIMDKINIIML</td>
<td>Udorn/74</td>
<td>+</td>
<td>A04088</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AIMDKNIIL</td>
<td>PR/8/34</td>
<td>+</td>
<td>J02150</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AIMDKDIL</td>
<td>HK/156/97</td>
<td>-</td>
<td>AF036360</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AIMDKDIL</td>
<td>HK/483/97</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td>B44</td>
<td>7-15</td>
<td>VETPIRNEW</td>
<td>PR/8/34</td>
<td>+</td>
<td>M10642</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VETLTRNGW</td>
<td>Chick/Pen/1370/83</td>
<td>-</td>
<td>J.Virol. 65:5491</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VETLTRNGW</td>
<td>HK/156/97</td>
<td>-</td>
<td>AF036358</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VETLTRNGW</td>
<td>HK/483/97</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>B27</td>
<td>383-391</td>
<td>SRYWAIRTR</td>
<td>PR/8/34</td>
<td>+</td>
<td>J02147</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SRYWAIRTR</td>
<td>NJ/8/76</td>
<td>+</td>
<td>M63754</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SRYWAIRTR</td>
<td>HK/156/97</td>
<td>+</td>
<td>AF036359</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SRYWAIRTR</td>
<td>HK/483/97</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>A2.1</td>
<td>58-66</td>
<td>GILGFVFTL</td>
<td>PR/8/34</td>
<td>+</td>
<td>M10642</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GILGFVFTL</td>
<td>HK/156/97</td>
<td>+</td>
<td>AF036358</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GILGFVFTL</td>
<td>HK/483/97</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NP</td>
<td>B27</td>
<td>174-184</td>
<td>RRSGAAGAAVK</td>
<td>PR/8/34</td>
<td>+</td>
<td>J02147</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RRSGAAGAIAK</td>
<td>HK/156/97</td>
<td>+</td>
<td>AF036359</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RRSGAAGAIAK</td>
<td>HK/483/97</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>NS1</td>
<td>DR3</td>
<td>34-42</td>
<td>DRLRDQKS</td>
<td>PR/8/34</td>
<td>+</td>
<td>J02150</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DRLRDQKS</td>
<td>HK/156/97</td>
<td>+</td>
<td>AF036360</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DRLRDQKS</td>
<td>HK/483/97</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>DR1</td>
<td>17-31</td>
<td>SGPLKAQIAQRLEDV</td>
<td>PR/8/34</td>
<td>+</td>
<td>M10642</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SGPLKAQIAQRLEDV</td>
<td>HK/156/97</td>
<td>+</td>
<td>AF036358</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SGPLKAQIAQRLEDV</td>
<td>HK/483/97</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

*The letter c designates unpublished observations by K. Subbarao. Changes in aa are bold and underlined. SIL is specific immune lysis of greater than 15%. SIL= specific immune lysis, lysis of uninfected targets subtracted from infected or peptide pulsed targets.
CHAPTER V

IN VITRO AND IN VIVO T CELL RESPONSES TO FLU-ISCOM VACCINATION

A. IN VITRO CTL LYSIS OF TARGET CELLS PULSED WITH FLU-ISCOM.

We wanted to determine whether Flu-Iscom (inactivated influenza vaccine incorporated into Iscom particles) could be presented to influenza specific CTL lines in vitro. The Iscom particle is thought to be a vehicle for entering antigen into the class I pathway via endocytosis and entry of antigen into the cytoplasm. A CD8+ HA-specific cell line was tested for recognition of B-LCL targets pulsed with either monovalent formalin-inactivated, detergent-disrupted A/Texas (H1N1) vaccine alone or incorporated into Iscom particles. As expected, there was no lysis of targets pulsed with the vaccine alone (1.4%), but there was specific lysis of target cells pulsed with the Flu-Iscom formulation (76.6%) (Table V-1). Although the background for both of these targets was high, it did not increase the specific lysis since the lysis of A/Texas vaccine-pulsed targets was negative (1.4%). There was also lysis of live virus-infected target cells, which were positive controls, and there was no lysis of target cells exposed to Iscomatrix alone without Flu-vaccine which was the negative control.

Next we tested the ability of another CD8+ CTL line to lyse targets pulsed with an influenza A viral protein alone or mixed with Iscomatrix. The NS1 protein was mixed
with Iscomatrix for 24 hours at 4°C before addition to the target cells for another 24 hours. This protein is not in high abundance in the inactivated vaccine preparation, so this would determine whether any influenza virus protein activates T cells in an Iscom vaccine. However, this NS1 protein was mixed with Iscomatrix. The CTL line 10-2C2 recognizes aa 122-130 of NS1 and is cross-reactive to human subtypes of influenza H1, H2, and H3. In Table V-2 lysis of target cells pulsed with the 122-130 peptide was high (88.4%) which served as a positive control. The negative control with uninfected cells had no specific lysis. There was also no lysis of target cells exposed to either the NS1 protein alone or Iscomatrix alone, but there was lysis of target cells exposed to the mixture of NS1 protein and Iscomatrix (19.6%). Target cells pulsed with the NS1 protein without Iscoms were not lysed, but this was not due to a failure by the target cells to present the NS1 protein, because a CD4+ CTL line specific for another epitope on the NS1 protein lysed the NS1 protein-pulsed targets (data not shown).
Table V-1. Recognition by HLA B18 Restricted HA-Specific Human CD8+ CTL Cell line of A/Texas H1N1 Flu-Iscom Vaccine Pulsed BLCL.

<table>
<thead>
<tr>
<th>HA-Specific cell line</th>
<th>PR/8/34 Virus</th>
<th>A/Texas Virus</th>
<th>Isomatrix*</th>
<th>A/Texas Vaccine†</th>
<th>A/Texas Flu-Iscom§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1 10-2F5</td>
<td>53</td>
<td>33.8</td>
<td>-1.6</td>
<td>1.4</td>
<td>76.6</td>
</tr>
<tr>
<td>Min/Max</td>
<td>12.8</td>
<td>12.1</td>
<td>17.4</td>
<td>41.3</td>
<td>44.6</td>
</tr>
</tbody>
</table>

*Saponin concentration used was 337.5 μg/ml.

†HA concentration used was 82 μg/ml.

§HA concentration used was 57.5 μg/ml; saponin concentration was 337.5μg/ml. The E:T ratio = 10:1.
Table V-2. Recognition by Human HLA A2.1 Restricted NS1 aa 122-130 Specific CD8+ CTL Cell line of Recombinant NS1 Protein and Iscomatrix Pulsed BLCL.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Uninfected</th>
<th>NS1 Protein (aa 122-130)*</th>
<th>NS1 Protein‡</th>
<th>NS1 Protein + Iscom‡,§</th>
<th>Iscom-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1 10-2C2</td>
<td>-6.2</td>
<td>88.4</td>
<td>-0.7</td>
<td>19.6</td>
<td>-11.0</td>
</tr>
<tr>
<td>Min/Max</td>
<td>17.8</td>
<td>19.6</td>
<td>17.3</td>
<td>29.9</td>
<td>21.3</td>
</tr>
</tbody>
</table>

*Peptide used at 25 ng/ml (killing observed down to 0.025 μg/ml also).

‡Protein used at 35 μg/ml.

§Saponin used at 100 μg/ml.

◊Saponin used at 70 mg/ml. The E:T ratio was 10:1.
B. HUMAN CTL RESPONSES TO FLU-ISCOM VACCINES.

In order to determine whether humans would be able to mount a T cell response to influenza A by vaccination with the Flu-Iscom vaccines we decided to analyze responses in vivo. Therefore, a clinical study was performed on 55 healthy adults (ages 18-45 years), and CTL assays were performed on the PBMC obtained from samples of blood drawn on day 0 (pre-vaccination), 14, and 56 after vaccination with a single immunization of a formalin-inactivated vaccine ± Iscoms or Iscomatrix. John Cruz tested samples of PBMC obtained on days 0, 14, and 56 from each donor together in the same assay to eliminate variability of CTL assays performed on different days. In addition, he included as a positive control in each assay a well-characterized CTL cell line to ensure that the virus-infected target cells were suitable for detecting specific lysis in the CTL assay. All assays were performed and interpreted under code.

We defined an increase in the post-vaccination sample of ≥ 10% specific immune lysis (SIL) above the day 0 level in at least 2/3 E:T ratios was a ++ response; an increase of ≥ 5% (a + response) was uniformly found to be statistically significant. Increases in the percentage lysis of <5% were considered negative although some values were still statistically significant. The CTL data were sent to the clinical monitor who organized the results into the vaccine groups. Table V-3 summarizes the increases in influenza A virus CTL memory detected in the three vaccine groups. The CTL assay results showed statistically significant (P < 0.01) increases in influenza A-specific CTL memory on days 14 or 56 (5.0-37.5% increases) in approximately 50% of the assays performed on individuals who received one dose of vaccine formulated into Iscoms and in about 30%
of the assays of individuals given vaccine mixed with the Iscomatrix. However, only 5% of the CTL assays of the recipients of the standard vaccine had an increase in influenza A-specific CTL memory.
Table V-3. CTL Responders in the Flu-Iscom Phase 1 Trial*.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Flu-Iscom[^1]</th>
<th>Flu-Iscomatrix</th>
<th>Fluzone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50/45</td>
<td>75/45</td>
<td>50/45</td>
</tr>
<tr>
<td>H1 (A/Tx/36/91)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonresponders</td>
<td>4</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>5-10%</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>&gt;10%</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Responders</td>
<td>7</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>H3 (A/Jhn/33/94)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonresponders</td>
<td>7</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>5-10%</td>
<td>2</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>&gt;10%</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Responders</td>
<td>4</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

*Subjects showing from 5-37.5% increases in specific immune lysis in at least two E:T ratios by the day 14 or day 56 samples above the level of lysis by the day 0 samples.

