NOVEL ROLE FOR HEPARAN SULFATE IN THYMIC DEVELOPMENT OF CD8⁺ T LYMPHOCYTES

DAVID ANAK SIMON DAVIS

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE AUSTRALIAN NATIONAL UNIVERSITY

DECEMBER 2015
Dedicated to my parents Mune Robert and Simon Davis and
to my fiancée Tan Ai May, family, friends and mentors
Declaration

The experimental work presented in this thesis constitutes original work by myself unless otherwise stated in the text. Mr Cameron Jack assisted with the bioinformatics screening for putative HS/heparin-binding proteins (Table 1.1). Dr. Craig Freeman synthesised and donated the heparin mimetics used in this work (Table 2.2). Dr. Craig Freeman also assisted in the purification of HRG from human plasma (Section 2.2). Ms Lora Jensen assisted with the in situ detection of heparan sulfate expressing cells on thymic sections (FIG. 5.1).

This thesis conforms to The Australian National University guidelines and regulations. The work contained within has not been submitted for the purpose of obtaining any other degree at this or other universities.

David Anak Simon Davis
(Author, PhD candidate)

Christopher R. Parish
(Supervisor)

Cancer and Vascular Biology Group
Department of Cancer Biology and Therapeutics
The John Curtin School of Medical Research
The Australian National University

Benjamin J. C. Quah
(Co-supervisor)

DECEMBER 2015
Acknowledgements

My utmost thanks and sincerest gratitude go to my supervisors, Chris Parish and Ben Quah. A special thanks to Chris for giving me the chance to undertake a PhD project in his laboratory, the endless support and guidance (scientific and otherwise) and for the intellectual flexibility that he allowed me throughout this journey. I could not have wished for better supervisors!

I would also like to express my gratitude to my advisory panel Craig Freeman, Chris Goodnow and Carola Vinuesa, and also Steve Daley, Andrew Ziolkowski and Ivan Poon for valuable advice and insights in various aspect of the project. Moreover, I am also thankful to those who have very kindly donated various mouse strains and reagents that allowed me to venture further into this work.

I am also utmost indebted to all the members (past and present) of the Parish group, including Anna Bezos, Anna Browne, Lucy, Sarah, Euan, Megan, Jinsoo and Mayank for the help and constructive discussions (scientific and otherwise). Special thanks also go to Harpreet, Mick, Anne and Cathy of the Imaging & Cytometry Facility for the assistance with flow cytometry, microscopy and histology and extreme patience. Furthermore, I am also thankful to Anna Cowan, Charani and Wendy for managing the student affairs (mine and others). My extended gratitude goes to the animal husbandry staff of the Australian Phenomics Facility that provided excellent animal care. I would also like to acknowledge The John Curtin School of Medical Research, The Australian National University and The Australian Government for financially supported by PhD.

Most importantly, I would like to thank my fiancée Tan Ai May and my parents Mune Robert and Simon Davis for your unconditional love, care, patience and support to embark on this study in pursuit of my professional goal.

David Anak Simon Davis
Abstract

The thymic stromal microenvironment is crucial for MHC-restricted positive and negative selection of T cells in the thymus to generate functional and self-tolerant αβ T cells. The studies outlined in this Thesis provide new insights into this important process.

It has been known for decades that murine CD4⁺CD8⁺ double positive (DP) thymocytes form multicellular conjugates with autologous erythrocytes in vitro, a phenomenon termed autorosetting. In Chapter 3 the molecular basis of autorosetting is defined. Using a flow cytometry assay it was confirmed that DP thymocytes are the major autorosetting population in the thymus, whereas peripheral T lymphocytes do not autorosette. The autorosetting assay also revealed that CD8β molecules are the main autorosetting receptor on DP thymocytes, an anti-CD8β mAb strongly inhibiting autorosetting, a finding confirmed using CD8β.KO and CD8α.KO mice. Heparanase treatment of erythrocytes greatly diminished autorosetting, thus indicating that heparan sulfate (HS) is the key autorosetting ligand on erythrocytes. This finding is consistent with HS-like molecules, such as heparin and dextran sulfate (DxS, 5 and 500 kDa), inhibiting autorosetting and blocking the binding of mAb to CD8β, with the autorosetting inhibition by heparin being 6-O-sulfate dependent. Also, it was found that in the periphery, α2-3 O-sialylation of CD8β molecules substantially inhibited autorosetting, as α2-3-specific neuraminidase treatment restored autorosetting by peripheral CD8⁺ T lymphocytes. In addition, it was found that serum-derived histidine-rich glycoprotein (HRG) totally inhibited CD8β-mediated autorosetting by masking HS on erythrocytes. In serum extracted from HRG.KO mice, however, another HRG-like molecule appeared to inhibit autorosetting. Thus, O-sialylation of CD8β molecules, combined with circulating HRG, strictly regulates the CD8-HS interaction by peripheral CD8⁺ T lymphocytes.

Experiments described in Chapter 4 investigated the functional consequences of the CD8-HS interaction by DP thymocytes. Consistent with earlier reports, HS-like molecules, particularly DxS₅₀₀ kDa can induce a sustained uptake of extracellular Ca²⁺ in DP thymocytes, but not in peripheral T lymphocytes. It was found that, like autorosetting, the DxS₅₀₀ kDa-induced Ca²⁺ flux was severely impaired in CD8β.KO DP thymocytes, and further diminished in CD8α.KO thymocytes. Furthermore, treatment of
peripheral CD8\(^+\) T lymphocytes with an \(\alpha_2\)-3-specific neuraminidase restored the DxS\(^{500\text{ kDa}}\)-induced Ca\(^{2+}\) flux, this T cell population otherwise being non-responsive to DxS\(^{500\text{ kDa}}\) stimulation. Using mutant mouse strains it was found that the DxS\(^{500\text{ kDa}}\)-induced Ca\(^{2+}\) flux was independent of TCR-induced proximal signalling, being unaffected by Zap70-deficiency, but was almost totally eliminated in Slp76-deficient DP thymocytes. Moreover, DxS\(^{500\text{ kDa}}\) markedly enhanced thymocytes activation by an immobilised anti-CD3\(\varepsilon\) mAb, as indicated by CD69 upregulation. Collectively, these observations suggest that the CD8-HS interaction, which mediates autorosetting, induces a sustained Ca\(^{2+}\) flux in DP thymocytes that lowers the threshold required for TCR-mediated activation.

Finally, in Chapter 5 the true physiological relevance of the CD8-HS interaction in T cell development was examined. It is well known that selection of the T cell repertoire in the thymus requires DP thymocytes to scan cortical thymic epithelial cells (cTEC) for self-peptide/MHC complexes. To survive, DP thymocytes need to carry a TCR that binds weakly to self-peptide/MHC complexes and generates a weak TCR signal, a process known as positive selection, with failure to generate an adequate positively selecting TCR signal resulting in DP thymocytes dying by neglect. Using flow cytometry it was discovered that highly sulfated HS expressed on the surface of cTEC participates in the rosetting between cTEC and thymocytes, this process being CD8-dependent and being markedly inhibited by HS-like molecules, such as heparin and DxS. Furthermore, the interaction of thymocyte CD8 with cTEC HS induced a sustained Ca\(^{2+}\) flux in the rosetting thymocytes, consistent with this interaction lowering the signalling threshold required to positively select DP thymocytes.

In conclusion, this study demonstrates that the CD8-HS interaction (1) enhances the interaction of DP thymocytes with cTEC expressing self-peptide/MHC complexes and (2) triggers unique CD8-dependent accessory signals, additional to TCR signals, that lower the threshold required for positive selection of TCR clones for self-peptide/MHC complexes.
Table of Contents

Declaration III
Acknowledgements IV
Abstract V
List of Figures X
List of Tables XI
Abbreviations XII
Publication resulting from this thesis meeting abstract XVI

Chapter One: Literature review 1
1.1 The roles of heparan sulfate in the mammalian immune system 2
  1.1.1 Introduction 2
  1.1.2 Different cellular location of HS chains 2
  1.1.3 Prevalence of HS in the mammalian immune system 3
  1.1.4 Functional roles of HS in immunity 7
    1.1.4.1 Regulator of cell adhesion 7
    1.1.4.2 Modulator of cytokine and chemokine function 9
    1.1.4.3 A sensor of tissue injury 10
    1.1.4.4 Physical barrier to leukocyte migration 11
  1.1.5 Concluding remarks 12
1.2 Intrathymic TCRαβ T lymphocyte development 13
  1.2.1 TCRαβ T lymphocytes 13
  1.2.2 The thymus 15
  1.2.3 Intrathymic TCRαβ T lymphocyte development 16
    1.2.3.1 DN stage: ETP settling, TCRαβ lineage selection and β-selection 16
    1.2.3.2 DP stage: repertoire selection and CD4/CD8-lineage choice in the cortex 19
      1.2.3.2.1 TCR signalling pathways involved in repertoire selection 21
      1.2.3.2.2 Positive and negative selection of DP thymocytes 25
      1.2.3.2.3 CD4/CD8-lineage choice in post-selected DP thymocytes 30
    1.2.3.3 SP stage: establishing central tolerance, final maturation and egress 31
  1.2.4 Concluding remarks 33
1.3 Experimental aims 34

Chapter Two: Materials and methods 35
2.1 General reagents 36
  2.1.1 Reagents, media and buffers 36
  2.1.2 Sulfated polysaccharides 36
  2.1.3 Antibodies 36
2.2 Purification of human plasma histidine-rich glycoprotein (hHRG) 36
2.3 Bradford protein assay 37
2.4 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) 37
2.5 Coomassie blue protein staining 37
2.6 Western blotting 40
2.7 Animals 41
2.8 Preparation of mouse blood 41
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8.1</td>
<td>Collection of mouse blood</td>
<td>41</td>
</tr>
<tr>
<td>2.8.2</td>
<td>Isolation of mouse serum</td>
<td>42</td>
</tr>
<tr>
<td>2.9</td>
<td>HRG detection by enzyme-link immunosorbent assay (ELISA)</td>
<td>42</td>
</tr>
<tr>
<td>2.10</td>
<td>Depletion of mouse serum albumin</td>
<td>42</td>
</tr>
<tr>
<td>2.11</td>
<td>Depletion of mouse serum HRG</td>
<td>43</td>
</tr>
<tr>
<td>2.12</td>
<td>Lymphocyte preparation</td>
<td>43</td>
</tr>
<tr>
<td>2.12.1</td>
<td>Preparation of single-cell lymphocyte suspension</td>
<td>43</td>
</tr>
<tr>
<td>2.12.2</td>
<td>Manual enrichment of peripheral lymphocytes</td>
<td>43</td>
</tr>
<tr>
<td>2.12.3</td>
<td>Cell counting and viability</td>
<td>44</td>
</tr>
<tr>
<td>2.13</td>
<td>Antibody labelling and analysis of leukocytes</td>
<td>44</td>
</tr>
<tr>
<td>2.13.1</td>
<td>Blocking of non-specific antibody binding FcR</td>
<td>44</td>
</tr>
<tr>
<td>2.13.2</td>
<td>Cell surface antibody labelling</td>
<td>44</td>
</tr>
<tr>
<td>2.13.3</td>
<td>Multiparametric analysis of cells by flow cytometry</td>
<td>45</td>
</tr>
<tr>
<td>2.14</td>
<td>Lymphocyte-erythrocyte autorosetting assay</td>
<td>45</td>
</tr>
<tr>
<td>2.14.1</td>
<td>Fluorescent labelling of lymphocytes</td>
<td>45</td>
</tr>
<tr>
<td>2.14.2</td>
<td>Preparation of CFSE-labelled erythrocytes</td>
<td>45</td>
</tr>
<tr>
<td>2.14.3</td>
<td>Standard autorosetting assay</td>
<td>45</td>
</tr>
<tr>
<td>2.15</td>
<td>Lymphocyte-erythrocyte autorosetting inhibition experiments</td>
<td>46</td>
</tr>
<tr>
<td>2.16</td>
<td>Analysis of cell surface heparan sulfate on erythrocytes</td>
<td>46</td>
</tr>
<tr>
<td>2.16.1</td>
<td>Heparanase treatment of erythrocytes</td>
<td>46</td>
</tr>
<tr>
<td>2.16.2</td>
<td>Assessment of erythrocyte cell surface heparan sulfate</td>
<td>47</td>
</tr>
<tr>
<td>2.17</td>
<td>Assessment of lymphocyte sialylation profiles</td>
<td>47</td>
</tr>
<tr>
<td>2.17.1</td>
<td>Neuraminidase treatment of lymphocytes</td>
<td>47</td>
</tr>
<tr>
<td>2.17.2</td>
<td>Cell surface lectin labelling</td>
<td>47</td>
</tr>
<tr>
<td>2.18</td>
<td>Inhibition of antibody binding by sulfated polysaccharides</td>
<td>47</td>
</tr>
<tr>
<td>2.19</td>
<td>Heparin binding assay</td>
<td>48</td>
</tr>
<tr>
<td>2.20</td>
<td>Flow cytometric intracellular Ca(^{2+}) flux assay</td>
<td>48</td>
</tr>
<tr>
<td>2.20.1</td>
<td>Loading of lymphocytes with Ca(^{2+}) indicator dye</td>
<td>48</td>
</tr>
<tr>
<td>2.20.2</td>
<td>Measurement of intracellular Ca(^{2+}) flux by flow cytometry</td>
<td>48</td>
</tr>
<tr>
<td>2.21</td>
<td>Thymocyte activation assay</td>
<td>49</td>
</tr>
<tr>
<td>2.22</td>
<td>Identification of rosetting thymic stromal cells</td>
<td>49</td>
</tr>
<tr>
<td>2.22.1</td>
<td>Enzymatic digestion of thymic fragments</td>
<td>49</td>
</tr>
<tr>
<td>2.22.2</td>
<td>Enrichment of pre-formed thymic rosettes</td>
<td>50</td>
</tr>
<tr>
<td>2.22.3</td>
<td>Cell surface labelling of stromal cells</td>
<td>50</td>
</tr>
<tr>
<td>2.22.4</td>
<td>Assessment of stromal cell surface heparan sulfate</td>
<td>50</td>
</tr>
<tr>
<td>2.23</td>
<td>Stromal-thymocyte rosetting assay</td>
<td>51</td>
</tr>
<tr>
<td>2.23.1</td>
<td>Manual enrichment of EpCAM(^{+}) thymic epithelial cells (TEC)</td>
<td>51</td>
</tr>
<tr>
<td>2.23.2</td>
<td>Fluorescent labelling of thymocytes</td>
<td>51</td>
</tr>
<tr>
<td>2.23.3</td>
<td>Stromal-thymocyte rosetting assay</td>
<td>52</td>
</tr>
<tr>
<td>2.24</td>
<td>Analysis of intracellular Ca(^{2+}) flux in rosetting thymocytes</td>
<td>52</td>
</tr>
<tr>
<td>2.25</td>
<td>Statistical analysis</td>
<td>53</td>
</tr>
</tbody>
</table>

**Chapter Three: Mechanisms and regulators of murine autorosetting**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Abstract</td>
<td>55</td>
</tr>
<tr>
<td>3.2</td>
<td>Introduction</td>
<td>56</td>
</tr>
<tr>
<td>3.3</td>
<td>Results</td>
<td>57</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Establishment of a flow cytometry-based autorosetting assay</td>
<td>57</td>
</tr>
</tbody>
</table>
3.3.2  Organ distribution of autorosetting lymphocytes 58
3.3.3  Identification of CD8 as the autorosetting receptor on lymphocytes 60
3.3.4  Identification of the autorosetting ligand on erythrocytes 64
3.3.5  Direct evidence that CD8 interacts with HS-like molecules 67
3.3.6  Sialylation influences the interaction of CD8 with HS-like molecules 70
3.3.7  Autorosetting inhibition by serum derived HRG 74
3.4  Discussion 76

Chapter Four: Functional significance of murine autorosetting 81

4.1  Abstract 81
4.2  Introduction 82
4.3  Results 83
4.3.1  Induction of a Ca\(^{2+}\) flux in lymphocytes by DxS\(^{500\,kDa}\) 83
4.3.2  The molecular basis of the DxS\(^{500\,kDa}\)-induced Ca\(^{2+}\) flux in thymocytes 86
4.3.3  Contribution of sulfated polysaccharide recognition to thymocyte activation 90
4.4  Discussion 92

Chapter Five: Physiological relevance of murine autorosetting 97

5.1  Abstract 97
5.2  Introduction 98
5.3  Results 100
5.3.1  Identification of HS-expressing cells in the murine thymus 100
5.3.2  The molecular basis of cTEC-thymocyte rosetting 104
5.3.3  Functional significance of cTEC-thymocyte rosetting 109
5.4  Discussion 111

Chapter Six: Final discussion and future directions 115

6.1  Introduction 116
6.2  CD8-HS interaction: important structural consideration 117
6.2.1  Location of HS binding site on CD8 molecule 117
6.2.2  Sialylation of the CD8 molecule 117
6.2.3  Does CD4 interact with HS? 119
6.3  Implications of CD8-HS interaction: the big perspective 119
6.3.1  Role of CD8-HS interaction in positive selection 120
6.3.2  Role of CD8-HS interaction in negative selection 121
6.3.3  Role of CD8-HS interaction in the periphery 124
6.4  Future research directions 125
6.4.1  Establishing the structural requirements for the CD8-HS interaction 125
6.4.2  Establishing the role of the CD8-HS interaction in positive and negative selection of DP thymocytes 126
6.4.3  Establishing the role of the CD8-HS interaction in peripheral CD8\(^{+}\) T lymphocyte effector function 127
6.5  Concluding remarks 128

References 131
## List of Figures

### Chapter One: Literature review

| FIG. 1.1 | The structure of HSPG. | 3 |
| FIG. 1.2 | Comparison of TCR-peptide/MHC-CD8 and TCR-peptide/MHC-CD4 ternary complexes. | 14 |
| FIG. 1.4 | Overview of thymic T cell development. | 17 |
| FIG. 1.5 | Stromal cell interactions during T cell development. | 20 |
| FIG. 1.6 | Canonical TCR signal transduction to Ras-MAPK in T cell development. | 22 |
| FIG. 1.7 | Co-receptor basis of MHC-specific thymic selection. | 27 |
| FIG. 1.8 | The kinetic signalling model for CD4/CD8-lineage choice. | 31 |

### Chapter Three: Mechanisms and regulators of murine autorosetting

| FIG. 3.1 | Multicolour flow cytometric lymphocyte-erythrocyte autorosetting assay. | 58 |
| FIG. 3.2 | Configuration of flow cytometer for detection of rosettes. | 59 |
| FIG. 3.3 | Flow cytometric analysis of autorosetting between thymocytes and autologous erythrocytes. | 60 |
| FIG. 3.4 | Lymphoid organ and lymphocyte subset distribution of autorosetting lymphocytes. | 61 |
| FIG. 3.5 | Contribution of leukocyte surface molecules to autorosetting. | 62 |
| FIG. 3.6 | Contribution of CD8 molecules to thymocytes autorosetting. | 63 |
| FIG. 3.7 | MHC-I molecules are dispensable for autorosetting. | 64 |
| FIG. 3.8 | Heparan sulfate on the surface of erythrocytes acts as an autorosetting ligand. | 65 |
| FIG. 3.9 | Analysis of anti-CD45, anti-CD4, anti-CD8α and anti-CD8β mAb binding to thymocytes in the presence of the sulfated polysaccharides heparin and dextran sulfate (5 & 500 kDa). | 68 |
| FIG. 3.10 | Binding of heparin to thymocytes requires CD8 molecules. | 69 |
| FIG. 3.11 | Analysis of the influence of the sialylation status of lymphocytes on their heparin binding ability and autorosetting capacity. | 72 |
| FIG. 3.12 | Analysis of the contribution of CD8β to the sialylation status of CD8+ T cells and impact of CD8β-deficiency on heparin binding and autorosetting following desialylation. | 73 |
| FIG. 3.13 | Surface HS is required for erythrocytes to autorosette with neuraminidase-treated T cells. | 74 |
| FIG. 3.14 | Autorosetting inhibition by histidine-rich glycoprotein (HRG) and by a compensatory HRG-like serum protein. | 75 |

### Chapter Four: Functional significance of murine autorosetting

| FIG. 4.1 | High but not low molecular weight sulfated polysaccharides are able to induce a Ca2+ flux in thymocytes that is dependent on the uptake of extracellular Ca2+. | 84 |
| FIG. 4.2 | The sulfated polysaccharide DxS500 kDa specifically induces a Ca2+ flux in subpopulations of thymocytes. | 85 |
| FIG. 4.3 | Contribution of CD8 molecules to the DxS500 kDa-induced Ca2+ flux in thymocytes. | 88 |
| FIG. 4.4 | Zap70 but not Slp76 is dispensable for the DxS500 kDa-induced Ca2+ flux in thymocytes. | 89 |
| FIG. 4.5 | Neuraminidase treatment restores the CD8β-dependent DxS500 kDa-induced Ca2+ flux in peripheral CD8+ T cells. | 90 |
FIG. 4.6: DxS^{500 kDa} enhances anti-CD3ε mAb-mediated thymocyte activation in a CD8β-dependent manner.

Chapter Five: Physiological relevance of murine autorosetting

FIG. 5.1: Immunohistochemical detection of cells expressing high levels of heparan sulfate (HS) in the cortex of the mouse thymus.

FIG. 5.2: Identification of different subsets of thymic stromal cells.

FIG. 5.3: Heparan sulfate is highly expressed by thymic stromal cells.

FIG. 5.4: Fluorometric assay for detection of thymocytes rosetting with thymic stromal cells.

FIG. 5.5: Thymocyte CD4 and CD8 molecules, cTEC MHC and sulfated polysaccharides contribute to rosetting between cTEC and syngeneic thymocytes.

FIG. 5.6: Synergistic contributions of MHC and HS on cTEC to thymocyte rosetting capacity.

FIG. 5.7: Rosetting between thymocytes and cTEC induces a prolonged Ca^{2+} flux in thymocytes.

