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Functional characterization and distribution of lymphokine secreting cells following influenza virus infection

Huneycutt, Brandon Scott, Ph.D.

The Ohio State University, 1990

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FUNCTIONAL CHARACTERIZATION AND DISTRIBUTION OF LYMPHOKINE SECRETING CELLS FOLLOWING INFLUENZA VIRUS INFECTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University by

Brandon Scott Huneycutt, B.S.

The Ohio State University 1990

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To My Parents, Joel and Carolyn Huneycutt, whose unconditional love
and trust inspired my thirst for knowledge even under
conditions of self-doubt and distrust.
ACKNOWLEDGEMENTS

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PUBLICATIONS


FIELDS OF STUDY

Major Field: Medical Microbiology and Immunology
# TABLE OF CONTENTS

DEDICATION ...................................................................................................................... ii  
ACKNOWLEDGEMENTS ................................................................................................. iii  
VITA ..................................................................................................................................... iv  
LIST OF TABLES ............................................................................................................... vii  
LIST OF FIGURES ............................................................................................................ viii  
LIST OF ABBREVIATIONS ............................................................................................... x  
PREFACE ............................................................................................................................... 1  
INTRODUCTION .................................................................................................................. 3  

<table>
<thead>
<tr>
<th>CHAPTER.</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. ACCUMULATION OF INFLUENZA VIRUS SPECIFIC CELLS IN DRAINING LYMPH NODES FOLLOWING PRIMARY INFECTION</td>
<td>18</td>
</tr>
<tr>
<td>Introduction</td>
<td>18</td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>23</td>
</tr>
<tr>
<td>Results</td>
<td>29</td>
</tr>
<tr>
<td>Discussion</td>
<td>88</td>
</tr>
<tr>
<td>II. LYMPHOKINE PRODUCTION IN DRAINING LYMPH NODES FOLLOWING REINFECTION WITH INFLUENZA</td>
<td>98</td>
</tr>
<tr>
<td>Introduction</td>
<td>98</td>
</tr>
<tr>
<td>Methods and Materials</td>
<td>101</td>
</tr>
<tr>
<td>Results</td>
<td>103</td>
</tr>
<tr>
<td>Discussion</td>
<td>128</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Kinetics and Distribution of IL-2 Secreting Cells from Lymphoid Tissues Following Primary Infection with Influenza Virus.</td>
<td>68</td>
</tr>
<tr>
<td>2.</td>
<td>Mononuclear Cell Recovery from Draining and Non-draining Lymphoid Tissues Following Primary Infection with Influenza Virus.</td>
<td>69</td>
</tr>
<tr>
<td>3.</td>
<td>Distribution and Frequency of IL-2 Secreting T Cells Following Primary Infection with Influenza Virus.</td>
<td>86</td>
</tr>
<tr>
<td>4.</td>
<td>Distribution and Frequency of IL-2 Secreting T Cells Following Reinfection of Influenza Virus Seropositive Mice with Influenza Virus.</td>
<td>124</td>
</tr>
<tr>
<td>5.</td>
<td>Total Cell Recovery from Draining and Non-draining Tissue Following Reinfection with Influenza Virus.</td>
<td>125</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Influenza Virus-Specific Lymphoproliferation of Spleen Cells</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Lymphoproliferative Response of Cells from Lymphoid Tissue 3 Days Post Infection with Influenza Virus.</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>Influenza Virus-Specific Lymphoproliferation of Cells from Draining and Non-draining Lymphoid Tissue 5 Days Post Infection with Influenza Virus.</td>
<td>37</td>
</tr>
<tr>
<td>4</td>
<td>Influenza Virus-Specific Lymphoproliferation of Cells from Draining and Non-draining Lymph Nodes 7 Days Post Infection with Influenza Virus.</td>
<td>40</td>
</tr>
<tr>
<td>5</td>
<td>Influenza Virus-Specific Lymphoproliferation of Cells from Draining and Non-draining Lymph Nodes 15 Days Post Infection with Influenza Virus.</td>
<td>43</td>
</tr>
<tr>
<td>6</td>
<td>PR8 Antigen Dose Response for IL-2 Secretion.</td>
<td>47</td>
</tr>
<tr>
<td>7</td>
<td>Specificity of CTLL-20 Cells for IL-2.</td>
<td>49</td>
</tr>
<tr>
<td>8</td>
<td>In Vitro Secretion of IL-2 by Draining and Non-Draining Lymph Node cells 3 Days Post Infection with Influenza Virus.</td>
<td>52</td>
</tr>
<tr>
<td>9</td>
<td>In Vitro Secretion of IL-2 by Draining and Non-draining Lymph Node Cells 5 Days Post Infection with Influenza Virus.</td>
<td>54</td>
</tr>
<tr>
<td>10</td>
<td>IL-2 Response of Draining and Non-draining Lymph Node Cells 7 Days Post Infection with Influenza Virus.</td>
<td>58</td>
</tr>
<tr>
<td>11</td>
<td>IL-2 Response of Draining and Non-draining Lymph Node Cells 15 Days Post Infection with Influenza Virus.</td>
<td>60</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>12. IL-2 Response of Draining and Non-draining Lymph Node Cells 30 Days Post Infection with Influenza Virus</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>13. Transformation of Net CPM to IL-2 Units.</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>14. In Vitro Kinetics of IL-2 Secretion by Cells from Lymphoid Tissues 15 Days Post Infection with Influenza Virus</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>15. Scatter Plots of the Frequency of Virus-Specific T Cells Able to Secrete IL-2 In Vitro from Lymphoid Tissues 7 Days Post Infection with Influenza Virus</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>16. Frequency of Virus-Specific T Cells That Secrete IL-2 In Vitro from Lymphoid Tissues Following Primary Infection with Influenza Virus</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>17. IL-2 Response of Spleen Cells from Influenza Virus Seropositive Mice Reinfected with Influenza Virus</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>18. IL-2 Response from Superficial Cervical Lymph Nodes 3 Days Post Reinfecction with Influenza Virus</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>19. IL-2 Response by Cells from Mediastinal Lymph Nodes 3 Days Post Reinfecction with Influenza Virus</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>20. Scatter Plots of Frequency of Virus-Specific IL-2 Secreting T Cells from Lymphoid Tissues 40 Days Post Primary Infection and 3 Days Post Reinfecction</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>21. Frequency of Virus-Specific IL-2 Secreting T Cells from Lymphoid Tissues 40 Days Post Primary Infection and 3 Days Post Reinfecction</td>
<td>121</td>
<td></td>
</tr>
</tbody>
</table>
**LIST OF 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>AXIL</td>
<td>Axillary lymph node</td>
</tr>
<tr>
<td>BRA</td>
<td>Brachial lymph node</td>
</tr>
<tr>
<td>CD4⁺</td>
<td>MHC class II-restricted T cells (helper T cells)</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>MHC class I-restricted T cells (cytolytic T cells)</td>
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<td>CTL</td>
<td>Cytolytic T lymphocyte</td>
</tr>
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<td>DCV</td>
<td>Deep cervical lymph node</td>
</tr>
<tr>
<td>DLN</td>
<td>Draining lymph node</td>
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<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
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<td>HA</td>
<td>Hemagglutinin</td>
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<td>HAU</td>
<td>Hemagglutination unit</td>
</tr>
<tr>
<td>HEV</td>
<td>High endothelial venules</td>
</tr>
<tr>
<td>IFN-a/b</td>
<td>Interferon-alpha/beta</td>
</tr>
<tr>
<td>IFN-gamma</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-2R</td>
<td>IL-2 receptor</td>
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<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>ING</td>
<td>Inguinal lymph node</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<td>-------------</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal infection</td>
</tr>
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<td>i.p.</td>
<td>Intraperitoneal injection</td>
</tr>
<tr>
<td>LHR</td>
<td>Lymphocyte homing receptor</td>
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<td>LIF</td>
<td>Leukocyte migration inhibitory factor</td>
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<td>M</td>
<td>Matrix protein</td>
</tr>
<tr>
<td>MED</td>
<td>Mediastinal lymph node</td>
</tr>
<tr>
<td>MEL-14</td>
<td>Lymphocyte homing receptor</td>
</tr>
<tr>
<td>MES</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NDLN</td>
<td>Non-draining lymph node</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>PR8</td>
<td>Influenza virus strain A/Puerto Rico/8/34</td>
</tr>
<tr>
<td>P1</td>
<td>Polymerase 1</td>
</tr>
<tr>
<td>P2</td>
<td>Polymerase 2</td>
</tr>
<tr>
<td>P3</td>
<td>Polymerase 3</td>
</tr>
<tr>
<td>SCV</td>
<td>Superficial cervical lymph node</td>
</tr>
<tr>
<td>SPL</td>
<td>Spleen</td>
</tr>
<tr>
<td>Tdth</td>
<td>T cells mediating delayed-type hypersensitivity</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>Ts</td>
<td>T suppressor cell</td>
</tr>
</tbody>
</table>
PREFACE

Influenza virus infects structurally distinct regions of the respiratory tract. Viral replication occurs in both the upper respiratory tract composed of ciliated columnar epithelium, and the lower respiratory tract composed of single cells of epithelium. As a consequence of the immune response to viral antigen, an interstitial pneumonia develops which is characterized by a mononuclear cellular infiltration and consolidation of the lung parenchyma (1). Due to the architecture-dependent function of the lung, this accumulation of cells adds to the severity of the infection and increases the mortality.

Lymphocytes are not stationary in lymphoid tissues, rather they continuously recirculate from blood to tissues and back again and accumulate at sites of inflammation (2, 3, 4). The elimination of virus during a localized viral infection is dependent on the recruitment of virus-specific effector cells to the site of infection and the secretion of lymphokines by activated lymphocytes (5, 6). In experimental animal models of influenza viral infection, pathology is caused, in part by CD4+ lymphocytes that induce a delayed-type hypersensitivity response (DTH), a lymphokine-mediated event (6). Lymphokines have been suggested to play a role in the development of the immunopathologic response in the lung, however, the mechanism that regulates lymphokine production during the course of infection remains unknown. IL-2, a lymphokine secreted by T lymphocytes, is essential for clonal expansion and differentiation of antigen-activated lymphocytes and the development of immunologic memory (7, 8, 9). Antigen-specific, IL-2-secreting T lymphocytes are induced during infection by
influenza virus. IL-2 serves as a sensitive and specific marker for an investigation of the role of lymphokine-secreting T lymphocytes in the induction of immunopathology during an influenza viral infection. The purpose of this study was two-fold: 1) to determine the kinetics of accumulation, distribution, and frequency of influenza virus-specific, IL-2-secreting T lymphocytes during a primary infection, and 2) to examine the effect of reinfection on the distribution and activation of IL-2 responses in influenza virus-seropositive mice. It was hypothesized that if lymphokine-secretion contributed to the pathology that develops in the lung during influenza viral infection, then there should be a preferential distribution of virus-specific, IL-2-secreting T cells to the nodes that drain the sites of infection during the peak of the immunopathology.

These data suggest that virus-specific cells accumulate and secrete IL-2 preferentially in the draining nodes at the peak of immunopathology, however, IL-2 is not detectable in the lung. Lymphokine expression by IL-2-secreting cells is dependent on an undefined population of cells or microenvironmental conditions that are restricted to the draining nodes at the peak of the response. Reinfection, however, induces a differential distribution of lymphokine expression among draining nodes. Lymph nodes draining the lung parenchyma preferentially accumulate or activate non-IL-2-secreting cells and therefore demonstrate reduced IL-2 responses.
INTRODUCTION

It was observed that an illness occurred in epidemic (and pandemic) proportions throughout the centuries with a series of common clinical symptoms; sore throat, respiratory discomfort, and fever. In the late 1800's and again in 1918, life threatening pandemics arose with these symptoms resulting in the deaths of millions of men and women (10, 11). It was not until the 1930's, with the advent of viral isolation techniques, that the etiological agent was identified (12). The virus isolated was called influenza. Today, serial outbreaks of influenza occur annually resulting in high rates of morbidity and, depending on the virus and the immune status of the host, increased mortality (13, 14, 15). Because of the potential for increasing mortality in the elderly and immunosuppressed populations, great efforts have been made to produce vaccines against influenza virus, however, success has been limited due to the dynamic interactions of the virus with the immune system (16).

Studies on the mechanisms of viral infection and the immune response to influenza virus have provided valuable information concerning the dynamics of viral infection and interaction with the immune system, however, such studies have raised more puzzling questions than answers. For instance, it has been observed that the severe interstitial pneumonia associated with high mortality is exacerbated by the immune response to the virus, an effect that is in conflict with the function of the immune system, that of protection (1, 6, 17-20). Though, much has been learned about the protective role of the immune response to influenza virus following primary infection, little is known about the regulation
of immunopathology. Reinfection of seropositive mice, however, demonstrates mechanisms that may reduce the detrimental influences of the immune responses found in primary responses.

The immunopathology of reinfected mice is diminished compared to the primary response as are the DTH and lymphoproliferative responses (19). The production of small factors that regulate the production of lymphokines has been implicated in reduction of immune responsiveness. This reduction of immune responses following reinfection suggests mechanisms for reducing immunopathology and is therefore an appropriate model for determining how the immune response is regulated in maintaining an equilibrium between successful elimination of virus while minimizing immune-mediated tissue damage. Therefore, the purpose of this study was to define the mechanisms that participate in development of immunopathology following primary infection and mechanisms that may reduce immunopathology following reinfection.

**Characteristics of Influenza Virus**

The influenza virus, a member of the orthomyxoviridae, is an enveloped, negative-stranded RNA virus composed of 8 segments of RNA. The genome codes for at least 10 viral proteins: the envelope proteins; hemagglutinin, neuraminidase, and matrix, and the internal proteins; nucleoprotein, P1, P2, P3, and NS (21-27). The core proteins, represented by the nucleoprotein (NP), and the matrix proteins (M), are associated with the single stranded RNA molecules and the internal side of the viral envelope, respectively. Of these proteins, the hemagglutinin and neuraminidase are the best characterized, particularly at the molecular level (28-30). Influenza virus enters the cell via the fusion of the viral envelope with the host cell membrane (31-33). Once the nucleoprotein-nucleic acid enters the cell, the nucleic acid migrates to the nucleus (34, 35). Associated with the nucleoprotein are the matrix protein and the transcriptase/replicase
enzymes, P1 and P2. Within the nucleus the negative-sense RNA is transcribed into mRNA by the use of host cell 10-mer primers (26, 36). These primers are derived from host cell mRNA by the enzymatic cleavage induced by viral endonuclease. The endonuclease cleaves the 5' capped end of host mRNA resulting in 5' capped viral mRNA. Each vRNA has identical 3' ends that are partially homologous with their 5' ends. Each viral genomic RNA is replicated independently from the other nucleic acid segments. The consistent homology of the 3' end suggests that this is a common binding site of the viral replicase.

Based on antigenic determinants expressed by the matrix and nucleoproteins, the influenza viruses are classified into three major types; A, B, and C (25, 37-41). All types infect the human hosts, however, types A and C infect both animals and man (41, 42). One of the salient features of the influenza virus is its ability to undergo antigenic change within the hemagglutinin and neuraminidase (43, 44). This is due presumably to the ability of the virus to undergo genetic reassortment with animal virus variants (though direct evidence is sparse) and by the generation of point mutations induced by imprecise viral mRNA transcription (45-48). Collectively, there are 13 HA variants and 9 NA variants. To date, however, only 3 HA and 2 NA variants have been isolated from humans.

There are two types of antigenic change that contribute to the cyclical infections of the population; antigenic drift and antigenic shift (43, 44, 48, 49). Antigenic drift is characterized as small restricted point mutations within the hemagglutinin and neuraminidase proteins. Such changes, however, do not necessarily abrogate the ability of the host's immune system to cross-react with the variants that cause disease in man. Antigenic shift, however, is characterized as "major" antigenic changes with little or no cross-reactivity within the HA and NA proteins, a consequence of genetic reassortment. Antigenic drift causes epidemics, while antigenic shift causes pandemics.
It is postulated that the combination of antigenic variation within the viral proteins and the absence of subtype specific antibodies in the human population is the mechanism by which such pandemic and epidemic conditions of influenza occur. The generation of antigenically distinct viruses is due to the ability of influenza virus to naturally infect and replicate in not only the human population but also in domesticated and wild animals, including chickens, turkeys, horses, swine, and waterfowl (27, 42, 45, 47, 48). These diverse sources of influenza virus create reservoirs in which the virus can reside and undergo mutations that can evade the human population's immune defenses. The frequent and periodical outbreaks of influenza have been postulated to occur as the virus crosses from the animal population back to the human population. The result is the generation of antigenically distinct and sometimes more virulent progeny virus.

**Infection by Influenza Virus**

In humans and experimentally in ferrets, the virus infects, via inhalation of infected mucosa droplets, the upper respiratory tract epithelium lining the nasal mucosa, larynx, and tracheobronchial tract (1, 50-53).

This infection pattern is a multiple stage event that is dependent on the viral coded hemagglutinin and neuraminidase. The hemagglutinin, the membrane bound trimer, is composed of monomers that form a long spike with a globular head (29, 30). This protein is responsible for attachment and fusion of the virus with the host cell membrane, specifically the sialic acid residues expressed on their surface (32, 33). These functions are carried out by the cleavage of each monomer of the trimer into a HA1 and HA2 protein connected by a disulfide link (54, 55). This modified hemagglutinin binds to the hosts sialic acid residue and induces the fusion of virus with host cell membrane. This occurs once the hemagglutinin has been cleaved and a fusion protein within the confines of the
hemagglutinin trimer is activated. It is the hemagglutinin that confers infectivity, and it is only the enzymatically modified protein that makes the virion infectious.

Upon initiation of viral replication, host cell RNA synthesis and protein synthesis are curtailed, cellular DNA is degraded, and the cell membrane disintegrates. The result is the generation of progeny virus, the desquamation of the epithelial cells, and the induction of host defense systems (56-64). The generation of progeny virus and the removal of the protective epithelium allows the virus to spread throughout the respiratory tract. The disruption of the airway lining inhibits the ability of the respiratory tract to remove additional pathogens and allows infection of the exposed basement membrane by bacteria. The consequences to the host are increased irritability of the respiratory tract to environmental irritants, depressed T cell function and granulocyte chemotaxis, poor gas exchange, and an inability to clear bacterial infection. Clinically, the infection can present as an inapparent infection to a severe complicated interstitial pneumonia with pharyngitis and tracheitis (64, 65). It is the severe interstitial pneumonia that creates increased mortality, particularly in the elderly.

In severe complicated influenza viral infections in the human population and, experimentally, in mice, the virus is not restricted to the upper respiratory tract but rather involves the lower airways and alveoli (1, 66-68). The result is the development of an interstitial pneumonia characterized by cellular infiltrates and consolidation of the lung parenchyma. In mice, the infection occurs in both the upper and lower respiratory tract with virus replication predominantly occurring in the alveolar cells (67, 68). In these animals, the pathology and progress of disease parallels that of human pneumonia making the mouse an excellent model for dissecting mechanisms of influenza virus induced immunopathology.
Non-Specific Immune Responses

The first lines of defense in the early stages of virus infection are the non-specific immune responses. These responses are activated within the first 24-48 hours post infection and functions primarily to retard viral replication long enough for the antigen-specific immune response to be activated (69-72). The major constituents of non-specific immune responses are cytokines, NK cells, and complement (69, 73-76).

Cytokines, cellular secretory products, are the earliest anti-viral component expressed. The interferons, cytokines expressed by lymphocytes and virus-infected cells, are known to play a major role in anti-influenza virus responses (77). All classes of interferon have been demonstrated to play a role in retarding influenza viral replication, interferon-a/b (IFN-a/b) and IFN-gamma (76-79). The IFN-a/b, secreted by virus-infected host cells, block viral replication and, in doing so, limit the spread of virus early during infection. Intranasal inoculation of anti-interferon along with virus in mice has been demonstrated to exacerbate the viral replication (78). IFN-gamma, an interferon secreted predominantly by NK cells during the early response, and also by T lymphocytes, also retards viral replication, however, the predominate effect of this interferon is the induction of major histocompatibility complex (MHC) class II glycoproteins on pulmonary macrophages and endothelial cells. With the expression of these antigens, antigen presentation to the specific arm of the response is enhanced and the ability of precursors of the effector cells to be activated is also enhanced.

