ANALYSIS OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS ANTIGENS PRESENTATION BY MACROPHAGES

by

Attiya Alatery

A thesis submitted to the Department of Microbiology and Immunology
in conformity with the requirements for the degree of
Doctor of Philosophy

Queen’s University
Kingston, Ontario, Canada
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ABSTRACT

The activation of cytotoxic T-cell (CTL) responses requires antigen presentation by professional antigen presenting cells. Macrophages (MØ) can regulate CTL responses but little is known about the role played by splenic macrophages (Sp-MØ) in antigen cross-presentation.

Here, we established, and characterized, an efficient culture method for generating Sp-MØ. By monitoring MØ markers, we found that 7-days Sp-MØ resembles the red pulp macrophages (RPMØ) phenotypic characteristics. The phagocytic capacity of Sp-MØ was increased as the cells become more differentiated. Thus, increased differentiation of Sp-MØ in vitro can be macrophage-colony stimulating factor (M-CSF) driven, which allows for an optimal condition to increase the yield of the spleen-derived MØ.

As a result of examining the antigen presentation of Sp-MØ during differentiation, we reported that Sp-MØ down-regulated their ability to cross-present the cell-associated lymphocytic choriomeningitis virus nucleoprotein (LCMV-NP) but not the soluble OVA proteins without altering their capacity to directly present LCMV antigens after infection. Mechanistically, we defined the cytosolic pathway as the dominant cross-presentation pathway used by Sp-MØ. Further analysis revealed a direct relationship between Sp-MØ differentiation, phagosomal acidification, and antigen cross-presentation. As Sp-MØ become more mature, their vesicular phagosomal system acquired high acidic characteristics, which adversely affected antigen cross-presentation due to potent and enhanced antigen degradation.

We also addressed the capacity of diverse LCMV antigens, generated during virus infection, to induce LCMV-specific CTL responses via cross-presentation by employing antigen donor cells (ADCs) that provide sufficient LCMV antigens after virus inactivation with no possible direct antigen presentation. Our results demonstrated that the ADCs induced LCMV-specific CTL responses in vitro and in vivo. Out of the four CTL epitopes tested (NP396, NP205, GP33, and GP276), in vitro cross-presentation were dominated by LCMV-NP396 epitope; while the in vivo cross-priming has shifted towards LCMV-GP33 and NP396 epitopes.

Collectively, the data presented in this thesis have defined for the first time important factors that influence Sp-MØ culturing in vitro and highlighted a potential role for the Sp-MØ in regulating CTL
responses via cross-presentation, and characterized how different epitopes from LCMV are cross presented

in vitro and in vivo.
HYPOTHESES

**General hypothesis**

Macrophages can cross-present LCMV antigens, and that cross-priming is needed for CTL activation in vivo.

**Hypothesis for chapter # 1**

Sp-MØ require macrophage-colony stimulating factor (M-CSF) for optimal growth and further in vitro differentiation & maturation.

**Hypothesis for chapter # 2**

Cross-presentation of cell-associated antigens by Sp- and BM-MØ is regulated during differentiation.

**Hypothesis for chapter # 3**

LCMV antigens can be cross-presented with different efficiencies by different pAPCs subsets.
ACKNOWLEDGEMENTS

First, I thank God immensely for giving me strength, patience, confidence, and optimism through all tests in the past.

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Hypotheses</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>Table of contents</td>
<td>vii</td>
</tr>
<tr>
<td>List of tables</td>
<td>xiii</td>
</tr>
<tr>
<td>List of figures</td>
<td>xiv</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>xvi</td>
</tr>
<tr>
<td>CHAPTER 1: Induction of CD8+ T-cells responses against virus antigens</td>
<td>1</td>
</tr>
<tr>
<td>1.1 overview of immunity against virus infection</td>
<td>2</td>
</tr>
<tr>
<td>1.2 CD8+ T lymphocytes</td>
<td>3</td>
</tr>
<tr>
<td>1.3 The primary T cell responses to virus infection</td>
<td>4</td>
</tr>
<tr>
<td>1.3.1 CD8+ T cells activation</td>
<td>4</td>
</tr>
<tr>
<td>1.3.2 CD8+ T cell phenotype during immune responses</td>
<td>8</td>
</tr>
<tr>
<td>1.4 Antigen processing</td>
<td>9</td>
</tr>
<tr>
<td>1.5 Antigen presentation</td>
<td>15</td>
</tr>
<tr>
<td>1.5.1 Direct presentation</td>
<td>15</td>
</tr>
<tr>
<td>1.5.2 Cross-presentation</td>
<td>16</td>
</tr>
<tr>
<td>1.5.3 Exogenous antigen uptake</td>
<td>20</td>
</tr>
<tr>
<td>1.6 viral models of antigen cross-presentation</td>
<td>22</td>
</tr>
<tr>
<td>1.6.1 Lymphocytic choriomeningitis virus (LCMV)</td>
<td>22</td>
</tr>
<tr>
<td>1.6.2 Polio virus (PV)</td>
<td>23</td>
</tr>
<tr>
<td>1.6.3 Adenovirus (Ad)</td>
<td>24</td>
</tr>
<tr>
<td>1.6.4 Vaccinia virus (VV)</td>
<td>24</td>
</tr>
<tr>
<td>1.6.5 Influenza A virus (IAV)</td>
<td>24</td>
</tr>
<tr>
<td>1.6.6 Epstein - Barr virus (EBV)</td>
<td>25</td>
</tr>
</tbody>
</table>
1.6.7 Human immunodeficiency virus (HIV) ................................................................. 25
1.6.8 Herpes simplex virus 1 (HSV-1) ......................................................................... 26
1.7 The characteristics of cross-presented antigens .................................................... 27
1.8 Cellular requirements for cross-presentation ...................................................... 29
  1.8.1 Dendritic cells (DCs) ......................................................................................... 30
    1.8.1.1 Conventional DCs (cDCs) ...................................................................... 31
    1.8.1.2 Plasmacytoid DCs (pDCs) ...................................................................... 33
  1.8.2 Macrophages (MØ) ......................................................................................... 34
    1.8.2.1 Spleen macrophages (Sp-MØ) .............................................................. 37
  1.8.3 B cells ............................................................................................................. 38
  1.8.4 Neutrophils .................................................................................................... 41
1.9 Mechanisms of cross-presentation ........................................................................ 42
  1.9.1 The cytosolic pathway .................................................................................. 42
  1.9.2 Vacuolar pathway ......................................................................................... 46
  1.9.3 Endosomes to ER pathway ........................................................................... 50
  1.9.4 Gap junction pathway (cytosol-to-cytosol pathway). ..................................... 50
1.10 LCMV infection model ....................................................................................... 51
1.11 Research objectives ............................................................................................ 57
  1.11.1 Objective #1. Culturing and investigating the differentiation of spleen
      macrophages (Sp-MØ) in vitro ........................................................................ 57
  1.11.2 Objective #2. Investigating the effect of differentiation on the capacity of spleen
      (Sp-MØ) and bone-marrow-derived macrophages (BM-MØ) to cross-present
      soluble and cell-associated antigens .................................................................. 57
  1.11.3 Objective #3. Analysis of cross-presented epitopes from LCMV-NP and GP after
      virus infection ..................................................................................................... 58
References .................................................................................................................. 59
CHAPTER 2: An efficient culture method for generating large quantities of mature mouse splenic macrophages

2.1 Preface ........................................................................................................................................... 91
2.2 Abstract ........................................................................................................................................ 92
2.3 Introduction ................................................................................................................................... 93
2.4 Materials and methods ................................................................................................................... 95
   2.4.1 Mice, cells, and media .......................................................................................................... 95
   2.4.2 Preparation of bone marrow-derived macrophages and dendritic cells ..................... 95
   2.4.3 Preparation of spleen macrophages .................................................................................... 96
   2.4.4. Immunofluorescence microscopy & lysosomal detection ............................................. 97
   2.4.5. Phagocytosis assays ........................................................................................................... 97
   2.4.6. Flow cytometry analysis .................................................................................................... 98
2.5 Results ..................................................................................................................................... 100
   2.5.1. Analysis of Sp-MØ development in conditioned medium ........................................... 100
   2.5.2. Increased number of recovered Sp-MØ is dependent on M-CSF ................................. 103
   2.5.3. Microscopic examination of macrophages’ lysosomal content CSF ............................... 103
   2.5.4. Phenotype analysis of Sp-MØ cultured in CM ................................................................. 106
   2.5.5. Sp-MØ maturation in medium supplemented with M-CSF associates with certain surface marker expression ................................................................. 113
   2.5.6. Analysis of the phagocytic capacity and the uptake mechanisms of SP-MØ cultured in CM ........................................................................................................... 113
2.6 Discussion ................................................................................................................................ 120
References ..................................................................................................................................... 124

CHAPTER 3: Cross, but not direct, presentation of cell-associated virus antigens by spleen macrophages is influenced by their differentiation state ........................................ 129

3.1 Preface ........................................................................................................................................ 130
3.2 Abstract .................................................................................................................................... 131
3.3 Introduction ............................................................................................................................. 132

3.4 Materials and methods ............................................................................................................... 133

3.4.1. Mice & Cells ........................................................................................................................ 133

3.4.2. Preparation of BM-MØ, BM-DCs, Sp-MØ, & ADCs .................................................... 133

3.4.3. Flow cytometry analysis .................................................................................................... 134

3.4.4. Phagocytosis and antigen degradation assays ................................................................. 134

3.4.5. Cytoplasmic staining for LCMV proteins .......................................................................... 135

3.4.6. Intracellular cytokine staining (ICS) .................................................................................. 135

3.4.7. Induction of CD8+ T cell lines and antigen presentation assays ....................................... 135

3.4.8. Proteasome activity measurements ................................................................................... 136

3.4.9. RT-PCR ............................................................................................................................ 137

3.4.10. Western Blotting .............................................................................................................. 137

3.5. Results ................................................................................................................................... 138

3.5.1. Characterization of Sp-MØ .............................................................................................. 138

3.5.2. Direct antigen presentation by Sp-MØ and BM-MØ is consistent ................................. 138

3.5.3. Cross-presentation capacity of Sp-MØ and BM-MØ varies with their differentiation state .................................................................................................................. 141

3.5.4. Cross-presentation of soluble antigens by Sp-MØ and BM-MØ is not affected by the differentiation in culture ............................................................................................................. 144

3.5.5. Mechanisms employed by Sp-MØ and BM-MØ to cross present cell-associated antigens ........................................................................................................................................ 147

3.5.6. Proteasomal activities in Sp-MØ and BM-MØ increase due to exogenous antigen uptake ...................................................................................................................................... 150

3.5.7. Phagosomal-degradation in Sp-MØ directly affects cross-presentation ......................... 150

3.6. Discussion ................................................................................................................................ 156

References ....................................................................................................................................... 162
CHAPTER 4: The outcome of cross-priming during virus infection is not directly linked to the ability
of the antigens to be cross-presented .......................................................... 169

4.1 Preface .............................................................................................................. 170
4.2 Abstract ............................................................................................................ 171
4.3 Introduction ...................................................................................................... 172
4.4 Materials and methods .................................................................................... 174
   4.4.1. Mice, cells and reagents ........................................................................... 174
   4.4.2. Preparation of bone marrow-derived macrophages (BM-ΜΟ) and dendritic cells
          (BM- DC) .............................................................................................................. 174
   4.4.3. Preparation of ADCs for cross-presentation ............................................. 175
   4.4.4. Detection of intracellular viral proteins and intracellular cytokine staining (ICS) 175
   4.4.5. Antigen presentation assays employing peptide-specific CD8+ T cells ...... 176
   4.4.6. Tetramer staining ..................................................................................... 177
   4.4.7. Statistical analyses .................................................................................. 177
4.5. Results ............................................................................................................. 178
   4.5.1. Characterization of LCMV-infected and UV irradiated antigen donor cells ...... 178
   4.5.2. Cross-presenting efficiencies of NP and GP epitopes differ and increase with time
          post infection .................................................................................................. 181
   4.5.3. Influence of RNA on cross-presentation of the LCMV-infected LyUV-ADCs ... 184
   4.5.4. LCMV-cell associated antigens are mainly cross-processed via the classical
          cytosolic pathway and require tightly-regulated phagosomal acidification ...... 188
   4.5.5. Cross-priming of infected ADCs induces CTL populations dominated by two
          epitopes with contrasting efficiencies in cross-presentation .......................... 191
   4.5.6. Cross-priming influences the magnitude of immunodominant T cells during
          subsequent virus infection .............................................................................. 194
4.6. Discussion ....................................................................................................... 197
References ............................................................................................................. 201
**LIST OF TABLES**

Table 1.1: Changes that occur during activation of naïve CD8+ T cells with virus antigens .................. 5

Table 2.1: Conditioned medium increases Sp-MØ numbers in culture ................................................... 104

Table 3.1: Cross-presentation ability of Sp-MØ and BM-MØ is influenced by their antigens degradation .................................................................................................................................................... 157
LIST OF FIGURES

CHAPTER 1

Figure 1  Pathways involved in the generation of MHC class I-presented peptides ......................... 10
Figure 2  Cytosolic pathways of cross-presentation. ........................................................................... 17
Figure 3  Vacuolar or endocytic pathway of cross-presentation. .......................................................... 47
Figure 4  LCMV replication cycle........................................................................................................... 55

CHAPTER 2

Figure 1  Generation of Sp-MØ in large quantities in vitro ................................................................. 101
Figure 2  Conditioned medium increases Sp-MØ numbers in culture.................................................. 104
Figure 3  Microscopic examination of the lysosomal content in macrophages..................................... 107
Figure 4  Analysis of surface marker expression on macrophages....................................................... 110
Figure 5  Staining profiles of Sp-MØ specific markers cultured in either CM or medium supplemented
          with recombinant M-CSF .................................................................................................................. 114
Figure 6  The phagocytic capacity of Sp-MØ increases with time in culture......................................... 117

CHAPTER 3

Figure 1  Comparison of Sp-MØ and BM-MØ cultured in vitro for 7 days .............................. 139
Figure 2  Direct antigen presentation profiles of LCMV epitopes by Sp-MØ and BM-MØ during
          differentiation are identical ............................................................................................................... 142
Figure 3  The ability of Sp-MØ and BM-MØ to cross-present cell associated antigens inversely
          correlates with their differentiation state ....................................................................................... 145
Figure 4  Cross-presentation of soluble antigens by Sp-MØ and BM-MØ is not affected by their
          differentiation in culture .................................................................................................................. 148
Figure 5  Mechanisms of cross-presentation employed by Sp-MØ and BM-MØ ............................ 151
Figure 6  Analysis of proteasomal activities in Sp-MØ and BM-MØ ................................................... 154
Figure 7  Cross-presentation ability of Sp-MØ and BM-MØ is influenced by their antigens degradation efficiency .................................................................................................................................. 157

CHAPTER 4

Figure 1  LCMV-infected and UV-irradiated ADCs are efficient source of exogenous antigens.  ... 179
Figure 2  Following virus infection LCMV epitopes are differentially cross-presented .................... 182
Figure 3  Cross-presentation of LCMV proteins is not abolished by RNA degradation ...................... 185
Figure 4  Mechanisms governing cross-presentation of LCMV proteins using infected LyUV ADCs...
.............................................................................................................................................................................. 189
Figure 5  Cross-priming of LCMV epitopes after virus infection and LyUV treatment ..................... 192
Figure 6  Cross-priming shapes immunodominance following LCMV infection .............................. 195

CHAPTER 5

Figure 1  Proposed schematic model of the relationship between Sp-MØ and their precursors ....... 211
Figure 2  Proposed schematic model for antigens cross-presentation by MØ during differentiation .. 216
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
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<tr>
<td>ADCs</td>
<td>Antigen donor cells</td>
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<tr>
<td>AEP</td>
<td>Asparagine endopeptidase</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>α-DG</td>
<td>α-dystroglycan</td>
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<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>Bcl-2</td>
<td>Anti-apoptotic marker</td>
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<tr>
<td>BFA</td>
<td>Brefeldin A</td>
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<td>BMA</td>
<td>Macrophage cell line</td>
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<tr>
<td>BM-DC</td>
<td>Bone marrow-derived dendritic cells</td>
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<td>BM-MØ</td>
<td>Bone marrow-derived macrophage</td>
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<tr>
<td>BNLF2a</td>
<td>EBV Lytic phase protein</td>
</tr>
<tr>
<td>β2m</td>
<td>Beta 2-microglobuline</td>
</tr>
<tr>
<td>B3Z</td>
<td>OVA-specific CD8+ T cells</td>
</tr>
<tr>
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<td>C57BL/6 mice (Black mice)</td>
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<td>Cath.</td>
<td>Cathepsin</td>
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<tr>
<td>CCD</td>
<td>cytochalasin D</td>
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<td>CCL</td>
<td>CC-chemokine ligands</td>
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<td>CCR</td>
<td>CC-chemokine receptor</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<td>A cell containing CD8 co-receptor</td>
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<td>cDCs</td>
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<td>CDR</td>
<td>Complementarity-determining region</td>
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<td>5,6-carboxyfluorescein diacetate succinimidi ester</td>
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<td>Cytosine-guanosine dinucleotide</td>
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<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
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<td>d</td>
<td>Day</td>
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<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>DC2.4</td>
<td>dendritic cell line</td>
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<tr>
<td>DEC-205</td>
<td>Multilectin endocytic receptor</td>
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<tr>
<td>DC-SIGN</td>
<td>DC-specific C-type lectin receptor</td>
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<tr>
<td>DLN</td>
<td>Draining lymph node</td>
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<tr>
<td>DMA</td>
<td>Dimethyl amiloride</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DNAse</td>
<td>Deoxyribonuclease</td>
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<tr>
<td>dnDCs</td>
<td>Double negative dendritic cells</td>
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<tr>
<td>DPI</td>
<td>Diphenylamine iodonium</td>
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<tr>
<td>DRiPs</td>
<td>Defective ribosomal products</td>
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<tr>
<td>dsRNA</td>
<td>Double stranded ribonucleic acid</td>
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<tr>
<td>EBNA</td>
<td>Epstein-Barr Virus Nuclear Antigen</td>
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<tr>
<td>EBV</td>
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<td>ERAB</td>
<td>ER-associated aminopeptidases</td>
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<td>E:T</td>
<td>Effector to target</td>
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<tr>
<td>FBS</td>
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</tr>
</tbody>
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FCS  Fetal calf serum
EDTA  Ethylenediaminetetraacetic acid
FcyR  Fc gamma receptors for IgG binding
FCM  Flow Cytometry
FITC  Flourescin-isothiocyanat
GEN3  Plasmacytoid cell line
GFP  Green fluorescent protein
GM-CSF  Granulocytes-macrophages colony stimulating factor
GP  Glycoprotein
GPC  Glycoprotein precursor
h  Hour
HCMV  Human cytomegalovirus
HEK293  Human embryonic kidney epithelial cell line
HEK-NP  LCMV-NP-transfected HEK293 cells
HEL  Hen egg lysosome
HEV  High endothelial venules
HIV  Human immunodeficiency virus
Hsp  Heat shock proteins
HSV  Herpes simplex virus
IAV  Influenza A virus
ICS  Intracellular cytokine staining
iDCs  Immature dendritic cells
intDCs  Intermediate dendritic cells
IFN  Interferon
Ig  Immunoglobulin
IL  Interleukin
IL-15Ra  Interleukin-15 receptor a chain
IMDM  Iscove’s modified Dulbecco’s medium
i.p.  Intra-peritoneal
ISCOMS  immune-stimulating complexes
i.v.  Intra-venous
Kb  Kilo base
KDa  Kilo Dalton
lacZ  Beta-D-galactosidase
LCs  langerhans cells
LCMV  Lymphocytic choriomeningitis virus
LMP  Low molecular mass polypeptide
LPS  Lipopolysaccharide
LyUV  Lysis followed by UV treatment
L929  Murine fibrosarcoma cell line
MØ  Macrophage
mAAb  Monoclonal antibody
M-CSF  Macrophage-colony stimulating factor
MC57  C57BL/6-derived fibrosarcoma cell line
MDDCs  Monocyte-derived dendritic cells
mDC  Myeloid dendritic cells
ME  2-mercaptoethanol
MECL-1  Multicatalytic Endopeptidase Complex-Like 1
MG-132  Reversible proteasome inhibitor (peptide aldehydes)
MHC-I  Major histocompatibility molecules class I
MHC-II  Major histocompatibility molecules class II
min  Minute
mM  Milli molar
µM  Micro molar
MMMØ  Marginal zone metallophilic macrophages
m.o.i.  Multiplicity of infection
<table>
<thead>
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<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>VV</td>
<td>Vaccinia virus</td>
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<tr>
<td>WE</td>
<td>LCMV-WE strain</td>
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<tr>
<td>WT HEK</td>
<td>Wild type HEK293 cells</td>
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CHAPTER 1

Induction of CD8+ T-cell responses against virus infection
1.1 Overview of immunity against virus infection

The immune response to virus infections consists of different phases ranging from the simple innate immune response to the more sophisticated adaptive or acquired immune response. The innate immune response is the first defence line against viruses. It is not specific to a particular pathogen and comes into play within hours of infection. Innate antiviral immunity is mediated by specific cells of haematopoietic origin located throughout the various tissues such as macrophages (MØ) and dendritic cells (DCs). These immune cells possess sensory molecules called pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). Expression of PRRs can be either extra-cellular or intra-cellular depending on the type of the PRR in order to recognize and bind specific foreign material such as viral RNA or DNA [1, 2].

The interaction of viral products with TLRs (TLR3, 7, 8, and 9) leads to induction of interferon-α and β (IFN-α and β) antiviral genes. Both IFN-α and β are called type I interferons that induce apoptosis of virus-infected cells and inhibit virus replication. Furthermore, induction of co-stimulatory molecules such as CD80, CD86, and CD40, and secretion of pro-inflammatory cytokines such as tumour necrosis factor (TNFα), interleukin 12 (IL-12), and IL-6 by the cells of the innate immune system during virus infection directly influences adaptive immune responses. Induction the proliferation and activation of cytotoxic T lymphocytes (CTLs) and production of interferon-γ (IFN-γ) that promote T helper lymphocytes 1 (Th1) cellular responses are considered some of the most important functions of these effector molecules [1-3].

On the other hand, the adaptive immune response is the specific component of the immune system, as it shows a high degree of specificity and requires more time to develop. Its function is to eliminate and terminate pathogenic challenges and generate more specific and long lasting protection mechanisms (memory) against viral infections. The adaptive immune system acts via two non-overlapping mechanisms: the humoral and cell-mediated immunity [4-6]. The humoral immunity is mediated by B cells, which secrete antibodies that can specifically and efficiently recognize the viral antigens [6-8]. Cellular immunity involves immune cells that specifically recognize and eliminate virus-infected cells in addition to other innate
cells such as MØ and natural killer (NK) cells. The immune cells employed by the adaptive immune system can be divided into two subpopulation; cytotoxic T (CTL) and T helper lymphocytes (Th). While CTL destroy virus-infected cells by secreting cytotoxic mediators like perforin and granzyme B [9], Th cells secrete several regulatory cytokines such as IL-4, IL6, IL10, and IFN-γ that regulate the activation of CTL [3]. These subpopulations can be distinguished from one another by the presence of either CD8 or CD4 co-receptors on their surfaces. T cells are adapted to distinguish between self vs. non-self antigens. They achieve this by recognizing virus antigens in the form of small peptides presented on the major histocompatibility (MHC) molecules by their T cell receptors (TCRs) [10, 11].

It is known that stimulation of adaptive immune responses require a foreign antigen being presented by cells like DCs and MØ. These cells (DCs and MØ) have the ability to modulate adaptive immune responses to pathogens by processing and presenting antigens on MHC molecules and secreting cytokines like IL-12 that have several impacts on CD8+ and CD4+ T cell responses [1, 2].

1.2 CD8+ T lymphocytes

The lymphocytes CD8+ T cells (also known as cytotoxic T cell, Tc, CTL, T-Killer cell, cytolytic T cell, or killer T cell) are capable of attacking and killing infected cells, and tumor cells. CD8+ T lymphocytes arise from the bone marrow, from the haematopoietic stem cells of the lymphoid lineage, and mature in the thymus. In the thymus, the TCR is generated due to genetic rearrangement process in the α and β-chains genes to form the TCR. This results in the generation of different T cells with different TCRs specificities. The specificity of T cells to their cognate antigens is confined to the extracellular part of α & β variable regions of TCR [12-15].

During the maturation stages in the thymus, thymocytes (immature lymphocytes in the thymus) are exposed to two kinds of selection: the positive selection, to select for T cells capable of binding with MHC molecules, and the negative selection, to remove T cells that strongly bind with self peptide-MHC complex. These processes are important to select for only those T cells that bind to the MHC-self-antigen complexes weakly [16-20]. Differentiation into single-positive T cells (CD4+ or CD8+) is another differentiation
process that happens in the thymus. The TCR of double-positive T cells (CD4+CD8+) recognizes either peptide/MHC-I complexes to produce CD8+ T cells, or peptides/MHC-II complexes to give rise to CD4+ T cells. However, it is the CD8+ T cells that will mature and become CTL following their activation with MHC-I-restricted antigens. After becoming fully mature, they leave the thymus and reside in the secondary lymphoid organs (the spleen, lymph nodes, and Peyer’s patches) as mature naïve CD8+ T cells [21, 22].

In general, TCRs of virus-specific CD8+ T cells recognize and bind only to MHC-I molecules bound to peptides of 8-10 amino acids length [23]. Expression of co-receptor such as CD8 and CD3 by CTL are indispensable for signal transduction, and CTL activation [24]. CD8+ T cells can be found in three different states; naïve, effector, and memory cells based on the presence and absence of immunological stimuli. These CD8+ T cell subsets differ profoundly with regard to their cell surface molecules (markers), anatomical distribution, activation requirements, and life span [5, 25]. The markers and phenotype are summarized in Table 1.

1.3 The primary T cell responses to virus infection

1.3.1 CD8+ T cells activation

Naïve CD8+ T cells are defined as the cells that have not encountered their cognate antigens. Generally, naïve antigen-specific CD8+ T cells, of any particular specificity (recognizing any given peptide) exist at very low frequencies [26, 27]. They are continuously circulating among the lymphoid organs, which is controlled by T cells-homing receptors CD62L and CC-chemokine receptor 7 (CCR7). The priming of naïve CD8+ T cells requires the activity of the bone marrow-derived professional antigen presenting cells (pAPCs), mainly antigen-bearing DCs expressing CCR7 that can encounter T cells in the secondary lymphoid organs [28-31]. Besides DCs, MØ has also been shown to reach lymph nodes upon subcutaneous injection and prime naïve CD8+ T cells [31]. The pAPCs have the ability to present peptides derived from pathogens as MHC-I/peptide complexes along with co-stimulatory molecules such as CD80, CD86 and CD40, which are crucial for priming naïve CD8+ T cells. It has been clearly shown that upon
Table 1: Changes that occur during activation of Naïve CD8+T cells with Virus antigens. Antigenic stimulation causes naïve CD8 T cells to proliferate and expand, and the generation of effector CD8 T cells. Phenotypic and functional changes that occur the naïve effector → memory transition are summarized in the table.
### Homing

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<th>Naïve</th>
<th>Effector</th>
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<tr>
<td>2nd Lymphoid organs</td>
<td>++++</td>
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<td>Peripheral tissues</td>
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### Effector functions

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<td>CTL</td>
<td>—</td>
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<tr>
<td>Perforin</td>
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<tr>
<td>Granzyme</td>
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### Rapid recall

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<tr>
<td>IFN-γ/TNF-α</td>
<td>— / +</td>
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<td>IL-2</td>
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### Proliferation

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<tr>
<td>Homeostatic</td>
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### Phenotype characteristics

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<th>Naïve</th>
<th>Effector</th>
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<td>CCR7</td>
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<td>CD27</td>
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<td>CD127</td>
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<td>Bcl-2</td>
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<td>CCD44</td>
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viral infection many danger signals are released that result in licensing pAPCs to initiate priming of naïve T cells [32].

As a consequence of infection or immunization, naïve antigen-specific CD8+ T cells become activated and undergo differentiation into effector antigen-specific CTLs. Entering the effector phase, requires two signals: i) the first signal provided by the interaction between pAPCs, displaying an agonistic MHC-I/peptide complex and naïve T cells, bearing TCR for this peptide [33]. The second signal generated by the interaction of co-stimulatory molecules, like CD80 and CD86, expressed on pAPCs with CD28 molecules on naïve T cells. These signals support the IL-2 dependent clonal expansion of high affinity antigen-specific CTLs to ensure more efficient and effective immune responses [33-35]. The IL-2 cytokine is needed by naïve T cells to enter in the effector stage [25, 36]. At this stage, effector CD8+ T cells are characterized by significant and dramatic proliferation and differentiation capacities. The rapid expansion of CTL during immune responses has been shown by the adaptive transfer of 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled epitope-specific-CTL in mice injected with soluble OVA, cell-associated OVA [37], or infected with LCMV [38, 39]. Other investigations have estimated that CD8+ T cells undergo rapid cell division (one division every 6–8 h) and dramatic increase in numbers, with one precursor giving rise to more than 1000-10,000 daughter cells during the course of response (5-8 days) [40, 41].

The activated CTL down-regulate the chemokines receptors (CCR7) and adhesion molecules (CD62L), leave the secondary lymphoid organs, and migrate into the infected areas [42, 43]. Upon encountering infected cells, mature effector cells significantly up-regulate the production of several cytokines like IFN-γ and TNF-α, and intra-cytoplasmic granules containing cytotoxic molecules (such as Perforin and Granzyme B), which are the most important effector cytotoxic molecules [44]. The cytotoxic immune responses, carried out by CD8+ T cells, play a central role in eliminating virus infection. Such function requires cooperation between CD8+ T cells and antigen-presenting cells [45].

Switching the effector CTL into a memory phase is accompanied by clearance of infection, silencing the immune response and substantial reduction in the activated CTL populations. Generally, this phase is called cell death or the contraction phase, in which about 90-95% of antigen-specific effector CTLs
die by apoptosis. The number of antigen-specific CD8+ T cells that survive the cell death phase (about 5-10%) will be turned into antigen-specific memory CD8+ T cells. Memory CD8+ T cells are long-lived lymphocytes and are highly maintained in the absence of antigens (antigen-independent cells). They can undergo very fast immune responses upon re-encountering their cognate antigens [5, 25].

1.3.2 CD8+ T cell phenotype during immune responses

The phenotypic and functional steps involved in CD8+ T cells activation can be detected by relying on stage-specific markers such as CD44 (adhesion molecules), CD25 (IL-2 receptor α chain), CD62L (L-selectin, cell adhesion molecules), CD69 (activation marker), CD122 (IL-2 receptor β chain), CD127 (IL-7 receptor α chain), CD27 (TNF receptor), Ly6C, and CCR7 (Chemokine receptor 7). In addition, ex vivo effector functions and production of IL-2, IFN-γ/TNFα can also be used as parameters to distinguish naive, effector and memory CD8+ T cells from each other (Table 1) [5, 46].

Naïve CD8+ T cells are characterized by high expression of CD62L and CCR7 that allow them to home preferentially to the lymphoid organs. Naïve CD8+ T cells can also express high levels of CD127 which mediates essential signals for their survival in lymphoid organs [47]. Effector CD8+ T cells are characterized by low CD62L, CCR7, CD127, and CD27 expression levels. Instead, they up-regulate all effector markers such as CD44, CD122, CD25, CD69, and IL-15Rα. The CD8+ T cells effector phase is also characterized by production high quantity of IFN-γ/TNFα, Granzyme B, and Perforin [5, 48]. Moreover, effector CD8+ T cells are exclusively distributed in the peripheral tissues and express low levels of Bcl-2 (anti-apoptotic marker) [46].

Memory CD8+ T cells are heterogeneous in terms of phenotype, function, and anatomical distribution and they are divided into two major subsets: central- and effector-memory CD8+ T cells. While central memory is classified as CD62L hi CCR7 hi, effector memory CD8+ T cells are CD62L lo CCR7 lo. In recent published data [49, 50] CD127 and CD62L have been employed to separate memory CD8+ T cells into effector (CD62L− CD127−), effector memory (CD62L− CD127+) and central memory (CD62L+ CD127+). Krishna and Ahmed have shown that upon challenging with LCMV virus the cells up-regulated most of effector/memory markers such as CD44, CD122, CD69, CD25, CD71, and CD132 [41].
Expression of effector molecules such as granzyme B and perforin is highly restricted to effector memory but not central memory CD8+ T [46]. The central memory CD8+ T cells found in the spleen, blood, and lymph nodes, whereas the effector memory also found in the spleen and blood but not in lymph nodes. Memory CD8+ T cells express high levels of CD127 (IL-7 receptor) and IL15Rα (IL-15 receptor) as these cytokines are critical for memory CD8+ T cells homeostatic turnover which work as survival signals for memory lymphocytes [47, 51].

Since the initiation of CD8+ T cells responses against virus infection requires antigen presentation by pAPCs, understanding the mechanism(s) by which CD8+ T cells are activated against viruses that cannot infect or interact with pAPCs presentation machineries is an important objective. Additionally, while much is known about activation of CD8+ T cells via direct presentation pathway, participation of cross-presentation pathway in generating CTL responses against viral antigens is still poorly understood.

1.4 Antigen processing

Antigen is defined as any substance that can be recognized by the adaptive immune system and cause immune responses. Classically, antigens presented on MHC-I molecules and activate CD8+ T cells are generated from endogenous proteins [52, 53], or acquired exogenously from other viral-infected antigen donor cells (cross-priming) [54, 55]. Recent cumulative data have proven that the vast majority of the endogenous proteins are regularly degraded in the cytosol by the ubiquitin-proteasome proteolytic machinery [56-60]. This proteolytic machinery is highly organized and sophisticated system, which degrades the undesirable proteins into small polypeptides and supplies the cellular immune system with peptides of the appropriate quality and quantity [61, 62]. However, processing of cytosolic proteins for loading on MHC-I passes through several stages such as: 1) Ubiquitination, 2) proteasome degradation, 3) endoplasmic reticulum (ER) translocation and MHC-I loading (Figure 1).

For antigen degradation to occur by proteasome, antigens must be ubiquitinated. Ubiquitin is a small, highly conserved regulatory protein (8.5 kDa) found in large quantities in the cytoplasm and nucleus of all eukaryotic cells [63, 64]. Ubiquitination (or ubiquitylation) is attaching of ubiquitin monomers to
Figure 1: Pathways involved in the generation of MHC class I-presented peptides. The endogenous antigens (proteins) generated during virus infection are subjected to the ubiquitin-proteasome pathway of protein degradation. Ubiquitination process involves conjugation of four ubiquitin molecules to proteins destined to be degraded by proteasome. The protein-ubiquitin complexes are then recognized by the 26S proteasome where the ubiquitin chain is removed and the protein is linearized and injected into the central core of the proteasome, where it is digested to peptides. The resultant peptides are transported to ER through TAP system, loaded on MHC-I molecules, exported through protein secretory pathway and utilized in antigen presentation for the activation of CD8+ T cell responses.
MHC-I/peptide complex

Golgi apparatus

Ubiquitin

Proteasome

Peptides

Viruses

Proteins

Nucleus
lysine residues of targeted proteins forming polyubiquitin of 4 ubiquitin molecules [65]. Although most of the ubiquitin targets are short-lived endogenous proteins [57, 66], one can propose that exogenous proteins can also be ubiquitinated since they are processed by the proteasome [67]. The role of ubiquitination is to serve as recognition elements for cytosolic 26S proteasome complexes.

The 26S proteasome, known as the constitutive proteasome, is an ATP-dependent multi-subunit proteolytic system composed of two complexes: the catalytic barrel-shaped 20S core which is flanked by two 19S-regulator caps responsible for the binding and unfolding of ubiquitylated substrates. 26S proteasomes degrades ubiquitinated proteins as part of their cellular regular function and play a critical role in the generation of the majority of MHC-I- peptides [68]. This was evident after experiments where treating antigen presenting cells with reversible (MG132) [69], or irreversible (lactacystin) proteasome inhibitors [70] resulted in a profound decrease in induction of CTL responses and a reduction on the number of MHC complexes.

The crystal structure of the 20S proteasome revealed a stack of four rings, each containing either seven α (α1-α7; the outer 2 rings) or seven β (β1-β7; the inner 2 rings) subunits, with the rings arranged in the order αββα [71]. The protease inhibitor–binding studies indicated that the inner β subunits bear the active proteolytic centre of the 20S proteasome. Interestingly, only three out of the seven β subunits β1, β2, and β5, located in both of the two heptametric β-rings are the active hydrolyzing units [72]. Proteasome inhibitors such as lactacystin and epoxomycin have been shown to block the catalytic site of active β subunits via binding to β subunits-N-terminal threonine residue [73, 74].

The three active proteolytic sites or subunits of proteasomes differ markedly in their preference in cleavage site usage. Consequently, the proteolytic activities of β1, β2, and β5 are referred to as caspase-like, trypsin-like, and chymotrypsin-like activities, respectively [75-77]. The caspase-like site cleaves preferentially after acidic residues, and the trypsin-like site cleaves after basic residues, whereas the chymotrypsin-like site cleaves peptide bonds after hydrophobic residues [78]. It has been documented that MHC-I molecules typically bind peptides that have hydrophobic or basic residues at the C-terminus, suggesting the importance of the trypsin and chymotrypsin-like activities in MHC-I-peptide complexes generation [79].
After proteasomal degradation, the resultant peptides are most of the time about 3-22 amino acids length [80]. The generated long peptides exist in the cells for short time and undergo further trimming by the cytoplasmic aminopeptidases and/or within the ER by a newly discovered aminopeptidases and endoplasmic reticulum aminopeptidases I (ERAP1). These proteolytic peptidases trim extra amino acids off the longer precursors allowing for the correct size of amino acid residues, to transport to ER via transporter associated with antigen presentation (TAP) and bind MHC molecules [81-83]. It has been demonstrated that peptides, which fit in MHC-I binding groove, are 8-10 residues long [84, 85].