Chapter Six: Final discussion and future directions

FIG. 6.1. Predicted implications of the CD8-HS interaction for intrathymic MHC-I-restricted TCRαβ CD8^{+} T lymphocyte development and function.

List of Tables

Chapter One: Literature review

Table 1.1: Hypothetical HS-interacting proteins.

Table 1.2: Stages of T cell development correlate with specific location in the thymus, distinct cell-surface phenotypes and TCR requirement.

Chapter Two: Materials and methods

Table 2.1: Reagents used in experimental procedures.

Table 2.2: Sulfated polysaccharides used in experimental procedures.

Table 2.3: Details of primary antibodies used in experimental procedures.

Table 2.4: Mouse strains used in experimental procedures.

Table 2.5: MAb cocktails used in manual MACS enrichment of peripheral lymphocytes.

Table 2.6: Calcium indicator dyes.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AF</td>
<td>Alexa fluor</td>
</tr>
<tr>
<td>AIRE</td>
<td>Autoimmune regulator</td>
</tr>
<tr>
<td>AM</td>
<td>Acetoxymethyl ester</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocynin</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6 mouse strain</td>
</tr>
<tr>
<td>Bcl</td>
<td>B cell lymphoma</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 interacting mediator of cell death</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BV</td>
<td>Brilliant violet</td>
</tr>
<tr>
<td>C</td>
<td>Coincidence</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>Calcium</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemotactic chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemotactic chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFDA-SE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>CMJ</td>
<td>Cortico-medullary junction</td>
</tr>
<tr>
<td>Co^{2+}</td>
<td>Cobalt</td>
</tr>
<tr>
<td>CPD_{670}</td>
<td>Cell Proliferation Dye eFluor® 670</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium release-activated calcium channel</td>
</tr>
<tr>
<td>cTEC</td>
<td>Cortical thymic epithelial cell</td>
</tr>
<tr>
<td>Ctrl</td>
<td>Control</td>
</tr>
<tr>
<td>CTV</td>
<td>CellTrace™ Violet</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC chemokine motif ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine motif receptor</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyanine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DLL4</td>
<td>Delta-like ligand 4</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>CD4⁺CD8⁻ double-negative</td>
</tr>
<tr>
<td>DP</td>
<td>CD4⁺CD8⁺ double-positive</td>
</tr>
<tr>
<td>DxS</td>
<td>Dextran sulfate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>$E_{m}$</td>
<td>Emission wavelength</td>
</tr>
<tr>
<td>ENU</td>
<td>N-ethyl-N-nitrosourea</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial cell adhesion molecule (CD326)</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ETP</td>
<td>Early thymic progenitor</td>
</tr>
<tr>
<td>Ext</td>
<td>Exotoses</td>
</tr>
<tr>
<td>$E_{x}$</td>
<td>Excitation wavelength</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>$F_{c}$</td>
<td>Fragment crystallisable region</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FIG.</td>
<td>Figure</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Flt-3</td>
<td>Fms-like tyrosine kinase 3</td>
</tr>
<tr>
<td>FoxN1</td>
<td>Forkhead box N1</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>$g$</td>
<td>Unit gravity</td>
</tr>
<tr>
<td>$g$</td>
<td>Gram</td>
</tr>
<tr>
<td>$G$</td>
<td>Gauge</td>
</tr>
<tr>
<td>Gads</td>
<td>Grb2-relared adapter downstream of Shc</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GATA</td>
<td>Globin transcription factor 1</td>
</tr>
<tr>
<td>Glce</td>
<td>D-glucuronyl C5-epimerase</td>
</tr>
<tr>
<td>GMFI</td>
<td>Geometric mean fluorescence index</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>H2-K</td>
<td>H2 class I histocompatibility antigen, K region</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balance Salt Solution</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HMGB</td>
<td>High mobility group box</td>
</tr>
<tr>
<td>HPNSEC</td>
<td>Heparinase-1/heparitinase-III</td>
</tr>
<tr>
<td>HPSE</td>
<td>Heparanase</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HRG</td>
<td>Histidine/proline-rich glycoprotein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>Hs2st</td>
<td>HS 2-O-sulfotransferase</td>
</tr>
<tr>
<td>Hs6st</td>
<td>HS 6-O-sulfotransferase</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate proteoglycan</td>
</tr>
<tr>
<td>$IC_{50}$</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-R</td>
<td>Interleukin receptor</td>
</tr>
<tr>
<td>$IP_{3}$</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITK</td>
<td>IL-2-inducible T cell kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker of activated T cell</td>
</tr>
<tr>
<td>Lck</td>
<td>Lymphocyte-specific protein tyrosine kinase</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
</tr>
<tr>
<td>LPC</td>
<td>Lymphoid progenitor cell</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>m</td>
<td>Metre</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>MAL-II</td>
<td><em>Maackia amurensis</em> lectin II</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mcl</td>
<td>Myeloid cell leukaemia</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Mililitre</td>
</tr>
<tr>
<td>mM</td>
<td>Milimolar</td>
</tr>
<tr>
<td>mTEC</td>
<td>Medullary thymic epithelial cells</td>
</tr>
<tr>
<td>n</td>
<td>Nano</td>
</tr>
<tr>
<td>NCC</td>
<td>Neuro crest cells</td>
</tr>
<tr>
<td>Ndst</td>
<td>HS N-deacetylase/N-sulfotransferase</td>
</tr>
<tr>
<td>Neu</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappaB</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cell</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>Nur77</td>
<td>Growth factor-inducible immediate early gene nur/77-like receptor</td>
</tr>
<tr>
<td>p</td>
<td>Phosphorylated</td>
</tr>
<tr>
<td>P</td>
<td>P-value</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E synthase</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKCθ</td>
<td>Protein kinase C theta</td>
</tr>
<tr>
<td>PLC</td>
<td>Phosphoinositide phospholipase C</td>
</tr>
<tr>
<td>PNA</td>
<td>Peanut agglutinin</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination-activating gene</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor kappa-β</td>
</tr>
<tr>
<td>RasGRP</td>
<td>Ras guanyl nucleotide-releasing protein</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rosewell Park Memorial Institute medium</td>
</tr>
<tr>
<td>$R_{\text{raw}}$</td>
<td>Uncorrected rosetting value</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RUNX</td>
<td>Runt-related transcription factor</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SCZ</td>
<td>Subcapsular zone</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SIRPa</td>
<td>Signal-regulatory protein alpha</td>
</tr>
<tr>
<td>Slp76</td>
<td>SH2 domain containing leukocyte protein of 76 kDa</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store-operated calcium entry</td>
</tr>
<tr>
<td>So</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>SP</td>
<td>Single-positive</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>STIM</td>
<td>Stromal interaction molecule</td>
</tr>
<tr>
<td>Sulf</td>
<td>Endosulfatase</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEC</td>
<td>Thymic epithelial cell</td>
</tr>
<tr>
<td>Tespa1</td>
<td>Thymocyte-expressed positive selection associated 1</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Tumour growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T lymphocyte</td>
</tr>
<tr>
<td>Th-POK</td>
<td>T-helper-inducing POZ/Kruppel-like factor</td>
</tr>
<tr>
<td>Themis</td>
<td>Thymocyte-expressed molecules involved in selection</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNC</td>
<td>Thymic nurse cell</td>
</tr>
<tr>
<td>TOX</td>
<td>Thymocyte selection-associated HMGB</td>
</tr>
<tr>
<td>TRA</td>
<td>Tissue-restricted antigen</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T lymphocyte</td>
</tr>
<tr>
<td>UEA-1</td>
<td><em>Ulex europaeus</em> agglutinin 1</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>V(D)J</td>
<td>Variable-Diversifying and Joining</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VGSC</td>
<td>Voltage-gated sodium channel</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigen</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>Zap70</td>
<td>Zeta-chain (TCR) associated protein kinase 70 kDa</td>
</tr>
<tr>
<td>$\beta_2$m</td>
<td>Beta-2-microglobulin</td>
</tr>
<tr>
<td>$\mu$g</td>
<td>Microgram</td>
</tr>
<tr>
<td>$\mu$L</td>
<td>Microlitre</td>
</tr>
<tr>
<td>$\mu$M</td>
<td>Micromolar</td>
</tr>
</tbody>
</table>
Publication resulting from this thesis

Meeting abstract
Chapter One

Literature Review

The first part of this Chapter provides a general introduction to the contribution of heparan sulfate to the mammalian immune system. The second part of this Chapter reviews T lymphocytes development and the contribution of the thymus to this process.
1.1 The roles of heparan sulfate in the mammalian immune system

1.1.1 Introduction

Heparan sulfate (HS) is a glycosaminoglycan (GAG) that is ubiquitously expressed on cell surfaces and in the extracellular matrix (ECM) and basement membrane (BM). Each HS molecule is a linear polysaccharide composed of repeating disaccharides of hexuronic acid and D-glucosamine that can exhibit immense structural diversity due to substitution to varying extents with sulfate groups and epimerization of glucuronic acid to iduronic acid, with areas of high sulfation and glucuronic acid epimerisation being co-located in ‘hot spots’ throughout the molecule (FIG. 1). HS is structurally related to heparin, an extremely highly sulfated form of HS that is restricted to mast cells. The biosynthesis and modification of HS chains is thought to take place within the endoplasmic reticulum, Golgi apparatus and trans-Golgi network, which in the end produce unique HS chains that are covalently attached to a range of core proteins to form HS-proteoglycans (HSPG) (FIG. 1) (Kreuger and Kjellen, 2012, Esko and Selleck, 2002). After synthesis HS chains can be modified by the endoglycosidase, heparanase (Peterson and Liu, 2010), and endosulfatases, Sulf1 and Sulf2 (Nagamine et al., 2012, Hossain et al., 2010, Uchimura et al., 2006), to regulate HS availability and function. Although the core proteins can function independently of the HS chains they carry (Kramer and Yost, 2003), HS predominantly dictates the ligand-binding capability and therefore the biological roles of HSPG (Sarrazin et al., 2011). Furthermore, while different cell types may express similar core proteins, the HS chains these core proteins carry can be markedly distinctive, resulting in HSPG with highly divers yet specialised roles in mammalian physiology (Sarrazin et al., 2011, Bernfield et al., 1999). In this section, some of the contributions of HS to the functioning of the immune system, notably leukocyte development, leukocyte migration, immune activation and inflammatory processes will be discussed.

1.1.2 Different cellular location of HS chains

In general, cell surface HSPGs include members of the transmembrane syndecans (syndecan 1-4) and glycosylphosphatidylinositol (GPI)-linked glypicans (glypican 1-6). ECM/BM associated HSPGs are comprised of perlecan, collagen type XVIII and agrin. These HSPGs are collectively termed ‘full-time’ HSPGs. ‘Part-time’ HSPG include cell surface CD44 (isoform 3 is HS-linked) and extracellular betaglycan, testican and
neuropilin (Sarrazin et al., 2011, Parish, 2006). Secretory vesicle-associated serglycin is a HSPG that is exclusively expressed intracellularly, particularly in mast cells (Kolset and Pejler, 2011). Furthermore, HSPGs can also be localised in the nucleus where they potentially regulate gene transcription (Richardson et al., 2001, Fedarko and Conrad, 1986, Buczek-Thomas et al., 2008, Purushothaman et al., 2011, Chen and Sanderson, 2009).

**FIG. 1.** The structure of HSPG. HS chains (blue line) are linear polysaccharides composed of repeating disaccharide subunits, which in their unmodified form are D-glucosamine and D-glucuronic acid (blue box). During synthesis, HS chains are covalently attached to core proteins (open circles) at serine (S) residues. A single HSPG molecule may carry multiple HS chains or contain other glycosaminoglycans other than HS (not shown). HS modifications include various degrees of O- and N-sulfation and epimerisation of D-glucuronic acid to D-iduronic acid by HS-modifying enzymes (red box) (Kreuger and Kjellen, 2012). The modifications occur in regions (hot spots) along the polysaccharide chain, these hot spots being separated by regions of low sulfation. Post-synthesis structural alterations are mainly mediated by the endo-β-glucuronidase, heparanase, which cleaves HS chains within highly sulfated regions (cleavage site indicated by red scissors and arrow) (Peterson and Liu, 2010).

### 1.1.3 Prevalence of HS in the mammalian immune system

Due to the structural similarities between HS and heparin, the latter is often used as an experimental model for biochemical studies of HS-protein interactions and predicting potential HS binding partners. Several heparin-binding proteins are known to carry the ‘consensus’ heparin/HS-binding motifs XBBXBX or XBBBXXBX (B being the basic amino acids arginine, lysine or histidine and X being one of a range of aliphatic/aromatic amino acids) (Hileman et al., 1998). If correctly displayed in the secondary structure and optimally positioned within the three-dimensional conformation of polypeptides, these sequences are hypothetically capable of facilitating strong ionic
interactions with negatively charged glycosaminoglycans (Cardin and Weintraub, 1989, Hileman et al., 1998). Based on this simple amino acid sequence criterion, the Parish Laboratory, assisted by Mr Cameron Jack (Genome Discovery Unit, JCSMR, ANU) recently screened for protein sequences matching selected GO terms in the Ensembl database (release 72) with a custom Python script for murine gene products that carry these motifs and are listed on the UniProt database (www.uniprot.org) as being reported to have immunological functions. We identified a total of 235 HS-binding proteins in the mouse genome (Table 1.1), a list that includes known HS-binding proteins and many potential new ligands for HS. An intriguing feature of this analysis was that 66% of the molecules that potentially bind HS are expressed intracellularly, with only 18% being exclusively expressed on the cell surface and only 10% being in the extracellular compartment. Remarkably, only one HS-binding protein, the HS degrading enzyme heparanase, was identified that can be expressed in intracellular and extracellular compartments as well as being able to associate with plasma membranes. This finding is consistent with the multiple functional roles of the enzyme.

Despite earlier reports claiming that HS negatively regulates gene transcription primarily by repressing the activity of p300 and pCAF histone acetyltransferase (Buczek-Thomas et al., 2008, Purushothaman et al., 2011), the bioinformatics screen implies that intracellular HS plays a more elaborate role in dictating cellular responses to various stimuli. Thus, it is predicted that HS interacts with several regulators of histone-modifying enzymes, such as Jarid2 (motif: MKRRHI), Kat6a (LHHLRM, KKVKK, RRVRK), and Mll1 (LRRFRA, IKKLRA, LKKAKA, VHRIRV, KKVKR, RHLKK) alongside key molecules that are involved in signal transduction and regulation of gene transcription, notably Vav1 (VKHIKI), STAT5A (KRIKR), STAT-6 (KKIKR), Bel-6 (WKKYKF), Bel-11a (KHMKK), Ciita (LKRLKL), PTBP3 (VHRVKI, HRFKK), Lck (VKHYKI), IRAK1 (RRAKK), IRAK4 (HHIHR), Foxj1 (FKKRRRL), Syk (RKAHH), ITK/TSK (IKHYHI), Card11 (KRFRK), Zap70 (KKLFLKR), Jak3 (IHKLKA, AKKLKF, RRIRR) and Cbl-b (RHFFH) and some components of the NF-κβ signalling pathway including NFKB2 (YHKMKI), IKBKG (MRKRHV) and Nkap (RRAKK, KKA, KKYKK). Interestingly, in the RAG-1 protein, the presence of a HS-binding motif adjacent to a site critical for DNA binding (560D, UniProt) (AKRFRY), and overlapping the site that is essential for DNA hairpin
formation (971F and 972R, UniProt (Lu et al., 2006)) (RRFRK), may imply a role for HS in regulating V(D)J recombination.

Table 1.1: **Hypothetical HS-interacting proteins.**

<table>
<thead>
<tr>
<th>Cellular Location</th>
<th>Total</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular</td>
<td>154</td>
<td>Nlrp3, Nkap, Lrp5, Lyst, Lck, Irak4, Sla2, Mx2, Syk, Oas2, Tcf3, Myo1e, Alp7a, Cplx2, Tusc2, Ak7, Vav3, Blink, Spta1, Skap1, Fgr, Pmaip1, Aim2, Shb, Ddx60, Dapk3, Nfkβ2, Apobec3, Ripk2, Nod1, Sptb, Oas1d, Mapk14, Ptpb3, March8, Dapk1, Zfp385a, Bcl2, Mecom, Chd7, Gpam, Ap3d1, Lcp2, Arid4a, Poir3a, Tnfaip1, Kat6a, Ung, Myo9b, Bcl6, Eprs, Sp3, Bag6, Plocg1, Nbn, Pml, Kif1, Rb1, Sfxn1, Bcl11a, Farp2, Jarid2, Xrcc6, Itk, Myo1f, Nirc5, Cyp27b1, Ikerf1, Ddx58, Pip4k2a, Ctla, Foxj1, Rnf168, Prkdc, Pmis2, Mil1, Stk3, Mef2c, Acbstf, Prkx, Rag1, Batf3, Map3k14, Ilf2, Herc6, Card11, Card9, Dlg1, Itch, Dyrk3, Tnip3, Cdk6, Irmg1, Rnf31, Apc, Unc13d, Tr13, Tr18, Nedd4, Msh6, Pcid2, Sh2b2, Aicda, Myh9, Pik3cd, Zap70, Vav1, Stat5a, Tgrp1, Mxi1, Enpp2, Dock2, Pgm3, Unc93b1, Plcg2, Stxbp2, Ifi44l, Zfp35, Inpp5d, Oas3, Cblb, Ostn1, Bcl11b, Eps8, Prkd1, Ctnnb1, Poir3b, Samhd1, Nirc4, Tec, Tel2, Map3k5, Pou1f1, Ncaph2, Stat6, Smarca4, Fnip1, Jak3, Cactin, Dicer1, Atm, Ikbkg, Satb1, Eif2ak2, Stap1, Msh3, Sgp1, Cdk13, Foxe1, Zc3h8, Spib, Mhea, March1, Anik1, Mink1</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>42</td>
<td>Mpzl2, Adam10, Ntrk1, Icosl, Cxcr5, Cd97, Tr16, Pde2a, Adam9, Tlr1, Ccr3, Trem1, Tril, Tek, Lrcc8a, Il2ra, Selp, Fas, Hfe, Cd83, Cd22, Ccpgb2, Cta4, Tlr2, Kire1, Gpr183, Ccr7, Abcc9, Hoxb4, H2-M5, Thsd1, Dcstamp, Il7r, Proc, Amica1, Chrbn2, Tnfrsfl3c, Csf1r, Tlr4, Tyr, Cd93, Eda, Cd40lg</td>
</tr>
<tr>
<td>Extracellular</td>
<td>24</td>
<td>Masp1, Cdl25, Osm, Il9, Bmp4, Inhba, Pdgfb, Scg2, Hc, Fam20c, Wnt2b, Lrrc17, Gas6, C7, Il1a, Wnt5a, Cxcl12, A182371, C4b, Cxcl5, C8b, Ccl17, Serping1, Ccl28</td>
</tr>
<tr>
<td>Intracellular, Plasma membrane &amp; Extracellular</td>
<td>1</td>
<td>Hpse</td>
</tr>
<tr>
<td>Intracellular &amp; Plasma membrane</td>
<td>3</td>
<td>Bk, Flt1, Flt3</td>
</tr>
<tr>
<td>Intracellular &amp; Extracellular</td>
<td>2</td>
<td>Isg15, Prg4</td>
</tr>
<tr>
<td>Plasma membrane &amp; Extracellular</td>
<td>8</td>
<td>Tgfb1, Vegfa, Ennp3, Ctsg, C8a, Ptpor, Adam17, Ennp1</td>
</tr>
</tbody>
</table>

List of murine proteins that carry ‘consensus’ heparin/HS-binding motifs. The motifs adapted for bioinformatics screens are the standard motifs, which are primarily XBBBXXBX or XBBXXBX (Hileman et al., 1998) (http://gduserv.anu.edu.au/~cameron/protein.html). Short motifs are truncated versions of the standard motifs XBBBBX, BBXBB or BBXXBB (B = basic amino acids R, K, H; X = A, V, I, L, M, F, Y, W). Only proteins in the UniProt database (www.uniprot.org) that are known to play immunological roles are listed.
Chapter 1

It is not surprising that cytokines including IL-1\(\alpha\) (LKKRL), IL-9 (HRVKR), TGF-\(\beta\)1 (V KRKRI) and chemokines such as CCL17 (KHKVK), CCL19 (RLLKK), CCL25 (ARKRLVHM), CCL28 (VKRRRI), CXCL5 (KKAKR), and CXCL12 (VHHLKI) also carry HS-binding motifs, presumably allowing HS to act as their atypical receptors. Moreover, HS-binding motifs are also present on the cognate receptors for soluble factors such as the cytokine receptors IL-2R\(\alpha\) (HRWKR) and IL-7R (KKVKH) and the chemokine receptors CCR3 (WKFHA), CCR7 (AHRRHA) and CXCR5 (YYRRRL), indicating new roles for HS in regulating leukocyte homeostasis and trafficking. In addition, HS may regulate the availability of crucial components in the immunological synapse as indicated by the presence of HS-binding motifs in the leader sequences of ICOSL (WKHLHV) and CTLA-4 (LRRYKA). The presence of HS binding motifs in CD22 (KKARR) and CD40L (KKLKR) also suggests additional roles for HS in cell-cell communication and T cell costimulation.