Concomitant with the production of the interferons, NK cells accumulate in the lung (69, 70). These cells are not antigen-specific nor are they MHC-restricted in their activity, but they are capable of lysing infected cells. With the accumulation of these cells at sites of viral replication and their ability to lyse infected cells, fully infectious progeny virus are kept from forming, thus retarding viral spread (70, 80).
Complement has also been connected with the non-specific responses (73, 74). This activity has been demonstrated to be independent of antibody and it appears that viral enzymes are capable of causing complement activation through both the alternate (74) and classical pathways (73). The result is the production of chemotaxins and the resultant accumulation of inflammatory cells at the site of influenza virus antigen deposition.

The non-specific response is characterized by the lack of antigen specificity and early activation. However, the role of these responses in defense against infection is temporal and its effect incomplete. For complete removal of virus and the generation of long-term memory and protection, the antigen-specific response is necessary.

**Specific Immune Responses**

During the course of a natural infection, both humoral and cell-mediated responses are induced (81-93). The viral hemagglutinin and neuraminidase molecules appear to be immunodominant proteins in the generation of protective immune responses. However, all the viral antigens are immunogenic including the core proteins, NP and M (5, 84, 85, 89, 91). The humoral response is subtype-specific for the conformational epitopes of the hemagglutinin and neuraminidase, while the cell-mediated response is directed to the conserved portions of the hemagglutinin spike (92, 93) and/or to the conserved residues of internal proteins, particularly NP and M (85, 91). Studies of T helper cell (Th) clones have shown that these cells are specific for both external and internal viral proteins (84, 85, 89, 91). However, the T cells preferentially respond to internal proteins that have both hydrophobic and hydrophilic domains (89). From such studies on influenza virus and in other peptide studies it has been found that T cells recognize distinct epitopes that are spatially distinct from B cell epitopes, though they are expressed on the same macromolecule (28).
The humoral response, mediated by B cells, functions to protect against initial infection. Virus-specific immunoglobulins, predominantly specific for the hemagglutinin, neutralize influenza infection by either blocking the attachment of infectious virions to the host cells or by interfering with the transcription of the viral nucleic acid (88, 94). Three major classes of immunoglobulin are generated by an influenza viral infection; IgM, IgG, and IgA (28, 95-97). Serum immunoglobulin of the IgM class is synthesized by day 5 of infection with isotypes of IgG and IgA being generated by day 7 of infection (98). The circulating antibodies are able to afford protection in the deeper lung, however, the presence of serum antibody does not prevent infection of the upper respiratory tract (99). The role of secretory IgA in both the upper and lower airways is less understood although both IgA and IgG secreting antibody cells are found in the lung following primary infection with influenza (100, 101). Studies on the specificity of the humoral responses in neutralizing infection have demonstrated that hemagglutinin-specific antibodies transferred into infected animals protected the host from infection (88). In contrast, anti-neuraminidase only partially protected animals from infection in vivo and only high concentrations of anti-neuraminidase antibody blocked in vitro infection of susceptible cells.

The major arm of the immune response in the clearance of an ongoing influenza viral infection is the cell-mediated immune response. Unlike the humoral response, the cell-mediated effector T cells are predominantly type-specific and cross-react with all subtypes within a particular virus type (102). The cell-mediated response, characterized by T lymphocytes, functions primarily to clear ongoing infection by the action of cytolytic T cells and delayed-type hypersensitivity cells (1, 5, 81, 87, 83, 103-107). All functional and phenotypic T cell subsets are activated in the anti-viral response; T helper (Th), T suppressor (Ts), T cytolytic (CTL), and T delayed-type hypersensitivity (Tdth) (6, 17, 18, 20,
The primary cells participating in anti-influenza viral immune responses are the inflammatory T cells (DTH) and CTL effector cells. The Tdth cells function by secreting lymphokines, in particular IFN-gamma and IL-2 (108-110). These cells induce the recruitment of mononuclear cells to sites of viral replication and thereby induce the inflammatory response. Two classes of functional Tdth cells have been identified in the virus model, MHC class I- and MHC class II-restricted cells (87). The development of these two restricted Tdth cells is dependent on the virulence of the virus. If mice are infected with live virulent virus, both restricted subclasses of Tdth cells are generated. However, if mice are primed with an inactivated virus, only the class II-restricted Tdth cells are produced. It has not yet been concluded as to the role of CD8+ cells in DTH responses.

The CTL specific for influenza virus have been well characterized in the mouse models (111-115). These cells recognize viral proteins expressed on the surface of infected cells in association with either MHC class I or, less frequently, MHC class II antigens.

The role of these cells in clearing the virus and providing protection to infection is controversial. Adoptive transfer experiments demonstrated that influenza virus-specific CTL were proficient in reducing the viral titers of the infected host (1, 90). In contrast, Tdth cells were not effective in clearance of virus but instead enhanced pathology and increased mortality. Collectively, these data suggested that the CTL, and not the Tdth cells, were the primary effector cell for clearing virus and affording protection against influenza. Further investigation found that CTL, like the Tdth cells, were also capable of secreting lymphokines (116, 117). This observation raised the question of whether CTL functioned to clear virus by lysis of the infected cells or by the elaboration of lymphokines, just as the Tdth cells. In addition, in vivo kinetic studies examining the development of class I and class II-restricted CTL and Tdth
following infection with influenza virus, showed that the first cells to develop during the course of infection were the class II-restricted Th (111). In studies exploring these confounding functions between Tdth and CTL, it was found that an interaction between Tdth cells and CTL was necessary for a completely functional immune response to influenza virus (107). Schiltkenecht et al. examined the effect of cyclosporin A on the development and effector function of both CTL and Tdth cells in the mouse (118, 119). They found that in cyclosporin-treated animals, the CTL activity was induced but only poorly. In contrast, Tdth cells in the treated animals were induced, however, their activity was not demonstrated until the cells were adoptively transferred to non-treated hosts. Two conclusions were derived from these studies. First, the development of CTL was dependent on the production of lymphokines in vivo, whereas in vitro their effector function was independent of lymphokines. Second, and in contrast to the CTL cells, Tdth cells required less if any production of lymphokines for induction but for effector function they required lymphokines.

The principal regulatory cells of the immune system are the CD4+ cells or inducer T cell (Ti), cells which, upon antigen stimulation elaborate lymphokines (107, 120, 121). Based on the species of lymphokines secreted, two T cell subsets have been provisionally defined; Th1, the inflammatory T cells (Tdth), and Th2, the helper T cells (120, 121). The Th1 cells, those cells regulating localized inflammatory responses, elaborate the lymphokines IL-2 and IFN-gamma (121). Th2, or helper T cells, elaborate the lymphokines responsible for B cell maturation, IL-4 and IL-5 (120). Collectively, Th cells regulate the expansion and differentiation of influenza virus-specific Tdth cells and B cells (106).

The regulation of anti-influenza viral immune responses by T suppressor cells has also been observed in a few studies. Liew and Russell described an Lyt 1+ (CD4+) T cell that, when adoptively transferred with Tdth cells, blocked immunopathology (17, 20). These T suppressors were found to be specific for
portions of the hemagglutinin molecule.

The study of the regulation of influenza viral infection by the immune response has concentrated on the cytotoxic T cells, and T cells regulating humoral responses. The role of lymphokine-secreting cells in regulating immune responses is less well characterized.

**Immunopathology of Influenza Viral Infection**

The function of the immune system is to recognize foreign antigens, clear them from the host, and to generate memory cells that can protect against re-exposure to the eliciting antigen. However, during an influenza viral infection, the immune response contributes to pathogenesis of the infection (1, 18, 20).

Histologically, the inflammatory response in the lung mimics the histology of a cutaneous delayed-type hypersensitivity reaction. The lung parenchyma consolidates and becomes packed with cellular infiltrates (104). Because of the extensive cellular infiltration into the interstitium and alveolar sacs, the fine architecture of the lung is disrupted. Due to the architecture dependent function of the lung, this immune response, though a normal function in containing viral spread and activating lymphocytes, exacerbates the infection.

Early studies to ascertain the mechanisms responsible for the observed immunopathology have been contradictory. It was observed that Tdth cells which function to recruit lymphocytes, monocytes, and granulocytes to sites of antigen deposition were the cells that induced the immunopathology (20). Liew and Russell demonstrated that adoptive transfer of inflammatory cells into syngeneic hosts infected intranasally with a virulent strain of influenza virus demonstrated both histopathology and increased mortality. However, further investigation into the role of CTL in the protection and resolution of viral infection revealed that these cells, like Tdth cells, were capable of producing lymphokines that play a role in cell extravasation, i.e. IFN-gamma (116, 117). These data suggested that
the immunopathology seen with influenza viral infection is due, in part, to the localized elaboration of lymphokines.

It has been postulated that some form of regulatory mechanism must exist to reduce or minimize the immunopathology that results from influenza viral infection. Studies by Beck and Sheridan have identified a small molecular mass factor that is generated in reinfected mice by spleen (SPL) cells and mononuclear cells from the lung (19). This factor blocks the biological expression of the lymphokines IL-2 and leukocyte inhibitory factor (LIF) but not their secretion. These data suggest that one mechanism for maintaining equilibrium between tissue damage and clearance of virus is the modulation of lymphokine production.

**Lymphocyte Recirculation Patterns**

Lymphocytes are not restricted to a specific lymphoid tissue but rather migrate from one tissue to the other via the circulatory and lymphatic system (2, 3). However, recirculation of lymphocytes is not a random event. There is evidence indicating that lymphocytes, depending on subset, state of activation, and lymphoid source, will preferentially localize or be retained in certain lymphoid tissues (4, 122-129). Stevens et al., by using fluorescein labelled lymphocytes, observed that T cells and B cells, homed preferentially to different tissues (123). This group found that T cells localized in the peripheral lymph nodes while B cells localized in the SPL and Peyer's patches. The divergent trafficking of lymphocytes to specific organs is not restricted only between the B and T cells but also between subsets of T cells (122, 129). T cells with the helper phenotype (CD4+) appear to be preferentially located in mucosal tissues over that of non-mucosal tissues (122).

Blood born lymphocytes enter lymph nodes through two routes (2, 125). Lymphocytes leave the blood and enter tissues through the capillaries within the
tissue and then enter the lymph node through afferent lymphatic vessels. Alternatively, lymphocytes enter lymph nodes directly by passing through specialized columnar endothelial cells within the node parenchyma, called high endothelial venules (HEV) (130-132). The binding of lymphocytes is a prerequisite for their subsequent extravasation into the node. The mechanisms by which lymphocytes traffic are not fully understood, however, evidence has revealed that adhesion molecules expressed on both lymphocyte cell membranes and endothelial cells, particularly HEV, are necessary constituents for lymphocyte extravasation. Two of these proteins have been identified serologically, the lymphocyte adhesion molecule, MEL-14 in the mouse, (HERMES-1 and 3 in humans) and the endothelial cell adhesion molecules, MECA 325 (133-135). Other proteins that have also been implicated in regulating lymphocyte extravasation/homing are the MHC and the leukocyte function antigen (LFA) proteins (136, 137).

Lymphocyte homing receptors (LHR) have been identified serologically (133). From such work, a 85-90 kilodalton protein or proteins have been described. Gallatin et al. determined that MEL-14, a monoclonal antibody specific for the reputed LHR, blocked the binding of mouse lymphocytes to lymph node high endothelium. In correlated studies of human peripheral blood lymphocytes (PBL), the antigens defined serologically as HERMES-1 and HERMES-3 were instrumental in binding to different tissue HEV (130). PBL stained with HERMES-1 were prevented from binding to HEV from peripheral lymph nodes but were not inhibited in binding to mucosal HEV or sites of chronic inflammation. In contrast, PBL stained with HERMES-3 were blocked in binding to mucosa and inflamed HEV, but were not affected in binding to peripheral lymph node HEV. Both the HERMES antibodies precipitated a 85-90 kilodalton glycoprotein. These data suggest that a conserved family of related proteins regulates the extravasation of lymphocytes to different tissues, however, either
by an association with other proteins or variations within these proteins, serologically distinct molecules are generated.

In studies addressing the role of adhesion molecules on HEV, Duijvestijn et al. found that serologically distinct endothelial adhesion molecules are expressed on endothelial cells located at sites of chronic inflammation (134). They have been defined as MECA-217, and MECA-325. The MECA-217 proteins are expressed on virtually all endothelial cells of every tissue, however, the MECA-325 was found exclusively on endothelial cells of lymphoid tissues. At sites of cutaneous granulomatous reactions, the neovasculature developed both MECA-217 and MECA-325 but in a time dependent fashion. Within a few days of granulomatous reaction, the MECA-217 was found. The MECA-325 antigen, postulated to be the adhesion molecule important for the extravasation of lymphocytes into lymphoid tissues, was detected but only after 7 days of inflammation, the peak of the inflammatory response. This suggested that the expression of MECA-325 was associated with immunological extravasation, particularly at sites of inflammation.

Rationale

Studies on the regulation of immunopathology in influenza viral infection in animal models have concentrated on the role of Tdth and CTL activity. Most of these studies have concentrated on adoptive transfer of cells or have studied particular immune functions from a restricted cross-section of lymphoid tissues. With the increasing knowledge of the immune system, it is apparent that immune functions are distinctly different depending on the particular lymphoid tissue examined. This has been determined by both the differences in antigen presentation requirements between lymph nodes and spleen, and the differential trafficking of lymphocytes to lymphoid and non-lymphoid tissues (4, 121-128, 138).
This study addresses two general questions. 1) Are lymphokines differentially expressed in individual lymph nodes draining structurally-distinct sites during primary and secondary viral infection? and 2) Where do virus-specific cells accumulate following infection?

The specific aims of this study are to determine the kinetics of the accumulation of influenza virus-specific cells that secrete lymphokines in draining and non-draining lymphoid tissues during a primary immune response; and to study the production of IL-2 in lymphoid tissues following reinfection, a time when factors are produced that may regulate the biological expression of lymphokines.

The data provide evidence that influenza virus-specific T cells that secrete lymphokines are able to recirculate to both draining and non-draining lymph nodes but that proliferation and secretion of IL-2 requires the environment of the draining lymph nodes at the peak of the response. The draining lymph nodes appear to contain localized, as yet, undefined factors/cells that provide the stimuli for IL-2 secretion. Furthermore, the data provide evidence that influenza virus-specific IL-2-secreting cells are of two subpopulations, one population requires multiple cell interactions for the secretion of IL-2 while the other does not. The multifactor dependent population is restricted to draining lymph nodes and SPL and requires complex cellular interactions for the production of IL-2 at the peak of the immune response. Reinfection of seropositive mice, however, induces a series of regulatory mechanisms specifically within the lymph nodes draining the lung parenchyma that selects for the accumulation of cells that do not express IL-2, or alternatively selects for cells that produce lymphokine and soluble factors which block lymphokine production.
CHAPTER I

ACCUMULATION OF INFLUENZA VIRUS-SPECIFIC CELLS IN DRAINING LYMPH NODES FOLLOWING PRIMARY INFECTION

INTRODUCTION

Influenza virus replicates in several regions of the respiratory tract (1, 50-53, 60, 62, 64, 67, 68) which are structurally distinct. The oropharynx and trachea, which are composed of columnar ciliated epithelium, and the lung parenchyma, composed of one cell thick epithelium. In the mouse, the upper respiratory tract tissue communicates with two major regional lymph nodes, the SCV, located by the salivary glands and DCV, located on either side of the trachea (139). The relationship of the nodes with lymphoid aggregates in the tissues of the upper respiratory mucosa is currently ill-defined as are the migratory behavior of cells to these nodes (140, 141). The lower respiratory tract is drained by the MED, located within the mediastinal cavity. The MED, like the MES, is anatomically unique (142-146). These nodes are believed to receive cells from the bronchus-associated lymphoid tissue (BALT), lymphocyte aggregates that are distributed diffusely throughout the lung parenchyma (144-146), and from the broncho-alveolar cells within the alveoli of the lung, cells believed to be immigrants from the circulatory system (147, 148).

Immune responses differ among lymphoid tissues and the sites that they drain (80, 138, 146, 149-159). This has been attributed, in part, by the cell
populations that traffic through the various regions (122-126, 129, 132, 160). Bishop et al. comparing the distribution of CTL and IL-2-secreting T cells following allografts found that activated CTL and Th were differentially distributed between the allograft, draining nodes, and spleen (153). Activated CTL were found in allografts, with little activity found in the draining nodes and spleen. In contrast, the activated Th were comparably distributed between the various regions. This supports previous work which showed that the recruitment of cells to sites of inflammation, as well as the recruitment of cells to other tissues, is a non-random event and is dependent on the state of cell activation, the tissue of inflammation, and localization of antigen (161-165).

However, little is known about the initial site of influenza virus-specific cell activation, trafficking of virus-specific cells within the different lymphoid tissues, or the regulation of lymphokine secretion by the environment following influenza viral infection.

Studies of lymphocyte trafficking have relied primarily on the adoptive transfer of fluorescein isothiocyanate (FITC)-labelled cells or cells differentially labeled with radioisotopes during short time intervals (125). The antigen specificity and the capacity of cells in various lymphoid tissues to express lymphokines remains obscure, particularly in regard to respiratory tract viral infection. Recent studies investigating mechanisms of inflammation in rheumatoid arthritis, have defined phenotypically-activated cells in the synovium, however, the production of lymphokines within these sites appears to be depressed (166-168). In contrast, such cells were able to proliferate in response to the exogenous addition of lymphokines. The dichotomy between the localization of activated cells at sites of inflammation able to respond to lymphokines and the lack of detectable lymphokine secretion implies that the environment plays a role in specifically regulating the production of lymphokines.
Most studies on influenza have not investigated the relationship of the microenvironment in regulating the development of lymphokine production, but rather have focused on the regulation and development of the effector CTL, Tdth cells, and to a lesser extent cells capable of proliferating from a limited cross-section of lymphoid tissue (81, 82, 169, 159). Functional characterization of virus-specific cells have concentrated on adoptive transfer studies or in vitro effector assays. Under these conditions, however, the cells are either removed from their initial environment or additional cell components are added, such as the case in pooling cells from various lymphoid tissues.

Lipscomb et al. reported that, in the guinea pig, influenza virus-specific cells that proliferate to influenza viral antigen specifically accumulated in the lung and hilar lymph nodes (draining the lung parenchyma) following intratracheal inoculation with virus (169, 170). Furthermore this group found that virus-pulsed bronchial alveolar cells induced the recruitment of these cells. Unlike these studies however, little is known about the regulation of lymphokine secretion by the environments of individual nodes draining regionally distinct sites, nor the trafficking of lymphokine secreting cells during the development of the immune response to influenza viral infection. Where are influenza virus-specific T cells that secrete lymphokines activated? Where do influenza virus-specific cells that secrete lymphokines traffic? And are virus-specific cells able to express lymphokines in any lymphoid tissue?

Influenza viral infection in the mouse is a dynamic process. Virus replicates to peak titers in the lung by day 4 post infection and is subsequently eliminated by day 10 (104). Concomitant with the virus replication, is the induction of immune responses and immunopathology. Cellular infiltrates are detected in the lung by day 3 post infection and reach a peak by day 7 as does the CTL activity. By day 15 post infection, virus-specific cells with CTL activity are diminished in the lung as are the cellular infiltrates (5). During the interval from day 1 to day
40 post infection, the development of progeny virus and the development and maturation of the immune response can be monitored. The trafficking of lymphocytes is also a dynamic process. To determine development of IL-2 secretion and the distribution of cells as the immune response develops, lymph node cells were removed at various times post infection correlating with the peak of viral replication, development of immunopathology, and the resolution of both immunopathology and viral infection.