Another important element related to the proteasome is an immunologically defined enzyme known as the immunoproteasome. Upon virus infection that induces IFN-γ, an immunomodulatory cytokine secreted by activated Th1, CTL, and NK cells, key changes occur to the proteasome that are associated with IFN-γ induction. As a result, the proteolytically active β-subunits designed β1 (δ), β2 (MC14, Z) and β5 (MB1) of the constitutive proteasomes are replaced by inducible subunits named β1i (LMP2), β2i (MECL-1), and β5i (LMP7) during the assembly of 20S proteasome. This newly formed proteasome is referred to as immunoproteasome [86]. Remarkably, immunoproteasome are constitutively expressed in some tissues, like spleen, thymus and lungs [86, 87]. Recently, it has been shown that immunoproteasomes can also be induced in hepatocytes as an early response to virus-induced type I IFN [88].

In contrast to the constitutive proteasomes that have half-life of about 120 h, the immunoproteasomes have shorter half-life of only 21 h and it is independent of the presence of cytokines [89]. Eleuteri et al., has clearly shown that immunoproteasomes enhanced cleavage of peptides after hydrophobic and basic residues and reduced cleavage after acidic residues; as a result, they increased production of peptides sequences that bind preferentially to MHC-I molecules in a manner that would favour their function in antigen processing [77]. Interestingly and in opposite to the previous results, mutant cells that lack LMP-2 and LMP-7 subunits reduced their capacity to cleave peptides after hydrophobic and basic residues, instead, they favoured cleavage after acidic residues and minimize the quantity of peptides for loading MHC-I molecules [90, 91].

The influence of immunoproteasomes on antigen presentation has extensively been studied on viral epitopes. Significant enhancements of CD8+ T cell responses against some viruses have been
connected to the up-regulation of the three inducible immunoproteasomes subunits, LMP2, LMP7, and MECL-1 [92-94]. As compared to constitutive proteasomes, immunoproteasomes create epitope precursor peptides with more extended N-terminal sequences that will enhance TAP transport [95]. On the other hand, immunoproteasome has been shown to interfere with presentation of certain viral epitopes and thus influence immunodominance hierarchies of antiviral CD8+ T cells.

Immunodominance is an important immunological phenomenon whereby the immune system responds differently to different epitopes generated from a foreign protein. For instance, although immunoproteasomes enhanced presentation of immunodominant LCMV-NP396 and GP33 epitopes, they reduced or decreased the presentation of the subdominant LCMV-GP276 epitope. The previous observation was due to the fact that immunoproteasome led to a destruction of GP276 epitope [96]. In support of the previous observations and by using well-defined CTL clones specific for two murine leukemia virus (MuLV)-derived peptides, Van Hall et al., has found that the presentation of the immunodominant MuLV gag-leader epitope was enhanced, whereas the presentation of the subdominant MuLV env epitope was not affected in the presence of immunoproteasome [94]. Additionally, immunoproteasome played a critical role in determining or shaping the immunodominance hierarchy of influenza virus-specific CD8+ T cell repertoire [97]. Therefore, immunoproteasomes appear to play a vital role in determining and shaping the final CTL responses during virus infection.

After proteasomal degradation, translocation of peptides, whether they are 8-10 amino acids or longer, into the ER lumen is a highly organized process dependent on a specific peptide transport system, called TAP. TAP is a heterodimeric protein complex consisting of the TAP.1 and TAP.2 subunits located in the ER membrane [98]. Several in vitro studies have demonstrated that the TAP system is responsible for transporting the cytosolic-processed peptides to bind MHC-I molecule in the ER [99-101]. Importantly, Shepherd et al. [99], and Daniel et al. [102], have demonstrated that peptide translocation via the TAP system is a highly selective and specific process whereby the N-terminal and C-terminal residues of short peptides are the most important determinants. Particularly, mouse TAP has a strong preference for peptides with hydrophobic C-terminal amino acids [103, 104]. TAP preferentially translocates peptides of 8-12 amino acids in length, although it also handles peptides of up to 40 amino acids [105, 106].
It has been shown that TAP−/− cells are inefficient in generating MHC-I/peptide complexes, thus limiting CTL activation [107, 108]. Generally, up-regulation of TAP expression is positively influenced by treatment with IFN-γ, which is an important cytokine for adaptive immunity [109]. Notably, several viral proteins such as herpes simplex virus (HSV) ICP47 protein, human cytomegalovirus (HCMV) US6 protein, and EBV BNLF2a protein have been shown to block TAP-mediated peptide transport to inhibit antigen presentation to cytotoxic T-cells [110]. In agreement with these findings, Kurts and colleagues have shown that cross presentation of exogenous OVA was diminished in the presence of the TAP inhibitor US6, a human cytomegalovirus protein [111].

In the ER membrane, stable MHC-I/peptide complexes are formed. The initial process, involving folding and assembly of MHC-I molecules and peptides is a highly sophisticated and controlled process regulated by various molecular chaperons such as calnexin, calreticulin and tapasin [112]. After peptides are loaded on MHC-I molecules in the ER, the MHC-I-peptide complexes are then transported to the cell surface for display and recognition by CD8+ T cells [56]. Brefeldin A (BFA) has been shown to interfere with protein transportation through the Golgi apparatus and blocks the presentation of newly assembled MHC-I/peptide complexes [67, 113-115]. Generally, endogenous and exogenous proteins can provide peptides that go through this system of antigen processing, as we will discuss later.

1.5 Antigen presentation

1.5.1 Direct presentation

Traditionally CD8+ T cells recognize viral peptides generated during the degradation of endogenously synthesized viral proteins through a process called the direct antigen processing and presentation pathway (Figure 1). In this process, the antigens are generated, processed, and presented by the infected cells as MHC-I/peptide complexes. MHC-I molecules principally function to express endogenous antigens derived from viruses to trigger CD8+ T cell responses. Although all nucleated cells can express MHC-I molecules and activate CTL responses, priming or initiation of naïve virus-specific CD8+ T cells responses requires the action of pAPCs, like DCs and MO which can cross- or directly present the antigens
of interest [54, 116]. This seems to be important because it is now appreciated that direct presentation may not be optimally utilized in inducing CD8+ T cell responses against tumors that do not reach the lymph nodes [117]. Furthermore, it may be inefficient against viruses such as human cytomegalovirus (HCMV) and herpes simplex virus (HSV) that have evolved various strategies to inhibit the direct presentation pathway and prevent the display of viral peptides [110, 118]. Therefore, cross-presentation appears to be the main responsible mechanism for inducing CTL responses if direct presentation cannot be initiated. However, new evidences are accumulating that show both pathways can work independently of each other depending on the source and the nature of the antigen provided [54, 67, 119]. Thus, a key factor in antiviral responses is the balance between the direct and cross-presentation pathways, which could be determined by the nature of the antigen itself (figure 1).

1.5.2 Cross-presentation

As highlighted above, CD8+ T cells can also be primed or activated by pAPCs presenting peptides generated from exogenous antigens through a sophisticated mechanism called cross-presentation [54]. The cross-presentation pathway is carried out by cells of haematopoietic originate professional antigen-presenting cells (pAPCs) like DCs and MØ. These cells possess an extraordinary capacity to phagocytose antigens in endosomes or phagosomes whether it is in a soluble or cell-associated form for MHC-I-processing. Importantly, these pAPCs can express different arrays of co-stimulatory molecules such as CD80, CD86, and CD40 required for CTL priming upon triggering with certain stimuli derived from foreign pathogens [54, 120] (figure 2).

In vivo cross presentation leads to cross-priming. The cross-priming phenomenon was first reported in 1976 by Bevan when he showed that the host CTLs were elicited against the minor histocompatibility antigens of grafts transferred from donor allogenic mice [121, 122]. Accordingly, he termed the resultant phenomenon of T-cell priming as ‘cross-priming’. It was later understood that cross priming refers to acquiring exogenous antigens and presenting them on MHC I.

Several years later, reports demonstrated that cross-presentation can occur in vivo under physiological conditions resulting in activation of naïve T cells [54, 123]. As one of the earliest
**Figure 2. Cytosolic pathways of cross-presentation.** During cross-presentation pathway, exogenous antigens derived from different sources (infected or tumour cells) are taken up by pAPCs, (dendritic cells or macrophages) and processed mainly in the cytosol by proteasomes into several peptide fragments. Phagosomes acquire MHC class I, TAP, Sec61 and other ER molecules through fusion with the ER. Antigen are internalized into phagosomes and then exported (possibly by Sec61) to the cytosol and processed by proteasomes and loaded on MHC-I molecules via one of two pathways. 1) phagosome-to-cytosol-to-ER pathway in which the resulting peptides are transported by the transporter associated with antigen processing (TAP) into the lumen of endoplasmic reticulum (ER), loaded on MHC-I molecules, and exported to the cell membrane through the protein secretory pathway for presentation to T cells. 2) phagosome-to-cytosol-to-phagosome pathway which is a postulated mechanism in which the resulting peptides are re-imported into phagosomes by TAP, where they bind MHC class I molecules in the phagosome and exported to the surface via unknown mechanism.

pAPCs; Professional antigen presenting cells, ER; Endoplasmic reticulum, MHC-I; Major histocompatibility class I molecules, Sec61; ER membrane protein translocator (translocon).
Proteasome Peptides
MHC-I/peptide complex
Nucleus
Golgi apparatus
Sec61
Phagosome
Exogenous antigens
MHC-I/peptide complex
TAP
pAPCs
Proteasome
Sec61
Peptides
TAP
ER
Nucleus
observations, Wraith et al., has shown an interesting observation where he found that immunization of mice with purified nucleoprotein from X31 (H3N2) influenza A virus could efficiently enhance CTL responses and protected the mice from a consequence lethal influenza A infection [124]. Similarly, Staerz et al., has demonstrated the capacity of exogenous OVA soluble proteins to induce CTL responses in vivo and in vitro [125]. This phenomenon was further supported by the observation that the generation of detectable peptide-MHC-I complexes required up to 6 h when soluble OVA protein was used as antigens [126]. Providing OVA proteins as a cell-associated antigenic form could also trigger CTL responses in vivo [127]. Moreover, Harding and Song have documented clearly that Latex-OVA (particulate antigens) has high capacity to elicit CTL responses than soluble OVA [128]. In this study, they have found that Latex-OVA was at least 100- to 1000-fold more efficient at cross presentation than soluble OVA [128]. Several additional studies confirmed this observation when comparing particulate antigens to soluble antigens in cross-presentation [37, 129, 130].

Based on extensive studies done during the last three decades, there is strong accumulating evidence to suggest that all cell-associated antigens including apoptotic [131, 132], necrotic [133], opsonized tumour cells [134, 135], immune complexes (IC) [136, 137], LyUV (lysis followed by UV treatment)- cells [55, 67], virus like particles (VLPs) [138], antigens coupled to beads [129] and fragments of live cells [139] are efficient antigenic forms for studying cross-presentation.

Generally, cross-presentation of viral antigens represent an efficient mechanism to induce immune responses due to several factors: first, it enables pAPCs to present viral antigens derived from viruses that do not infect them directly [140, 141]; second, it is critical to transport viral antigens from the periphery to the secondary lymphoid organs where the pool of naïve CD8+ T cell circulates [22]; third, some viruses might interfere with the direct presentation pathway (e.g. HCMV, HIV, EBV and HSV) and require cross-priming during T cell activation [110]; and fourth, many viruses have cytopathic effect (HIV-1, vaccinia and influenza virus) and can inhibit DC maturation (e.g. vaccinia and herpes simplex virus [HSV]) [142]. Collectively, the work cited above suggest that cross-priming, in which antigens expressed in non-pAPCs need to be taken up by pAPCs, plays an important role in the initiation of CD8+ T cell responses.
1.5.3 Exogenous antigen uptake

MØ and immature DCs (iDCs) are efficient at taking up different antigenic forms ranging from soluble proteins to large cellular fragments from dying cells. In general, exogenous antigens are mainly internalized by such phagocytic cells via different mechanisms including receptor-mediated endocytosis, phagocytosis or macropinocytosis. While endocytosis, phagocytosis are responsible for internalizing large particulate materials such as debris from dying cells, macropinocytosis is used for the uptake of soluble and small antigens from the surroundings [143, 144]. Interestingly, Krysko et al., has reported that apoptotic cells were taken up via phagocytosis while necrotic cell debris were entering via macropinocytosis in a mouse MØ cell line [145, 146].

With the aid of specific inhibitors that interfere with receptors or the mechanisms of engulfing, it has been shown that phagocytic cells can utilize vast arrays of pathways to target exogenous antigens to MHC-I pathway. Blocking certain phagocytic functions with agents like cytochalasin D, F-actin cytoskeleton polymerization inhibitors [147, 148] or Fucoidin A, scavenger receptor class-A inhibitor [149, 150] resulted in profound inhibition in the cross-presentation of particulate antigens [67, 128]. With regard to the latter, a recent in vivo study has demonstrated that SR-A−/− mice could initiate antiviral CTL responses and cross-prime CD8+ T cells with cellular antigens or molecular chaperone-peptide complexes [151]. This would indicate the presence of other uptake mechanisms could compensate the lacking of SR-A. The functional macropinocytosis process is also a useful mechanism to uptake small fragments from dead cells. Blocking macropinocytosis using Dimethyl amiloride (DMA) resulted in the complete inhibition of the cross-presentation of apoptotic and necrotic cells [152].

Albert et al., showed distinct receptors used by DCs and MØ to recognize and uptake dying cells. While DCs used CD36 and αβ5 integrins, MØ use only αβ3 integrins [153]. However, other related observations reported that the phagocytosis of dead cells and cross-presentation of cell-associated antigens was normal in CD8α− DCs obtained from CD36-deficient mice or αβ5/β3 double KO mice [154, 155].

Other receptors such as Mannose receptor (MR) has been detected in several cell types including DCs and MØ. Recent studies by Burgdorf et al., have shown clearly that MR expressed on DCs and MØ was essential for the uptake and cross-presentation of soluble OVA protein but not cell-associated OVA in
vitro and in vivo. Interestingly and in contrast to DCs, even thought MR−/− MØ internalized large amount of soluble OVA protein, possibly via other mechanisms, it failed to cross-present OVA protein. These observations suggested strongly that cross-presentation of soluble OVA by MØ was entirely MR-dependent [156, 157].

Employing antibody-mediated antigen targeting to some receptors by coupling them to receptors-specific antibodies strongly augments cross-presentation by DCs and MØ. For instance, it has been shown that targeting antigens via Fcγ receptors has two main advantages: very efficient up-take of the antigen (1000-fold higher) and maturation of the DCs [158], and robust CTL responses can be induced by DCs loaded with Ab-bound antigen in very low concentrations [159]. Related to this targeting concept, Rodriguez et al., showed that BM-DCs and D1 dendritic cell line but not MØ could uptake immune-complexed OVA (OVA-ICs) by utilizing FcγR and activate B3Z hybridism (OVA-specific CD8+ T cells) [126]. In addition, some other cell-associated antigens were also efficiently cross-presented when targeted to FcγR [158, 160, 161]. Considering DEC205 receptor, which is expressed at high levels on lymphoid tissue DCs (CD8+DEC205+DCs), targeting anti-DEC205-complexed with OVA proteins to the DEC-205 endocytic receptor greatly enhances the efficiency of antigen presentation and CTL proliferation in vivo and in vitro [162, 163].

Another antigen targeting approach has been tested to target DCs to modulate immune responses by modifying the antigens and allow them to bind specific receptors. With regard to this, DC-SIGN transgenic mice that express human DC-SIGN (DC-specific C-type lectin receptor) under the control of CD11c promoter has been used recently by Singh et al., to address the contribution of DC-SIGN receptor in cross-presentation. Interestingly, Singh et al., has found that targeting of glycosylated OVA proteins to DC-SIGN resulted in significant enhancement of OVA cross-presentation and OT-I T cells activation, 10 fold more than soluble native OVA proteins [164]. Overall, cross-presentation can be enhanced by either modulating and targeting antigens to specific set of receptors or providing them as particulate antigens which are more efficient than soluble antigens in generating MHC class I epitopes [37, 128].
1.6 viral models of antigen cross-presentation

Exogenous proteins or antigens (i.e. not synthesized by the pAPCs) constitute one of most important sources of peptide ligands for priming CD8+ T-cell responses. A complete understanding of the antigenic sources and its characteristics is critical for analyzing the cross-presentation pathway. Generally, in vivo, the antigens for cross-presentation can be supplied from virally-infected cells [165], tumours [166], or self-tissues to maintain cross tolerance [167].

Importantly, the cross-presentation of viral antigens supplied by infected dying cells has been reported in vitro and in vivo for many viruses include vaccinia virus (VV) [67, 168, 169], Epstein-Barr virus (EBV) [140, 141, 170], human cytomegalovirus (HCMV) [171-173], HIV-1 [174], and herpes simplex virus (HSV-1) [175, 176]. Basta et al. [67], and Dunbar et al. [55], have shown that LCMV-NP could be efficiently cross-presented in vivo and in vitro when it was supplied from dying cells such as apoptotic, necrotic, or LyUV-treated HEK-NP. Virus antigens can also be provided from live cells as evidenced by the fact that live, vaccinia virus-infected cells could significantly supply antigens for cross-presentation [108]. Furthermore, several models have been employed to address cross-presentation efficiencies of different viral antigens. Beside using several transgenic mice, xenogenic or allogenic [67], syngeneic [177], or MHC-I-deficient cells [178] expressing viral antigens have provided further insight into the ability of exogenous viral antigens to activate CTL responses in vivo and in vitro. The following summarizes data obtained in various virus models.

1.6.1 Lymphocytic choriomeningitis virus (LCMV)

A previous study carried by Zinkernagel group failed to show any significant data with regard to cross-presentation capacity of LCMV proteins [179-181]. In contrast, Basta et al., were able to detect both cross-presentation, and cross priming when they employed stably transfected human embryo kidney cells (HEK293) with LCMV-NP and used them to address the same question. Interestingly they found that LCMV-NP expressed as the full NP in the cytosol of antigen donor cells was efficiently cross-presented by MO [67]. This observation was further supported by Dunber et al., have demonstrated remarkable
capability of HEK-NP to induce LCMV-NP-specific CTL responses that efficiently enhanced viral clearance in vivo. The introduction of HEK-NP cells as a source of NP antigen for the cross-priming pathway caused the immunodominance hierarchy to shift; NP396 has become the single most immunodominant epitope [55]. Cross-priming capacity of LCMV-GP was further addressed by Ochsenbein’s group who utilized tumour cells transfected with the LCMV-GP. Interestingly, they found that cross-priming of LCMV-GP was dominated the immunodominant epitopes LCMV-GP33 [182].

Recently Zinkernagel et al., [177] have found that LCMV-GP expressed as a C-terminal truncated (modified to escape degradation), non-cleavable construct by tumour cells could efficiently cross-prime specific CTL responses in vivo better than wild-type LCMV-GP. The cross-priming of LCMV-GP was shifted toward the immunodominant epitopes LCMV-GP33 as evidenced by induction of high percentages of LCMV-GP33-specific CTL responses [177]. In a separate study, GP33 peptide was expressed as an epitope in virus-like particles which allowed for an efficient cross-presented to occur [138]. This implies that virus epitopes may be able to cross-present with various efficiency depending on the mode that they are expressed before uptake by pAPCs. It is important to note that none of the investigators in the above cross-presentation models have examined the contribution of several epitopes after virus infection in vivo and in vitro at the same time.

1.6.2 Polio virus (PV)

Cross-priming and generation of Polio virus-specific CD8+ T cell responses has been demonstrated by Sigal et al., who employed transgenic mice that express human receptor for poliovirus (PVR). He generated bone marrow chimeras (B6cPVR) in which pAPCs were of B6 origin and, therefore, cannot be infected with PV, instead only non-bone-marrow-derived cells were susceptible. Upon challenging with PV-OVA, these chimeric mice generated strong anti-OVA CTL responses, but they failed to do so when infected with a recombinant polio expressing an irrelevant protein [183]. However, Recently, Freigang et al., has argued that B6 mice could induce strong CTL responses when infected with high PV dose, excluding the possibility that exogenous virion proteins were cross-presented by non-infected pAPCs [184].
1.6.3 Adenovirus (Ad)

Adenovirus (Ad) has been shown to infect wide variety of cell types including pAPCs [185]. Ad is a widely used as a vector due to its ability to accommodate relatively large segments of foreign DNA [186]. However, to better understand the contribution of Ad viruses in cross-presentation, Prasad *et al.*, have generated recombinant adenoviruses (rAdV) that express influenza A-NP (IAV-NP) under the control of a promoter that is either active in all cell types or in a tissue-specific manner. Therefore by changing the route of immunization, they could determine the cross-presentation ability of adenovirus (AdV)-encoded antigens. IAV-NP-specific CD8+ T cells were strongly primed with recombinant adenoviruses (rAds-NP) vectors that only infected non-immune cells. Thus, the most likely suggestion is that CD8+ T cells were primed with IAV-NP cross-presented by pAPCs [187]. So it appears that Ad proteins can be cross-presented and activated CTL responses.

1.6.4 Vaccinia virus (VV)

Vaccinia virus has been reported to inhibit DC maturation and causes extensive apoptosis of infected cells [188, 189], yet it is highly immunogenic. Using recombinant vaccinia virus encoding the influenza matrix protein as model vector (VAC-MP), Larsson *et al.*, have found that both apoptotic and necrotic vaccinia-infected cells are sources of antigens for cross presentation. He demonstrated that DCs were more efficient than monocytes in stimulating CD8+ T cells to secret IL-2 and IFN-γ, proliferate and develop into cytotoxic effectors T cells [190]. In another related study, Basta *et al.*, used rVV expressing human cytomegalo virus (HCMV) proteins US2 or US11 glycoproteins that interfere with newly synthesized MHC-I molecules in the infected cells [191]. Because the data showed that the responses were lower but not absent, it was suggested that both direct priming and cross-priming are involved in the activation of local and systemic VV-specific CD8+T cell responses following i.p. infections [192].

1.6.5 Influenza A virus (IAV)

It has been shown that purified influenza A virus nucleoprotein (IVA-NP) could efficiently enhance CTL responses and protected the mice from a consequence lethal influenza A infection [124].
Later, a study by Albert et al., employed apoptotic IVA-infected monocytes as a model ADCs suggested that IVA antigens gained access to MHC-I of DCs but not MØ and efficiently cross-primed IVA-specific CD8+ T cells [131]. Cho et al., have addressed the cross-presentation potential of IAV by comparing the immunogenicity of infectious vs. heat-inactivated IAV. Interestingly, the primary CTL responses to heat-inactivated virus were sufficiently strong to be detected and that the responses include many of the peptides recognized by CTLs induced by infectious virus. Moreover, heat-inactivated IAV induced a more balanced CD8+ T immunodominance hierarchy than infectious virus [193].

1.6.6 Epstein - Barr virus (EBV)

Epstein-Barr virus (EBV), a human gamma herpesvirus encodes six nuclear proteins EBNAs 1-6 and two membrane proteins LMPs1 and 2 [194]. This virus causes persist infection and gain efficient strategies to escape the immune system by; 1) blocking the proteasome processing with EBNA1 [195], and 2) inhibiting TAP-mediated peptide transport via binding of BNLF2a to TAP and limit the supply of peptides to the ER and, therefore, impair the maturation of MHC class I molecules [196]. Accordingly, it has been suggested that to establish effective immune responses against EBV, virus proteins should be processed and presented via cross-presentation pathway. Although EBNA-1 could avoid CD8+ T cells detection by preventing its own degradation and generation of EBNA-1 epitope by proteasome, Blake et al., have shown that EBNA-1specific CTL have been identified in some individuals. Furthermore, it has been shown that full length EBNA-1 protein can be processed and presented by DCs to activate EBNA-1specific CD8+ T cells [197]. Notably, DCs could efficiently cross-present several EBV latent antigens (proteins) such as EBNA-1, EBNA-3A and LMP-2 and activates CD8+ T cell responses [140, 141].

1.6.7 Human immunodeficiency virus (HIV)

It has been reported that HIV induces apoptosis of infected and non-infected cells, in vivo and in vitro [198]. It is intriguing that HIV-1 Nef protein can evade CTL detection by down-regulating the expression of MHC-I molecules by divert the trafficking of MHC-I molecules directly from the Golgi network to an endocytic compartment before they reach the cell surface [199, 200]. Based on facts
mentioned earlier, cross-presentation seems to be crucial in generating efficient CTL responses against HIV proteins.

Bahardwaj et al., have shown that DCs could internalize and cross-present antigens from apoptotic CD4+ T cells, either infected with life or chemically inactivated non-replicating HIV-1. Moreover, when immature DCs incubated with HXB2/3gpt cell line (cell line that produces Gag, Env, Tat, Rev and Nef proteins, but not detectable viral reverse transcriptase, mature virions or infectious virus) in presence of maturation stimuli they could significantly induce HIV-specific CTL responses [174].

Furthermore, Shi et al., have shown that immunization of B6 mice with HIV-gp120 bound to latex beads or with allogenic 3T3 cells transfected with HIV gp120 resulted in priming of CD8+ T cells responses to a cell-associated HIV gp120 antigen [201]. In another study, Monocytes-derived DCs could efficiently uptake yeast-derived HIV-1 Gag virus-like particles (VLPs) and induce Gag-specific CD8+T cells responses in vitro [202, 203].

1.6.8 *Herpes simplex virus 1 (HSV-1)*

HSV-1 infects and replicates in many cell types and causes life-long latent systemic infection [204]. Herpes simplex virus (HSV) ICP47 protein is well known to bind and inhibit the function of TAP, consequently, interferes with the peptides translocation into the ER and loading on MHC-I, thereby preventing CD8+ T cell recognition [205, 206]. Additionally, HSV has been shown to induce apoptosis to DCs and decrease their viability in culture [207, 208]. Therefore, the ability of pAPCs to take up dead or dying HSV-infected cells and cross present them to cognate CTL may be the key to induce effective immune responses against HSV. With this regard, Bosnjak et al., have shown that HSV infection of human MDDCs resulted in apoptosis and subsequent phagocytosis by uninfected DCs, which, in turn, stimulated HSV-specific CD8+ T cells by cross-presentation [209].

Jermo et al., have utilized an elegant system in which HSV1-ΔgH, HSV-1 lacking gp H, a glycoprotein that is essential for viral cell entry [210] was employed to address the cross-presentation capacity of HSV-1 proteins. HSV1-ΔgH was generated using a complementary cell line that provided gp H for the virion production. Therefore, the single round of infection will result in production of HSV1-ΔgH
particles lacking the gp H gene but containing gp H proteins in their envelope. These particles were used to inoculate bm1 splenocytes, which then were co-cultured with the respective DCs population and gBT-I cells. However, bm1 splenocytes, which cannot directly present HSV gB498–505 epitope, was used as ADCs and the virus could synthesis all HSV-1 proteins except for gp H glycoproteins, thus unable to produce infectious virions. As a result of these in vitro conditions, only the CD8+DCs could perfectly cross-present HSV-1 gp B proteins from HSV1-ΔgH-infected splenocytes and induce gBT-1-specific T cells [176].

Collectively, results mentioned in the previous sections imply strongly that virus proteins can gain access to MHC-I via cross-presentation mechanisms even for those viruses whose proteins interfere and block the endogenous MHC-I presentation pathway.

### 1.7 The characteristics of cross-presented antigens

Based on several studies used diverse experimental protocols, it has been concluded that cellular antigens are cross-presented much more efficiently than soluble antigens in vitro and in vivo [37, 211]. In an elegant system, Norbury et al., have employed OVA protein provided in different constructs to discriminate between the impacts of stable vs. degradable-OVA protein on cross-priming. They used rVV as a vector to express different forms of stable vs. degradable-OVA protein on cross-priming. They used rVV as a vector to express different forms of OVA antigens with different antigenic and stability properties. Interestingly, out of all constructs used only allogenic P815 infected with rVV expressing a metabolically stable chimeric protein (GFP-S) could work as substrates for cross-priming and induced OVA-specific CTL responses. In such investigation, blocking the proteasome activity by treating the rVV-, or IVA-infected ADCs with lactacystin enhanced the cross-presentation capacity of OVA and IVA-NP proteins, respectively [178].

In another model, Basta et al., have worked to study the viral proteins form responsible for cross-priming CTL responses. Basta et al., have generated two models that express LCMV-NP as stable proteins (designed HEK-NP) or unstable ubiquitin-LCMV-NP fusion protein (designed as HEK-Ub-NP) which is rapidly degraded by the proteasome in the ADCs. Upon challenging B6 mice with these two xenogenic
transfectants, they noticed that only HEK-NP but not HEK-Ub-NP efficiently supplied LCMV-NP and induced NP396-specific CTL activation. Therefore, the results strongly suggested that the long-lived form of NP is a better source for cross-priming than the unstable ubiquitin (Ub)-NP fusion protein [67]. In supporting to the previous studies, Shen and Rock have shown that depletion of protein antigens from cell fractions resulted in almost complete inhibition in their cross-priming activity [211]. Collectively, the previous observations strongly suggest that cross-presentation requires transfer of metabolically stable, long-lived proteins and it is enhanced in the absence of proteasomal degradation of protein antigens. Besides the proteins stability, the abundance of proteins in the ADCs seems to be another important factor during cross-presentation.

Considering other forms of antigens it appears that minimal peptides expressed from minigenes in ADCs (human cell line that produced cytosolic OVA257-264 from a transfected plasmid) [178] or defective ribosomal products (DRiPs) [67] failed to be cross-presented due to their rapid degradation inside the antigen donor cells. In addition, short lived proteasomal products generated in the antigen-carrying cells are inefficient in cross-priming [178]. Moreover, T cell epitopes that are located in signal sequences of proteins destined for cell membranes are also inefficient at cross-presentation due to their rapid degradation [212]. On the other hand, and based on microscopic study, proteasomal products (the 9-mer peptides) have been shown to be transferred from the cytosol of one cell into neighbouring cell through gap junctions [213]. The later observation might suggest a possible transferring of 8-10 amino acids peptides from one cell and presented on another cells.

Binder and Srivastava have demonstrated that peptides coupled and chaperoned by heat-shock proteins (Hsp) have been shown to be efficiently cross-presented [214]. Of a great importance to notice that depletion of Hsp [214], using Hps90 inhibitors [67], or using Hsp1-deficient cells [215] resulted in remarkable decreased in some antigens cross-priming liability. The study examining cross-presentation of LCMV-NP has shed light on a possible role of Hsp90, a member of the Hsp family, in enhancing cross-presentation of cytosolic proteins [67]. This observation was based on the significant reduction of LCMV-NP cross-presentation when the chaperone functions were interfered with using the Hsp90 family inhibitors, geldanamycin and herbimycin [67]. Another group investigating the in vivo consequences of reduced
expression of Hsp90 in heat shock factor 1 knock-out mice have also proposed a role for Hsp90 in cross-presentation [215]. Along this line of evidence, another study found that the heat treatment of tumour cells (ADCs) permits enhanced cross-priming [216]. By investigating the difference in the proteomic profile of heat-treated and unheated tumour bodies, the authors found a significant over-expression of Hsp70 that may have contributed to this enhanced cross-priming [216].

Against this observation, recent data show that the ER chaperon GRP94 (gp96) gene knockdown showed no effect on in vivo cross-priming of viral antigens supplied from HEK-293 cells infected with VV, IAV or vesicular stomatitis virus (VSV) [217]. Furthermore, Binder and Srivastava have shown that lysates depleted individually of gp96, Hsp90, Hsp70 or calreticulin had no effect on cross-priming and were equally capable of eliciting strong CTL responses [214]. Altogether, these findings point to a possible role of Hsp in cross-presentation that may aid cell-associated proteins during cross-priming. However, it seems that there are different factors could influence the role of proteins in the cross-presentation pathway: the amount, steady-state levels, sub-cellular location, and/or physical form (e.g. membrane-associated versus soluble) of the proteins.

1.8 Cellular requirements for cross-presentation

Cross-presentation is achieved by a bone marrow-derived APC [117, 218]. Efficient presentation of exogenous antigens was originally attributed to MØ [219]. However, other APC, such as DCs [67, 220], B cells [221, 222], and neutrophils [223], have also been shown to possess the ability to cross-present antigens. Interestingly, as we will discuss here, it appears that the ability of pAPCs to cross-present antigens may vary and depend on different factors such as: (i) their ability to acquire antigens from the external environment; (ii) their ability to travel between the sites of infection and lymphoid organs; and (iii) their ability to express MHC-I/peptide complex and co-stimulatory molecules (CD80 and CD86) on the surface. Below essential data are summarized that examine the involvement of various cell types in cross-presentation of exogenous antigens.
1.8.1 Dendritic cells (DCs)

DCs are the most potent and crucial pAPCs for regulating CD8+ T cell responses for both immunity and tolerance. They are distinct populations, which develop and differentiate from circulating bone marrow-derived precursors and become resident in peripheral tissues. Generally, DCs are a heterogeneous group of cells distributed all over the body and can be distinguished \textit{in vivo} based on their progenitors, surface markers, and distribution [224, 225].

Based on studies done on mice, DCs are broadly categorized into two types: the conventional DCs [(cDCs), CD11c^{high}, MHC-II^{high}], and plasmacytoid Dcs [(pDCs), CD11C^{low}, MHC-II^{low}, and B220^+]. Conventional DCs (cDCs) are further divided into two groups based on their origin. The first group, derived from blood and that reside in peripheral lymph nodes and the spleen, comprises three subpopulations based on the differential expression of CD4 and CD8α markers (CD4^+, CD8^+, and CD4^-CD8^-) [224, 226, 227]. The second is derived from tissues that include migratory epidermal langerhans cells (LCs) and dermal DC [227-229]. Although each subset of cDCs serves a unique role in the immune system, their primary role in the immune system appears to be in antigen presentation and induction of specific immune responses.

It has been concluded that, in the peripheral tissues, DCs exist as immature cells and exhibit both high levels of antigens uptake capacity through different routes, and low CD8+ T-cell activation potential [230, 231]. Interestingly, once these cells meet the inflammatory stimuli in the periphery, they undergo maturation and differentiation into mature dendritic cells. During this process, DCs migrate to the local lymphoid organs and increase expression of MHC-I & II, adhesion, and co-stimulatory molecules such as CD80, CD86 and CD40, which are crucial for priming naïve CD8+ T cells [133, 153, 232]. Movement of these antigen-loaded DCs from potential sites of inflammation from the periphery towards the CD8+ T cell area of lymphoid organs is highly dependent on the up-regulation of the chemokine receptor CCR7, which is commonly associated with DCs maturation [233, 234].

In general, DCs exhibit higher cross-presentation activities than other pAPCs; their intracellular machinery is more adapted to process and present exogenous antigens in the context of MHC-I molecules rather than degrade and destroy them [116, 235, 236]. This could be explained based on the observations that DCs have limited phagosomal acidification and degradation of ingested antigens than MØ, which
protects antigenic peptides [236]. Recently it was demonstrated that DCs recruit more nicotinamide adenine dinucleotide (NADH) oxidase to the phagosome membrane, which results in an elevation of the phagosomal pH (alkalinization), thus limiting the hydrolytic activity of phagosomes [237].

Conversely, disruption of the endocytic compartmental pH by using diphenylamine iodonium (DPI) (an inhibitor of NADH oxidase) results in the suppression of cross-presentation in BM-DCs [237, 238]. Additionally, DCs have been shown to express cathepsin S inhibitors cystatin C [239-241] which might also play role in enhancing antigen cross-presentation by DCs. Supporting this theory, recent reports has shown that mature DCs could internalize high quantity of Ab-bound Ovalbumin (IgG-OVA) and conserve it for long time in what is called lysosome-like compartments. These storage compartments serve as an antigen source for continuous supply of MHC-I ligands to maintain CD8+ T cell cross-priming. In such experiment, significant proliferation of OVA-specific naïve transgenic OT-1 cells was observed even 14 days after injection of DCs loaded with IgG-OVA [242].

However, expression of exogenous antigens on MHC-I (cross-presentation) is a tightly regulated process during DC maturation in vitro. For example, it has been shown that intermediate BM-DCs (int-DCs) have higher cross-presentation efficacy than immature and mature BM-DCs prepared under the same conditions in vitro [130]. This is because of the fact that the int-DCs have a high phagocytic capacity, transport high amount of antigens into the cytosol, and specifically up-regulate the level and/or activity of certain proteasome subunits such as LMP2, LMP7, PA28α and PA28β [130, 243]. These observations interestingly raise the possibility that several factors may be involved in regulating cross-presentation during maturation and differentiation stages of pAPCs in general.