Examination of the innate immune system reveals that, although HS is already known to interact with TLR-4 (Johnson et al., 2002), it appears that HS may also be recognised by other TLRs, including cell surface TLR-1, TLR-2, and TLR-6, and endosomal TLR-8 and TLR-13. To be more precise, the HS binding motifs appear to be in the extracellular domains of TLR-4 (RHIFWRR) and TLR-2 (IRRLHA) and in the cytoplasmic domains of TLR-1 (HRARH) and TLR-6 (YHKLRA and HRARH). For the endosomal TLRs, both motifs (LKKLHL and LKKKHF) are luminal facing for TLR-8, and cytoplasmic (HRLRK) and luminal (LKRLKI) for TLR-13. It is possible that HS is involved in regulating downstream signalling when the motifs are present in the TLR cytoplasmic domains. In contrast, when these motifs are facing the luminal or extracellular space, HS may be a ligand or a regulatory component that modulates the interaction between a TLR and its cognate ligands. Furthermore, HS-binding motifs are also present on inflammasome components such as Nlrp3 (LKKFKM), Nlr4 (LKKMRL, RHIHR) and Aim2 (LKRFKY), implicating a role for HS in regulating the activation of inflammatory caspases. In another aspect of innate immunity, several studies have reported that heparin and HS are able to interact with several components of the complements system, including C1 (Wuillemin et al., 1997), C1q, C1 inhibitor, C2, C4, C4b, C4bp, C6, C8, C9, Factor B, Factor D, Factor H (Sahu and Pangburn, 1993), MASP-1/2 (Presanis et al., 2004) and complement receptors CR3 (CD11b/CD18) (Diamond et al., 1995) and CR4 (CD11c/CD18) (Vorup-Jensen et al., 2007). In support
of these observations, we have identified the presence of HS binding motifs in complement proteins such as C4b (FRKFHL), Hc/complement C5 (FHKYKV), C7 (KRLYLKR), C8a (WRKLRY) and C8b (KRYRH) as well as regulatory components of the complement machinery including MASP-1 (KHWRR), Serping1 (HKIRK) and CD93/complement component C1q receptor (YHKRRA), further highlighting the role of HS as a major modulator of the complement system (Zaferani et al., 2013).

However, it should be noted that more in-depth analyses are required to assess the validity of these predicted interactions, in particular the demonstration that the predicted HS-binding sites are correctly presented within the secondary structure and final three-dimensional conformation of the putative HS-binding proteins. Also, there are known HS-binding proteins that lack the heparin/HS binding motifs used in this analysis, suggesting that the list of binding proteins identified in this screen may, in fact, be an underestimate. Thus, at face value, the data set implies that a wide range of HS-binding proteins participate in the immune system.

1.1.4 Functional roles of HS in immunity

Despite the previous section suggesting that there are many unknown HS-protein interactions that may control the immune system, there are a number of well-established functional roles for HS in immunity. Indeed HSPGs, through their HS chains, are involved in a broad spectrum of biological processes, profoundly influencing development (Hacker et al., 2005), homeostasis (Bishop et al., 2007) and the progression of many diseases (Lindahl and Kjellen, 2013). In the case of the immune system, HSPGs are fundamentally involved in regulating cell adhesion, cytokine and chemokine function, sensing tissue injury, and mediating inflammatory reactions. Each of these functional roles will be briefly discussed below, with specific examples given which highlight each function.

1.1.4.1 Regulator of cell adhesion

Cell adhesion molecules are important to facilitate and regulate cell-cell signalling, migration and activation of leukocytes during development, homing and recruitment to inflammatory sites. For example, of particular relevance to leukocytes development in the bone marrow is the receptor complex on hematopoietic stem cells (HSC) comprised of CD45 and Mac-1 (CD11b/CD18) that has been shown to bind to surface HS on bone
marrow-derived stromal cells and facilitate strong adhesion (Coombe et al., 1994). In a related study, the HSPG glypican-3 enhances the antagonising effect of tissue factor pathway-inhibitor (TFPI) on CD26, the stromal-bound ectopeptidase that is involved in cleaving surface CXCL12, a typical ligand for CXCR4 on HSC. As a result, glypican-3 indirectly supports the directional homing of grafted HSC towards, and their retention in, the bone marrow (Khurana et al., 2013). Similarly, in the thymus a particular subset of cortical epithelial cells known as thymic nurse cells are reported to express high levels of highly sulfated HS that is thought to aid thymocyte adhesion and facilitate T cell development (Werneck et al., 1999, Werneck et al., 2000, Oliveira-dos-Santos et al., 1998, Oliveira-dos-Santos et al., 1997).

During an inflammatory response, HS positively regulates the recruitment of inflammatory cells at three different stages based on the following observations. First, endothelial surface HS can reduce neutrophil rolling velocity via L-selectin-mediated cell adhesion (Wang et al., 2005). Second, once attached, the HS-mediated Mac-1-CD44v3 interaction enhances the binding of leukocytes to the endothelial surface to drive extravasation (Zen et al., 2009). Finally, within the endothelial ECM/BM, collagen type XVIII promotes leukocytes infiltration in again an L-selectin-dependent manner (Kawashima et al., 2003, Celie et al., 2005). However, the role of HS in the adhesion of leukocytes to the endothelium can occur paradoxically, in a biphasic manner. Under physiological conditions, glycocalyx HSPGs of pulmonary endothelial cells are known to impede neutrophil adhesion (Schmidt et al., 2012). Following the induction of an experimental sepsis model of acute lung injury, the localised production of TNF-α activates endothelial cells to produce heparanase, which in turn catalyses the partial degradation of HS constituents of the glycocalyx. The loss of HS results in a significant increase in neutrophils binding (Schmidt et al., 2012), presumably via neutrophils L-selectin binding to residual endothelial HS and via cytokine-induced endothelial P- and E-selectin (Giuffre et al., 1997). Additionally, reduction (in wild-type mice) or deletion (in knockout mice) of syndecan-1 from murine endothelial cells strongly accentuates antigen-specific lymphocytes infiltration into inflammatory sites during a delayed-type hypersensitivity reaction (Kharabi Masouleh et al., 2009).
1.1.4.2 Modulator of cytokine and chemokine function

Soluble factors, such as cytokines and chemokines, are crucial to support growth, maintain homeostasis, and orchestrate immune cell trafficking across various locations. However, some of these molecules are inactive or susceptible to degradation in their native, soluble form. Furthermore, these factors need to be timely presented at the right site to exert their anticipated functions. HSPGs have been implicated in modulating various aspects of cytokine and chemokine function (Handel et al., 2005, Coombe, 2008).

HSPGs interact with various cytokines primarily on target cells or act as atypical cytokine receptors on cytokine presenting cell. The former situation enables HPSGs to regulate the availability and influence the interaction between cytokines and their cognate receptors on target cells. For example, the binding of cell surface HS to cytokines such as IL-7 and IFN-γ proved critical to protect them against proteolysis (Clarke et al., 1995, Lortat-Jacob and Grimaud, 1991). Furthermore, the lack of HSPG expression on the mouse pro-B cell surface severely impairs IL-7-dependent maturation towards pre-B cells suggesting that in this situation HS acts as a primary IL-7 receptor. In addition, HSPG can contribute to IL-7 biological activity by presenting IL-7 on stromal cells to promote lymphopoiesis in the bone marrow (Borghesi et al., 1999). In the thymus, however, the ability of stromal cell HSPGs to bind IL-7 and aid thymocytes development is dispensable (Banwell et al., 2000). Depending on the degree of sulfation, cell surface HSPG has also been shown to potentiate the IFN-γ-IFN-γ-receptor interaction (Sadir et al., 1998). Importantly, HSPG also facilitate cytokine localisation in specific niches, forming depots where they can be made available to target cells. For instance, perlecan binds IL-2, sequestering it from the circulation and subsequently depositing the cytokine in the marginal zone and red pulp of the murine spleen to modulate murine T lymphocyte homeostasis (Miller et al., 2008). HSPGs also facilitate the storing of IL-2 within the vascular smooth muscle wall where cytokine availability is regulated through heparanase-mediated ECM degradation (Miller et al., 2012).

The ability to bind and present a chemokine to target cells is insufficient to drive cell migration, the hallmark of chemokine function. HSPGs are not only capable of binding and assisting in inducing conformational changes in bound chemokines (Proudfoot et
al., 2003, Salanga and Handel, 2011), but also contribute to the establishment of immobilised (haptotactic) chemokine gradients in tissues (Laguri et al., 2008). For example, HSPG facilitate the oligomerisation of bound RANTES/CCL5, CXCL8, MCP-1, and MIP-1, thereby allowing better recognition by their cognate G-protein-linked transmembrane receptors (Hoogewerf et al., 1997). In addition to the sequestration of CXCL2 (Massena et al., 2010), HSPG also mediate transcytosis of CXCL8 across the endothelium, presenting both chemokines on the luminal side and establishing haptotactic gradients that aid neutrophil recruitment during inflammation (Wang et al., 2005). In a separate study, the migration of tissue dendritic cells (DC) to regional lymph nodes and the local positioning of DC within lymph nodes was also found to be mediated by a HS-dependent haptotactic gradient of CCL21 (Weber et al., 2013). A similar interaction is believed to facilitate lymphocytes homing through the high endothelial venules into peripheral lymphoid organs (Bao et al., 2010, Tsuboi et al., 2013), although it is unclear if HS also influences local positioning in specific niches. Also, shedding of HSPG, such as syndecan-1 (Hayashida et al., 2009) and removal of glucosamine 6-O-sulfate by the endosulfatase, Sulf2 (Uchimura et al., 2006) has been implicated in regulating the interaction between HS and various chemokines. Furthermore, the tissue-specific inactivation of HS-modifying enzymes can modify neutrophil binding to the endothelium, Ndst1 (HS N-deacetylase/N-sulfotransferase) deletion severely impairing (Wang et al., 2005), while Hs2st (HS 2-O-sulfotransferase) deletion significantly augmenting (Axelsson et al., 2012), neutrophil binding. These studies support the concept of regulating HS function by altering the availability of enzymes that are involved in HS biosynthesis.

1.1.4.3 A sensor of tissue injury

Tissue injury may induce cell necrosis, an event that is often associated with the release of various endogenous damage-associated molecular pattern (DAMP)-containing molecules that are potent inducers of inflammatory responses and initiators of tissue repair mechanisms (Kono and Rock, 2008). Both surface bound HSPG and soluble HS participate in sensing tissue injury and also in repair mechanisms. For example, endothelial cell surface HS mediates the oligomerisation of the receptor for advanced glycation endproducts (RAGE) (Xu et al., 2013) and together form a receptor complex that efficiently recognises the chromatin protein, high-mobility group protein B1 (HMGB1) released from necrotic cells (Xu et al., 2011). As part of the tissue repair
mechanism, HS on the surface of professional phagocytes also assists in the clearance of necrotic cells (Poon et al., 2010). In fact, soluble HS itself can also function as a DAMP (Ihrcke et al., 1993) by interacting with TLR-4 on leukocytes (Johnson et al., 2002). This interaction has been shown to modulate the release of pro-inflammatory cytokines by macrophages (Wrenshall et al., 1999) and markedly induce the maturation of DC, as indicated by the up-regulation of MHC-II, CD40, ICAM-1, CD80, CD86 and reduced antigen uptake, a typical phenotype of a professional antigen presenting cells (Kodaira et al., 2000). Although this is beneficial in triggering immune activation following an insult (Johnson et al., 2002), it is also implicated in the underlying mechanism of disease progression which can occur in experimental pancreatitis (Akbarshahi et al., 2011), sepsis-like syndrome (Johnson et al., 2004), hyperacute rejection in graft-versus-host disease (GvHD) (Brennan et al., 2012) and cardiac injury (Strand et al., 2013).

1.1.4.4 Physical barrier to leukocyte migration

The ECM/BM-associated HS is crucial to form a temporary depot of HS-binding soluble factors and to form a physical barrier that supports tissue integrity. In order to migrate, particularly through blood vessel walls, leukocytes need to break down the ECM/BM barrier and heparanase is primarily involved in this process (Vlodavsky et al., 1992, Parish, 2006). For example, tissue DC increase the availability of cell surface heparanase to aid ECM degradation before migrating into lymphatic vessels leading towards regional lymph nodes where they induce antigen-specific responses (Benhamron et al., 2006). Subsequent studies have suggested that the matrix metalloproteinase, MMP-14, cooperatively works with heparanase to more efficiently degrade ECM/BM barriers (Benhamron et al., 2012). During inflammation, infiltrating monocytes and neutrophils also exhibit similar modes of degrading ECM/BM barriers to aid their extravasation (Matzner et al., 1985, Sasaki et al., 2004). It has also been demonstrated that heparanase derived from infiltrating leukocytes is primarily responsible for the destruction of the pancreatic islet β-cells that produce insulin, thereby providing a novel explanation for the underlying immunopathology of autoimmune Type 1 diabetes (Ziolkowski et al., 2012). In a separate study, leukocytes were shown to also use endogenous myeloperoxidase to produce oxidants that degrade the core protein of perlecan, releasing soluble factors and allowing leukocyte migration across ECM/BM barriers (Rees et al., 2010). Although HS can also be degraded by
nitric oxide (Vilar et al., 1997, Mani et al., 2000) and reactive oxygen species (Rao et al., 2011, Singh et al., 2013, Raats et al., 1997), their direct relevance in the degradation of ECM/BM–associated HSPGs and therefore their contribution to leukocytes extravasation is yet to be elucidated.

1.1.5 Concluding remarks

The evidence presented in the first part of this Literature Review corroborates the fundamental importance of HS in the mammalian immune system. HSPGs, primarily through their HS side chains, regulate various aspects of the immune system ranging from haematopoiesis to homing of leukocytes to peripheral tissues and, most importantly, regulating the elicitation of immune responses. Perturbing HS function or availability has been proven to result in various abnormal immune phenotypes. Furthermore, based on a simple bioinformatics screen presented in Section 1.1.3, it is suggested that HS may in fact interact with many more components of the immune system than previously realised. A better understanding of HS function across various systems is fundamental to exploit its potential in boosting beneficial immune responses and also in finding treatments for related immunopathologies.
1.2 Intrathymic TCRαβ T lymphocyte development

As experiments described in this thesis investigated the role of HS in T lymphocyte development, in this section the major events in intrathymic T lymphopoiesis that through positive and negative selection produce self-tolerant and functional MHC-restricted TCRαβ T lymphocytes, are reviewed.

1.2.1 TCRαβ T lymphocytes

A functional T lymphocyte antigen receptor carried by TCRαβ T lymphocytes is composed of an α-chain and a β-chain, each containing a variable (V) and a constant (C) region, the V regions being generated via somatic gene recombination during T cell development in the thymus. With the exception of the TCRαβ on Natural Killer T (NKT) cells that recognises non-MHC ligands (Rossjohn et al., 2012), the vast majority of TCRαβ T lymphocytes recognise MHC molecules and can be subcategorised into MHC-I restricted CD8+ cytotoxic T lymphocytes (CTL) (Zhang and Bevan, 2011) and MHC-II-restricted CD4+ helper T lymphocytes, the latter often being further classified, based on the production of signature cytokines following activation, into the Th1 (IFN-γ), Th2 (IL-4 and IL-13), Th17 (IL-17a, IL-17 and IL-22), Tfh (IL-4 and IL-21) and Treg (IL-10) subsets (O'Shea and Paul, 2010, Swain et al., 2012). MHC-I is expressed on all nucleated cells and notably at high levels on professional antigen presenting cells (APC), such as DCs. The MHC-I molecule is comprised of heterodimeric polypeptide chains, namely non-polymorphic β2-microglobulin non-covalently linked to a polymorphic α chain, the latter containing α1, α2 and α3 domains. Intracellular derived peptides (8-10 amino acids in length) are usually presented by the α1-α2 pocket of MHC-I and this allows peptide/MHC-I complexes to be screened by different TCRαβ, whereas the α3 loop of MHC-I mediates non-cognate binding to CD8αβ coreceptor molecules (FIG. 1.2A), these two binding events being collectively termed Signal 1. During an adaptive immune response against an infection, pathogen-derived peptides presented by MHC-I on DC provide Signal 1, alongside the ligation of costimulatory molecule, such as CD28 on CD8+ T lymphocytes with CD80/CD86 on DC providing Signal 2. The resultant activation generates CD8+ T lymphocytes (CTL) that can kill target cells expressing cognate peptide/MHC-I complexes via apoptosis. Unlike MHC-I, MHC-II expression is restricted to professional APC, including DCs, macrophages and B lymphocytes. MHC-II is comprised of a heterodimer of polymorphic α and β
FIG. 1.2: Comparison of TCR-peptide/MHC-CD8 and TCR-peptide/MHC-CD4 ternary complexes. (A) Hypothetical model of the TCR-peptide/MHC-CD8 complex oriented as if the TCR and CD8 molecules are attached to the lymphocytes at the bottom and the MHC-I molecules is attached to an opposing APC at the top. The model was constructed by superimposing the CD8αβ-H-2D^d complex (Protein data Bank code 3DMM) onto a TCR-H-2D^d complex (3PQY) through the MHC-I molecule. A portion of the CD8β stalk region was visible in the crystal structure and points toward the T cell membrane. The C termini of CD8α and CD8β chains are labelled. (B) Crystal structure of a TCR-peptide/MHC-CD4 complex in the same orientation as in (A). TCRα, blue; TCRβ, cyan; MHCα, gold; MHCβ/β2m, grey; CD8α, green; CD8β, violet; CD4 (D1-D4 domains), red. Adapted from Figure 3, Li et al., (2013).

polypeptides, the α chain containing α1 and α2 domains and the β chain β1 and β2 domains, with extracellular derived peptides (15-24 amino acids in length) usually being presented in a groove composed of the α1-β1 domain. During a CD4^+ T lymphocyte immune response, TCRαβ interacts with cognate peptide/MHC-IIα1-β1 complexes and CD4 molecules stabilise this interaction by binding to the β2 domain of MHC-II (FIG. 1.2B). As with CD8^+ T lymphocytes, Signal 2 is provided by costimulatory interactions, such as CD28-CD80/CD86 ligation. Effector CD4^+ T lymphocytes can encourage antibody production by B lymphocytes and produce cytokines to orchestrate various inflammatory responses as well as ‘helping’ the induction of CD8^+ CTL responses. By the action of regulatory T lymphocytes (Treg), CD4^+ T lymphocytes can also inhibit T lymphocyte immune responses against both self and foreign antigens. Intracellularly, the TCRαβ lacks a signal transduction domain and, consequently, is associated with a collection of related signalling molecules termed the CD3 complex. Furthermore, a close association of the TCR/CD3 complex with the coreceptor molecules CD8 and CD4 is imperative to initiate TCR signalling (FIG. 1.3) (Huppa and Davis, 2003, Neefjes et al., 2011, Li et al., 2013, Gascoigne, 2008).
1.2.2 The thymus

The thymus is a highly organised epithelial organ with distinct anatomical regions, each providing unique microenvironments to facilitate the development of functional MHC-restricted and self-tolerant TCRαβ T lymphocytes (Miller, 1961, Ciofani and Zuniga-Pflucker, 2007). The thymus originates from the endodermal gut tube. In mice the thymus primordium, indicated by the presence of FoxN1+ thymic epithelial cells (TEC), appears between embryonic days 10.5 (E10.5) to E11.5 from the third pharyngeal pouch. At this stage the surrounding neural crest-cells (NCC) that eventually form the mesenchymal capsule provide signals for the formation, patterning and outgrowth of the thymic anlage (Rodewald, 2008, Gordon and Manley, 2011). Early differentiation of the progenitor FoxN1+ TEC into cortical or medullary TEC occurs from E12 onwards in an autocrine-dependent manner, despite the thymus rudiment still lacking the distinct cortical and medullary regions of an adult thymus (Rossi et al., 2006, Hamazaki et al., 2007). The establishment of the cortical and medullary TEC lineages requires thymocyte-derived signals. This coincides with the colonisation of the embryonic thymus by lymphoid progenitor cells (LPC) at E12 onwards. The migration of LPC across the mesenchymal capsule into the foetal thymus is guided by CCL21 and CCL25 (expressed by the Gcm2+ parathyroid primordium and FoxN1+ TEC), which are the cognate ligands for CCR7 and CCR9 on LPC (Liu et al., 2005, Liu et al., 2006). TEC-thymocyte ‘crosstalk’ promotes TEC lineage commitment (Ritter and Boyd, 1993, van Ewijk et al., 1994, Su et al., 2003), however, thymocyte-derived signals are dispensable.
at the initial stage, as blocking initial LPC colonisation has a negligible effect on TEC development, but is more important in subsequent stages of TEC development (Klug et al., 2002). Thus, the committed cortical-TEC (cTEC: CD205+, β5t+ and keratin K8+) and medullary-TEC (mTEC: lectin UEA-1+, keratin K5+, AIRE+/−, MHC-II+, CD80+, MTS10+, ERTR5+ and claudin cld-3,4+) (Gray et al., 2002, Seach et al., 2012, Anderson and Takahama, 2012) inhabit and define the cortical and medullary regions of the thymus, respectively. Finally, the functional thymus architecture is co-inhabited by fibroblasts, endothelial cells, DCs and macrophages (Gray et al., 2002). At this stage onwards LPC appear to enter the thymus in distinct waves rather than in a continuous manner via the vasculature (Foss et al., 2001). The process of extravasation via the vasculature is mediated by the interaction of endothelial P-selectin with LPC P-selectin glycoprotein 1 (PGSL-1) and endothelial VCAM-I with the LPC α4β2/VLA4 integrin (Rossi et al., 2005, Scimone et al., 2006). Furthermore, the seeding of the thymus at the corticomedullary junction (CMJ) is guided by chemokines produced by thymic stromal cells (Krueger et al., 2010, Zlotoff et al., 2010).

1.2.3 Intrathymic TCRβ T lymphocyte development

T lymphocytes are generated from bone marrow (BM) hematopoietic stem cells (HSC) that migrate to the thymus (Orkin and Zon, 2008) (FIG. 1.4). Multipotent LPC/early thymic progenitors (ETP) have the potential to differentiate into natural killer (NK) cells, DCs, macrophages and B lymphocytes (Bell and Bhandoola, 2008, Wada et al., 2008). In the context of an adult thymus, LPC/early thymic progenitors (ETP) enter at the CMJ (Lind et al., 2001) and migrate outwards to the subcapsular zone (SCZ), then back inwards to the cortex and into the medulla, guided by chemokines and the ECM (Savino et al., 2002, Savino et al., 2004), while intimately interacting with stromal cells which provide distinct cues for sequential developmental stages (FIG. 1.5) (Lind et al., 2001, Anderson and Jenkinson, 2001, Takahama, 2006, Klein et al., 2014).