Virus-specific cells can be detected by the in vitro stimulation of lymphocytes with viral antigen. Virus-specific cells recognizing the stimuli will express IL-2 and undergo proliferation (171). IL-2, a lymphokine expressed predominantly by CD4+ cells, regulates the cell activation of both T and B cells (120, 121). This lymphokine induces the proliferation and differentiation of antigen-activated cells into T effector cells (CTL, Tdth, Th cells) and regulates the production of other lymphokines, i.e., IFN-gamma (7, 8). Due to the broad regulatory spectrum of IL-2, and the fact that several lymphokines are coordinately expressed, the production of IL-2 indicates a marker for lymphokine expression (9). IL-2 can be quantitated by the proliferation of an IL-2 dependent cell line, CTLL-20. Proliferation of these cells in response to IL-2 is proportional to the concentration of IL-2. Both proliferation and IL-2 activity can be detected by the incorporation of tritiated-thymidine.

To determine the distribution of virus-specific cells and the secretion of lymphokines, cells from individual draining lymph nodes (SCV, DCV, MED) and individual non-draining nodes (AXIL, BRA, ING, MES) were assayed for the ability to proliferate and secrete IL-2 at several times during infection.

Lymphoproliferation and the production of IL-2, however, can be suppressed by cytokines and lymphokines produced by suppressor cells in the cell population. Limiting dilution analysis is an assay that can determine the frequency of antigen-specific cells at a sensitivity upwards of 1 cell out of 500,000, and can, in
most systems, detect cells in the presence of suppressor cells. Due to the sensitivity of the assay, limiting dilution analysis of IL-2 secretion by lymph node cells was also employed to enumerate virus-specific cells.

The data presented in this chapter demonstrate that virus-specific cells that secrete IL-2 are distributed preferentially to draining lymph nodes and to a lesser extent in non-draining lymph nodes both at the peak of the immune response and the resolution of infection. In addition, lymphokine production is dependent on multiple factors or cells that are restricted to the draining nodes at the peak of immunopathology, which appears to reflect two subpopulations of IL-2-secreting cells. The data suggests that lymphokine-secreting cells that may participate in the development of immunopathology in the lung are primarily activated and clonally expanded in the environment of draining lymph nodes and that these cells then enter the lung by the circulatory system. However, the production of lymphokines appear to be regulated differently in the lung either by specifically inhibiting the secretion of IL-2 or by rapid adsorption of IL-2 by activated lymphocytes expressing IL-2 receptors.
METHODS AND MATERIALS

Mice: Virus antibody-free male C57BL/6 mice at four to six weeks of age were purchased from either Charles River Inc. (Wilmington, MA) or Harlan-Sprague-Dawley Inc. (Indianapolis, IN). They were housed in filtered positive-pressure cabinets in specified rooms containing only pathogen-free animals in Wiseman Hall at the Ohio State University. Animals were housed at 5 mice per cage and fed and watered ad libitum. An enzyme-linked immunosorbent assay (ELISA) specific for influenza virus was used to screen the serum to ensure seronegativity (104).

Virus: Mouse-adapted influenza virus strain A/Puerto Rico/8/34 was purchased from the American Type Culture Collection (Rockville, MD). Virus was propagated in the allantoic sac of 10 day-old fertile chicken eggs by injection of 0.1 HAU in a total volume of 0.100 ml in PBS. Infectious allantoic fluid was harvested 48 hours after incubation at 37°C and stored at -70°C until use. Quantitation of virus was accomplished by the hemagglutination of type "O" human red blood cells (RBC) with each HAU representing the reciprocal of the dilution of 50% hemagglutination. Titers of 1280 HAU/ml were routinely achieved.

Infection: Each mouse was lightly anesthetized intramuscularly with 0.05 ml of a ketamine-xylazine solution (ketamine; Bristol Labs, Syracuse, NY :xylazine;
Haver-Lockhart, Shawnee, KS) in PBS containing 4.0 ug of ketamine and 0.50 ug of xylazine. Fifty microliters of infectious allantoic fluid containing a total of 32 HAU of virus was placed on the snouts of each mouse and then allowed to be aspirated. This dose caused mortality of 40-50% of infected mice.

Recombinant Interleukins and Culture Supernatants: Murine recombinant IL-2 (Genzyme, Boston, MA) and murine recombinant IL-4 (Genzyme) were derived from bacterial expression vectors. Units are expressed as defined by Genentech, 1 unit of IL-2 is 1/2 maximal proliferative response of the HT-2 cell line, 1 unit of IL-4 is 1/2 maximal proliferative response anti-IgM stimulated B cells. Recombinant interleukins were stored at -70°C in phosphate buffered saline.

Culture supernatants of rat spleen cells stimulated with Concanavalin A (Sigma, St. Louis, MO) exhibit high levels of lymphokines. These culture supernatants were used for the maintenance of the CTLL-20 cell line. Spleen cells from normal Lewis rats (Harlan-Sprague-Dawley) were macerated through a fine mesh screen. Single cell suspensions were washed 3 times in RPMI 1640 (Gibco, Grand Island, NY) (supplemented with 1% penicilin-streptomycin (Gibco), 1% HEPES (Gibco), 1% sodium pyruvate (Gibco), 0.4% L-arginine-HCL (2.9% solution), 5 x 10⁻⁵ M 2-mercaptoethanol (Kodak), 0.75% L-glutamine (Gibco), 0.06% folic acid/0.36% asparagine (Gibco) and centrifuged at 100 x g. Single cell suspensions of 5 x 10⁶ cells/ml were cultured horizontally in culture flasks (Corning, ) in a total of 40 ml at 37°C in 5% CO₂. Cultures were stimulated for 40 hours with 2.5 ug/ml of Concanavalin A (Sigma, St. Louis, MO). Cells and debris were removed by centrifugation and cell free supernatants stored at -70°C.

Single Cell Suspensions: Mice were sacrificed by cervical dislocation at the indicated times post infection. To prevent contamination of the internal milieu by dander, mice were rinsed in 70% ethanol. To remove lymphoid tissues, a
midline incision from the distal inguinal to the proximal portion of the salivary glands was made and tissue removed from muscle fascia. Lymph nodes within the cutaneous tissues (SCV, AXIL, BRA, and ING) were removed first. The DCV and MED were removed following dissection of the rib cage. The MES and SPL were removed by a midline incision of the abdominal wall extending from the inguinal to the distal end of the thoracic cavity. Draining lymph nodes (SCV, DCV, MED), non-draining lymph nodes (AXIL, BRA, ING, and MES), and spleen were removed aseptically at the indicated times post infection by teasing lymph nodes away from connective tissue by forceps removing as much fatty tissue as possible. Explants were placed in 10 ml petri dishes containing 5 ml complete serum-free Dulbecco's minimal essential media (Gibco, Grand Island, NY) (DMEM). Media was supplemented with 1% penicillin-streptomycin (Gibco), 1% HEPES (Gibco), 1% sodium pyruvate (Gibco), 0.4% L-arginine-HCL (2.9% solution), 5 x 10^{-8} M 2-mercaptoethanol (Kodak), 0.75% L-glutamine (Gibco), 0.06% folic acid/0.36% asparagine (Gibco). Single cell suspensions were prepared from each region by macerating tissues through a fine mesh wire screen in 5 ml of complete serum-free DMEM. Lung mononuclear cells were isolated similarly by macerating tissue through fine mesh wire screens, however, mononuclear cells were isolated from lung tissue by centrifuging cell suspensions over ficoll-metazoate solution (density=1.086) at 100 x g for 30 minutes. Mononuclear cells were removed from the interface. Lymph node, splenic, and lung mononuclear cells were washed three times and centrifuged at 100 x g for 10 minutes. Cells were stained with crystal violet and counted, and cell concentration adjusted to 2 x 10^6/ml in complete DMEM containing 4% fetal bovine serum (Gibco). Cells were kept at 4°C throughout preparation to retard cell death. Greater than 95% viability as determined by trypan blue exclusion was routinely achieved.
**Proliferation Assay:** Single cell suspensions (2 x 10^6/ml) from the indicated lymph nodes were placed in triplicates into wells of 96 well round-bottom microtiter plates (Flow Laboratories, McLean, VA). Each well contained 2 x 10^5 cells in a total volume of 0.100 ml. To each set of replicates 0.050 ml of medium or influenza virus (13 HAU/ml) was added with a final volume of 0.15 ml. Cultures were then incubated at 37°C at 10% CO₂. Proliferation was determined by the incorporation of tritiated-thymidine (ICN Biochemicals, Irvine, CA). Briefly, microcultures were incubated for 3, 5, and 7 days. At each of these time points, each well was pulsed 4 hours with 0.8 uci of tritiated-thymidine. Each plate was harvested by a multi-well Phd cell harvester (Cambridge, MA) onto glass fiber discs. Incorporation of the radionuclide was determined by scintillation spectroscopy. Values represent mean counts per minute +/- one standard deviation.

**IL-2 Overlay Assay:** Secretion of IL-2 was determined by the overlay of the IL-2 dependent cell line CTLL-20 into microcultures of lymph node or spleen cells stimulated with influenza virus. Microcultures of lymph node, spleen, and lung mononuclear cells were stimulated with Concanavalin A (2 ug/ml), influenza virus PR8 (13 HAU/ml), or medium alone and were cultured for 24, 48, and/or 72 hours. After incubation, each plate was irradiated with 2000 rads of gamma rays (172). To each well, 1 x 10⁴ CTLL-20 cells in log phase of growth (cultured 24 hours prior to assay with optimal doses of IL-2 and subsequently washed 3 times) were overlaid on the irradiated responder cells in a final volume of 0.20 ml. CTLL-20 cells were incubated for an additional 24 hours with a terminal 8 hour pulse of 0.8 uci of tritiated-thymidine. Values represent mean cpm of CTLL-20 proliferation in triplicates +/- one standard deviation.
Limiting Dilution Analysis: The limiting dilution analysis determines the frequency of antigen-specific cells in a given population of cells. Within the assay, all antigen presenting cells and optimal concentrations of antigen are provided. The limiting component is the cell population being examined. Under these conditions, the percent reactive cells as a function of serial dilutions of the test population fits a Poisson distribution. From these conditions, the frequency of cells can be determined.

The frequency of influenza virus specific IL-2 secreting cells in the lymph nodes and spleen was determined by a modification of the high density microcultures of the IL-2 overlay assay described previously. Normal spleen cells served as the antigen presenting cell population. Each well of a microtiter plate contained $2 \times 10^5$ normal spleen cells pulsed with 26 HAU/ml of influenza virus for 1 hour at $37^\circ C$ in a volume of 0.100 ml. This protocol allows for antigen to associate with the antigen presenting cells within the population. Stimulators were then irradiated with 2,000 rads of gamma rays to prevent incorporation of tritiated-thymidine.

Single cell suspensions of lymph node cells serving as the responder population were serially diluted two-fold in complete DMEM in 5 ml snap cap tubes. Each dilution was overlaid in replicates of 12 to 24 with cell concentrations ranging from $5 \times 10^4$ cells/well to $4 \times 10^2$ cells/well in a final volume of 0.150 ml. Cultures were incubated for 30 hours at $37^\circ C$. Following culture, cultures were irradiated and then overlaid with $1 \times 10^3$ CTLL-20 cells. Cultures were incubated for an additional 24 hours with a terminal 8 hour pulse of 0.8uci/well of tritiated thymidine. Cells were harvested and incorporation determined by scintillation spectroscopy. Frequencies were determined by a chi-square minimization analysis (173). Values represent the 95% confidence intervals and the chi-square probabilities. Chi-square probabilities > 0.050 were considered a fit to the Poisson distribution.
**Statistical Analysis.** Student's t test analysis or one way analysis of variance followed by Fisher's protected least significant difference were performed where indicated.
RESULTS

Distribution of Virus-Specific Cells Following Primary Infection

Studies of anti-influenza virus immune response have concentrated on regionally pooled lymph node cells or cells from a single immunologic active site i.e. lung, MED lymph nodes (6, 70, 76, 81, 159). Such studies did not distinguish the function or relationship of individual lymph nodes with other lymphoid tissues and have assumed that the regionally defined lymph nodes functioned identically. Comprehensive data on the development of virus-specific immune responses in regionally distinct lymphoid tissue, particularly lymphokine production, are sparse (76, 81, 82). To determine the distribution of virus-specific lymphocytes in distinct nodes, proliferation and secretion of lymphokines were examined in the SPL, SCV, DCV, and MED (draining nodes) and the AXIL, BRA, ING, and MES (non-draining nodes) at the peak of viral antigen expression, peak of immunopathology, and the peak of lymphocyte maturation, days 3, 5, 7, and, 15 post infection respectively.

First, to determine the optimal in vitro culture conditions for the detection of influenza virus-specific cells that proliferate, splenocytes from i.p. primed mice, a source rich in antigen specific cells, were cultured in microtiter plates in the presence or absence of PR8 antigen concentrations ranging from 5-52 HAU/ml. Proliferation of these cells was determined by the incorporation of tritiated-thymidine at 3, 5, and 7 days post culture. The proliferative responses demonstrated peak incorporation of label by day 5 of culture with all doses of virus examined (Figure 1). At these doses, peak incorporation was observed at
Figure 1. Influenza virus-specific lymphoproliferation of spleen cells. Cohorts of five male C57BL/6 mice 4-6 weeks of age were primed by an intraperitoneal injection of 320 HAU of influenza virus strain A/Puerto Rico/34/8 (PR8) in a total volume of 0.20 ml. Thirty days p.i. mice were sacrificed. Single cell suspensions were prepared and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained 2 x 10^5 cells in a total volume of 0.150 ml. Each set of replicates were either non-stimulated or stimulated with, 6, 13, 26, or 52 HAU/ml of PR8 antigen. Cultures were incubated for A) 3, 5, and 7 days or B) 5 days with the indicated dose of PR8 at 37°C in 10% CO2.

Proliferation was determined by the incorporation of 0.8 uCi/well of tritiated-thymidine in a four hour pulse. Values represent mean cpm +/- one standard deviation. Background incorporation of non-stimulated cultures was 5,000 cpm.
Figure 1A.
[\text{\[^3\text{H}\text{-Tdr incorporation (CPM)}\]}

\text{\textbf{Virus Concentration}}

Figure 1B.
day 5 with little incorporation on day 3 and day 7 of culture. The optimal dose for the proliferative response was determined to be 13 to 26 HAU/ml of influenza PR8.

To determine the kinetics of accumulation and distribution of influenza virus-specific cells following intranasal infection, proliferative responses from lymph nodes and spleen were examined 3, 5, 7, and 15 days post infection. Three days post intranasal instillation of influenza virus, a time when virus undergoes replication and small but detectable cell infiltrates in the lung are observed, no significant proliferation in either the MED, AXIL, or BRA nodes was observed on any in vitro day studied (Figure 2). This is consistent with the observations made in the LCMV model described by Doherty et al. (141). In these studies, antigen-activated cells as determined by immunofluorescence did not accumulate above values of non-stimulated animals within the draining node early post infection. However, this was in contrast to the increase in the recruitment of CD4+ lymphocytes. The initial response to infection induced an inflammatory response however, no cell activation was observed. In the present study, the proliferative studies substantiate the LCMV studies. It appears that even though infiltration of lung, and most probably the MED occurs at this time, there is no substantial cellular activation. The lack of responses in the non-draining nodes also indicates that the initial stimulation with influenza virus does not involve the lymphoid tissues distant from the site of infection. The SPL in contrast, did demonstrate low but detectable proliferative responses on days 3 and 5 of culture (Figure 2). The results indicate that early during the infection, no virus-specific cells were activated following in vitro stimulation with influenza virus within sites that drain the tissues of infection, however activated lymphocytes were found in the SPL. This might be due to the dissemination of virus to the SPL following the initial infection. Virus may directly enter the circulatory system from the lung as the virus replicates, however, the draining nodes may not take
up virus until the antigen load increases enough to enter the draining nodes in immunogenic concentrations. This is consistent with studies by Streilein et al., where radiolabelled and infectious virions were detected in the SPL by day 2 of intratracheal inoculation of virus (80).

Five days post infection, cells from the MED (which drain the lung and internal contents of the thoracic cavity) and cells from the SPL (which communicates directly with the circulatory system) demonstrated detectable proliferative responses to influenza virus (Figure 3). These cells demonstrated antigen-specific proliferation 3 times that of non-stimulated controls at day 3 of culture and continued to incorporate tritiated-thymidine through day 7 of culture. Proliferation of lymphocytes from these regions were higher by day 5 of culture for SPL and on day 7 for MED (p< 0.05) compared to in vitro incorporation on days 3 and 5. The presence of reactive cells in both the SPL and draining nodes is consistent with other influenza virus studies where both CTL and Tdth cells are detected by day 5 of infection (5).

The non-draining lymph nodes, AXIL and BRA, did not contain antigen-reactive lymphocytes that were detectable in vitro through day 5 of culture. However, on day 7 the BRA cells showed a small but significant response above non-stimulated controls (p< 0.05). Cells from the BRA proliferated significantly less than the draining lymph nodes but were not significantly different from the non-draining lymph nodes. This is an indication that the BRA and AXIL nodes, though associated with the thorax, do not communicate directly with either the sites of infection.

The results indicate that as the virus titer increases, the draining lymph node and SPL contain the necessary components for T lymphocytes to become activated and proliferate. At this time, the non-draining lymph nodes did not contain antigen-specific lymphocytes. These results indicate that cells reactive to influenza virus have access to the circulatory system but that they do not enter
Figure 2. Lymphoproliferative response of cells from lymphoid tissues 3 days p.i. infection with influenza virus strain A/Puerto Rico/8/34 (PR8). Cohorts of five male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Three days p.i. mice were sacrificed and lymph nodes draining the site of infection and distant sites were surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained $2 \times 10^5$ cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 3, 5, and 7 days at 37°C in 10% CO$_2$. Proliferation was determined by the incorporation of 0.8 uCi/well of tritiated-thymidine in a four hour pulse. Values represent mean cpm +/- one standard deviation. Background incorporation of non-stimulated cultures was 5,000 cpm.
Figure 2: 

DAYS IN CULTURE

[H]-TdR INCORPORATION (Thousands)

SPF
BR
AXIL
MED
Figure 3. Influenza virus-specific lymphoproliferation of cells from draining lymph nodes and spleen 5 days p.i. with influenza virus strain A/Puerto Rico/8/34 (PR8). Cohorts of five male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Five days p.i. infection mice were sacrificed and lymph nodes draining the site of infection and distant sites were surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained $2 \times 10^5$ cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 3, 5, and 7 days at 37°C in 10% CO$_2$. Proliferation was determined by the incorporation of 0.8uCi/well of tritiated-thymidine in a four hour pulse. Values represent mean cpm +/- one standard deviation. Background incorporation of non-stimulated cultures was 5,000 cpm.
Figure 3.

- MED
- AXIL
- BRA
- SPL
the non-draining nodes at this time. The in vitro proliferation kinetics of cells from the SPL and draining lymph nodes indicate further that there are differences between the cells from the lymphoid tissue draining the site of infection and the lymphoid tissue that has direct communication with the circulatory system. This may also be an indication that the frequency of antigen-reactive cells is still not optimal, and that it may require longer times for the frequencies to increase.

The proliferation of the MED cells on day 7 of culture suggests that the draining lymph nodes either have lower frequency of reactive cells or that the full complement of regulatory components is not present.