### 1.8.1.1 Conventional DCs (cDCs)

It is well known that the initiation of CTL responses requires activation provided mainly by DCs. Depletion of cDCs from draining lymph node (DLN) by anti-CD11c magnetic beads completely inhibited the elicitation of CD8+ T cells against infection with herpes simplex virus (HSV), whereas the depletion of MØ , or B , had no effect [167, 244]. In addition to priming, cDCs can also induce peripheral tolerance by presenting self-antigens to and energizing autoreactive T cells [245, 246].
With regard to cross-presentation, cDCs have shown high capacity to cross-present antigens derived from different sources including apoptotic, necrotic cells [247, 248], soluble antigens [249], immune complexes (IC)[130], antigen bound to latex beads [148], antigens from healthy cells [139], antigens from vaccinia-infected cells [183, 250], and virus-like particles (VLPs) [251]. Depletion of cDCs from DTR-CD11c⁺ transgenic mice using DT (diphtheria toxin) demonstrated an essential in vivo role for CD11c⁺DC in the priming of CTL responses to cell-associated antigens (cross-priming) [252]. However, recently, MØ have been shown to express the CD11c marker [253-257], which might have been depleted upon treatment with DT. Probst et al., has shown that a single injected of DT into DTR-CD11c⁺ transgenic mice resulted in complete and protracted depletion of marginal zone MØ (MZMØ) and metallophilic marginal zone MØ (MMMØ)[258]. Therefore, it is difficult to attribute cross-presentation to only DCs since MØ may also be involved in the cross-presentation of cell-associated antigens in vivo.

Although almost all of the DCs subsets have a similar capacity to uptake antigens from the surrounding, they vary substantially in their antigen-handling properties during cross-presentation. It has been shown in several studies that CD8⁺DCs cross-presented different antigenic forms (soluble, cell-associated, or antigen bound to latex beads) much more efficiently than CD8⁻DCs [148, 259, 260]. These observations suggested that CD8⁺DCs possess specialized machinery to deliver different forms of antigen to the cross-presentation pathway that is expressed poorly by CD8⁻DCs. Indeed, the cross-presentation capacity of CD8⁻DCs can be improved via triggering of FcγR by OVA/anti-OVA immune complexes (OVA-IC) [137], lipopolysaccharide (LPS, a microbial maturation stimuli) treatment [259], or incubation with virus-like particles [261]. Furthermore, double negative DCs (dnDCs) can acquire, under the influence of LPS, a limited ability to cross-present soluble OVA protein in vivo [259]. The later observations have obviously suggested that CD8⁻DCs and dnDCs might turn out to be a highly cross-presenting pAPCs if they encounter pathogens or are stimulated with any of their products.

Based on current knowledge, CD8⁺DCs and CD8⁻DCs are found in lymph nodes (LN) and the spleen, and they do not appear to circulate through the blood or lymph to other secondary lymphoid organs. These observations suggest that CD8⁺ and CD8⁻DCs fulfill their specific immune response by capturing blood and/or lymph-born antigens, or antigens that have been transported and provided by migratory cDCs.
In this regard, skin migratory cDCs (LCs and dermal DCs) play a central role in immune defences against antigens that gain access to the peripheral tissues. Subsequent studies, based on experimental models that allow LCs in vivo tracking, concluded that the principle function of LCs is to carry antigens to the LNs where they are processed and presented by lymphoid tissues-resident cDCs [262-265].

1.8.1.2 Plasmacytoid DCs (pDCs)

The second major type of DCs is Plasmacytoid DCs (pDCs), which are characterized by their ability to produce large quantities of type-I interferon (IFN-α/β) during viral infection [266, 267]. Although mouse pDCs have been shown to prime CTL responses when infected by cytomegalovirus [268], it has been claimed that they are unable to cross-prime naive CD8+ T cells in vivo [269]. Recently, a study has concluded that toll like receptor-ligands or viral stimulation induces efficient cross-presentation leading to in vivo cross-priming of naive CD8+ T cells by pDCs [270]. However, the ability of mouse pDCs to capture, process, and cross-present exogenous antigens has not been thoroughly investigated. The expression of high level of inhibitory molecules and low levels of stimulatory molecules on pDCs’ surface prevents them from priming T cells [271], therefore, they might induce tolerance instead. In a marked contrast, expression of the LN homing receptor CCR7 and co-stimulatory molecules like CD80 and CD40 by pDCs and GEN3 (plasmacytoid cell line) has been reported recently [272].

A recent report by Pucchio et al., have shown that human pDCs could efficiently cross-present antigens generated from UV-inactivated influenza virus. Interestingly, pDCs were more efficient than myeloid DCs (mDCs) at inducing matrix protein (MP)-specific CD8+ T cell proliferation within the first 4-6 hours of incubation, suggesting that pDCs cross-present influenza virus antigen more rapidly and efficiently than mDCs [273].

Moreover, pDCs retained the capacity to cross-present antigens and induce proliferation of influenza A-specific CD8+ T cells even in the absence of type I interferon [273]. Interestingly, Tough et al., have demonstrated that infection with LCMV induced type I interferon production and enhanced cross-priming of soluble OVA [274]. Moreover, both human pDCs and GEN3 (a plasmacytoid cell line) were employed by Lui et al., to study cross-presentation of influenza virus antigens provided from B cell
incubated with formaldehyde-inactivated influenza virus (flu-B cells) as ADCs model. Strikingly and beside their capacity to uptake high quantity of flu-B cells, GEN3 and primary pDC induced a highly significant increase of IAV-mp-specific CD8+ T cells responses as measured by IFNγ secretion [272]. Accordingly, pDCs seem to participate in the cross-presentation either via cross-presenting antigens or secreting type I interferon that enhances the cross-presentation capacity of other pAPCs.

1.8.2 Macrophages (MO)

A related cell to DCs is MO are heterogeneous mononuclear phagocytic cells. Generally, MO provide innate and adaptive immune surveillance for every tissue in the body. They are widely distributed in the body and exist as tissue residents or freely circulating MO. Interestingly, the heterogeneity reflects the variable functions that are carried out by MO according to requirements of the tissues in which they reside [275].

MO differentiate from hematopoietic, self-renewing stem cells in bone marrow through multiple pathways summarized in the following. The first pathway involves some MO populations derived from monocytic cells and their precursors (macrophage colony-forming cells; M-CFCs) originating from myeloid precursors in the bone marrow. The differentiation and maturation of these mediated cells into mature MO is controlled by several cytokines, particularly the macrophage-colony stimulating factor (M-CSF)[276, 277]. M-CSF is a hematopoietic growth factor controlling survival, proliferation, and differentiation as well as other functions of cells of the monocyte/macrophage lineage. It is produced by numerous cell types including monocytes, MO, fibroblasts, endothelial cells, thymic and osteoblasts [278].

The Second pathway involves the release of bone marrow macrophage progenitor cells directly into the circulation where they then migrate into peripheral tissues, and differentiate into mature tissue-specific MO. These populations do not require M-CSF for their maturation and differentiation; instead, they migrate and differentiate into tissue macrophages in response to various cytokines and chemotactic factors [275, 279]. The previous classification was supported in detailed studies done on osteopetrotic mice (MCSFop/MCSFop or op/op mice) that have a mutation in the gene that encodes M-CSF. As a result, tissue
and, particularly, spleen MØ (Sp- MØ) have been classified into two broad groups based on their requirements to M-CSF; the M-CSF-dependent and M-CSF-independent MØ [280-282].

MØ have a broad capacity to capture antigens from the surrounding environment, present it on their surface MHC-I molecules, activate CTL responses and generate strong protective immunity [283]. MØ have been documented to express and use a broad array of surface receptors [e.g. scavenger receptor A (SR-A), CD36, FcR, C-type lectine, mannose receptor (MR), integrin (CR3), CD14, Dectin-1, and CD136] and mechanisms [phagocytosis, endocytosis, and macropinocytosis] in recognizing and engulfing a wide range of exogenous materials [145, 253, 257]. Indeed, the expression of peptide antigens on MHC-I molecules and costimulatory molecules (e.g. CD80, CD86, and CD40) by MØ is required not only for priming of CD8+ T cells, but also for the generation of long lasting memory immune responses. It has been shown that macrophages can play a crucial role in the initiation of naïve CD8+ T cell responses in vivo [127, 284-286].

MØ have been shown to reside in the T-cell-containing regions of the lymphoid organs, where they come in direct contact with T cells [287, 288]. Since they are distributed in both lymphoid and non-lymphoid organs, MØ may have a great opportunity to present the antigens to the circulating lymphocytes. Interestingly, the evidence for migratory properties of MØ has come from direct in vivo studies using fluorescent tracking dye system in which the adoptive transfer of live PKH26-labeled inflammatory MØ rapidly emigrated intact from the inflamed peritoneum specifically to the draining LN [289]. In addition, the CFSE fluorescent labelling system has revealed that subcutaneously injected bone marrow-derived MØ (BM-MØ) can migrate to the draining lymph node, although less efficiently than DCs. In such experiments the s.c. injected MØ required 10-fold more antigens compared with DCs to prime naïve CD8+ T cells to proliferate and mature into both effector and memory cells when they are injected in same numbers [31]. In a related study, during an inflammatory response, the largest pAPCs fraction recruited to the site of injection (intra-peritoneal), 80-90%, were monocytes/macrophages, followed by NK cells and DCs, each constituting 5-10% [290]. Moreover, the use of intravital microscopy to visualize pAPCs-T cell interaction in lymph nodes has demonstrated that antigen specific CD8+ T cells are clustered around CD11c+ and CD11c- antigen-presenting pAPCs [291]. The previous observations would allow us to propose that MØ are
as efficient as DCs in the presentation of antigens during inflammation site and the induction of a specific immune response.

Although most cross-presentation studies have been carried out using DCs (derived from the bone marrow and spleen) [129, 130, 176, 292], MØ (cell lines or BM-MØ) have also been reported to present exogenous antigens in the context of MHC-I molecules [67, 127, 156, 293]. Due to the distribution of MØ throughout the body, along with their phagocytic activity, this enables them to play a critical role in eliminating invasive microbes and in generating strong protective responses [283].

MØ have been shown to cross-present antigens derived from particulate antigens and proteins bound to beads [128], recombinant proteins expressed by bacteria [294], live virus-infected cells [250], soluble antigens[295], and LyUV-treated transfected cells [67]. MØ have the capacity to cross-present cell-associated antigens derived from apoptotic or necrotic cells [247, 248, 293, 296], although it has been shown that MØ use different routes to uptake apoptotic and necrotic cells[145, 146]. In addition, injection of LCMV-GP33-pulsed MØ [31] or MØ incubated with apoptotic cells [293] resulted in the stimulation of naïve CD8+ T cells responses. The ability of MØ cell lines (such as BMA and BMC-2) [67, 295] and primary MØ [293, 295-297] to cross-present antigens has provided insight into the role that MØ play during immune responses.

The fact that MØ fail to cross-present antigens from cell-associated antigens in some studies [30, 247, 248, 298-300] may be due to the fact that they are acting as a degradation machine to eliminate any large exogenous antigens. An extensive study by Delamarre et al., has demonstrated that the lysosomal protease activity are more potent in peritoneal MØ compared to splenic DCs (20-60 fold higher). In addition, the content of cathepsin S, D, B, L, lamp and AEPs are much higher in BM-MØ as compared with BM-DCs (immature and mature), and the lysosomal asparagine endopeptidase (AEP) and cathepsin D (cat D) are remarkably higher in 6-day than in 2-day cultured BM-MØ [235]. Collectively, these observations indicate that MØ have high antigens degradation capacity than DCs, and as MØ become more mature, their degradation property increases. In a related study, the efficient property of degrading antigens in phagosomes of MØ is enhanced by interferon gammas (IFN-γ) which up-regulate productions of toxic substances like superoxide and nitric oxide that act as digestive materials [301].
Based on the above mentioned result, the highly active proteases and toxic mediators in MØ could destroy exogenous antigens and interfere with generation of 8-10 amino acid peptides for loading on MHC-I molecules. On the other hand, the capacity of BM-MØ and BM-DCs to take up antigens from the surrounding environment to induce CTL responses have been demonstrated [250, 293, 302]. As a result, even though MØ have extremely active degradation machinery, they are competent to cross-present antigens as DCs preferentially do. Therefore, it is of a great importance to address the antigen presentation capacities of MØ during differentiation stages to reveal the changes occur in MØ that might determine direct and cross-presentation.

**1.8.2.1 Spleen macrophages (Sp-MØ)**

Similar to BM-MØ, Spleen-resident macrophages (Sp-MØ) show a high degree of heterogeneity in their origins, morphology, surface markers profiles, functions and regional specialization [275]. Sp-MØ are distributed in two regions: i) the red pulp region which contains red pulp macrophages (RPMØ); and ii) the white pulp region which contains the marginal zone (MZMØ), metallophelic marginal zone macrophages (MMMØ) [257, 275]. While MØ distributed in the white pulp region are classified as M-CSF-dependent population, the RPMØ are considered to be M-CSF-independent population [280, 281]. These populations exhibit totally different functions: while RPMØ plays a major role in removing old and dead erythrocytes from the blood circulation, the MMMØ and MZMØ are restricted to involvement in either the innate or adaptive immunity [303].

Insights into the role played by Sp-MØ in eliciting immune responses against some viruses are derived from many reports. For example, it has been shown that MZMØ & MMMØ-depleted mice failed to control a systemic lymphocytic choriomeningitis virus (LCMV) infection [304]. The results were further supported by utilizing osteopetrotic (op−/−) mice that fail to develop MZMØ and MMMØ in the spleen [280]. Infection of op−/− mice with cytopathic vesicular stomatitis virus (VSV) resulted in generating low antiviral protection responses against VSV which critically depended on the presence of MZMØ [305]. Moreover, in the same study, when the mice were challenged with 100 pfu non-cytopathic LCMV, the LCMV titers were 100–10,000 times higher than in C57BL/6 controls [305].
Studies done to examine the cross-presentation capacity of MØ relied on BM-MØ [156, 295], peritoneal MØ [293, 297], and MØ cell lines [67, 295, 296]. Surprisingly, there are no available data on the ability of Sp-MØ to cross-present antigens, whether in soluble or cell-associated forms. Notably, the participation of Sp-MØ in antigen-presentation in vivo was noticed by Debrick et al., where reducing the pool of Sp-MØ by injecting silica and carageenan intravenously, and destroying MØ in a rather specific fashion, completely diminished CTL responses against sonicated OVA-coated spleen cells. This effect seemed to be caused by a lack of functional MØ in the spleen, since i.v. infusion of peritoneal MØ restored the CTL responses[127].

This observation provided insight into the role that could be played by Sp-MØ to present antigens in vivo. However, the question as to whether reconstitution with Sp-MØ can also induce same immune responses remains unanswered, since the replenished cells were from the peritoneal cavity. In contrast, depletion of MZMØ and MMMØ (CD169+ cells) in CD169-DTR mice by diphtheria toxin administration caused remarkable delayed clearance of circulating apoptotic cells and the induction of tolerance to these cells was severely impaired, suggesting that Sp-MØ induced immune tolerance [287]. In a recent in vivo investigation, MMMØ has shown significant capacity to capture mAb-OVA complex and blood-borne adenoviruses-encoded antigens and exclusively transfer them to splenic CD8+ DCs for cross-presentation and for the activation of CTL [306]. Collectively, some subsets of Sp-MØ might play a vital role, either direct or indirect, in antigen-presentation of blood-born antigens and induction of specific CTL responses.

1.8.3 B cells

B cells internalize antigens mainly via their surface immunoglobulin receptors, also called B-cell receptor (BCR), as a part of their primary function in antibody secretion. The immunoglobulin receptors of B cells directly recognize complex folded proteins (antigens) and deliver them into endocytic compartments for processing and presentation [307]. The activation of the immunoglobulin receptors accelerates the intracellular trafficking of the receptors-antigens complexes and increases the expression level of co-stimulating molecules like CD80 and CD86, which enhances antigen-presenting ability of B cells [308, 309]. In general, it is well known that B cells express costimulatory and adhesion molecules that can
stimulate CD+ T cell response. In addition, B-lymphocytes have been shown to induce tolerance via activation and expansion of regulatory T cells in vivo [310].

In spite of the fact that B cells can generate MHC-I-peptide complexes [221, 222], the presentation of exogenous antigens on MHC-I molecules by B cells is not fully understood. Recently, the presentation of exogenous antigens on MHC-I molecules by B cells has gained much interest especially in gene gun immunization studies [221, 311]. This was due to previously reported observations that this immunization process generates CD8+ T-cell responses via the cross-priming pathway [312]. More importantly, the contributions of in vivo-formed antigen–antibody immune complexes (IC) to cross-priming, as a result of B-cell activation, warrant further studies in this field. Such complexes were shown to be effective in enhancing MHC-I-restricted Ag presentation to CD8+ T cells through the engagement of FcγRI and FcγRIII on DC [136, 137].

Mice immunized i.v. with CD40L-activated, LCMV-GP33 (MHC-I restricted)-pulsed B-cells and challenged with a tumour cell line expressing the LCMV-GP33 epitope, showed a marked delay in tumour growth. In such experiment, CD40L-activated, LCMV-GP33-pulsed B-cells were capable of inducing significant activation and proliferation of GP33-specific CD8+ T-cells in vitro, and in vivo. In fact, culturing of B-cells in the presence of CD40L, LPS and IL4 caused a marked elevation in CD80 and CD86 markers within 48 h [313].

B-cells, loaded with CpG-DNA-OVA complex (OVA covalently linked to CpG-DNA), have been shown to cross-present OVA protein and induce SIINFEKL-specific CTL responses in vitro and in vivo. This efficiency has been related to two factors: i) the efficient uptake and processing of OVA protein; and ii) activation of TLR9 by the immunostimulatory CpG-DNA [221]. The ability of resting and activated B cells to induce T cell expansion in vivo [221, 308, 311] has been recently supported by Hon et al. has found that B cells were activated when mice were injected with CpG-DNA-OVA antigen, and resulted in the cross-priming of naïve OVA-specific CD8+ T cells. The induction (priming) of OT.1 T cells was about 3-fold lower than the DCs isolated from the same draining lymph node. In the same study Hon et al. demonstrated that cross-presentation was not impaired in B-cell-deficient mice (μMT−/− mice) [311].
Although B cells could internalize CpG-DNA-OVA via DNA-receptor-mediated endocytosis and can also internalize many varieties of antigens via their Ig receptors, the exact mechanism through which B cells generate MHC-I-peptide complex is poorly understood. A recent study by Robson et al., have demonstrated that HEL-specific B-cells could uptake OVA-HEL (OVA-hen egg lysosome) protein, either alone or incorporated in immune-stimulating complexes (ISCOMS) [314]. HEL-specific B cells stimulated CD8+ T cell responses in vitro to the same extent as BM-DCs, presumably due to efficient Ig-mediated endocytosis of antigens. Cross-presentation by HEL-specific B cells required endosomal acidification, proteasomal processing and classical MHC class I/peptide transport. Moreover, HEL-specific B cells from OVA-HEL ISCOMS-immunised mice could efficiently cross-present OVA to naive OVA-specific CD8+ T cells in vivo [314].

In a recent work, spleen follicular (FO) and marginal zone (MZ) B cells were employed to address their capacity to present viral antigens derived from LCMV-GP33 (MHC-I restricted) and GP13 (MHC-II restricted) epitopes genetically fused to VLPs derived from HBcAg. Although, and in similarity to spleen DCs, they could capture and internalize VLPs and efficiently presented the MHC class II-restricted peptide GP13 to Th cells, they failed to present GP33 epitope in association with MHC class I molecules and activate CTL in vivo and in vitro. [315].

Thus, B cells readily take up exogenous materials via mainly receptor-mediated endocytosis and deliver them to MHC-I pathway. It seems that triggering of B cells during cross-presentation is an important factor. Additionally, B cells may aid cross-priming on two fronts: by directly cross-processing antigens after cross-linking their receptors and by secreting antigen-specific antibodies which form IC that empower other pAPCs to cross-prime CD8+ T cells. We should also take in consideration that B cells produce high level of MHC-II molecules, and prefer to target exogenous antigens to MHC-II pathway rather than to generate MHC-I-peptide complexes. Therefore, this may explain why B cells are less efficient in cross-priming of CD8+ T cells than DCs.
1.8.4 Neutrophils

Neutrophils are professional polymorphonuclear phagocytes that rapidly migrate, in high numbers, to the inflamed sites and accumulate locally more than any other cell type. Neutrophils are similar to MØ in that they exhibit potent phagocytic properties [316], but they have higher proteolytic activity than macrophages and DCs [236]. Neutrophils have been shown to express MHC-I [317-319]. The phagocytic capacity of Neutrophils along with their capability to express MHC class I molecules propose that MHC-I antigen-processing mechanisms could allow the processing of antigens that are phagocytosed by neutrophils [318, 320].

Initially, murine neutrophils from peritoneal exudates were shown by Harding’s group to generate MHC-I–peptide complexes from internalized bacteria in vitro via the vacuolar pathway [320]. Consequently, Harty’s group have proposed a role for neutrophils as substrates in cross-priming to activate CD8+ T cells to bacterial antigens in vivo. This seems probable because activated neutrophils die within hours, as a result of ingesting bacteria, providing rich material for cross-presenting DC [223]. In support of this hypothesis, depletion of Neutrophils, with a low dose of Ly-6G-specific mAbs, reduced CD8+ T cell priming against non-secreted antigen, but not recombinant or control secreted epitopes [223, 321].

Recently, Beauvillain et al., have demonstrated that murine neutrophils isolated from peritoneal exudates (PENs) or from bone marrow (BMNs) were as efficient as MØ at cross-presenting soluble and particulate OVA protein to OVA-specific CD8+T cells (B3Z) cells. Cross-presentation of soluble and particulate OVA protein in vitro by neutrophils was TAP and proteasome dependent. Additionally, using an in vivo model system, in which pAPCs do not express functional MHC I molecules, Beauvillain et al., have show that injection of antigen-pulsed PENs and BMNs induces naive CD8+ T-cell to proliferate and express effector functions (IFN-γ production and cytolysis) [322].

Collectively, the low antigen presentation capacity by Neutrophils may be due to the fact that soon after phagocytosis, the proteolytic and digestion activities of Neutrophils’ phagosomes become very high. This is due to the low pH environment and accumulation of several proteases that directly alter the antigens integrity and enhance their digestion and further degradation [236].
1.9 Mechanisms of cross-presentation

Based on studies that have been conducted thus far, there are two major proposed mechanisms to explain how exogenous antigens can be processed and presented on MHC class I molecules in pAPCs: the first one requires trafficking of antigens from phagosomes into the cytosol where they are then degraded by proteasomes into small peptide fragments [37, 67, 152, 323, 324]. The other mechanism involves generation of MHC-I peptides by proteases within the phagosome itself [152, 325]. The first process is called the cytosolic pathway (proteasome and TAP-dependent), and the second is called vacuolar pathway (proteasome and TAP-independent). Interestingly, cell-associated viral antigens have been shown to be processed either in the cytosol by the proteasome [326], or in the acidic endosomal compartments by Cathepsin D [152] and cathepsin S [325]. The exact mechanism that controls the selection between these mechanisms remains unknown, but it has been concluded that most of the phagocytic antigens are directed to the cytosol and processed by the proteasome [123]. Several other postulated mechanisms are described below.

1.9.1 The cytosolic pathway

This model, also known as the proteasome-dependent pathway, proposes that the internalized antigens are exclusively processed by the proteasomes after being translocated from the phagocytic compartments to the cytosol of the pAPCs. Importantly, antigens processed via this pathway should first be translocated to the cytosol, before cross-presentation can take place. The cytosolic processing mechanism passes through several critical steps: first, ubiquitination followed by proteasomal degradation to generate small peptide fragments; second, peptide transport from the cytosol into the ER through TAP, where they are subjected to further trimming in order to obtain the correct peptide size (8-10 a.a) for MHC-I loading in lumen of ER; and third, peptide loading onto MHC-I molecules and expression on the cell surface (figure 2).

However, the pathways governing or controlling the transfer of antigens across phagosomes membranes for proteolysis in the cytosol are not well defined. On the basis of several results it has been
shown that exogenous antigens can reach the cytosol through the ER-associated degradation (ERAD) pathway translocon, Sec61 channel [327, 328] and/or Derlin1 [329, 330]. The presence of protein Sec61 translocon may be involved in the transfer of proteins from the phagosome to the cytosol and vice-versa [331]. The crystal structure of Sec61 [332] has shown that the Sec61 pore forms an opening of 20Å in diameter, which is permeable to molecules up to 20 kDa. But the ability of the large dextran molecules (40-kDa) [126] and the large protein HLA class I heavy chains (40-kDa) [333] to migrate to the cytoplasm after phagocytosis has pointed to other translocation mechanism(s) that may be involved in shuttling antigens between ER and cytosol, or indicative of Sec61 capacity to transport long polypeptide chains under specific conditions. Also, it has been known that Sec61 acts to remove unstable or accumulating proteins from the ER for proteolysis by the proteasome, a process deemed ER-associated degradation. Rupture of phagosomal membranes or passive egress through leaky phagosomal membrane has also been suggested [126].

How can phagosomes acquire Sec61 protein? Interestingly, three independent studies have demonstrated that the ER membrane forms the phagosomal membrane during cross-presentation; therefore, phagosomes contain most of the antigenic-presenting compartments and act as antigen-presenting organelles [334-336] (figure 2); such ER phagosome fusions have been termed ‘ergosomes’. Thus, ergosomes should contain all major elements of the MHC-I loading complex such as TAP, tapasin, calnexin, MHC-I heavy chain, ER chaperones, ER-aminopeptidase associated with peptide trimming (ERAP) and peptide translocation channel (Sec61). The introduction of ER components into phagocytic organelles after phagocytosis enables internalized antigens to escape into cytosol, where they become susceptible to proteasome degradation, and subsequently can be loaded onto MHC-I molecules.

Rodriguez et al., have showed that exogenous antigen internalized by phagocytosis can escape lysosomal degradation by translocation into the cytosol in a murine DC cell line [126]. This observation was further supported with antigens from internalized cytomegalovirus infected fibroblast in human DCs [171] and vaccinia virus-infected fibroblasts in murine MØ and DCs [250]. The transferring of ER membranes to nascent phagosomes during phagocytosis by murine MØ and DCs supports the idea that internalized proteins could be translocated from phagosome to the cytosol.
A recent study has shown that antigen release kinetics from the phagosomes influences the efficiency of cross-presentation occurring via the cytosolic pathway. With regard to this, cathepsin S has been shown to improve cross-presentation of some antigens by facilitating their transport and processing in the cytosol. Importantly, the rate of antigen release is a highly productive mechanism if it is within a time of around 20 min post-phagocytosis. The combination between the cathepsin S, in the phagosome, and proteasomes, in the cytosol, suggest that particulate antigens need two kinds of treatments: partial degradation by phagosomal proteases and release into the cytosol, followed by the proteasomes processing in the cytosol [337]. This observation was further supported because the processing of soluble OVA by BMA was independent of acidification [219]. The last mentioned observation could be explained due the fact that OVA are soluble proteins, which can escape quickly and easily into the cytosol without any endosomal treatments.

However, several recent studies have argued against the ER-phagosome fusion theory and its role in cross-presentation [338, 339]. However, once antigens enter the cytosol, through any of the systems previously mentioned, the proteolytic machinery called proteasome will digest them. The resulting oligopeptides might be re-imported by TAP system into the phagosome and loaded onto MHC-I molecules within the vacuole (phagosome-to-cytosol-to-phagosome pathway), or they might be imported into the ER by TAP system and loaded onto MHC-I molecules (phagosome-to-cytosol-to-ER pathway) [340]. The distinction between these mechanisms is based on the ways of MHC-I/peptide complexes transferring to the surface. However, while the former pathway is sensitive to lactacystin (proteasome inhibitor) only, the later one is sensitive to lactacystin and BFA (protein transport inhibitor), since they express the MHC-I/peptide complex through the Golgi apparatus. Notably, the majority of studies done so far have demonstrated that the phagosome-to-cytosol-to-ER pathway is the main mechanism involved in cross-presentation by pAPCs (figure 2).

Therefore, three active systems are exclusively critical for the cytosolic pathway during cross-presentation: the proteasome, the TAP system, and transportation via the Golgi apparatus. The critical role of the proteasome during cross-presentation has been proven, as the induction of CTL responses was totally blocked when pAPCs were treated with lactacystin during co-incubation with ADCs. Additionally,
treatment with proteasome inhibitors resulted in significant reduction of MHC-I/peptide complexes surface expression and a blocking of peptide-presentation derived from many proteins [57, 341-343].

The proteasome-generated peptides are then translocated by a specialized transporter into the ER for loading on MHC-I via TAP system [54]. An interesting study, using both DCs from B6 (TAP\(^{-/-}\)) mouse, and proteasome inhibitor-treated DCs from B6 (TAP\(^{+/+}\)) mouse, has shown that these cells could not cross-present 65-P1 to naive 2C (CD8+ T cells) cells [344]. We can extract the idea that the requirements for TAP and proteasomal activity are important for cross-presentation of 65-P1 protein. Indeed, the movement of peptides into ER for loading on MHC-I is TAP-dependent, as demonstrated by both an antibody against TAP and the inhibitory peptide ICP47, derived from herpes simplex virus [345]. In addition, Brefeldin A, an inhibitor of protein secretion, blocked the MHC-I/peptide complex transportation through the Golgi apparatus and interfered with the cross-presentation process in different studies [250, 346].

The inhibition of cross-presentation, either by blocking proteasomal degradation, activity by lactacystin[126], inhibition of TAP system or in TAP deficient pAPCs [260], or by inhibition of the secretory pathway and the trans-Golgi network using Brefeldin A [346], support the idea that, after internalization into pAPCs, antigenic peptides can be degraded in the cytosol by proteasomes and inter ER lumen via TAP system.

Notably, ammonium chloride increases the pH in the intracellular vacuoles from 5.4 to 5.8-6.4. In addition, it has been shown to slow but not prevent the proteolytic processing of the lysosomal enzymes [347]. Treatments with ammonium chloride have shown significant enhancement in both antigen export into the cytosol and the restoration of the cross-presentation capacity of BM-DCs [348]. Furthermore, chloroquine and \(\text{NH}_4\text{Cl}\) have been shown to cause direct lysosomal membrane permeabilization, with the subsequent release of lysosomal products (i.e., cathepsins) from the lysosomal lumen into the cytosol [349].

In a summary, the cytosolic pathway seems to be the most efficient mechanism during cross-presentation which is controlled mainly by antigen export to the cytosol and the proteasomes activity to generate 8-10 amino acids peptides.
1.9.2 Vacuolar pathway

The vacuolar pathway, also called the TAP-independent or proteasome-independent pathway is where antigens are internalized into phagosomes and are degraded by the proteases therein. The endocytic compartment-resident antigens are prototypically treated by the lysosomal proteolysis to generate 8-10 amino acids peptides, which are then loaded onto MHC-I molecules through an unknown mechanism (figure 3) [294, 350-352].

However, phagosomal-antigen breakdown is likely to be controlled not only by protease specificity, but also by protease accessibility where the vast majority of phagosomal proteases are active at the acidic pH [236]. As an exception of other proteases, Cathepsin S has unique ability among the other cathepsins to be catalytically active at neutral pH [353]. It is well known that the maturation of phagosomes to phagolysosomes results in the generation of extremely acidic organelles that acquire high degradation properties due to the presence of several active proteases [236, 354]. Therefore, the effective antigen presentation via the vacuolar pathway requires a balance between liberation and destruction of CD8+ T cell epitopes. The phagosomal proteases may be important in generating T cell determinants but may also destroy them as the organelles become more acidic [337].

In addition to what has been mentioned above about the role of Cathepsin S in the cytosolic pathway, it has been found to play the major degradation process in vacuolar pathway [325, 355]. But, is Cathepsin S able by itself to generate the presented peptide from different antigenic sources? Shen et al., have found that Cathepsin S could generate SIINFEKL signal sequence when was incubated in vitro with OVA protein [325], although conditions in vivo may not be mimicked by simple mixing of antigen and proteases. Interestingly, the resultant sequence was capable of binding with MHC-I molecules [325]. Also, Cathepsin S has been found to generate immunodominant epitopes from influenza nucleoprotein (NP) and polymerase (PA) [325]. Although maturation of the vesicle into a phagolysosome leads to proteolysis and a decrease in the quantity of ER components, it has been noticed that Cathepsin S could generate peptide epitopes within the phagolysosome when high levels of antigen were used [193, 325]. Fonteneau et al., have demonstrated that human DCs have utilized the vacuolar pathway to cross-present influenza A virus matrix protein (IAV-MP) supplied from apoptotic or necrotic monocytes infected with recombinant
Figure 3. Vacuolar or endocytic pathway of cross-presentation. In the vacuolar pathway, antigens are internalized into phagosomes where it is degraded into oligopeptides by phagolysosomal proteases rather than proteasomes. The resulting peptides are probably loaded on the recycled MHC-I molecules that have trafficked into the vesicle from the plasma membrane or from the endoplasmic reticulum (ER), either by internalization, transport or ER–phagosome fusion. The peptide/MHC-I complexes are then expressed on the surface to induce CD8+ T-cell responses
Exogenous Ags

CD8+ T cells
MHC-I/pep/complex

Phagosome

Proteases
Peptides

MHC-I

ER

MHC-I
vaccinia virus expressing IAV-MP. Interestingly, the aspartic protease Cathepsin D was essential for antigen proteolysis and cross-presentation of IAV-MP [152]. In the both studies mentioned earlier [152, 325] the cross-presentation was also reduced by using TAP<sup>−/−</sup> [325] and lactacystin and BFA [152]. These observations strongly suggest that; 1) cathepsins in the phagocytic compartments may be required for partial antigen degradation into sizes that can be transported out to the cytosol and processed by the proteasomes, or 2) exogenous antigens are processed in the phagosomes and in the cytosol as well.

In contrast to the previous observations [152, 325], cross-presentation of UV-inactivated IAV-MP by pDCs was via the vacuolar pathway, as it was insensitive to neither lactacystin nor BFA. Treatments with chloroquine and cathepsin inhibitor FMK resulted in profound inhibition of IAV-MP-specific CTL proliferation and IFN-γ production. Moreover, cross-presentation of IAV-MP was sensitive to the recycling endosomal inhibitor primaquine, indicating that MHC-I/peptide complexes do not require contribution of de novo synthesized MHC class I [273]. However, the possible mechanism(s) by which vacuolar pathway acquires MHC-I molecules are not fully understood.

Based on some investigations, the processes whereby phagosomes acquire MHC class I may involve; i) empty MHC class I molecules that traffic from the ER into phagocytic compartments, ii) cross-presented peptides may be loaded onto MHC-I molecules [356], or iii) MHC class I molecules are carried into the phagosome from invagination of the plasma membrane during phagocytosis, or MHC-I molecules may be internalized into endosomes that could then be fused with phagosomes.

Interestingly, work done by Gromme et al, have shown that peptides on MHC-I molecules can be easily exchanged at pH 4.5-5.5, which matches to the late endosomal pH [357]. In support of this observation, compounds such as NH₄Cl and chlororquine, which elevate the endocytic compartments’ pH (make it more alkaline), should efficiently inhibit presentation by recycling MHC-I molecules [358, 359]. However, NH₄Cl and chloroquine have been shown to enhance soluble antigen cross-presentation via enhancing their migration to the cytosol [360]. These results suggested a possible role of loading peptide for MHC-I presentation via the endocytic pathway (figure 3).

The vacuolar pathway is dependent on the phagosomal proteases that are influenced by the phagosomal pH. Cathepsins are the most important proteases in this pathway without excluding their
participation in the cytosolic pathway. However, whether CTLs activated by peptides produced exclusively by the vacuolar pathway are able to respond to the same peptides when they are generated by proteasome-to-cytosol pathway is still unknown.

1.9.3 Endosomes to ER pathway

This pathway was recently introduced to attempt to explain the cross-presentation of soluble proteins. The observations [327, 361] that DCs could internalize some exogenous soluble proteins and direct them to the ER correlates with the capacity of DCs to cross-present exogenous soluble antigens. The role of ER-associated degradation pathway (ERAD) in cross-presenting soluble proteins has provided evidence that soluble antigens can be degraded in the cytosol after they are internalized into endosomes through the endosome to ER pathway, but whether the ERAD components are operating in the phagosome or the ER has yet to be studied. Ackerman et al., have documented that the ER donation of membranes to nascent endocytic vesicles (early phagosomes) also functions in DCs macropinocytosis, allowing soluble antigens to have access to the ER for loading onto MHC class I molecules [335].

In contrast to the previous observations, several studies [362, 363] have proved that MHC-I molecules encounter and bind exogenously derived peptides in post-Golgi or endolysosomal compartments before being transported to the cell surface. In addition, cross-priming of some virally or bacterially derived antigens has been documented to occur in the absence of TAP [358, 364, 365], suggesting that these antigens are either loaded onto MHC class I molecules within endosomes or phagosomes. These contrasting observations are mainly showing the effect of the acquiring mechanisms on processing inside the APCs, indicating many other possible ways by which MHC-I molecules can reach the processed peptides, as well as pointing to big question: are there any signals controlling the direction of the MHC-I molecules to their special peptides outside the lumen of the ER?