These experiments were performed by John Cruz.

[^1]: Numerator = dose of Iscomatrix/ denominator = dose of virus HA.
C. PEPTIDE SPECIFIC RESPONSES WERE DETECTED IN FLU-ISCOM VACCINE RECIPIENTS.

We studied the PBMC of two HLA-typed vaccine recipients that had received the Flu-Iscom vaccine. As demonstrated by John Cruz, the PBMC of these donors had increased levels of influenza-specific cytotoxic activity post-vaccination. Using the ELISPOT assay, we examined whether their PBMC post-vaccination had increases in the peptide-specific CTL that secrete IFN-γ. Donor 54 had an increase in the precursor frequency of CTL that recognized aa 383-391 of nucleoprotein (NP) on days 14 and 56 (Fig. V-1B). This is a HLA-B27 restricted T-lymphocyte epitope, and this donor has the HLA-B27 allele. Donor 54 also had a modest increase in IFN-γ secreting T cells that recognize an HLA-A2.1 restricted epitope aa 58-66 of matrix 1 protein (M1), but no increases were detected against a second HLA-B27 restricted epitope NP aa 174-184 (Fig. V-1A, V-1B). Therefore, the number of peptide-specific CTL secreting IFN-γ in response to at least two epitopes that utilize different HLA alleles for presentation were boosted in this donor. Both of these increases occurred by day 14 and remained high at day 56 after vaccine administration. This indicates that the number of IFN-γ secreting CTL specific for influenza A virus subtype cross-reactive epitopes were increased up to at least 56 days post-vaccination.

Donor 8 had a delayed response in which CTL specific for epitopes on influenza were undetectable on days 0 and 14. However, by day 56 the number of IFN-γ secreting cells specific for aa 44-52 on NP increased from undetectable to 1/41,667 (Fig. V-1D). This epitope is HLA-A1 restricted. Likewise, CTL specific for aa 58-66 on M1 increased
from undetectable to 1/53,763 (Fig. V-1C). Neither of these donors’ PBMC had
detectable responses to the control allantoic fluid or to a control peptide derived from
Hantavirus (data not shown). These results indicate that the Flu-Iscom vaccine
augmented the numbers of CTL that recognize epitopes on M1 and NP, two internal,
conserved viral proteins.
Fig. V-1. Peptide-Specific Responses of Two Donors Who Received the Flu-Iscom Vaccine.

The PBMC from A, B) donor 54 and C, D) donor 8 were stimulated with 10μg/ml of peptide in ELISPOT assays.
D. INCREASES IN VIRUS-SPECIFIC IFN-γ SECRETION AND IN VIRUS-
SPECIFIC CTL ACITIVITY.

We next tried to detect changes in the numbers of influenza virus-specific CTL that secrete IFN-γ by using live influenza virus as a stimulating antigen in ELISPOT assays.

Three general trends in virus-specific CTL secreting IFN-γ were detected. Some vaccine recipients had very low numbers of precursors specific for influenza virus on days 0 and 14, but by day 56 the numbers were significantly increased. The PBMC of donor 8 showed this increase; there were no detectable IFN-γ secreting cells that recognized A/Johan/94 on days 0 and 14, but by day 56 a significant increase in IFN-γ secreting CTL was detected (Fig. V-2A). Other vaccine recipients had a boost in influenza-specific IFN-γ secreting CTL in their PBMC above pre-vaccine levels detectable by day 14 after vaccination. This was observed in donor 45, where an increase in the number of IFN-γ secreting T cells cross-reactive for both A/Johan/94 (H3N2) or A/Texas/91 (H1N1) was detected (Fig. V-2B, 3B). Donors 7 and 53 also had a boost in CTL specific for A/Texas/91 that was sustained until day 56 (Fig. V-3A, 3C). Donor 53 had a different type of response to A/Johan/94 with an increase in precursor frequency on day 14 (Fig. V-2C), but by day 56 the number of IFN-γ secreting T cells had dropped almost to pre-vaccine levels.

In most of the vaccine recipients, when there were increases in the number of IFN-γ secreting cells there were also increases in cytotoxic T cell levels of specific lysis by bulk culture effector cells that were described previously (Section B). Tables V-4 and V-5 contain both the frequencies of IFN-γ secreting T cells and the increases in cytotoxic
activity. In general, when the numbers of IFN-γ secreting cells determined by ELISPOT assay increased, there was also an increase in CTL cytotoxicity. In Table V-4 the only time point that did not correlate between increases in the number of IFN-γ secreting cells and cytotoxic lysis to A/Johan/94 was the PBMC of donor 23 on day 56. Although the number of IFN-γ secreting cells remained high, the CTL lysis did not increase when compared to day 0. Usually the frequency of T cells that secrete IFN-γ in response to A/Texas/91 (Table V-5) also increased when cytotoxic activity increased. In donors 7 and 45, however, an increase in IFN-γ secreting cells was detected at day 14 without a corresponding increase in cytotoxicity; but increases in both IFN-γ secreting cells and bulk culture CTL activity are detectable by day 56. Donor 30 maintained an increased IFN-γ secreting cells at day 56, but did not exhibit an increase in CTL lysis on that day.

Overall, when there were increases in the levels of bulk cytotoxic activity, the ELISPOT assay also quantitated increases in IFN-γ secreting CTL-specific for influenza. When differences between the two assays were observed, changes were detected by ELISPOT assay, but not by the chromium release assay, suggesting that the ELISPOT assay may be more sensitive. To be certain that the population of cells that secrete the IFN-γ were T cells, we used intracellular IFN-γ staining. Using two-color FACS we found that approximately 78% of the IFN-γ secreting cells were CD8+, 22% were CD4+, and less than 1% were CD56+ cells (data not shown).
Three donors with increases in the numbers of cells secreting IFN-γ in response to A/Johan/33/94. Donor 8 (A) received Flu-Iscom 75/45, donor 45 (B) received Flu-Iscom 50/45, and donor 53 (C) received Flu-Iscom 50/45. Bars represent ELISPOT # SFC per 1x10^6 PBMC, the line represents the % specific lysis previously discussed. The * = p<.05 and ** = p<.01 by student t-test. SFC stands for spot forming cells.
Three donors with increases in the numbers of cells secreting IFN-γ in response to A/Texas/31/91. Donor 7 (A), donor 45 (B), and donor 53 (C) received Flu-lscom 50/45. Bars represent ELISPOT # SFC per 1x10⁶ PBMC, the line represents the % specific lysis previously described. The * = p<.03 and ** = p<.005 by student t-test. SFC stands for spot forming cells.
Table V-4. Precursor Frequency of Influenza Virus (A/Johan/97, H3N2)-Specific T Cells in Recipients of Adjuvanted Vaccines Pre and Post Vaccination.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Vaccine Received</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 56</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pCTL</td>
<td>pCTL</td>
<td>Increase in CTL lysis</td>
<td>pCTL</td>
</tr>
<tr>
<td>8</td>
<td>FluIscom 75/45</td>
<td>1/115,385</td>
<td>1/150,000</td>
<td>-</td>
</tr>
<tr>
<td>29</td>
<td>FluIscom 75/45</td>
<td>1/8,475</td>
<td>1/6,727</td>
<td>++</td>
</tr>
<tr>
<td>54</td>
<td>FluIscom 75/45</td>
<td>1/1,042</td>
<td>1/857</td>
<td>+</td>
</tr>
<tr>
<td>53</td>
<td>FluIscom 50/45</td>
<td>1/4,886</td>
<td>1/2,308</td>
<td>++</td>
</tr>
<tr>
<td>45</td>
<td>FluIscom 50/45</td>
<td>1/3,513</td>
<td>1/3,036</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>FluIscomatrix 75/45</td>
<td>&lt;1/150,000</td>
<td>1/7,389</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>FluIscomatrix 75/45</td>
<td>&lt;1/150,000</td>
<td>1/88,235</td>
<td>+</td>
</tr>
</tbody>
</table>