Maximal gross pathology of the lung illustrated by extensive cellular infiltrates and disruption of the lung architecture is evident seven days post infection (1, 67, 104). At this time the SCV, DCV, MED, and SPL cells demonstrated proliferative responses to stimulation above that of non-stimulated controls by day 3 of in vitro stimulation and maximal responses by day 5 of culture (Figure 4A). The SCV, DCV, MED, and SPL cells proliferated significantly higher on day 3 and 5 compared to day 7 of in vitro culture (p<0.05) indicating mature in vitro proliferative responses. The BRA, ING, and AXIL cells demonstrated no detectable reactivity throughout the in vitro culture period (Figure 4B). There were no significant differences between the non-draining lymph nodes. These data indicate that at the peak of pathology, both the draining node and SPL have the full complement of factors and antigen presenting components necessary for mature proliferative responses. This is in contrast to the immature in vitro proliferative responses of the MED cells on day 5 of infection (Figure 3). These data indicates that either the frequency of reactive cells increased from day 5 post infection to day 7 post infection or that the immune cells matured to create the optimal conditions for mature proliferative responses.
Figure 4. Influenza virus-specific lymphoproliferation of cells from draining and non-draining lymph nodes 7 days p.i. with influenza virus strain A/Puerto Rico/8/34. Cohorts of five male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Seven days p.i., mice were sacrificed and lymph nodes draining the site of infection (A) and distant sites (B) were surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained $2 \times 10^5$ cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 3, 5, and 7 days at 37°C in 10% CO$_2$. Proliferation was determined by the incorporation of 0.8uCi/well of tritiated-thymidine in a four hour pulse. Values represent mean cpm +/- one standard deviation. Background incorporation of non-stimulated cultures was 5,000 cpm. Data presented are representative of 2 independent experiments.
Figure 4A.
Figure 4B.
Figure 5. Influenza virus-specific lymphoproliferation of cells from draining and non draining lymph nodes 15 days p.i. with influenza virus strain A/Puerto Rico/8/34. Cohorts of five male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Fifteen days p.i. mice were sacrificed and lymph nodes draining the site of infection (A) and distant sites (B) were surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained $2 \times 10^5$ cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 3, 5, and 7 days at 37°C in 10% CO$_2$. Proliferation was determined by the incorporation of 0.8uCi/well of tritiated-thymidine in a four hour pulse. Values represent mean cpm +/- one standard deviation. Background incorporation of non-stimulated cultures was 5,000 cpm. Presented are data representative of 2 independent experiments.
Figure 5A.
Fifteen days post intranasal instillation of influenza virus, infectious virions are not detected and the histology of lung has almost returned to normal (104). Following the resolution of the infection, the MED, SCV, DCV, and SPL proliferated in response to influenza virus (Figure 5A). All the draining lymph nodes studied and SPL incorporated label maximally by day 5 of culture. The draining lymph nodes demonstrated peak responses at day 5 with significant decline by day 7 (p<.0.05). The SPL cells maintained responses from day 5 through day 7 of culture. These data indicated that the full complement of conditions for mature proliferative responses were present 15 days p.i.

The AXIL, BRA, and ING lymph nodes demonstrated low but detectable responses by day 5 of culture (Figure 5B). By day 7 of culture, the non-draining nodes reached significantly higher proliferative responses compared to days 3 and 5 post culture indicating immature in vitro proliferative profiles (Figure 5B). These data indicate that the non-draining lymph nodes acquire conditions or cells that enable virus-specific cells to respond to in vitro stimulation with the influenza virus following the resolution of infection whereas such conditions do not exist at the peak of immunopathology (day 7 post infection).

Distribution of IL-2-Secreting Cells Following Primary Infection

The absence of proliferative responses in the non-draining lymph nodes at the peak of pathology suggested the absence of virus-specific cells in these nodes, however, it was possible that cells were present and were able to secrete lymphokines but were not able to proliferate optimally under the experimental conditions used in vitro (Figure 4B). Due to the central role of IL-2 in activating immune responses (8, 120) and therefore its potential for exacerbating the immunopathology induced by influenza viral infection, secretion of IL-2 by cells from various lymphoid tissues during the course of infection was examined. IL-2 secretion was determined by the in vitro stimulation of cells for 24 hours in
Figure 6. PR8 antigen dose response for IL-2 secretion by spleen cells to influenza virus strain A/Puerto Rico/8/34 (PR8). Cohorts of 3 male C57BL/6 mice 4-6 weeks of age were primed by an intraperitoneal injection of 320 HAU of influenza PR8. Thirty days p.i. mice were sacrificed and spleens were surgically removed. Single cell suspensions were cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained $2 \times 10^5$ cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with 6, 13, 26, or 52 HAU/ml of PR8 antigen. Cultures were incubated for 24 and 48 hours at 37°C in 10% CO$_2$. Following culture, plates were irradiated with 2,000 rads of gamma rays and overlaid with $1 \times 10^4$ CTLL-20 cells. Cultures were additionally incubated for 24 hours with a terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/- one standard deviation. Background incorporation of non-stimulated controls was 5,000 cpm.
Figure 6.

[3H]-TdR INCORPORATION (CPM)
(Thousands)

0 10 20 30 40 50 60

0 6 13 26 52

HAU/ML

24 HOURS

48 HOURS
Figure 7. Specificity of CTLL-20 cells for IL-2. CTLL-20 cells were cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained $1 \times 10^4$ cells in a total volume of 0.150 ml. Each set of replicates were either non-stimulated or stimulated with two-fold serial dilutions of either recombinant IL-2 (Genzyme) or recombinant IL-4 (Genzyme). Units are expressed as defined by Genentech. Cultures were incubated for 24 at $37^\circ C$ in 10% CO$_2$. Following culture, CTLL-20 cells were given terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/- one standard deviation.
Figure 7.

[3H]-TdT INCORPORATION (CPM)
(Thousands)

UNIT SHEET
in microculture followed by irradiation. The irradiated cultures were then overlaid with the IL-2 dependent cell line, CTLL-20. The presence of IL-2 was subsequently determined by the proliferation of CTLL-20 cells.

To determine the optimal dose of viral antigen to stimulate the production of IL-2, two-fold serial dilutions of PR8 antigen were used to stimulate influenza virus-primed mice (Figure 6). The IL-2 responses to doses of 6 through 26 HAU/ml were not significantly different at both 24 and 48 hours post culture, however, maximal IL-2 production these doses occurred at 24 hours. Influenza virus concentrations used for the determination of IL-2 production was 13 HAU/ml. Cell lines considered to be dependent on IL-2 for growth have been recently identified as being responsive to IL-4, in particular those cells and cell lines derived from Th1 cells (174, 175). To determine the specificity of the CTLL-20 cell line for IL-2, CTLL-20 cells were cultured in three-fold serial dilutions of recombinant murine IL-2 or recombinant murine IL-4 (Figure 7). CTLL-20 cells in the present study responded preferentially to IL-2, with peak proliferation at 40 units/ml. Responses to IL-4 over the concentration range of 1000 to 20 units/ml demonstrated a response of 20% of the maximum IL-2 response at 1000 units/ml. This suggested that the proliferative responses by the CTLL-20 cell line were due primarily to IL-2 and not IL-4.

Three days p.i., the nodes draining the upper and lower respiratory tract, SCV, DCV, and MED, demonstrated small but significant IL-2 production above non-stimulated controls (Figure 8). There were no significant differences between these tissues suggesting an equal distribution of viral antigen throughout the respiratory tract. These data show that, unlike the proliferative responses (Figure 2), detectable activation of draining lymph node cells was found as early as day 3 p.i. Furthermore, the high background in the non-stimulated control cultures indicated the possible presence of cell constitutively expressing IL-2. This high background may reflect the presence of activated cells and the carry
Figure 8. In vitro secretion of IL-2 by draining lymph node cells 3 days p.i. with influenza virus strain A/Puerto Rico/8/34 (PR8). Cohorts of 5 male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Three days p.i. mice were sacrificed and lymph nodes draining the site of infection and distant sites were surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained 2 x 10^5 cells in a total volume of 0.150 ml. Each set of replicates were either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 24 hours at 37°C in 10% CO2. Following culture, plates were irradiated with 2000 rads and overlaid with 1 x 10^4 CTLL-20 cells. Cultures were additionally incubated for 24 hours with a terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/- one standard deviation. Levels of significance were determined by Student's t test. Asterisk represents p<0.05.
Figure 8.
Figure 9. \textit{In vitro} secretion of IL-2 by draining and non-draining lymph node cells, and spleen cells 5 days p.i. infection with influenza virus strain A/Puerto Rico/8/34. Cohorts of three male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Five days p.i. mice were sacrificed and lymph nodes draining the site of infection and distant sites were surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained $2 \times 10^5$ cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 24 hours at 37°C in 10% CO$_2$. Following culture, plates were irradiated with 2,000 rads and overlaid with $1 \times 10^4$ CTLL-20 cells. Cultures were additionally incubated for 24 hours with a terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/− one standard deviation. Levels of significance were determined by Student's t test. Asterisk represents $p<0.05$. 
Figure 9.
over of sufficient antigen at this time. It appears at the time of maximum viral replication that the cells are stimulated to produce IL-2.

Five days p.i., a time when virus titers decline and the presence of CTL is first detected (5, 104), the DCV and MED node cells expressed detectable IL-2 responses, whereas the SCV did not (Figure 9). The MED node cells with 8,000 cpm above background were significantly higher than DCV node cells with a mean cpm of 2,000 cpm. The SPL did express detectable IL-2 above background with net cpm of 1,500. This response was significantly lower than the DCV and the MED nodes. In contrast to the MED node, which drains the lung parenchyma, mononuclear cells from the lung did not express detectable levels of IL-2. The non-draining nodes, like the lung did not produce detectable levels of IL-2. It appears that as the immune response develops, there is a shift in the stimulation of IL-2-secreting cells, from the SCV and DCV to the MED and SPL. This may reflect the spread of virus from the upper to the lower respiratory mucosa and that virus antigen enters the circulation and stimulates the SPL.

Seven days following intranasal instillation of PR8, a time when the cellular infiltrate peaks in the lung and infectious virus titer has declined, all the draining lymph nodes including DCV, MED, and SCV, and the SPL responded to in vitro stimulation with virus and were able to secrete IL-2 (Figure 10). The SCV demonstrated incorporation of 87,000 cpm, which was significantly lower than the incorporation of the DCV (115,000 cpm). The MED demonstrated 50,000 cpm which was significantly lower that either the SCV or DCV but was higher than the SPL (45,000 cpm). The non-draining lymph nodes, with the exception of the AXIL, did not produce IL-2. Though the net incorporation was statistically significant in the AXIL (p<0.05), the change was slight with net incorporation of 1,000 cpm. As observed at day 5 p.i., the lung mononuclear cells did not express detectable levels of IL-2. At the peak of the immunopathology, cells able to express IL-2 are preferentially localized in tissues that drain the site of infection.
but not the site itself (lungs). The non-draining nodes had comparatively little, if any, response to influenza virus stimulation.

Following resolution of infection and resolution of the immunopathology (15 days p.i.) the distribution of IL-2-producing cells shifted to include non-draining nodes (Figure 11). The lymph node cells, both draining and non-draining, expressed IL-2 above non-stimulated controls. The SCV response demonstrated net incorporation of 60,000 cpm which was significantly lower than the DCV and MED nodes which demonstrated net cpm of 80,000. The SPL demonstrated net incorporation of 50,000 cpm which was not significantly different from the SCV but was lower than either the MED or DCV. The non-draining nodes (BRA and ING) demonstrated significant IL-2 production with the BRA having net incorporation of 10,000 cpm and the ING demonstrating 13,000 cpm. The BRA node responses were lower than the ING. Comparison of the magnitude of the response between the draining and non-draining lymph nodes demonstrated that the non-draining nodes had values 1/3-1/4 of the draining nodes. These data indicate that following resolution of infection and the concomitant recovery of the lung parenchyma from the localized inflammatory response, IL-2-producing cells redistribute to non-draining lymph nodes.

This pattern of IL-2 response was consistent through the study period (30 days p.i.), though the magnitude of the responses was different (Figure 12). The SCV cells demonstrated net incorporation of 30,000 cpm which was not different from the MED node cells (40,000 cpm). The SPL response was 5,000 cpm above non-stimulated controls and was significantly lower that the draining nodes. All the non-draining nodes examined demonstrated responses above non-stimulated controls however, the BRA and ING demonstrated values above that of the AXIL. The magnitude of the response in the ING nodes was not significantly different from either the SPL or AXIL, but was lower than the ING. These data showed that, like the lymphoproliferative response (Figure 4A and Figure 5B), the non-
Figure 10. IL-2 response of draining and non-draining lymph node cells 7 days p.i. with influenza virus strain A/Puerto Rico/8/34. Cohorts of 10 male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Seven days p.i., mice were sacrificed and lymph nodes draining the site of infection and distant sites were surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained 2 x 10^5 cells in a total volume of 0.15 ml. Each set of replicates was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 24 hours at 37⁰C in 10% CO₂. Following culture, plates were irradiated with 2,000 rads and overlaid with 1 x 10⁴ CTLL-20 cells. Cultures were additionally incubated for 24 hours with a terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/- one standard deviation. Levels of significance were determined by Student's t test. Asterisk represents differences between non-stimulated controls and PR8 antigen stimulated cultures at p<0.05. Data presented are representative of 5 independent experiments.
Figure 10.
Figure 11. IL-2 response of draining and non-draining lymph node cells 15 days p.i. with influenza virus strain A/Puerto Rico/8/34. Cohorts of three male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Fifteen days p.i. infection mice were sacrificed and lymph nodes draining the site of infection and distant sites were surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained 2 x 10^5 cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 24 hours at 37°C in 10% CO_2. Following culture, plates were irradiated with 2,000 rads of gamma rays and overlaid with 1 x 10^4 CTLL-20 cells. Cultures were additionally incubated for 24 hours with a terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/- one standard deviation. Levels of significance were determined by Student's t test. Asterisk represents differences between non-stimulated controls and PR8 antigen stimulated cultures at p<0.05. Data presented are representative of 5 independent experiments.
Figure 11.
draining nodes acquired responsive cells able to secrete IL-2, but only after the resolution of infection.

To summarize, the kinetics of the accumulation of viral antigen-specific lymphocytes in the various nodes following primary infection and to normalize the magnitude of the responses from individual experiments, IL-2 responses were transformed into fractional values/arbitrary units. The units are defined as the fractional response of the maximal responses of CTLL-20 cells to saturating concentrations of IL-2 (Figure 13).

The non-draining lymph nodes, AXIL, BRA, and ING did not express IL-2 prior to day 15. The MES lymph node cells demonstrated small but detectable IL-2 levels by day 7 of infection, however, like the other non-draining lymph node cells, did not express strong responses until day 15 p.i. (Table 1). Analysis of the BRA and ING lymph node cells in terms of IL-2 secretion by one way analysis of variance followed by Fisher’s least significant difference, demonstrated significant IL-2 secretion on day 15 when compared to day 7 at a p= 0.005. Though significant changes in the BRA and ING cells were observed on day 15 these changes were significantly less than the draining lymph nodes.

The inability of cells in the non-draining nodes to express IL-2 at day 7 p.i., the peak of the cellular accumulation in the lung, suggested that cells were recruited from the recirculating pool of mononuclear cells. This would account for the lack of responses in the non-draining nodes. Examination of the total mononuclear cell yields per regional lymph node demonstrated that cells were removed from the recirculating pool at the peak of pathology. The draining nodes demonstrated a 1-2-fold decrease in total cell yields from day 7 to day 15 p.i., while, in contrast, the non-draining nodes demonstrated a 1-3-fold increase (Table 2). The recruitment of total mononuclear cells however may not determine the functional capacity of the cells. It is possible that the antigen specific cells were evenly distributed to other tissues even though the total
Figure 12. IL-2 response of draining and non-draining lymph node cells 30 days p.i. with influenza virus strain A/Puerto Rico/8/34 (PR8). Cohorts of 10 male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Thirty days p.i. infection mice were sacrificed and lymph nodes draining the site of infection and distant sites were surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained $2 \times 10^5$ cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 24 hours at 37°C in 10% CO$_2$. Following culture, plates were irradiated with 2,000 rads of gamma rays and overlaid with $1 \times 10^4$ CTLL-20 cells. Cultures were additionally incubated for 24 hours with a terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/- one standard deviation. Levels of significance were determined by Student's t test. Asterisk represents differences between non-stimulated controls and PR8 antigen stimulated cultures at p<0.05. Data presented are representative of 5 independent experiments.
Figure 12.
Figure 13. Transformation of net cpm to IL-2 units. CTLL-20 cells were cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained $1 \times 10^4$ cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with two-fold serial dilutions of rat Con A culture supernatant. Cultures were incubated for 24 at 37°C in 10% CO₂. Following culture, CTLL-20 cells were given terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Units in experiments are expressed as the net cpm of influenza virus strain A/Puerto Rico/8/34 antigen-stimulated lymph node cultures divided by the maximum proliferative response of CTLL-20 under saturating concentrations of Con A culture supernatant multiplied by 100. Values represent mean cpm +/- one standard deviation.
Figure 13.

[Graph showing dilution vs. [3H]-Thd incorporation (CPM) in thousands]
mononuclear cells were not.

The continued in vitro lymphoproliferative responses of non-draining node cells on day 7 post culture, 15 days p.i, suggested that the non-draining nodes had lower frequencies of reactive cells compared to the draining nodes (Figure 5B). To address a possible difference in frequency, the in vitro kinetics of IL-2 secretion was examined by culturing cells for 24, 48, and 72 hours. Both draining and non-draining nodes demonstrated detectable IL-2 secretion by 24 hours post culture, however, the AXIL, BRA, and ING lymph node cells expressed continually higher levels of IL-2 from 24 to 72 hours post culture. In contrast, the SCV, MED, DCV, and SPL demonstrated a continual decline of IL-2 secretion within the same time period (Figure 14).

These data suggest that at different times post infection the lymph nodes contained different frequencies of antigen-reactive T cells. To examine the frequency, lymph node cells from draining and non-draining nodes were systematically studied by limiting dilution analysis.

Frequencies of IL-2-Secreting Cells in Draining and Non-Draining Lymph Nodes Following Primary Infection.

The observation that influenza virus-specific cells were unable to secrete detectable IL-2 in the non-draining lymph nodes prior to the resolution of infection, and that following the resolution of infection the in vitro IL-2 kinetic profiles demonstrated continued incorporation of label at day 3 of culture, suggested that the frequency of virus-specific cells might vary between the various lymphoid tissues. Conversely, the activation of IL-2-secreting lymphocytes might have no relevance to changes in frequency, but rather might be due to micro-environmental signals specifically located in the various lymph nodes. To test these possibilities, a limiting dilution analysis for influenza virus-specific lymphocytes that produce IL-2 was performed. On days 7, 15, and 40
Table 1

Kinetics and Distribution of IL-2-Secreting T Cells from Lymphoid Tissue Following Primary Infection with Influenza Virus Strain A/Puerto Rico/8/34

<table>
<thead>
<tr>
<th>Region</th>
<th>Days Post Infection</th>
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<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>SCV</td>
<td>20b(9)c</td>
</tr>
<tr>
<td>MED</td>
<td>22 (12)</td>
</tr>
<tr>
<td>AXIL</td>
<td>2 (3)</td>
</tr>
<tr>
<td>BRA</td>
<td>0</td>
</tr>
<tr>
<td>ING</td>
<td>0</td>
</tr>
<tr>
<td>MES</td>
<td>N.D.</td>
</tr>
<tr>
<td>SPL</td>
<td>0</td>
</tr>
</tbody>
</table>

a. Cohorts of 10-15 male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Lymph node and spleen cell suspensions were prepared at the indicated times post infection and were cultured for 24 hours in high density microcultures with 13 HAU/ml of influenza virus PR8. IL-2 secretion was determined by the proliferation of CTLL-20 cells in an overlay assay as described in methods.

b. Values represent arbitrary units of IL-2 determined by experimental net cpm (triplicate)/maximum cpm of CTLL-20 cells stimulated with saturating concentrations rat Con A supernatant times 100. Non-stimulated cultures incorporated 5,000-10,000 cpm. Data for day 7, 15, and 30 are representative of 3 experiments.

c. Values in parentheses represent one standard deviation.

d. Underlined values represent positive responses.

e. Not determined.
Mononuclear Cell Recovery from Draining and Non-Draining
Lymphoid Tissue Following Primary Infection
with Influenza virus

<table>
<thead>
<tr>
<th>Region</th>
<th>Exp.</th>
<th>Day 7</th>
<th>Day 15</th>
<th>Day 15/Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCV</td>
<td>1</td>
<td>8.30</td>
<td>8.00</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>12.00</td>
<td>6.45</td>
<td>0.54</td>
</tr>
<tr>
<td>MED</td>
<td>1</td>
<td>2.33</td>
<td>2.75</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.47</td>
<td>1.80</td>
<td>0.73</td>
</tr>
<tr>
<td>BRA</td>
<td>1</td>
<td>0.33</td>
<td>1.00</td>
<td>3.03</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.40</td>
<td>1.20</td>
<td>0.85</td>
</tr>
<tr>
<td>ING</td>
<td>1</td>
<td>0.42</td>
<td>1.50</td>
<td>3.57</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.47</td>
<td>2.30</td>
<td>1.56</td>
</tr>
</tbody>
</table>

a. Single cell suspensions of each lymph node were prepared from a total of 10-15 mice 7 and 15 days post primary infection. Total number of mononuclear cells/region/mouse was calculated by counting total mononuclear cells from each region in crystal violet and dividing the total by the number of mice used in each experiment. Values represent $10^{9}$ cells/region. Data for days 7 and 15 p.i. represent paired groups of 5 to 15 mice.

b. Ratio of cell yields from day 15 and day 7 p.i.
Figure 14. **In vitro** kinetics of IL-2 secretion by cells from lymphoid tissues 15 days p.i. with influenza virus strain A/Puerto Rico/8/34. Cohorts of 5 male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Fifteen days p.i. mice were sacrificed and lymph nodes draining the site of infection and distant sites were surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained $2 \times 10^5$ cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 24, 48, and 72 hours at 37°C in 10% CO$_2$. Following culture, plates were irradiated with 2,000 rads of gamma rays and overlaid with $1 \times 10^4$ CTLL-20 cells. Cultures were additionally incubated for 24 hours with a terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/- one standard deviation. Levels of significance were determined by Student's t test. Asterisk represents differences between non-stimulated controls and PR8 antigen stimulated cultures, and differences between 24 and 72 hour cultures at p<0.05. Data presented are representative of 3 experiments.
Figure 14.
days p.i., cells from the various lymphoid tissues were prepared and the frequency of virus-specific IL-2-producing lymphocytes determined.