1.9.4 Gap junction pathway (cytosol-to-cytosol pathway).

Gap junctions are small intracellular pores formed between adjacent cells by forming channels of 6 connexins proteins on each side [366]. These channels are generally employed by the cells to the transport
of intracellular nutrients and other small molecules [367]. Neijssen et al., have documented that a 9-mer FL-peptide [stable fluorescently labelled peptides] diffused into surrounding cells expressing Connexin 43 (Cx43) protein. Gap junctions are abundantly expressed and found among different antigen presenting cells, such as DCs and monocytes, in addition to normal tissue [213, 368, 369]. This might suggest that amino acids between 8-10 amino acids can be transferred through Cx43 gap junctions, and provides other possible processes by which pAPCs can acquire peptide fractions from peripheral infected cells. Collectively, the actual amount of the peptides that can be transferred between cells, the effect of the many peptidases in the acceptor cells, as well as the mechanism(s) by which transferred peptides are loaded onto MHC-I molecules in the acceptor cells, may determine the efficiency of gap junction processes on cross-presentation.

1.10 LCMV infection model

Lymphocytic Choriomeningitis virus (LCMV) is a member of the arenaviridae family. Almost all of the viruses belonging to this family are commonly associated with rodent-transmitted disease in humans. LCMV is a non-cytopathic agent that can cause very little damage to the infected cells. Generally, LCMV is an enveloped, negative single-stranded RNA virus composed of 2% nucleic acids, 70% proteins (structural and nonstructural), 8% carbohydrates, and 20% lipids. The average diameter of the virus particle is 90-120nm [370, 371].

The viral nuclear material consists of two single stranded RNA genomics, the large segment (L; 7.2 Kb) and small segment (S; 3.4 Kb). The structural proteins of the virus, encoded by S RNA segment, include 558 amino acids nucleoproteins (NP; 63kDa) and 498 amino acids glycoprotein (GP) precursor (GP-C; 75kDa). The viral NP encapsidates the nuclear material (S and L segments) and represents the most abundant protein in virions and infected cells. The L RNA segment codes for the virus RNA-dependent RNA polymerase (L, ca 200 kDa) and a small (11-kDa) RING finger protein (Z) [370-374].

The NP and viral polymerase are complexed with the genomic viral RNA to form ribonucleoprotein (RNP) complexes, which are active in virus transcription and replication. As with other negative stranded RNA viruses, this RNP is the minimum unit of LCMV infectivity. The virus
glycoproteins precursor is posttranslationally processed into GP1 (aa 1-262) and GP2 (aa 263-498). GP-1 plays role in binding the virus to its receptor (α-dystroglycan; α-DG) on the target cells and GP-2 contains the fusion peptide and the transmembrane domain [370, 371, 373-375].

It has been established that cellular receptor that can enhance infectivity for lymphocytic choriomeningitis virus (LCMV) is alpha-dystroglycan (alpha-DG), which is expressed in many cells such as DCs, fibroblast, and macrophages [375, 376]. Notably, LCMV entry can also be alpha-dystroglycan-independent [377]. The GP-1 and GP-2 proteins are noncovalently linked and are responsible for virus attachment and fusion with host cell membrane and consequent virus cell entry via receptor-mediated endocytosis into smooth-surfaced vesicles [375, 378]. The neutralization activity of monoclonal antibodies to GP-1 have protected mice against lethal intracranial infection with LCMV [7]. After entering the endosomes, the cell membrane merges (fuses) with the endosome membrane and so the virus components are released. This step is pH-dependent fusion step in intracellular vesicles as evidenced by the observation that the lysosomotropic weak bases (chloroquine and ammonium chloride) and carboxylic ionophores (monesin and nigericin) inhibited virus entry [378, 379].

In the endocytic vacuoles the viruses lose their envelopes and are therefore released into the cytoplasm as genomic material coated with capsid structure. It is well known that for the all enveloped viruses the uncoating (removal of the capsid structures) of viral nucleic acid and mRNA transcription process takes place in the cytoplasm. For brief description of RNA and protein synthesis during LCMV replication, I will summarize the general feature of arenavirus (-single stranded RNA) replication cycles. Once LCMV genomic material exposed into the cytoplasm, the RNA-dependent RNA polymerases (RdRp), which already attached with upcoming virion will act to make the plus strands mRNA (figure 4).

The newly generated mRNAs will act to comprise two fundamentally different processes; 1) to translate all virus-encoding proteins necessary for infectivity, and 2) as templates to copy the plus strands RNA several times to generate complementary negative stranded viral RNAs. After that capsid proteins will assemble with viral nucleic acid to form the ribonucleoprotein core (RNA coated with NP) the virion in the cytoplasm. LCMV budding takes place at the surfaces (plasma membrane) of infected cells. The budding
process has been shown to be dependent on the interaction between the ribonucleoprotein core (RNA coated with NP) and the virus-encoded transmembrane GP.

Interestingly, because they have shown different affinities toward α-DG receptor, LCMV strains are divided into two major groups; high-affinity α-DG receptor binders which include strains that cause persistent infection (LCMV clone 13, WE54, and Traub) [380]; and low-affinity α-DG receptor binders like LCMV-Armstrong (LCMV-Arm) and E350 which do not establish persistent infection in vivo [376, 381, 382]. In particular, low-affinity binders have been shown to utilize alternative mechanisms involved other receptors (proteins or protein-bound entities) [377]. It has been shown that LCMV targets different cell types of the immune system including APC such as macrophages, B cells, and DC [380, 383, 384] and replicates efficiently in secondary lymphoid organs [385]. Challenging mice with LCMV resulted in induction of immunopathology state, which involves infiltration by lymphocytes, activation of antigen presenting cells, and expression of inflammatory cytokines [386-388]. Infection of B6 (H-2b) mice with LCMV induces a strong and protective CTL response. Based on tetramer binding and measuring of IFN-γ production by LCMV-specific T cell, Murali-Krishna et al., has found that LCMV-specific CTL responses were detected early within 3 days and reached the highest effector stage by 7–8 days after infection [40]. A high percentage of CD8+ T cells at the peak of LCMV infection appear to be specific for GP33-41/Db, GP34-41/Kb, NP396-404/Db, GP276-286/Db, GP92-101/Db, GP118-125/Kb, and NP205-212/Kb epitopes [40, 389-391]. Notably, presentation of the GP33 peptide to CTL by the Kb molecules has also been reported [392].

Experiments performed to delineate the relationship among LCMV clones and the preferential activated epitope-specific CTL have found remarkable differences in the hierarchies of the activated LCMV-specific CTLs. Infection of C57BL/6 mice with LCMV-Armstrong (Arm) resulted in immunodominance of NP396-specific CTL [40, 393], whereas infection with faster replicating strains such as LCMV-WE, LCMV-Docile (Doc), or LCMV-Arm Clone13 resulted in immunodominance of gp33-CTL [341, 391]. Interestingly NP-derived CTL epitopes are generated faster than GP-derived epitopes [394].

In this project, I am investigating the cross-presentation of LCMV proteins generated after virus infection by different pAPCs. Several studies using different antigen modules have proven that the LCMV-
NP396 expressed as the full NP in the cytosol of xenogenic antigen donor cells [67], GP33 expressed as an epitope in virus-like particles [395], and GP33 expressed as a stable GP construct in syngeneic cells [177] can be cross-presented. But none of them has shown the contribution of these epitopes in cross-presentation after virus infection in vivo and in vitro.

In summary, LCMV has been historically used as an effective research model for studying the immune response to virus infection and helped in discovering the role of MHC in regulating CTL responses [396].
**Figure 4: LCMV replication cycle.** LCMV have negative-strand RNA genomes. The GP1 and GP2 mediate the virus binding to cellular receptors and entry via clathrin-independent endocytosis. After entry, LCMV are taken into large, smooth-walled endocytic vesicles. After uncoating in the endocytic compartments, the viral genome is released into the cytoplasm where the viral replication takes place. Direct manipulation of RNA virus genomes depends on the ability to produce recombinant RNAs that are accepted as template by the particular viral RNA-dependent RNA polymerase (RdRp). Replication and transcription of the genome occur in the cytoplasm of an infected cell and both take place within ribonucleoprotein complexes. During genome replication, a full-length copy of genomic S and L RNAs is synthesized, yielding the corresponding antigenomic S and L RNAs. Due to the ambisense coding strategy, both genomic and antigenomic RNA serve as templates for the transcription of viral mRNA. The viral envelope glycoproteins are synthesized in cells as a single mannose-rich precursor molecule, which is proteolytically cleaved and processed to contain complex glycans during transport to the plasma membrane. Virions mature by budding at sites on the surface of cell.
Endocytosis

Smooth vesicle (Endosome)

Lysosome

Virus genome

Transcription

+ strands RNA

Replication

- strands RNA

Assembly

Ribonucleoprotein

Translation

Nucleoprotein & L (polymerase)

Glycoprotein precursor (GP-C)

Cleavage

GP1 & GP2

Budding

Virus release

LCMV receptor

Small segment

Large segment

Polymerase

Glycoprotein (GP1 and 2)
1.11 Research objectives

Cross-presentation is an alternative pathway for activation of CTL responses. Research has shown that viruses that interfere with direct presentation via blocking any of MHC-I processing and presentation machineries in the infected cells can be still be exposed to immune system through the cross-presentation pathway by the pAPCs. Importantly, recent observations have provided important insights into the role of pAPCs in inducing virus-specific CTL responses via the cross-presentation pathway. Tissue-resident pAPCs and their precursors have formed the important sources for studying cross-presentation. Although recent research has provided important insight into the role of DCs in cross-presentation, research on MØ is still far behind, particularly Sp-MØ.

The principle aims of this project are to: 1) delineate the potential relationship between MØ differentiation over time and their capacity to cross-present soluble and cell-associated antigens, and 2) analyze the cross-presentation capacity of LCMV proteins generated during virus infection.

1.11.1 Objective #1. Culturing and investigating the differentiation of spleen macrophages (Sp-MØ) in vitro

Spleen is the biggest secondary lymphoid organ in the body. It filters blood to remove blood-born antigens. Macrophages (MØ) among so many other immune cells reside in the spleen and interact with other effector immune cells such as T and B cells. Spleen macrophages (Sp-MØ) are heterogeneous populations distributed in different anatomical compartments and perform distinctive immunological tasks.

The optimization of an efficient culture method that allows for isolation and differentiation of Sp-MØ in vitro was a must to initiate these projects.

Therefore, first we optimized a method to culture and differentiate Sp-MØ in vitro in order to study their phenotypic and functional changes during maturation stages.

1.11.2 Objective #2. Investigating the effect of differentiation on the capacity of spleen (Sp-MØ) and bone-marrow-derived macrophages (BM-MØ) to cross-present soluble and cell-associated antigens.
Macrophages have been shown to cross-present antigens from either soluble or cell-associated antigens. Most of the data are obtained with either using MØ cell lines, or thioglycolate-elicited MØ. Notably, little is known about cross-presentation capacity of Sp-MØ. Furthermore, effects of maturation and differentiation stages on cross-presentation capacity of MØ have never been addressed.

In this objective we have evaluated both Sp- and BM-MØ in order to address: 1) how MØ handle different forms of exogenous antigens (soluble vs. cell-associated antigens), 2) relationship between antigens processing vs. degradation machineries of MØ during maturation stages, 3) effects of different specific inhibitors to distinguish between the endocytic and cytosolic pathways of cross-presentation, 4) the extent of functional changes of immunoproteasomes during the differentiation stages. Collectively, results from this objective define a novel strategy to elucidate the contribution of the Sp- and BM-MØ in cross-presentation.

1.11.3 Objective #3. Analysis of cross-presented epitopes from LCMV-NP and GP after virus infection

It has been shown that LCMV targets different cell types of the immune system including pAPC such as MØ, B cells, and DCs [380, 383, 384] and replicates efficiently in secondary lymphoid organs [385]. Infection of B6 mice with LCMV induces a strong and protective CTL response dominated by GP33-41/Db, NP396-404/Db, GP276-286/Db, and NP205-212/Kb epitopes [40, 389-391]. Several studies using different non-infectious models have proven that the LCMV proteins expressed in could be cross-presented.

Here, we studied the contribution of LCMV proteins in cross-presentation after virus infection in vivo and in vitro. Impact of LCMV proteins accumulated during the course of infection was addressed using an efficient antigen donor cells (ADCs) model system. The capacity of different pAPCs to induce LCMV-specific CTLs responses through cross-presentation pathway was fully addressed by involving cell lines and primary pAPCs. We also investigated the mechanisms involved in cross-presentation of LCMV proteins to clarify whether the cytosolic or endocytic pathway is involved in processing and presenting the engulfed LCMV proteins on MHC-I molecules. We further investigated the effect of in vivo cross-priming of cell-associated LCMV proteins on immunodominant hierarchies of LCMV-specific CD8+ T cells during subsequent virus infection.
References


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CHAPTER 2

An efficient culture method for generating large quantities of mature
mouse splenic macrophages
2.1 Preface

Spleen macrophages are important pAPCs in eliciting CD8+T cell responses against virus infections. They are also involved in clearing the blood from debris and blood-born pathogen. However, little is known about their functions during differentiation and their dependence on M-CSF therein. The aim of this project was to isolate and characterize splenic macrophages (Sp-MØ) during in vitro differentiation. We approached this aim with the following hypothesis; Sp-MØ require macrophage-colony stimulating factor (M-CSF) for optimal growth and further in vitro differentiation & maturation. By employing this cytokine for in vitro culturing of spleen-derived macrophages, our investigation of such cells has revealed the following:

- Most of spleen macrophages (Sp-MØ) likely exist as a semi-mature cells or macrophage precursors in the spleen.
- Culturing the splenocytes in M-CSF-containing medium over time allows for the generation of more differentiated spleen macrophages.
- Culturing splenocytes in M-CSF-containing medium resulted in up-regulation of most of macrophages’ surface and intracellular markers.
- In contrast to BM-MØ, Sp-MØ have shown remarkable increase in their capacity to uptake antigens during differentiation.
- Similar to BM-MØ, Sp-MØ can utilize different internalizations mechanisms to taken-up dead cells.

The work in this study was planned and executed by myself. The results described in this chapter were published in:

An efficient culture method for generating large quantities of mature mouse spleen macrophages.

2.2 Abstract

In this study, we established an efficient in vitro culture method for generating mature splenic macrophages (Sp-MØ). Splenocytes were cultured in the presence of conditioned medium containing macrophage colony-stimulating factor (M-CSF) for 7 days post isolation and the generated Sp-MØ were characterized phenotypically and functionally. Through this method, $9 \times 10^6$ / mouse Sp-MØ were obtained in comparison to $2 \times 10^5$ / mouse when MØ were cultured in regular medium. In addition, the purity of these cells was as high as 80% by day 5 and >90% by day 7 of culturing, confirmed with MØ-specific markers. The increased Sp-MØ yields, in the presence of M-CSF, point towards the existence of a precursor population in the spleen that can be influenced to differentiate into Sp-MØ. Moreover, we compared the maturation of generated Sp-MØ to conventional bone marrow-derived MØ (BM-MØ) in vitro. Interestingly, Sp-MØ exhibited lower capacity to phagocytose dead cells after 3 days of maturation, but showed similar internalizing capacity after 5 and 7 of maturation to BM-MØ cultured for the same time period. Importantly, Sp-MØ up-regulated the expression of several intracellular markers such as MOMA-2 and CD68 while downregulating SIGN-R1 after 7 days, indicating that these Sp-MØ undergo further maturation in vitro due to culturing in M-CSF. Taken together, we describe and validate a method for generating Sp-MØ in large quantities and high purity. These data should prove valuable in future studies characterizing the functions and maturation of Sp-MØ.
2.3 Introduction

Macrophages (MØ) are some of the most important accessory cells to the immune system; they play a central role in both innate and adaptive immunity. Macrophages belong to the mononuclear phagocyte system and differentiate from blood monocytes (1). Initially, these monocytic cells develop from myeloid precursors in the bone marrow and eventually undergo maturation into MØ upon migration into tissues. At the early stages of development, pro-inflammatory immune-specific signals may influence MØ functional and phenotypic heterogeneity (2-6).

Macrophages are heterogeneous cell populations, which perform diverse functions depending on their maturation state in vivo. In vitro, the transition from either bone marrow-derived cells or blood monocytes into MØ as well as their maturation is influenced by cytokines or media supplements such as the macrophage colony-stimulating factor (M-CSF). M-CSF is a hemopoietic growth factor that specifically causes the proliferation and differentiation of mononuclear phagocytic cells and is produced by several cell types including fibroblasts and endothelial cells (7, 8).

Macrophage maturation can be monitored by functional assays and the expression of specific surface markers (1, 9-11). Recently, it was shown that the antigen-presenting capacities as well as the expression of different MØ surface markers were significantly affected by the addition of cytokines such as IFNγ and IL-4 to in vitro-derived bone marrow MØ (12).

Splenic MØ (Sp-MØ) are some of the most intriguing MØ populations in the body. They are classified according to the distinct anatomical locations they reside in; red pulp MØ, marginal zone MØ, and marginal zone metallophilic MØ. Although these Sp-MØ are found in one organ, they are heterogeneous and vary in their surface markers expression (2). Nonetheless, in vitro culturing of Sp-MØ has not been widely employed possibly because of their low yield. In this report, we describe the characterization of Sp-MØ cultured in conditioned medium supplemented with M-CSF from murine fibrosarcoma cell (L929) supernatant (8, 13, 14) and provide evidence for the validation of this protocol as a more efficient culturing
method. In addition, our data suggest that Sp-MØ exist as semi-mature cells and that further signals from cytokines are needed for them to fully differentiate.
2.4 Materials and Methods

2.4.1. Mice, Cells, and Media

C57BL/6 (H-2b) mice were purchased from Taconic farms (Germantown, NY, USA). All mice were kept under specific pathogen-free conditions and were used between 6 to 8 weeks. Animal experiments were carried out in accordance with the guidelines of the Canadian council of animal use. As controls, the macrophage cell line BMA, developed by inhibiting cell death in bone marrow derived macrophages, (kindly provided by Dr. K. Rock, University of Massachusetts Medical School, Worcester, MA), was used (15, 16). All media were purchased from Invitrogen (Ontario, Canada). The HEK 293 cells, human embryonic kidney cell lines, were grown in DMEM 10% FBS, and BMA cells were grown in RPMI 10% FBS.

To obtain peritoneal macrophages, resident peritoneal cells were collected from peritoneal cavities by injecting 10 ml cold PBS. The harvested cells (3-5 x 10^6) were cultured overnight in RPMI 10% FBS in 6 well-plates (Fisher, Whitby, ON, Canada), at 37 ºC. The non-adherent cells were removed and adherent cells were supplemented with fresh RPMI 10% FBS and cultured for the indicated time.

2.4.2. Preparation of bone marrow-derived macrophages and dendritic cells

Femurs and tibias from 6-8 weeks old C57BL/6 mice were collected and the marrow was flushed with warm PBS using a 26g 3/8 sterile needle (Becton-Dickinson, Rutherford, N.J, U.S.A). Bone marrow cells were washed twice with warm PBS and re-suspended in lysis buffer (1.66% w/v ammonium chloride) for 5 min with gentle shaking to lyse red blood cells. The cells were then washed twice with warm PBS. Debris was removed by passing the cells suspension through a metal sieve. Approximately 4-6 x 10^7 cells were obtained per mouse.

To generate BM-MØ, bone marrow cells were prepared as previously described (14). Cells were cultured in 6-well tissue culture plates (3-5 x 10^6 cells/ well in conditioned medium (CM), consisting of RPMI containing 10% FBS and 20% of L929 supernatant as a source of M-CSF or 5 ng / ml of recombinant mouse M-CSF (Shenandoah Biotechnology, PA). After 3 days, the non-adherent cells were washed out and
fresh medium was added. The medium was changed every 2 days and the cells were harvested with 1x Trypsin-EDTA and tested at the indicated times.

To generate bone marrow dendritic cells (BM-DCs), bone marrow cells were prepared as previously described (17) with some modifications. Bone marrow cells were cultured in 6-well tissue culture plates (3-5 x 10^6 cells/well) in RPMI medium containing 10% FBS, 50 µM 2ME (Bioshop Canada Inc., Burlington, Ontario), 10 ng/ml GM-CSF (Cedarlane, Ontario, Canada), and gentamicin 3 µg/ml (Invitrogen, Ontario, Canada). The medium (2 ml) was removed every 2 days and replaced with fresh medium. At day 6 the non-adherent cells were transferred into a new 6 well-plate and left for at least 24 h at 37°C. The loosely adherent cells (highly enriched CD11c^+ MHC-II^+ BM-DCs) were harvested for FCM analysis.

2.4.3. Preparation of spleen macrophages

Macrophages were either isolated from single individual spleens or from 3 pooled spleens if higher cell numbers were required. To prepare Sp-MØ, homogenized splenocytes were passed through a metal sieve to remove debris, and the cells were suspended for 3-5 min at 37°C in lysis buffer (1.66% ammonium chloride) to lyse the red blood cells. After two washing steps with warm RPMI 10% FBS medium, the cells were cultured in recombinant M-CSF (5 ng/ml) or CM as described above. After 3 days of culture, non-adherent cells were removed as the entire medium was replaced on day 3 to enrich for adherent cells. This was repeated again on day 6 and adherent cells were harvested on day 7. Using this method, we obtained mature macrophages after 7 days of culture. The cells were collected with 1x Trypsin-EDTA and tested at the indicated times of culture. In certain experiments as indicated, the macrophages were cultured in RPMI medium only.

For estimating cell counts and yield of MØ, 10^6 splenocytes were cultured in a special culture dish (2mm Grid, 60x15MM, Corning Inc.) in either 20% M-CSF medium or RPMI containing 10% FBS to assess how they grew in a specific surface area. At day 7 post isolation, MØ were counted in each square under the microscope. In addition, total Sp-MØ counts per spleen were calculated after removing non-adherent cells at each time-point indicated.
2.4.4. Immunofluorescence microscopy & lysosomal detection

In order to examine the distribution of the lysosomal vesicles in the macrophages, we made use of the acridine orange dye to stain such organelles. This dye localizes to lysosomes, resulting in red fluorescence accumulation therein (18). Cells were seeded into 24-well plate onto 12mm circular glass cover slips (Fisher Scientific, Ontario, Canada) at a density of $2 \times 10^5$ / well (PECs, bone marrow cells) and $10^6$ cells/well in the case of splenocytes. The cells were cultured as described above and were allowed to mature for 7 days. Non-adherent cells were removed by washing twice with warm PBS. Macrophages were stained with acridine orange (50 μM) for 1 min at room temperature to stain for acidic lysosomal vesicles. Cells were washed twice with PBS and then examined using fluorescence microscopy (Leica DM IRE2, Germany) at a 60x magnification. Images were acquired using the filter system N2.1S with a Leica DFC340 cooled monochrome digital camera with a resolution of 2 Mpxels.

2.4.5. Phagocytosis assays

After 7 days in culture, BM-MØ and Sp-MØ were prepared in round-bottom 96-well plates at $1 \times 10^5$ cells/well. The MØ were then co-cultured with antigen donor cells (ADCs) at a ratio of 3:1 (ADCs: MØ). ADCs were HEK cells labeled with the fluorescent marker carboxyfluorescein diacetate succinimidyl ester (CFSE). The cells were labeled according to the manufacturer’s instruction (Invitrogen). Briefly, HEK cells were harvested and resuspended in PBS followed by incubation with CFSE dye 0.2 µg/ml at 37 °C for 15 minutes. The cells were washed twice following incubation and resuspended in fresh DMEM medium.

The CFSE-labeled HEK cells (ADCs) were treated to undergo death by a treatment we refer to as LYUV (19). To achieve this the cells were snap frozen (lysed) in liquid nitrogen and UV irradiated using a CL-1000M UV cross-linker [Ultraviolet Products, Upland, CA] at a radiation intensity of 200,000 μJ/cm² (maximum intensity) for 5 min. Treated HEK cells were incubated with either BM-MØ or Sp-MØ for 1 h at 37 °C in absence or presence of Fucoidin A (100 μM) – a ligands for scavenger receptor class-A (SR-A) on phagocytic cells (20, 21), Cytochalasin D (10μM), which prevents F-actin polymerization, and thus inhibits both phagocytosis and macropinocytosis (22), Dimethyl amiloride (DMA) (150μM), a macropinocytosis inhibitor (23), and the microtubule inhibitor Vinblastin A (30 μM) which leads to microtubule
depolymerization and inhibits endocytosis (24). The above inhibitors were purchased from (Sigma, Oakville) and were initially added to the macrophage cultures for 45 min prior to co-culturing the HEK cells during the 1 h assay. Sp-MØ and BM-MØ were then stained with anti-mouse H-2K\textsuperscript{b} R-PE antibody for 15 min at 4 °C to distinguish them from the antigen donor cells. Cells were analyzed by FCM and the percentage of phagocytosis was calculated based on the number of double positive macrophages that indicated uptake of the CFSE-labeled LyUV-treated HEK cells. Trypan blue (0.04%, v/v) was added to tubes after initial acquisition to quench the signal from CFSE-labeled cell fragments adsorbed on the cell surface but not yet internalized as described previously (10, 25).

In addition, splenocytes were seeded into 24-well plates onto 12mm circular glass cover slips at a density of 2 x 10\textsuperscript{5} cells/well. The cells were cultured in CM as described above and were allowed to mature for 7 days. Non-adherent cells were removed by washing twice with warm PBS. CFSE-labeled dead HEK cells were added to the adherent cells and left for 1 hour before removing the media, and acquiring images with a fluorescence microscope (Leica DM IRE2) using 60x and 20x magnifications. Images were acquired using the filter system I3 for green fluorescence, and the bright field view for overlays.

\subsection*{2.4.6. Flow Cytometry analysis}

The macrophages were stained directly with fluorochrome-labeled Abs against surface markers; PE-conjugated anti-mouse MHC-I, H-2K\textsuperscript{b} (clone CTKb), FITC-conjugated anti-mouse MHC-II, I-Ab, (clone 25-9-17s) PE-Cy5-conjugated anti-mouse CD11b (clone M1/70.15), PE-conjugated Hamster anti-mouse CD11c (clone N418), PE-conjugated anti-mouse CD80 (clone RMMP-1), PE-conjugated anti-mouse CD86 (clone RMMP-2), and FITC-conjugated anti-mouse F4/80 (clone C1:A3-1) were purchased from Cedarlane, (Ontario, Canada), and the specific anti-macrophage marker (clone MOMA-2), anti-SIGN-R1 (clone ER-TR9), anti-CD169 (clone MOMA-1) and anti-CD68 (clone FA-11) were purchased from AbD Serotec (NC, USA).

For cell surface staining, the cells were stained for 15-20 min at 4°C. After two washing cycles with cold PBS buffer, the cells were prepared for measurement with flow cytometry (FCM). For intracytoplasmic staining, cells were fixed with 1% paraformaldehyde for 20 min, washed twice in PBS and then
permeabilized with 1% saponin for 1 h before incubation with FITC-conjugated mAbs for MOMA-2 and CD68 for 1 h at room temperature. Data were acquired with the Epics XL-MCL flow cytometer and analyzed with the Expo 32 Advanced Digital Compensation Software package (Beckman Coulter, Miami, FL).
2.5 Results

2.5.1. Analysis of Sp-MØ development in conditioned medium

Splenic macrophages (Sp-MØ) are recognized as tissue resident MØ that have most likely developed from circulating monocytes. Since Sp-MØ are usually isolated to test their functions ex vivo, we sought to compare their activities after in vitro culturing over several days and relate this to their maturation state. We initially tried RPMI 10% FBS, which regularly yielded very few Sp-MØ (Fig. 1a, left panel). We then tried different culture conditions and found that having Sp-MØ cultured in 20% M-CSF medium was a very efficient approach to obtain high yield of in vitro cultured cells from spleens rapidly (Fig. 1a, middle panel). When we compared these cells morphologically to those derived from bone marrow over a 7 day period, we observed that they grew in a similar manner but Sp-MØ reached a higher density at day 7 (Fig. 1a, right panel). It was interesting that these Sp-MØ cells appeared morphologically similar to BM-MØ 7 days post culturing.

Next, we calculated the influence of CM on the yield of in vitro cultured Sp-MØ compared to regular RPMI with two different methods. First, we cultured the cells in 2mm grided TC-treated dishes so that we could easily count the number of the cultured Sp-MØ per square in the grid (Fig. 1b). As it can be observed in figure 1b, even though cells were cultured for 7 days in vitro, the CM had significantly increased the number of counted MØ from approximately 3 to 130 cells per square in the TC-treated dishes.

In parallel, we assessed the total number of Sp-MØ obtained per spleen using either CM or regular RPMI after 7 days in culture. Out of 3-5 x 10⁷ splenocytes, we could successfully isolate and grow approximately 9 x 10⁶ Sp-MØ by day 7 when CM was used, which would account to a yield of 20-25%. The purity of these cells was >90% by day 7 as determined by flow cytometric detection of surface markers useful for the broad detection of MØ such as F4/80 and MOMA-2 (figure 4 & 5). This high yield was in sharp contrast to the mere 2 x 10⁵ Sp-MØ (0.6% yield) obtained using regular RPMI (Fig. 1c). Both figures 1b and 1c clearly show that the increase in yield of Sp-MØ was approximately 40-fold more when CM was used instead of regular medium.
Figure 1. Generation of Sp-MØ in large quantities in vitro. (a) Splenocytes (1 x 10⁶) from naïve B6 mice were cultured on cover slips in RPMI 10% FBS (left panel), or in conditioned medium (CM; RPMI 10%FCS, 20% M-CSF) (middle panel) for 7 days in 24-well plates at 37°C. In the right panel, 10⁶ bone marrow-derived cells were cultured also in the same CM. Cell morphology was examined via microscopy (Leica DM IRE2, 30x magnification, bright field). (b) Splenocytes (1 x 10⁶) were cultured in either CM or RPMI 10% FBS medium onto grided TC-treated dishes to examine cell growth in situ. After 7 days in culture, only adherent macrophages were counted in 10 squares and the results are presented as the average per square from three independent experiments ± std. (c) To compare the recovery yield of macrophages per spleen between the two conditions, splenocytes were cultured in a 6-well plate (5 x 10⁶ / well) in either CM or RPMI 10% FBS medium. The cells were incubated at 37 °C for 7 days and only adherent macrophages were counted. Data shown is from one spleen and is representative of four independent trials.
2.5.2. Increased number of recovered Sp-MØ is dependent on M-CSF

Having observed this remarkable effect of CM on the differentiation and maturation of Sp-MØ we asked if this effect is dependent on the continual presence of splenic-MØ in CM. Therefore, we tested the effect of CM removal every 24h from the Sp-MØ cultures post isolation. Splenocytes (1 x 10^6) cultured in CM for one day followed by culture in medium with regular RPMI for 6 more days (Fig. 2a,i) resulted in yielding fewer Sp-MØ compared to those obtained after 6 days in CM (Fig. 2a,vi). Furthermore, we observed that culturing the Sp-MØ in CM for at least 4 days was sufficient to support a substantial increase in cell numbers compared to regular medium (compare with Fig. 1a, left panel).

In addition to the morphological observation in figure 2a, we also quantified how the yield of Sp-MØ was affected as a result of CM removal. To perform these experiments, we seeded 5 x 10^6 splenocytes/well in 6-well plates. In a similar manner to 2a, we replaced the CM from individual wells after 24h with regular medium, and left the cells for a total of 7 days in culture. In addition, cell counts of adherent cells post isolation (4h) was performed, to reveal the potential number of cells that can give rise to Sp-MØ. As it can be seen in figure 2b (The average numbers are shown in 2c), CM has a significant impact on increasing the number of Sp-MØ cultured in vitro. We obtained high yields if we cultured the cells for 6days in CM / 1day RPMI or for 7days CM. Thus, CM was required for at least 6 days for cells to reach their maximum proliferative potential. Either way, the yield was similar to that observed in figure 1 reaching approximately 20-25%.

Importantly, by considering the data from figures 1 & 2, it appears that the Sp-MØ progressively expanded in numbers during the course of culture as a result of CM. This would imply that either the medium was critical in reducing cell death during the early phases, induced Sp-MØ to proliferate, or that it aided uncommitted precursor cells in the spleen to differentiate towards MØ.

2.5.3. Microscopic examination of macrophages’ lysosomal content

The above findings provided important information with regard to the key requirements for CM for culturing Sp-MØ in vitro. Since lysosomal activities are critical to differentiated MØ in degrading engulfed
Figure 2. Conditioned medium increases Sp-MØ numbers in culture. (a) Sp-MØ (1 x 10⁶) were cultured on cover slips in vitro in 24-well plates in presence of CM (RPMI 10%FCS, 20% M-CSF). Every 24h one of the wells had its medium changed to regular RPMI (i, represents 24h, ii, 48h etc.). The total incubation time was 7 days (vi, represents 6 days in CM and 1 day in regular RPMI). Cell morphology was examined as in fig.1 (30x magnification, bright field) under the Leica microscope. This experiment was repeated twice in addition to the one presented here. (b) Splenocytes cultured in 6-well plates (5x10⁶ cells/well) and every 24h one of the wells had its medium changed to regular RPMI until day 6. Only adherent cells (macrophages) were counted (c) at the end of the 7 day period. In addition, splenocytes were cultured for 4 h in RPMI 10% FCS. After that the non-adherent cells were removed and the adherent cells were counted (4 h). Data shown is representative of three independent trials.
materials, it was important to examine the lysosomal content of Sp-MØ grown in CM. In order to examine this characteristic, we made use of fluorescence microscopy to observe MØ obtained from spleen, bone marrow and peritoneal lavages that were treated with acridine orange. The fluorescent vital dye, acridine orange, is a weak base that becomes protonated in acidic compartments such as lysosomal compartments in the cell. As more protonated acridine orange accumulates in a compartment, the color of the compartment will change to red/orange.

We cultured peritoneal lavages in regular RPMI, Sp-MØ, and BM-MØ for 7 days in CM followed by the removal of non-adherent cells. After harvesting the adherent cells we added acridine orange, and examined the cells under the microscope. We wanted to check if the CM treatment had affected Sp-MØ to mature in a manner similar to other macrophages (e.g. BM-MØ and PEC-MØ). Figure 3 shows that the round acidic lysosomal compartments where the dye accumulates in perinuclear regions (indicated by white arrows) appeared quite similar when comparing all three cells types. The presence of abundant lysosomes in the cytoplasm of MØ is a characteristic of such scavenger phagocytic cells.

2.5.4. Phenotype analysis of Sp-MØ cultured in CM

From the above results we envisaged that these Sp-MØ were differentiating in a similar manner to BM-MØ which is somewhat unexpected because the BM-MØ are derived over seven days from precursor stem cells whereas the Sp-MØ ought to be mature cells in the spleen. In order to verify this issue, we tested surface markers expression during the one week culture period over different time points. Thus, we compared BM-MØ, Sp-MØ cultured in CM as well as Sp-MØ cultured in recombinant MCSF over 7 days. We also tested a macrophage cell line (BMA) and the total splenocytes immediately after their isolation as well as adherent cells that were harvested after 4 hours of culturing the splenocytes (Fig. 4a).

From figure 4a it is clear that classical macrophage surface marker F4/80, was only expressed on a minor population in splenocytes, which was more apparent when the adherent cells were analyzed after 4h. However, as cells were allowed to differentiate and mature in culture the expression of this marker increased significantly reaching levels similar to those found on mature BM-MØ and the BMA cell line (Fig. 4a-b, F4/80 panel). By examining this marker one could conclude that a high purity of 80% day 5 and
Figure 3. Microscopic examination of the lysosomal content in macrophages. Macrophages from different sources (bone marrow; BM-MØ 2x10⁵, peritoneal lavages 2x10⁵, and spleen; Sp-MØ 1x10⁶) were cultured on cover slips in 24 wells for 7 days and were then treated with the lysosomal marker acridine orange for 1 min at room temp. The cells were then transferred to glass slides and examined with fluorescence microscopy (60x magnification). The bright round organelles indicated by white arrows represent the acidic lysosomal vesicles where the acridine orange dye accumulates.
>90% by day 7 was attained. This pattern of expression was also observed (Fig. 4, CD11b panel) when we tested for the expression CD11b (the alpha subunit of Mac-1), as well as the type I transmembrane protein CD11c (Fig. 4, CD11c panel). Thus, both CD11b and CD11c expression increased by day 5-7 to levels similar to those expressed by BM-MØ.

As expected, both BM-MØ and Sp-MØ were either negative or exhibited a very low expression for MHC-II (Fig. 4, MHC-II panel), while the expression of MHC-I was detected at lower levels by Sp-MØ, after 3 days in culture compared to BM-MØ. As Sp-MØ continued their maturation in vitro, their MHC-I expression increased by day 5-7 reaching similar levels to those expressed by BM-MØ and the BMA cell line (Fig. 4, MHC-I panel). Interestingly, there was a population that expressed MHC-II, which was detectable during early isolation steps as well as the 4 h adherent step (Fig. 4a MHC-II panel). These MHC-II⁺ cells were most likely not enriched during the culture period, as day 7 MØ were almost negative for MHC-II (Fig. 4b MHC-II panel). In comparison, BMDC cultured with GM-CSF expressed high levels of MHC-II (data not shown).

Examination of costimulatory molecules of Sp-MØ and BM-MØ matured for different time points has provided evidence that there are differences in their kinetics of expression. It appears that Sp-MØ upregulated the expression of CD80 (low levels) and CD86 throughout the culture period with CD86 having the higher intensity of expression. This was in contrast to BM-MØ that had actually down regulated their CD86 expression (though it remained positive) after 7 days (Fig. 4, CD80/86 panels).