A ++ signifies an increase in specific immune lysis of greater than 10% as compared to day 0, + represents an increase of greater than 5%, and - represents no increase in specific immune lysis. All increases in CTL lysis are in comparison to day 0.
Table V-5. Precursor Frequency of Influenza A Virus (A/Texas/97, H1N1)-specific T Cells in Recipients of Adjuvanted Vaccines Pre and Post Vaccination.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Vaccine Received</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Day 56</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pCTL</td>
<td>pCTL</td>
<td>Increase in CTL lysis</td>
<td>pCTL</td>
</tr>
<tr>
<td>3</td>
<td>FluIscom 75/45</td>
<td>1/10,122</td>
<td>1/7,289</td>
<td>+</td>
</tr>
<tr>
<td>29</td>
<td>FluIscom 75/45</td>
<td>1/65,217</td>
<td>1/26,316</td>
<td>++</td>
</tr>
<tr>
<td>54</td>
<td>FluIscom 75/45</td>
<td>1/1,320</td>
<td>1/939</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>FluIscom 50/45</td>
<td>1/50,000</td>
<td>1/8,197</td>
<td>-</td>
</tr>
<tr>
<td>53</td>
<td>FluIscom 50/45</td>
<td>1/3,778</td>
<td>1/1,515</td>
<td>++</td>
</tr>
<tr>
<td>45</td>
<td>FluIscom 50/45</td>
<td>1/4,732</td>
<td>1/1,777</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>FluIscomatrix 75/45</td>
<td>&lt;1/150,000</td>
<td>1/88,235</td>
<td>++</td>
</tr>
<tr>
<td>30</td>
<td>FluIscomatrix 75/45</td>
<td>&lt;1/150,000</td>
<td>1/88,235</td>
<td>+</td>
</tr>
</tbody>
</table>

A ++ signifies an increase in specific immune lysis of greater than 10% as compared to day 0, + represents an increase of greater than 5%, and - represents no increase in specific immune lysis. All increases in CTL lysis are in comparison to day 0.
E. FLUZONE CONTROL VACCINE RECIPIENTS DID NOT HAVE INCREASES IN THE PRECURSOR FREQUENCY OF INFLUENZA A-SPECIFIC CTL.

The recipients of the standard vaccine (Fluzone), without being incorporated into Iscom particles or being mixed with Iscomatrix, did not have increases in bulk culture CTL activity after immunization. The PBMC from 3 Fluzone recipients, selected based on availability of PBMC samples, were tested pre and post vaccination, and there was no change in the precursor frequency of influenza A-specific CTL after vaccination (Fig. V-5 and Table V-6).
Table V-6. Precursor Frequency of Influenza-A Virus specific T Cells of Three Fluzone Vaccine Recipients Pre and Post Vaccination.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Virus Recognized</th>
<th>pCTL Day0</th>
<th>pCTL Day 14</th>
<th>Increase in CTL lysis</th>
<th>pCTL Day 56</th>
<th>Increase in CTL lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>A/Johan/94</td>
<td>1/62,500</td>
<td>1/115,385</td>
<td>-</td>
<td>&lt;1/150,000</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>A/Johan/94</td>
<td>1/2,830</td>
<td>1/3,606</td>
<td>-</td>
<td>1/3,171</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>A/Texas/91</td>
<td>1/2,600</td>
<td>1/3,546</td>
<td>-</td>
<td>1/3,061</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
<td>A/Johan/94</td>
<td>1/4,630</td>
<td>1/4,087</td>
<td>-</td>
<td>1/4,839</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
<td>A/Texas/91</td>
<td>1/5,618</td>
<td>1/5,495</td>
<td>-</td>
<td>1/6,438</td>
<td>-</td>
</tr>
</tbody>
</table>

A ++ signifies an increase in specific immune lysis of greater than 10% as compared to day 0, + represents an increase of greater than 5%, and - represents no increase in specific immune lysis.
Fig. V-5. Number of A/Johan/33/94-Specific SFC in Fluzone Vaccine Recipients.

The responses of three Fluzone vaccine recipients specific for A/Johan/33/94 in ELISPOT assays.
CHAPTER VI

γδ T CELL ACTIVATION IN RESPONSE TO INFLUENZA A INFECTION IN HUMANS

A. γδ T CELLS ARE ACTIVATED TO SECRETE IFN-γ IN RESPONSE TO INFLUENZA INFECTION OF HUMAN PBMC.

After determining that humans have a broad CTL repertoire specific for influenza A, we became interested in the other T cell population called γδ T lymphocytes. We wanted to analyze the interactions of influenza A virus and the γδ T lymphocytes in the PBMC. Although γδ T cells only make up 1-10% of the PBMC, they are in much higher proportion in the mucosal areas. These T cells have the ability to secrete cytokines and lyse cells, and can home to the site of infection (Carding et al., 1990; Eichelberger et al., 1991; Allan et al., 1992; Boullier et al., 1997; Vincent et al., 1998). To observe whether they could be activated to secrete IFN-γ in response to influenza infection, we used intracellular IFN-γ staining of PBMC. After the PBMC were infected for 8-10 hours, the cells were stained and analyzed by flow cytometry. The T cells were gated on the γδ+ population and the CD8+/CD8-, IFN-γ+/IFN-γ- cells were analyzed.

The % of γδ+ T cells in the PBMC of donor 1 that were IFN-γ+ increased from 2.9% to 9.68% after exposure to PR/8/34 (H1N1) virus in vitro (Fig. VI-1). More noticeable was the CD8- population that increased almost 5 fold. We also found an
increase in response to A/Japan/57 (H2N2) influenza virus, but not in response to A/Port Chalmers/1/73 (H3N2) influenza virus (data not shown). There seemed to be activated CD8+ T cells, but the \( \gamma^\delta \) T cells did not secrete IFN-\( \gamma \) in response to this H3N2 subtype of influenza. In order to determine whether this increase was a donor-specific phenomenon we analyzed the PBMC of two other donors. Donor 2 had an increase in the \( \gamma^\delta{+} \)IFN-\( \gamma{+} \) population from about 4% in the Ig control to 30% after exposure to PR/8/34 virus (Fig. VI-2). This increase in activated \( \gamma^\delta \) cells was again most noticeable in the CD8- population where there was more than a 12-fold increase in IFN-\( \gamma \)-secreting \( \gamma^\delta \) cells. Donor 5 had more activated \( \gamma^\delta \) cells in the PBMC prior to infection; however, a clear increase in IFN-\( \gamma \)-secreting \( \gamma^\delta \) cells was apparent (Fig. VI-3). The percent of IFN-\( \gamma{+}\gamma^\delta{+} \) cells went from about 24% to over 56%. Again, the greatest increase in IFN-\( \gamma{+} \) cell was observed in the CD8- \( \gamma^\delta \) cell population with over a 3 fold increase.

In order to determine whether the virus infection was an important factor in this \( \gamma^\delta \) T cell activation, PR/8/34 virus was inactivated by boiling and then added to the cells for the 8-10 hour incubation. In all three donors, the inactivated virus abolished most, if not all, IFN-\( \gamma \) secretion by the \( \gamma^\delta \) cells. Donor 1 maintained a slight increase, but it was substantially lower than the increase seen after exposure to live influenza virus (Fig. VI-1). Donor 2 had a 3-fold increase with the inactivated virus stimulation, but it did not reach the 12-fold increase observed with live virus infection (Fig. VI-2). Donor 5 had a similar percent of activated \( \gamma^\delta{+} \)CD8- T cells, with inactivated virus stimulation as with compared to no stimulation (Fig. VI-3).
Although the \( \gamma \delta + \text{CD8} + \) T cells also produce IFN-\( \gamma \) upon influenza infection in the three donors tested, it is less clear whether inactivated virus also stimulates these cells. In donor 1 the inactivated virus stimulated PBMC to a similar degree as the live virus (Fig. VI-1). However, in donors 2 and 5 there were fewer \( \gamma \delta + \text{CD8} + \) T cells activated by inactivated virus as compared to live virus infection (Fig. VI-1, VI-2).
Fig. VI-1. Donor 1 γδ+ T Cells Respond to Influenza Infection.

Control Ig as FL2

Uninfected

PR/8 Virus

Inactivated PR/8

The data are gated by γδ+ lymphocytes. In this donor 2.5% of the PBMC are γδ+. 3000 events were plotted. 80μl of both PR/8/34 and inactivated PR/8/34 were added.
Fig. VI-2. Donor 2 γδ T Cells Respond to Influenza Infection.

Control Ig as FL2

Uninfected

PR/8 Virus

Inactivated PR/8

IFN-γ

The data are gated by γδ+ lymphocytes. In this donor 1.26% of the PBMC are γδ+. 1500 events were plotted. 80μl of both PR/8/34 and inactivated PR/8/34 were added.
Fig. VI-3. Donor 5 γδ T Cell Respond to Influenza Infection.

Control Ig as FL2

Uninfected

PR/8 Virus

Inactivated PR/8

The data are gated by γδ+ lymphocytes. In this donor 2.35% of the PBMC are γδ+. 1500 events were plotted. 80μl (moi of 10) of both PR/8/34 and inactivated PR/8/34 were added.
B. TIME COURSE OF INF-γ PRODUCTION BY γδ T CELLS AFTER EXPOSURE TO INFLUENZA VIRUS AND DOSE RESPONSE OF VIRUS DILUTIONS.