The draining nodes, non-draining nodes, and SPL cells were examined for the frequency of IL-2-secreting cells following in vitro stimulation with PR8 antigen. This was carried out by culturing a series of 2-fold dilutions of lymph node cells with a constant number of irradiated SPL cells pulsed with PR8 antigen. The inclusion of the SPL cells was to ensure that the antigen presenting cell population was not limiting. To ensure that our limiting dilution assay was under conditions where only the responder cells were limiting, chi-square minimalization analysis was performed (Figure 15). This analysis provides a 95% confidence interval. The chi-square probability (a measure of goodness of fit with a Poisson distribution) should be equal to or more than 0.05 if conditions are such that a single factor (such as the responder cell) is limiting.

As a negative control, SPL cells from uninfected mice were assayed for the frequency of IL-2-secreting lymphocytes. From such studies, it was determined that the frequency of virus specific T lymphocytes from uninfected mice was below the sensitivity of the assay (judged to be 1/500,000 - 1/1,000,000).

To determine the frequencies of cells expressing IL-2 constitutively and virus-specific cells expressing IL-2 following stimulation with antigen, both non-stimulated and PR8 antigen-stimulated cells were assayed in the limiting dilution assay. The data are presented as scatter plots of the cpm of tritiated-thymidine from individual wells and as chi-square linear regression curves. Cultures were considered responsive if incorporation of label exceeded 3 standard deviations above the mean cpm of cultures containing only PR8 antigen pulsed stimulators.

Scatter plots of values from individual wells of non-stimulated cultures of both the SCV and MED demonstrated values accumulating at 1,500 cpm, which were below the cutoff of 2,000 cpm (Figure 15A and Figure 15C). At cell concentrations of 25,000 cells/well, 8% of the responding wells (1 well of 12)
were positive for IL-2 production with values of 2,500 cpm. No positive wells were found at cell concentrations of 12,500 cells/well. Unlike the SCV, however, the MED showed a resurgence of responding cells at 6250 cells/well with 16% positive responders (2 wells of 12). Cell concentrations below 6,250 were again negative for IL-2 secretion. The results indicated that few cells were present in the cell population that were able to secrete IL-2 without further stimulation by PR8 antigen. Determination of frequencies from assays where there is one useful point (25,000 cells/well) is considered inaccurate, therefore the frequencies of cells constitutively secreting IL-2 under the current experimental conditions were considered below the sensitivity of the assay. The recurrence of positive wells as the cells become more dilute is not uncommon in this assay, however interpretation of its cause is controversial. Proponents of active suppressor cells suggest that the resurgence of activity is due to the dilution of suppressor cells. However, the magnitude of these responses at the more dilute concentrations suggests an alternative. It is probable that the resurgence is due to a statistical phenomena. Since determination of the percent reactive cells translates to the whole population, the fact that we only sample a small proportion of the total population with relatively few test wells (i.e. 24 wells/dilution), makes it likely that the increase in responses is due to random probability of picking up positive cells that were missed at the comparable higher concentrations.

Scatter plots of draining lymph nodes stimulated with viral antigen demonstrated virus-specific cells. The SCV nodes cultured at 25,000 cells/well with PR8 antigen showed an even distribution of values ranging from 12,000 to 3,000 cpm with 100% positive wells (Figure 15B). At 12,500 cells/well, the distribution of values ranged from 6,000 cpm to 1,500 cpm with 30% negative wells. The distribution of values continued to accumulate below the cutoff at cell concentrations of 3,125 cells/well. At this concentration, 95% negative wells was observed. Responding wells were again detected at 1,563 cells/well as
Figure 15. Scatter plots of the frequency of virus-specific cells able to secrete IL-2 in vitro from lymphoid tissues 7 days p.i. with influenza virus strain A/Puerto Rico/8/34 (PR8). Cohorts of 15 male C57BL/6 mice were infected intranasally with 32 HAU of infectious virus. Seven days p.i., mice were sacrificed and lymphoid tissues surgically removed. Single cell suspensions of superficial cervical (SCV) (A and B), mediastinal (MED) (C and D), and inguinal (ING) (E and F) were prepared and two fold serial dilutions made. The frequency of cells was determined by a limiting dilution analysis. Briefly, 2.5 x 10^4 to 2 x 10^2 responder cells from the indicated tissues were placed in each well in replicates of 24 containing 2 x 10^5 PR8 antigen pulsed irradiated normal spleen cells (B, D, and F) or non-pulsed stimulator (A, C, E). Cells were cultured for 30 hours and irradiated with 2,000 rads of gamma rays. CTLL-20 cells were then added at 1 x 10^3 and cultured an additional 24 hours with a terminal 12 hour pulse. Values represent CTLL-20 proliferation for each well. Non-stimulated cultures contained replicates of 12. Cutoff points were determined by replicates of 12 that contained PR8 antigen pulsed normal splenocytes only. Cutoff line was calculated by the mean cpm incorporation plus 3 standard deviations. Data presented are representative of 3 independent experiments.
Figure 15A.
Figure 15B.
Figure 15C.
Figure 15D.
Figure 15F.
observed for the non-stimulated cultures. The gradual increase of the number of wells accumulating below the cutoff through a broad range of cell concentrations was indicative of a Poisson distribution. Therefore, the presence of SCV lymph node cells was the limiting factor for IL-2 secretion in response to PR8 antigen stimulation.

The MED node cells cultured at 25,000 cells/well with PR8 antigen viral antigen showed an even distribution of values ranging from 10,000 to 6,000 cpm above the cutoff point of 2,000 cpm (Figure 15D). At 12,500 cells/well the distribution ranged from 8,000 to 2,000 cpm with an accumulation of values at 6,000 to 3,000 cpm. This represented 4% negative wells. At 6,250 cells/well, approximately 76% of the wells were negative with the distribution ranging from 3,000 to 1,800 cpm, with an accumulation at 1,800 cpm. Unlike the SCV nodes, the MED cell cultures accumulated rapidly below the cutoff with accumulations of negative wells ranging from 4% to 76% between cell concentrations of 12,500 cells/well to 6,250 cells/well. The rapid drop of the fraction of responder wells indicated that the responder cells were not the only limiting factor. The non-draining node, i.e. the inguinal, contained PR8 antigen-specific IL-2-secreting cells at 7 p.i. No detectable frequencies of constitutively secreting cells (Figure 15E and Figure 15F) were found at this time. At the highest dilutions used, 25,000 cells/well, 24% of the wells were positive following PR8 viral antigen stimulation (Figure 15F). The distribution was small ranging from 4,000 cpm to 2,000 cpm with an accumulation below the cutoff line.

These data are depicted graphically by linear regression analysis (chi-square minimization) (Figure 16). Frequencies are determined by the intersection of the lines generated plotting % negative wells against the total number of responder cells/well at 37% negative wells. The higher the frequency of responding cells, the steeper the slope of the regression line. In contrast to the high density cultures of non-draining lymph node cells (where IL-2 is not detected until day
15 p.i.), detectable frequencies of cells are found by day 7 and remained relatively unchanged through day 15.

At 7 days p.i., the SCV, MED, and SPL contained influenza virus-specific cells as expected from the data obtained from the high density bulk cultures (Figure 16 and Table 3). The SCV nodes had frequencies ranging from 1/6,000 to 1/12,000. The SPL demonstrated comparable frequencies ranging from 1/8,000-1/13,000. When compared to non-draining nodes, the draining nodes and SPL had frequencies that were generally 10-fold higher. Analysis of the goodness of fit to the Poisson distribution demonstrated that the draining nodes required more than one factor. The MED and the SPL demonstrated chi-square probabilities less that 0.05 which indicated that the frequency estimates were not accurate. However, based on the frequency analysis of day 15 and day 40 (where the chi-square was >0.100), it was suggested that the day 7 frequencies were probably a fair estimation. This multi-factor requirement of day 7 cultures was not observed on 15, or 40 days p.i.

Following resolution of infection and through day 40 of infection, the frequencies of cells in the draining nodes and SPL did not change from the day 7 frequencies, however, the chi-square goodness of fit probabilities did (Table 3). Compared to day 7, the draining nodes became a one hit kinetics model by day 15 indicating that the requirement for multiple factors was overcome following the resolution of infection. This pattern continued through day 40 p.i. The non-draining nodes demonstrated no change in frequencies from day 7 to 15, with the exception of the BRA node which changed from non-detectable frequencies to 1/130,000-500,000 cells. However from day 15 to day 40, the ING and MES nodes demonstrated an estimated 2-fold increase in frequencies of cells. These cells, however, deviated from the one hit kinetics model assumption unlike the BRA. The different accumulation pattern in the BRA may reflect different accumulation rates within the BRA nodes. In sheep, certain lymph nodes
Figure 16. Frequency of virus-specific cells that secrete IL-2 in vitro from lymphoid tissues at 7 (A) and 15 (B) days p.i. with influenza virus strain A/Puerto Rico/8/34 (PR8). Cohorts of 15 male C57BL/6 mice were infected intranasally with 32 HAU of infectious virus. Seven days p.i., mice were sacrificed and lymphoid tissues surgically removed. Single cell suspensions of lymphoid tissue were prepared and two fold serial dilutions made. Frequency of cells was determined by a limiting dilution analysis. Briefly, 2.5 x 10^4 to 2 x 10^2 responder cells from the indicated tissues were placed in each well in replicates of 24 containing 2 x 10^3 virus pulsed irradiated normal spleen cells or non-pulsed stimulator. Cells were cultured for 30 hours and irradiated with 2,000 rads of gamma rays. CTLL-20 cells were then added at 1 x 10^3 and cultured an additional 24 hours with a terminal 12 hour pulse. Values represent CTLL-20 proliferation for each well. Non-stimulated cultures contained replicates of 12. Virus stimulated cultures represent 24 replicates. Cutoff points (A. 1990 cpm and B. 945 cpm) were determined by replicates of 12 that contained PR8 antigen pulsed normal splenocytes only. X-axis represents total number of responder cells/well. Y-axis represents % negative wells. Linear regression lines were determined by chi-square minimization analysis. Data presented are from experiments presented in Figure 15.
Figure 16A.
Figure 16B.
Table 3

Distribution and Frequency of IL-2-Secreting T Cellsa Following Primary Infection with Influenza Virus.

<table>
<thead>
<tr>
<th>Region</th>
<th>Time Post Infection</th>
<th>C. I. (1/f)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAY 7 b</td>
<td>DAY 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCV</td>
<td>6,950-12,902c</td>
<td>9,252-17,251</td>
</tr>
<tr>
<td></td>
<td>(0.188)d</td>
<td>(0.326)</td>
</tr>
<tr>
<td>MED</td>
<td>6,706-12,113</td>
<td>8,932-14,746</td>
</tr>
<tr>
<td></td>
<td>(0.004)</td>
<td>(0.111)</td>
</tr>
<tr>
<td>BRA</td>
<td>NEGe</td>
<td>132,121-531,926</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.363)</td>
</tr>
<tr>
<td>ING</td>
<td>46,205-136,372</td>
<td>57,308-146,173</td>
</tr>
<tr>
<td></td>
<td>(0.722)</td>
<td>(0.059)</td>
</tr>
<tr>
<td>MES</td>
<td>77,140-396,636</td>
<td>106,827-367,985</td>
</tr>
<tr>
<td></td>
<td>(0.987)</td>
<td>(0.363)</td>
</tr>
<tr>
<td>SPL</td>
<td>9,475-18,099</td>
<td>11,223-20,490</td>
</tr>
<tr>
<td></td>
<td>(0.040)</td>
<td>(0.458)</td>
</tr>
</tbody>
</table>

a. Cohorts of 5-15 male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. At the indicated times p.i. infection, two-fold serial dilutions of lymph node and spleen responder cell suspensions were cultured with 2 x 10⁵ irradiated virus pulsed normal splenocytes in 96 well round-bottom microtiter plates. Dilutions in replicates of 24 ranged from 5 x 10⁵ responder cells/well to 3 x 10⁴ cells/well. IL-2 secretion was determined 24 hours post culture for each well by the overlay of CTLL-20 cells as described in methods. Values were derived from experiments presented in figures 15 and 16.

b. Day 7 frequency representative of 3 experiments.

c. Values represent 95% confidence intervals of the reciprocal of frequency as determined by chi-square minimalization.

d. Chi-square probability. P>0.050 were considered to fit a Poisson distribution.

e. Frequency below limits of chi-square minimization analysis.
accumulate cells at a faster rate than other peripheral lymph nodes (125). In the current study, the BRA may function differently from the other non-draining nodes. Whether the different accumulation patterns and the retention of a single hit kinetics model throughout the study period is a reflection of the BRA communicating with the lung is not understood.

These data indicate that detectable IL-2-secreting virus-specific cells were present in both draining and non-draining lymph nodes by 7 days p.i., however, it was only in the environment of the draining nodes that the production of IL-2 was detected. It appears that there is a subsequent redistribution of IL-2-secreting T lymphocytes (that require multiple factors for the production of IL-2) from the draining nodes to other peripheral nodes 40 days p.i.
DISCUSSION

The accumulation of cells at sites of inflammation is well documented (1, 5, 119, 125, 67, 141, 148, 153, 161-170, 176-178, 159), however, the mechanisms of recruitment and the regulatory influences of the environment on the secretion of lymphokines are less well understood, particularly in respiratory tract viral infections. The regulation of lymphokine secretion may play the dominant role in the development of immunopathology and not just the recruitment of cells to sites of inflammation.

In this chapter, the kinetics of the accumulation and distribution of influenza virus-specific cells were examined to address the questions; how do influenza virus-specific cells that secrete IL-2 traffic or distribute themselves in lymphoid tissues? And do cells express IL-2 differentially depending on the environment in which such cells accumulate? Four observations were made. First, in high density cultures, influenza virus-specific lymphocytes that secreted IL-2 and proliferated to in vitro stimulation with PR8 antigen preferentially accumulated in the SCV, DCV, MED, and SPL by day 7 of infection and were maintained through day 30 (Figures 4, 10, and 12). Second, SPL and draining lymph node cells demonstrated that IL-2-secreting T cells were limited by multiple factors (Table 3). Third, and in contrast to the high density cultures, limiting dilution analysis demonstrated that influenza virus-specific cells distributed to non-draining nodes by day 7 of infection and were maintained through day 15 (Tables 1 and 3). Fourth, virus-specific cells that secrete IL-2 in the non-draining lymph nodes were limited by multiple factors 40 days p.i..
Limiting dilution analysis demonstrated that SCV, MED, and SPL, accumulated apparent frequencies of IL-2-secreting cells 10-100-fold above uninfected SPL cells by day 7 p.i. The frequencies ranged from 1/5,000-1/14,000 and remained unchanged throughout day 40. However, in these nodes the analysis did not fit the expected Poisson distribution as indicated by the chi-square probability of less than 0.05. The non-draining nodes, ING and MES, demonstrated frequencies of 1/50,000-1/140,000 at both day 7 and 15 p.i. but did conform to the expected Poisson distribution. The 10-fold greater frequency of cells in the draining nodes at the peak of the response suggests that the influenza virus-specific cells that are able to secrete IL-2 preferentially home to draining lymph nodes. However, they did not appear to localize to the site of infection (lung) or if they did, their production of IL-2 was down-regulated or not detectable.

The high density cultures for IL-2 secretion and lymphoproliferation responses of draining lymph node cells support previous observations made by Lyons et al. who reported that influenza virus-specific T cells which were able to proliferate to antigen in vitro specifically localized in the lung and hilar lymph nodes of guinea pigs infected intratracheally with influenza virus (169). In the current study, however, antigen specific cells were also detected in the non-draining lymph nodes, although the frequencies were 7-fold less than the draining nodes. The persistence of antigen within the draining lymph nodes might be responsible for the preferential accumulation of IL-2 secreting T cells in the draining lymph nodes. Astry et al. found that influenza viral antigen is present in the respiratory tract weeks after the resolution of infectious virus (52). In addition, Lipscomb found that antigen presenting cells derived from the lungs of guinea pigs infected with influenza virus were effective stimuli for in vitro proliferative responses for up to one week post infection (170). However, another explanation for the preferential accumulation of cells might be organ specific
recruitment of activated lymphocytes. It has been demonstrated that lymphocytes, particularly activated lymphocytes, will home to either their tissue of origin or lymphoid tissues of similar structure, i.e., peripheral lymph nodes versus mucosal tissue (160). This organ-specific recruitment of cells in addition to the retention of viral antigen may be significant in inducing the immunopathology following primary infection with influenza by continually activating recirculating T cells that home to the draining nodes. This continual activation might induce the exaggerated production of lymphokines which may contribute to immunopathology.

The dichotomy between the detection of IL-2-secreting T cells in non-draining lymph nodes in the limiting dilution cultures at day 7 p.i. and the undetectable secretion of lymphokines in the bulk culture experiments suggested that the microenvironments of the lymph nodes (draining and non-draining node) differed in their ability to support IL-2 secretion and further suggested that the accumulation of cells in lymphoid tissues does not necessitate lymphokine secretion. Firestein et al. found that in patients with rheumatoid arthritis, even though lymphocytes were present in the synovium and T cell activation markers, i.e. IL-2R and Ia, were expressed, there was no detectable lymphokine production, but rather the majority of cellular products were derived from monocytes (166). However, in similar studies by Burmester et al., the T cells expressing IL-2R within the synovium, were able to proliferate to in vitro stimulation with IL-2 (168). These data suggest that lymphokine production at sites of inflammation is regulated independently of the state of cell activation and responsiveness but, rather by the environment of the site of inflammation. These environmental differences might be due to alterations in the distribution of antigen-specific B cells or mononuclear cells acting as antigen presenting cells (170), the development of CD8+ suppressor cells, or the differential distribution of regulatory T cells that regulate the production of IL-2.
Under limiting dilution conditions, the production of IL-2 by cells from the draining lymph nodes demonstrated a transient requirement for several cells or factors at the peak of the response (Table 3). This was indicated by the rapid decline of % positive wells in the LDA. The scatter plot of the MED lymph node cells demonstrate a sharp slope. This rapid decline does not conform to the expected Poisson distribution of a single hit kinetics assumption. Because of this, the data indicate that, other, as yet undefined, factors are limiting. This multifactor requirement has been observed in *Trichinella spiralis* infections of rats (158).