Collectively the above markers used for characterizing the Sp-MØ have clearly established them as MØ that were still undergoing a certain degree of maturation, differentiation, and proliferation due to the increase in their numbers noted above. From figure 4, it appears that these Sp-MØ were similar to BM-MØ and the BMA cell line, yet they exhibited subtle differences that distinguished them from each other. In addition, by examining Sp-MØ cultured in recombinant MCSF, we observed similar markers to those cultured in CM, with a lower expression of CD11b, CD11c, and F4/80 in the case of recombinant MCSF on day 3 of cultures. However, by day 5 and 7 the markers’ expression was nearly identical (Fig. 4b).
**Figure 4. Analysis of surface marker expression on macrophages.** Histograms show staining with monoclonal antibodies specific for MHC-I, MHC-II, CD11b, CD11c, F4/80, CD80, and CD86 after incubation with the specific antibodies on ice for 15 min. a) Total splenocytes after spleen isolation (left panel), adherent cells after 4h of culturing and removal of non-adherent cells (middle panel). A macrophage cell line (BMA) was also included in these tests for comparison (right panel). Negative controls are depicted by filled histograms. b) Macrophages (spleen; Sp, bone marrow; BM) were tested for the above surface markers after 3, 5, and 7 days of *in vitro* culturing (key illustrated in legend). Left panel depicts Sp-MØ cultured in rec. M-CSF, compared with Sp-MØ cultured in CM (RPMI 10%FCS, 20% M-CSF) (middle panel). Right panel depicts bone marrow; BM-MØ cultured in CM over the same number of days. Data was acquired by FCM and is presented as overlay plots to reflect the different time points. This is one representative experiment from 3 independent trials.
2.5.5. Sp-MØ maturation in medium supplemented with M-CSF associates with certain surface marker expression

To further characterize these Sp-MØ cultured with either CM or recombinant M-CSF, we focused our analysis on more specific markers such as CD169 (MOMA-1) that are known to be expressed mostly on metallophilic marginal zone Sp-MØ (2, 3, 26). We observed that CD169 expression was low (Fig 5b) on Sp-MØ cultured in vitro for 3 days, probably reflecting expression on a minor subset. However, as cells were cultured for longer time periods, the expression of this marker increased significantly. When we examined freshly isolated splenocytes as well as the 4 h adherent cells (Fig 5a), we could see that CD169 expression was low at the beginning, which would suggest that CD169 expression correlates with Sp-MØ maturation.

CD68 is another macrophage specific marker which is associated with the lysosomal-associated membrane protein family (2, 3). It is usually very low or negative on dendritic cells. This MØ marker exhibited a very significant increase over time in culture (Fig 5b) and could be clearly observed on a small population after 4h adherence (Fig 5a). Again, this is another intracellular marker defined in this study that directly associates with MØ maturation.

Interestingly, these Sp-MØ showed enhanced expression for the general MØ marker MOMA-2 throughout the culture period. However, they downregulated the expression of SIGN-R1 (normally expressed on marginal zone Sp-MØ) during the same maturation period over 7 days (Fig 5b). The above data identify the criteria to determine the state of Sp-MØ maturation in vitro.

2.5.6. Analysis of the phagocytic capacity and the uptake mechanisms of Sp-MØ cultured in CM

Having examined the morphological characteristics and surface marker expression of Sp-MØ in comparison to other MØ, we next addressed an important character of MØ; their ability to function as scavenger cells by phagocytosing dying cells.

In this study, we used dead HEK cells labeled with CFSE as ADCs. The ADCs were prepared as described above in the materials and methods section. In figure 5a (left panel) we observe that the instant mixing of MØ, defined by anti H-2 Kβ antibodies (y-axis), with CFSE-labeled ADCs before acquisition in
Figure 5. Staining profiles of Sp-MØ specific markers cultured in either CM (RPMI 10%FCS, 20% M-CSF) or medium supplemented with recombinant M-CSF. Histograms show staining with monoclonal antibodies specific for the macrophage markers: (MOMA-1), SIGN-R1 (surface markers), MOMA-2 and CD68 (intra-cytoplasmic staining). a) Total splenocytes after spleen isolation (left panel), adherent cells after 4 h of culturing and removal of non-adherent cells (right panel). b) Spleen macrophages (Sp-MØ) were cultured in either recombinant M-CSF (left panel), or CM supplemented medium (right panel) and tested for the above macrophage markers after 3, 5, and 7 days of in vitro culturing (key illustrated in legend). Data is a representative experiment from 3 independent ones.
the flow cytometer did not demonstrate significant phagocytosis. Similar observations were noted if we had incubated the cells together for 1 h at 4 °C or in PBS at 37 °C (4 °C panel shown as an example), implying that double-positive cells were the result of an active uptake mechanism by the MØ.

When we incubated the cells for 1 h at 37 °C, we observed a significant double-positive population indicating that the MØ had taken up fragments of these CFSE-labeled ADCs (Fig. 6a middle panel, 1h). By gating on the H-2Kb positive MØ, we calculated the percentage of phagocytic cells that were able to internalize ADC fragments within 1 h (Fig. 6a right panel, in this example 70 % of MØ engulfed ADCs). We also added trypan blue after the initial FCM acquisition to quench extracellular fluorescence (Fig. 6a, T. blue). The number of double-positive MØ remained largely unchanged indicating that most of the signal recorded was reflecting material inside the cytoplasm rather than adsorbed material. Addition of cytochalasin D (phagocytic inhibitor) reduced the signal obtained to about 14-15 % (Fig. 6a, CCD). This was reduced further to 10% when trypan blue was added; indicating that about 4 % of the signal corresponded to material adsorbed on the surface – a finding that is not unexpected (Fig. 6a, T. blue + CCD).

We have monitored this phagocytic capacity in both Sp-MØ and BM-MØ over 7 days of culture (Fig. 6b). It was interesting to note that after 3 days in culture, BM-MØ were superior to Sp-MØ with regard to their phagocytic ability. Interestingly, by day 5 as well as day 7, Sp-MØ exhibited comparable phagocytic capacities to BM-MØ, with approximately 50% of the cells in the assay being highly phagocytic. Overall, both cell types increased their phagocytic capacity over the 7 day period with Sp-MØ exhibiting the largest augmentation considering the values obtained at the early time point.

We have examined the uptake of ADC fluorescent fragments using fluorescence microscopy to ensure that the fragments were not merely adsorbed to the cell surface. In figure 6c (i) an image (acquired with low magnification) is shown where fluorescence is localized to numerous round organelles in the cytoplasm. A higher magnification (x60) is also shown of an image acquired as (ii) bright field, (iii) fluorescence, and (iv) a merged image overlay. By comparing the three fields it appears quite clear that the bright fluorescent fragment is contained within a phagosomal structure inside the cell. These images helped to confirm that our data reflect materials engulfed by the MØ.
Figure 6. The phagocytic capacity of Sp-MØ increases with time in culture. (a) Illustration of data acquisition during the assay. Macrophages were incubated with dead CFSE-labeled HEK cells for 1 h at 37°C. The left dot plot (top panel) presents data acquired after instant mixing of the two cell populations to compare with the regular experiment in middle dot plot. Gates (dotted line) were set based on MHC-I expression to calculate the % of macrophages that were able to engulf the HEK cells (right dot plot). In the bottom panel, the left dot plot represents a control experiment carried out at 4 ºC. Trypan blue was added after FCM acquisition at 1 h at 37 ºC to quench extracellular fluorescence (T. blue, dot plot). Cytochalasin D treatment of macrophages (CCD, dot plot) shows significant inhibition of the uptake. The addition of trypan blue to Cytochalasin D treated cells reduces the signal further (T. blue + CCD, dot plot). (b) Percentage of phagocytosis was calculated as in (a) above over a 7-day period comparing Sp-MØ, and BM-MØ. Data shown are the average from 3 independent experiments ± std. (c) Localization of HEK cell fragments engulfed by MØ. Multiple round vesicles identifying engulfed CFSE-labeled HEK cell fragments can be seen in (i) as indicated by white arrows when examined under low magnification (30x). Using high magnification (60x), bright field (ii), and epifluorescence images of the same field, the engulfed fluorescent material can be clearly seen to be contained within the cell membrane (white arrows). Panel iv shows the merged image. (d) Uptake of HEK cells by Sp-MØ, BM-MØ in the presence of the following inhibitors; Fucoidin A (Fuc, 100 μM), Cytochalasin D (CCD, 10 μM), vinblastine (Vin, 30 μM), and dimethyl amiloride (DMA, 150 μM). Data shown are the average from 3 independent experiments ± std.
Since both Sp-MØ and BM-MØ reached similar high phagocytic capacities by day 7, we performed inhibitory studies to compare their uptake mechanisms of dying cells. In figure 6d, where we applied inhibitors (during the phagocytosis assay) to both Sp-MØ and BM-MØ we observed similar effects on both types of MØ indicating that common pathways were used during the internalization process. In general, Fucoidin A (SR-A competitive inhibitor) that interferes with phagocytosis through the SR-A receptor, cytochalasin D (CCD, general phagocytic inhibitor), vinblastine (Vin, endocytic inhibitor), and DMA (macropinocytosis inhibitor) significantly reduced the ability of both MØ to internalize dead cells or their fragments when compared to untreated cells (Fig. 6d). Individually, these inhibitors have reduced the uptake ability of MØ by at least 50% (up to 85% inhibition in the case of CCD and Vin, fig 6d), indicating that more than one mechanism of uptake was being employed by the MØ. Thus, the above data helped to characterize the splenic MØ cultured in vitro and relate such functions to the markers detected above.
2.6 Discussion

Macrophages are fundamental elements of the immune system. Much of our knowledge about the participation of MØ in innate and adaptive immune responses has been acquired from studies performed on BM-MØ peritoneal cells or freshly isolated Sp-MØ (27). Apart from their regular functions in innate immunity, MØ play an important role in direct and cross-presentation (19, 28-31). However, unlike their close cousins, dendritic cells, studies on MØ have not progressed at the same rate, partly because of difficulties in purifying and isolating these cells with high efficiency, especially from the spleen. Therefore, we developed a protocol for isolating and studying Sp-MØ in vitro to facilitate future studies exploring MØ functions. We have chosen to focus our study on three major properties of MØ: morphological aspects, expression of MØ surface markers, and their phagocytic capacity to internalize dead cell fragments.

Phenotypic heterogeneity in Sp-MØ reflects their complex differentiation mechanisms (2, 3, 6, 9, 32). Several MØ subpopulations have been characterized in vivo in the spleen. These heterogeneous MØ subpopulations show different localization and function. For example, there are distinguishable F4/80-positive red pulp MØ, MOMA-1-positive marginal metallophilic MØ, ER-TR9-positive marginal zone MØ, and MOMA-2-positive white pulp MØ in mice. The splenic red pulp MØ, which resemble the cells we isolated in culture, function in the uptake and degradation of aged cells in the tissues. They also play a role in immune responses and produce various cytokines. The other types of spleen MØ have been extensively reviewed recently (2, 3).

In the above reported experiments, splenocytes were cultured for 7 days in M-CSF containing medium (CM), which resulted in obtaining an excellent yield of highly pure cells. This is clearly a simple procedure that does not involve laborious cell depletion or purification steps. The relatively abundant yield of Sp-MØ obtained by using CM, suggests a critical role for M-CSF in growth and maturation of in vitro cultured Sp-MØ. If this cytokine did not influence Sp-MØ proliferation, then two other possible premises can explain the increase in MØ numbers in vitro. The first is that the presence of M-CSF during the early stages of culturing would divert uncommitted myeloid populations in the spleen to differentiate into MØ instead of dendritic cells. This argument can be supported in part by the view that MØ and dendritic cells
are closely related cells (33). The second rationale may be related to an anti-apoptotic effect of M-CSF that allows MØ to survive efficiently in culture. This effect could be similar to that of a recent report in which M-CSF was shown to protect cardiomyocytes from cell death (34).

Previously, Takahashi et al. provided evidence for the in vivo existence of an M-CSF-dependent spleen MØ subpopulation. Because the mutant mice were deficient in M-CSF they showed a reduced presence of red pulp MØ and an absence of the marginal metallophilic MØ and marginal zone MØ when compared to normal littermates. The study proposed that the development and expansions of marginal metallophilic MØ and marginal zone MØ as well as a subpopulation of the red pulp MØ are M-CSF-dependent (35). Considering the data presented in our study, it is plausible that our in vitro-derived MØ are the M-CSF-dependent subpopulation. In addition, because the cells are F4/80, MOMA-1 positive, and SIGN-R1 low/negative, they could be mainly representing the red pulp MØ as previously characterized (35, 36).

Morphologically, the in vitro derived MØ exhibited distinguished characteristics, including the classical firm attachment to the surface of the flask. Over time in culture, the MØ acquired markedly larger size indicating greater cell maturation. Moreover, the MØ increased their expression of surface differentiation markers such as F4/80 indicating macrophage development and maturation (2, 3, 9, 32). It is also conceivable that these acquired MØ features are CSF-driven effects rather than being purely associated with splenic MØ maturation in vivo. But if this was the case, one would not expect to see increases in such classical markers of differentiation and maturation during the in vitro culture since the cells isolated from the spleen are presumably fully mature.

Interestingly, Sp-MØ shared certain features with BMDCs when their surface markers were examined, including the expression of CD11c and CD11b, but they lacked MHC II constitutive expression which confirms previous publications that reported low or negative MHC II expression on Sp-MØ, peritoneal MØ and bone marrow-derived MØ (12, 35, 37). This MHC II constitutive expression is typically exhibited by BMDCs cultured in GM-CSF. Importantly, expression of CD11c by MØ, monocytes and T cells has been reported previously (3, 38-40), yet it is considered as a classical DC marker. Occasionally, only this marker has been used to refer to, or isolate pure DC populations ex vivo, which is clearly sub-
optimal without the use of other appropriate markers. The MØ also exhibited typical lysosomal distribution showing round acidic organelles that generally cluster in the perinuclear regions of cells when compared to MØ obtained from the peritoneum or the bone marrow. Likewise, surface and intracellular markers associated with MØ were confirmed. Accordingly, the present study highlights the changes in certain macrophage specific marker expression such as (MOMA-2 and CD68) during this in vitro maturation period.

The capacity to internalize, degrade, and eliminate antigens are crucial functions of MØ. In order to address the phagocytic activities of Sp-MØ we used an in vitro assay to monitor the uptake of dead target cells by the MØ. We observed that culturing the Sp-MØ in CM enhanced this activity between 3 and 7 days to levels comparable to those obtained by mature BM-MØ. The increase in the phagocytic capacity by Sp-MØ combined with changes in the expression of integrins, MHC-I and CD86 molecules reflects changes in maturation and differentiation stages of Sp-MØ and suggests that they may exist in a physiologically less mature status in vivo than was previously thought.

Since several pathways are utilized by MØ to internalize exogenous antigens (41), we deemed it imperative to compare the uptake mechanisms of dead cells by Sp-MØ and BM-MØ. With the aid of specific inhibitors that interfere with receptors or the mechanisms of engulfing, we performed phagocytosis assays of CFSE-labeled dead cells (42) to define such processes. In our model, we tested the different pathways that can be exploited by Sp-MØ to uptake LyUV-treated cells (19). By employing fucoidin A to define the involvement of scavenger receptor class-A in the uptake process (20, 21), we found that the Sp-MØ use these scavenger receptors in the uptake of dead cells.

Clearly the uptake was mainly phagocytosis-dependent as Cytochalasin D, which disrupts F-actin cytoskeleton polymerization had a significant impact on the process. Nevertheless, since Cytochalasin D inhibits both phagocytosis and macropinocytosis (22) we employed dimethyl amiloride (DMA) to identify the involvement of macropinocytosis in the process (23). Our data clearly showed that besides the regular phagocytic process, macropinocytosis - traditionally used by Sp-MØ to internalize huge quantities of soluble and small antigens - was also involved in the uptake as demonstrated by DMA inhibition. Additionally, endocytosis was also implicated by the Sp-MØ because the endocytosis inhibitor vinblastine
A (24) had clearly inhibited the uptake of antigens by these cells. Vinblastine binds tubulin, leading to inhibition of microtubule assembly needed to facilitate the movement of membrane-bound components from the plasma membrane to early endosomes.

Thus, the inability of a single inhibitor by itself to totally block the uptake of dead ADCs by Sp-MØ suggests that the internalization was taking place through several routes, including SR-A dependent uptake, phagocytosis, endocytosis, and macropinocytosis. This is probably because such dead cells could be a source of different antigenic compositions varying from intact cellular organelles or membrane fragments, to small soluble molecules or proteins. It is possible that each process will be differentially employed depending on the manner cell death has ensued prior to the removal by the MØ(43).

In this study, we have characterized isolated Sp-MØ in M-CSF-containing medium over different time points and have optimized the condition for obtaining high-yield pure Sp-MØ cultures. We have provided initial evidence to support two important concepts: firstly, freshly isolated Sp-MØ are not fully mature, but can be influenced to do so under such culture conditions highlighted above, and secondly, M-CSF may actually exert anti-apoptotic effects on Sp-MØ or influence uncommitted myeloid cells in the spleen that may express CD11c to differentiate into Sp-MØ. This work should enhance the development of much needed knowledge regarding Sp-MØ interactions with pathogens that evade the immune system (44-47).
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CHAPTER 3

Cross, but not direct, presentation of cell-associated virus antigens by spleen macrophages is influenced by their differentiation state
3.1 Preface

Antigen presentation and CTL activation is mediated via pAPCs that have the capacity to generate MHC-I/peptide complexes from both endogenous and exogenous antigens sources. The most efficient pAPCs that start CTL activation are dendritic cells and macrophages. However, macrophages are heterogeneous populations and among these populations are spleen macrophages that are subdivided into different groups. Antigen cross-presentation by spleen macrophages has not been addressed before. The aim of this project was to study the antigen presentation capacity of Sp-MØ in relation to their MCSF-dependent differentiation. We approached this aim with the following hypothesis; cross-presentation of cell-associated antigens by Sp- and BM-MØ is regulated during differentiation. In this study we have revealed the following:

- Sp-MØ and BM-MØ were susceptible to LCMV infections and directly presented the major LCMV epitopes in a similar fashion.
- Cross-presentation of Sp-MØ and BM-MØ inversely correlates with their differentiation state.
- Sp-MØ and BM-MØ use mainly the cytosolic pathway to cross-process cell-associated antigens.
- Cross-presentation by Sp-MØ is more dependent on aspartic peptidases (cathepsin S) in comparison to BM-MØ.
- The cross-presentation capacity of MØ is greatly influenced by the changes in the phagosomal acidification during differentiation.

The works contained in this study, were planned and executed by myself. Sarah Saddiqui helped with RT-PCR results (figure 1c), Matthew Chan helped with western plot results (figure 6b), and Agnieszka Kus provided general help as a fourth year student in optimizing the antigen presentation assays. Dr. Elaine O. Petrof. assisted with figure 6a dealing was measuring proteasome activity assays. The data described in this chapter was published in:

3.2. Abstract

The initiation of T cell immune responses requires professional antigen presenting cells. Emerging data point to an important role for macrophages in the priming of naïve T cells. In this study we analyzed the efficiency and the mechanisms by which macrophages derived from spleen (Sp-MØ) or bone marrow (BM-MØ) present Lymphocytic choriomeningitis virus (LCMV) antigens to epitope-specific T cells. We demonstrate that due to phagosomal maturation, Sp-MØ down-regulate their ability to cross-present cell-associated but not soluble antigens as they further differentiated in culture without altering their capacity to directly present virus antigens after infection. We propose that Sp-MØ are extremely efficient at direct and cross-presentation. However, if these cells undergo further M-CSF dependent maturation, they will adapt to be more scavenger and phagocytic and concurrently reduce their cross-presenting capacity. Accordingly, Sp-MØ can play an important role in regulating T cell responses via cross-presentation depending on their differentiation state.
3.3. Introduction

Macrophages (MØ) are heterogeneous cells, which perform diverse functions important in immunity (1, 2). They are needed in regulating adaptive immune responses (1-3), and play a vital role during the early stages of virus-host interactions (4-7). In vivo, MØ can initiate T cell responses against various antigens (8-12). The capacity to prime T cells by MØ is supported by studies showing that the depletion of MØ suppresses T cell responses (9, 10, 13, 14). Their ability to express costimulatory molecules, as well as MHC-II to interact with helper T cells, allow MØ to participate effectively in such immune mechanisms (3, 10, 12).

To initiate CD8+ T cell responses, antigens are processed and presented by professional antigen presenting cells (pAPCs) via two pathways - direct and cross-presentation (15-18). MØ are able to cross-present particulate and soluble exogenous antigens to generate MHC-I/peptide complexes (11, 19-24). A central population of MØ in the spleen (Sp-MØ) plays a critical role in controlling pathogens circulating in the blood (3, 25, 26). We have recently described the characterization of Sp-MØ cultured in vitro and provided evidence that further signals from M-CSF are needed for Sp-MØ to fully differentiate giving rise to an M-CSF dependent population (27).

MØ and dendritic cells (DCs) were reported to possess competent phagosomes needed for optimal cross-presentation (28, 29). In contrast to DCs, the processing pathways within MØ are apparently geared towards antigen degradation, which would explain why DCs are more efficient at cross-presentation (30, 31). However, such observations are based on studies performed using bone marrow derived MØ (BM-MØ) with little or no data examining other types of MØ, especially Sp-MØ. Furthermore, hardly any studies have assessed the role of MØ differentiation on MHC class I-restricted antigen presentation. In this report, we analyzed the differentiation of Sp-MØ in relation to antigen presentation and illustrate for the first time that Sp-MØ are extremely efficient in cross-presenting cell associated antigens, but downregulate this ability when they differentiate into fully competent scavengers.
3.4. Materials and methods

3.4.1. Mice & Cells

C57BL/6 (H-2b) mice were purchased from Charles River (St. Constant, QC, Canada), and were used between 6 to 8 weeks. Animal experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care. The cell lines BMA and DC2.4 (kindly provided by Dr. K. Rock, University of Massachusetts Medical School, Worcester, MA), were cultured in RPMI 5% FBS (23, 32). HEK-NP (LCMV-NP transfected HEK293 cells) were cultured in DMEM supplemented with puromycin as previously described (20, 33). The HEK293 cells were grown in DMEM 5% FBS. All media were purchased from Invitrogen (Burlington, ON, Canada).

3.4.2. Preparation of BM-MØ, BM-DCs, Sp-MØ, & ADCs

Femurs and tibias from mice were collected and the marrow was flushed with PBS, re-suspended in lysis buffer (1.66% w/v ammonium chloride) for 5 min. Debris was removed by passing the cell suspension through metal sieve. To generate BM-MØ, cells were prepared as previously described (27, 34). Cells were cultured in 6-well tissue culture plates (3-5 x10^6 cell/ well in conditioned medium (CM), consisting of RPMI containing 20% of L929 supernatant as a source of M-CSF and 10% FBS. After 3 days, fresh medium was added, and MØ were harvested with 1x Trypsin-EDTA. To generate BM-DCs as previously described (27, 35), cells were cultured in 6-well tissue culture plates (3-5 x10^6 / well) in RPMI medium containing 10% FBS, 50 µM 2ME, 10 ng/ml GM-CSF, and gentamicin 3 µg/ml. At day 5 the non-adherent cells were transferred into a new 6 well-plates and left for 24 h at 37°C. The loosely adherent cells (highly enriched CD11c^+ MHC-II^+ BM-DCs) were harvested for FCM analysis.

Sp-MØ were isolated and prepared as previously described (27), and adherent cells were harvested with 1x Trypsin-EDTA and tested at the indicated times of culture (3-7 days). HEK-NP cells (ADCs) were treated as previously described (33). The cells were exposed to three cycles of freezing at -180°C and thawed at 37°C to induce necrosis. The LyUV condition was performed by snap freeze/thaw followed by UV irradiation for 10 minutes at 120,000 microjoules / cm^2 (Ultraviolet Products, Upland, CA). Apoptosis
was induced by exposing the cells to a 5 min UV treatment as above, followed by incubation for 3 h at 37ºC.

3.4.3. Flow cytometry analysis

The MØ were stained directly with fluorochrome-labeled Abs against surface markers; PE-conjugated anti-mouse MHC-I, H-2Kb (clone CTKb), FITC-conjugated anti-mouse MHC-II, I-Ab, (clone 25-9-17s) PE-Cy5-conjugated anti-mouse CD11b (clone M1/70.15), PE-conjugated Hamster anti-mouse CD11c (clone N418), and FITC-conjugated anti-mouse F4/80 (clone C1: A3-1) were purchased from Cedarlane, (Ontario, Canada). For cell surface staining, the cells were stained for 15-20 min at 4ºC. After washing with PBS, the cells were prepared for measurement with FCM. Data were acquired with the Epics XL-MCL flow cytometer and analyzed with the Expo 32 Advanced Digital Compensation Software package (Beckman Coulter, Miami, FL).

3.4.4. Phagocytosis and antigen degradation assays

To examine phagocytosis, BM-MØ and Sp-MØ were processed as previously described (27). The cells were then co-cultured with CFSE-labeled antigen donor cells (ADCs) at a ratio of 3:1. The CFSE-labeled HEK cells (ADCs) were induced to undergo death by a treatment we refer to as LyUV (20). Sp-MØ and BM-MØ were then stained with anti-mouse H-2Kb R-PE antibody for 15 min at 4 ºC to distinguish them from the ADCs (27). In parallel experiments, we investigated the effects of phagosomal activities on antigen degradation using CFSE-labeled, LyUV-treated HEK cells as ADCs. The CFSE-labeled ADCs were co-incubated with MØ for 1 hour before extensive (3x) washing of the wells with PBS to remove excess ADCs. The cells were chased for 12 hours to monitor antigen degradation where the loss of fluorescence is indicative of antigen degradation due to the action of phagosomal acidification and enzyme activation.
3.4.5. Cytoplasmic staining for LCMV proteins

BM- and Sp-MØ were infected overnight at a multiplicity of infection (MOI) of 3 at 37ºC. The cells were harvested as before, fixed with 4% Formalin-PBS for 30 min at RT, and permeabilized with 1% Triton X-100 (Fisher, New Jersey, USA) for 20 min at RT, followed by incubation for 1 hr at RT with rat anti-LCMV-NP mAb (clone VL4) or mouse anti-LCMV-GP mAb (clone KL25). After washing, the appropriate secondary antibodies were added for 1 hr at RT.

3.4.6. Intracellular cytokine staining (ICS)

For measuring IFN-γ production by CD8+ cytotoxic T cells, ICS was performed in peptide restimulation assays (20, 33). Effector cells were incubated with APC-loaded peptides (0.1 µM) at a ratio of 1:1 in the presence of brefeldin A (10 µg/ml). The APCs were loaded with one of the following synthetic peptides GP33-41 (KAVYNFATC), GP276-286 (SGVENPGGYCL), NP205-212 (YTVKYPNL), and NP396-404 (FQPQNGQFI) or an irrelevant peptide control. The peptides (purity >90%) were synthesized at CPC Scientific (San Jose, CA).

To determine the frequency of activated T lymphocytes, cells were stained with a TRI-COLOR conjugated, rat anti-mouse CD8α clone 5H1 (Cedarlane Hornby, ON) at 4°C, then fixed with 1% paraformaldehyde, washed twice before adding FITC-conjugated anti-IFN-γ antibody, clone XMG1.2 (Cedarlane) prepared in PBS; 0.1% saponin overnight at 4°C. The following day, data were acquired with FCM as described above.

3.4.7. Induction of CD8+ T cell lines and antigen presentation assays

Peptide-specific T cell lines were generated as previously described (20). Briefly, mice were infected with LCMV-WE and splenocytes were isolated 4 weeks later and cultured together with γ-irradiated peptide-loaded APCs. In all assays, CTLs specificity corresponded to at least 75% when tested with the specific peptide (100 nM). For direct presentation, BM-MØ or Sp-MØ were infected with LCMV-WE at a multiplicity of infection (moi) of 3. The infection was allowed to proceed for 24 h at 37ºC before co-
incubation (ratio 1:1) with LCMV epitope-specific T cells. To study cross-presentation, the HEK-NP ADCs (5x10^5 cells/well) were co-incubated with BM-MØ or Sp-MØ (1x10^5 cells/well) overnight before the addition of NP396-specific CTL lines (20).

Where indicated, the following inhibitors were used in a range of doses: BFA (0-15 µg/ml), chloroquine (0-120 µM), leupeptin (0-250 µM), pepstatin A (0-350 µM), lactacystin (0-12 µM), and ammonium chloride (0-80 mM). Lactacystin was purchased from Biomol (PA, USA) and the other inhibitors from Sigma. As previously described (20), controls were peptide-labeled drug-treated cells to ensure that MHC-I molecules are not affected by the drug treatment and are presenting peptides appropriately.

The B3Z T-cell hybridomas (kindly provided by Dr. Shastri, University of California) contain a *lacZ* reporter gene linked to the nuclear factor of activated T cells (36) and were used to study the capacity of Sp-MØ to cross-present the soluble OVA protein antigen (Sigma, Oakville, Ontario, Canada). B3Z contains a *lacZ* reporter gene linked to the nuclear factor of activated T cells. Recognition of the SIINFEKL peptide in the context of class I by the T-cells results in activation of the enzyme and conversion of the chromogenic substrate into a colored compound that can be detected by absorbance spectrophotometry at 415nm. A total of 10^5 Sp-MØ were incubated with varying concentrations of OVA protein overnight at 37ºC before washing with PBS and addition of 10^5 B3Z cells. After 24 h of incubation, the cells were processed and the absorbance was read at 415 nm using a microplate reader (Varioskan, Thermo Electron Co.).

### 3.4.8. Proteasome activity measurements

Sp-MØ and BM-MØ were incubated for 5 hours with LyUV-treated HEK-NP cells at 37 ºC before being resuspended in ice-cold lysis buffer (10 mM Tris / pH 7.4, 5 mM MgCl₂, 50U/ml DNase and RNase) and complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Proteasome activity was determined with a fluorogenic substrate assay as previously published (37). These fluorescent substrates are cleaved by the proteasome and define three types of protease activities; chymotrypsin-like activity, caspase-like activity, and the trypsin-like activity, respectively. The proteasome inhibitor, MG-132 (25 µM) (Biomol) was used as an inhibitor control.
3.4.9. RT-PCR

MØ were starved overnight in regular medium before treating with 1 µg/ml LPS (E. coli 055:B2, Sigma) for 5h. Total RNA was isolated and RT-PCR was performed with the Sprint Thermal Cycler. The following primers were purchased from Integrated DNA Technologies (Coraville, Iowa): IL-10 Forward 5’-ATTAGATCCCTGGTGAGAA-3’, IL-10 Reverse 5’-ACACCTTTGCTTGGAGCTTATTAA-3’, IL-6 Forward 5’-CCTCTCTGAAGAGACTTCC-3’, IL-6 Reverse 5’-GCACAACCTTTTCTCATTCC-3’, TNF-α Forward, 5’-CAGGGGACACCACGCTCC-3’, TNF-α Reverse 5’-TGACCTCAGCGCTGAGCTTGT-3’, 18S rRNA Forward 5’-AAACGGCTACCACTCAAG-3’, 18S rRNA Reverse 5’-CCTCAAATGGATCTCGTTA-3’.

3.4.10. Western Blotting

To prepare cell extracts for protein gels, cells (6x10⁶) were lysed on ice for 20 minutes in cell lysis buffer (50mM HEPES, 100mM NaF, 1mM EGTA, 150mM NaCl, 1.5mM MgCl₂, 10% glycerol, and 1% Triton X-100) and 1X protease inhibitor cocktail (0.1% Aprotin, 0.1% Leupeptin, 0.035% Pepstatin A, and 8.5% PMSF from Biovision, Mountain View, California). Protein content was determined and 50 µg was loaded onto a 10% SDS-PAGE gel. Proteins were transferred to a 0.2 µm Biotrace Pure Nitrocellulose membrane (Pall, Mississauga, Ontario). Blots incubated overnight at 4°C with the mouse mAb (0.5 µg/ml in 5% BSA) specific for the immunoproteasome subunit LMP7 (ab58094 from Abcam, Cambridge, MA), or mouse mAb (0.2 µg/ml in 5% BSA) for β-actin (3598-100 from Biovision, Mountainview, CA) and detected by enhanced chemiluminescence with Amersham’s ECL Advance kit (Amersham, Piscataway, NJ).
3.5. Results

3.5.1. Characterization of Sp-MØ

We have previously shown that M-CSF is needed by both SP-MØ and BM-MØ for further differentiation in culture (27). First, we tested surface antigen expression during the culture period as depicted in figure 1a (3, 5 and 7 days). Both cell types expressed similar levels of the F4/80 antigen as well as other intracellular MØ markers (data not shown). By testing for the expression of CD11b, we concluded that the cells were of high purity reaching >90% by day 7. With regard to MHC expressions, by day 7 in culture, we detected significant levels of MHC-I but failed to detect any MHC-II molecules (figure 1a). We also tested their ability to initiate cytokine production as a result of TLR ligand stimulation and to phagocytose dead ADCs - an important property that contributes to antigen cross presentation (16, 38). In figure 1b, we incubated the ADCs with MØ for 1 h at 37 °C and observed a significant uptake of the CFSE-labeled ADCs by both MØ populations, which was comparable by day 7 in culture.

We next tested for the induction of cytokines mRNA by RT-PCR analysis for IL-10, IL-6 and TNFα after 6 h stimulation with the TLR4 ligand LPS (1 µg/ml). We replaced the M-CSF medium that cells were cultured in with regular RPMI 24 h prior to the stimulation with LPS to exclude the effects of M-CSF on the cytokine induction. Figure 1c shows that LPS induced a similar cytokine profile of induction in both cell types with regard to IL-10, IL-6 and TNFα compared to untreated cells and the control 18S rRNA. Thus, M-CSF-dependent Sp-MØ exhibit a classical MØ phenotype, able to phagocytose cell debris and upregulate certain cytokines in response to bacterial stimulus.

3.5.2. Direct antigen presentation by Sp-MØ and BM-MØ is consistent

One major aim of this study was to examine how the maturation and differentiation of MØ can influence antigen presentation after virus infection. We proceeded by infecting both cell types at MOI of 3 throughout different days after in vitro culture. The cells were then examined for de novo synthesis of the two major LCMV proteins (NP and GP) after 24 h of infection. Figure 2a shows that both Sp-MØ and BM-
Figure 1. Comparison of Sp-MØ and BM-MØ cultured in vitro for 7 days. MØ were cultured for different time points and tested for the expression of MHC-I, MHC-II, CD11b, and F4/80 (a). In (b) the same cells were examined for their abilities to phagocytose CFSE-labeled dead HEK cells for 1 h at 37°C. By gating on MHC-I expression, as a marker for the mouse cells, the % of MØ that were able to engulf the HEK cells were plotted. Data shown are the average from 3 experiments ± SEM. In (c) we compared the induction of IL-10, IL-6 and TNFα in Sp-MØ, and BM-MØ cultured for 7 days after stimulation with LPS for 6h. PCR assay cycles were as follows: 30 cycles for IL-6 and IL-10, 25 cycles for TNFα, and 15 cycles for 18S rRNA. The PCR products were visualized on 1.2% agarose gels and ethidium bromide staining. One experiment representative of three is shown.
MØ were highly susceptible to LCMV infection, evident by the abundant expression of LCMV-NP (top panel) as well as LCMV-GP (bottom panel).

To detect direct antigen presentation, we tested for the major epitopes derived from the GP and NP proteins of LCMV, by utilizing epitope-specific CD8+ T cell lines (specific for GP33-41, GP276-286, NP205-212, and NP396-404). After LCMV infection both cell types were co-incubated with peptide specific T cells for a total period of 3 h to allow for IFN-γ synthesis in stimulated T cells. As seen in figure 2b, with the exception of the NP205 peptide, the other three major epitopes were presented in a comparable manner by both MØ populations. Furthermore, this level of presentation was within a similar range (60-80 %) throughout the seven-day culture period, whereas, the NP205 peptide was presented with lower efficiency (30-40 %). However, the NP205 presentation after infection was comparable in the two populations. This lower peptide presentation compared to other epitopes was not due to the quality of the peptide specific T effector cells. This was confirmed because the same effector CD8+ T cells were tested in a peptide control assay using the same MØ populations pulsed with the NP205 peptide (figure 2c).

**3.5.3. Cross-presentation capacity of Sp-MØ and BM-MØ varies with their differentiation state**

We next focused our attention on the ability of those cells to cross-present cell associated antigens. For this purpose, we made use of HEK-NP cells as ADCs in antigen cross presentation assays as published previously (20). We utilized NP396-specific CD8+ T cells to detect antigen cross presentation of LCMV-NP by the MØ throughout the differentiation period *in vitro*.

In figure 3, the three different conditions of ADCs (as described in the material and methods) were employed in the assay at a ratio of 5:1 (ADC: APC). After 3 days in culture, Sp-MØ were efficiently able to cross-process cell associated antigens from ADCs (LyUV), better than from ADCs that were undergoing apoptosis or necrosis (fig 3a). Significantly, there was an observable trend of a reduced cross-processing ability of the Sp-MØ during differentiation especially when comparing them between days 3 and 7 in culture. This observation was consistent throughout the different conditions used to prepare the ADCs and was most prominent in the LyUV condition. There was a similar trend when analyzing the cross-processing
Figure 2. Direct antigen presentation profiles of LCMV epitopes by Sp-MØ and BM-MØ during differentiation are identical. (a) Infection of both cell types by LCMV results in a similar de novo synthesis of viral proteins 24 h post infections. At different stages of in vitro culturing (days 3, 5, 7), both cell types were infected with an MOI of 3 before testing for the expression of LCMV-NP or LCMV-GP 24h later. (b) In parallel, the same infected cells were assayed for direct antigen presentation by assessing the activation of NP396, NP205, GP33, or GP276-specific CD8+ T cells and measuring IFN-γ production after in vitro restimulation. (c) Peptide-loaded (10^{-7} M) Sp-MØ and BM-MØ were used as positive controls. The data shown are the average from 3 independent experiments ± SEM.
abilities of BM-MØ however; this was less evident when apoptotic or necrotic ADCs were examined (fig 3b).