To look at the number of IFN-γ secreting γδ T cells after influenza infection over time, we added influenza virus at time 0 and harvested the cells at various time points thereafter. We analyzed the CD8- population of activated γδ T cells. Around 4-6 hours post influenza infection there was a peak in IFN-γ secretion in donor 1, 8-10 hours with the brefeldin A treatment included (Fig. VI-4). Therefore, the CD8-γδ+ T cell population secretes IFN-γ relatively quickly, in the first 10 hours after exposure to influenza virus. This is similar to αβ T cells previously found to secrete IFN-γ by 6 hours post infection (Murali-Krishna et al., 1998).

Next we analyzed the dose of virus which generated the highest number of CD8-IFN-γ secreting γδ T cells. At a 1:11 dilution of virus (approximately an moi of 8), the peak IFN-γ response occurred, while further dilutions produced decreasing numbers of IFN-γ secreting cells (Fig. VI-5). Both the 8-10 hour total time point and the 1:11 dilution of virus were used in the subsequent experiments.
Fig VI-4. Time Course of IFN-γ production by CD8-γδ+ cells in the PBMC of Donor 1.

The hours indicated on the X axis are the number of hours prior to the addition of brefeldin A for 4 hours. The γδ+ CD8- population was gated.
Fig IV-5. Dose Response of Influenza Virus Infection of PBMC.

Virus was diluted and added to PBMC for 8-10 hours. The γδ+ CD8- population was gated.
C. EXPRESSION OF ACTIVATION MARKERS ON γδ T CELLS IN RESPONSE TO INFLUENZA INFECTION.

In order to determine whether γδ T cells increase the surface expression of T cell activation markers in response to influenza virus, expression of CD69 and CD25 were analyzed. CD69 and CD25 are early activation markers expressed on αβ T cells. γδ T cells have been shown to express CD25 and CD69 also (Moretta et al., 1991; Wallace et al., 1995). PBMC were stimulated with influenza virus for a total of 8-10 hours and two-color flow cytometry was performed. In donor 1 there was a large increase in CD69 expression of γδ T cells in response to influenza virus. The percent of γδ+ T cells expressing high levels of CD69 went up almost 85%, and the mean level of fluorescence increased (Fig. VI-6).

To further analyze the type of γδ T cell that is upregulating CD69, we used three-color flow cytometry to distinguish between the CD8+ and the CD8- populations of γδ T cells. In donor 1 there was an increase in the percent of both CD8+ and CD8- CD69+ γδ T cells. The CD8+ CD69+ T cells increased from approximately 52% in uninfected to 88% in the PR/8/34 infected cells. This percentage was 86% of the CD8+ γδ T cells exposed to the inactivated virus. The CD8- population is about three times the size as the CD8+ population, and it also had an increase in surface CD69 expression in response to PR/8/34. The CD8-γδ+ cells went from 40% CD69 expression positive in the uninfected population to 92% in the influenza infected cells. There was a slight decrease in the percentage of cells expressing CD69 (10%) when stimulated with inactivated virus. The
data on CD69 expression were not as different between live and inactivated virus in this donor.
Fig. VI-6. CD69 Expression on γδ T Cells Infected with PR/8/34 (H1N1) in Donor 1.

Ig Control

Uninfected

PR/8/34 Virus

PBMC was γδ+ gated, and CD69 expression assessed. Between 450 and 700 events were plotted as histograms.
Fig. VI-7. Surface Expression of CD69 on CD8+ or CD8- γδ+ T cells from Donor 1.

Uninfected

PR/8 Virus

Inactivated Virus

The PBMC is gated on γδ+ cells with either CD8+ or CD8-. Between 1700 and 3000 events were plotted on the histograms.
CD25 expression was analyzed in the PBMC of donor 5. The γδ population in this donor is comprised of approximately 15% CD8+ and 85% CD8- T cells. Therefore, more cells could be analyzed with the CD8- phenotype. Similar results as with the CD69 surface expression were observed. First of all, the CD8+ and CD8- γδ+ T cells increased CD25 surface expression in response to influenza infection (from 5% to 41% for CD8+; from 1.6% to 32% for CD8-) (fig. VI-8). For both cell types the inactivated virus did not stimulate a CD25 high population, but did stimulate some increased CD25 expression on γδ cells.
Fig. VI-8. CD25 Surface Expression on the γδ T Cell Population From Donor 5.

**Uninfected**

**PR/8/34 Virus**

**Inactivated Virus**

The PBMC was γδ+ gated and either CD8+ and CD8-. 2000 CD8- events or 250 CD8+ events were plotted in histograms.
D. DETERMINATION OF WHETHER IFN-γ SECRETION BY γδ T CELLS CAN BE INHIBITED BY ANTI-CLASS I MONOCLONAL ANTIBODIES.

Some groups have found that γδ T cells are restricted by MHC alleles, but, most groups have found that γδ T cells are non-MHC restricted. To determine whether the activation is MHC restricted we incorporated an anti-class I mAb into the intracellular IFN-γ staining assay. Antibody was added prior to the addition of influenza virus or inactivated virus particles. No inhibition was found for the CD8- γδ+ T cell population (Fig. VI-9). There were comparable numbers of IFN-γ secreting cells with or without the antibody (3.9%). However, the CD8+ γδ+ T cells were inhibited by the antibody by greater than 50% (from 23% down to 9.2%). To be sure that the antibody was added at the proper concentration, we analyzed the CD8+ population as a whole and observed an inhibition in IFN-γ secretion upon addition of the antibody (data not shown). This indicates that some CD8+ γδ+ T cells may use the MHC class I molecule to become activated. However, the CD8- γδ+ T cells become activated to secrete IFN-γ by a different means.
Fig. VI-9. Anti-Class I Antibody Inhibits CD8+ γδ+ T Cell IFN-γ Secretion, But Not CD8- γδ+ T Cells.

The γδ+ lymphocytes were gated. There were 1800 events plotted on the histograms. The mAb was used at a dilution of 1:1000 for 30 minutes prior to addition of virus to the PBMC of donor 1.
E. VACCINIA VIRUS DOES NOT ACTIVATE $\gamma^8+$ CELLS IN PBMC AS WELL AS INFLUENZA.

To determine whether any virus would give us similar results, we infected PBMC with vaccinia virus ($3 \times 10^8$ PFU/ml) instead of influenza virus. Although there was the characteristic increase in $\gamma^8+$CD8- cells that produce IFN-$\gamma$ in response to influenza infection, there was no increase detected with vaccinia virus infection (Fig. VI-10).

There was some activation of the $\gamma^8+$CD8+ cells (.65%) but it did not reach the amount detected with influenza infection (4.40%). To be certain that the vaccinia virus was live and at a high enough titer, we analyzed the entire CD8+ T cell population in the PBMC and found that approximately 3% of the CD8+ T cells (probably $\alpha\beta$) in the lymphocyte population responded to vaccinia virus as compared to the .59% by uninfected cells (data not shown).
Fig. IV-10. Donor 1 γ/δ T Cells Do Not Respond to Vaccinia Virus. 

Uninfected  

PR/8 Virus  

The γδ+ lymphocytes were gated. There were 2500 events plotted on the histograms.

Vaccinia virus dilutions of either 1:200 or 1:100 were added to PBMC.
CHAPTER VII

DISCUSSION

A. THE HUMAN CYTOTOXIC T CELL REPERTOIRE SPECIFIC FOR INFLUENZA A VIRUS.

The influenza virus-specific T-cell repertoire was analyzed in healthy adults from the United States. The PBMC of five donors recognized influenza-infected targets when stimulated by PR/8/34 (H1N1) in bulk cultures. Previously, it was reported that humans have bulk culture CTL responses to targets expressing NP, M1, and PB2 influenza viral proteins (Gotch et al., 1987). We found that NP expressing targets were recognized by all donors, PB1- and HA- expressing targets were lysed by the PBMC of donor 1, and NS1-expressing targets were lysed by the PBMC of donors 2 and 3 in bulk culture. Since influenza A virus-stimulated bulk cultures recognized multiple influenza A virus proteins, we decided to analyze the CTL at the clonal level to determine whether humans have a multispecific CTL memory response specific for influenza A virus.

Previously, in the murine system, the CTL repertoire specific for influenza A was found to be limited (Pala and Askonas, 1986; Bennink and Yewdell, 1988; Vitiello et al., 1996). For example, targets infected with vaccinia virus constructs that express NP were lysed by bulk culture CTL from virus-immune mice with either the Kk or K0 allele in common, but not by mice with only the Dk, D0, or L0 alleles in common. Furthermore, targets infected with vaccinia virus constructs expressing PB2 were only lysed by CTL
from mice with the $D^d$ allele (Bennink and Yewdell, 1988). This indicated that only mice with certain MHC alleles would recognize epitope(s) on a certain viral protein. After that initial finding, many groups have reported immunodominant epitopes on influenza viral proteins (e.g. NP 366-374 in H-2$^b$ mice; NP 147-155 in H-2$^d$ mice). Nonimmunodominant epitopes have been reported in the H-2$^b$ mouse; the CTL specific for these epitopes were detected after several peptide immunizations and were not observed in mice after infection with virus (Oukka et al., 1994; Oukka et al., 1996). However, mice immunized with both the immunodominant peptide and the subdominant peptide were protected better against influenza challenge than immunization with the immunodominant epitope alone (Oukka et al., 1996). This suggests that mice mount only a limited CTL response to influenza A, and it may be beneficial to have a broad multispecific CTL response.