The deviation from a Poisson distribution has been observed in studies of influenza virus-specific CTL (83). The factor that was limiting was determined to be IL-2. Supplementing these cultures with IL-2 or Th cells shifted the curves back to the Poisson distribution. In the current study, it appears that just as the CTL cells need additional factors from Th cells, so do IL-2-secreting cells. It is possible that IL-2 secretion requires another helper T cell elaborating lymphokines, which are initially restricted to the draining nodes. Studies investigating the requirements for in vitro induction of effector T cells that express DTH reactivity, have also found that Th cells are important (106). The current studies may reflect the differential distribution and secretion of IL-2 between Tdth and another IL-2-secreting Th cell. Mosmann et al. have postulated that CD4\(^+\) cells represent two distinct categories of functional T cells (120, 121). One, the T inflammatory cell which expresses IL-2 and IFN-gamma, and two, the Th2 cell which expresses IL-4 and IL-5. However, more recent work has discovered that Th1 cells can be further subdivided into non-inflammatory and inflammatory subsets (179). One subset may regulate the production of IL-2 by the other i.e., one may be a helper cell. The detection of one factor dependent cells in the non-draining nodes may be a reflection of the different distribution of Th1 cells that mediate DTH and Th1 cell that don't. Conversely, in the
draining nodes, both cells may be present. The combination of both of these cells restricted to the nodes might explain why high density cultures express IL-2 from the draining nodes while the high density cultures of non-draining nodes do not. During the peak of the immune response, the localization of Th cells required for the production of IL-2 may induce the immunopathology.

Investigation of priming requirements between the two Th cell subsets has revealed different requirements for antigen presentation (181, 138, 154, 180). The Th1, inflammatory cell, requires B-cells, macrophages, and dendritic cells along with an undefined, cell-associated factor for activation, whereas a Th2 cell, requires primarily macrophages and IL-1. The selective recruitment and activation of Th1 cells by B cells that are restricted to the draining nodes and perhaps lungs suggests that pathology may be induced by the recruitment of T cells by B cells. Janeway et al. (138, 181) found that peripheral lymph node cells from primed mice do not proliferate to antigenic stimuli if B cells were depleted either in vitro or in vivo. This response could not be reconstituted by lymph node macrophages. The antigen-specific response could be reinstated, however, by supplementing the B cell-depleted cells with either splenic adherent and or non-adherent macrophages. In the current study, it is possible that the lack of either proliferative response or IL-2 secretion in the non-draining lymph nodes at the peak of the anti-viral immune response, even in the presence of detectable frequencies of virus-specific cells, is due to the restricted localization of antigen-specific B cells to the draining lymph nodes. Following influenza virus infection, virus-specific B cells that are necessary for activation of resident cells might be retained in the respiratory mucosa at the peak of the response, or restricted in their recirculation. Studies on the distribution of IgA-secreting B cells of the gut mucosa demonstrated a preferential homing to mucosal tissues over that of peripheral lymph nodes (4, 122). Influenza virus specific B cells may be initially stimulated in the respiratory tract and therefore be restricted to sites draining
the respiratory mucosa. It is not until the infection has resolved that these cells are then able to recirculate to other tissues. This speculation may point to a role for B cell migration in the regulation of T cell-mediated immunopathology of respiratory tract viral infections.

Hamann et al., studying the role of activation on the expression of MEL-14, the reputed lymphocyte homing receptor (LHR), found that stimulation of lymphocytes with optimal concentrations of Con A reduced the expression of LHR (182). Studies with non-Hodgkins lymphoma cell lines (tumor cells believed to be frozen in specific stages of activation and or differentiation) have revealed a correlation between lymphoma dissemination and LHR expression (183, 184). It has been reported that lymphomas that disseminate to various lymph nodes have a high level of expression of LHR. If the LHR was removed or down-regulated, then dissemination might be expected to be restricted (183, 184). In the present study, draining lymph nodes may generate a subpopulation of regulatory mononuclear cells that express modified LHR which are specific for the draining lymph nodes and or SPL and therefore restrict their circulation patterns during the early course of the infection. The restricted recirculation of cells may cause repeated or continual activation of lymphokine-secreting cells with the tissues of virus replication. This continual activation may induce exaggerated cellular extravasation to lung or exaggerated proliferation of influenza virus-specific effector cells in the draining nodes and contribute to the pulmonary pathology.

As the immune response matures and differentiates to memory cells and returns back to the resting state, it is possible that the once modified or removed LHR are re-expressed. The result is the ability of cells to again enter the non-draining lymph nodes. This is supported by the detection of IL-2 secretion in high density cultures within the non-draining BRA and ING lymph nodes at day 15 p.i., the time the pathology has resolved and the full generation of memory cells is complete (Figure 4B and Figure 5B).
A second explanation for the inability of the non-draining lymph nodes to support IL-2 secretion in high density cultures may be the transient presence of suppressor cells in the recirculating pool of virus-specific cells. Suppressors generated in low frequencies in the draining lymph nodes might co-migrate with IL-2-secreting cells to non-draining lymph nodes and thereby block the production of IL-2 or proliferative responses at the peak of pathology. In oral tolerance studies (155, 156), it has been demonstrated that oral administration of either soluble antigens or alloantigens induced systemic suppression of both T and B cell responses. These studies indicate that suppressor cells activated at the site of antigen deposition are able to circulate to peripheral tissues. In the current study, the fact that no observable diminution of IL-2 or proliferative responses was detected in the draining nodes or SPL 7 days p.i. may be due to the relative proportions of suppressor cells to helper cells in the given lymphoid tissue.

An explanation for the production of IL-2 and accumulation of mononuclear cells in the non-draining lymph nodes following the resolution of infection may be the expression of vascular addressins on HEV of the non-draining lymph nodes. Immunogenic doses of antigen may enter either or both draining and non-draining nodes and subsequently induce the production of lymphokines which induce the expression of vascular addressins (136, 137). Yu et al. found that cell surface addressins expressed on HEV are induced by the lymphokine IFN-gamma and the cytokines IL-1 and TNF (136). These immunologic hormones may play a role in the extravasation of regulatory lymphocytes or APC from the circulation to the lymph nodes.

Work done by Streilein has determined that detectable viral antigen is present in the lung, hilar lymph nodes, and SPL (80). It is possible that antigen is carried to non-draining nodes following the resolution of infection by mononuclear cells leaving the lung parenchyma. This is supported by the 3-fold
increase in total mononuclear cells recovered on day 15 p.i. (Table 2). This influx of cells in the recirculating pool may represent monocytes or lymphocytes leaving the site of infection and therefore carrying antigen from the site to other tissues following infection. Furthermore the limiting dilution analysis in the non-draining nodes 40 days p.i. demonstrated a 2-3-fold increase in frequency of cells that did not fit the chi-square distribution. The deviation from the chi-square distribution indicated that factors or cells other than the IL-2-secreting cells were limiting in the non-draining nodes, an observation that was made in the draining nodes at day 7 p.i. This accumulation of T cells with the requirement for multiple factors for the production of IL-2 suggested that cells were being endogenously activated (like draining nodes at day 7 p.i.), perhaps by the recruitment of antigen-presenting cells carrying antigen from the lung parenchyma (Table 2).

An area of primary interest in the regulation of local immune responses is the origin of the activated cells entering the site of infection. It is not known whether the cells in a localized inflammatory response of non-lymphoid tissues are activated and clonally expanded at the site or whether the cells require the lymph nodes for their development. In the current study, lung cells in contrast to the draining lymph nodes did not express detectable IL-2 in high density bulk cultures at either 5 or 7 days p.i. The lack of IL-2 production in this tissue at a time when Tdth cells have been reported to be localized suggests that virus-specific cells do not proliferate in an IL-2-dependent manner at the site of viral replication, but requires the environment of the draining lymph node. Virus-specific cells may require initial activation at the site but for the full differentiation and clonal expansion of cells, such cells might need to be educated in the draining node. This has been reported in IgA-secreting B-Cells (143). These cells are activated in the Peyer's patches but do not become fully mature until they recirculate to the mesenteric nodes and spleen. In the current
study, virus specific IL-2-secreting T cells might undergo differentiation in the draining nodes. From the node, fully activated inflammatory cells, perhaps expressing IL-2R, may then recirculate to the lung.

The multi-factor dependent production of IL-2 within the draining nodes supports the possibility of the state of activation of the cell. Two populations of cells differing in the expression of IL-2R may be present following activation. A subset of T cells, either T helper cells or CD8+ cytotoxic cells may express high levels of IL-2R whereas the other population of T cells may predominately secrete IL-2. In effect one cell population may act as an IL-2 sponge by consuming IL-2 and it is not until the population of IL-2-secreting cells become limiting that IL-2 is rapidly adsorbed (185). The lack of detectable IL-2 in the lung at either day 5 or day 7 may be a reflection of rapid IL-2 consumption by IL-2R-expressing T cells that preferentially localized to the lung (186). In the current study, the lack of IL-2 in the lung, might be due to this rapid consumption which may then preferentially activate the production of IFN-gamma at the site thereby induce the inflammatory extravasation of cells while adsorbing IL-2 (7, 8, 187). Functional characterization of mononuclear cells derived from normal human lung and peripheral blood have shown an inverse relationship in their ability to express either IL-2 or IFN-gamma (157). It was demonstrated that the blood contained a proportionally elevated number of IL-2-secreting cells over IFN-gamma whereas the lung demonstrated a proportionally elevated number of cells expressing IFN-gamma over that of IL-2-secreting cells. It has been demonstrated using T cell clones that mRNA for IFN-gamma is activated by IL-2 interacting with IL-2R (7, 8, 187). These data suggest that at the site of viral replication, the lung selects for one lymphokine activity over the other.

A third possibility is the presence of IL-2 suppressors that are specifically located within the lung parenchyma during the primary response. It has been
documented in several animal species including mice, rats and canines, that alveolar macrophages are poor activators of \textit{in vitro} lymphoproliferative responses (181). This has been attributed to the production of soluble suppressor factors. These same factors may be acting \textit{in vivo} to prevent the clonal expansion of cells at the site. Whether this effect also prevents activation of cells and the elaboration of lymphokines is not known, however, studies in the guinea pig, which do not contain such suppressor activities are able to induce LPR \textit{in vitro}. This suggests that alveolar macrophages are capable of presenting antigen and therefore activating cells (169, 188).

These data demonstrate a distinct anatomical localization and compartmentalization of influenza virus-specific T cells that secrete IL-2. It appears that helper T cells recirculate at the peak of the response but require the environment of the draining lymph nodes for the production of IL-2. At the site of viral replication there appears to be no detectable IL-2 production, thus suggesting that clonal expansion and differentiation of CD4\(^+\) T cells does not occur at the inflammatory site, but rather is restricted to the environment of draining lymph nodes. This suggestion is also supported by the recent work of Allan et al. who found activated CD8\(^+\) T cells but not CD4\(^+\) cells were essential for clearance of influenza virus from the lung (159).

The data presented in this chapter suggest that the immunopathology observed following primary infection correlates with the preferential and differential accumulation of IL-2-secreting T cells in lymph nodes draining the site of viral replication. The localization of lymphokine-secreting cells requiring multiple factors in the draining nodes at the peak of pathology suggests that IL-2-secreting cells may be associated with immunopathology.
CHAPTER II

LYMPHOKINE PRODUCTION IN DRAINING LYMPH NODES FOLLOWING REINFECTION WITH INFLUENZA

INTRODUCTION

Chronic or multiple re-exposure of primed hosts to a priming antigen can attenuate instead of accentuate immune responsiveness. Chronic infection with *Schistosoma japonicum* or the reactivation of Herpes simplex virus (HSV) have been reported to induce the expression of soluble factors by lymphocytes that both depress DTH and lymphoproliferative responses. Horohov et al. (189) and Sheridan et al. (190) both reported diminished anti-HSV responses due to the expression of soluble lymphocyte products. Gao et al. (191) reported the partial characterization of lymphocyte products that suppressed *in vitro* DTH responses following infection with trypanosomes.

Recently, Beck et al. (104, 19) found that reinfection of seropositive mice with the homologous influenza strain A virus induced spleen and lung mononuclear cells to secrete low molecular mass factors that interfered with the biological expression of lymphokines. Following reinfection, a transient depression of DTH responses, depressed lymphoproliferation of splenocytes, and a diminished cellular infiltrate in the lung was observed. It was postulated that reinfection induces immunological mechanisms that maintain a balance between viral clearance and immunopathology by blocking the biological expression of
lymphokines at sites of viral replication.

Influenza virus infects the columnar epithelium of the upper respiratory tract and the alveolar cells in the lower respiratory tract in the mouse (68, 50-53). The structure and function of the two regions are distinct. The upper respiratory tract epithelium functions primarily as a protective barrier against foreign agents. This is reflected by the ciliated epithelial cells, which function to expel particulate antigens by the motion of the cilia away from the lower respiratory tract, and by the keratinization of the oral mucosa of the mouse (192). In contrast, the lower airways are composed of single cells of alveolar epithelium which function primarily in gas exchange between the host and the environment. This function is dependent on the architecture of the alveoli.

The tissues between the upper and lower respiratory tract also differ immunologically. The lung possesses lymphoid aggregates of the BALT which are interspersed within the lung (145, 146). Studies on the phenotype and distribution of mononuclear cells along the upper respiratory and lower respiratory tract in rats demonstrated that two phenotypically-distinct dendritic cells (DC) were differentially distributed between the upper and lower airways (192). The cells of the trachea stained with the rat DC marker ED-1, however, the DC of the alveolar septa stained with another DC marker, ED-2. The regions of the respiratory tract are also distinct in that lower airways receives circulating serum antibodies through the transudation of blood born antibodies which upon infection are able to afford protection against fatal pneumonia whereas under the same conditions the upper respiratory tract generates tracheitis.

Inflammatory responses in connective tissues of the skin are characterized by cellular infiltrates, pain, and localized tissue damage, however, because of the function of skin the intense influx of cells rarely causes fatal outcomes. However, in mucosal tissues where the tissue functions to exchange environmental agents between the hosts internal milieu and the environment,
such inflammatory responses can cause severe outcomes and increased mortality (1, 18). This is demonstrated in influenza viral infection of the lung. The inflammatory response in this site disorganizes the architecture dependent function therefore impeding exchange of gases. The intensity of this inflammatory response can increase mortality (18).

The differences in both the consequences of inflammatory responses and structure of the various regions of the respiratory tract suggests that immune responses may be regulated differently between nodes draining different regions of the tract. In this chapter, reinfection of seropositive mice was used to ask if the production of lymphokines among nodes draining structurally distinct sites are regulated differently following reinfection? And, whether the distribution patterns and frequency of virus-specific cells are altered following reinfection? And finally, to determine if CD4+ T cells which secrete factors which interfere with lymphokine production are distributed to lymph nodes.

To determine the distribution of influenza virus-specific cells and the production of IL-2, the draining lymph node cells (SCV and MED) and the non-draining lymph nodes (AXIL, BRA, ING) were assayed for IL-2 production in high density bulk cultures (SCV and MED) and limiting dilution analysis (SCV, MED, BRA, ING, AXIL) at 40 days post primary infection and 3 days post reinfection of seropositive mice.

The data demonstrated that IL-2 secretion in lymph nodes draining the upper respiratory tract (SCV) were increased following reinfection whereas the nodes draining the lung parenchyma demonstrated depressed IL-2 activity. This depressed IL-2 was not due to the lack of inflammatory responses or IL-2-secreting cells but rather to active suppressive mechanisms located preferentially in the MED lymph nodes. Collectively, these data indicate that reinfection induces mechanisms for regulating lymphokine production preferentially in lymph nodes that specifically communicate with the lung parenchyma.
METHODS AND MATERIALS

Mice.
As for Chapter 1

Virus.
As for Chapter 1

Infection of Mice: Seropositive mice 30 days post primary infection were lightly
anesthetized as described in Chapter 1. Each mouse was reinjected with 0.05 ml
of a total of 32 HAU of infectious influenza virus A/PR8 as described in
Chapter 1. Three days post reinfection, mice were sacrificed by cervical
dislocation followed by removal of draining and non-draining lymph nodes (see
Chapter 1).

Cell Suspensions:
As for Chapter 1

Cell Culture.
As for Chapter 1

Generation of Culture Supernatants: IL-2 was detected from bulk culture
supernatants of spleen cells. Single cell suspensions of spleens from mice 30 days
post primary infection or 3 days post reinfection were prepared by macerating
splenic explants through a fine mesh wire screen with a 10 ml syringe plunger. Single cell suspensions were washed 3 X at 100 g for 10 mins in 5 ml of complete DMEM with 4% heat inactivated FBS (Gibco). Single cells suspensions of $2 \times 10^6$ cells/ml in 1 ml volumes were cultured with either 13 HAU/ml of PR8 antigen or media alone for 72 hours at 37°C in 10% CO2. Supernatants were cleared of cells and debris by centrifugation at 100 x g for 10 min.

**IL-2 Assay:**

As for Chapter 1

**Limiting dilution analysis:**

As for Chapter 1
RESULTS

Distribution of IL-2-Secreting T cells in Lymph Nodes Draining the Respiratory Tract Following Reinfection.

The secretion of the lymphokine, IL-2 by T lymphocytes was examined in the draining (SCV and MED), non-draining (BRA, ING, MES) lymph nodes and SPL. SPL cells from mice reinfected with influenza virus PR8 demonstrated a decreased IL-2 response compared to seropositive memory mice (Figure 17). The response was diminished from 50 to 70 percent of the memory values. This depression in IL-2 was independent of time in culture; IL-2 was decreased from day 1 to day 3 in culture. These data indicate that either there was an active suppression of the IL-2 response in the SPL, or that following reinfection the frequency of antigen-reactive cells was diminished.

As previously shown in the primary response, 30 days p.i. infection, both the draining and non-draining nodes exhibited IL-2 production in high density bulk cultures and demonstrated frequencies of antigen-specific cells of 1/10,000 to 1/50,000 respectively (Chapter 1, Tables 1 and 2). Reinfection of seropositive mice demonstrated the same distribution of IL-2 secreting cells in high density bulk cultures, however, the magnitude of the response was either increased or decreased depending on the site which the respective lymph nodes drained (Figures 18 and 19). SCV lymph node cells, which drain the upper respiratory tract and oropharynx were cultured with influenza for 24, 48, and 72 hours. Cells from primed mice showed IL-2 secretion with net incorporation of 30,000 cpm at 24 hours post culture (Figure 18). In contrast, cells from reinfected hosts
demonstrated significantly higher IL-2 secretion than the cells from primed mice with net incorporation of 52,000 cpm, a 30% increase. In experiment two, the same pattern was observed. Cells from primed mice gave IL-2 responses of 20,000 cpm while cultures from reinfected mice had significantly higher responses at 42,000 cpm, a 50% increase. In both experiments, there were no significant differences between cells from primary infected mice or cells from reinfected mice at either 48 or 72 hours post culture.