1.2.3.1 DN stage: ETP settling, TCRβ lineage selection and β-selection

Uncommitted ETP lack CD4 and CD8 molecules, hence, collectively they are termed double negative (DN) thymocytes. DN cells are classified into four major subsets (DN1, DN2, DN3 and DN4), based on CD117 (c-Kit), CD44, CD25, CD24 (heat stable antigen, HSA) and CD27 expression (Godfrey et al., 1993, Koch and Radtke, 2011). The DN2 and DN3 subsets can be further subdivided into two additional developmental
FIG. 1.4: **Overview of thymic T cell development.** The individual stages of T cell maturation from earliest lymphoid progenitor cells (LPC) to the CD4 single positive (SP) and CD8 SP thymocytes. The expression of CD4 and CD8 surface molecules separates the CD4+CD8+ double positive (DP) and SP negative from the CD4CD8 double negative (DN) cells. The surface CD44 and CD24 expression characterises the four major DN cell populations: CD44+CD25- (DN1), CD44+CD25+ (DN2), CD44CD25+ (DN3) and CD44CD25- (DN4) cells. The earliest LPC is derived from a hematopoietic stem cell (HSC) in the bone marrow. HSCs undergo a gradual differentiation process and become more restricted to the lymphoid lineage. LPC arrive at the thymus and develop to the early thymic progenitor (ETP) cell stage, a subpopulation of the heterogeneous DN1 subset that retains the potential to give rise to dendritic cells (DCs), natural killer (NK) cells and macrophages (MΦ). The DN2 and the DN3 cells can be subdivided further into two additional developmental cell stages on the basis of CD117 and CD27 cell surface marker expression, respectively (Table 1.2). Critical checkpoints during T cell maturation are indicated in bracketed numbers and are the (1) DN1 checkpoint, where Notch signalling inhibits alternative cells fate potentials; (2) the β-selection checkpoint, where the transition from DN3a to DN3b marks the progression along T cell receptor αβ lineage; and finally (3) the positive and negative selection checkpoint, where DP thymocytes commit to either MHC-I-restricted CD8 SP or MHC-II-restricted CD4 SP cell fate. Dashed arrows indicate differentiation routes that have been elucidated mainly by *in vitro* differentiation assays. Adapted from Figure 1, Koch and Radtke (2011).
commit to the DN2 stage as a result of DLL4-induced Notch1 signalling which supresses NK and B cell lineage genes (Schmitt et al., 2004, Garcia-Ojeda et al., 2013) and drives RAG-mediated TCRγ, TCRδ and TCRβ gene rearrangements, the former two preceding monoallelic Vβ gene assembly (Capone et al., 1998, Livak et al., 1999, Agata et al., 2007).

Table 1: Stages of T cell development correlate with specific location in the thymus, distinct cell-surface phenotypes and TCR requirement.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Cell surface phenotype</th>
<th>Location</th>
<th>TCRβ rearrangement</th>
<th>TCRα rearrangement</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP / DN1</td>
<td>CD117⁺CD44⁺CD25⁺CD24⁻CD27⁻</td>
<td>CMJ</td>
<td>Germline</td>
<td>Germline</td>
</tr>
<tr>
<td>DN2a</td>
<td>CD117⁺CD44⁺CD25⁺CD24⁺CD27⁻</td>
<td>Cortex</td>
<td>Germline</td>
<td>Germline</td>
</tr>
<tr>
<td>DN2b</td>
<td>CD117⁺CD44⁺CD25⁺CD24⁺CD27⁺</td>
<td>Cortex</td>
<td>DJH</td>
<td>Germline</td>
</tr>
<tr>
<td>DN3a</td>
<td>CD117⁺CD44⁺CD25⁺CD24⁺CD27⁺</td>
<td>SCZ</td>
<td>DJH, VDJH</td>
<td>Germline</td>
</tr>
<tr>
<td>DN3b</td>
<td>CD117⁺CD44⁺CD25⁺CD24⁺CD27⁺</td>
<td>SCZ</td>
<td>VDJ⁺</td>
<td>Germline</td>
</tr>
<tr>
<td>DN4</td>
<td>CD117⁺CD44⁺CD25⁺CD24⁺CD27⁺</td>
<td>SCZ</td>
<td>VDJ⁺</td>
<td>Germline</td>
</tr>
<tr>
<td>DP</td>
<td>CD4⁺CD8⁺TCRβ⁺preTCRα⁺</td>
<td>Cortex</td>
<td>VDJ⁺</td>
<td>VJ</td>
</tr>
</tbody>
</table>

Abbreviation: CMJ, corticomedullary junction; D, diverse; DN, double negative; DP double positive; ETP, early thymic progenitor; H, heavy chain; J, joining; SCZ, subcapsular zone; TCR, T cell receptor; V, variable.

Adapted from Table 1, Koch and Radtke (2011).

Studies have indicated that strong IL-7 and TCRγδ signals favour the TCRγδ lineage, whereas Notch1 and weak pre-TCRαβ signals favour the TCRαβ lineage choice (Laky et al., 2003, Garbe et al., 2006, Kreslavsky et al., 2008). Thus, a strong TCRγδ signal or a weak pre-TCRαβ signal results, respectively, in prolonged or transient ERK activation, thus skewing the commitment towards either the TCRγδ or TCRαβ T lineage (Lee et al., 2014). This selection process occurs as DN2 thymocytes progress into the T-lineage committed DN3 stage upon arriving at the SCZ and this migration is guided by thymocyte CCR9 interacting with CCL25 displayed by cTEC (Benz et al., 2004). DN3 thymocytes express the pre-TCR complex, which is comprised of a monoallelic TCRβ chain, an invariant pre-TCRα chain and the CD3 complex (Saint-Ruf et al., 1994). Studies have shown that the pre-TCR signal (cell autonomous after dimerisation of the TCRβ and pre-TCRα chains (FIG. 1.6B) (Kortum et al., 2013)), the cTEC-derived Notch1 signal and the CXCR4 signal are indispensible, in combination with the IL-7 signal (Boudil et al., 2015), for the survival, proliferation and differentiation (β-
Literature Review

selection) (Ciofani and Zuniga-Pflucker, 2005, Golec et al., 2013, Kreslavsky et al., 2012, Maillard et al., 2006, Trampont et al., 2010, Mandal et al., 2008) of DN3 thymocytes into DN4 stage thymocytes and, subsequently, into the CD4⁺CD8⁺ double positive (DP) stage, these developmental stages coinciding with Rag gene reactivation to facilitate V(D)J recombination at the TCRα locus (Petrie et al., 1993, Shinkai et al., 1993, Wilson et al., 1994, Yannoutsos et al., 2001).

1.2.3.2 DP stage: repertoire selection and CD4/CD8-lineage choice in the cortex

In addition to its contributions to early thymopoiesis, the thymic cortex is renowned for its role in the deletion of ‘useless’ TCRαβ clones, repertoire selection and the deletion of potentially autoreactive TCRαβ T lymphocytes (Shah and Zuniga-Pflucker, 2014, Klein et al., 2014). DP (CD3⁺TCRβlo preTCRα⁺) thymocytes undergo continuous TCRα gene rearrangement until the pre-TCRα is MHC-selectable (Petrie et al., 1993). Pre-selection DP thymocytes undergo a slow (3-8 µm/min) ‘random walk’ in the cortex (Witt et al., 2005, Bousso et al., 2002), which is guided by cTEC-derived CXCL12 and CCL25 gradients (Love and Bhandoola, 2011), while scanning self-peptides being presented by MHC molecules on cTEC (Capone et al., 2001, Laufer et al., 1996) and on migratory SIRPα⁺CD8α⁻ DCs (Wu and Shortman, 2005). Unique antigen presentation machineries bestow cTEC with distinct abilities to process low affinity ‘private’ self-peptides from macroautophagy-derived self-antigens for presentation on MHC-I and MHC-II molecules to DP thymocytes (Klein et al., 2014). For example, the expression of the thymoproteosome subunit, β5t (Psmb11), at high levels in cTEC is essential for the positive selection of CD8⁺ T lymphocytes (Murata et al., 2007, Nitta et al., 2010) by increasing the repertoire of TCR ligands for presentation by MHC-I (Sasaki et al., 2015). In fact, a recent study reported that peripheral CD8⁺ T lymphocytes generated in a β5t-deficient environment exhibited aberrant homeostasis and immune responses to infections (Takada et al., 2015). Similarly, the lysosomal proteases cathepsin-L and thymus-specific serine protease (TSSP), which are highly expressed by cTEC, are found to be essential for the positive selection of CD4⁺ T lymphocytes by similarly by increasing the repertoire of TCR ligands for presentation by MHC-II molecules (Nakagawa et al., 1998, Honey et al., 2002, Gommeaux et al., 2009, Klein et al., 2014).
FIG. 1.5: Stromal cell interactions during T cell development. (A) Successive stages of DN T cell development are accompanied by an outward movement of thymocytes towards the subcapsular zone. Subsequent to β-selection at the DN3 stage, DP thymocytes 'randomly walk' through the outer cortex, which possibly facilitates the 'scanning' of cortical thymic epithelial cells (cTECs) for positively selecting ligands. At this stage, DP thymocytes may be engulfed by cTECs and form so-called thymic nurse cells; however, the molecular control and physiological relevance of this process remains to be established. Interactions between DP thymocytes and cortical conventional dendritic cells (cDCs) may lead to negative selection. It remains unknown whether these cortical cDCs exclusively belong to the migratory signal-regulatory protein-α (SIRPα)-expressing subset. Positively selected CD4 or CD8 lineage-committed thymocytes relocate into the medulla by directed migration. Upon reaching the medulla, SP thymocytes again assume a 'random walk' motion pattern. Through this random migration, SP thymocytes may now 'scan' resident and migratory cDCs, plasmacytoid dendritic cells (pDCs), medullary thymic epithelial cells (mTECs) and B cells. It is estimated that SP thymocytes engage in around five contacts with antigen-presenting cells (APCs) per hour, so that during their 4- to 5-day residency in the medulla, thymocytes may serially interact with several hundred APCs. Solid arrows indicate main migratory pathways that are involved in thymocyte selection. Dashed arrows indicate other relevant migratory pathways. (B) Key functional properties of thymic APCs that are discussed in this literature review. AIRE, autoimmune regulator; BCR, B cell receptor; TRA, tissue-restricted antigen. Adapted from Figure 1, Klein et al., (2014).
1.2.3.2.1 TCR signalling pathways involved in repertoire selection

The quality of TCR signalling is determined by the strength of the TCRαβ/coreceptor-self-peptide MHC interaction (FIG. 1.6), which is the key determinant in repertoire and lineage selection (Werlen et al., 2003, Fu et al., 2014). The TCR complex consists of TCR αβ chains and the CD3 complex (composed of CD3γε, CD3δε and CD3ζζ subunits), with the CD3ζ subunit containing an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. The Src family PTK p56Lck (Lck) is constitutively phosphorylated (Nika et al., 2010) and is key to the initiation of TCR signalling. TCR binding to self-peptide/MHC complexes recruits Lck-bound to CD4/CD8 to the proximity of the TCR/CD3 complex, with resultant phosphorylation of the CD3ζ chain ITAM. This provides a docking site for Zap70 recruitment, which is phosphorylated by Lck (Y319) and trans-phosphorylated by adjacent Zap70 (Y493), with pZap70 then phosphorylating the adaptor protein LAT. Subsequently, pLAT recruits two sets of molecular complexes to regulate Ras activation, namely the [PLC-γ1-Gads-ITK-Slp76] complex and the [Grb2-Sos] complex. Slp76, Gads and ITK regulate the phosphorylation of PLC-γ1, pPLC-γ1 then cleaving PIP2 into IP3 and DAG. IP3 initiates the release of ER-stored Ca2+, which can trigger the uptake of extracellular Ca2+. The resultant rise in intracellular Ca2+ activates the phosphatase calcineurin to dephosphorylate the transcription factor pNFAT and NFAT then translocates into the nucleus and dimerises with AP-1 family transcription factors to regulate gene transcription. The increase in intracellular Ca2+ also induces DAG to recruit PKCθ and RasGRP1 to the plasma membrane, PKCθ activating the NF-κB pathway and RasGRP1 activating the downstream Ras-MEK-MAPK/ERK1/2 cascade. The Grb-Sos complex can form a positive feedback loop to activate Ras thus promoting the Ras/ERK1/2 pathway and augmenting AP-1 activity in regulating gene transcription (Gascoigne and Palmer, 2011, Fu et al., 2014, Kortum et al., 2013, Feske et al., 2012).

TCR signalling in pre-selection DP thymocytes is extremely sensitive, small changes in upstream pathways significantly tipping the signalling threshold either for or against cell survival (Davey et al., 1998). At the cell surface, N-glycosylation enhances the retention of CD4 and CD8 molecules at the plasma membrane consequently augmenting the recruitment of CD4/CD8-bound Lck to initiate TCR signalling and activation of the downstream ERK1/2 cascade following an encounter with low affinity TCR ligand thereby promoting positive selection (Zhou et al., 2014). Biphasically, through an
FIG. 1.6: Canonical TCR signal transduction to Ras-MAPK in T cell development. (A) After TCR binding and engagement of the upstream kinases Lck and Fyn activated Zap70 triggers phosphorylation of LAT. Phosphorylated LAT (pLAT) associates with two molecular complexes that regulate Ras activation: [PLC-γ1–GADS–ITK–SLP-76] and [Grb2-Sos] complexes. On pLAT, pPLC-γ1 cleaves PIP2 generating IP3 and DAG, the former stimulates release of Ca2+ from intracellular stores and DAG then recruits PKCθ and RasGRP1 to the membrane, and the combined actions of Ca2+, DAG, and PKCθ activate RasGRP1. The Ras guanine nucleotide exchange factors (GEFs) Sos1 and Sos2 are also recruited to LAT via the adapter Grb2. Sos proteins have basal RasGEF activity, which can be enhanced by the binding of activated Ras (Ras-GTP) to an allosteric binding pocket on Sos. Ras-GTP binding to Sos potentially allows for the engagement of a positive feedback loop, primed by either RasGRP1 or basal Sos activity, to produce high levels of Ras activation. Ras signals to multiple downstream effector pathways including the Raf–MAPK/ERK kinase MEK cascade. (B) Integrated model of Ras activation during thymocyte development. At the DN3 stage, ligand-independent pre-TCR signals are transmitted to Ras and ERK by the combined actions of Sos1, RasGRP1, and RasGRP4 to stimulate proliferation and differentiation to the DP stage. In the thymic cortex, positively selecting signals are transmitted from the TCR to Ras via RasGRP1. Downstream of Ras, ERK activation is required for efficient positive selection. To promote negative selection, higher-potency ligands trigger Ras activation via either RasGRP1 or Sos1. Here, activation of ERK seems to be dispensable, and activation of other MAPKs; JNK, p38, and ERK5 are likely more important. RasGEFs whose individual deletion blocks thymocyte development for a given signal are shown in red, whereas RasGEFs that require combined deletion with a second RasGEF to cause a developmental block are shown in blue. Proteins whose role at a given developmental checkpoint have not been demonstrated by developmental studies are shown in green. Adapted from Figure 1 and Figure 2, Kortum et al., (2013).
unknown mechanism, N-glycosialylation also appears to attenuate persisting TCR-induced Ca\(^{2+}\) signalling when encountering high affinity TCR ligands, thus inhibiting negative selection (Zhou et al., 2014). Intracellularly, it has been shown that low affinity TCR ligands generate weak signals by favouring intermittent Zap70 and LAT activation, slow and low level intracellular Ca\(^{2+}\) rises and, hence, moderate Ras/ERK activation, as the Grb-Sos complexes remain localised within the Golgi apparatus. In contrast, high affinity/avidity TCR ligands induce a strong signal, characterised by rapid activation of Zap70 and LAT, robust yet transient intracellular Ca\(^{2+}\) rises and accentuated Ras/ERK activation, implicitly due to an exaggerated pLAT-bound Grb-Sos complex-mediated positive feedback loop. Downstream, high affinity/avidity TCR ligands induce robust activation of the MAPKs ERK1/2, JNK1/2, p38 and ERK5, the latter three being strongly associated with cell death. Thus, only low affinity TCR ligands promote DP thymocytes survival and maturation into SP thymocytes (FIG. 1.6B) (Daniels et al., 2006, Hernandez et al., 2010, Kortum et al., 2013).

Several signalling molecules are appreciated for their roles in fine-tuning the intracellular translation of analogue-to-digital TCR signalling in DP thymocytes. Thus, Grb2-bound Tesp1 in the LAT signalling platform is implicated in the release of ER-stored Ca\(^{2+}\) required for effective induction of the ERK/AP-1 and Ca\(^{2+}\)/NFAT pathways essential for T cell survival (Wang et al., 2012). Also, the LAT-Grb2-bound Themis-SHP1/2 complex expands the threshold for weak TCR ligands to promote T cell survival by exerting a negative feedback which dampen upstream Lck activity (Fu et al., 2013, Fu et al., 2014, Paster et al., 2015). Downstream, the calcineurin/NFAT-pathway favours T cell survival by enhancing ERK activity in response to very weak, but not strong, TCR signalling (Gallo et al., 2007, Neilson et al., 2004).

As TCR on DP thymocytes encounter compatible self-peptide/MHC complexes, the thymocytes cease their ‘random walk’ and begin to tightly interact with cTEC (Witt et al., 2005, Bousso et al., 2002). In addition to providing TCR signals (FIG. 1.6A) cTEC express unique cell surface molecules that can provide ‘accessory signals’ to aid the selection of DP thymocytes and their development into CD4 SP and CD8 SP thymocytes (Anderson et al., 1994). It is likely that these accessory signals are delivered as DP thymocytes tightly interact with cTEC to form rosette-like structures (Kyewski et al., 1982, Oliveira-dos-Santos et al., 1997), the most extreme example of this interaction.
being thymic nurse cells (TNC) (FIG. 1.5) (Wekerle and Ketelsen, 1980, Wekerle et al., 1980), despite TNC formation not being required for positive selection (Nakagawa et al., 2012). This heterotypic cell-cell interaction, however, is not restricted to cTEC, as other stromal cells, including DCs and macrophages, have been observed to rosette with thymocytes (Shortman et al., 1989, Shortman and Vremec, 1991, Oliveira-dos-Santos et al., 1998). The molecular mechanisms underpinning these interactions are not fully understood, although thymocyte Thy-1/CD90 (He et al., 1991), cTEC CD326/EpCAM (Nelson et al., 1996) and several thymocyte-cTEC cell adhesion receptor-ligand pairs have been implicated, such as the α4β1/VLA4 integrin-VCAM-1 (Salomon et al., 1997), CD2-LFA-3 and LFA-1 integrin-ICAM-1 interactions (Singer, 1990, Paessens et al., 2008). A separate study showed that thymocytes unable to synthesise a TCR (Rag-1−/−, p56lck−/−, TCRβ−/− and to a lesser extent, TCRα−/− mice), CD44 or CD4 resulted in reduced TEC-thymocyte rosette formation (Oliveira-dos-Santos et al., 1998). In addition, as mentioned in Section 1.1.4.1, an in vitro study implicated highly sulfated HS expressed on cTEC in thymocyte adhesion, although the HS-specific receptor on thymocytes and the physiological role of this interaction in thymocyte development is yet to be elucidated (Britz and Hart, 1983, Werneck et al., 1999, Werneck et al., 2000).

### 1.2.3.2.2 Positive and negative selection of DP thymocytes

The Bcl-2 family of anti-apoptotic proteins, including Bcl-2, Bcl-XL and Mcl-1, are critical to maintain mitochondrial outer membrane integrity (Ma et al., 1995, Harris and Thompson, 2000, Dunkle et al., 2010), and the survival of pre-selection DP thymocytes, with pre-selection DP thymocytes having limited time to attain the much needed TCR survival signals as they test the ability of their nascent TCRαβ to recognise self-peptide/MHC complexes. Also, at this time pre-selection DP thymocytes gradually lose their sensitivity for cTEC-derived IL-7 signalling (Ribeiro et al., 2013), as SOCS-1 suppresses IL-7R expression by these cells (Yu et al., 2006), consequently reducing the expression of Bcl-2 (Munitic et al., 2004). In the absence of TCR signals downregulation of Bcl-2, Bcl-XL and Mcl-1 results in oligomerisation of the pro-apoptotic proteins Bax and Bak on the mitochondrial wall, favouring the release of mitochondrial cytochrome c and activation of the intrinsic cell death pathway (Hernandez et al., 2010, Zhang et al., 2005). The majority of the nascent TCRαβ molecules either fail to bind or very weakly bind to self-peptide/MHC complexes such that an inadequate TCR survival signal is generated and they are destined towards
apoptosis-mediated ‘death by neglect’ (Starr et al., 2003b, Szondy et al., 2012, Yates, 2014), a fate that can be enhanced by glucocorticoids produced by cTEC and thymocytes (Wyllie, 1980). Studies have also revealed that TCR ligation by a non-MHC ligand (anti-TCR cross-linking) without a concomitant CD4/CD8 signal (Van Laethem et al., 2007), or CD8 ligation (anti-CD8 cross-linking) in the absence of a TCR signal (Grebe et al., 2004), may also favour T cell death by neglect. Thus, the deletion of CD4 (Rahemtulla et al., 1991) or CD8 (Fung-Leung et al., 1991) molecules, whilst retaining an intact TCR that permitted the survival and further development of post-DN thymocytes the resultant T cells lacked helper or cytotoxic functions, respectively. Collectively, these observations emphasise the importance of simultaneous TCR and CD4/CD8 coreceptor engagement by self-peptide/MHC complexes for the survival and subsequent lineage specification of DP thymocytes (FIG. 1.7) (Werlen et al., 2003, Van Laethem et al., 2012). Also, it should be noted that throughout the thymus, F4/80+ macrophages play a key role in clearing dying T cells and maintaining a non-inflammatory milieu by producing TGF-β, PGE₂, PAF and adenosine (Surh and Sprent, 1994, Fadok et al., 1998, Koroskenyi et al., 2011, Szondy et al., 2012).