Human CTL that recognize influenza A virus-infected targets have been reported, but most groups have only focused on a single HLA allele (such as A2.1) or viral protein (such as NP), and the CTL were not usually isolated from the same donor (McMichael et al., 1986; Gotch et al., 1987; Huet et al., 1990; Cerundolo et al., 1991; DiBrino et al., 1993; Sutton et al., 1993; Man et al., 1995). There has been one report of multiple NP epitopes found in one individual specific for the much more conserved influenza B virus (Robbins et al., 1997). However, there have been few reports on the breadth of the memory response to influenza A and recognition of the other 9 viral proteins in addition to NP. The previously identified CTL specific for influenza A are summarized in Table I-3. Human CTL epitopes have been more thoroughly characterized in other systems,
such as the HIV system; however, most of the HIV-1 epitopes were also identified in different individuals (Johnson and Walker, 1994). Moreover, HIV is a persistent viral infection and may have a different type of CTL repertoire than an acute self-limited infection with influenza would induce.

Many influenza A virus-specific CTL lines were isolated from three donors we studied in detail (Table VII-1). We isolated both CD4+ and CD8+ CTL from each donor. In donor 1, there were 10 CTL lines characterized. These CTL recognized seven different viral proteins: NP, NS1, HA, PB1, PB2, M1 and NA. This proves that there is a broad pattern of influenza A virus recognition by the CTL from this donor reflected in the number of viral proteins recognized. Isolated from this donor were even more CTL lines which were not characterized in such detail, but also reflected this very broad CD8/CD4 CTL recognition of multiple influenza A virus proteins (data not shown). This is the first report of human CD8+ CTL lines that recognize epitopes on the PB2 and HA proteins of influenza A virus. From donor 2, five cell lines were characterized. These CTL lines recognized four different viral proteins: HA, NP, NA, and M1 (Table VII-1). Two cell lines were characterized from donor 3, specific for NS1 and M2 (Table VII-1). This is the first characterization of a human CD8+ CTL line specific for an epitope on M2, and a CD4+ CTL line specific for NS1. Our results suggest that humans have a broad multispecific response with no single immunodominant epitope, unlike what has been reported in the murine system for influenza and other viral diseases (such as LCMV) (Van Bleek and Nathenson, 1990; Vitiello et al., 1996; Hudrisier et al., 1997). Due to the
marked antigenic variation of influenza A viruses, a polyclonal response to several epitopes may be beneficial to help clear virus and decrease morbidity.
Table VII-1. Summary of CTL Lines Isolated from All Three Donors.

<table>
<thead>
<tr>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Donor 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL Line</td>
<td>CD4/CD8</td>
<td>Influenza Protein Recognized</td>
</tr>
<tr>
<td>10-1C4</td>
<td>CD8</td>
<td>NP</td>
</tr>
<tr>
<td>10-1B7</td>
<td>CD8</td>
<td>PB2</td>
</tr>
<tr>
<td>4-30E11</td>
<td>CD8</td>
<td>M1</td>
</tr>
<tr>
<td>10-1G5</td>
<td>CD8</td>
<td>HA</td>
</tr>
<tr>
<td>1-2F8</td>
<td>CD8</td>
<td>PB1</td>
</tr>
<tr>
<td>4-10D9-1</td>
<td>CD4</td>
<td>NA</td>
</tr>
<tr>
<td>10-2C2</td>
<td>CD8</td>
<td>NS1</td>
</tr>
<tr>
<td>1-7-K</td>
<td>CD8</td>
<td>M1</td>
</tr>
<tr>
<td>1-3</td>
<td>CD4</td>
<td>M1</td>
</tr>
<tr>
<td>1-1</td>
<td>CD8</td>
<td>NP</td>
</tr>
<tr>
<td>3F4</td>
<td>CD8</td>
<td>HA</td>
</tr>
<tr>
<td>3G11</td>
<td>CD8</td>
<td>NP</td>
</tr>
<tr>
<td>3E9</td>
<td>CD4</td>
<td>NA</td>
</tr>
<tr>
<td>10E7</td>
<td>CD4</td>
<td>M1</td>
</tr>
<tr>
<td>3E5</td>
<td>CD4</td>
<td>NP</td>
</tr>
<tr>
<td>124</td>
<td>CD8</td>
<td>M2</td>
</tr>
<tr>
<td>77</td>
<td>CD4</td>
<td>NS1</td>
</tr>
</tbody>
</table>

Dash indicates that the epitope was not localized or the HLA restriction was not determined. A * indicates that this epitope was previously reported.
There were a variety of different HLA alleles utilized by the CTL isolated from these donors (Table VII-1). In donor 1 CTL lines were restricted by A2.1, B27, B18, and DR1. CTL lines from donor 2 were restricted by B62 and an unidentified class II allele, while the CTL lines from donor 3 were restricted by B44 and DR3. Furthermore, HLA alleles were able to present more than one epitope within an individual. For example, there were HLA B27-restricted epitopes on PB1, PB2, and NP in donor 1. This donor also presented epitopes from M1 and NS1 on HLA A2.1. Similarly the CTL lines from donor 2 recognized epitopes on NP and HA that were both presented on B62.

To determine whether the CTL lines that we isolated were rare cell lines in the PBMC of the donors, we used an ELISPOT assay to count the number of IFN-γ secreting CTL in the PBMC. The ELISPOT assay measures single-cell IFN-γ secretion by PBMC stimulated with a peptide. This method has been used by several groups to detect influenza specific precursor CTL in humans (Lalvani et al., 1997) and LCMV-specific precursor CTL in mice (Murali-Krishna et al., 1998). We detected the highest number of CTL precursors directed at the NP 174-184 epitope, with a frequency of 1 in 4,156. However, aa 58-66 of M1, aa 17-31 of M1, and aa 383-391 of NP had precursor frequencies of 1 in 31,250, 1 in 26,316, and 1 in 16,447 respectively indicating that they are not rare CTL. These memory CTL precursor frequencies specific for influenza A are similar to those previously reported in other donors (Lalvani et al., 1997).

Subtype cross-reactive and subtype specific CD8+ CTL have been isolated from mice (Bennink et al., 1982; Braciale et al., 1986; Kuwano et al., 1990) and subtype cross-reactive CD8+ CTL have been isolated from humans (McMichael et al., 1986).
Furthermore in humans, subtype cross-reactive and subtype-specific lymphocyte proliferation was previously reported (Lamb et al., 1982). All of the CTL lines that we characterized were H1N1, H2N2, and H3N2 subtype cross-reactive if they recognized epitopes on the internal conserved proteins of influenza such as NP, NS1, PB1, PB2, and M1. Having memory CTL that recognize the internal conserved proteins of influenza may help clear virus rapidly when another subtype of virus infects. The CTL lines that recognized the external glycoproteins HA and NA were either subtype specific (only recognize H1N1) or partially cross-reactive (recognize H1N1 and H2N2). The H1 and H2 hemagglutinins are closer in homology than the H1 and H3 glycoproteins. An H1-H2 cross-reactive CD8+ epitope in H-2d mice was detected on the HA transmembrane region by our laboratory (Kuwano et al., 1988) and CD4+ HA and NA subtype specific CTL have been previously identified in humans (Sterkers et al., 1985), but this is the first report of HA subtype-specific human CD8+ CTL.

We isolated and characterized six CD4+ CTL lines. The role of CD4+ CTL in virus infections, including influenza, is not as well defined as CD8+ CTL. β2-microglobulin-deficient mice have delayed viral clearance and increased mortality after a virulent influenza A virus infection (Bender et al., 1992). However, these mice can survive infection with a less virulent influenza A virus and can eliminate virus from the respiratory tract, whereas infection of nude mice or mice treated with antibodies to both CD4 and CD8 eventually leads to death (Eichelberger et al., 1991). These results lead us to speculate that CD4+ CTL can help compensate for the loss of CD8+ CTL during a viral infection. During acute LCMV infection the CD4+ T helper splenic population
increases up to 1 in 600 splenocytes, and remains as long-lived memory at a precursor frequency of 1 in 1200 (Varga and Welsh, 1998). We were able to also characterize the memory CD4+ precursor frequency specific for M1 17-31 of influenza A. The precursor frequency of these CD4+ CTL was 1 in 26,316 PBMC, which is higher than the well characterized M1 58-66 CD8+ A2.1-restricted epitope in the same donor. These results add to the growing evidence that CD4+ CTL are stable into long-term memory.

Healthy adults have probably had more than one exposure to influenza A because primary infections generally occur during childhood (Glezen, 1980). The primary infection in a nonimmune host would result in extensive virus replication and would be expected to stimulate the influenza A virus-specific naïve T cell repertoire. Secondary and subsequent influenza A virus infections would stimulate memory CTL which recognize epitopes on conserved proteins.