The MED, unlike the SCV nodes, receive lymph directly from the lung. Within this lymph are immigrant cells and cells from the specialized mucosa associated lymphoid follicles in the lung, the BALT (144, 145). In contrast to the SCV, IL-2 secretion in high density cultures of cells from the MED 3 days post reinfection demonstrated depressed IL-2 levels compared to primed mice (Figure 19). At 24 hours post culture, experiment 1 showed a significant depression of IL-2 responses with net incorporation of 30,000 cpm while cells from primed mice demonstrated net incorporation of 45,000 cpm, a 33% change. At 48 hours post culture, cells from primed mice demonstrated net incorporation of 50,000 cpm while reinfected demonstrated incorporations of only 10,000 cpm, a 60% depression. Experiment 2 demonstrated similar depression of IL-2, however, the suppression was not significant until 48 hours post culture. At 24 hours, both the cultures from primed mice and reinfected mice demonstrated net incorporation of 30,000 cpm. However, at 48 hours, the cultures from primed mice demonstrated net incorporation of 50,000 cpm while the cells from reinfected mice demonstrated 20,000 cpm, a 50% change. Though the two experiments were variable, a pattern of IL-2 suppression was seen by 48 hours post culture. The variability of this response may be more of a reflection of the variability of the dose of virus that is reaching the lung. In experiment 1, it was observed that the total increase in cell yields was 2-fold over the memory animals (data not shown). In this experiment suppression of IL-2 was found at both 24 and 48 hours. In
Figure 17. IL-2 response of spleen cells from influenza virus seropositive mice reinfected with influenza virus strain A/Puerto Rico/8/34 (PR8). Single cell suspensions of spleen cells derived from either I.P. primed C57BL/6 or I.P. primed mice reinfected with influenza virus PR8 were cultured in 5 ml culture tubes at a concentration of $2 \times 10^6$ cells/ml in a total of 1 ml. Each set of replicate tubes was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen for 24, 48, or 72 hours at 37°C in 10% CO2. Following culture, supernatants were harvested and assayed for IL-2 by the IL-2 dependent cell line CTLL-20. CTLL-20 cells at $1 \times 10^4$ cells/well were cultured with a 1/2 dilution of culture supernatants in a 96 round bottom microtiter plate. Cultures were then incubated for 24 hours with a terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/- one standard deviation. Levels of significance were determined by Student’s t test. Asterisk represents differences between cells from primary and reinfected hosts at p<0.05.
Figure 17.
Figure 18. IL-2 response by cells from superficial cervical nodes 3 days post reinfection with influenza virus strain A/Puerto Rico/8/34 (PR8). Cohorts of 7-15 male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Thirty days post primary infection mice were reinfected with 32 HAU of virus. Three days post reinfection mice were sacrificed and lymph nodes surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained 2 x 10^6 cells in a total volume of 0.150 ml. Each set of replicates were either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 24 and 48 hours at 37°C in 10% CO₂. Following culture, plates were irradiated with 2,000 rads of gamma rays and overlaid with 1 x 10^4 CTLL-20 cells. Cultures were additionally incubated for 24 hours with a terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/- one standard deviation. Levels of significance were determined by Student's t test. Asterisk represents differences between IL-2 responses from cells of primary and reinfected hosts at p<0.05.
Figure 18.
Figure 19. IL-2 response by cells from mediastinal lymph nodes 3 days post reinfection with influenza virus strain A/Puerto Rico/8/34 (PR8). Cohorts of 7-15 male C57BL/6 mice 4-6 weeks of age were infected intranasally with 32 HAU of influenza PR8. Thirty days post primary infection mice were reinfected with 32 HAU of virus. Three days post reinfection mice were sacrificed and lymph nodes surgically removed. Single cell suspensions were prepared from the indicated nodes and cultured in 96 well round bottom microtiter plates in replicates of four. Each well contained 2 x 10^5 cells in a total volume of 0.150 ml. Each set of replicates was either non-stimulated or stimulated with 13 HAU/ml of PR8 antigen. Cultures were incubated for 24 and 48 hours at 37°C in 10% CO₂. Following culture, plates were irradiated with 2,000 rads of gamma rays and overlaid with 1 x 10⁴ CTLL-20 cells. Cultures were additionally incubated for 24 hours with a terminal 8 hour pulse of 0.8uCi/well of tritiated-thymidine. Values represent mean cpm +/- one standard deviation. Levels of significance were determined by Student's t test. Asterisk represents differences between cells from primary and reinfected hosts at p<0.05.
Figure 19.
experiment 2, suppression was not seen until 48 hours post culture. The
difference between experiment 1 and 2 might be related to the total increase in
mononuclear cells. Where experiment 1 demonstrated a 2-fold increase in cells,
extperiment 2 demonstrated an almost 7-fold increase. This extensive
inflammatory response in experiment 2 might have caused such a vigorous
activation and recruitment of cells that there was a proportional increase of IL-2
cells over the suppressive mechanisms.

**The Frequency Distribution of IL-2 Secreting Cells Following Reinfection.**

The depression of the IL-2 response in the MED and SPL might have been a
consequence of the redistribution of antigen-specific T cells to other sites in the
host, i.e. the circulatory system or the lung (Chapter 1, Table 2). To test whether
the depressed IL-2 response was due to a change in frequency in the lymph nodes,
limiting dilution analysis was performed and the total number of mononuclear
cells determined following reinfection of memory animals.

Frequency analysis of the SCV and MED nodes at days 40 p.i. infection fit
the expected Poisson distribution, thus indicating that the responder cell
population was the only limiting factor (Figure 20A and Figure 20C). Scatter
plots of the analysis of primary SCV node cells at 50,000 cells/well demonstrated
a distribution of values ranging from 10,000 cpm to 4,000 cpm with an
accumulation at 60,000 cpm. At a concentration of 25,000 cells/well an even
distributions of 5,000 cpm to 3,500 cpm was apparent with all values above the
cutoff line. Values below the cutoff was first detected at 6,250 cells/well with
12% negative wells.

Cells from the SCV from reinfeected mice, however, demonstrated increased
values for each responding well concentration compared to day 40 p.i. cultures
with distribution of 11,000 to 8,000 cpm (Figure 20A and Figure 20B). The
distribution range remained consistent through responder cell concentrations of
25,000 cells/well. It was not until responder cell concentration of 12,500 cells/well that the distribution increased, ranging from 10,000 to 4,000 cpm with a tight accumulation at 8,000 cpm. Negative wells were not detected until 3,125 cells/well.

Scatter plots of the analysis of the primary MED node cells (day 40 p.i) at the lowest responder cell dilution, 50,000 cells/well, had a distribution of values similar to the SCV (day 40 p.i.) ranging from 7,000 cpm to 2,000 cpm with an accumulation at 3,500 cpm, all above the cutoff point (Figure 20C). At 25,000 cells/well the distribution of responding wells ranged from 6,000 to 1,000 cpm. Negative wells did not occur until the cell concentration dropped to, or below, 12,500 cells/well. Analysis of cells from reinfected mice (3 days post reinfection) again fit the Poisson distribution, however, the magnitude and distribution of the values in each responding well were increased as might be expected in secondary immune response (Figure 20D). At 50,000 cells/well, the distribution of responding wells demonstrated a narrower range than the day 40 study, ranging from 8,000 to 5,500 cpm with an accumulation at 7,000 cpm. The distribution range, however, increased at responder concentrations of 25,000 cells/well, ranging from 7,500 cpm to 3,000 cpm (like day 40 cultures) with an accumulation at 6,800 cpm. Negative wells were not detected until 6,250 cells/well unlike the cells from the primed mice where negative wells were first detected at 12,500 cells/well.

The scatter plot of the ING lymph node 40 days p.i. demonstrated 100% positive wells at the highest concentration of responder cells, 50,000 cells/well. At this concentration, the values were evenly distributed with incorporation ranging from 1,500 cpm to 3,500 cpm. The percentage of positive wells rapidly decreased to 20% at 25,000 cells/well, thus indicating that as yet undefined factors were limiting other than the responder cell population. In contrast, the scatter plot of the ING from reinfected mice demonstrated a gradual decrease in
Figure 20. Scatter plots of the frequency of virus-specific IL-2-secreting T cells from lymphoid tissues 40 days p.i. (A, C, and E) and 3 days post reinfection (B, D, and F) with influenza virus strain A/Puerto Rico/8/34 (PR8). Cohorts of 15 male C57BL/6 mice were infected intranasally with 32 HAU of infectious virus. Forty days post infection and 3 days post reinfection, mice were sacrificed and lymphoid tissues surgically removed. Single cell suspensions of superficial cervical (SCV) (A and B), mediastinal (MED) (C and D), and inguinal (ING) (E and F) were prepared and two-fold serial dilutions made. Frequency of cells was determined by a limiting dilution analysis. Briefly, $2.5 \times 10^4$ to $2 \times 10^5$ responder cells from the indicated tissues were placed in each well in replicates of 24 containing $2 \times 10^3$ PR8 antigen pulsed irradiated normal spleen cells (B and D) or non-pulsed stimulator (A and C). Cells were cultured for 30 hours and irradiated with 2,000 rads of gamma rays. CTLL-20 cells were then added at $1 \times 10^3$ and cultured an additional 24 hours with a terminal 12 hour pulse. Values represent CTLL-20 proliferation for each well. Non-stimulated cultures contained replicates of 12. PR8 antigen stimulated cultures represent 24 replicated. Cutoff points were determined by replicates of 12 that contained PR8 antigen pulsed normal splenocytes only. Cutoff line was calculated by the mean incorporation plus 3 standard deviations. Primary infection and reinfection assays demonstrated cutoffs of 867 cpm and 1440 cpm, respectively.
Figure 20A.
Figure 20B.
Figure 20C.
Figure 20F.
percent positive wells as the cell concentration decreased. At 50,000 cells/well, 50% of the wells were positive with incorporation ranging from 2,500 to 900 cpm. These scatter plots suggested that reinfection reduced the frequency of cells present in the ING nodes. In addition, the change in the slope of the distribution of incorporation of wells indicated that the multi-factor dependent cell population was preferentially removed following reinfection.

These results are depicted graphically by the line plots (Figure 21). Both the SCV and MED lines rotate towards the y axis following reinfection, however, the SCV advances towards the Y-axis to a greater extent than does the MED. In contrast to the draining nodes, the non-draining nodes and SPL rotates toward the x axis. The shift in the slope of these lines indicates changes in the frequencies of IL-2 secreting T cells. It appears that cells leave the recirculating pool while accumulating in the draining nodes, and that both SCV and MED accumulate cells following reinfection.

Using chi-square minimization analysis, the 95% confidence intervals and chi-square probabilities were determined. The calculated frequencies of cells in the SCV increased 1/9,000 to 1/800 following reinfection, a 7-8 fold change whereas the MED frequencies increased from 1/9,000 to 1/4500, a 1.5-2 fold above that of the primed animals (Figure 21 and Table 4). Both the SCV and MED nodes demonstrated a 2-3 fold increase in total mononuclear cells recovered indicating local inflammatory responses were induced by antigenic stimulation. Concomitant with these changes in the draining lymph nodes were small changes in the SPL frequency changing from 1/15,000 to 1/20,000. The non-draining nodes demonstrated a 2-3 fold decrease following reinfection. The BRA changed from 1/40,000 to 1/120,000 with chi-square probabilities changing from 0.356 to 0.661. The ING and MES also demonstrated similar changes however unlike the BRA, the day 40 chi-square probabilities deviated from the one hit kinetics assumption with values of 0.002. Following reinfection, the chi-square
Figure 21. Frequency of virus-specific IL-2-secreting cell from lymphoid tissues
(A) 40 days post primary infection and (B) 3 days post reinfection with influenza
virus strain A/Puerto Rico/8/34 (PR8). Cohorts of 15 male C57BL/6 mice were
infected intranasally with 32 HAU of infectious virus. Three days post
reinfection, mice were sacrificed and lymphoid tissues surgically removed.
Single cell suspensions of lymphoid tissue were prepared and two-fold serial
dilutions made. Frequency of cells was determined by a limiting dilution
analysis. Briefly, 25,000 to 195 responder cells from the indicated tissues were
placed in each well in replicates of 24 containing 2 x 10^5 virus pulsed irradiated
normal spleen cells or non-pulsed stimulator. Cells were cultured for 30 hours
and irradiated with 2,000 rads of gamma rays. CTLL-20 cells were then added at
1 x 10^3 cells/well and cultured an additional 24 hours with a terminal 12 hour
pulse. Values represent CTLL-20 proliferation for each well. Non-stimulated
cultures contained replicates of 12. Virus stimulated cultures represent 24
replicates. Cutoff points were determined by replicates of 12 that contained
virus pulsed normal splenocytes only. X-axis represents total number of
responder cells/well. Y-axis represents % negative wells. Linear regression lines
were determined by chi-square minimization analysis. Data are from
experiments presented in Figure 20.
Figure 21A.
Figure 21B.
Distribution and the Frequency of IL-2-Secreting T Cells Following Reinfection of Influenza Virus Seropositive Mice with Influenza Virus.

<table>
<thead>
<tr>
<th>Region</th>
<th>Time Post Infection</th>
<th>C. I. (1/f)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAY 40 (Primary)</td>
<td>DAY 3 (Reinfection)</td>
</tr>
<tr>
<td></td>
<td>SCV</td>
<td>6,094-11,343(^b)</td>
</tr>
<tr>
<td></td>
<td>MED</td>
<td>5,959-11,314 (0.346)</td>
</tr>
<tr>
<td></td>
<td>BRA</td>
<td>28,476-55,439 (0.356)</td>
</tr>
<tr>
<td></td>
<td>ING</td>
<td>28,020-55,185 (0.004)</td>
</tr>
<tr>
<td></td>
<td>SPL</td>
<td>10,488-19,317 (0.480)</td>
</tr>
</tbody>
</table>

a. Cohorts of 15 male C57BL/6 mice 4-6 weeks of age were either infected or reinfected intranasally with 32 HAU of influenza PR8. At the indicated times post infection and reinfection, two fold serial dilutions of lymph node and spleen responder cell suspensions were cultured with 2 x 10^5 irradiated virus pulsed normal splenocytes in 96 well round-bottom microtiter plates. Dilutions in replicates of 24 ranged from 5 x 10^2 responder cells/well to 3 x 10^2 cells/well. IL-2 secretion was determined 24 hours post culture for each well by the overlay of CTLL-20 cells as described in methods. Each well was scored positive 3 standard deviations above cultures containing only PR8 antigen pulsed stimulators. Values were derived from experiments presented in Figures 20 and 21. Ninety-five percent confidence intervals for MES frequency determinations were 1/39,985-1/79,914 forty days p.i. with a p (x^2) of 0.002.

b. Values represent 95% confidence intervals of the reciprocal of the frequency as determined by chi-square minimization.

c. Chi-square probability. P> 0.05 were considered to fit a Poisson distribution.

d. Frequency determinations using complete series of responder cell concentrations above the limit of chi-square minimization analysis. Values represent frequency determinations using four dilution series ranging from 3,125 to 390 responder cells/well.
<table>
<thead>
<tr>
<th>Region</th>
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<th>Day 30 (Primary)</th>
<th>Day 3 (Reinfection)</th>
<th>Reinfection/Primary</th>
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</thead>
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<tr>
<td>SCV</td>
<td>1</td>
<td>2.50</td>
<td>3.75</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.70</td>
<td>7.50</td>
<td>2.78</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.67</td>
<td>7.07</td>
<td>2.65</td>
</tr>
<tr>
<td>DCV</td>
<td>1</td>
<td>0.33</td>
<td>0.67</td>
<td>2.03</td>
</tr>
<tr>
<td>MED</td>
<td>1</td>
<td>0.50</td>
<td>3.38</td>
<td>6.76</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.57</td>
<td>2.86</td>
<td>1.82</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.00</td>
<td>2.25</td>
<td>2.25</td>
</tr>
<tr>
<td>AXIL</td>
<td>1</td>
<td>1.70</td>
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</tr>
<tr>
<td>BRA</td>
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<td>1.13</td>
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<td>0.44</td>
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<tr>
<td></td>
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a. Regional lymphoid tissue pooled from a total of 10-15 mice 30 days post primary infection and 3 days post reinfection were counted in crystal violet. Total number of mononuclear cells/region/mouse was calculated by counting total mononuclear cells from each region and dividing the total by the number of mice used in each experiment. Values represent 10⁶ cells/region from 3 experiments. Data represent values from paired groups of 5-15 mice.

b. Ratio of cell yields from day 3 post reinfection and day 30 post primary infection.
probabilities attained values of 0.661 again fitting the one hit kinetics assumption.

**Distribution of Mononuclear Cells Following Reinfection.**

The determination of frequencies of cells as defined by a single parameter, i.e. IL-2 secretion, only defines the relative number of cells within the non-responding cell population. An observed change in frequency can occur either by direct changes in the cells being examined, i.e., IL-2 secretors, or by changes in the non-responder population. For example, an increase in cell frequency may be due to the removal of the non-responding population and not an increase in the responder population. Conversely, a decrease in frequencies may be a reflection of increased frequencies of the non-responder population. To determine which event was occurring following reinfection, the total number of mononuclear cells was determined from each region following reinfection (Table 5).

The total number of mononuclear cells recovered from both the SCV lymph nodes and MED increased 2-3 fold following reinfection. The SCV lymph nodes at 30 days post infection yielded $2.5 \times 10^6$ cells/mouse. In contrast, the SCV nodes yielded $4.7 \times 10^6$ cells/mouse following reinfection, a 2.5 fold increase. The MED at 30 days post infection, yielded $3.5 \times 10^5$ cells/mouse. Reinfection, however, induced a 2-6 fold increase with cell yields ranging from $1.25-3 \times 10^6$ cells/mouse. The DCV showed $3.30 \times 10^5$ cells at day 40 p.i. and increased 2-fold to $6.7 \times 10^5$ following reinfection.

In contrast to the draining nodes, the total mononuclear cell yields from the non-draining nodes of reinfected mice demonstrated decreased cell yields. The AXIL node cell yields dropped from $1.7 \times 10^6$ to $8 \times 10^5$ following reinfection, a 0.40 decrease. In addition, the BRA node cell yields dropped from $2 \times 10^6$ to $9 \times 10^5$ following reinfection, again a 0.40 decrease. The ING similarly changed 1.1-0.40 fold following reinfection with numbers changing from $1.253 \times 10^6$ (day 40
p.i.) to $1.93 - 0.5 \times 10^6$ cells following reinfection.

These results indicate that both the SCV and MED nodes recruit mononuclear cells from the recirculating pool of cells (from the BRA and ING) following reinfection. The relatively smaller increase of frequency of IL-2-secreting cells in the MED node following reinfection combined with an increase in total cell yield suggests that there is either an expansion or recruitment of non-IL-2 producers in the draining node. In contrast, the comparatively increased frequencies of cells in the SCV with the increase in cell yields suggests that, unlike the MED, IL-2 producers are specifically expanded or recruited to this site over non-producers.
DISCUSSION

In Chapter 1, it was demonstrated that influenza virus-specific T lymphocytes that secrete IL-2 preferentially accumulated in the nodes draining the sites of viral replication and, to a lesser extent, in the non-draining nodes (Chapter 1, Table 3). However, at the peak of the immune response, IL-2 secretion was observed only in the draining nodes where, as yet undefined limiting factors for the production of IL-2 were located (Chapter 1, Tables 1 and 3). The mononuclear cells recovered from the lung did not demonstrate IL-2 secretion (Chapter 1, Figures 9 and 10). These data suggested that the cells mediating immunopathology may be induced within these nodes and, where upon differentiation, enter the circulation that then transports the cells back to the lung. This localization of both lymphokine-secreting cells and the factors necessary for the production of IL-2 within the node parenchyma suggested that the draining nodes might play a primary role in developing immunopathology following primary infection. Under conditions of reinfection, however, the immunopathology is reduced, as are the lymphoproliferation and DTH responses (104). This suggested that unique mechanisms might regulate lymphokine production and the development of immunopathology following reinfection. Depressed lymphocyte functions following reinfection may be regulated by one of two mechanisms, an active suppressive mechanism such as those induced by suppressor factors, and/or the removal of antigen specific lymphocytes from the site. Studies by Beck et al. found that CD4+ T cells from the spleen and lung mononuclear cells of reinfected mice, expressed small molecular mass factors that
modulated the production of lymphokines (19, 104). These studies did not examine the distribution of the CD4+ T cells in lymph nodes, where during the primary response, cells elaborating lymphokines are localized. Where the regulation of lymphokines may develop following reinfection and how these may function in regulating immunopathology is still unclear. In this chapter, the secretion of IL-2 and the frequency of IL-2-secreting T cells among the draining and non-draining nodes were studied to address the questions; does reinfection change conditions within the lymph nodes draining the lung parenchyma for the secretion of IL-2? And do CD4+ IL-2-secreting T cells traffick to the draining nodes following reinfection?

Three major observations were made. First, mononuclear cells and antigen-specific T cells were recruited away from the non-draining lymph nodes following reinfection. Second, IL-2 production was depressed specifically in the lymph node draining the lung (MED), whereas IL-2 was enhanced in the lymph nodes draining the upper respiratory tract (SCV). Third, the frequency of IL-2-secreting cells was increased 7-fold in the SCV whereas the MED increased only 1.5-2-fold.