The binding of weak to intermediate affinity TCRs and adequate CD4/CD8 coreceptors to self-peptide/MHC complexes generates weak to moderately strong TCR signals (FIG. 1.6B) that favour the survival of DP thymocytes (‘positive selection’) (Daniels et al., 2006). This leads to the upregulation of IL-7R expression which restores the sensitivity of the T cells to cTEC-derived IL-7, thus leading to the upregulation of Bcl-2 expression (Linette et al., 1994, Yu et al., 2006, Munitic et al., 2004). A recent study confirmed that recruitment of CD8/CD4-bound Lck to the proximity of the TCRαβ/CD3 complex initiates TCR signalling that favours survival of DP thymocytes with weak to intermediate TCRαβ-MHC interactions that generate weak to adequate TCR-signals (FIG. 1.7) (Van Laethem et al., 2012, Van Laethem et al., 2013). These TCR signalling events coincide with the repression of Rag to cease TCRa gene rearrangements (Borgulya et al., 1992, Wilson et al., 1994) and an increase in TCRαβ, CD69 and CD5 expression levels with CD69 and CD5 expression being directly proportional to TCR signal strength and TCR affinity for self-peptide/MHC complexes, respectively (Yamashita et al., 1993, Merkenschlager et al., 1997, Davey et al., 1998, Azzam et al., 1998, Hare et al., 1999).
FIG. 1.7: Co-receptor basis of MHC-specific thymic selection. TCR signalling requires the juxtaposition of Lck with TCR (FIG 1.3 and FIG. 1.6A). In immature pre-selection DP thymocytes, all available tyrosine kinase Lck is co-receptor-associated and bound to the tails of both CD4 and CD8 co-receptors. Consequently, TCRs on pre-selection DP thymocytes can only access Lck when they bind to the identical peptide/MHC complexes as CD4 and CD8 co-receptors. TCRs that bind MHC-independent native ligands cannot access co-receptor-associated Lck because the CD4 and CD8 co-receptors only bind to extracellular MHC complexes. Thus, only MHC-specific TCRs transduce thymic selection signals with the result that thymic selection is strictly MHC-specific. However, genetic deletion of both CD4 and CD8 co-receptors makes Lck freely available to all TCRs, so that TCRs that bind to any ligand in the thymus can transduce selection signals and be expressed on mature T cells in the periphery. Consequently, deletion of both CD4 and CD8 co-receptors allows the generation of mature T cells expressing MHC-independent TCRs with antibody-like recognition specificities. According to this perspective, the TCR repertoire is intrinsically diverse with specificity for both native and MHC-dependent ligands. However, MHC restriction is imposed on this diverse TCR repertoire by CD4 and CD8 co-receptors during thymic selection because of their dual specificity for intracellular Lck and extracellular MHC. Adapted from Figure 2, Van Laethem et al., (2012).
Another notable change in DP thymocytes during and after positive selection is the level of cell surface \( N \)- and \( O \)-glycosialylation (Daniels et al., 2002, Bi and Baum, 2009). As mentioned in Section 1.2.3.2.1 the marked increase in \( N \)-glycosialylation from the DN to the DP stage is strongly associated with the enhanced retention of Lck-bound CD4 and CD8 molecules at the plasma membrane to potentiate and fine-tune TCR proximal signalling. Subsequently, SP thymocytes and naïve peripheral T lymphocytes showed lower levels of \( N \)-glycans to maintain peripheral tolerance (Zhou et al., 2014, Rabinovich and Toscano, 2009) that can be reversed by T cell activation (Comelli et al., 2006). Moreover, of relevance to this thesis, CD8\(\alpha\beta\) molecules are heavily \( O \)-sialylated following positive selection. The addition of \( \alpha_2-3 \) sialic acids to the core-1-\( O \)-glycans, associated with the stalk region of the CD8\(\beta\) chain by the activity of ST3Gal-I sialyltransferase is strongly associated with a reduced ability of the CD8 molecules on post-selection DP thymocytes to bind MHC-I molecules (Moody et al., 2001, Daniels et al., 2001, Moody et al., 2003, Merry et al., 2003). Similar to \( N \)-glycosialylation, \( O \)-sialylation can be reversed by activation, consequently restoring the coreceptor function of CD8 molecules (Pappu and Shrikant, 2004, Starr et al., 2003a). Additional to its role in peripheral tolerance, \( O \)-sialylation of CD8 molecules is crucial to maintain normal homeostasis of peripheral CD8\(^+\) T lymphocytes (Priatel et al., 2000).

High affinity TCR clones are potentially autoreactive and are destined to clonal deletion to avoid autoimmunity, a process termed ‘negative selection’ (Palmer, 2003). Studies have implicated strong TCR signals and an extended CD4/CD8 coreceptor-bound Lck recruitment following a high affinity TCR\(\alpha\beta\)-self-peptide/MHC complex interaction to favour negative selection (FIG. 1.6B) (Daniels et al., 2006, Stepanek et al., 2014). This strong interaction eventually leads to activation-induced apoptosis in DP thymocytes with the pro-apoptotic proteins Bim (also known as Bcl2l11) and the nuclear steroid receptor Nur77 playing major roles in the process, independently antagonising the anti-apoptotic activity of Bcl-2 (Bouillet et al., 2002, Calnan et al., 1995). A strong TCR signal results in Bim neutralising the Bcl-2 pro-survival signal in a dose-dependent manner (O’Connor et al., 1998) and Nur77 recruiting a related member protein, Nor-1, translocating to the mitochondria and together interacting with Bcl-2 and altering the conformation of the protein such that it becomes a pro-apoptotic molecule (Thompson and Winoto, 2008). Although negative selection is thought to mainly take place in the thymic medulla, an early study revealed a high proportion of thymocytes actually
undergoing active apoptosis in the cortex in situ (Surh and Sprent, 1994). While this may reflect DP thymocytes dying by neglect, recent observations revealed that approximately 55-75% of these apoptotic cells are TCR-signalled DP thymocytes undergoing negative selection and, in fact, this population is greater than that observed undergoing medullary clonal deletion (Daley et al., 2013, Stritesky et al., 2013). Thus, this finding implicates cortical thymic stromal cells in instigating the screening for and deletion of potentially autoreactive TCR clones. The migratory CD11c⁺SIRPα⁺CD8α⁻ classical DC (FIG. 1.5) (Wu and Shortman, 2005) is believed to mediate this important role due to its efficiency in capturing and presenting blood-borne self-antigens to DP thymocytes in the thymus. In fact, ablating the CCR2/CCL8-dependent migration of these DCs to the thymus substantially abrogated negative selection of T cell clones specific for blood-borne antigens (Baba et al., 2009). Another study revealed that negative selection in the cortex is mainly occurring in the vicinity of these DCs, the targeted ablation of DCs resulting in severely abrogated deletion of strongly self-reactive TCR clones, with cTEC being incapable of substituting for this function (McCaugtry et al., 2008).

Apparently, however, not all DP thymocytes experiencing strong TCR signals face negative selection. An early observation revealed an escape route from negative selection, in which high affinity transgenic TCRs became internalised before undergoing secondary TCRα rearrangement (McGargill et al., 2000), although a recent study insists that negative selection is the most physiological outcome for high affinity transgenic TCR clones (Kreslavsky et al., 2013). Another study revealed that β5t⁺ cTEC facilitate TCR rearrangement in DP thymocytes associated with TNC, but this feature is prominent with non-transgenic/polyclonal TCR thymocyte populations (Nakagawa et al., 2012). Thus it was hypothesised that this phenomenon is relevant to rescuing DP thymocytes from death by neglect or mediating ‘facilitated’ positive selection of TCR clones with an inadequate affinity for self-peptide/MHC complexes rather than allowing DP thymocytes to escape from negative selection (Klein et al., 2014).
1.2.3.2.3 CD4/CD8-lineage choice in post-selected DP thymocytes

Positively selected DP thymocytes that survive negative selection undergo CD4/CD8-lineage choice, a process elegantly explained by the ‘kinetic signalling’ model of CD4-CD8 differentiation (FIG. 1.8) (Singer et al., 2008). Despite CD4/CD8 coreceptor signals dictating the outcome of the positive selection process, the same signals however, are insignificant for CD4/CD8-lineage commitment (Erman et al., 2006). Post-selection TCRαβhiBcl-2hi DP thymocytes (Punt et al., 1996) cease expression of the Cd8 gene, but not the Cd4 gene and enter a CD4+CD8+ transcriptional stage (CD4hiCD8lo surface phenotype). Initially, a persisting TCRαβ/CD4-self-peptide/MHC-II signal biases the thymocytes towards CD4-lineage commitment by preventing the suppression of CD4-lineage genes and upregulating the transcription factors GATA3, TOX and Th-POK; Th-POK being the key suppressor of CD8-lineage genes and the transcription factor, RUNX3 (Luckey et al., 2014). Consequently, CD4+CD8lo DP thymocytes commit and differentiate into CD4 SP thymocytes. Alternatively, TCRαβ recognition of self-peptide/MHC-I ceases downstream TCR signalling, which leads to the downregulation of GATA-3 and Th-POK expression. Signals from IL-7, IL-15 (McCaughtry et al., 2012) and possibly other common γc cytokines dominate to trigger ‘coreceptor reversal’, leading to the downstream effect of terminating CD4-lineage gene expression and upregulation of RUNX3 expression (Brugnera et al., 2000). This, in turn results in the suppression of Th-POK activity and the reinitiation of CD8-lineage gene expression and, thus, the CD4+CD8lo DP thymocytes commit and differentiate into CD8 SP thymocytes (Brugnera et al., 2000, Yu et al., 2003, Singer et al., 2008, Park et al., 2010).

Positively selected thymocytes also regain enhanced motility (>13 µm/min) that allows them to migrate to the medulla (Witt et al., 2005, Bousso et al., 2002). It has been reported that post-selection thymocytes show a marked increase in PlexD1 expression, which upon its interaction with its medullary-TEC (mTEC)-derived ligand, Sema3e, induces cessation of CCR9-mediated thymocytes migration in the cortex (Choi et al., 2008) and relaxes VLA4-VCAM-1-mediated adhesion of thymocytes to cTEC (Choi et al., 2014). Coincidentally, the upregulation of CCR7 and CCR4 expression by TCRαβhi SP thymocytes drives chemotaxis towards the mTEC-derived CCL19/CCL21 and CCL5/CCL17/CCL22 chemokine gradients, respectively (Ueno et al., 2004, Laan et al.,
Literature Review

2009), and this migration is thought to be aided by a passive inward flow of interstitial fluid (Takahama, 2006).

![Diagram of CD4/CD8 lineage choice](image)

**FIG. 1.8:** The kinetic signalling model for CD4/CD8-lineage choice. Regardless of the specificity of their TCR, positively selecting TCR signals induce DP thymocytes that are transcriptionally $\text{Cd}4^-\text{Cd}8^+$ to terminate $\text{Cd}8$ gene expression and to convert into $\text{Cd}4^-\text{Cd}8^-$ intermediate thymocytes. Because of the absence of $\text{Cd}8$ gene transcription, $\text{Cd}4^-\text{Cd}8^-$ intermediate thymocytes appear phenotypically as $\text{Cd}4^-\text{Cd}8^{\text{lo}}$ cells, and these are the cells in which lineage choice is made. Persistence of TCR signalling in $\text{Cd}4^-\text{Cd}8^-$ intermediate thymocytes blocks IL-7-mediated signalling and induces differentiation into mature CD4 SP thymocytes. Cessation or disruption of TCR signalling in $\text{Cd}4^-\text{Cd}8^-$ allows IL-7-mediated signalling, which induces $\text{Cd}4^-\text{Cd}8^-$ intermediate thymocytes to undergo co-receptor reversal, gain a $\text{Cd}4^-\text{Cd}8^+$ phenotype and differentiate into CD8 SP thymocytes. Adapted from Figure 3, Singer et al., (2008).

**1.2.3.3 SP stage: establishing central tolerance, final maturation and egress**

In addition to the aforementioned mTEC derived signals relocating SP thymocytes to the medulla, signals received by mTEC from positively selected DP and SP thymocytes contribute to the formation and maintenance of a functional thymic medulla (Anderson and Takahama, 2012). Thus, RANKL derived from positively selected DP thymocytes and CD40L and LTβ from SP thymocytes are crucial for the formation of a thymic...
medulla, these molecules binding to their cognate receptors RANK, CD40 and LTβR, respectively, on mTEC, perturbation of these signals being associated with autoimmune phenotypes in mice (Hikosaka et al., 2008, Akiyama et al., 2008, Boehm et al., 2003). The thymic medulla is the key to establishing central tolerance due to the participation of mTEC, DCs and thymic B lymphocytes in the clonal deletion of autoreactive SP thymocytes or the deviation of CD4 SP thymocytes towards MHC-II-restricted CD4⁺CD25⁺FoxP3⁺ natural regulatory (Treg) lymphocytes (Xing and Hogquist, 2012, Hsieh et al., 2012, Klein et al., 2014, Hadeiba and Butcher, 2013).

In establishing central tolerance, AIRE regulates the promiscuous gene expression of tissue-restricted antigens (TRA) by mTEC. The presentation of MHC-restricted TRA in combination with the appropriate cytokine and costimulatory signals can induce the negative selection of high affinity TRA-reactive SP thymocytes (Liston et al., 2003, Anderson et al., 2005). AIRE also controls the transfer of mTEC-derived TRA to thymus resident SIRPα⁺CD8α⁺ DCs (Perry et al., 2014) that can cross-present TRA to CD8 SP thymocytes and induce clonal deletion (Gallegos and Bevan, 2004, Proietto et al., 2008). The transfer of mTEC-derived TRA to SIRPα⁺CD8α⁺ DCs is physiologically feasible due to their close proximity (Klein et al., 2014), this colocalisation being due to mTEC-derived XCL1 (lymphotactin) attracting DCs to the medulla near mTEC via its receptor, XCR1, which is unique to SIRPα⁺CD8α⁺ DCs. The study also showed that perturbing the XCL1-XCR1 interaction leads to the mislocalisation of SIRPα⁺CD8α⁺ DCs, alters Treg cellularity and the deficient mice develop autoimmune phenotypes (Lei et al., 2011). In addition to the ability of the migratory SIRPα⁺CD8α⁺ DCs to mediate negative selection of TCR clones strongly reactive to blood-borne self-antigen (Section 1.2.3.2.2 and FIG. 1.5) (Baba et al., 2009), a separate study showed that SIRPα⁺CD8α⁺ migratory DC are superior to SIRPα⁺CD8α⁺ resident DC in inducing Treg development (Proietto et al., 2008). Moreover, plasmacytoid DC can also acquire blood-borne antigens from the periphery and migrate to the thymus in a CCR9-dependent manner to exert central tolerance via negative selection but lack the ability to support Treg development (Hadeiba et al., 2012). Finally, thymic B lymphocytes also share functional redundancies with mTEC and thymic DCs in inducing central tolerance. For example, a recent study proposed that AIRE peripheral B lymphocytes homing to the thymus initiate a cognate interaction (TRA-specific) with autoreactive CD4 SP thymocytes to gain a CD40 signal, which leads to the upregulation of AIRE, MHC-II.
and CD80, the B lymphocytes thus becoming ‘licensed’ to delete cognate autoreactive CD4 SP thymocytes (Yamano et al., 2015).

Immature SP thymocytes that survive negative selection then undergo final stages of maturation towards becoming long-lived and functionally competent MHC-restricted T lymphocytes in the periphery. Hogquist et al. (2015) separates these highly complex maturation process into distinct phases: semi-mature (SM: CD69⁺MHC-I⁻, susceptible to activation-induce apoptosis), mature-1 (M1: CD69⁻MHC-I⁺, cells able to survive antigen induced-apoptosis and proliferate on antigenic stimulation) and mature-2 (M2: CD69⁻MHC-I⁻, competent to proliferate upon antigenic stimulation and express surface molecules that allow thymus egress) (Hogquist et al., 2015). Eventually, M2 SP thymocytes express Krupple-like factor (KLF)-2 (Carlson et al., 2006) that induces the expression of sphingosin-1-phosphate (S1P) receptor-1, the receptor for S1P. High expression levels of S1P on the endothelium of the thymic periphery drives SP thymocytes to egress from the thymus and undergo further maturation in the periphery (Matloubian et al., 2004).

1.2.4 Concluding remarks

Based on the findings summarised above it is clear that T lymphocyte development and, in particular, positive and negative selection, is a highly complex process that requires diverse extracellular cues, with distinct microenvironments in special anatomical regions of the thymus being critical to guide and drive T cell development in a timely manner. Perturbing the normal symbiotic ‘cross-talk’ between the developing thymocytes and thymic stromal cells results in the impaired development of all thymic components, uncontrolled development of lymphoid malignancies, severe immunodeficiency and autoimmune diseases. Understanding the optimal conditions for T cell development is important to characterise factors predisposing individuals to immunodeficiencies and autoimmunity, and developing therapies that reverses these undesirable processes.
1.3 Experimental aims

Previous studies by my supervisor’s laboratory (the Parish laboratory) demonstrated that HS mimetics inhibit a MHC-restricted interaction (i.e., autorosetting) between DP thymocytes and autologous erythrocytes (Parish et al., 1984). In this PhD Thesis it is postulated that autorosetting is mimicking an important HS-dependent cell adhesion process in the thymus that plays a role in promoting a tight interaction between thymocytes and TECs (Werneck et al., 1999), particularly in the cortex, where DP thymocytes reside. In another study, my supervisor’s laboratory demonstrated that HS mimetics could trigger a sustained intracellular Ca\(^{2+}\) flux in a subset of DP thymocytes (Tellam and Parish, 1987, Weston et al., 1991). Hence, it was predicted that the HS-induced Ca\(^{2+}\) flux and the autorosetting phenomenon are linked, the interaction of DP thymocytes with HS on cTEC providing an ‘accessory signal’ (Anderson et al., 1994) that is important in T lymphocyte development.

Therefore, the main aims of this PhD project were:

a) To identify the receptor(s) on DP thymocytes that bind HS and mediate autorosetting.

b) To determine the functional significance of HS binding by DP thymocytes.

c) To investigate the physiological role of HS recognition by thymocytes on T lymphocyte development.
Chapter Two

Materials and Methods

This chapter describes the materials and methods used for experiments described within this thesis.
2.1 General reagents

2.1.1 Reagents, media and buffers

Details of reagents, including cell-labelling fluorescence dyes, enzymes, culture media, kits, buffers and anticoagulant solutions used in this thesis are as listed in Table 2.1.

2.1.2 Sulfated polysaccharides

Sulfated polysaccharides used in this thesis are listed in Table 2.2. Modified heparins were synthesised and donated by Dr. Craig Freeman (JCSMR, ANU, Canberra).

2.1.3 Antibodies

Table 2.3 details all the antibodies used in the experimental procedures.

2.2 Purification of human plasma histidine-rich glycoprotein (hHRG)

Human Histidine-Rich Glycoprotein (hHRG) was purified from human plasma, as previously described (Rylatt et al., 1981). Whatman P-11 phosphocellulose (50 g) (Whatman, Kent, UK) was rehydrated in a mixture of 0.2 M HCl and ethanol (1:1 vol/vol) with occasional stirring for 60 min. The phosphocellulose was sequentially washed with 1 L of distilled-distilled water (ddH₂O), 1 L of NaOH (0.1 M) and 1 L of ddH₂O before being resuspended in 1 L of 1 mM EDTA for 30 min with occasional stirring. The phosphocellulose was then washed in 1 L of ddH₂O and resuspended in 0.5 M NaCl loading buffer (10 NaH₂PO₄, 1 mM EDTA, pH6.8). The subsequent steps were performed at 4°C to prevent protein degradation. The phosphocellulose was loaded onto a Econo-Column® (2.5 x 20 cm) (Bio-Rad, Richmond, CA) and sequentially washed with fresh 90 mL of 0.5 M NaCl loading buffer containing 50 mg/mL BSA, then three times with 90 mL of 0.5 M NaCl loading buffer (without BSA), 90 mL of 2 M NaCl loading buffer, before final equilibration with 0.5 M NaCl loading buffer. Perfabloc (200 µg/mL) (Roche Diagnostics, Indianapolis, IN), 4 µg/mL of aprotinin (Boehrhringer, Mannheim, Germany), 1 mM EDTA and 0.5 M NaCl was added to 0.5 L of human plasma (Red Cross, The Canberra Hospital, Canberra, Australia) and the plasma centrifuged (20,000 g, 20 min, 4°C) in a Sorval RC-5B Centrifuge (Dupont, Wilmington, DE). The plasma supernatant was collected and mixed with an equal volume of 0.5 M NaCl loading buffer and then loaded onto the equilibrated phosphocellulose P-11 column. Unbound proteins were sequentially washed from the
column with 1 L of 0.5 M NaCl loading buffer and then 0.3 L of 0.7 M NaCl loading buffer. Finally, column-bound HRG was eluted with 2 M NaCl loading buffer and then concentrated in 0.5 M NaCl/10 mM NaH$_2$PO$_4$ storage buffer using a Diaflo Concentrator 202 with YM-30 Diaflo Ultrafiltration Membrane (Amicon, Baverly, MA). The purified human HRG was aliquoted and stored at -80°C until use.

2.3 **Bradford protein assay**

The Bradford Protein assay was used to estimate protein concentration. BSA standards and protein samples were diluted in PBS to a final volume of 100 µL and loaded onto a flat-bottomed 96-well microplate (NUNC™, Thermo Fisher Scientific, Waltham, MA). A 100 µL aliquot of concentrated Bradford Reagent dye (Bio-Rad, Richmond, CA) was added to each BSA standard and protein sample and the plate incubated at RT for 5 min. The absorbance at 595 nm was measured on Thermomax microplate reader using SoftMaxPro 4.0 software (Molecular Devices, Sunnyvale, CA).