Because M-CSF has been reported to influence the ability of cells to cross-present antigens (39), we next compared the ability of Sp-MØ and BM-MØ to cross-present cell associated antigens in the presence of M-CSF (figure 3c and 3d). When examining figure 3 (c, d) and comparing it to figure 3 (a, b), one can observe an identical profile with regard to LyUV-treated and necrotic ADCs. However, in the presence of M-CSF, the apoptotic ADCs were cross-presented at a higher rate and exhibited a similar trend to the LyUV condition.

Evidently, it was important to compare the cross-processing abilities of these cells to dendritic cells - considered to be the major cells able to cross-process antigens. For this purpose, we employed two different cell types; conventional BMDCs cultured in vitro for 7 days and the cloned dendritic cell line, DC2.4, which has the ability to cross-process exogenous antigens (32). We cultured BMDCs for 7 days as previously reported (27) and confirmed their expression of CD11c and MHC-II (data not shown). When BMDCs or DC2.4 were tested in the cross-presentation assay (fig 3e), we observed patterns of data similar to those obtained with the Sp-MØ and BM-MØ cells (day 3-5 in culture). This indicated that both MØ cell populations (Sp and BM) at the early time points of differentiation were competent in antigen cross-presentation given that they were able to cross-present antigens similar to BMDCs tested on day 7 of culture.

3.5.4. Cross-presentation of soluble antigens by Sp-MØ and BM-MØ is not affected by the differentiation in culture

Since soluble antigens and cell-associated antigens can access different compartments and be cross-processed intracellularly (16), we examined the patterns of soluble antigen (OVA) cross-presentation during MØ differentiation. The efficiency of OVA cross-presentation was evaluated by monitoring the activation of the B3Z hybridoma in a standard colorimetric assay. B3Z cells was also added to wells containing SIINFEKL-pulsed MØ (figure 4b), medium alone, or MØ alone as positive and negative controls respectively. Cross-presentation of OVA was observed throughout the various OVA
Figure 3. The ability of Sp-MØ and BM-MØ to cross-present cell associated antigens inversely correlates with their differentiation state. HEK-NP cells (ADCs) were incubated with Sp-MØ (a) or BM-MØ cells (b) cultured at different time points (3-7 days) and were left overnight in regular RPMI medium before the addition of NP396 specific T-cells. In (c), and (d), the same experiments were carried out, but RPMI was supplemented with M-CSF. Data represent the means of three wells ± SEM from one experiment with 4 independent repeats. HEK-NP cells were used in three diverse conditions. Apoptotic (black-filled histograms), necrotic cells (unfilled histogram) and LyUV-treated cells (hatched). NP396-specific CTL responses were determined by measuring IFN-γ induction. (e) BMDCs (7 days) and DC2.4 were employed in a cross-presentation assay before the addition of NP396 specific T-cells. This is one representative experiment from 3 independent trials.
concentrations, with optimal activation occurring at the highest OVA doses (figure 4a). B3Z activation was very similar with either Sp-MØ or BM-MØ throughout the culture period; however, at the higher OVA doses, BM-MØ did show slightly better cross-presentation. Importantly, no significant differences were observed between the different days of culture in either cell population, indicating that in contrast to cell-associated antigens, soluble OVA cross-presentation was not influenced by MØ differentiation. Furthermore, we made use of the protein OVA (20 mg/ml) to compare the best cross-presenting (3 day Sp-MØ) cells to those of classical BM-DCs and examine their abilities to cross-prime naïve CTL in vivo after injecting $10^6$ cells (Sp-MØ or BM-DCs) i.v. We isolated the splenocytes after 7 days, and expanded SIINFEKL-specific CTL. Remarkably, we found that the 3 day spleen-derived MØ were equally competent to the classical BM-DCs in priming these CTLs as shown (figure 4c).

3.5.5. Mechanisms employed by Sp-MØ and BM-MØ to cross present cell-associated antigens

We next investigated the possibilities that these two MØ populations employ different processing pathways to cross-process cell-associated antigens. For this purpose, we employed well-characterized inhibitors that are known to interfere with various steps in the cross-presentation pathway in dose dependent assays. The presentation of NP396 was significantly diminished with lactacystin and BFA (fig 5a,) suggesting that both Sp-MØ and BM-MØ utilize the cytosolic (proteasome-dependent) pathway for degrading cell-associated antigens.

To assess the relative contribution of the proteasome-independent vacuolar-processing pathway, we incubated the APCs with various inhibitors of the endo/lysosomal vacuolar-processing pathway. We observed a difference in the degree of inhibition of cross-presentation between the two MØ populations with Sp-MØ being more susceptible when serine and cysteine proteases were inhibited by leupeptin (fig 5a). However, the aspartate proteases inhibitor, pepstatin, did not exert any effects on NP396 generation during cross-presentation (fig 5a).

Initial endosomal processing is critical in these two MØ populations for the final outcome of cross-presentation. We confirmed this by interfering with the vacuolar acidification using chloroquine, a specific inhibitor of the vacuolar pathway, which reduced cross presentation to near background levels. However,
Figure 4. Cross-presentation of soluble antigens by Sp-MØ and BM-MØ is not affected by their differentiation in culture. (a) The soluble protein ovalbumin was added to Sp-MØ (filled square) or BM-MØ (open circles) overnight before the addition of B3Z T-cell hybridoma. B3Z T-cell activation is measured in the assay by detection of absorbance at 415nm as described in the M&M. (b) As a positive control, Sp-MØ and BM-MØ were loaded with SIINFEKL. The data shown are the average from 3 independent experiments ± SEM. (c) The protein OVA (20 mg/ml) was incubated with 3 day Sp-MØ or classical BM-DCs for 1 h, then supernatant were removed and the cell pellets were injected i.v. (10⁶ cells) to test for T cell priming in vivo. After 7 days, splenocytes were isolated and re-stimulated with γ-irradiated, SIINFEKL-pulsed BMA cells for further 8 days. ICS specific for IFN-γ production was performed by incubating the expanded SIINFEKL-specific CTL with peptide-labeled BMA cells. The gate was sat where the negative control value was less than 1%. The experiment shown is one trial from three independent repeats showing similar results.
interference with cytosolic pH using NH₄Cl did not have any significant inhibitory effects on cross presentation by the two MØ populations (fig 5a), because it allows for efficient antigen release in the cytosol as has been reported previously (20, 40). As controls, we tested the peptide-loaded MØ in the same inhibition studies, and found no negative effects on T-cell responses (fig 5b).

3.5.6. Proteasomal activities in Sp-MØ and BM-MØ increase due to exogenous antigen uptake

The activity of the proteasome was examined during exogenous antigen processing. We tested the three enzymatic activities of the proteasome in Sp-MØ and BM-MØ after incubation with LyUV-treated HEK-NP as ADCs. As can be seen in figure 6a, exposure of either MØ populations to exogenous antigen in the form of cellular debris had a marked induction of all three activities (chymotrypsin-like, trypsin-like, and caspase-like activities) of the proteasome. Thus, a possible link between proteasomal activities and the reduced ability to cross present cell-associated antigens was ruled out in this model.

Since immunoproteasomes were reported to play an important role in controlling cross-priming (41), we have explored the possibility that basal expression of the immunoproteasome during MØ differentiation may be affected. Thus, using western blot analysis we examined the expression of LMP7, one of the key catalytic subunits of the immunoproteasome (fig. 6b) by Sp-MØ and BM-MØ during day 5 and day 7 in culture, when cross-presentation was significantly altered (figure 3). As a control for LMP7 detection we employed IFN-γ-stimulated BMA cells and β-actin as a loading control. Steady state expression of the LMP7 subunit was observed in both the day 5 and day 7 MØ populations (fig 6b). There were no significant changes in the immunoproteasome expression levels during the different days tested when comparing each population.

3.5.7. Phagosomal-degradation in Sp-MØ directly affects cross-presentation

Phagocytosis and phagosomal processing are critical for cross-presentation. Therefore, we focused our analysis on exploring the role of phagosomal activities during cross-presentation. We made use of our CFSE-labeled HEK cells that we already employed in the phagocytosis assay (fig 1b). Here, we chased the degradation of the engulfed CFSE-labeled HEK cells over 12h. A shift in fluorescence after one hour (1h)
Figure 5. Mechanisms of cross-presentation employed by Sp-MØ and BM-MØ. a) The ADCs (LyUV-treated) expressing LCMV-NP were incubated with either Sp-MØ (filled square) or BM-MØ (open circles) in the presence of various inhibitors overnight as indicated in the individual graphs. The APCs were then incubated with NP396-specific T cells to measure IFN-γ induction. b) As controls both MØ (open histograms) were pulsed with the NP396 peptide 20 h post treatment with inhibitors employed at the highest concentration as in 5a, to control for antigen presentation. Data represent the means of three experiments ± SEM.
of incubation was detected (fig. 7a). This fluorescence shift was directly proportional to the ratio of ADCs: MØ employed in the assay. In the top panel (fig 7a), the ratio was 3:1 whereas in the bottom panel, the data was obtained at a ratio of 1:1 showing total CFSE degradation.

In order to confirm the premise that phagosomal maturation and acidification are related to the antigen degradation in these MØ, we carried out the assay (1:1 ratio) in the presence of chemicals that can regulate phagosomal maturation and acidifications. In these assays, we used Diphenyleneiodonium chloride (DPI), which inhibits NADPH oxidase (NOX2) system elevating acidification of the phagosome, and Concanamycin A (Con.A) to interfere with V-ATPase activities reducing the ability of the phagosome to efficiently acidify. The data in figure 7b are mean fluorescence values obtained from different treatments during the antigen degradation assay. We found that DPI accelerated the degradation of the CFSE in MØ to a level that was almost similar to the negative control (fig. 7b, compare 0h, 1h, and 1h DPI). After 12 hours the values corresponded to the results obtained with no treatment. On the contrary, Con.A delayed the degradation in the phagosomes as evident by the already higher values obtained at 1 and 12 h post treatment (figure 7b, 1h, 1h Con.A, and 12h Con.A).

We next addressed the correlation between the phagosomal acidification/maturation and antigen cross-presentation in these MØ and compared them to BMDCs. We chose day 7 MØ because our data showed that cross-presentation was lowest in these cells. We repeated the cross presentation assays and treated the MØ with either DPI or Con.A throughout the assay using three different populations. There was a significant increase in cross-presentation with Con.A and a decrease with DPI when compared to the untreated cells (figure 7c, BMDC). This phenomenon was also observed with day7 BM-MØ. However, cross-presentation in day7 Sp-MØ was largely unaffected by the DPI treatment and increased significantly with Con.A treatment. These data indicate that even though phagosomes in mature Sp-MØ undergo maturation/acidification during phagocytosis and cross-presentation, they do not rely extensively on NOX2 to regulate phagosomal functions. The inhibitor did not affect MHC antigen presentation of the treated cells since peptide-pulsed treated cells behaved in a similar manner to peptide-pulsed untreated cells (fig. 7c, right panel).
Figure 6. Analysis of proteasomal activities in Sp-MØ and BM-MØ. (a) Both cell types were cultured for either 3 or 7 days and analyzed for the three proteasomal chymotrypsin-like, trypsin-like, and caspase-like activities post ADC co-incubation. Macrophages without ADCs were the background controls. The proteasome activities were expressed as relative fluorescence unit over time (RFU/min). Data are expressed as means of 3 repeats of one experiment. (b) Cell lysates from Sp-MØ and BM-MØ cultured for either 5 or 7 days were analyzed by western blot for the expression of the immunoproteasome subunit LMP7. As a positive control, BMA cell lines were treated with IFNγ.
3.6. Discussion

Macrophages are known to be potent antigen presenting cells and have been reported to prime CD8+ T cell responses (8, 9). Much of our understanding about the participation of MØ in antigen presentation has been acquired from studies performed on BM-MØ or activated peritoneal cells (2), but more studies are needed to investigate their role as pAPCs (2, 3, 15, 42). Interestingly, it has recently been reported that monocytes (the circulating precursors for tissue MØ and DCs) are able to sample antigen from the bone marrow environment and present them after they leave the bone marrow where they may differentiate into DC at the sites of inflammation (43-45).

With regard to MØ role in antiviral immune responses, it has been shown that mice, deficient in marginal zone MØ failed to clear infections with the cytopathic vesicular stomatitis virus while infections with low doses of non-cytopathic lymphocytic choriomeningitis virus usually resulted in a carrier state in the majority of mice (46). Moreover, a crucial role of marginal zone MØ and marginal zone metallophils in the clearance of lymphocytic choriomeningitis virus infection was previously reported (47). However, the regulation of cross-presentation in Sp-MØ is not well-defined especially when considering their activation status. Thus, we set our objectives to study their capacity to present viral antigens via the direct or cross-presentation pathway.

We have recently reported that Sp-MØ cultured in the presence of MCSF-enriched medium undergo further differentiation in vitro (27). Concerning cross-presentation of cell-associated antigens, we focused on the immunodominant LCMV epitope (LCMV-NP396) which is cross-presented in vitro and in vivo (15, 20, 33). This epitope is derived from a very stable protein important for cross-priming (20, 48).

We compared M-CSF dependent Sp-MØ with BM-MØ and found them to be similar phenotypically and functionally including mRNA induction of IL-10, IL-6 and TNFα when treated with LPS. This cytokine profile induced by Sp-MØ treated with LPS is similar to previous reports with BM-MØ, where an increased expression of pro-inflammatory cytokines IL-6 and TNF-α (49) as well as the Th2 cytokine, IL-10 was observed (50). Both cell populations were permissive to LCMV infection and allowed efficient de
Figure 7. Cross-presentation ability of Sp-MØ and BM-MØ is influenced by their antigens degradation efficiency. (a) Comparison of antigen degradation in Sp-MØ and BM-MØ. Using CFSE-labeled, LyUV-treated HEK cells co-incubated with MØ, antigen fate was traced for 12 hours by monitoring the loss of fluorescence. In the top panel, the ratio was 3:1 (ADCs:MØ), whereas in the bottom panel it was 1:1. (b) Values for mean fluorescence intensities were determined in the absence or presence of the inhibitors DPI (12µM) and Con.A (0.5nM) in day7 cells. The ratio was 1:1 between ADCs and MØ. (c) The ability of BMDC, Sp-MØ and BM-MØ to cross-present cell associated antigens were examined in the presence or absence of DPI (12µM) and Con.A (0.5nM). HEK-NP cells were incubated with the various pAPCs overnight in regular RPMI medium before the addition of NP396 specific T-cells. NP396 cross-presentation was determined by measuring IFN-γ induction in the NP396-specific T cells. In the right panel the pAPCs were pulsed with the NP396 peptide 20 h post treatment with inhibitors to control for antigen presentation. Data represent the means of three experiments ± SEM.
novo synthesis of the LCMV proteins NP and GP similar to the reports showed with peritoneal MØ (46, 47).

As a result of MØ infection with LCMV and by employing peptide-specific T cells to test for antigen presentation, we found that the four major T cell epitopes were processed and presented in a similar manner by both cells types independent of time in culture. NP205 was the least efficient in stimulating high numbers of the T cell line, most likely due to competition for MHC molecules after infection rather than an intrinsic binding property for MHC, because NP205 has been shown to have high MHC binding affinity (51).

Thus, one would predict after LCMV infection that either cell type can directly prime CD8+ T cells specific for LCMV antigens even if its MHC II expression that is normally needed for helper T cell interactions is low or absent. This is particularly relevant since certain infectious agents such as LCMV can prime T cells in the spleen without the need for MHC II expression or CD4 help (52). Such priming mechanism can be achieved by TLR stimulation (e.g. TLR3) on pAPCs that initiate CD40-CD40L interactions, thereby bypassing the need for CD4+ T cell help (53, 54). Nonetheless, it is well established that MØ can readily induce MHC II expression and co-stimulatory molecules in the presence of IFN-γ (1, 55).

MØ were reported to be capable of cross-presentation in many different systems employing mainly BM-MØ or MØ cell lines (11, 19-24, 28). Furthermore, BM-MØ and BM-DCs are known to possess organelles optimized for cross-presentation of exogenous antigens (56), and are able to use mannose receptors to efficiently cross-present soluble antigens (57). Interestingly, soluble antigen cross-presentation was found to involve early endocytic compartment trafficking and is aided by TAP recruitment to such early endosomes due to signaling through TLR4 and MyD88 (58). Our data presented here show for the first time several interesting observations with regard to Sp-MØ by examining the cross-presentation of the immunodominant epitope (NP396) derived from a very stable virus protein that has been shown to cross prime in vivo and alter T cell immunodominance hierarchies during future virus infections (33).

We observed that Sp-MØ were efficient in cross-presenting this NP396 epitope and behaved in a similar manner to BM-MØ during differentiation in culture. We also obtained equivalent data when we
compared the most efficient cross-presenting MØ (day 3-5 MØ) to the classical BMDC. With regard to the latter cell population, cross-presentation of the exogenous antigens was reported to vary during maturation (59), and were best carried out by BM-DCs that were classified as not fully mature (40, 60). Interestingly, we did not detect significant reduction in the ability of MØ (day 3, 5 and 7) to cross-present the soluble protein OVA antigens. This could be due to the different processing pathways involved when small soluble proteins versus large cellular fragments are taken up by different APCs (16-18). When we compared 3-day Sp-MØ with classical BMDC to uptake OVA, injected i.v. to prime CD8+ T cells in vivo, we obtained remarkably analogous levels of CD8+ T cells activation. These observations substantiate previous data showing that peptide-pulsed BM-DCs and MØ exhibited comparable abilities to stimulate CTL proliferation (8).

Similar to BM-MØ, the Sp-MØ relies mainly on the cytosolic pathway for cross-processing these antigens. This cytosolic pathway is currently recognized to be the main mechanism operating under physiological conditions (42). We examined three different treatments of ADCs; apoptosis, necrosis and LyUV-treated cells. When Sp-MØ (cultured for 3 days) were allowed to undergo further differentiation in vitro, we observed a steady decrease in their ability to cross-present NP396. This was very clear in the LyUV condition, however when the assay was carried out in the presence of M-CSF, cross-presentation of apoptotic ADCs increased significantly at day 3 and consequently declined during the culture period in a similar manner to the LyUV condition. It is possible that M-CSF sustains an optimal expression of receptors such as the SR-A I/II by the MØ. These receptors are known to be involved in the uptake of apoptotic cells (25). We investigated the activities of the proteasome responsible for degrading cellular proteins in the cytosol and generating antigenic peptides (61). The proteasome core particle has three different active sites, chymotrypsin-like, trypsin-like and caspase-like activities (62). Although there was an increase in such activities due to exogenous antigen processing, we could not identify specific activities responsible for reduction in cross-presentation. We also tested for the induction of immunoproteasomes which can alter the generation of MHC-binding peptides (63, 64), as well as regulate cross-presentation (41), but levels of expression did not vary significantly during the culture period.
We next focused our analysis on the degradation of antigens in the phagosomal compartments during MØ differentiation (65, 66). Since phagosomal degradation is dependent on acidification and acid-dependent enzymes, we treated the cells with DPI, which inhibits the NOX2 system and renders the phagosomes more acidic. As a result, this treatment accelerated the degradation of the CFSE-labeled antigen (BM more than Sp-MØ) and also reduced the cross-presentation in (BM more than Sp-MØ). In contrast, Con.A (V-ATPase inhibitor) delayed the degradation of engulfed antigens and enhanced cross-presentation in mature MØ. As controls for these assays, we treated BMDCs with DPI which reduced their ability to cross-present the NP396 epitope. The data obtained with BMDCs and BM-MØ concur with those recently reported by the group of Amigorena (30) and fit nicely with their model (67). In this model, it is proposed that the NOX2 system is an important mechanism operating more efficiently in BMDCs than in BM-MØ to limit phagosomal acidification. However, our data indicate that unlike BM-MØ, Sp-MØ do not utilize this NOX2 system in the same manner (since cross-presentation was not affected by DPI treatment), probably because the kinetics of acidification do not occur at the same rate as in BM-MØ.

The role of MØ in adaptive immunity, antigen presentation is less defined in comparison to dendritic cells (2, 3, 12, 15, 42, 68). Our data provide important new insights into the mechanisms of cross-presentation of virus cell-associated antigens with respect to the differentiation of Sp-MØ. These Sp-MØ are efficient at cross presentation and this ability is regulated in relation to their phagosomal differentiation and rapid degradation of ingested antigens. Remarkably, this cross-presentation phenomenon does not hold for spleen B cells, which were recently shown to be unable to cross-present VLPs (69).
dendritic cells or macrophages prime murine MHC class I-restricted cytotoxic T lymphocytes in vivo. *J Immunol* 155:3313-3321.


CHAPTER 4

The outcome of cross-priming during virus infection is not directly linked to the ability of the antigen to be cross-presented
4.1 Preface

Much of what we know about cross-presentation of virus antigens has been done with a single transfected epitope or protein. As shown in the previous chapter, LCMV-NP supplied from transfected HEK-NP as an exogenous antigen could gain access to MHC-I molecules. In this chapter, we addressed the capacity of LCMV proteins generated during virus infection to be cross-presented in vivo and in vitro. The aim of this chapter was to investigate the cross-presentation and cross-priming of LCMV proteins after virus infection. We approached this aim with the following hypothesis; LCMV antigens can be cross-presented with different efficiencies by different pAPCs subsets. Our investigation on cross-presentation capacity of LCMV proteins after virus infection has revealed the following:

- Cross-presentation of cell-associated LCMV proteins in vitro was dominated by NP396
- Cross-presentation of cell-associated LCMV proteins was influenced by proteins accumulation during the course of infection and it was RNA-independent
- Cell-associated LCMV proteins were cross-presented via the cytosolic pathway
- In vivo cross-priming of cell-associated LCMV proteins was dominated by NP396 and GP33.

The work involved in this project were planned, and executed by myself. Esther Tarrab and Dr. Alain Lamarre assembled and provided us with tetramers. The results in this chapter are submitted to Eur J Immunol. (Revised version recently submitted)

The outcome of cross-priming during virus infection is not directly linked to the ability of the antigen to be cross-presented.

4.2. Abstract

The initiation of CD8+ T cell immune responses by professional antigen presenting cells can occur via cross-priming. Recent data suggested a possible relationship between cross-presentation and immunodominance of epitope-specific T cells. In this study we tested if the ability of an epitope to be cross-presented would enable it to assume an immunodominant position during cross-priming. Employing the lymphocytic choriomeningitis virus (LCMV) infection model, we demonstrated that the cross-presentation ability of LCMV proteins was RNA-independent and was dominated by LCMV-NP396 but not LCMV-NP205, both in vitro and in vivo when analyzing the LCMV-NP protein. Whereas with LCMV-GP, cross-presentation was dominated by LCMV-GP276 in vitro, and cross-priming was dominated by LCMV-GP33 in vivo. Importantly, although NP396 was significantly more efficient than GP33 in cross-presentation, cross-priming of their specific CTLs was comparable. Yet, on a subsequent virus challenge after cross-priming was induced, GP33-specific CTLs dominated the response. Accordingly, based on our data, the ability of viral epitopes to be cross-presented in vitro does not entirely reflect what would occur in cross-priming. Thus, in designing immunotherapeutic vectors it is important to consider the ability of an epitope to be cross-presented and estimate its naïve T cell precursor frequencies to maximize the response during cross-priming.
4.3. Introduction

The immune response to virus infections relies on different mechanisms involving innate and adaptive immune defenses. These mechanisms require CD8+ T-cell responses to eliminate viruses before they spread and establish persistent infections. The priming of CD8+ T cells is initiated by bone marrow-derived professional antigen presenting cells (pAPCs), such as dendritic cells and macrophages (1-5). This priming is achieved via MHC-I presentation of endogenous “direct-presentation” and exogenous “cross-presentation” antigens (6-8).

Virus-infected cells undergoing necrosis or apoptosis are considered to be a major source of antigens in cross-presentation (9-11), although the contribution of multiple epitopes from viral proteins to this cross-presentation pathway after infections is not well-understood. In fact, much of what is known about cross-presentation of virus antigens is derived from studies relying on antigen donor cells (ADCs) transfected with a single virus protein (12-15). Although these reports represent valuable data, they do not always reflect what happens during virus infection and they do not compare in vitro and in vivo information.

With regard to the nature of the ideal antigens for cross-presentation, the emerging dogma is pointing to an intrinsic property related to the stability of the antigen and its ability to escape cytosolic degradation (12, 16-19). Interestingly, it appears that the ability of an epitope to access cross-priming may support its immunodominance when considering the overall hierarchy (13, 15, 20). Collectively, these findings would seem to conflict with the immunodominant status of GP33 after virus infection because this epitope is located in signal sequence of lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) (21), which would imply a plausible lack of cross-priming (18).

In addressing these issues, we report for the first time on the cross-presentation and cross-priming capacity of lymphocytic choriomeningitis virus (LCMV) antigens after virus infection and subsequent inactivation in ADCs. We have tested four epitopes, NP396, NP205, GP33, and GP276 derived from two different viral proteins where LCMV infection would normally elicit a substantial CD8+ T cell response to these determinants (22, 23). Our results clearly demonstrate that the cross-presentation abilities of
immunodominant and subdominant epitopes do not directly correlate with their cross-priming \textit{in vivo} and may explain why certain cross-presentation models do not replicate \textit{in vivo} (24). The data point to a significant role contributed by the naïve CTL precursor frequency that could compensate for poor antigen cross-presentation.
4.4. Materials and methods

4.4.1. Mice, cells and reagents

C57BL/6 (H-2b) mice were purchased from Charles River (St. Constant, QC, Canada), and were used between 6 to 8 weeks of age. Animal experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care. HEK293 is a human embryonic kidney cell line. HEK293-NP is HEK293 cells transfected with LCMV-NP cultured in DMEM supplemented with puromycin as previously described (12, 13). As antigen-presenting cells, the cell lines BMA and DC2.4 (kindly provided by Dr. K. Rock, University of Massachusetts Medical School, Worcester, MA), were cultured in RPMI 5% FBS (25, 26). All media were purchased from Invitrogen (Ontario, Canada).

For inhibitory studies we employed lactacystin, brefeldin A (BFA), leupeptin, pepstatin A, and chloroquine purchased from Sigma (Oakvilla, ON, Canada). Murine recombinant granulocyte-macrophage colony-stimulating factor (rmGM-CSF) was purchased from Cedarlane Laboratories (Hornby, ON, Canada). Diphenyleneiodonium chloride (DPI) was purchased from Calbiochem. The Ribonuclease A from bovin pancrease (RNase A, R4875, 10µg/ml) was purchased from Sigma. LCMV-WE was originally obtained from F. Lehmann-Grube (Germany) and propagated in L929 cells. For in vivo virus titration, spleens were homogenized in 1ml DMEM, and supernatants were titrated onto MC57 monolayers by an immunofocus assay as previously described (13, 27). In certain experiments we used the above method to test for virus replication after HEK cells were infected in vitro and UV-irradiated.

4.4.2. Preparation of bone marrow-derived macrophages (BM-MØ) and dendritic cells (BM-DC)

Femurs and tibias from 6–8 weeks old C57BL/6 mice were collected and the marrow was flushed with warm PBS and resuspended in lysis buffer (1.66% w/v ammonium chloride) for 5 min to lyse red blood cells. After washing with PBS, debris was removed by passing the cells suspension through a metal sieve. To generate BM-MØ, cells were prepared in conditioned medium (CM) containing 20% of L929 supernatant as a source of M-CSF as previously described (28). After 3 days, the non-adherent cells were
removed and fresh medium was added. The medium was changed 2 days later and the cells were harvested with 1× trypsin-EDTA and tested after 5 days of culture.

To generate BM-DCs, bone marrow cells were prepared as previously described (28). Briefly, bone marrow cells were cultured in 10% FBS, 50 μM 2-mercaptoethanol (ME; Bioshop Canada Inc., Burlington, Ontario), 10 ng/ml GM-CSF (Cedarlane, Ontario, Canada), and gentamicin 3 μg/ml (Invitrogen, Ontario, Canada). The medium (2 ml) was removed every 2 days and replaced with fresh medium. At day 6 the non-adherent cells were transferred into a new 6-well plate and left for 4 h before the loosely adherent cells (highly enriched CD11c⁺ MHC-II⁺) were harvested and used at this day in the assay.

4.4.3. Preparation of ADCs for cross-presentation

HEK293 cells were infected with LCMV-WE at a multiplicity of infection (moi) of 1 for different time points at 37°C. After that, the cells were subjected to 1 round of freeze/thaw (liquid N₂), followed by UVB radiation using a CL-1000M UV cross-linker (Ultra-Violet Products Ltd., Cambridge, UK) at a radiation intensity of 200,000 µJ/cm² (maximum intensity) for 1 hour to inactivate LCMV. Following UV exposure, cells were collected and used directly after treatment. These cells are termed LyUV-treated cells (referred to as ADCs henceforth). HEK-NP cells were treated similarly, except they were UV-irradiated for only 15 min.

4.4.4. Detection of intracellular viral proteins and intracellular cytokine staining (ICS)

For LCMV-NP detection, LCMV-infected HEK cells were harvested, fixed with 1% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% saponin solution for 20 min at room temperature, followed by incubation for 1 hr at room temperature with rat anti-LCMV-NP (VL4) followed by FITC-conjugated goat anti-rat IgG Ab (lot 66497, 1 µg/mL; Cedarlane, Hornby, Ontario, Canada) for 1 h to detect NP expression. In a similar fashion, the cells were incubated with mouse anti-LCMV-GP (KL25) followed by Alexa488-goat anti-mouse IgG1 (lot 53419A, Invitrogen, Oregon, USA) to stain for LCMV-GP. Data were acquired with the flow cytometer (FCM) and analyzed with the Expo32 software package (Beckman Coulter, Miami, FL).
For *ex vivo* analysis of T cell activation, IFN-γ production by CD8+ cytotoxic T cells was measured by performing intracellular cytokine staining (ICS) in peptide re-stimulation assays. Effector splenocytes were incubated with peptide-loaded (0.1 μM) APCs (2 x 10^5/well) at a ratio of 1:10 (APCs:splenocytes) in round-bottom 96-well plates (Fisher, Whitby, Ontario, Canada). The APCs were loaded with one of the following synthetic peptides: GP33-41/Db (KAVYNFATC), GP276-286/Db (SGVENPGGYCL), NP205-212/Kb (YTVKYPNL), and NP396-404/Db (FQPQNGQFI), or an irrelevant peptide control (SIINFEKL). The peptides (purity > 90%) were synthesized at CPC Scientific (San Jose, CA). After 2 h of incubation at 37°C, BFA (10 μg/mL) was added for another 3 h. Cells were stained with a TRI-COLOR conjugated, rat anti-mouse CD8α clone 5H1 (Cedarlane) at 4°C, then fixed with 1% paraformaldehyde, washed before adding FITC-conjugated anti-IFN-γ antibody, clone XMG1.2 (Cedarlane) prepared in PBS, and 0.1% saponin. The samples were prepared in FACS buffer and data were acquired with FCM as described above. The cells were analyzed with a live gate on the CD8+ cells after 100,000 gated cells were acquired.

**4.4.5. Antigen presentation assays employing peptide-specific CD8+ T cells**

Peptide-specific CD8+ T cells were generated as described previously (12). Briefly, B6 mice were injected i.v. with 200 pfu LCMV and left for 4-6 weeks before spleens were removed and lymphocytes were purified by ficoll-gradient centrifugation with lymphocytes separation medium (Fisher, Whitby, On). Purified splenocytes were then re-stimulated with peptide-pulsed (10^-7 M) γ-irradiated BMA cells in the presence of IL-2 (20 U/ml). On day 5 or 6 post stimulation, an additional ficoll-gradient centrifugation step was conducted 2 days before testing in the antigen presentation assays. At this stage, the cells were found to be specific for the peptide used for stimulation with purity reaching > 80% as determined with ICS assays.

For direct presentation assays, APCs were pulsed with the peptide of interest for 1 h in serum free medium and then co-incubated with peptide-specific CD8+ T cells for 3 h in presence of BFA 10 μg/ml. To study cross-presentation, the LyUV-treated LCMV-infected HEK cells (5 x 10^5 cells/well) were co-incubated overnight in 96-well round-bottom plates with pAPCs (DC2.4, BMA, BM-MØ or BM-DCs) at 1 x 10^5 cells/well. Where indicated, inhibitors were used to address the cross-presentation pathways.
employed. The inhibitors were added to the APCs 45 min before adding the ADCs and maintained during the incubation periods. In certain experiments RNase treatment of ADCs was performed. ADCs were lysed and treated with 10 µg/ml of RNase for 20 min at RT followed by two washing steps before UVB treatment. ADCs were then incubated with pAPCs in a regular cross-presentation assay as described above.

To test for cross-priming, B6 mice were injected intraperitoneally (i.p.) with HEK293 resuspended in PBS (negative control) or LCMV-infected, LyUV- treated HEK cells (7 x 10⁶). After 7 days splenocytes were obtained and stained with tetrameric MHC-I-peptide complexes. Alternatively, epitope-specific cytotoxic T lymphocytes (CTLs) were expanded in vitro before performing ICS assays as previously described (12).

4.4.6. Tetramer staining
Splenocytes (5 x 10⁵/well) were stained using 0.5-1 µg of PE-labeled tetramers. NP396, NP205 and GP276 tetramers were obtained from the NIH tetramer facility and GP33 was prepared as previously described (29). Splenocytes were then stained with a TRI-COLOR-conjugated, rat anti-mouse CD8α at 4°C for 30 min. Cells were washed twice and data were acquired using FCM as described above.

4.4.7. Statistical analyses
Statistics were performed using the paired, two-tailed t tests and differences in results between treatment conditions were deemed significant when p < 0.05.
4.5. Results

4.5.1. Characterization of LCMV-infected and UV irradiated antigen donor cells

We employed HEK293 in our experimental model to study the cross-presentation of LCMV proteins because these cells can not directly present antigens to mouse T cells after infections. First, we tested the susceptibility of HEK cells to LCMV infection. HEK cells were infected with LCMV (moi) of 1 for 24 h and the expression levels of LCMV-NP and LCMV-GP were evaluated as an indication of viral protein de novo synthesis due to replication. We found that HEK cells are highly permissible to LCMV infection because we could detect more than 90% infected cells after 24 h as indicated by LCMV-NP and GP staining (Figure 1a, i-HEK). We also tested for both proteins after lysis treatment (Ly) as well as with UV treatment to inactivate the virus (LyUV). We did notice a reduction in the intensity of protein expression as detected by flow cytometry (FCM), but it was still significantly positive and thus allowed us to use these infected LyUV-ADCs in our experiments (Figure 1a, i-HEK-Ly and i-HEK-LyUV). This reduction in protein content may occur due the partial lysis that occurs after treatment as observed with trypan blue staining (data not shown). Thus this treatment allows the cells to be intact for longer period and thus they are acquired easily in FCM.

Next we examined the effect of UV inactivation on virus replication to ensure that no possible direct presentation can occur as a result of virus infection of pAPCs. We assessed this in vitro (Figure 1b) and in vivo (Figure 1c). In vitro, we incubated L929 cells (highly susceptible to LCMV) with supernatants from both Ly and LyUV-treated LCMV-infected HEK cells. The data obtained when we examined LCMV-NP expression in L929 indicate that the supernatant taken from Ly- treated, but not LyUV- treated cells contained live virus particles that were able to replicate in the L929 cells (Figure 1b, compare solid line with dotted line histograms). As a positive control, the expression of LCMV-NP was tested in infected L929 cells (Figure 1b, filled dark gray histogram). Uninfected L929 cells labeled with primary and secondary antibodies or infected cells incubated with secondary antibody only served as a negative control (Figure 1b, filled light gray histogram). We confirmed these observations in vivo by performing titration assays from mice injected with either condition. Our results (Figure 1c) show that no virus replication was
Figure 1. LCMV-infected and UV-irradiated ADCs are efficient source of exogenous antigens. a) LCMV-NP (left panel) and LCMV-GP (right panel) protein expression in LCMV-infected HEK cells before and after LyUV treatment. HEK cells were infected at an moi of 1 for 24 h (gray histogram) and were either exposed to a lysis cycle (Ly, solid line), or a lysis cycle followed by UV treatment for 1 h (LyUV, dotted line). b) In vitro detection of LCMV replication after LyUV treatment. Supernatants from infected HEK cells after the above treatments (Ly; solid line or LyUV; dotted line) were added to L929 cells and LCMV-NP was detected 24 h later. Supernatants from uninfected HEK were added to L929 as negative control (gray histogram) and as a positive control L929 cells were infected overnight with LCMV (black histogram). c) in vivo determination of LCMV replication after LyUV treatment. Cell lysates (5 x 10^6) from LCMV-infected HEK cells that were either Ly or LyUV-treated were injected i.p. Seven days later, spleens were harvested to perform virus titration using a focus-forming assay with 200 pfu of LCMV-WE employed as a positive control. This is one experiment representative of two independent trials where the data is the average ±SD from 3 mice per condition. d) cross-presentation capacity of NP396 generated from LyUV-treated infected HEK cells and HEK-NP. Infected HEK cells were treated as above to inactivate live virus and were added to DC2.4 (3:1 ratio) and incubated overnight before the addition of NP396-specific CTLs in presence of 10 μg/ml BFA. ICS was performed by staining for IFN-γ and gating on CD8+ T cells to quantify the % activated double positive peptide-specific T cells. The data depicted are from one experiment with three independent trials.
detected after injection with LyUV-treated-infected ADCs when compared to the Ly-treated condition or with LCMV injected-mice.