These observations suggested that within the lymph nodes draining the respiratory tract, the nodes draining the lung parenchyma preferentially develop mechanisms that inhibits the activation or accumulation of IL-2-secreting cells following reinfection, and that CD4+ T cells that modulate the production of lymphokines might specifically develop in these nodes.

The depressed secretion of IL-2 by cells from the MED nodes supported this possibility (Figure 19). However, a number of possible mechanisms exist that may modulate IL-2 secretion in the MED. First, cells may traffick away from the MED nodes. Second, non-IL-2-expressing cells may be preferentially activated and clonally expanded over IL-2-secreting T cells. Third, IL-2R expression may be enhanced thereby increasing the consumption rate of IL-2, and fourth, the
CD4+ lymphocytes that secrete low molecular factors which interfere with lymphokine production might preferentially locate in MED following reinfection.

Beck and Sheridan previously reported that reinfection induced a transient depression of DTH responses in footpads challenged with virus and that lymphoproliferative responses in the spleen were decreased (104). In the current study, it was observed that mononuclear cells and virus-specific cells that secrete IL-2 were recruited from the recirculating pool of lymphocytes to the draining nodes (Tables 4 and 5). The depressed DTH responses under the same conditions might be a reflection of the preferential recruitment of cells away from the site of challenge thus inhibiting the extravasation of DTH cells into the footpad and nodes draining the foot. This may also explain the depressed lymphoproliferative responses (104) and the depressed IL-2 secretion in the spleen (Figure 17), though the present study demonstrated only slight decreases in the frequency of cells following reinfection (Table 4). These data suggest that the redistribution of cells may be the primary mechanism of immune regulation in distant tissues following reinfection.

At the site of infection and in the spleen, however, suppressor cells may be the predominant mechanism for regulating lymphokine secretion and proliferation. CD4+ T cells from the spleen express both large and small molecular mass factors that inhibit mitogen-induced lymphoproliferation and the biological expression of lymphokines, respectively (19, 104). It is possible that the CD4+ T cells elaborating suppressor factors preferentially home to sites where cells accumulate, i.e. draining lymph nodes and lung, in order to down-regulate immune responses where lymphokine-secreting cells accumulate and thereby inhibit immunopathology.

To test the possibility that active suppression of IL-2 was occurring at sites preferentially draining the lung parenchyma, lymph node cells were assayed under limiting dilution analysis. Advantages of this assay are its ability
enumerate the cells contributing to IL-2 secretion and its ability to detect responsive cells in a population of cells that may contain regulatory suppressor cells.

Following reinfection, the frequency of cells from the MED changed from 6,000-11,000 (day 40 p.i.) to 3,234-6,211 (day 3 post reinfection), a 1.5-2-fold increase. In contrast, the SCV lymph nodes demonstrated a frequency change from 5,967-11,251 (day 40 post infection) to 783-1462 following reinfection, a 10-fold increase. The differences in the frequencies between the two nodes might be explained by differences between the upper and lower respiratory tract to support viral replication or to clear virus by antibody-dependent mechanisms. However, in the present study, the total number of mononuclear cells recovered from both the SCV and MED lymph nodes increased by 2-6-fold following reinfection (Table 5). These data indicated that sufficient antigen for the activation of local inflammatory responses were reaching both the SCV and MED nodes and that antigen-specific cells were present in both of these sites. These data collectively precluded the elimination of virus as the sole cause for the frequency differences between the two nodes but rather suggested the presence of different environmental conditions between lymph nodes that drain the lung and lymph nodes draining the other regions of the respiratory tract. Furthermore, these data indicated that both the SCV and MED nodes possessed the same number or more antigen-specific T cells following reinfection and that the depletion of antigen-reactive cells in the draining MED was not the cause of the depressed IL-2 response.

The MED, like the MES, receives lymphocytes from both the extravasation of cells from the circulation into the lymph node parenchyma by HEV and, based on indirect evidence, from mucosa associated lymphoid follicles found dispersed throughout the lung parenchyma (130, 131, 142-146). In contrast, peripheral lymph nodes receive cells from tissues that have no specialized lymphoid
follciles, but rather receive cells that have entered the surrounding tissues. The association of the SCV node with lymphoid follicles lining the oropharynx remains obscure. It is possible that the depressed IL-2 within the MED is a reflection of cells that transit through the lung prior to their entrance into the MED node. Recirculating cells that transit through the lung may be selectively retained in the lung parenchyma or "educated" by the mucosa associated lymphoid follicles which then regulate lymphocyte function in the draining node. This is consistent with the postulates made by Kees et al. who studied the phenotype and frequencies of lymphokine-expressing cells between lung mononuclear cells and peripheral blood in normal human subjects (157). This group found that compared to the peripheral blood T lymphocytes the lymphocytes within the lung demonstrated lower frequencies of cells able to express IL-2 than frequencies of IFN-gamma-producing cells. In contrast, the peripheral blood demonstrated an inverse relationship to the lung. Frequencies of IL-2-secreting cells were higher than IFN-gamma producing cells. It is possible that cells (or their precursors) that regulate the production of lymphokines such as those cells reported by Beck (19), reside in the respiratory mucosa associated follicles. Reinfection of these hosts may then activate these resident cells which then migrate to the MED node and the spleen where they depress IL-2 responses. This is supported by the fact that IL-2 production was depressed specifically in the lymph node draining the lung (MED), whereas IL-2 was enhanced in the lymph nodes draining the upper respiratory tract (SCV) (Figures 18 and 19). Conversely, such precursor cells may become activated within the bronchus associated lymphoid tissue (BALT) but then migrate to the MED where further activation and differentiation occurs (145, 146).

In the human lymphocyte population, two phenotypes of T cells have been identified, CD45R- and CD45R+. The CD45R+ cells are reputed to represent virgin lymphocytes while the CD45R- cells represent memory cells. Hirohato et
al. reported the ability of CD45R⁻ and CD45R⁺ cells to regulate the secretion of lymphokines differently (186). This group found that activated virgin cells expressed higher levels of IL-2 whereas activated memory cells did not. Following anti-Tac treatment, however, the CD45R⁻ cells expressed IL-2 to similar levels of the virgin cell population. This suggested that the memory cells were regulating IL-2 production by selective adsorption. In addition, the CD45R⁻ cells expressed higher levels of IFN-gamma compared to the CD45R⁺ population.

In the present study, reinfection of mice may induce the expression of IL-2R that selectively adsorb IL-2 in an activated memory cell population that accumulates in the MED nodes. This may reflect the depressed IL-2 in the MED nodes. This is supported by the in vitro kinetics of IL-2 in high density cultures. In the MED cultures, depressed IL-2 compared to primary infection cell cultures was most pronounced at 48 hours instead of 24 hours. This may represent an increased rate of IL-2R expression which adsorbs IL-2. Though the present study does not clarify this point it does suggest that the IL-2R-expressing cells may preferentially accumulate in the MED nodes and that lymphokine induced production of lymphokines may play a role in regulating immunopathology following reinfection. In Chapter 1, the deviation from the chi-square one hit kinetics model in the limiting dilution analysis during the primary response suggested that IL-2R-expressing cells were competing for IL-2 under limiting dilution conditions. In the reinfection, however, the chi-square was within the p>0.05 but the chi-square dropped from 0.388 to 0.111 suggesting that there was a proportional increase of IL-2R-expressing cells following reinfection. The CD4⁺ T cells that express the small factors reported by Beck and Sheridan (19, 104) may represent cells that are dependent on lymphokine receptor interaction for the induction of factors. It is possible that IL-2 induces the production of these factors which in turn modulates the production of IL-2. This negative feed back loop may be of primary importance in maintaining immunological homeostasis
and therefore regulating immunopathology of the lung.

The relatively unchanged frequency of IL-2-secreting cells in the MED following reinfection might be a reflection of decreased transit time through the lymph node. Recirculating IL-2-secreting cells may transit through both the lung and lymph node at a faster rate due to the production of factors that reduce the retention of lymphocytes in the lung. This may be why the degree of lung consolidation is reduced compared to the primary response. The small molecular weight factors expressed by lung mononuclear cells as described by Beck and Sheridan may increase motility of the IL-2-secreting cells (19). This is suggested by the fact that these factors increase the random migration of polymorphonuclear cells even in the presence of LIF, which retards the random migration of cells. Though the effect of these factors on lymphocytes are unknown it is possible that it may also increase lymphocyte migration.

A component secreted by the CD4+ T cell described by Beck (104), is a large molecular weight factor that inhibited the mitogen-induced proliferation of lymphocytes. These cells may accumulate in the MED node and express such factors. These factors may in turn retard IL-2-secreting cell proliferation. The relatively smaller changes in frequency of helper T cells in the MED nodes with an increase in total mononuclear cells following reinfection suggested an additional expansion or recruitment of non-IL-2-expressing cells as compared to the SCV.

These cells may represent clonal progeny (or recruitment) of antigen specific Th2 cells (cells that secrete IL-4 and provide help for B-cell differentiation), B-cells, CD8+ cells, or antigen non-specific mononuclear cells that do not express IL-2. Reinfection with influenza virus appears to select for an additional functional cell subset within the lower respiratory tract that does not express IL-2. Turcotte et al. found that in mice infected with BCG, the peripheral lymph nodes demonstrated a proportional increase in Ig+ cells with that of T cells (193).
Within these nodes, the production of IL-2 was also diminished. In the current study, the MED nodes may also accumulate non-T cells.

Lymph nodes accumulate lymphocytes through two portals of entry, the HEV found within the node parenchyma, and cells in lymph entering the afferent lymphatic vessels. It has been documented that the extravasation of cells into nodes or inflamed tissues is dependent on their binding to receptors expressed on the endothelium (130-136). These endothelium receptors in the mouse have been identified serologically (MECA-325) (135). Unlike most peripheral lymph nodes, the MED may receive cells by afferent lymphatics that are derived from the MALT (144, 145), however, it is unclear as to the association of the BALT with the MED nodes. The comparatively lower accumulation of IL-2-secreting cells in the MED in relation of the SCV may be a reflection of different trafficking patterns of influenza specific cells between these two nodes, primarily influenced by differences in the HEV receptors. IL-2-secreting cells may enter the inflamed tissues of the upper respiratory tract via the endothelium receptors at a greater rate than the lung parenchyma. HEV receptors for lymphocytes in the lung may be modified to resist extravasation of cells of IL-2-secreting nature whereas in the more complicated tissues of the respiratory tract cells are able to enter.

The activation of one type of immune response over another has been documented in several animal models (194-198). Heinzel et al. demonstrated that certain species of mice when primed to an antigen, selectively generated either humoral responses or cellular humoral responses. This phenomenon was attributed to the preferential activation of either Th1 cells or Th2 cells. It is possible that the environment of the MED induces the preferential stimulation of Th2 cells over the Th1. This stimulation of Th2 over Th1 may be due to either the concentration of antigen expressed, the type of antigen expressed, or the association with different MHC antigens. With the presence of circulating antibody, certain epitopes of the viral antigen may be expressed that stimulate
Th2 cells. The differences between the accumulation of IL-2-secreting cells between the SCV and MED may reflect the different mechanisms of viral neutralization by secretory IgA in the upper tract and the IgG in the lower tract. Taylor et al. reported that secretory IgA blocked viral infection in host cells by blocking internalization whereas circulating IgA and IgG neutralized virus by blocking events after the internalization of binding of virus to the surface of cells (94). Thus, different mechanisms may cause different viral antigen associations with host cell surfaces and therefore activate different virus-specific cell functions. Manca et al. demonstrated that antigen-presenting cells could present suboptimal concentrations of antigen if complexed with specific antibodies (199). In addition, this group found that of the T cell clones examined, the successful activation was dependent on the specificity of the antibody used for antigen presentation. It has been postulated that the binding antibody regulates antigen processing by the macrophage and thereby selects for the production of specific antigen epitopes necessary for T cell activation. Certain antibody was efficient at presenting certain epitopes. In the influenza study, the circulating antibody may complex with the virus and present specific epitopes to the T cells that preferentially activates T cells other than Th1 cells. The fact that anti-hemagglutinin is the predominant antibody produced following primary infection, along with the fact that both hemagglutinin specific T cells and internal protein T cells are activated, suggests that the binding of antibody may select for one of the other T cells (hemagglutinin vs internal protein). Conversely, it has been reported that Th1 cells require high density ligands for stimulation such as class II MHC antigens on activated macrophages. Reinfection may select for other MHC antigen interactions.

Another cell subset that may be preferentially activated is the CD8⁺ subset. CD8⁺ cells have reported different activation signals compared to Th1 cells (200, 201). Kim et al. studying PMA activation found that CD8⁺ cell clones were
stimulated more efficiently than Th clones (201). In addition, CD8+ cells have demonstrated the ability to proliferate in an IL-2-independent fashion (200). The reduced expansion of IL-2-secreting cells and the diminished production of IL-2 in high density cultures of MED nodes may be a reflection of an IL-2-independent expansion of CD8+ cells. Beck and Sheridan found that in vitro virus-specific induction of CTL was not inhibited by the small molecular weight factors, however, the expression of effector function of CTL was inhibited (104). The data support that in the presence of these suppressor cells, CTL (CD8+) cells may be activated and proliferate. The relatively lower change in frequency of IL-2-secreting cells may reflect the presence of the CD4+ suppressor cells that block IL-2 secretion while allowing for CTL maturation.

Unlike the primary response, reinfection demonstrated the production of IL-2 differentially among the draining nodes and the SPL (Figures 18 and 19). The diminished production of IL-2 and the diminished increase in the frequencies between these two nodes as determined by high density cultures and limiting dilution analysis respectively, suggested that following reinfection, mechanisms were induced to retard either the clonal expansion or recruitment of IL-2-secreting T cells in nodes that drain the lung tissue while still allowing for lymphocyte activation (Table 3). It appears that lymph nodes that drain the lung, though able to recruit mononuclear cells, develop an environment that selectively inhibits the production of IL-2 while activating other functional subsets of lymphocytes. It is possible that the CD4+ cells that modulate the biological expression of lymphokines (19, 104) may preferentially be localized in these nodes to retard both the production of inflammatory lymphokines and the proliferation of IL-2-secreting cells.
SUMMARY

It appears that not all lymphokine-secreting cells defined by their potential to secrete IL-2 are able to express lymphokines, but rather environmental factors, found in tissues where the cells reside, influence the production of lymphokines. In this study, the data suggests that IL-2 secretion may be regulated by either the type of antigen presentation, i.e. B cells versus macrophages, or by the state of T cell activation/maturation. The observation that IL-2-secreting cells were present in the non-draining nodes at the peak of pathology and that high density cultures did not express IL-2, suggests a restriction in the cellular components required for IL-2 secretion other than virus-specific T cells. The recruitment of mononuclear cells to the non-draining nodes 15 days post infection, along with the concomitant ability of T cells to express IL-2 in bulk culture suggested that within the total mononuclear cell population an APC was present. The fact that the major difference between the two culture conditions was the source of stimulators further supported the possibility that an antigen presenting cell was lacking in the non-draining nodes. In the current study, the APC may be virus-specific B cells. During the peak of the response, B cells may be specifically retained in the draining nodes. Following the resolution of infection, these cells may then migrate to the non-draining nodes. The restricted localization of virus-specific B cells maybe of importance in the generation of immunopathology during the primary response. The localization of antigen-specific B cells in the lung parenchyma may preferentially activate Th1 cell, those cells necessary for inflammatory responses.
The dichotomy between the lung and the draining nodes may be due to differences in the proportion of cells expressing high levels of IL-2R and those cells that do not. Cells in the lung may represent a relatively homogenous population of IL-2R-expressing cells. This may explain the fact that IL-2 is not detected in the lung. Conversely, the production of IL-2 in the draining nodes may represent a heterogenous population of cells. The chi-square probability values of the limiting dilution analysis of the draining nodes supports this possibility. The fact that the production of IL-2 is dependent on multiple factors may reflect the ratio of IL-2-secreting cells to IL-2R⁺ cells that adsorbs lymphokines.

To date it is unresolved as to where cells in the respiratory tract receive signals for proliferation and differentiation. In the influenza model, it appears that cells undergo proliferation and differentiation primarily in the draining nodes. The cells then enter the circulation where activated cells are preferentially retained in the lung tissue.

A model for the development of immunopathology is as follows: Recirculating IL-2-secreting precursor cells enter the lymph nodes draining both upper and lower respiratory tract containing viral antigen which was carried to these tissues by tissue macrophages or dendritic cells. There, the cells are activated by either macrophages or B cells. The lymphokine IL-2 is then expressed, which in turn induces the proliferation and differentiation of two types of IL-2-secreting Th cells, one population requiring B cells for activation and restricted to the draining nodes, and spleen and the other population requiring macrophages and IL-1, which can migrate to other tissues, perhaps as memory cells. The restricted Th cells expressing IL-2R then migrate preferentially to the lung while the single factor cell distributes to other tissues. The restricted cell, where upon stimulation by antigen within the lung tissue, expresses IFN-gamma by an IL-2-IL-2 receptor mechanism. This interaction
rapidly adsorbs IL-2 while expressing IFN-gamma, therefore resulting in immunopathology. As the immune response diminishes and the virus is eliminated both the multi-factor Th cell and the single factor Th cell migrate to the non-draining lymph nodes perhaps as memory cells, while leaving memory cells in the BALT. Antigen then may also be carried to non-draining nodes by the cells leaving the lung parenchyma.

In contrast to the primary response, where cells accumulate and express IL-2 in all three draining nodes, reinfection induces a differential pattern of lymphokine production among these same nodes. By examining the distribution of IL-2-secreting T cells following reinfection, it was found that IL-2 production was differentially distributed between the lymph nodes that drain the upper respiratory tract and lymph nodes that drain the lung parenchyma. It was observed that the MED node cells had depressed IL-2 secretion following reinfection while the SCV node cells had either equivalent or enhanced IL-2 production. Compared to the MED, their was a 10-fold increase in the frequency of cells in the SCV nodes while the MED demonstrated a 2-fold increase. The retarded expansion or recruitment of IL-2-secreting cells in the mediastinal node suggests a specialized function of lymph nodes draining the mucosal surface of the lung parenchyma over that of nodes draining the upper respiratory tract. Following reinfection, non-IL-2-secreting cells (Th1, B cells, CD8+ cells) may preferentially be activated by the differential expression of epitopes of viral antigen. Circulating antibodies entering the lung an nodes may modulate the antigen processing that then selects for other non-IL-2-expressing cells. Manca et al. found that specific antibodies altered the ability of antigen presenting cells to activate certain T cell clones specific for the antigen. The selective activation of T cells by the modulation of antigen processing may select for Th2 cells over Th1 cells. This is suggested by the 2-6-fold increase in total mononuclear cells with a 1.5 fold increase of IL-2-secreting cells. The preferentially activated cells then
recruit or expand in the draining nodes at a faster rate than IL-2-secreting cells.

A model for the regulation of immunopathology following reinfection is as follows: Reinfection induces the activation of resident memory cells within the BALT which migrate to the draining nodes. In the superficial nodes specific antigens expressed and modulated by either low levels of antibody or specifically by secretory IgA activate a large proportion of IL-2-secreting memory cells and perhaps virus-specific virgin cells that were not activated following primary infection. This may be due to increased antigen expression in the upper respiratory tract due to the ability of virus to preferentially replicate in the upper respiratory tract. In contrast, circulating IgG and or circulating IgA which transudates into the lung parenchyma in the deeper respiratory tract modulates another set of antigens that activates predominately non-IL-2-secreting cells and CD4+ cells expressing small molecular weight factors by modulating the antigen processing mechanisms of antigen presenting cells. The activated cells then migrate to the lung where, with the elaboration of these factors decreases the retention time of IL-2-secreting cells that enter the lung and specifically inhibits proliferation of IL-2-secreting cells. The decreased retention time of IL-2-secreting cells may reduce the immunopathology following reinfection.

Collectively, the data indicates that infection with influenza induces environments within the draining nodes that modulate the production of IL-2. In addition, nodes that drain sites that function in an architecture dependent fashion develop specialized functions for selecting non-inflammatory immune responses. These functions or mechanisms may be the primary component of regulating immunopathology, and be instrumental in maintaining a balance between effective viral clearance and immunopathology in tissues that depend on their structure for function.
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