2.4 **Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

Protein samples were mixed with an equal volume of reducing SDS (2x) sample buffer (125 mM Tris-HCl, 20 % Glycerol, 4% SDS, 0.01% bromophenol blue, 10% dithiothreitol, pH 6.8) and boiled for 5 min. Samples were electrophoresed (80 volts, 90 min) on a 4-20 % gradient pre-cast polyacrylamide MiniGel (NuSep, Homebush NSW, Australia) using Mini-Protean II apparatus (Bio-Rad, Richmond, CA) in SDS-HEPES running buffer (NuSep, Homebush NSW, Australia). To estimate the molecular weight of protein samples, 5 µL of pre-stained protein standards (Bio-Rad, Richmond, CA) were simultaneously run.

2.5 **Coomassie blue protein staining**

Following SDS-PAGE the polyacrylamide gel was stained with Coomassie Blue staining solution (0.4% Coomassie Blue, 50% methanol, 10% acetic acid) for 30 min at RT. The gel was sequentially destained with destaining solution-I (50% methanol, 10% acetic acid) for 30-60 min and then destaining solution-II (7% ethanol, 5% acetic acid) until the protein bands were discretely observable.
Table 2.1: Reagents used in experimental procedures.

<table>
<thead>
<tr>
<th>Name</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General reagents</strong></td>
<td></td>
</tr>
<tr>
<td>2-mercaptoethanol (2-ME)</td>
<td>Gibco BRL, Grand Island, NY</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA) ≥98%</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Complete EDTA-free protease inhibitor</td>
<td>Roche Diagnostics, Indianapolis, IN</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Cambridge Isotope laboratories, Tewksbury, MA</td>
</tr>
<tr>
<td>Foetal calf serum (FCS)</td>
<td>Serana/Fisher Biotec, Australia. Heat-inactivated (56°C/30 min).</td>
</tr>
<tr>
<td>HEPES</td>
<td>Gibco BRL, Grand Island, NY</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td>Recombinant mouse Histidine-Rich Glycoprotein (rec. mHRG)</td>
<td>R&amp;D Systems, Minneapolis, MN</td>
</tr>
<tr>
<td>Streptavidin-FITC</td>
<td>BD Bioscience, San Jose, CA</td>
</tr>
<tr>
<td>Streptavidin Microbeads</td>
<td>Miltenyi Biotec, Bergisch Gladbach, Germany</td>
</tr>
<tr>
<td><strong>Cell labelling dyes</strong></td>
<td></td>
</tr>
<tr>
<td>CFDA-SE</td>
<td>Ex/Eµm; 495/519 nm. Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td>Cell Proliferation Dye eFluor® 670 (CPD670)</td>
<td>Ex/Eµm; 647/670 nm. eBioscience, San Diego, CA</td>
</tr>
<tr>
<td>CellTrace Violet (CTV)</td>
<td>Ex/Eµm; 405/450 nm. Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td>Hoechst-33342</td>
<td>Ex/Eµm; 350/461 nm. Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td>Hoechst-33258</td>
<td>Ex/Eµm; 350/461 nm. Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td><strong>Digestion enzymes</strong></td>
<td></td>
</tr>
<tr>
<td>Collagenase Type IV (filter-sterilised)</td>
<td>Worthington, Lakewood, NJ</td>
</tr>
<tr>
<td>Collagenase/Dispase</td>
<td>Roche Diagnostics, Indianapolis, IN</td>
</tr>
<tr>
<td>DNAse I</td>
<td>Roche Diagnostics, Indianapolis, IN</td>
</tr>
<tr>
<td>Heparanase (human platelet)</td>
<td>Purified by Dr. Craig Freeman (Freeman and Parish, 1997)</td>
</tr>
<tr>
<td>Heparinase I (Flavobacterium heparinum)</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Neuraminidase Type II (Vibrio cholerae)</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td><strong>Kits</strong></td>
<td></td>
</tr>
<tr>
<td>Qproteome Murine Albumin Depletion Kit</td>
<td>QIAGEN, Limburg, Netherlands</td>
</tr>
<tr>
<td>ReadiLink 647/674 Antibody Labelling Kit</td>
<td>Bio-Rad, Richmond, CA</td>
</tr>
<tr>
<td><strong>Lectins</strong></td>
<td></td>
</tr>
<tr>
<td>Maackia amurensis lectin II (MAL-II), biotinylated</td>
<td>Vector Laboratories, Burlingame, CA</td>
</tr>
<tr>
<td>Peanut agglutinin (PNA), biotinylated</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Ulex europaeus agglutinin I (UEA-I), Fluorescein and Rhodamine Red-conjugated</td>
<td>Vector Laboratories, Burlingame, CA</td>
</tr>
<tr>
<td><strong>Media, buffers and anticoagulant solution</strong></td>
<td></td>
</tr>
<tr>
<td>Alsever’s Solution</td>
<td>D-glucose 20.5 g/L, Na₂C₄H₇O₂·2H₂O 8g/L, NaCl 4 g/L, Citric acid 0.55 g/L, pH 6 ±1 in ddH₂O. Filter-sterilised (0.22 µm)</td>
</tr>
<tr>
<td>Dulbecco’s Phosphate Buffered Saline (PBS)</td>
<td>Ca²⁺/Mg²⁺-free. Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>FACS buffer</td>
<td>PBS. 0.1 % BSA (weight/vol) or 0.5 % FCS (vol/vol), pH 7.0-7.4</td>
</tr>
<tr>
<td>HEPES buffered saline (HBS)</td>
<td>NaCl 8 g/L, KCl 0.4 g/L, CaCl₂ 0.2 g/L, MgCl₂.6H₂O 0.2 g/L, D-glucose 1.8 g/L, HEPES 10 mM in ddH₂O</td>
</tr>
<tr>
<td>Hank’s Balanced Salt Solution (HBSS)</td>
<td>NaCl 8 g/L, KCl 0.4 g/L, MgSO₄.7H₂O 0.1 g/L, MgCl₂.6H₂O 0.1 g/L, CaCl₂ 0.112g/L, D-glucose 19g/L, phenol red 0.02 g/L, NaH₂PO₄·2H₂O 0.078 g/L, KH₂PO₄ 0.06 g/L in ddH₂O</td>
</tr>
<tr>
<td>MACS buffer</td>
<td>PBS. 2 mM EDTA, 0.5% FCS (vol/vol), pH 7.7</td>
</tr>
<tr>
<td>Minimal Essential Medium (MEM-F15)</td>
<td>F15 MEM powder (Gibco BRL, Grand Island, NY) 9.61 g/L and NaHCO₃ 2.2 g/L in ddH₂O</td>
</tr>
<tr>
<td>Normal saline</td>
<td>NaCl 9 g/L in double-distilled water</td>
</tr>
<tr>
<td>BD Pharm Lyse™ Erythrocyte Lysing Buffer (10X)</td>
<td>BD Bioscience, San Jose, CA Diluted to 1x in ddH₂O</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Life Technologies, Carlsbad, CA</td>
</tr>
<tr>
<td>Complete RPMI-1640 medium (Supplemented with)</td>
<td>10% FCS (vol/vol), 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol</td>
</tr>
</tbody>
</table>
### Table 2.2: Sulfated polysaccharides used in experimental procedures.

<table>
<thead>
<tr>
<th>Name</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran sulfate 5 kDa</td>
<td>∞</td>
</tr>
<tr>
<td>Dextran sulfate 500 kDa</td>
<td>∞ Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Heparin (Bovine Lung)</td>
<td>∞</td>
</tr>
<tr>
<td>Heparin, fluoresceinated</td>
<td>∞ Provided by Dr. Craig Freeman</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfated polysaccharides were solubilised</td>
<td>in ddH2O to 20 mg/mL.</td>
</tr>
<tr>
<td>Fluoresceinated heparin powder derived</td>
<td>from porcine mucosa heparin (Calbiochem, La Jolla, CA) was solubilised to 20 mg/mL in ddH2O, then aliquoted and stored at -20°C.</td>
</tr>
<tr>
<td>All modified heparins were synthesised</td>
<td>from porcine mucosa heparin (Calbiochem, La Jolla, CA) and provided at 20 mg/mL in ddH2O. Stored at -20°C.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Modified heparins (Synthesised and donated by Dr. Craig Freeman) §</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil <em>(Unmodified precursor)</em></td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>De-COO⁻</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>De-2S</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>De-NS/re-NAc</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>De-2S, de-NS/re-NAc</td>
<td><img src="image5" alt="Structure" /></td>
</tr>
<tr>
<td>De-6S</td>
<td><img src="image6" alt="Structure" /></td>
</tr>
<tr>
<td>De-NS</td>
<td><img src="image7" alt="Structure" /></td>
</tr>
<tr>
<td>De-S, re-NS</td>
<td><img src="image8" alt="Structure" /></td>
</tr>
<tr>
<td>De-S</td>
<td><img src="image9" alt="Structure" /></td>
</tr>
</tbody>
</table>

*∞* Sulfated polysaccharides were solubilised in ddH2O to 20 mg/mL.
*⌘* Fluoresceinated heparin powder derived from porcine mucosa heparin (Calbiochem, La Jolla, CA) was solubilised to 20 mg/mL in ddH2O, then aliquoted and stored at -20°C.
*§* All modified heparins were synthesised from porcine mucosa heparin (Calbiochem, La Jolla, CA) and provided at 20 mg/mL in ddH2O. Stored at -20°C.

**Heparin modification:** de-COO⁻=decarboxylated, de-2S=2,6-O-desulfated, de-2S=2-O-desulfated, de-6S=6-O-desulfated, de-NS=N-desulfated, re-NAc=N-reacetylated
Table 2.3: Details of primary antibodies used in experimental procedures.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Isotype</th>
<th>Clone</th>
<th>Format</th>
<th>Conc. Used (final)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD2</td>
<td>Rat IgG₂b</td>
<td>RM2-5</td>
<td>Biotin, FITC</td>
<td>5 µg/mL =</td>
<td>BD</td>
</tr>
<tr>
<td>CD3ε</td>
<td>Ar. Ham IgG₁</td>
<td>145-2C11</td>
<td>Purified, FITC</td>
<td>5 µg/mL =</td>
<td>BD</td>
</tr>
<tr>
<td>CD4</td>
<td>Rat IgG₂a</td>
<td>RM4-5</td>
<td>Biotin, FITC, PE, AF700</td>
<td>1 µg/mL</td>
<td>BD, Biolegend</td>
</tr>
<tr>
<td>CD8α</td>
<td>Rat IgG₂a</td>
<td>53-6.7</td>
<td>Biotin, FITC, APC, APC-eF780</td>
<td>1 µg/mL</td>
<td>BD, eBioscience</td>
</tr>
<tr>
<td>CD8β.2</td>
<td>Rat IgG₁</td>
<td>53-5.8</td>
<td>Biotin, FITC</td>
<td>1 µg/mL</td>
<td>BD, Biolegend</td>
</tr>
<tr>
<td>CD11a</td>
<td>Rat IgG₂a</td>
<td>M17/4</td>
<td>Purified</td>
<td>5 µg/mL =</td>
<td>BD</td>
</tr>
<tr>
<td>CD11b</td>
<td>Rat IgG₂b</td>
<td>M1/70</td>
<td>Biotin, AF700,</td>
<td>1 µg/mL</td>
<td>BD, Biolegend</td>
</tr>
<tr>
<td>CD11c</td>
<td>Ar. Ham IgG₁</td>
<td>N418</td>
<td>Biotin, APC-Cy7</td>
<td>5 µg/mL =</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD16/CD32</td>
<td>Rat IgG₂b</td>
<td>2.4G2</td>
<td>Purified</td>
<td>5 µg/mL =</td>
<td>BD</td>
</tr>
<tr>
<td>CD44</td>
<td>Rat IgG₂b</td>
<td>IM7</td>
<td>Purified</td>
<td>5 µg/mL =</td>
<td>BD</td>
</tr>
<tr>
<td>CD45</td>
<td>Rat IgG₂b</td>
<td>30-F11</td>
<td>Purified</td>
<td>5 µg/mL</td>
<td>BD</td>
</tr>
<tr>
<td>CD45.1</td>
<td>Mouse IgG₂a</td>
<td>A20</td>
<td>FITC, PE-CF594</td>
<td>0.4 µg/mL</td>
<td>BD</td>
</tr>
<tr>
<td>CD45.2</td>
<td>Mouse IgG₂a</td>
<td>104</td>
<td>PerCP-Cy5.5</td>
<td>0.4 µg/mL</td>
<td>BD</td>
</tr>
<tr>
<td>CD45R/B220</td>
<td>Rat IgG₂a</td>
<td>RA3-6B2</td>
<td>Biotin</td>
<td>5 µg/mL</td>
<td>BD</td>
</tr>
<tr>
<td>CD62L</td>
<td>Rat IgG₂a</td>
<td>MEL-14</td>
<td>Biotin</td>
<td>5 µg/mL =</td>
<td>BD</td>
</tr>
<tr>
<td>CD69</td>
<td>Ar. Ham IgG₁</td>
<td>H1.2F3</td>
<td>PE, BV605</td>
<td>1 µg/mL</td>
<td>BD, Biolegend</td>
</tr>
<tr>
<td>CD90.2</td>
<td>Rat IgG₂a</td>
<td>53-2.1</td>
<td>Biotin, PE</td>
<td>5 µg/mL =</td>
<td>BD, Biolegend</td>
</tr>
<tr>
<td>CD326/EpCAM</td>
<td>Rat IgG₂a</td>
<td>G8.8</td>
<td>Biotin, PE-Cy7</td>
<td>2 µg/mL</td>
<td>BD, Biolegend</td>
</tr>
<tr>
<td>H2-K²</td>
<td>Mouse IgG₂a</td>
<td>AF6-88.5</td>
<td>PE, FITC</td>
<td>1 µg/mL</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>Mouse IgM</td>
<td>F58-10E4</td>
<td>Purified, FITC, ReadiLink 647/674⁴</td>
<td>Varied</td>
<td>Seikagaku Corp., US Biological, AMSBIO</td>
</tr>
</tbody>
</table>

* Purified anti-HS mAb (50 µL aliquot, 1 mg/mL) conjugated to ReadiLink 647/674 (Bio-Rad) using custom Ab conjugation kit according to manufacturer’s instructions.

Application

Fluorescence flow cytometry

Surface marker blocking

MACS-enrichment

Fc-Block (blocking of non-specific antibody binding)

2.6 Western blotting

Following SDS-PAGE (Section 2.4) protein samples were electrophoretically (100 volts, 60 min) blotted onto a Hybond ECL nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) using a Mini-Protean II apparatus and a Mini Tran-Blot Electrophoretic Transfer Cell Kit (Bio-Rad, Richmond, CA) in transfer buffer containing 5 mM Tris, 38.5 mM glycine, 20% methanol. The membrane was incubated in PBS/5% skim milk powder at 4°C for 16 hr before the target protein was probed with specific Ab diluted in PBS/0.1% BSA at 4°C for 90 min. The membrane was
extensively washed in PBS/0.5% Tween-20 before being incubated with HRP-conjugated detection Ab, diluted in PBS/0.1% BSA at 4°C for 90 min. After the membrane was extensively washed in PBS/0.5% Tween-20, immunoreactive proteins were detected using an ECL™ Western blotting reagent (Amersham Biosciences, Buckinghamshire, UK) and imaged using a LAS-1000 Chemiluminesence and a Fluorescence Imaging System (Fuji Photo Co. Ltd, Japan).

2.7 Animals

The strain, phenotype and sources of mice used in this thesis are detailed in Table 2.4. Mice were housed in a certified PC2 facility and daily maintained by the staff of Australian Phenomics Facility (APF) or the candidate. All animal procedures were performed under approval from the Animal Experimentation Ethics Committee of the Australian National University, Canberra.

Table 2.4: Mouse strains used in experimental procedures.

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Phenotype / Reference</th>
<th>Source/Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 (B6)</td>
<td>Wild type. CD45.2</td>
<td>APF, ANU</td>
</tr>
<tr>
<td>B6.SJL</td>
<td>Wild type. CD45.1</td>
<td></td>
</tr>
<tr>
<td>CD4&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>CD4 deficient ENU mutant (unpublished)</td>
<td>Dr. Anselm Enders, APF/ANU</td>
</tr>
<tr>
<td>CD8α.KO</td>
<td>CD8α chain deficient (Mak et al., 1992)</td>
<td>Jackson Laboratories, USA</td>
</tr>
<tr>
<td>CD8β.KO</td>
<td>CD8β chain deficient (Crooks and Littman, 1994)</td>
<td>Prof. Alfred Singer, NIH, USA</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;-/-&lt;/sup&gt;×CD8β.KO</td>
<td>CD4 and CD8β double deficient (unpublished)</td>
<td>APF, ANU</td>
</tr>
<tr>
<td>CD45.KO</td>
<td>CD45 deficient (Kishihara et al., 1993)</td>
<td>Dr. Zuopheng Wu, APF/ANU</td>
</tr>
<tr>
<td>H2-K&lt;sup&gt;K&lt;/sup&gt;/D&lt;sup&gt;D&lt;/sup&gt;.KO</td>
<td>MHC-I deficient (Peramau et al., 1999)</td>
<td>Dr. G. Karupiah, APF/ANU</td>
</tr>
<tr>
<td>β2m.KO</td>
<td>MHC-I deficient (Koller et al., 1990)</td>
<td></td>
</tr>
<tr>
<td>β2mxMHC-II.KO</td>
<td>MHC-I/MHC-II double deficient (MHC-II.KO: (Madsen et al., 1999))</td>
<td>Dr. Stephen Daley, APF/ANU</td>
</tr>
<tr>
<td>Slp76&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Slp76 deficient ENU mutant (ENUstrain.T-wimp (Siggs et al., 2015))</td>
<td></td>
</tr>
<tr>
<td>Zap70&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Zap70 deficient ENU mutant (ENUstrain.MrTLess (Siggs et al., 2007))</td>
<td></td>
</tr>
</tbody>
</table>

2.8 Preparation of mouse blood

2.8.1 Collection of mouse blood

A maximum blood volume of 1% of total body weight or ~100-200 µL from a 20-25 g adult mouse (8-10 week old) was collected from the lateral canthus into a microfuge tube using a non-heparinised haematocrit tube (Hirschmann Läborgerate GmbH & CO. KG, Eberstadt, Germany).
2.8.2 Isolation of mouse serum

The blood was incubated at 37°C in a humidified cell incubator with 5% CO₂ in air for 30 min and allowed to clot before being centrifuged at 7000 g for 20 min. The serum was collected, aliquoted and stored at -80°C until use.

2.9 HRG detection by enzyme-link immunosorbent assay (ELISA)

Mouse serum was diluted 1/500 in PBS/1% BSA before being loaded (100 µL/well) into a nickel-coated (Ni-NTA) HisSorb 96-well plate (QIAGEN, Limburg, Netherlands) and plates then incubated at RT for 2 hr. Unbound proteins were removed by flicking out the supernatant from the plates and washing the plates three times with 200 µL per well of PBS/0.5% Tween-20. A goat anti-mouse HRG pAb (R&D Systems, Minneapolis, MN) in PBS/1%BSA was added to each well (0.5 µg/mL, 100 µL/well) and the plates incubated at RT for 2 hr. After extensive washing in PBS/0.5% Tween-20, HRP-conjugated rabbit anti-goat pAb (DAKO, Glostrup, Denmark) in PBS/1% BSA was added to each well (6.5 µg/mL, 100 µL/well) and incubated at RT for 45 min. Immunolabelled HRG was detected by adding ABTS Peroxidase Substrate (100 µL/well) (1 Component system) (KPL, Gaithersburg, MA) for 20-30 min at RT. The absorbance at 405 nm was measured on a Thermomax microplate reader using SoftMaxPro 4.0 software (Molecular Devices, Sunnyvale, CA).

2.10 Depletion of mouse serum albumin

Mouse serum albumin was removed from mouse serum using a Qproteome® Murine Albumin Depletion Kit (QIAGEN, Limburg, Netherlands) according to the manufacturer’s instructions. Briefly, 25 µL of mouse serum was diluted 1:4 in 75 µL of 50 mM NaCl binding buffer (supplemented with 50 mM Na₂HPO₄, 10 mM Imidazole and complete EDTA-free protease inhibitor (Roche Diagnostics, Indianapolis, IN)) and loaded onto a Qproteome® Depletion Spin Column that was pre-equilibrated in the same buffer. The column was incubated at RT for 5 min before briefly being centrifuged (500 g, 10 s). The albumin-depleted eluted serum fraction was collected, aliquoted and stored at -80°C until use.
2.11 **Depletion of mouse serum HRG**

Mouse serum HRG was depleted using Cobalt (Co\(^{2+}\))-affinity chromatography (Patel et al., 2013). All steps were performed at 4°C to prevent protein degradation. A 40 µL aliquot of Co\(^{2+}\)-Sepharose bead (TALON Metal affinity resin, Clontech, Palo Alto, CA) was washed by centrifugation (500 g, 4 min) to remove storage buffer. Beads were then equilibrated with 50 µL of 50 mM NaCl binding buffer (prepared as described in Section 2.10). The beads were centrifuged (500 g, 4 min) and supernatant removed ready for serum application. A 10 µL aliquot of mouse serum was diluted 1:6 by the addition of 50 µL of 50 mM NaCl binding buffer and this was then added to washed beads. In some experiments, the albumin-depleted serum fraction (prepared as described in Section 2.10) was used. The beads were incubated at 4°C for 16 hr then centrifuged (500 g, 4 min) before the supernatant was collected, aliquoted and stored at -80°C until use.

2.12 **Lymphocyte preparation**

2.12.1 **Preparation of single-cell lymphocyte suspension**

Animals were humanely euthanised by CO\(_2\) asphyxiation or cervical dislocation. The lymphoid organs were dissected out aseptically and placed in ice-chilled serum-free HBSS or RPMI-1640, gently pressed through a 70 µm cell strainer (BD, San Jose, CA) and pelleted via centrifugation (300 g, 5 min). Where necessary, erythrocytes were depleted by adding 5 mL of 1x BD Pharm Lyse™ lysing buffer, incubated at RT for 7 min followed by three washes (300 g, 5 min) in 10 mL RPMI-1640/5% FCS. The cells were resuspended in an appropriate assay buffer and kept on ice until use.