It is of interest that several novel T cell epitopes were defined. For example, cell line 10-1C4 from donor 1 recognizes aa 174-184 of NP, line 3E5 from donor 2 recognizes aa 256-266 of NP, line 77 from donor 3 recognizes aa 34-42 of NS1, and line 124 from donor 3 recognizes aa 7-15 of M2. We described a broad CTL repertoire that may benefit humans, who are one of the natural hosts for these antigenically highly variable viruses. In addition, our results suggest that many of the influenza virus structural and nonstructural proteins contain epitopes that may be useful to consider in vaccine development. It would be desirable to augment cross-reactive memory CTL to provide a second line of defense against influenza disease, especially where major antigenic variation occurs at the antibody binding sites of HA. Overall, these results
suggest that unlike the experimental models in inbred mice, humans have a broad repertoire of CTL that respond to influenza A.

**B. HETEROSUBTYPIC IMMUNITY TO INFLUENZA A VIRUSES IN HUMANS.**

We have demonstrated that adults living in the United States have influenza A-specific CD8+ and CD4+ memory T cells that recognize epitopes on influenza A virus strains derived from swine and avian species including the 1997 H5N1 virus strains isolated from patients in Hong Kong. This is the first report that characterizes the human CD8+ and CD4+ CTL responses to influenza A viruses of nonhuman species. The results clearly show that adult humans with no known exposure to swine or avian influenza A virus strains possess high levels of memory CTL capable of lysing swine or avian influenza virus infected targets at the bulk culture level following stimulation with a H1N1 human subtype influenza A virus. Recognition by bulk culture T cells of autologous target cells infected with the recent Hong Kong H5N1 isolates was similar to the level of lysis of targets infected with human influenza A viruses. These bulk culture results were in accord with the specific CTL lysis of target cells infected with the swine and avian-derived virus strains and the recent H5N1 isolates from Hong Kong by a panel of human CD8+ and CD4+ CTL lines, the majority of which are cross-reactive among the H1, H2, and H3 subtypes.

The cross-reactive recognition by the panel of human CD4+ and CD8+ CTL lines agrees with available sequence data. Epitopes on the swine-derived Hsw1N1, the older
avian virus H5N2 and H1N1 strains, and the recent H5N1 isolates from Hong Kong with no mutations or few conservative aa changes were recognized by the CTL lines. For example, the T→I change in the A/HK/156/97 strain at position 4 of the epitope recognized by the CD4+ CTL line 3E5 did not abrogate recognition. This aa change does not result in a different charge at that aa, however there is a loss of polarity. T cell lines failed to recognize other strains when certain nonconservative mutations occurred. For example, a N→D mutation at position 6 of the epitope on NS1 in the A/HK/156/97 strain abolishes recognition by cell line 10-2C2. Therefore, having an acidic aa at this position that is usually oriented toward the cleft top (Engelhard et al., 1993) abrogates recognition by this cell line. It is interesting that other avian viruses such as A/Duck/Ukraine/1/63 also have this mutation at aa 127 of NS1 (Man et al., 1995). Mutations that occur at CTL epitopes have the potential to lose MHC binding, become a partial agonist, or lose TCR recognition (competitive inhibitor). Mutations that cause an epitope to become a partial agonist has been observed in other systems including dengue virus infection (Zivny et al., 1999). However, we have not yet distinguished between these possibilities with the A/HK/97 virus epitopes.

The estimation of precursor frequency in PBMC using the IFN-γ ELISPOT assay was in accord with the results of the CTL assays performed with bulk cultures and with the T cell lines. Cross-reactive CTL which killed targets infected with human and avian species of influenza virus were detectable at the single cell level in PBMC at similar precursor frequencies following stimulation with either the human H1N1 peptide or the corresponding avian H5N1 peptide. This was observed with peptides based on the NP
epitope with aa 174-184, which has a conservative V→I change in the A/HK/156/97 strain and has a precursor frequency similar to that detected with the A/PR/8/34 sequence. On the other hand, peptides of the H5N1 virus with mutations in the epitope that resulted in no lysis of virus-infected or peptide-pulsed target cells had an undetectable number of IFN-γ secreting cells following stimulation with the H5N1 peptide. For example, the E→Q mutation at position 7 in the M1 epitope (aa 17-31) abrogated IFN-γ secretion. The number of SFC derived using the human H1N1 viral peptide stimulation was much higher than that of the A/HK/483/97 peptide. Therefore, replacing the acidic glutamic acid residue with the neutral glutamine residue at position 7 on this epitope decreased IFN-γ secretion by the PBMC of donor 1. It has previously been reported that changing this aa to an asparagine, along with a tyrosine at position 3 and a lysine at position 10 abrogates proliferation of T cell clones (Rothbard et al., 1988).

The role of cross-reactive memory T cells during infection with nonhuman influenza A viruses is not well understood. There was relatively little influenza A virus activity in the two decades prior to the 1918 influenza pandemic (Collins, 1957). An epidemic occurred in 1890 which seems to have been caused by a H3-like virus based on serological evidence in individuals born before 1890 (Masurel and Marine, 1973). The causes of the tremendous mortality during the 1918 pandemic are not known. Young adults, especially ages 25-35 years old, had very high mortality rates during the 1918 pandemic which is unusual in influenza; deaths usually occur in the very young and in older individuals (Collins, 1957). Thus, a W-shaped curve in age-related mortality with the peak deaths between the ages of 25-35 was described instead of the usual U-shaped
curve with deaths peaking only in the very young and old (Collins, 1957). In addition to
the unusual virulence of the 1918 influenza A virus, memory responses, including cross-
reactive influenza-specific T cell responses, may have been low or absent in some of the
young adult cohort because there was relatively little influenza A activity noted during
the two decades immediately before 1918 (Collins, 1957).

The impact of cross-reactive T cells during influenza epidemics and pandemics is
unknown. In 1957, most adults presumably had cross-reactive CTL memory to epitopes
on the H1N1 viruses, which circulated widely before the H2N2 pandemic of 1957.
Similarly, when the H3N2 “Hong Kong” virus emerged in 1968, adults and older
children would be expected to have had cross-reactive memory CTL as a result of
infection with the prior H2N2 virus strains, and adults would also have been exposed to
the earlier H1N1 strains. The pandemic of 1957 caused more deaths than that of 1968,
but fortunately this was only a fraction of the deaths that were observed in 1918.
Although it is clear that cross-reactive memory T cells did not prevent infections and
excess mortality in 1957 and 1968, these memory T lymphocytes may have contributed a
degree of partial protection by limiting the degree of viral replication, based on
experimental studies of CTL in mouse models (Wells et al., 1981; Taylor and Askonas,
1986; Kuwano et al., 1988; Kuwano et al., 1990).

Our results suggest that adults living in urban areas have CD8+ and CD4+
memory CTL as a result of prior infection with human influenza A viruses, and these
CTL are in large part cross-reactive to epitopes on influenza A virus strains derived from
swine and avian species. Although we detected mutations at several human CTL
epitopes, some mutant virus sequences were still recognized by these cell lines. Most of the human CTL cell lines were able to recognize entirely conserved or mutated viruses, consistent with the results of our bulk culture experiments. There is considerable conservation among the genes encoding the internal and nonstructural proteins of influenza A virus, which may explain our results. The presence of these memory T lymphocytes may play a role in helping to restrict virus replication, thereby decreasing morbidity and mortality to a degree. Our results support the hypothesis that cross-reactive T cells will be activated in humans by infection with a novel influenza A virus derived from another species, and the activation of these memory CTL by infection may result in reduced replication of the new infecting virus.

C. FLU-ISCOM VACCINES INCREASE CTL MEMORY IN VACCINATED HUMANS.

Our results verify that protein antigens can be formulated with Iscom particles to be presented by HLA class I molecules and be recognized by CD8+ and CD4+ CTL in vitro. These results extend earlier findings that demonstrate murine CD8+ T cell recognition of epitopes on proteins that entered cells treated with osmotic shock or formulated into particles (Schild et al., 1991). In these experiments we show that formalin-killed monovalent influenza A virus vaccine formulated into Iscom particles enabled the mixture to be recognized by a HA specific CD8+ CTL line. Furthermore, we were able to mix a recombinant NS1 protein with Iscomatrix and detect recognition of pulsed
targets by a human CD8+ CTL cell line, while the recombinant protein alone was not recognized.

Along with the in vitro data, in vivo work done by John Cruz in our laboratory, showed that the Iscom adjuvanted influenza vaccines increased influenza A virus CTL memory activity in humans after a single dose (Ennis et al., 1999). The increases were detected first by bulk culture CTL lysis of influenza infected target cells. The increases in cytolytic activity post vaccination were most apparent in the recipients of the Iscom-formulated vaccines, with a smaller number of individuals responding well to the vaccine mixed with Iscomatrix. Virtually no increases in bulk culture CTL activity were detected in the recipients of the influenza vaccine alone.