Next, we assessed the ability of the infected LyUV-ADCs in supplying LCMV proteins for cross-presentation by comparing them with the previously characterized HEK-NP cells (12, 13). For this purpose we used effector NP396-specific CTL (75-85 % positive when tested with the NP396 peptide, data not shown). The data (Figure 1d) indicate that the infected LyUV-ADCs supplied sufficient levels of LCMV-NP antigens to induce comparable NP396-specific CTL activation similar to HEK-NP cells. Collectively, the data provided evidence to use these infected LyUV-ADCs as an efficient system to provide LCMV proteins for cross-presentation due to complete virus inactivation without the interference of direct presentation.

4.5.2. Cross-presenting efficiencies of NP and GP epitopes differ and increase with time post infection

In order to evaluate how different epitopes from proteins generated after LCMV infection can be cross-presented, we determined LCMV protein expression (NP and GP) and cross-presentation of the four major epitopes at different time points (1, 3, 6, 12, and 24 h post infection [p.i.]). Using moi of 1 pfu, we could not detect either LCMV-NP or GP (Figure 2a, 1 h) after 1 hour of infection, which would represent mainly input virus. This lack of detection of virus input was expected and made it easy for us to detect de novo protein synthesis. Over the course of infection the levels of LCMV-NP and GP increased gradually and reached significant expression levels after 24 hours (figure 2a). Interestingly, and consistent with previous reports, LCMV-NP was detected earlier than LCMV-GP (30, 31).

We next analyzed the cross-presentation of four LCMV epitopes (NP396, NP205, GP33, and GP276) by four types of pAPCs of myeloid origin (primary and cell lines) over 4 time-points post infection of the ADCs (Figure 2b). All four pAPCs showed comparable abilities to cross-present the various antigens. Clearly, the NP396 epitope was the most efficient epitope to be cross-presented and that was more profound with increasing time post infection (compare 3 and 24 h p.i, Figure 2b). The other three epitopes were cross-presented with less efficiency with GP33 being the least efficient while GP276 being the best (Figure 2b). However, as with NP396, cross-presentation of the other three epitopes increased with time p.i.
Figure 2. Following virus infection LCMV epitopes are differentially cross-presented.  

a) Kinetics of LCMV proteins accumulation after infection. HEK cells were infected with LCMV-WE as in figure 1 and protein expression of NP (solid line) and GP (dashed line) were evaluated at 1, 3, 6, 12, and 24 h p.i. Mock-infected cells were employed as a negative control (gray histogram).  
b) Analysis of LCMV-WE proteins cross-presentation. HEK cells were infected as before for different time points then they were LyUV-treated to inactivate the virus before co-incubation with different pAPCs (DC2.4, BMA, BM-DCs, and BM-Mø) at a ratio of 5:1 (ADC: APC) to assess cross-presentation. As effector cells, NP396, NP205, GP33, and GP276-specific CTLs were added to the APC for 3 h in the presence of BFA 10 µg/ml and the percentage activated CD8+ T cells (IFN-γ +ve) were calculated.  
c) Kinetics of LCMV cross-presentation. HEK cells were infected overnight with LCMV-WE as before followed by LyUV treatment. The cells were then co-incubated with DC2.4 and BMA cell lines at a ratio of 5:1 (ADC: APC) for different time points followed immediately with NP396 and GP276-specific CTL in the presence of BFA 10 µg/ml. The percentage of the activated CTLs is calculated based on the number of double positive cells (CD8+, IFNγ +ve). The data in (b) and (c) are representative of one experiment from three independent trials where the error bars are the average of triplicate wells ±SD within one experiment.
 Overall, these results confirmed that cross-presentation of cell-associated LCMV proteins did occur with different efficiencies. The differences in the data were not due to different qualities or sensitivities of the effector epitope-specific CTLs. We confirmed this by testing them in a peptide restimulation assay (as explained in materials and methods) as a positive control and they were all activated to comparable levels (75-85% positive when tested with the specific peptide, data not shown).

Thus far, we found that NP396 and GP276 were the best cross-presented epitopes from LCMV-NP and GP, respectively. Since both epitopes are located in different proteins of the virus and are likely distributed in different cellular compartments, we asked if the two epitopes would be cross-presented with similar kinetics after a fixed time p.i. of ADCs. By incubating the LyUV ADCs with APCs for different time points before adding either NP396- and GP276-specific effector CTLs we could detect significant cross-presentation as early as 3 h post incubation (Figure 2c). Whereas cross-presentation of GP276 peaked around 12 h, optimal NP396 cross-presentation was reached within 18 hours of co-incubation (Figure 2c).

Interestingly the DC and MØ cell lines exhibited similar kinetics and efficiencies at cross-presenting both LCMV epitopes (Figure 2c), suggesting that LCMV proteins generated during virus infection can be cross-processed similarly by DC and MØ.

4.5.3. Influence of RNA on cross-presentation of the LCMV-infected LyUV-ADCs

Thus far, we have shown that LCMV-NP396 can be efficiently cross-presented by the infected LyUV-treated cells. We next addressed the question if the viral RNA, which would normally complex with LCMV-NP during virus assembly, is contributing to the efficiency of this cross-presentation. We approached this aim by treating LCMV-infected cells with the endonuclease RNase A to degrade the RNA in the ADCs after infection. To test for RNA integrity after RNase treatment, we isolated the RNA and checked for 28S and 18S rRNA. In figure 3a, these two bands are clearly visible in the intact RNA control samples (L1 and L2; similar to the untreated control samples in figure 3c, ii and iii), whereas in the treated sample (figure 3a, L3) only a lower molecular weight smear was obtained indicating RNA degradation. We also confirmed that the RNase treatment did not have adverse effects on LCMV proteins (Figure 3b).
Figure 3. Cross-presentation of LCMV proteins is not abolished by RNA degradation. a) RNA degradation by RNase A was visualized on a 1% native agarose gel by examining the 18S and 28S ribosomal RNA. In each lane 2 µg of total RNA were applied where L1 and L2 (represent control samples ii and iii in section C respectively) are control samples without RNase treatment. L3 is the test sample incubated for 20 min at RT with RNase A (10 µg/ml), and in L4 0.24-9.5 Kb RNA ladder was applied. b) LCMV proteins expression after RNase A treatment. LCMV-NP and LCMV-GP expression was detected in LCMV-infected HEK cells (moi of 1 for 24 h) treated with 10µg/ml RNase A as before (dotted line) and was compared to the positive LCMV-infected untreated control (solid line). c) Cross-presentation of NP396 and GP276 is not abolished by RNase treatment. Infected LyUV-treated HEK cells were either untreated (i), or treated with 10µg/ml RNase A (iv). As controls for the RNase treatment, cell pellets of LyUV-treated HEK cells were prepared under similar conditions (ii and iii). The ADCs were co-incubated with APCs at a ratio of 3:1 overnight before the addition of epitope-specific T cells as before. d) Effect of poly I:C on cross-presentation of RNase A treated ADCs. Infected LyUV-ADCs treated as in (c) were pre-incubated with poly I:C for 40 min before the cross-presentation assay commenced overnight, before adding NP396 and GP276-specific CTLs. The percentage of activated CTL was estimated as before. Data depicted are a representative experiment out of 3 independent trials.
clear that LCMV-GP was unaffected by the treatment, but there was some loss in LCMV-NP (Figure 3b), probably due to dissociation of the RNA from the NP protein.

In figure 3c, several conditions were performed while testing for cross presentation of two different epitopes (NP396 and GP276) from infected ADCs. The first condition was done as before with LyUV-treated cells and gave the expected results for all two tests (figure 3c, i). In order to use the RNAase, we had to spin down the pellet from the lysed infected cells, incubate at RT for 20 min and then treat with UV. Because we added these steps, we needed to perform other controls that varied slightly from the original condition in (i). With regard to the controls without RNase treatment (figure 3c, ii and iii) we experienced a decrease in cross-presentation of NP396 but not GP276 - probably because using only the cell pellet in the assays meant that any protein in supernatant preparations might have been lost. This supernatant may have been richer in the LCMV-NP than GP since the latter tend to be membrane bound. Treatment of infected HEK cells with RNase degraded the ADCs’ RNA as shown in figure 3a, L3, did cause a small but significant reduction in cross-presentation of NP396 but not GP276 (Figure 3c, iv). Thus, degrading RNA in the ADC preparations did not abolish cross-presentation and rules out a possible de novo protein translation occurring in APCs.

Since degradation of the double stranded (ds) RNA (known to affect cross-presentation) may have occurred as a result of the RNase treatment, we evaluated the effects of adding the synthetic viral dsRNA analogue poly (I:C) in our system after removal of the RNase. We co-incubated the DC2.4 and the ADCs in the presence of poly (I:C) and observed, as depicted in figure 3d, a small increase in the cross-presentation of both epitopes (396 and 276) that correlated with increasing concentrations of poly (I:C). When considering the values obtained under normal condition (figure 3c, iii), it is clear that the small reduction in cross-presentation of NP396 after the RNAase treatment was not due to degradation of the dsRNA, but could be due reduction in APC activation due to lack of TLR engagement.
4.5.4. LCMV-cell associated antigens are mainly cross-processed via the classical cytosolic pathway and require tightly-regulated phagosomal acidification

To investigate whether cross-presentation of LCMV proteins generated after infection required the same molecular components used by DC and MO, we tested different inhibitors with variable concentrations that targeted the intracellular processing pathways. Employing the proteasome inhibitor lactacystin and the ER protein transport inhibitor, BFA, we were able to interfere with cross-presentation in a dose-independent manner (Figure 4). This would suggest that the LCMV epitope generated by LCMV-infected HEK cells was cross-processed in both cell types by the proteasome, and was ER-dependent, which indicates the involvement of the cytosolic pathway.

To assess the relative contribution of the proteasome-independent vacuolar processing pathway, we applied leupeptin to inhibit serine and cysteine proteases (cathepsin B, L, and S), and pepstatin A to inhibit aspartic proteases (cathepsin D). Figure 4 shows limited interference with leupeptin that was more pronounced with DC2.4 than with BMA, whereas pepstatin A was ineffective with either cell types. These results would suggest that low/modest antigen processing (cathepsin D-independent) took place in the endosomal/phagosomal compartments.

To investigate if phagosomal acidification was needed in cross-presentation of LCMV-NP396 from the infected ADCs, we pre-treated the APCs with different doses of chloroquine up to 120 µM, which would inhibit the acidification of the phagosomes. An inhibitory effect (50%) of chloroquine was observed with DC2.4 only at the highest doses, whereas with BMA almost complete inhibition was evident (Figure 4). Another regulator of phagosomal acidification, NADPH oxidase (NOX2), which mediates the transfer of electrons across endocytic and plasma membranes, was required by the APCs. This was observed because cross-presentation of NP396 was somewhat reduced following treatment with DPI (NOX2 inhibitor). This treatment causes a quick and sharp increase in phagosomal acidification, which is also detrimental to epitope processing (Figure 4). As controls, we employed peptide-labeled APCs with all the inhibitors tested and did not find any significant adverse effect on activation of NP396-specific CTL when compared with untreated APCs (data not shown).
Figure 4. Mechanisms governing cross-presentation of LCMV proteins using infected LyUV ADCs. HEK cells were infected as before and were co-incubated overnight with either DC2.4 or BMA cell lines at a ratio of 5:1 (ADC: APC) in presence and absence of specific inhibitors. The APCs were pre-treated for 45 min with increasing concentration of the following inhibitors; lactacystin (0-12 μM), BFA (0-15 μg/ml), leupeptin (0-250 μM), pepstatin A (0-350 μM), chloroquine (0-120 μM), and DPI (0-10 μM), before adding the ADCs. The inhibitors were left throughout the incubation time and were removed before adding NP396 specific-CTLs as before. The data are representative of one experiment from three independent trials where the error bars are the average of triplicate wells ±SD within one experiment.
4.5.5. Cross-priming of infected ADCs induces CTL populations dominated by two epitopes with contrasting efficiencies in cross-presentation

To examine if the cross presentation results we obtained above translate in vivo, we sought to investigate the cross-priming capacity of LCMV proteins generated from LCMV-infected ADCs to elicit epitope-specific CD8+ T cell responses. Generally, four major epitope-specific CTLs are easily enumerated after infection of B6 mice with LCMV. These CTLs are mainly against two immunodominant epitopes, GP33, and NP396, and two subdominant GP276 and NP205 epitopes. We confirmed these data with a positive control study where 200 pfu of LCMV infection were introduced i.v. (Figure 5a, LCMV). Compared to the negative control (Figure 5a, naïve), we detected tetramer positive effectors where NP396 and GP33-specific CTL were the immunodominant cells in the spleen 8 days post infection (Figure 5a).

The results obtained in figure 2b would predict that cross-priming in vivo would result in mainly NP396-specific T cells being detected since it was the only efficiently cross-presented epitope. We analyzed cross-priming after mice received the LyUV-ADCs (7x10^6 cells/mouse) with either tetramer analysis directly ex vivo or by expanding the epitope-specific T cells and testing them in functional ICS assays. Using tetramer staining, the CTL were analyzed seven days post injections (Figure 5b, LyUV-i-HEK) and were found to be dominated by NP396 and GP33, although the values obtained were very low. With regard to NP205 and GP276, the values obtained were barely higher than the background staining of naïve mice (figure 5b, 0.3%). We also compared these data with peptide-pulsed APCs. We pulsed the APCs with the four peptides separately and then pooled them at equal ratios before injecting the cells intravenously (i.v.). Tetramer analysis was performed 7 days later and the positive CTLs were dominated by NP396 and GP33 epitopes (figure 5b, DC2.4-peptide), whereas NP205 and GP276 epitopes were very low similar to the cross-priming data (figure 5b).

To confirm these data, we expanded all four epitope-specific CD8+ T cells obtained 7 days after cross-priming was initiated, for further 8 days in culture with the peptide pulsed APCs in separate wells. When we tested these CTLs in a peptide re-stimulation ICS assay measuring IFN-γ, we found that the response was dominated by NP396- and GP33-specific CTLs, whereas very low numbers were detected for NP205 and GP276-specific CTLs (figure 5c). Splenocytes from mock-treated mice or mice immunized with 200
Figure 5. Cross-priming of LCMV epitopes after virus infection and LyUV treatment. a) Tetramer analysis of LCMV epitopes from naïve and LCMV-infected B6 mice. B6 mice were infected with 200 pfu LCMV or mock preparations and CTLs were analyzed 7 days later. The four major epitope-tetramer complexes were used to assay for peptide specific CD8+ T cells as described in the Material and Methods. In b) tetramers analysis of splenocytes from mice injected i.v. with peptide-pulsed DC2.4 (5x10^5) or with 7x10^6 LyUV-treated infected-HEK cells injected i.p. The data are representative of one experiment from three independent trials where the error bars are the average of triplicate wells ±SD within one experiment. c) Ex vivo expansion of epitope-specific T cells using peptide-labeled APCs with the four different peptides from mice injected with 7x10^6 LyUV-treated infected-HEK cells (i-HEK). As background controls mice were injected with control HEK cells. After peptide-specific splenocytes were cultured in vitro for 6 days, the cells were re-stimulated with the specific peptide and CTL were assessed for their capacity to produce IFN-γ with an ICS assay. The data depicted are from one experiment with three independent trials.
The images depict bar graphs comparing the percentage of tetramer (a) and IFN-γ (C) in different conditions.

**Graph a:**
- Naive and LCMV groups are compared for four different treatments (NP396, NP205, GP33, GP276).
- Error bars indicate variability.

**Graph b:**
- DC2.4-peptide and LyUV-1.HEK groups are compared for four different treatments (NP396, NP205, GP33, GP276).
- Error bars indicate variability.

**Graph C:**
- IFN-γ expression levels for NP396, NP205, GP33, and GP276 are shown.
- Expression levels are indicated for i.HEK and HEK cells.
- CD8 marker is used for comparison.

The graphs illustrate the immune response and tetramer expression under different conditions.
pfu of LCMV were also tested as negative and positive controls in all experiments (data not shown). These experiments indicated that cross-priming after LCMV infections favors the CD8+ T cell response towards GP33 and NP396.

4.5.6. Cross-priming influences the magnitude of immunodominant T cells during subsequent virus infection

To further test the finding that GP33 as well as NP396 dominate the response after cross-priming, we asked if inducing cross-priming could affect the immunodominance during subsequent viral infection. Employing different controls such as prior injections with PBS or WT HEK cells before introducing 200 pfu of LCMV did not have a significant impact on the immunodominance hierarchy, with GP33>NP396>GP276=NP205 (figure 6). If infected-HEK-LyUV were introduced first, it caused GP276 to be higher than NP205 with GP33 still >NP396 but with a larger difference between the two epitopes (figure 6, i-HEK-LyUV). We compared these data with LyUV-treated HEK-NP where NP396 was the main epitope being cross-presented. In the latter condition (figure 6, HEK-NP), where NP 396 was cross-presented, NP396-specific T cells were the major ones to be detected after virus infection whereas GP33-specific T cells were now subdominant possibly due to the prior expansion of NP396-specific CTLs competed out the naïve GP33-specific T cells. This did not occur when GP33 was cross-presented when infected-HEK-LyUV were tested (figure 6 i-HEK-LyUV).

These findings suggest that prior introduction of exogenous antigens will influence immunodominance hierarchy during a subsequent infection but this will depend on which epitopes were being cross-presented. Taken together, the data presented above indicate that the outcome of cross-priming during virus infection is only partially dictated by the ability of the antigen to be cross-presented and that the T cell precursor frequency plays a major role therein. Therefore, in the case of GP33, where cross-presentation was poor, the high precursor frequency of GP33 was compensatory. Thus, increasing this frequency would result in consolidating immunodominance and the magnetite of CTLs specific for the cross-presented epitopes after virus infection.
Figure 6. Cross-priming shapes immunodominance following LCMV infection. Mice were injected i.p. with PBS, control HEK cells, HEK-NP, or infected-HEK-LyUV as described in the materials and Methods. After 7 days mice were infected with LCMV 200pfu i.v. Splenocytes were collected on day 14 and assessed for the activation of NP396, NP205, GP33, or GP276-specific CD8+ T cells by measuring IFN-γ production after \textit{in vitro} peptide re-stimulation. The data are representative of one experiment from three independent trials where the error bars are the average of three mice ±SD from one experiment.
4.6. Discussion

Induction of protective immune responses against viruses or tumors can be achieved via cross-priming as previously reported by several groups investigating different antigenic models (6-8). An important question that remains unanswered is how the ability of an epitope to cross-present \textit{in vitro} relates to the final outcome during cross-priming. In this study we employed the well characterized LCMV infection model to study how cross-presentation of multiple epitopes translates when studying cross-priming.

By characterizing the infected ADCs, we found that an adequate accumulation of LCMV proteins was achieved 24 h p.i. We used LyUV treatment because it was suitable to fully inactivate the virus and allow for efficient uptake of the ADCs by the APCs. When we tried milder UV treatments or if we did not first induce lysis prior to UV treatment, we experienced insufficient viral inactivation (data not shown). Finally, we validated these infected ADCs by comparing them with HEK-NP cells, previously shown to supply sufficient antigen allowing for the cross-presentation of NP396 (6, 12, 13).

By utilizing this model we could now test for the cross-presentation of four major epitopes directly after virus infection of the ADCs. We confirmed the detection of both NP and GP 24 h post infection and expected that since significant proteins were accumulating in these cells that at least three epitopes should cross-present with high efficiencies except for GP33. We initially expected that the dominant LCMV-GP33 epitope would fail to cross-present because this epitope is located in a signal peptide that is prone to have a short half-life and, thus, would bias this epitope to be inefficient at cross-presentation (18). Unexpectedly, all four pAPCs employed in our assays yielded similar results demonstrating that NP396 was the only epitope efficiently cross-presented compared to NP205, GP33 and GP276. The ability of GP33 to cross-present might be due to its exceptionally long half-life of 6 hours (21) unusual for a signal sequence, but perhaps long-enough for cross-presentation to occur. In contrast, NP205 which is located in the long-lived nucleoprotein (32) was very poor at cross-presentation, possibly due to poor processing by the phagosomal/proteasomal machineries.
GP276 is another epitope that cross-presented with much lower efficiency when compared to NP396, probably reflecting differences in MHC binding affinities that may be more critical during MHC loading in cross-presentation versus direct presentation due to the limited supply of antigen. In a recent report examining the binding affinity values (IC50 nM), it was clear that NP396 binds MHC with a high affinity of 0.23 compared to GP276 that exhibited a lower affinity of 414 (33). Another possibility to account for the poor cross-presentation of GP276 could be due to involvement of immunoproteasomes, which were previously reported to down-regulate presentation of this subdominant epitope (34). We have detected LMP7 expression in DC2.4 (unpublished data) which concurs with previous data showing that immunoproteasomes are expressed in DCs irrespective of their maturation states (35). Thus, immunoproteasomes which have been implicated in cross-presentation (36) could be responsible for the poor cross-presentation of GP276.

Interestingly, we were able to detect significant cross-presentation as early as 6 h p.i. until 24 h p.i., which would indicate that a certain threshold of newly synthesized proteins was needed to allow for cross-presentation. This finding concurs with previously reported data employing controlled protein expression systems (12). Furthermore, considering published data examining direct antigen presentation of the NP and GP-derived epitopes, showing that NP epitopes can be detected as early as 2 h p.i. and that GP epitopes can be detected between 4-6 h p.i. (31), one can envisage that an infected ADC can provide antigen for cross-presentation in a time frame comparable to direct presentation. This time-frame would probably be dependent on the antigen-donating and stress status of the infected cell (37).

When we used ADCs that had accumulated proteins for 24 h and determined the time span for cross-presentation and subsequent T cell activation, we found that we could detect significant responses 3 h post incubation, which peaked around 12-16 h. With regard to the kinetics of cross-presentation, the data confirm recent studies using cell-associated antigen such as OVA and vaccinia (38, 39). Thus, our data further indicate that a virus-infected ADC is able to supply antigens for cross-presentation much earlier than the completion of a replication cycle by the virus. Moreover, depending on the location of pAPCs, at least another 3 h would be needed before significant T cell priming can occur.
Thus far, the data would imply that if these ADCs were to induce cross-priming one would expect NP396 to dominate the CD8+ T cell response and the other three epitope-specific T cells would be equally detectable but at very low levels. However, when we enumerated the T cells directly ex vivo we were surprised to find comparable NP396 and GP33 tetramer positive T cells but extremely low numbers of NP205 or GP276-specific T cells. By expanding epitope-specific T cells and testing for IFNγ induction, we could now clearly detect significant NP396 and GP33-specific T cells but still found only few activated NP205 and GP276-specific T.

Even though the cross-presenting ability of GP33 was poor in vitro and was worse than GP276, the CD8+ T cells were able to expand to levels similar to the most efficient cross-presenting epitope (NP396) and outperform GP276 as well as NP205. This finding was unexpected because recent data indicate that poor cross-presentation would directly lead to a subdominance position during T cell activation during cross-priming (20). However, the failure of NP205 and GP276 to efficiently cross-prime CTL responses in vivo is consistent with the findings of Otahal et al. This would imply that in addition to a certain threshold of cross-presentation, successful priming of exogenous antigens would require other in vivo properties.

When we consider the recently reported data examining how the difference in naïve precursor frequencies of CD8+ T cells affects immunodominance during infection, it is not difficult to extrapolate this finding to cross-priming. In a recent extensive analysis of precursor frequencies of naïve CD8+ T cells (33), it was found that GP33-specific naïve CD8+ T cells constituted the highest number (449), followed by NP396 (117), and NP205 (57). The fact that naïve GP33 T cells was 3x more than naïve NP396 may explain why GP33-specific T cells were able to expand to levels comparable to NP396-specific T cells even though cross-presentation was very different between the two epitopes.

To further confirm our observations we tested how cross-priming of these ADCs can affect immunodominance during a challenge of LCMV, we compared this to the HEK-NP cells for which a shift of the immunodominance in favor of NP396 was observed confirming previous observations (13). Accordingly, cross-priming of LCMV-NP396 alone modifies the immunodominance hierarchy favoring the immunodominance of NP396-specific CD8+ T cells after a virus challenge. This NP396-specific CD8+ T
cells expansion could adversely affect GP33-specific T cells expansion possibly due to competition for APCs between CD8+ T cells of different specificities (40-42).

If the data obtained for cross-priming with infected LyUV cells (figure 5c) would be influential in this experiment, one would expect to see a response dominated by GP33 and NP396. In fact, this is what we observed and it occurred at much higher magnitude compared to untreated mice. This immunodominance was different from the HEK-NP condition suggesting that the immunodominance of NP396 with HEK-NP can not be explained by a faster kinetics of virus clearance by NP396 cells (i.e. virus was cleared before GP33-specific T cells were fully activated) because GP33-and NP396-specific T cells expanded significantly in the infected LyUV condition.

The above observations are particularly important because they relate to real-life scenarios where inactivated virus preparations are given to the public on regular basis. In this case, the CD8+ T cell of the cross-priming epitopes would dominate in the host, provided that an initial respectable precursor frequency is present. Furthermore, according to our data, the immunodominance would be shaped by same cross-priming epitopes during a regular virus exposure. Thus, in this report, we analyzed how cross-presentation relates to cross-priming in the induction of CTL specific for four different epitopes of LCMV-GP and LCMV-NP after infection. The data demonstrate such cross-presentation of cell-associated antigens was occurring mainly via the cytosolic pathway, was protein-dependent and did not rely on RNA transfer. Finally, we showed that the ability to cross-prime CTL in vivo varies for different epitopes derived from the same viral protein. This ability did not directly translate from the capacity to cross-present in vitro, but instead appears to additionally rely on other factors such as the precursor frequencies of naïve CD8+ T cells.
Reference:


CHAPTER 5

Discussion, summary, and prospective work
5.1 Discussion

Immune cells such as DCs and MØ are needed to link the innate and adaptive immune systems in order to eradicate pathogens and generate long lasting memory responses. DCs and MØ perform many different immunological functions ranging from secreting cytokines, which can regulate the immune response, to ingesting microbes and pathogens before their destruction into peptides. The generated peptides can be then presented on MHC class I and II needed to activate both CD8 and CD4 T cells, respectively (1-3).

In relation to antigen presentation, it has been demonstrated that DC and MØ act as professional antigen presenting cells (pAPCs) to initiate CD8+ T-cell responses. The pAPCs can present viral antigens on MHC-I molecules via two different pathways. One is the direct presentation pathway where peptides are generated endogenously after infection, whereas the other is the cross-presentation pathway, which deals with internalized exogenous viral antigens (4-6). The overall theme of this thesis was to examine the cellular and molecular parameters, which can influence the outcome of antigen processing and presentation to CD8+ T cells.

In chapter 2 the objective was to establish an efficient method to culture spleen macrophages (Sp-MØ) in vitro in order to study their differentiation in relation to antigen presentation. It is known that Sp-MØ are heterogeneous and are distributed among three anatomically distinct locations: Red pulp macrophages (RPMØ), Marginal zone macrophages (MZMØ), and Marginal zone metallophelic macrophages (MMMØ). Although they are located in one organ, they vary substantially in their surface markers expression (e.g. F4/80, SIGN-R1, and CD169 (MOMA-1; sialoadhesin)) and immunological functions, such as the clearance of blood borne pathogens by RPMØ, and generating immune responses during viral infections by MZMØ and MMMØ (2, 7, 8).

Based on data obtained from mice models, Sp-MØ have been classified into two groups based on macrophage-colony stimulating factor (M-CSF) requirements: the M-CSF-dependent and M-CSF-independent MØ (9-11). M-CSF is a hematopoietic growth factor controlling survival, proliferation, and
differentiation as well as other functions of cells of the monocyte/macrophage lineage. It is produced by numerous cell types including monocytes, MØ, fibroblasts, endothelial cells, and osteoblasts (12). Under normal conditions, M-CSF is present in vivo in small amounts (1–5 ng/ml) (13), but it is elevated as a result of the onset of some diseases or during viral infection (14). Interestingly, the observation that HIV-1-infected human monocyte-derived macrophages (MDMs) resulted in a substantial increase in M-CSF production (15), suggests an in vivo association between virus infection and the need for M-CSF as a potent MØ survival factor in immune defences. However, while it is well-documented that M-CSF is needed to promote the generation and development of MØ from the bone marrow progenitor cells (16, 17), M-CSF requirements to culture and differentiate Sp-MØ in vitro had not been extensively tested before.

Several investigations underlined the importance of M-CSF in Sp-MØ development by studying osteopetrotic mice (MCSFsup/MCSFsup or op/op), which have a mutation in the gene that encodes M-CSF (9-11). These studies implicated that Sp-MØ differ in their differentiation and growth needs with regard to M-CSF. This is because both MZMØ and MMMØ were completely absent in op/op mice, whereas RPMØ were reduced to about 60% of those in the normal littermates. Therefore, these in vivo observations led us to hypothesize that Sp-MØ would likely need M-CSF for further differentiation during in vitro culturing. In our experiments, we studied splenocytes for 7 days in M-CSF condition medium (CM), and obtained excellent yields of highly pure MØ. The increased yield of Sp-MØ confirms a critical role for M-CSF in growth and maturation of in vitro cultured Sp-MØ.

In this study we used different criteria such as phenotypic, morphological, and functional properties, to investigate the in vitro cultured Sp-MØ. By assessing these parameters we were able to follow the changes occurring in Sp-MØ due to the effect of M-CSF over the period of culturing. This work revealed for the first time that the phenotypic characteristics of Sp-MØ resemble that of BM-MØ cultured under the same conditions. The data suggests that similarity in their differentiation profile was probably linked to M-CSF. Another important observation we noticed was the expression level of CD11c increased gradually during the differentiation stages. Several studies have relied on CD11c as DC specific markers, although it has been shown to be expressed by different kinds of cells including MØ (18). Related to this issue, a recent study examining the depletion of DCs using diphtheria toxin administration to CD11c-DTR
transgenic mice resulted in the complete ablation of the MZMØ, MMMØ, and F4/80-positive RPMØ (19). However, it is not clear if the MØ upregulated CD11c, and thus were affected, or if they simply cleared the toxin-containing debris.

If MØ and DCs are derived from a common precursor (18), it is easy to predict that CD11c will be expressed on these populations under different conditions that can affect differentiation. We were further able to analyze the different population by studying the expression patterns of MØ specific markers, such as SIGN-R1, MOMA-1, MOMA-2 and CD68. Our results revealed a reduction of the MZMØ-specific marker SIGN-R1 and up-regulation of other MØ-specific markers such as, MOMA-2, CD68 and F4/80 that belong to the group of RPMØ. Thus, our work extends on the data reported by other studies that examined Sp-MØ subpopulations in op/op mice following i.v. administration of rM-CSF. These studies showed that newly generated F4/80 and MOMA-2 positive RPMØ were detectable at about 7 days, while MOMA-1 and SIGN-R1 positive MØ slowly increased in the marginal zone of the spleen, and were still very low even after 4 weeks of daily rM-CSF administration (9, 10). The same kinetic patterns in the reappearance of Sp-MØ were also observed in wild type mice injected with liposome-entrapped dichioromethylene diphosphonate (DMDP) that causes the specific elimination of Sp-MØ (20). Based on these observations, and from our data, we propose and confirm that culturing of splenocytes in M-CSF for different time points will result in further differentiation of Sp-MØ subpopulations in vitro leading to M-CSF-dependent population.

Further evidence towards the importance of M-CSF in MØ development showed that the development of F4/80 positive cells in the spleen is regulated by M-CSF, where M-CSF restoration causes a 1.6-fold increase of RPMØ compared with op/op mice (11). Notably, in the spleen in contrast to MZMØ and MMMØ, the RPMØ express high levels of the F4/80 antigen (2). In summary, the in vitro-derived Sp-MØ in our experiments appear to be a M-CSF-dependent subpopulation and are most likely RPMØ as evidenced by the fact that they are F4/80, MOMA-2 positive, and SIGN-R1 low/negative, as previously characterized (9, 21).

The functional property of Sp-MØ and BM-MØ was addressed by investigating the changes in their phagocytic activity. Related to this function, RPMØ are efficient scavenger cells that rely on phagocytosis
to remove debris and dead cells (2, 8). The data examined 3, 5, and 7-day Sp-MØ and BM-MØ have shown significant up-regulation in the phagocytic capacity of Sp-MØ over 7 days in M-CSF medium, whereas BM-MØ cultured under the same conditions exhibited similar profile. This proved important to evaluate because the data confirmed that such cells are enhancing their phagocytic function, implying a progression in differentiation and ruling out the possibility that such cells were aging and thus may lose their functions. Combined with the phenotypic analysis, the increased phagocytosis further indicates that the 7-days Sp-MØ resemble the RPMØ population.

With regard to this point, the M-CSF-dependent Sp-MØ represents semi-mature MØ derived from macrophage-DC progenitor (MDP), or circulating monocytes that mature into typical MØ in the spleen, most likely under the influence of M-CSF. In a similar manner, Hume et al., has reviewed this issue extensively and concluded that a common progenitor cell in the bone marrow called the MDP serves as a precursor to give rise to either MØ or DCs (18). Therefore, it is possible that a certain myeloid uncommitted precursor destined to become Sp-DCs might differentiate into Sp-MØ once they are stimulated by M-CSF. The same local MØ progenitor may eventually differentiate into RPMØ, under conditions depending on the availability of M-CSF (9) (figure 5.1).

In conclusion, the results presented in this section reflect differences among Sp-MØ in response to M-CSF. Our results suggest that both Sp-MØ and BM-MØ respond to M-CSF signals, upregulate their macrophage markers and differentiate into mature macrophages in vitro. This study helped us to proceed forward with our work depicted in the next chapter to determine the contribution of Sp-MØ and BM-MØ in antigen presentation and the activation of CD8+ T cell-responses.

In the next chapter, we continued our investigations to determine how the differentiation stages influence the functional antigen presentation capacity of Sp-MØ and BM-MØ. It is known that pAPCs such as BM-MØ have the ability to activate CD8+ T-cell responses via one of two mechanisms: the direct or cross-presentation pathway (4). It is also known that Sp-MØ play a role in antiviral immune responses where, MZMØ and MMMØ are crucial in the clearance of viruses like LCMV and vesicular stomatitis virus (VSV) (22, 23). Although several studies have examined the abilities of BM-MØ, peritoneal MØ, and MØ cell lines to present antigens either via the direct or the cross-presentation pathway (17, 24-26), there is
Figure 5.1. Proposed schematic model of the relationship between Sp-MØ and their precursors. A) Development, differentiation, and maturation of Sp-MØ in vivo. It has been established that Sp-MØ are divided into two main groups based on M-CSF requirement; M-CSF-dependent and M-CSF-independent subpopulations. Monocytes and macrophage-dendritic cells progenitors (MDP) can differentiate into MZMØ and MMMØ under the influence of M-CSF (i), and also have potential to differentiate to RPMØ in presence of M-CSF (ii). Some of MZMØ and MMMØ might differentiate into RPMØ under the influence of M-CSF (iii). Additionally, precursor cells in the spleen can differentiate into or RPMØ (iv) MZMØ and MMMØ (v) or under the influence of M-CSF. Solid arrows represent pathway that are supported by established data, whereas dashed arrows represent pathways that are putative.

B) Possible pathways for the differentiation of spleen-derived MØ in vitro in our model. Based on our results, we propose that cells that enter or reside in the spleen (Monocytes, macrophage-dendritic cells progenitors (MDP), MZMØ, MMMØ, and local macrophage precursor cells) are differentiated into fully mature macrophages characterized by F4/80$^{hi}$, MOMA-1$^{hi}$, MOMA-2$^{hi}$, CD68$^{hi}$, and SIGN-R1$^{lo}$ when they are culture in M-CSF medium.
A

Peripheral blood
- Monocytes
- Macrophage-dendritic cells progenitor (MDP)

Spleen
(i)

Spleen
(ii)
(iii)

MZMØ & MMMØ

RPMØ

Local Sp-MØ progenitor

B

F4/80hi
MOMA-1hi
MOMA-2hi
CD68hi
SIGN-R1lo

Sp-derived MØ

Monocytes & MDP

Local Sp-MØ

MZMØ & MMMØ

7 days in M-CSF
little information about Sp-MØ that can be related to their role in regulating CTL responses. Recently, one such study implicated their role in suppressing or tolerizing T cells as a result of presenting antigens from dying apoptotic ADCs (27).