2.12.2 **Manual enrichment of peripheral lymphocytes**

Peripheral CD4\(^+\) (pCD4) and CD8\(^+\) (pCD8) T and B220\(^+\) B lymphocytes were enriched from pooled lymph nodes and spleens by negative selection via magnetic-activated cell sorting (MACS) (Quah et al., 2004). Briefly, erythrocytes-depleted leukocytes in 1 mL of MACS buffer were incubated with a biotin-conjugated mAb cocktail (Table 2.5) at RT for 15 min. The cells were washed three times (300 g, 5 min), resuspended in 0.5 mL of MACS buffer containing 14 µL of Streptavidin-Microbeads (Miltenyi Biotec, Germany) and incubated at RT for 15 min. Excess Streptavidin-Microbeads were
removed by pelleting cells by centrifugation (300 g, 5 min) and removal of the supernatant. Cells were then resuspended in 0.5 mL MACS buffer and loaded onto a magnetised LS Column (Miltenyi Biotec, Germany). The eluted cells were collected and pelleted via centrifugation (300 g, 5 min) and the supernatant removed. Cells were then washed three times, each by resuspending the cell pellet in 10 mL of buffer followed by centrifugation (300 g, 5 min, 4°C) and removal of the supernatant. The cells were resuspended in an appropriate buffer and kept on ice until use.

Table 2.5: MAb cocktails used in manual MACS enrichment of peripheral lymphocytes.

<table>
<thead>
<tr>
<th>Target cell</th>
<th>MAb cocktail (5 µg/mL each)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral CD4+ T cells (pCD4)</td>
<td>Anti-CD8α, CD11b, CD11c, B220, TER-119</td>
</tr>
<tr>
<td>Peripheral CD8+ T cells (pCD8)</td>
<td>Anti-CD4, CD11b, CD11c, B220, TER-119</td>
</tr>
<tr>
<td>Peripheral B cells (B220)</td>
<td>Anti-CD90, CD4, CD8α, CD11b, CD11c, TER-119</td>
</tr>
</tbody>
</table>

* All mAbs are biotinylated and are detailed in Table 2.3

2.12.3 Cell counting and viability

A 10 µL aliquot of cell suspension was diluted 1:2 in 10 µL of 0.1% Trypan Blue (BDH Poole Chemicals, London England) and loaded onto a haemocytometer. The cells were viewed at x40 magnification using a standard Olympus B light microscope (Olympus Optical, Tokyo, Japan) to determine cell number and viability. Alternatively, 10 µL of a cell suspension was diluted in 490 µL of PBS and loaded into a sample cup and cell number and cell viability determined using VI-CELL® cell viability analyser (Beckman Coulter, Pasadena, CA).

2.13 Antibody labelling and analysis of leukocytes

2.13.1 Blocking of non-specific antibody binding FcR

Cells were resuspended (10^6-10^7 cells/mL) in ice-chilled FACS buffer and incubated with a rat anti-mouse CD16/CD32 mAb (5 µg/mL) (Mouse BD Fc Block™, clone 2.4G2) on ice for 20 min in a total volume of 1 mL.

2.13.2 Cell surface antibody labelling

Cells were labelled with fluorochrome-conjugated mAbs (Table 2.3) in 1 mL of FACS buffer on ice and in the dark for 30 min. Cells were then pelleted by centrifugation and washed three times (300 g, 5 min). Samples were then resuspended in ice-chilled FACS
Materials & Methods

buffer ready for flow cytometry. Where necessary, Hoechst-33258 (1 µg/mL) was added to distinguish dead cells.

2.13.3 Multiparametric analysis of cells by flow cytometry

Cells were acquired at a constant event rate (<1000 events/sec) on a BD LSRFortessa™ flow cytometer using BD FACSDiva™ software (Franklin Lakes, NJ). A total of 1-5x10⁵ viable (Hoechst-33258⁻) cells were acquired and recorded after excluding cell debris and cells clumps based on forward (FSC-A) and side (SSC-A) light scattering. Post-acquisition analysis was performed using FlowJo software (version 8.8.7) (Tree Star Inc. Ashland, OR).

2.14 Lymphocyte-erythrocyte autorosetting assay

2.14.1 Fluorescent labelling of lymphocytes

Lymphocytes (4x10⁶ cells/mL) in HBSS/0.5% FCS (pre-equilibrated to RT) were incubated with Hoechst-33342 (4 µg/mL) (Life Technologies, Carlsbad, CA) at RT for 30 min and then used for autorosetting assay. In some experiments the cells were then labelled with fluorescent mAbs, as described in Section 2.13.2, and washed (300 g, 5 min and removal of supernatant) before being resuspended in HBSS/0.5% FCS.

2.14.2 Preparation of CFSE-labelled erythrocytes

An adult mouse (8-10 week old) was intravenously (i.v.) injected via its lateral tail vein with 25 µL of 20 mM CFDA-SE in DMSO (Life Technologies, Carlsbad, CA) mixed with 100 µL of sterile PBS using a BD Ultra-Fine™ 0.3 mL insulin syringe (12.7 mm, 31G gauge needle) (Coupland et al., 2010). Blood from mice was collected (as described in Section 2.8.1) 4-16 hr after CFDA-SE injection, into an equal volume of Alsever’s Solution, rested for 20 min at RT away from light before being washed (700 g, 5 min) four times in normal saline. The blood was resuspended (%) vol/vol in ice-chilled HBSS/0.5% FCS and was kept on ice until use.

2.14.3 Standard autorosetting assay

A fluorescence-based autorosetting assay was used, modified from a prior method used by the Parish Laboratory (Parish et al., 1982, Sia and Parish, 1980a). In U-bottomed 96-well microplates (Linbro®, MP Biomedicals, Santa Ana, CA) 50 µL of Hoechst-33342
labelled lymphocytes (4x10^6 cells/mL) and 50 µL of CFSE-labelled erythrocytes (2%, vol/vol) were mixed. To induce autorosetting the mixtures were briefly centrifuged (200 g, 1 min, 4°C) and the pelleted cells then incubated on ice for 45 min. The cell pellets were gently transferred to 1.2 mL cluster tubes using wide-bore pipette tips (Axygen® Corning, Tewksbury, MA) and were immediately analysed on a bench-top analytical flow cytometer. The cells were acquired at medium event rate (~3000 events/sec) while recording a minimum of 10,000 viable (Hoechst-33342lo) cells and autorosetting depicted by Hoechst-33342lo cells acquiring CFSE^+ profiles (％A_{raw}) gated to include ≥2 bound erythrocytes (1 CFSE^+ peak/erythrocyte) per thymocyte. Non-rosetting cell mixtures were also examined as a control, which involved analysis of lymphocyte and erythrocyte samples immediately after mixing the two populations (and therefore before true rosette formation) to measure background ‘coincidence’ (％C) values of cell mixtures (Snow, 2004). %True autorosetting was calculated by %A_{raw} - %C.

2.15 Lymphocyte-erythrocyte autorosetting inhibition experiments

Hoechst-33342 labelled lymphocytes (4x10^6 cells/mL) were incubated with either blocking mAbs (0.005-5 µg/mL) (Table 2.3) or sulfated polysaccharides (0.001-100 µg/mL) (Table 2.2) and CFSE-labelled erythrocytes (2%, vol/vol) were incubated with either purified hHRG or mouse serum at pre-determined concentrations. Cells were incubated with inhibitors on ice for 60 min. Cell autorosetting was then examined without removing the excess inhibitors.

2.16 Analysis of cell surface heparan sulfate on erythrocytes

2.16.1 Heparanase treatment of erythrocytes

CFSE-labelled erythrocyte suspensions (2%, vol/vol) were incubated with platelet-derived human heparanase (HPSE, 4 µg/mL) (provided by Dr. Craig Freeman, ANU) or bacterial heparinase I/heparitinase III from Flavobacterium heparinum (HPNSE, 5 U/mL) (Sigma-Aldrich, St. Louis, MO) in a 37°C/5% CO2 humidified cell incubator for 60 min and subsequently kept on ice until use.
2.16.2 Assessment of erythrocyte cell surface heparan sulfate

A CFSE-labelled erythrocytes suspension (0.06%, vol/vol) was incubated with a ReadiLink 647/674-conjugated anti-heparan sulfate mAb (0.125–4 µg/mL) (clone F58-10E4) on ice for 60 min. Cell surface HS labelling was examined by flow cytometry.

2.17 Assessment of lymphocyte sialylation profiles

2.17.1 Neuraminidase treatment of lymphocytes

Lymphocytes (3x10^7 cells/mL) in HBSS or RPMI-1640 (supplemented with 0.5% FCS) (pre-equilibrated to 37°C) were incubated with 0.1 U/mL of bacterial neuraminidase (Neu) type II from *Vibrio cholerae* (Sigma-Aldrich, St. Louis, MO) in a 37°C/5% CO₂ humidified cell incubator for 60 min (Moody et al., 2001, Daniels et al., 2001). The cells were washed three times (300 g, 5 min) and resuspended in the appropriate assay buffer.

2.17.2 Cell surface lectin labelling

Lymphocytes (1x10^7 cells/mL) in ice-chilled PBS/0.1% BSA were incubated with 0.2 µg/mL of biotin-conjugated peanut agglutinin (PNA) (Sigma-Aldrich, St. Louis, MO) or *Maackia amurensis* lectin II (MAL-II) (Vector Laboratories, Burlingame, CA) (Brennan et al., 2006) on ice for 30 min. The cells were washed twice (400 g, 5 min) in ice-chilled PBS/0.1% BSA and then Streptavidin-FITC (1 µg/mL) was added to the cells and incubated on ice for 20 min. The cells were then washed twice (400 g, 5 min) with ice-chilled PBS/0.1% BSA before resuspension in 50 µL of PBS/0.1% BSA. Lectin binding was analysed by flow cytometry.

2.18 Inhibition of antibody binding by sulfated polysaccharides

Lymphocytes (1x10^7 cells/mL) suspended in ice-chilled PBS/0.1% BSA were incubated with sulfated polysaccharides (0.1-100 µg/mL) for 60 min on ice prior to labelling with fluorescent mAbs (Table 2.3), including anti-CD45, anti-CD4, anti-CD8α or anti-CD8β mAbs (5 µg/mL each), for 30 min on ice. The cells were then washed four times (400 g, 5 min, 4°C) before being resuspended in 50 µL of ice-chilled PBS/0.1% BSA and analysed by flow cytometry. Inhibition of mAb binding by sulfated polysaccharides was
represented by ‘% control mAb binding’ calculated using the following formula (Parish and Warren, 1991).

\[
\frac{\text{GMFI mAb-reacted cells (with sulfated polysaccharide)}}{\text{GMFI mAb-reacted cells (without sulfated polysaccharide)}} \times 100
\]

### 2.19 Heparin binding assay

Lymphocytes (1x10^7 cells/mL) in ice-chilled PBS/0.1% BSA were incubated with 0.1 mg/mL of FITC-heparin (donated by Dr. Craig Freeman, ANU) on ice for 30 min. The cells were washed four times (400 g, 5 min) and resuspended in 50 µL of PBS/0.1% BSA before being analysed by flow cytometry.

### 2.20 Flow cytometric intracellular Ca^{2+} flux assay

The loading of lymphocytes with intracellular Ca^{2+} indicator fluorescent dyes and the flow cytometric analysis of intracellular Ca^{2+} flux were performed as detailed below.

#### 2.20.1 Loading of lymphocytes with Ca^{2+} indicator dye

Lymphocytes (3x10^7 cells/mL) in MEM-F15 or RPMI-1640 medium were incubated with Indo-1 AM (5 µM) (Tellam and Parish, 1987, Weston et al., 1991) at 37°C for 60 min. After three washes (300 g, 5 min and removal of supernatant) with MEM-F15 or RPMI-1640 medium supplemented with 5% FCS the cells were resuspended (4x10^6 cells/mL) in ice-chilled RPMI-1640/0.5% FCS (pH 7.4) supplemented with 10 mM HEPES. The cell suspension was kept on ice and used within 3 hr post-loading.

#### 2.20.2 Measurement of intracellular Ca^{2+} flux by flow cytometry

Intracellular Ca^{2+} flux was monitored on a BD LSR-1™ flow cytometer using BD CellQuest™ Pro software. Ca^{2+} flux assessed as an increase in the ratio of fluorescence (GMFI) of Ca^{2+}-bound over Ca^{2+}-unbound Indo-1 (Table 2.7). The cells were pre-equilibrated and maintained at 37°C during analysis using an external sheath connected to a heated waterbath. After the exclusion of cellular debris and clumped cells on the basis of FSC/SSC light scattering the basal Ca^{2+} level was monitored for 2 min before a stimulus was added and a change in Ca^{2+} level was monitored for 20 min at a constant
flow rate (~500 events/sec). Also, a 2 min (endpoint) intracellular \( \text{Ca}^{2+} \) level was measured after the kinetic assessment.

Table 2.6: Calcium indicator dyes.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Loading conc.</th>
<th>Flow cytometer</th>
<th>Excitation laser</th>
<th>Detection BP filter (nm)</th>
<th>Ca(^{2+}) Flux (Ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indo-1 AM</td>
<td>5 µM</td>
<td>LSR I</td>
<td>325 nm (UV)</td>
<td>424 ± 22</td>
<td>424/510</td>
</tr>
<tr>
<td>Fluo-4 AM</td>
<td>2.5 µM</td>
<td>LSRFortessa</td>
<td>498 nm (Blue)</td>
<td>530 ± 30</td>
<td>530/695</td>
</tr>
<tr>
<td>Fura-Red AM</td>
<td>5 µM</td>
<td>LSRFortessa</td>
<td>498 nm (Blue)</td>
<td>695 ± 40</td>
<td></td>
</tr>
</tbody>
</table>

Calcium indicator dyes (Life Technologies, Carlsbad, CA) were solubilised in DMSO to make 2 mM stock solution and stored at -20°C, protected from light.

2.21 Thymocyte activation assay

Thymocyte activation was assessed based on the upregulation of CD69 after an exposure to a stimulus (Davey et al., 1998, Yamashita et al., 1993). Thymocytes (2x10\(^6\) cells/mL) in RPMI-1640/10% FCS medium (supplemented with 25 mM HEPES, 1 mM L-glutamine/2 µM 2-ME) were loaded (200 µL /well), with or without sulfated polysaccharide, into a flat-bottomed 96-well plate (Linbro®, MP Biomedicals, Santa Ana, CA) and incubated at 37°C for 4 hr. For TCR/CD3 stimulation, 96-well microplates were pre-coated with an anti-CD3ε mAb (clone 145-2C11) diluted in PBS (50 µL/well, 0.4–10 µg/mL) (pH 8.0) at 4°C for 16 hr. Each well was rinsed three times with 200 µL of PBS to remove unbound mAb and thymocytes were then loaded, with or without sulfated polysaccharides and incubated at 37°C for 4 hr. The cells were washed three times (400 g, 5 min, 4°C) and subsequently labelled with a PE or BV605-conjugated anti-CD69 mAb (1 µg/mL) in ice-chilled PBS/0.1%BSA on ice for 20 min. Excess anti-CD69 mAb was removed via centrifugation (400 g, 5 min, 4°C) and removal of the supernatant. The cells were then resuspended in ice-chilled PBS/0.1%BSA containing Hoechst-33258 (1 µg/mL) to discriminate dead cells. CD69-labelling was analysed by flow cytometry.

2.22 Identification of rosetting thymic stromal cells

2.22.1 Enzymatic digestion of thymic fragments

Four thymuses from 3 week old C57BL/6 mice were isolated and placed in serum-free RPMI-1640 (RT) medium. Aseptically, the connective tissue was removed and each
lobe cut into smaller fragments and placed in 5 mL of RPMI-1640 in a BD Falcon™ 50 mL tube. The fragments were agitated using wide-bore transfer pipette and were allowed to sediment (~1 min) before removing the supernatant. The fragments were sequentially enzymatically digested (10 min/cycle), in which the uppermost 5 mL of the supernatant was collected after each 10 min cycle and fresh enzymes suspension applied to the remaining cell suspension. This was repeated until all fragments were fully disintegrated. The process included four cycles in type-IV collagenase solution (1 mg/mL) (Worthington, Lakewood, NJ) and two cycles in collagenase/dispase solution (1 mg/mL) (Roche Diagnostics, Indianapolis, IN), all in the presence of DNAse-I (100 µg/mL) (Roche Diagnostics, Indianapolis, IN) in 5 mL of serum-free RPMI-1640 at 37°C. Finally, the supernatants were pooled and pelleted (100 g, 10 min) before being resuspended in 1 mL of RPMI-1640.

2.22.2 Enrichment of pre-formed thymic rosettes

Pre-formed thymic rosettes were enriched via two-step unit gravity sedimentation procedure above a FCS cushion, as described previously (Kyewski et al., 1982, Oliveira-dos-Santos et al., 1997). Briefly, thymic digests (as prepared in Section 2.22.1) were gently applied to the top of a FCS (12 mL) layer in a 14 mL BD Falcon™ tube. Denser multicellular conjugates were allowed to sediment at unit gravity for 60 min, the pelleted cells collected and centrifuged once (100 g, 10 min), the supernatant removed and the cell pellet resuspended in ice-chilled PBS/0.1% BSA/2 mM EDTA buffer and kept on ice until analysis.

2.22.3 Cell surface labelling of stromal cells

Enriched thymic stromal cells (10^6-10^7 cells/mL) from Section 2.22.2 were incubated with mouse BD Fc Block™ (5 µg/mL) at 4°C for 20 min before being labelled with fluorescently conjugated anti-EpCAM mAb (2 µg/mL), anti-Ly-51 mAb (40 ng/mL) and the lectin UEA-1 (2 µg/mL) (Table 2.3). The cells were washed once (400 g, 10 min and removal of supernatant) before being resuspended (5x10^6 cells/mL) in ice-chilled RPMI-1640/0.5 % FCS, pH 7.0 and were kept on ice until use.

2.22.4 Assessment of stromal cell surface heparan sulfate

Enriched thymic stromal cells (10^6-10^7 cells/mL) from Section 2.22.2 were incubated with a ReadiLink 647/674-conjugated anti-heparan sulfate mAb (clone F58-10E4) (1
µg/mL) on ice for 60 min and washed once by centrifugation (300 g, 5 min and removal of supernatant) before being resuspended in ice-chilled PBS/0.1% FCS. Cell surface HS labelling was examined by flow cytometry.

2.23 Stromal-thymocyte rosetting assay

2.23.1 Manual enrichment of EpCAM⁺ thymic epithelial cells (TEC)

TEC were enriched from thymic digests (as prepared in Section 2.22.1) by two-step positive selection of EpCAM⁺ cells using MACS. Thymic digest in 1 mL of MACS buffer was filtered through a 100 µm cell strainer (BD, San Jose, CA) and incubated with a biotin-conjugated anti-EpCAM mAb (CD326, clone G8.8) (5 µg/mL) at RT for 15 min. After two washes (300 g, 5 min and removal of supernatant) the cells were incubated with Streptavidin-Microbeads (20 µL) in 0.5 mL of MACS buffer at RT for 15 min. Excess Streptavidin-Microbeads were removed by centrifugation (300 g, 5 min) and removal of supernatant and the cells resuspended in 0.5 mL of MACS buffer. Cells were then loaded onto a magnetised LS Column. Unlabelled cells were eluted three times with MACS buffer, the column detached from the magnet and the content flushed into a fresh pre-equilibrated magnetised LS column. Unlabelled cells were then similarly eluted with MACS buffer as above. Finally, the column bound cells were flushed from unmagnetised columns with 5 mL of MACS buffer and washed four times (300 g, 5 min and removal of supernatant) in RPMI-1640/5% FCS. The cells were resuspended in ice-chilled RPMI-1640/0.5% FCS (pH 7.0) and were kept on ice until use. The phenotype of enriched TEC was confirmed by flow cytometry, as described in Section 2.22.3 and 2.22.4.

2.23.2 Fluorescent labelling of thymocytes

Thymocytes (10⁸ cells/mL) in 1 mL of RPMI-1640/5% FCS (pre-equilibrated to RT) were incubated with Cell Proliferation Dye eFluor® 670 (CPD 670, 100 nM) or CellTrace™ Violet (CTV, 500 nM) at RT for 5 min (Quah and Parish, 2012). The cells were washed four times (300 g, 5 min and removal of supernatant) before being resuspended to 5x10⁷ cells/mL in ice-chilled RPMI-1640/0.5% FCS (pH 7.0). Cells were kept on ice until use.
2.23.3 Stromal-thymocyte rosetting assay

A stromal-thymocyte rosetting assay was used based on the standard autorosetting assay described in Section 2.14.3. In a U-bottomed 96-well microplate (Linbro® MP Biomedicals, Santa Ana, CA), equal volumes (50 µL) of CPD\textsuperscript{670} labelled thymocytes (5x10\textsuperscript{7} cells/mL) (Section 2.32.2) and pre-labelled EpCAM\textsuperscript{+} MACS-enriched stromal cells (5x10\textsuperscript{6} cells/mL) (Section 2.32.1) were mixed (10:1 cell ratio) and briefly centrifuged (200 g, 1 min, 4°C) before being incubated on ice for 45 min. The cell aggregates at the bottom of each well were gently transferred to 1.2 mL cluster tubes using wide-bore pipette tips and immediately analysed on a bench-top analytical flow cytometer using similar configurations to those used to examine autorosetting (Section 2.14.3), with at least 20,000 EpCAM\textsuperscript{+} cells being recorded whilst maintaining a constant flow rate (~3000 total events/sec). Rosetting was assessed as EpCAM\textsuperscript{+} stromal cells acquiring a CPD\textsuperscript{670+} profile (%R\textsubscript{raw}), equivalent to ≥3 bound thymocytes (1 CDP\textsuperscript{670+} peak/thymocyte) per stromal cell. In parallel, an equivalent non-rosetting cell mixture was analysed to determine the level of coincidence (%C) as described in Section 2.14.3. %True rosetting was calculated by %R\textsubscript{raw}-%C.