This led us to examine the precursor frequency of CTL pre and post vaccination with either the Iscom vaccine or the influenza vaccine alone. We observed increases in peptide-specific T cells secreting IFN-γ in the PBMC of several vaccine recipients indicating that a single dose of a Flu-Iscom vaccine was capable of boosting the numbers of influenza A virus-specific memory T cells. This expands upon previous work in the mouse where Iscom vaccines were shown to boost peptide-specific cytotoxicity in the BAL in chromium release assays (Sambhara et al., 1998). The murine CTL recognized aa 189-199 on HA2, which is conserved between H1N1 and H2N2 subtypes, but not the H3N2 subtype. The mice did not have an increase in CTL lysis to an immunodominant NP epitope (NP 147-158) that is usually induced during live infection. However, mice that received a DNA vaccine containing this epitope had an increase in CTLp frequency by limiting dilution assay (LDA) that correlated with the extent of protection against
challenge (Fu et al., 1999). In humans we identified increases in the precursor frequency of CTL that recognize epitopes on the M1 and NP proteins of influenza virus. Our laboratory has shown that these virus-specific CTL are able to be activated to lyse infected cells, proliferate, and secrete IFN-γ. Although in humans we cannot determine whether the precursor frequency of these influenza-specific CTL is high enough to provide protection against illness, mice with CTLp frequency of ≥1/100,000 spleen cells to NP aa 147-158 were protected after receiving a DNA vaccine (Fu et al., 1999).

Three types of changes in the number of IFN-γ secreting cells in the vaccine recipients’ were detected. Some donors had undetectable numbers of influenza A-specific precursors prevaccination, and the precursor frequency became detectable on day 14 or 56. A second pattern was a boost in which vaccine recipients had an increase in the number of IFN-γ secreting cells detectable on day 14 that remained high or increased further by day 56. We also observed that some donors had a short-term boost where the precursors increased at day 14 but went back to pre-vaccine levels by day 56. Aliquots of PBMC from all three time points were used in the same assay; therefore, any increases in IFN-γ secretion were due to increases in the number of T cells with influenza A specificity and not due to variation between assays. The viruses used in the ELISPOT assays were the same strains that were used in the vaccine, thus making it possible to quantitate all of the possible responding cells.

To make sure that the IFN-γ was secreted by CD8+ and CD4+ T cells, we used two-color flow cytometry. Other groups have found that direct infection of T cells does not induce significant IFN-γ production (Sareneva et al., 1998). It has previously been
shown in vitro that PBMC stimulated with influenza virus produce IFN-γ (Yamada et al., 1986). IFN-γ gene expression is controlled at the level of transcription, and IL-18 (IFN-γ inducing factor) can induce IFN-γ production in human PBMC (Ushio et al., 1996).

The results of our intracellular IFN-γ staining determined that mostly CD8+ T cells secrete IFN-γ, some CD4+ T cells secrete IFN-γ, while very few CD56+ NK cells secrete IFN-γ. LCMV infection of mice was also reported to induce IFN-γ secretion by CD8+ T cells, some CD4+ T cells, and very few NK cells (Nguyen and Biron, 1999). In our system, the IFN-γ production was the same whether the stimulator PBMC were infected with live virus and then irradiated and added as APCs to the ELISPOT assay, or the live virus alone was added to the PBMC in the ELISPOT assay. Furthermore, the infected, irradiated PBMC alone did not secrete any IFN-γ. All of these results indicate that the IFN-γ secretion is due to a specific interaction between the T cells and infected APCs. Evidence that the responses were specific is also shown by the lack of detectable IFN-γ production by the PBMC of several of the donors at day 0 and sometimes at day 14. In other studies we have also observed donors without influenza specific memory T cells using this assay, indicating that the virus infection alone does not nonspecifically activate IFN-γ secretion.

Quantitating the changes in the numbers of CTL that recognize epitopes on viruses or other pathogens is important in assessing vaccine immunogenicity. Our results with bulk culture cytotoxicity can thus be verified using the ELISPOT assay to measure changes in CTL responses to vaccines and determine the effectiveness of a vaccine in
boosting the number of CTL. Using the ELISPOT assay it is possible to be more quantitative because CTL activation is detected at the single cell level. To our knowledge this is the first human vaccination study to use the IFN-γ ELISPOT to measure changes in T-lymphocyte responses. One group used the ELISPOT assay to measure changes in CTLp frequency in the mouse to a multi-epitope vaccine for HIV and *Plasmodium falciparum* (Hanke et al., 1998), but only peptide-stimulation was used in the assay. In the past, the LDA has been used to measure changes in the CTLp frequency, but several groups have found this assay to underestimate the actual number of CTLp (Lalvani et al., 1997; Murali-Krishna et al., 1998; Larsson et al., 1999).

The results of the chromium release and the ELISPOT assays showed similar findings; however, when a discrepancy occurred, usually an increase in IFN-γ secreting cells was detected in the ELISPOT assay but not in the chromium release assay. Perhaps the stimulation time involved in bulk culture cytotoxic T cell analysis makes it more difficult for all virus-specific cells to remain viable. Thus, some CTL killing may have been observed on another day post-stimulation; we only tested at the day 7 time point. It will be important to confirm and extend observations comparing the chromium release assays and IFN-γ ELISPOT assays in future vaccine studies. We can not directly confirm that the IFN-γ secreting cells are the cytotoxic cells detected by the chromium release assay, but often when there was an increase in cytotoxicity post-vaccination there was usually an increase in the frequency of IFN-γ secreting cells and the majority of IFN-γ secreting cells are CD8+. Furthermore, the CTL epitopes on influenza proteins that were previously described all induce IFN-γ secretion by PBMC pulsed with peptide.
Developing an influenza vaccine that can protect humans against antigenically variant viruses of various subtypes will require more than the induction of neutralizing antibodies. These techniques may be useful in determining whether the newer cold-adapted influenza vaccines (Belshe et al., 1998) can boost CTL responses. Inducing and augmenting cross-reactive T cells that recognize and kill cells infected with variant influenza A viruses may provide this cross-subtype protection (Fig. VII-1). This Flu-Iscom vaccine has been shown to activate T cells in mice (Morein et al., 1984; Takahashi et al., 1990; Sambhara et al., 1998) and now in humans by stimulating CD8+ and CD4+ T lymphocytes. It is important that such cross-reactive memory T cells can be increased in number and able to perform anti-viral functions such as killing infected cells and secreting IFN-γ when vaccinated individuals become exposed to influenza viruses with variation in the surface glycoproteins (Fig. VII-1).
Fig. VII-1. Potential Role of Flu-Iscoms: Increasing Numbers of IFN-γ Secreted by T Cells Respond to Influenza Infection by a Novel Subtype.

1. Vaccination increases the number of memory CTL that respond to influenza:

   [Diagram showing Flu-Iscoms leading to increased numbers of IFN-γ secreting T cells.]

2. Novel strain of influenza virus infects the epithelial cells of the lung:

   [Diagram showing influenza A virus infecting epithelial cells.]

3. Rapid response by memory T cells to become activated in the lymph nodes and home to the site of infection to lyse virus-infected cells and secrete IFN-γ:

   [Diagram showing DC capturing antigen, presentation to T cells, and secretion of IFN-γ.]

   *Class I presentation to CTL by APC. (DC can capture antigen at site of infection and present it in lymph nodes).*

4. Eventually naïve B cells become effector plasma cells and produce neutralizing antibody to prevent infections.

   [Diagram showing B cells in the lymph node migrating to the respiratory tract.]
D. THE ROLE OF γδ T CELLS IN INFLUENZA A VIRUS INFECTION IN HUMANS.

The role of γδ T cells in virus infection has not been well defined. Since the ligand for the TCR has not been deciphered, little is known about what these T cells recognize. We have shown that there is a population of γδ T cells in human PBMC that can secrete IFN-γ and upregulate activation markers in response to influenza A virus infection. The γδ T cell activation was characterized using the PBMC of several donors. Although there were differences in γδ T cell number and the extent of activation, there were clear increases in activated γδ T cells in the influenza-infected PBMC of all donors tested.

The role of γδ T cells in defense from influenza virus infection has been analyzed in the mouse model. When a component of the mycobacterium cell wall (TDM) is administered i.v. to normal mice, they acquire resistance to lethal influenza infection (i.n.) (Hoq et al., 1997). These mice were depleted of γδ T cells with a monoclonal antibody, and the resistance was lost (Hoq et al., 1997). Recently, these findings were validated when TCR δ/- mice given TDM were found to have insufficient resistance to influenza infection (Hoq et al., 1999). These results suggest that γδ T cells can play a role in protection against influenza A virus infection. Furthermore, γδ T cells were shown to increase in the BAL late in infection, generally after the virus is cleared (Carding et al., 1990). TCR usage by the γδ T cells is first by Vγ4, and then later by a Vγ2/Vγ1 population (Carding et al., 1990). The γδ T cells recovered during influenza
infection are activated and transcribe many cytokines (Eichelberger et al., 1991). This supports our finding that there is a population of γδ T cells in human PBMC that can produce IFN-γ and upregulate activation markers in response to influenza virus infection.