Our investigations have shown that both Sp-MØ and BM-MØ, that differentiated in vitro in the presence of M-CSF for 3, 5, and 7 days, were equally susceptible and permissible to LCMV infections. Such data provide further evidence that the cells we allowed to culture in vitro were not aging cells that may have changed their susceptibility to virus infection. In a different model, Kalter et al., has shown that human monocytes cultured for 7 days in M-CSF medium were 400-fold more susceptible to HIV-I infection than monocytes cultured in medium alone, due to monocytes differentiation into MØ. Furthermore, in the same study, the susceptibility of monocytes to HIV infection increased gradually from day 0 to day 36 of culture (28). In related studies, treating human MØ with M-CSF resulted in significant increases of CD4 (29) and CCR5 (30), which in turn increased the susceptibility of MØ to HIV-1 infection. Thus, unlike the data reported with M-CSF and HIV, our model was not affected by culturing the MØ in M-CSF. This most likely was related to the mechanism by which the LCMV virus enters and replicates in the cells, that is clearly not M-CSF differentiation dependent.

The data presented in chapter 3 also demonstrated that Sp-MØ and BM-MØ, which were allowed to differentiate for 3, 5, and 7 days, were infected similarly with LCMV, and efficiently presented the four LCMV epitopes (NP396, NP205, GP33, and GP276). These epitope presentation results were expected to some extent because the differentiated MØ were equally susceptible to LCMV infections and express MHC-I molecules at similar levels.

These interesting observations were also made when we examined cross-presentation. Both Sp-MØ and BM-MØ down regulated their cross-presentation activity as they differentiated further in culture. This phenomenon occurred only during cross-presentation of particulate antigens but not soluble antigens. This observation suggested that cross-presentation of cell-associated antigens by MØ is highly regulated.

The inverse correlation between the differentiation and cross-presentation efficiency of MØ was not related to expression levels of MHC-I, since all conditions have shown similar MHC-I expression patterns. It does not appear to be directly related to antigen uptake since day 3 cells, which were less phagocytic,
were better at cross presentation. Therefore, we excluded the phagocytic property as an influential factor. In agreement with this concept, it was shown that the efficient cross-presentation capacity of a subset of spleen DCs (CD8+) did not relate to antigen capture property (31). Additionally, we could not relate this difference to altered proteasomal activities. On the other hand, we did manage to reveal that phagosomal acidification was an important factor, which appears to be regulating the fate of internalized antigens in MØ during differentiation, and thus directly regulating cross-presentation activities.

In general, the phagosomal acidification in pAPCs is controlled by the respective activities of two systems: 1) V-ATPase, which imports protons across the phagosomal membrane and makes the environment more acidic, and 2) NOX2, which transfers electrons from the cytosol to the phagosomal lumen and causes an increase in the phagosomal pH making it more alkaline (32). Based on the experiments carried out, maturation of phagosomes to highly acidic compartments seems to be associated with the degree of MØ differentiation, as 3-day MØ have less acidic compartments than 7-day MØ. We proved this premise by changing the vesicular pH where it was observed that a more alkaline environment decreased antigen degradation, and a reduced acidic phagosomal pH accelerated the degradation CFSE-HEK cells.

These results indicate that when Sp-MØ differentiate further, they tend to acquire more degradation capacity. Thus our work expands on the observation made by Delamarre et al., showing a direct relation between the differentiation state and antigen degradation capacity of BM-MØ. In relation to his work, and in contrast to 3-day BM-MØ, 6-day BM-MØ contained high level of proteases and rapidly degraded internalized proteins (33). Therefore we conclude that the differentiation of Sp-MØ enable them to deal with exogenous antigens differently, which could be related to the levels of M-CSF in the environment. The effect of phagosomal acidification on exogenous antigen presentation has been previously established for BM-DCs (34, 35). It has been documented that within 1-2 hours after phagocytosis, phagosomes of MØ become highly acidic allowing for the activation of acid-dependent proteases such as cathepsin D, B, L, and S, and dipeptidyl peptidase which cause degradation of antigens (32). Thus our data expand on the findings by Delamarre et al., (33), and allow us to substantiate that acidification of the phagocytic compartments play a vital role in determining the cross-presentation of cell associated antigen by Sp-MØ.
Because we did not observe significant differences in cross-presentation with the soluble OVA protein, it is possible that this antigen is released into the cytosol quickly after endocytosis and does not require potent acidic conditions for initial proteolysis and degradation, as seen in DCs (36, 37). Thus it was cross-presented equally by different MØ populations during differentiation. This scenario is supported by various reports that indicated small soluble proteins and large cellular fragments follow different processing pathways once they enter the cell (37-40).

On the other hand, cross-presentation of cell-associated antigens appears to be controlled by two degradation machineries: the phagosome and proteasome. The release kinetics of cell-associated antigen in the phagosome is determined by the abundance of the proteolytic proteases in the acidic compartments and susceptibility of exogenous antigens to these proteases (33, 41, 42). Supporting evidence come from studies examining phago-lysosomal proteases, which are active at the acidic pH (5-6), or on the lysosome after 45-60 minutes of antigen engulfment (32, 35, 43). Accordingly, it seems that proteases in 3-day, but not 7-day MØ limit the extent of proteins degradation and preserve the potential T cell epitopes for further proteasome processing upon transport out of phagosomes.

To put our observations into prospective we suggest a model depicted in figure 5.2 where soluble antigens are released rapidly with a similar rate into the cytosol in the MØ populations differentiated for 3, 5, and 7 days. In contrast, cell-associated antigens are highly dependent on phagosomal acidification and antigen transportation rates. One can presume that in 7-day MØ a portion of the antigen is partially degraded in early phagocytic compartments and released into the cytosol for proteasomal processing. However, the bulk of the antigen will be systematically degraded in the late acidic phago-lysosomal compartments.

The scenario is completely different in 3-day MØ, as they contain less proteases (33), and therefore there is a partial antigen degradation which may be essential for optimal proteasomal processing to generate the correct MHC I peptides. Thus, the intracellular machinery of 3-day MØ is more adapted to process exogenous antigens for MHC-I presentation molecules rather than degrade and destroy them. In
Figure 5.2. Proposed model for antigens cross-presentation by MØ during differentiation.

Cell-associated and soluble antigens follow different routes during cross-presentation. Soluble OVA proteins are released rapidly and equally into the cytosol in both 3 and 7-day MØ. In such model, the antigen processing, presentation, and activation of OVA-specific CTL response by 3 and 7-day MØ will be comparable. On the other hand, cross-presentation of cell-associated antigens takes place via different pathways in 3 and 7-day MØ. 3-day MØ possess less proteases and less acidic compartments, therefore, the majority of cell-associated antigens are partially degraded and released in the cytosol for proteasome processing (upper part). The scenario is different in 7-days MØ in that they contain more active proteases and highly acidic compartments. The active alkalization of phagosomes is transient and it does not last long in 7-days MØ after phagocytosis. Within this period, some of the exogenous antigens will be partially degraded and released into the cytosol to be available for proteasomal processing. Rapidly after this stage, the phagosomes will fuse with the lysosomes and form highly acidic phago-lysosomes. During acidification, lysosomal proteases are activated and antigens are extensively degraded which will result in releasing antigens fragments that have less potential to be utilized to generate peptides for MHC-I molecules (lower part).
Antigen release and processing

Soluble Ag

Cellular Ag

Endosome

Phagosome

Proteasome

Phago-lysosome

Degraded peptides

Peptides for presentation

Antigen degradation

Nucleus

Lysosome (Acidification)

Phagosomal Acidification

Ag degradation

Ag presentation

0 min

3 days
MØ

7 days
MØ

> 60 min

3 days
MØ

7 days
MØ

217
conclusion, the work presented in the third chapter has revealed a novel association of antigen cross-presentation to the differentiation of MØ and the fundamental role of phagosomal acidification.

In the final part of the thesis the focus of the work centred on how different LCMV epitopes are cross-presented in vitro and in vivo. Most of the studies examining virus antigen cross-presentation have been performed on cells transfected with a single gene encoded by the virus (44, 45). By utilizing the infection model LCMV-WE, we analysed the cross-presentation of LCMV epitopes from proteins generated during the course of virus infection.

In our study, we employed HEK cells as an ADC model because their MHC is not of mouse origin and they have high susceptibility to LCMV infection. Accumulation of LCMV proteins was monitored in HEK cells over 24 hours of infection, which reflects the viral replication cycle. Our infection kinetic results have shown that 24 hour-infected HEK cells have a high accumulation of LCMV-NP and GP, while early time points of infections demonstrated more NP accumulation than GP. This is consistent with published data showing that the expression kinetics of MHC molecules by the LCMV-NP396 epitope preceded LCMV-GP33 at about 6 hours during virus infection (46).

In our model, LCMV-infected HEK cells were LyUV treated to efficiently destroy any infectious virus, as without the Ly treatment some residual infectious virus could remain (unpublished data).

One of the most important factors affecting cross-presentation is the quantity and the stability of the antigen proteins transferred from ADCs to APCs. This was concluded from various studies, using different antigen modules including those with LCMV antigens. For example LCMV-NP396 was efficient at cross-presentation when expressed in the context of the full NP in the cytosol of ADCs (44). Also, when GP33 is expressed as an epitope in virus-like particles (47) or expressed as non-cleavable GP variant that retained LCMV-GP in the endoplasmic reticulum of ADCs (48). Notably, out of the four epitopes tested (NP396, NP205, GP33, and GP276), only the NP396 epitope was efficiently cross-presented by the pAPCs. This might be attributed to stability of peptides contained this epitope in ADCs due to virus infection.

218
As expected, the LCMV-GP33 epitope was inefficient at cross-presentation because it is located in a signal peptide which is only stable for a short time in ADCs (50). Two important factors could be responsible for the diminished cross-presentation capacity of GP276: the presence of immunoproteasome which naturally leads to a destruction of the GP276 epitope (51), and the low MHC-I binding affinity (IC50 nM) of this epitope, which is about 414 nM compared to 0.23 nM for NP396 (49). NP205 is characterized by its occurrence in the long-lived nucleoprotein (52), but its inefficiency might be due to poor processing by the phagosomal/proteasomal machineries.

During the course of infection, while LCMV-NP complexes with the viral genomic materials (RNA) to form intracellular ribonucleoprotein (RNP) complexes, the LCMV-GP are embedded into the cytoplasmic membrane of infected cells (53-55). As a result, these complexes might have an impact on the cross-presentation of LCMV proteins after infection. Sousa et al., has shown that immunization of mice with encephalomyocarditis virus(ssRNA)-infected cells or cells containing synthetic dsRNA leads to a remarkable increase in CTL cross-priming against cell-associated antigens, which is largely dependent on TLR3 expression by pAPCs (56). Thus, one would expect that binding of viral RNA and some membrane structures with LCMV-NP and GP, respectively, will enhance the cross-presentation capacity of LCMV proteins.

By digesting the viral RNA, we have noticed a significant decrease in cross-presentation activity of LCMV-NP but not GP. Thus, in contrast to what has been suggested (57), our data demonstrates that cross-presentation of cell-associated antigens is protein-dependent and does not need RNA transfer.

When considering in vivo cross-priming, our investigations have shown a significant domination of NP396 and GP33 epitopes over NP205 and GP276. Considering the recent analysis of precursor frequencies of naïve CD8+ T cells (49), which found that GP33-specific naïve CD8+ T cells frequencies were 3-times higher than naïve NP396,. One can explain why GP33-specific T cells were able to expand to levels comparable to NP396-specific T cells in vivo. This finding was unexpected, as recent data indicated that poor cross-presentation would directly lead to a subdominant position during T cell activation following cross-priming (58).
Importantly, the data obtained after examining cross-priming of the four epitopes correlate well with the estimated CTL precursor frequencies that have been calculated in naïve and LCMV-immune B6 mice (49, 59). Thus, it appears that the differences in the precursor frequencies among the tested epitopes may shape the final outcome of the cross-priming events. Our findings strongly suggest that while in vitro cross-presentation outcomes depend mainly on the stability of the proteins provided by degradation in the phagosome, in vivo cross-priming can be affected by other physiological factors such as the precursor frequencies of CD8+ T cells.

To expand upon these observations, we analyzed the impact that cross-priming of LCMV proteins has on the LCMV immunodominance hierarchy during subsequent virus infection, the changes of the LCMV-specific CTL repertoires before and after LCMV infection were evaluated. Although many factors affecting the regulation of CD8+ T cell immunodominance have been previously delineated, the role of cross-priming in this process has yet to be specifically addressed. Previously, our group has shown that LyUV-treated HEK-NP could significantly induce cross-priming in vivo, alter the immunodominance hierarchy and shift it towards NP396-specific CTL which significantly controlled a subsequent viral infection (60).

Here we tested four epitopes in our experiments (NP396, NP205, GP33, and GP276). From cross-priming data obtained with infected LyUV cells, we expected to see domination of GP33 and NP396-specific CTL responses after subsequent virus infections. In fact, this is what we observed and it confirmed how multiple epitopes within the same protein could affect the immunodominance during a viral challenge.

Our study of LCMV proteins after virus infection revealed many distinct parameters that can take place and be critical during cross-presentation following virus infection in vitro and in vivo. In particular, our findings validated the generation of effective antiviral protection mechanisms by exploiting the cross-presentation pathway. This is confirming by the fact that the virus clearance was more efficient after the cross-priming was initiated. Thus, utilization of exogenous antigens to initiate CTL responses via cross-presentation pathway would help in developing and establishing novel vaccine strategies. In order to achieve this goal, more research is needed to dissect the unknown cellular and molecular factors, which regulate cross-presentation mechanism during the initiation of CTL immune response.
5.2 Summary and Conclusions

In summary, in this thesis, we have established a new efficient culturing method for obtaining high-yield pure Sp-MØ cultures, which is dependent exclusively on the presence of M-CSF. Our results have provided valuable findings about the critical role of M-CSF for \textit{in vitro} culturing of Sp-MØ and the mechanism(s) by which MØ participate in activation of CTL responses. In this project we have established, characterized, and validated an efficient culture method for generating mature Sp-MØ in large quantity and high purity. This new methodology for growing Sp-MØ \textit{in vitro} has relied on using M-CSF and thus confirms for the first time that M-CSF is a crucial growth factor for further differentiation of Sp-MØ \textit{in vitro}. The quantity and the purity of Sp-MØ that we obtained over time suggest the existence of an uncommitted precursor(s) in the spleen that have potential to differentiate into Sp-MØ.

Our data provide important new insights into the mechanisms of cross-presentation of virus cell-associated antigens with respect to the differentiation of Sp-MØ. We have reported for the first time a direct relationship between MØ differentiation and phagosomal degradation and how they impact the capacity of MØ to cross-present cell-associated antigens and activate CD8+ T cells. Furthermore, based on our data, the ability of viral epitopes to cross-present \textit{in vitro} does not entirely reflect what would occur in cross-priming which is important when evaluating different epitopes in vaccine-related studies. We also proved that cross priming relying on a single dose of antigen is an efficient way to induce immunity and that it plays an important role in regulating and shaping the immunodominance during secondary virus infections. Thus, our results provide important insights into vaccine field, and allow us to expand our knowledge of CD8+ T cell activation via cross priming, which is important for immunotherapy.
5.3 Prospective work

As a follow up to this work, further analysis of Sp-MØ subpopulations will enhance our knowledge with regard to their role during immune responses. Further studies in this field could focus on the correlation between antigen cross-presentation, phagosomal acidification, and proteases contents during differentiation where the enzymatic activity of each protease could be monitored.

The future direction of this project should also focus on answering questions about cross-presentation of virus antigens generated during infections. For example, one of the most important aims that should be investigated is the effect of persistent virus infection on cross-presentation, \textit{in vitro} and \textit{in vivo}. It has been shown that during acute LCMV infections, cross-priming of the exogenous antigen ovalbumin (OVA) is increased due to IFNα production as a result of virus infection. These findings indicate that DCs require a special “license”, provided by type I IFN after virus infection, to present exogenous antigens to CTLs. The above studies raise many questions in other virus infection models such as in persistent infections. Is type I IFN licensing of DCs for cross-priming effective during chronic virus infections?

Accordingly, the immediate objective should focus on establishing and characterizing a novel experimental model to delve further into the cellular and molecular mechanisms involved in regulating cross-priming. It will be of great interest to determine which Sp-MØ subpopulation is involved in inducing CTL responses \textit{in vitro} and \textit{in vivo}. This can be carried out by injecting antigens \textit{in vivo} and investigating by flow Cytometry sorting which subpopulation of Sp-MØ is able to activate CTL \textit{ex vivo}. Furthermore, incubating Sp-MØ \textit{in vitro} with ADCs and monitor their capacity to induce naive CD8+ T cells responses is also possible. Addressing the changes that could occur in the phenotype and the quality of memory T cells will provide insight into the potential role of Sp-MØ in generating and shaping the long lasting immune responses.
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223
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Appendix 1

Delivery Of Exogenous Antigens To Induce Cytotoxic CD8+ T Lymphocyte Responses

Julia Kim, Vandana Ganbhir, Attiya Alatery and Sameh Basta

I participated in this review by providing some sections and making figure 1. This review is submitted to Journal of Biomedicine and Biotechnology (in press)
Abstract

Vaccines intended to induce a cytotoxic CD8+ T cell response are highly sought after. However some of these vaccines can be problematic if they replicate in the host. An alternative strategy is to exploit cross-presentation of exogenous antigens to express peptides on major histocompatibility complex (MHC) class I molecules. During cross-presentation, the delivered exogenous antigen can be taken up and processed through diverse mechanisms. Here, we will discuss recent advances regarding the complex nature of the cross-priming process and the models that reflect its relevance in vivo. Moreover, we summarize current data that explore potential adjuvants and vaccine vectors that deliver antigens to activate CD8+ T cells relying on cross-presentation.

Introduction

Naïve CD8+ T-cells become activated when their receptors recognize antigens presented by professional antigen presenting cells (pAPCs) in the context of MHC-I molecules. Upon recognition of target cells, such primed cytotoxic T-lymphocytes (CTLs) are able to limit the spread of virus infection through the lysis of host-infected cells, halting viral propagation in permissible cells. Moreover, along with helper T cells they orchestrate the induction of key cytokines such as interferon-γ (IFN-γ) and tumor-necrosis factor-α (TNF-α) needed for an optimal immune response (1). In addition to their importance in halting virus replication, CTLs play a central role in the specific immune response and are essential in the elimination of intracellular pathogens and limiting the potential escape of tumor cells (2, 3).

The cross-presentation pathway allows for exogenously-derived antigens to be presented on MHC-I molecules to CTLs (4-7). To induce anti tumor immune responses, or to prime CTLs for viruses that inhibit direct presentation, the immune system utilizes cross-presentation (8, 9). Thus, cross-presentation represents a promising mechanism for strategies that target the induction of CTL responses for vaccine development to induce both effector and protective memory T-cell responses. In order for us to discuss the
role of cross-presentation in future vaccine developments we need to explore antigen presentation in details.

The MHC-I Presentation Pathways

All nucleated cells express MHC-I molecules and are capable of presenting antigens to CTLs. However, the “priming” stage or activation of naïve CD8+ T-cells requires peptide-MHC class I complexes presented by the pAPC in addition to co-stimulatory signals such as interaction with B7 molecules, CD40, CD70 and the 4-1BBL family members (10), as well as the secretion of key cytokines such as IL-12 and IFN-α (11-13). These cytokines are also important in optimal CD8+ T-cells memory development (14). The CD8+ T-cells priming step can occur via two different mechanisms of antigen presentation: the direct- and cross-presentation pathways. In the direct or “endogenous” presentation pathway, antigens are derived from endogenously synthesized proteins, improperly translated proteins, and or unstable defective ribosomal proteins (15, 16). Such cytosolic proteins must be polyubiquitinated, before being tagged for degradation by the proteasome. Proteasomal degradation products are then transported into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP), and finally move through the Golgi complex to the cell surface (Fig. 1).

Alternatively, the cross- or the “exogenous” presentation pathway occurs when uninfected pAPCs present exogenously-derived antigens (4, 5, 7) after they uptake soluble or cell-associated antigens. The latter antigenic form can be derived from different sources such as cellular fragments (17-19), intracellular bacteria (20), virus-infected or tumor cells (21-26), and as reported recently from parasitic infections (27). It appears that protein stability in these models is critical for efficient cross-presentation to occur (17, 28-31). On the other hand, soluble proteins tend to be cross-presented, but with much lower efficiency than cell-associated proteins (32). Although efficient presentation of exogenous antigens was originally attributed to macrophages (33), it is now clear that such function can be achieved by different bone marrow-derived APCs, including, of course, DCs (34-36), as well as spleen-derived macrophages (37). Interestingly, it appears both spleen and bone marrow-derived macrophages down regulate their ability to cross present cell associated antigens during differentiation (37).
**Figure 1. Antigen processing pathways.** Direct presentation involves the processing of endogenously synthesized antigens (route A) by the proteasome (I) to break them down into smaller fragment (polypeptides) that are transported through the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (II) for loading onto newly synthesized MHC class I molecules. These peptide-loaded MHC class I molecules are then transported through the Golgi (III) to the cell surface for presentation to CD8+ T cells. Considering the major route for antigen cross-presentation (route B), exogenous antigens are internalized in the endosome/phagosome before they are released into the cytosol and degraded by the proteasome to be presented on MHC class I molecules.
In cross-presentation, several processing mechanisms have been proposed (38), including the canonical model where antigens inside the endosomal/phagosomal vesicles are translocated into the cytosol before they follow the regular proteasome/ER/TAP route (figure 1). In addition, it was proposed that pAPCs possess organelles such as the phagosome optimized for the cross-presentation of exogenous antigens (38) by functioning autonomously to generate of MHC class I–peptide complexes from proteins internalized into the same phagosome. Moreover, soluble antigen cross-presentation was recently found to involve early endocytic compartment trafficking that is aided by TAP recruitment and signaling through TLR4 and MyD88 (39). However, whether these models also hold true for other forms of exogenous antigens other than OVA proteins, has not been fully investigated.

**Induction of tolerance versus priming**

As this review focuses on exogenous antigen delivery in vaccine development, we will concentrate on cross-presentation. Regardless of the exact processing mechanism in play within the cell, the cross-presentation pathway can result in one of two conditions: cross-tolerance or cross-priming. Inadequate activation of CTLs may result in tolerance, which is an ideal situation for self-antigens. Thus, under normal circumstances, cross-presentation of peripheral self-antigens from normal healthy tissue will induce cross-tolerance (40, 41). This outcome could depend on many factors such as which APC is presenting the antigen (41-43), and how the antigen is being cross-presented (44, 45). On the other hand, if immunity is to be induced, signals necessary for T-cell activation (46), leading to cross-priming will be provided by pAPCs leading to clonal expansion, differentiation and establishment of robust memory cells.

Memory T-cells are long-lived cells that allow for an efficient adaptive immune response upon re-exposure to a pathogen (47). Such Memory cells can be divided into two categories depending on their trafficking and effector functions. The effector memory T cells (T_{EM}) are found in peripheral tissues, can respond rapidly to infections, and are characterized by the low expression of certain markers such as CD62L, CCR7 and CD27. Upon antigen recognition, they immediately produce effector cytokines, such as IFN-γ and TNF-α, conferring a state of immediate protection (47). In contrast, central memory T-cells
(T<sub>CM</sub>) are found mainly in secondary lymphatic organs such as lymph nodes, and express high levels of the CD26L, CCR7 and CD27 markers and are slow to exhibit their effector functions. An important goal of any vaccination protocol would be the induction of both sets of CD8+ T-cell memory (48).

Another critical factor to consider in T cell activation focuses on the profile of T-cell immunodominance hierarchy that a vaccine is likely to induce. With regard to immunodominance, epitope-specific CD8+ T cells can be organized into a hierarchy, in which certain immunodominant epitopes will cause a set of T cells to expand extensively compared to subdominant epitope-specific T cells (49). A CTL response could be more effective when generated against a greater number of epitopes, as it is more diverse and should lead to the formation of a wider number of specific memory CD8+ T-cell populations that can confer protective immunity.

In evaluating the physiological relevance of cross-priming in vivo to vaccine development, it has been demonstrated that cross-priming is a robust process that induces significant CTL responses to multiple epitopes both in viral and tumor models (21). In a related study, studying cross-priming of inactivated flu virus resulted in a broad and balanced CTL responses compared to live virus. Interestingly, it appears that the ability of an epitope to access cross-priming may support its immunodominance position when considering the overall hierarchy (50-52). During virus challenge experiments, it was found that an initial cross-priming with a lymphocytic choriomeningitis virus (LCMV)-nucleoprotein (NP) that normally cross-prime CTLs for the NP396 epitope (17), resulted in increasing the magnitude of NP396 epitope specific T-cells. Thus, the immunodominance hierarchy was modified so that the NP396 epitope, an immunodominant epitope found within the LCMV-NP, was favored in a subsequent virus challenge (50). These effects were maintained over time, and may have important implications for vaccination protocols such as the currently administrated inactivated flu vaccines. In this study, it was speculated that the initial cross priming with LCMV-NP resulted in the enhanced ability of NP396-specific clones but not the NP205-specific clones, to expand and out compete other T-cell clones specific for epitopes generated form other proteins (50). Importantly, these changes only occurred when cross-priming preceded viral infection and thus when T-cell frequencies competing for resources were lower. Such findings were not observed in the vaccinia virus model when peptides were used prior to infections to prime CTL responses (53). These diverse findings
may be related to the different replication profiles of both viruses considering that vaccinia does not replicate as efficiently as LCMV in mice. It would also be interesting to compare immunodominance data in a virus challenge experiment obtained after priming with peptide vs. proteins, where in the latter condition cross priming is expected to take place (50, 53).

Finally, with regard to cross-priming and vaccination, it seems that there is plenty of room and interest to discover and develop adjuvants that are able to regulate cross-presentation, for example, through the stimulation of toll-like receptors (TLRs). TLR signaling can result in DC maturation, leading to up-regulated MHC and co-stimulatory molecule expression, and increased priming of T-cells without the need for CD4 T-cell help (26, 54-57).

Thus, based on many models of cross-priming, researchers are logically aiming to develop novel vaccine vectors that are capable of inducing CTL responses \textit{in vivo} by utilization exogenous antigen delivery. In the latter part of this review, we will discuss some examples of such vectors and contemplate on other future developments in the vaccine field.

**Utilizing cross-presentation in the induction of CTL responses**

In vaccination, delivering exogenous antigens via cross-presentation should ideally induce both humoral and CTL responses to improve effective immunity. As the major pAPC in vivo, DCs are able to stimulate naïve CD8+ T-cells and are important when considering precise targeting of delivery vector. A key area of research involves the modification of vector surfaces with ligands for DC receptors, such as TLR ligands as well the application of natural substances that can allow for effective immune modulation. For example, the active metabolites of vitamin D3 can influence adaptive immune responses to peripherally administered antigens during vaccination/infection. In these experiments, the ability of murine DCs to migrate from skin sites of vaccination to mucosal lymphoid organs seem to be dependent on the production of active metabolites of vitamin D3 produced locally at the vaccination site, which was associated with the application of specific TLR (TLR3/TLR4) ligands (58, 59). This supports previous studies showing that both systemic and common mucosal immune responses developed in mature adult mice immunized
subcutaneously or intradermally with vaccines containing the active form of vitamin D3 may be represent potential novel adjuvant preparations (60).

Generally, vaccines should induce effective protective CTL immunity, and the production of immunological memory. Potent CTL responses have been reported to be limited to live attenuated viral or bacterial vaccines (48). However, the use of such vaccines is offset by the risk of reinitiating virulence. Thus, a key target of novel vectors, such as virus-like particles, microparticles and archaeosomes to name a few, is to efficiently access the cross-presentation pathway. As a result, these vectors will induce CTL effector and memory T-cell responses without risking the health of the host. Such vectors have shown interesting immunological observations, which we will discuss below, that enable them to be potent for inducing cross-priming.

**Virus-like particles**

Virus-like particles (VLPs) are formed from viral structural proteins that lack a viral genome and thus assemble into non-replicative particles (61, 62). VLPs are safe, stable, and extremely immunogenic due to their highly repetitive molecular structures (61, 62). In addition to their highly immunogenic nature, VLPs can be easily modified to increase their immunogenicity by packaging CpGs into the core antigen particle, which results in higher frequencies of peptide-specific CD8+ T cells being induced (63).

Since VLPs particles are exogenously acquired by pAPCs, they are able to use the cross-presentation pathway (64-66), and can induce both CTL and memory responses (67). For example, a recombinant porcine-parvovirus (PPV)-VLP, encoding a known LCMV CD8+ T-cell epitope was able to stimulate the immune system up to 8 months after the last immunization (62). This long-lasting effect was characterized by protection against a lethal viral (LCMV) infection, in addition to responses against peptide-coated or virus-infected target cells (62). The high particulate nature of PPV-VLPs was hypothesized to associate with its optimal delivery to the MHC-class I antigen presentation pathway resulting in high immunogenicity (62). This ability to induce CTLs via cross-priming was demonstrated in another study with HIV p55gag-VLPs immunizations (67). In this study, immunized animals were
efficiently primed (> 8.5 months) for p55gag-specific CTL responses that recognized multiple HIV p55gag epitopes. Altogether, these studies demonstrate that VLPs are able to prime strong MHC-I restricted CD8+ T-cell responses through cross-presentation.

The exact mechanism of cross-presentation in general is not fully defined; however recent data suggests that the pathway involved with VLPs may vary according to the type of vector employed. For example, cross-presentation of papaya mosaic VLPs which induces a robust CTL responses (68) was proteasome independent (64). In the case of parvovirus-like particles (PPV-VLPs) without employing adjuvant, exogenous antigen was localized in late endosomes of DCs (69). It is important to note that presence of potential adjuvants such as TLR ligands (39) enhances the efficiency of cross-presentation as it allows relocation of the TAP molecules to the early endosomes. As one would expect, the processing of the PPV-VLPs required vacuolar acidification, proteasome activity, and TAP translocation but not MHC class I molecule recycling. Therefore the combined data together shows cross-presentation of PPV-VLPs occur via an endosome-to-cytosol processing pathway (69). The uptake and capture of PPV-VLPs involved macropinocytosis and lipid rafts participation. Whereas, with hepatitis B virus (HBV), VLPs made of the viral core protein induced efficient CTL cross-priming by DCs, in either TAP-dependent or TAP-independent manner (66). The HBV (VLPs) are also taken up by macropinocytosis, but rely on endosomal processing and recycling MHC I molecules in DCs (70).

Thus, data concerning VLPs may require that each vector be evaluated independently because each case may be unique and could be closely associated with which pathogen or disease, it is meant to protect against. It is also important to note that not all VLPs are able to activate pAPCs by themselves. This was highlighted by data showing that a CTL epitope from LCMV (p33-VLPs) was efficiently processed for MHC class I presentation but induced weak CTL responses (63). The CTL response failed to mediate effective protection from viral challenge in the absence of external substances that activate APCs, such as anti-CD40 antibodies or CpG oligonucleotides, which engage TLR9 (63).
Microspheres (MSs)

Biodegradable microspheres (MSs) are useful to microencapsulated antigens alone or combined with adjuvants. Their ability to allow for the controlled release of the antigen or to allow for the formation of a depot at the site of injection is particularly useful (71). Poly (DL-lactide-co-glycolide) (PLGA) has been used to prepare microspheres that possess tremendous potential to release encapsulated antigens in a controlled manner, which facilitate the protection of antigen from immediate degradation in vivo (72). A key advantage of MSs is the flexibility in design, which allows for a variety of possibilities in the combining different antigens and adjuvants. Moreover, specific cell targeting is feasible through the ligands addition to the vehicle surface that bind specifically to receptors on the surface of the targeted cell type (71, 72).

MSs can deliver exogenous antigens to the cross-presentation pathway, but appear to require additional signals by employing adjuvants to trigger a CTL response which may involve T cell help (73). This help is needed to promote the co-stimulatory activation state of DCs, and can be provided via CD40 interactions with CD40L on the helper T-cells for optimal cross-priming (74, 75). As MSs are unable to trigger DC maturation by themselves (76), cross-presentation of antigens by immature DCs can result in T-cell ignorance (43).

Data examining this phenomenon concur that additional help signals are required to enhance the cross-priming of MSs. For example, MSs encapsulating an antigen with an immunodominant CD8+ T-cell epitope (B-OVAp) failed to induce specific cross-priming of antigen-specific CD8+ T-cells, unless they were co-injected with a recombinant PPV-VLP that served as an adjuvant to induce a potent CTL response (73). Interestingly, the PPV-VLPs enhanced this CTL response in MHC class II+ and CD40+ mice indicating that the CD4+ T-cell help was bypassed under these conditions. Such adjuvant activity did not require TLR2, TLR4, or TLR9 either.

More evidences along this line of work were reported recently examining the influence of including adjuvants within the antigens-containing MSs. These studies used OVA incorporated into MSs and examined cross-primed antigen-specific T-cells evaluated by the secretion of IFN-γ in peptide specific
assays (77, 78). The CTL response elicited when TLR ligands were co-encapsulated with OVA was stronger compared with OVA-MS alone or if antigen and adjuvants were separately encapsulated.

In one study, the co-encapsulation of OVA and a TLR9 ligand (CpG-ODN) found that both OVA and CpG-ODN were translocated into DC lysosomes for degradation (78). The resulting OVA peptides: MHC molecules and TLR9-dependent DC activation drove robust antigen-specific CD4+ and CD8+ T-cell proliferation (78). The processing route for MHC-I antigen presentation required endosomal acidification, TAP translocation, and proteasomal processing (78). In a separate study, it was found that CpG-ODN in the MSs induced DC maturation, as characterized by increased co-stimulatory molecule expression, increased secretion of cytokines, which was associated with increase cross-priming (77). In both reports (77, 78), induction of OVA specific T-cells were comparable to what is observed when live vectors encoding OVA are employed (79). In addition, the induced immunity was protective against a challenge of L. monocytogenes, vaccinia virus and protected against OVA expressing tumors (77, 78).

Clearly, this work or idea of including adjuvant activity with the antigens is not limited to MSs. For example, the vaccine adjuvant effects of liposomes complexed to TLR agonists were found to induce effective CD4+ and CD8+ T cell responses against peptide and protein antigens. In particular TLR3 or TLR9 agonists effectively cross-primed CD8+ T cell responses independently of CD4+ T cell help (55). Accordingly, despite what we know about the advantages of using MSs in vaccine delivery, additional data explaining the exact mechanisms underlying the processing of MSs vaccine vectors are needed and their protective potential with different antigenic determinants in different infections models. However, the simultaneous inclusion of antigen and adjuvant appears to be holding positive promises to optimize future vaccination strategies using MSs.

**Archaeosomes**

Archaeosome composition incorporates the polar lipids of Archaea, which leads to immune-stimulating interactions with APCs (80). Entrapment of Antigen in archaeosomes has been documented to facilitate MHC-I cross-presentation, resulting in the activation of long-lived CD8+ T-cell immunity (79-
For example, a single immunization with a Methanobrevibacter smithii-OVA (M. smithii) archaeosome was found to prime 2-5% of antigen-specific T-cells by day 7, while boosting on day 21 resulted in an expansion to about 20% (79). Furthermore, after two immunizations of low doses M. smithii-OVA archaeosomes, re-stimulated spleen cell effectors were able to exhibit strong cytolytic activity of target cells labeled with the specific peptides (83). This ability to activate CTLs with archaeosomes was demonstrated in CD4⁻ (84) and IL-12⁻ mice (83).

When examining the quality of CD8+ T-cell responses, primed by archaeosomes to entrapped cargo. It was reported that the responses are superior in magnitude to other particulate vesicular systems such as liposomes (81). In addition, a single injection of the vector induced a profound primary response leading to the formation of around 1% of CD8+ T_CM which exhibited a phenotype (CD44⁺CD62L⁺), typical of the central memory cells (79). The responses were comparable to vaccination with live vectors encoding OVA such as L. monocytogenes vector (79) and induced protective CD8+ T cells responses that were TLR2-independent.

Unlike the MSs delivery system we discusses above, archaeosomes can target antigen for cross-presentation, while simultaneously activating pAPC allowing for proper induction of co-stimulatory molecules and cytokine production (80). OVA entrapped in M. smithii archaeosome is the best-characterized model with respect to the archaeosome cross-presentation mechanisms. Due to the presence of exposed archaetidylserine head groups on their surfaces, M. smithii archaeosomes are taken up by receptor-mediated endocytosis (82). Thus, they are able to utilize the same pathway that relies on the phosphatidylserine (PS) receptor-mediated clearance of apoptotic cells by pAPCs (82). After uptake, acidification in the phagolysosomes help antigen escape from the archaeosomes eventually reaching the cytosol before undergoing cross-processing in a proteasome and TAP-dependent manner (82).

It is important to note that other archaeosome types lacking PS are also phagocytosed efficiently and induce strong CD8+ T cell immunity (80). Thus, diverse archaeosome vectors may be expected to utilize other receptors to gain entry into the cell. Overall, archaeosomes and its research field with regard to vaccine development appear to represent versatile, potentially universal, vaccine delivery vector, which would benefit tremendously if more research groups were involved in it.
Conclusion

Vaccines developed in the past have been critical in preventing dreadful infectious diseases where the successful majorities were designed as live attenuated vaccine strains. However, development of new generation non-replicating vaccine vectors should aim to efficiently target antigens exogenously to immune cells and allow cross priming rather than cross tolerance to take place. The attractiveness of targeting this alternative mechanism to induce CD8+ T cell responses is met by several hurdles on how to efficiently optimize the delivery vectors to maximize the immune response. In order to achieve this goal, more research is needed to dissect the unknown cellular and molecular factors, which regulate this mechanism during the initiation of CTL immune response. We have come far since the observations made by Edward Jenner and the field is now ripe with novel potential adjuvants to make the next step forward that improves on the initial discovery of the small pox vaccine.
References:


245