Although we could not completely elucidate the mechanism utilized by these γδ T cells to become activated by influenza A virus, we did rule out several possibilities. We inactivated the virus by boiling and observed that the cytokine secretion and CD25 upregulation substantially decreased. This suggests that either the virus infection is important or the boiling process destroys the recognition of a viral protein. The infection may either induce cytokines/factors that activate the γδ T cells or allow entry into the class I presentation pathway (Fig. VII-2). Since the activation of CD8- γδ cells was not blocked with an anti-class I antibody, we determined that the γδ T cells probably do not need interaction with the class I molecule to become activated. The specificity of γδ T cells remains ill-defined, since recognition has been demonstrated to classical and non-classical MHC, MHC-like molecules, heat shock proteins, viral glycoproteins, and non-peptidic mycobacterial antigens (Bluestone et al., 1995; Dieli et al., 1997; Sciammas and Bluestone, 1998).

If the virus infection itself induces the secretion of a molecule that is recognized by γδ T cells, it is important to determine whether any virus can have this effect. We infected the PBMC of donor 1 with another virus, vaccinia virus; however, there was no activation of the CD8- γδ T cells. This is in contrast to findings in a human clone where vaccinia virus infected targets were lysed by γδ T cell clones. Since there seem to be
various populations of γδ T cells in humans (natural killer inhibitory receptor + or -, cytokine + or -, and cytotoxic + or -), perhaps we are analyzing a different population of γδ T cells than the vaccinia virus-specific γδ T cell clones previously reported. In our laboratory it has been shown by Anuja Mathew that there is an increase in the number of γδ T cells in bulk culture when stimulated with dengue virus (unpublished data). It is possible that there are certain viruses that can induce γδ T cells while others cannot.

Several groups have suggested that γδ T cells recognize heat-shock proteins (HSP 65, HSP 60) secreted by the injured cells; however, the role of heat-shock proteins is not clear. Some speculate that they are superantigens, while others suggest that they may present antigen to the γδ cells. γδ T cells can also be activated by certain cytokines, such as IL-12 with TNF-α (Ueta et al., 1996).

For γδ T cells to promote recovery from infection with novel influenza virus strains, they need to be cross-reactive between influenza virus strains and subtypes. Whether this response would be cross-reactive memory to the virus or a more nonspecific response to factors from infected cells, we were not able to distinguish. However, we tested additional influenza virus strains/subtypes to look for stimulation of the γδ+CD8- T cells in the PBMC. The A/Japan/305/57 (H2N2) strain stimulated 1% more γδ+CD8- T cells than the PR/8/34 (H1N1) virus (approx. 5% to 4% of γδ T cells respectively). However, the A/Port Chalmers/1/73 (H3N2) virus did not stimulate IFN-γ production by γδ T cells, even though the CD8+ population as a whole did respond to this virus (approx. 1% of all CD8+). Different results were observed by Ponniah et al. using mouse γδ T cell
hybridomas that were stimulated to secrete IL-2 in response to X-31 (H3N2), PR/8/34 (H1N1), and B/HK/8/73 (influenza B) viruses (Ponniah et al., 1996). These results are somewhat conflicting with our findings, since they observed a response to an influenza H3N2 subtype virus while we did not. One hypothesis is that the infectivity of the virus is important to activate the γδ T cells, which may explain the results by Ponniah et al. and the results by our laboratory if the A/Port Chalmers/1/73 (H3N2) virus is not very infectious. Another possibility is that the viral proteins bind directly to the γδ TCR for stimulation. This has been shown with HSV-1 γδ clones that recognize the N-terminus of the surface glycoprotein (Sciammas and Bluestone, 1998). In our case, since the H3 and H1 molecules are very diverse, this would explain the loss of recognition we observe, but it would not explain the results by Ponniah et al. since they had recognition to all influenza subtypes (even type B).

Heterotypic immunity specific for influenza A viruses promotes clearance of antigenically distinct strains and subtypes of virus. It would be beneficial to have γδ T cells with heterotypic immunity, since γδ T cells can reside or localize to the site of infection, lyse infected cells, and secrete cytokines. Increases in γδ T cells have been detected in the BAL up to 5 days earlier upon secondary influenza virus infection (Carding et al., 1990), suggesting either a memory population of γδ T cells exists or a rapid secretion of chemokines by αβ memory T cells localizes γδ T cells to the site. Whether γδ T cells are activated by a direct signal, or an indirect signal (Fig. VII-2) they may help decrease virus titers by secreting IFN-γ, killing virus-infected cells, or
promoting tissue regeneration. Our results support the hypothesis that γδ T cells can respond to influenza A virus infection in a non-MHC class I restricted fashion, which may help promote a more rapid recovery.
Fig. VII-2. Models for γδ T Cell Activation.

1. Virus Infection Induces a Secreted Factor that Directly Activates γδ T Cells:
   - Virus → Factor or cytokine secreted → γδ
   - B, Mφ, Mo, DC, etc. → IFN-γ

2. Virus Infection Induces a Secreted Factor that Indirectly Activates γδ T Cells:
   - Virus → APC → T Cell → T Cell Activation → Mφ → Activation
   - IFN-γ → γδ → IL-12 → Activation of γδ cell

3. Viral Protein is Recognized, but Does Not Bind After Boiling of Virus:
   - Virus → Infects Cell, Replicates → Viral Protein Binds TCR
   - Viral Protein → IFN-γ

4. Viral Peptides are Presented by MHC Molecules to Activate γδ T Cells:
   - Virus → APC → γδ → IFN-γ
   - Infects Cell, Class I Pathway, Activates γδ T Cell
E. CONCLUSIONS.

The results in this thesis examine the T-lymphocyte responses to influenza virus infection and vaccine administration. The major conclusions of this thesis are:

1. Humans have a broad multispecific memory T cell response specific for influenza A. Responses to 7 of the 10 viral proteins were detected in the PBMC of donor 1, indicating that recognition of a variety of epitopes occurs in humans. This is unlike the T cell response observed in mouse models where very limited numbers of immunodominant epitopes were present. Both CD4+ and CD8+ T cell responses were detected in all donors.

2. There are T cells in normal adult donors that recognize autologous cells infected with human, swine and avian strains of influenza viruses. These responses were detected at the clonal level and the bulk culture level. Nonconservative mutations in epitopes from avian and swine strains resulted in a loss of recognition by T cells.

3. The increases in specific lysis previously detected in volunteers vaccinated with Flu-Iscom and Flu-Iscomatrix vaccines were due to an increase in the number of influenza-specific CTL post-vaccination. These increases were not detected in volunteers who received the Fluzone control vaccine. Increases in the number of influenza peptide-specific CTL were also detected post-vaccination.

4. Humans have CD8- γδ T cells in their PBMC which can secrete IFN-γ in response to live influenza A virus infection in vitro. The CD8- γδ T cells upregulate CD69 and CD25 in response to the virus infection. This response was not blocked by anti-class I monoclonal antibodies and does not occur after vaccinia virus infection.
This thesis analyzes the human T cell response to influenza virus infection, furthering our understanding of how the immune system responds to viruses. Although the mechanisms of $\alpha\beta$ and $\gamma\delta$ T cell activation are not fully uncovered, we now understand that they are activated in response to influenza infection in humans which may provide a second line of defense from variant influenza strains.
REFERENCES


chronic response are determined by avidity, CD8 variable contribution compensating for differences in TCR affinities. J. Immunol. 162, 6351-6358.


Clearance of influenza virus respiratory infection in mice lacking class I major

Influenza virus RNA in the lung and lymphoid tissue of immunologically intact and
CD4-depleted mice. J. Gen. Virol. 72, 1695-1698.

Engelhard, V., Appella, E., Benjamin, D. C., Bodnar, W. M., Cox, A. L., Chen, Y.,
Henderson, R. A., Huczko, E. L., Michel, H., Sakaguichi, K., Shabanowitz, J., Sevilir, N.,
associated with the human class I MHC molecules HLA-A2.1 and HLA-B7 and

Augmentation of human influenza A virus-specific cytotoxic T lymphocyte memory by


Fisch, P., Meuer, E., Pende, D., Rothenfusser, S., Viale, O., Kock, S., Ferrone, S., Fradelizi, D., Klein, G., Moretta, L., Rammensee, H., Boon, T., Coulie, P., and Bruggen,


bulk culture proliferation, clonal analysis and precursor frequency determination. J. Virol. 67, 5962-5967.


Hudrisier, D., Oldstone, M. B., and Gairin, J. E. (1997). The signal sequence of lymphocyte choriomeningitis virus contains an immunodominant cytotoxic T cell epitope that is restricted by both H-2D(b) and H-2K(b) molecules. Virology 234, 62-73.


Vincent, M. S., Roessner, K., Lynch, D., Wilson, D., Cooper, S. M., Tschopp, J., Sigal, L. H., and Budd, R. C. (1996). Apoptosis of Fas<sup>high</sup> CD4<sup>+</sup> synovial T cells by *Borrelia*-reactive Fas-ligand<sup>high</sup> γδ T cells in Lyme arthritis. J. Exp. Med. 184, 2109-2117.