2.24 Analysis of intracellular Ca\textsuperscript{2+} flux in rosetting thymocytes

Thymocytes were incubated with Fluo-4 AM (2.5 µM) and Fura-Red\textsuperscript{TM} AM (5 µM) dyes (Bailey and Macardle, 2006) in RPMI-1640/5%FCS at 37°C for 60 min and then labelled with the dye CTV (500 nM) at RT for 5 min. After three washes (300 g, 5 min and removal of supernatant) in ice-chilled RPMI-1640/10% FCS, the cells were resuspended in ice-chilled RPMI-1640/0.5% FCS/10 mM HEPES buffer. Stromal-thymocyte rosetting was induced as described in Section 2.23.3 in a U-bottomed 96-well microplate and the cells were gently transferred into a 1.2 mL cluster tube and the extent of rosetting and intracellular Ca\textsuperscript{2+} levels in bound thymocytes (gated as described in Section 2.23.3) at 5, 10 and 30 min post-incubation (1 min snapshot per time point) simultaneously monitored using BD LSRFortessa\textsuperscript{™} and BD FACSDiva\textsuperscript{™} software (Franklin Lakes, NJ). Ca\textsuperscript{2+} flux was assessed as an increase in the ratio of fluorescence (GMFI) of Ca\textsuperscript{2+}-bound Fluo-4 over Ca\textsuperscript{2+}-unbound Fura-Red (Table 2.7). To study rosette-induced Ca\textsuperscript{2+} mobilisation at 37°C, the cell suspensions were pre-heated at 37°C for 5 min, rosetting induced (200 g, 1 min) and cells then incubated in a 37°C/5% CO\textsubscript{2}
Materials & Methods

humidified cell incubator. During analysis, the cells were maintained at 37°C using an external sheath connected to a heated waterbath.

2.25 Statistical analysis

Statistical significance was measured using Student’s t-test (unpaired, 2-tailed) or One-way or Two-way ANOVA with GraphPad Prism 5.0 Software (GraphPad Software, San Diego, CA).
Chapter Three

Mechanisms and Regulators of Murine Autorosetting

3.1 Abstract

Autorosetting, the binding of lymphocytes to two or more autologous erythrocytes, is unique to thymocytes and much less evident with peripheral T lymphocytes. Previous studies have implicated MHC-I recognition and interaction with negatively charged polysaccharides on erythrocytes in autorosette formation. In this Chapter, a flow cytometry-based assay for quantifying autorosetting is described, the assay confirming that double positive (CD4⁺CD8⁺) thymocytes are the major autorosetting population in the thymus, whereas peripheral T lymphocytes do not autorosette. Using the new autorosetting assay a wide range of mAbs against different thymocytes cell surface antigens were tested and a CD8β-specific mAbs shown to strongly inhibit autorosetting, a finding confirmed with CD8α and CD8β knockout mice. It was also shown that heparanase treatment of erythrocytes prevented autorosetting, indicating that heparan sulfate (HS) is the autorosetting ligand on erythrocytes and consistent with sulfated polysaccharides, such as heparin and dextran sulfate, inhibiting autorosetting and
blocking the binding of mAbs to CD8β, with inhibition of autorosetting by heparin being \(6-O\)-sulfate dependent. Also it was found that sialylation of CD8β on peripheral CD8\(^+\) T lymphocytes substantially limits autorosetting, with neuraminidase treatment restoring autorosetting by these T lymphocytes. Additional studies confirmed that histidine-rich glycoprotein (HRG) from serum, a HS-binding molecule, inhibits autorosetting, although serum from HRG knockout mice still inhibited autorosetting via a HRG-like molecule. Thus, sialylation of CD8, combined with blocking of the HS ligand by serum-derived HRG and potentially related molecules, strictly regulates the CD8-HS interaction by peripheral CD8\(^+\) T lymphocytes.

3.2 Introduction

In mice, certain subsets of T lymphocytes are able to form rosettes with autologous and allogeneic erythrocytes, \textit{in vitro}, a phenomenon termed autorosetting. It has been previously demonstrated that autorosetting lymphocytes are predominantly immature, hydrocortisone-sensitive thymocytes or 20-50\% of the total thymocytes population. Moreover, peripheral lymphocytes usually display much lower levels of autorosetting (Charreire and Bach, 1975, Braganza et al., 1975, Kolb, 1977). Thus, at face value, autorosetting seems to be a developmentally regulated process.

The molecular basis of autorosetting is poorly understood. Early studies showed that murine autorosetting is more efficient when the lymphocytes and erythrocytes are MHC compatible, implying a level of MHC-I-restriction. However, autorosetting with allogeneic erythrocytes is clearly evident, indicating that the interaction is predominantly MHC-I independent (Primi et al., 1979, Charreire et al., 1980, Sia and Parish, 1980a). Furthermore, certain monosaccharides and complex sulfated polysaccharides can totally inhibit autorosetting by murine thymocytes (Parish et al., 1984), suggesting that besides MHC-I recognition, the binding of negatively charged glycans on the erythrocyte surface plays a major role in autorosetting.

Autorosetting can be regulated extrinsically by serum. Early studies revealed that mouse serum can totally inhibit autorosetting (Charreire and Bach, 1975, Kolb, 1977, Ghanta et al., 1978, Hsu et al., 1980), subsequent investigations showing that this inhibitory activity can be entirely ascribed to histidine-rich glycoprotein in serum (HRG) (Rylatt et
al., 1981). As HRG from several species totally inhibits autorosetting (Sia et al., 1982), it is likely to disrupt MHC-independent aspects of autorosetting.

Despite the information outlined above, little is known about the molecular basis of autorosetting, in particular the nature of the autorosetting receptor on T lymphocytes. This Chapter describes experiments that attempted to resolve this issue.

3.3 Results

3.3.1 Establishment of a flow cytometry-based autorosetting assay

Previously, chromogenic dyes were used to distinguish lymphocytes from erythrocytes and rosettes were manually scored using a light microscope (Lay et al., 1971, Baxley et al., 1973, Sandilands et al., 1974, Charriere and Bach, 1975, Sia and Parish, 1980a). Here, a multicolour flow cytometric autorosetting assay was developed in which erythrocytes were labelled in vivo with CFDA-SE (Coupland et al., 2010) (FIG. 3.1A), the DNA-binding fluorescent dye, Hoechst-33342, was used to label viable lymphocytes in vitro (FIG. 3.1B), the two cell populations mixed and autorosetting induced by centrifugation as previously described (Sia and Parish, 1980a) (FIG. 3.1C). Whilst the conventional flow cytometer configuration involves a narrow core stream, which facilitates analysis at the single-cell level (FIG. 3.2A), here the core stream was broadened to allow the detection of large rosettes at high speed (~3000 events/sec). Unfortunately, this increased the level of ‘coincidence’, namely lymphocytes and erythrocytes being simultaneously excited by the laser (Snow, 2004, Fent et al., 2008) and thus, being falsely identified as rosettes (FIG. 3.2B and C). To overcome this problem, lymphocytes and erythrocytes were mixed immediately before flow cytometry to obtain %coincidence values, true autorosetting values being determined by subtracting %coincidence from %uncorrected autorosetting values. Increasing concentrations of erythrocytes (0.03-2%, vol/vol) were allowed to autorosette with a constant concentration of thymocytes (2x10^6 cells/mL), true autorosetting being maximal with 1% erythrocytes (FIG. 3.3) and thus, being the default lymphocyte-to-erythrocyte ratio applied throughout this study.
FIG. 3.1: **Multicolour flow cytometric lymphocyte-erythrocyte autorosetting assay.** (A) Preparation of CFSE-labelled erythrocytes. On day 0, one C57BL/6 mouse (8 week old) was injected (i.v.) with 5 mM CFDA-SE (Ex<sub>λ</sub>/Em<sub>λ</sub>: 495/519 nm). The blood was retro-orbitally collected 16 hr later on day 1 into Alsever’s solution (pH 6.0) and the erythrocytes washed and resuspended in HBSS/0.5% FCS (4°C). (B) Preparation of fluorescently labelled lymphocytes in vitro. Lymphocytes from a syngeneic C57BL/6 mouse were labelled with 4 µg/mL of Hoechst-33342 (Ex<sub>λ</sub>/Em<sub>λ</sub>: 350/461 nm) in HBSS/0.5% FCS at RT, allowing live/dead discrimination of nucleated cells (see lower flow cytometry plot). (C) Induction of autorosetting. Equal volumes (50 µL) of lymphocyte (4x10<sup>6</sup> cells/mL) and erythrocyte suspensions (0.06-4%, vol/vol) were mixed in U-bottom 96-well plates, then briefly centrifuged (200 g, 1 min) before being incubated on ice for 60 min. Cell pellets were gently resuspended, transferred into cluster tubes and analysed on an analytical bench-top flow cytometer.

### 3.3.2 Organ distribution of autorosetting lymphocytes

It was previously reported that immature, cortisone-sensitive, thymocytes are the main autorosetting cells in the thymus, whereas peripheral lymphocytes autorosette at much lower levels (Charreire and Bach, 1975, Kolb, 1977, Sia and Parish, 1980a, Sia and Parish, 1980b). Using the flow cytometry based autorosetting assay, it was confirmed that thymocytes (54.6% autorosetting) were the main autorosetting population, whereas autorosetting by spleen (6.1% autorosetting) and various lymph node (LNs) populations, namely pooled axillary and brachial, inguinal and mesenteric LN derived lymphocytes, was low to negligible (1.8%, 0.2 % and 0.6% autorosetting, respectively)
(FIG. 3.4A). Furthermore, double positive (DP) and CD8 single positive (SP) thymocytes were the main autorosetting subsets (65.4% and 22.1% autorosetting, respectively), whereas double negative (DN), CD4 SP thymocytes, peripheral CD4\(^+\) and CD8\(^+\) T lymphocytes, and peripheral B lymphocytes (B220\(^-\)) autorosetted at low to negligible levels (2.5%, 0.4%, 4.8%, 4.9% and 1.2% autorosetting, respectively) (FIG. 3.4B).

**FIG. 3.2:** **Configuration of flow cytometer for detection of rosettes.** (A) Conventional setup for single-cell analysis. A flow cell with a narrow core stream allows separation and detection of individual cells. (B) Flow cell configuration for detection of multicellular conjugates in a lymphocytes rosetting cell mixture. (i) A broad core stream allows the entry of multicellular complexes. (ii) Representative histogram of autorosetting of viable Hoechst-33342-labelled thymocytes. Gate set to detect \(\geq 2\) erythrocytes/thymocyte (\%A\(_{\text{raw}}\)). (C) Measurement of background noise (‘coincidence’) in a non-rosetting cell mixture, which involves mixing lymphocytes and erythrocytes immediately before flow cytometry analysis. (i) A broad core stream allows simultaneous detection of signals from erythrocytes and lymphocytes, averaging 1 unbound erythrocyte per lymphocyte. (ii) Representative histogram of coincidence in a non-rosetting cell mixture, with the gate set to detect \(\geq 2\) erythrocytes/thymocyte (\%C). True autorosetting values represent \%A\(_{\text{raw}}\) - \%C (i.e., 68%-5.2%) = 62.8% in example shown.
60

Chapter 3

FIG. 3.3: Flow cytometric analysis of autorosetting between thymocytes and autologous erythrocytes. Equal volumes (50 µL) of erythrocyte (0.06-4%, (vol/vol)) and Hoechst-33342 labelled thymocyte (4x10^6 cells/mL) suspensions were mixed. Each set of cell mixtures was either immediately analysed by flow cytometry to provide a coincidence control (grey histogram) or induced to autorosette (red histogram) by brief centrifugation (200 g, 1 min) and incubation on ice for 60 min, prior to evaluation (i.e., viable Hoechst-33342^+ thymocytes acquiring CFSE^+ profiles) by flow cytometry. (A) Representative fluorescence histograms depicting autorosetting and coincidence plots for different erythrocyte concentrations. Gate includes thymocytes associating with ≥2 erythrocytes. (B) Percentage (%) of uncorrected autorosetting (A_{raw}), coincidence control values (C) and true autorosetting of thymocytes (A_{raw} - C) with increasing concentrations of erythrocytes (% vol/vol). Data are mean % autorosetting ± SD of triplicate samples.

3.3.3 Identification of CD8 as the autorosetting receptor on lymphocytes

Initially, a wide range of mAbs specific for lymphocytes cell surface antigens were examined for their ability to inhibit autorosetting. It was found that the presence of mAbs to CD11a, Ly-49G, NKG2D, CD44, CD62L, CD90.2, CD2, CD3ε, CD4 and CD45 individually did not inhibit autorosetting. Interestingly, only an anti-TCRβ mAb appeared to partially inhibit autorosetting compared to untreated control (50.2% vs. 64.3% autorosetting, P < 0.001) (FIG. 3.5A). Moreover, autorossetting was comparable between CD4.KO, CD45.KO and WT thymocytes (FIG. 3.5C), the data suggesting that besides TCRβ, these molecules do not independently contribute to autorosetting.
FIG. 3.4: Lymphoid organ and lymphocyte subset distribution of autorosetting lymphocytes. (A) Autorosetting by lymphocytes from various lymphoid organs. (Lymph node (LN): Axil./Brac. = axillary/brachial (pooled); Ing. = inguinal; Mes. = mesenteric). (B) Comparison of the ability of various lymphocyte subsets to autorosette. (Subset: DN = double negative (CD4-CD8-); DP = double positive (CD4+CD8+); SP = single positive (CD4+ or CD8+) thymocytes; pCD4 = peripheral CD4+ cells; pCD8 = peripheral CD8+ cells; B220 = predominantly peripheral B cells). Data are mean % autorosetting ± SD of triplicate samples.

Based on the lymphoid organ and T lymphocyte subset distribution of autorosetting cells (Section 3.3.2) and the potential involvement of MHC-I in autorosetting, it was hypothesised that the CD8 molecule that is known to bind MHC-I (Norment et al., 1988), could be involved in autorosetting. This hypothesis was tested by examining the ability of anti-CD8 mAbs to inhibit autorosetting. Indeed, an anti-CD8β mAb significantly inhibited autorosetting, compared to an isotype control (10.5% vs. 65.4% autorosetting, \( P < 0.001 \)), and to a lesser extent, an anti-CD8α mAb was also significantly inhibitory, compared to an isotype control (46.8% vs. 62% autorosetting, \( P < 0.001 \)). Moreover, combining the anti-CD8α and anti-CD8β mAbs was significantly more inhibitory than the anti-CD8β mAbs alone and diminished autorosetting to background levels, i.e., 2.6% autorosetting (FIG. 3.6A). Moreover, both anti-CD8 mAbs inhibited autorosetting in a concentration-dependent manner (FIG. 3.6B). To unequivocally demonstrate that CD8 plays a major role in autorosetting, autorosetting by CD8.KO thymocytes was examined. It was found that both CD8α.KO and CD8β.KO thymocytes showed a dramatically and significantly reduced ability to autorosette, compared to the WT control (3% and 7.8% vs. 64.3% autorosetting, \( P < 0.001 \)), with the CD8α.KO thymocytes showing significantly lower autorosetting than the CD8β.KO.
thymocytes ($P < 0.05$) (FIG. 3.6C). There was also significantly reduced autorosetting by DP and CD8 SP thymocytes from CD8β.KO mice, compared to DP and CD8 SP thymocytes from WT mice (6.6% and 3% vs. 65.4% and 22.1% autorosetting, $P < 0.0001$), highlighting the significant role of CD8β in autorosetting (FIG. 3.6D).

**FIG. 3.5: Contribution of leukocyte surface molecules to autorosetting.** (A) Analysis of autorosetting thymocytes in the absence (i.e., untreated control) or presence of 5 µg/mL of anti-CD11a, anti-Ly-49G, anti-NKG2D, anti-CD44, anti-CD62L, anti-CD69, anti-CD90.2, anti-CD2, anti-CD3ε or anti-TCRβ mAbs. Data are mean ± SD of triplicate samples. ***, $P < 0.001$. (1-way ANOVA with Dunnett’s multiple comparisons test to untreated control). (B) Thymocytes autorosetting in the presence of 5 µg/mL of anti-CD4 or anti-CD45 mAb, compared to isotype controls. In (A) and (B), thymocytes were pre-incubated with mAb/isotype controls on ice for 30 min prior to the autorosetting assays which were performed in the presence of the mAbs. (C) Autorosetting by WT, CD45.KO and CD4.KO thymocytes. Data are mean % autorosetting ± SD of triplicate samples. NS, not significant (Student’s $t$-test).
FIG. 3.6: **Contribution of CD8 molecules to thymocytes autorosetting.** Autorosette inhibition by anti-CD8α and anti-CD8β.2 mAb, individually and combined at (A) 5 µg/mL, or (B) titrated at 0.005-5 µg/mL, compared to isotype controls. (C) Autorosetting by WT, CD8α.KO and CD8β.KO thymocytes. (D) Autorosetting by WT and CD8β.KO, DP and CD8 SP thymocytes. (E) Autorosetting by CD8β.KO thymocytes in the presence of 5 µg/mL of an anti-CD8α mAb or an isotype control. In (A), (B), and (E) thymocytes were pre-incubated with mAb/isotype controls on ice for 30 min prior to the autorosetting assays which were performed in the presence of the mAbs. An additional control was untreated cells (control). Data are mean % autorosetting ± SD of triplicate samples. NS, not significant. *, P < 0.05. ***, P < 0.001. ****, P < 0.0001 (1-Way ANOVA with Tukey’s multiple comparisons test).

Furthermore, addition of an anti-CD8α mAb to CD8β.KO thymocytes further reduced autorosetting (2.6% vs. 7.7% of isotype control, P < 0.05), corroborating the supportive
role of CD8α in autorosetting (FIG. 3.6E). Collectively, the data suggest that the CD8αβ heterodimer is the key autorosetting receptor, with CD8β being the major participant in autorosetting but being aided by CD8α. In this context, it should be noted that the cell surface expression of CD8β is dependent on CD8α (Blanc et al., 1988, Gorman et al., 1988, Norment and Littman, 1988), which explains why autorosetting by CD8α.KO thymocytes is so dramatically reduced.

3.3.4 Identification of the autorosetting ligand on erythrocytes

Based on earlier data suggesting that H-2L/H-2D molecules on erythrocytes contribute to autorosetting (Sia and Parish, 1980a) it was postulated that MHC-I on the erythrocyte surface is a potential autorosetting ligand. To test this hypothesis, the ability of MHC-I deficient, H-2Kβ/Dβ.KO and β2-microglobulin.KO erythrocytes to autorosette was evaluated. Surprisingly, both H-2Kβ/Dβ.KO and β2m.KO erythrocytes were comparable to WT erythrocytes in their ability to autorosette (FIG. 3.7). Hence, despite MHC-I being implicated in autorosetting, it is most unlikely to be the primary autorosetting ligand on erythrocytes.

FIG. 3.7: MHC-I molecules are dispensable for autorosetting. Comparison of the ability of WT, β2m.KO or H-2Kβ/Dβ.KO erythrocytes to autorosette with syngeneic thymocytes. Data are mean % autorosetting ± SD of triplicate samples. NS, not significant. (1-Way ANOVA with Dunnett’s multiple comparisons test to WT).
**FIG. 3.8:** Heparan sulfate on the surface of erythrocytes acts as an autorosetting ligand. (A) Concentration-dependent autorosetting inhibition by the sulfated polysaccharides heparin and DxS (5 & 500 kDa) (IC$_{50}$ = concentration of sulfated polysaccharide required to inhibit autorosetting by 50%). (B) Autorosetting by bacterial heparinase-I (HPNSE) and mammalian heparanase (HPSE)-treated erythrocytes. Erythrocytes were treated with 5 U/ml of HPNSE (*F. heparinum*) or 4 µg/mL of platelet-derived HPSE at 37°C for 60 min. Data in (A) and (B) are mean % autorosetting ± SD of triplicate samples. (C) Availability of surface HS on HPNSE and HPSE-treated erythrocytes, as measured by anti-HS (clone 10E4) mAb binding at 2 µg/mL. Data are GMFI ± SD of triplicate samples. ***, $P < 0.001$ (1-way ANOVA with Dunnett’s multiple comparisons test to mock treated control). (D) Mapping of structural requirements of heparin to inhibit autorosetting. Thymocytes, untreated control or pre-treated with bovine lung heparin, porcine intestinal mucosa heparin or modified porcine intestinal mucosa heparins (10 µg/mL) were examined for autorosetting (Modification: de-COO$^-$ = decarboxylated; de-S = desulfated (complete); de-2S = de-2-O-sulfated; de-6S = de-6-O-sulfated; de-NS = de-N-sulfated; re-NAc = re-N-acetylated; re-NS = re-N-sulfated). In (A) and (D) thymocytes were pre-incubated with inhibitors on ice for 30 min prior to the autorosetting assays which were performed in the presence of the inhibitors. NS, not significant. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$ (1-way ANOVA with Dunnett’s multiple comparisons test to untreated control in (i), or porcine intestinal heparin in (ii)). Data are mean % autorosetting ± SD of triplicate samples.
In a separate study, autorosetting receptors were previously shown to have high affinity for sulfated polysaccharides, particularly heparin and dextran sulfate (DxS), with their presence totally inhibited autorosetting (Parish et al., 1984). These findings suggested that the autorosetting ligand is most likely to be a negatively charged polysaccharide. Indeed, it was consistently shown using the flow cytometry based autorosetting assay that heparin and DxS (5 and 500 kDa) inhibited autorosetting, with DxS\textsuperscript{500kDa} (IC\textsubscript{50} = 1.5 µg/mL) being the most inhibitory, followed by heparin (IC\textsubscript{50} = 1.8 µg/mL) and DxS\textsuperscript{5kDa} (IC\textsubscript{50} = 38.9 µg/mL) being the least inhibitory (FIG. 3.8A). Heparin and Dxs are physiologically not membrane-bound molecules and thus it was postulated that erythrocyte surface heparan sulfate (HS) (Trybala et al., 1993, Vogt et al., 2004), which closely resembles heparin in structure, is most likely to be the autorosetting ligand. To test this hypothesis, erythrocytes were treated with bacterial heparinase-I (HPNSE) or human platelet heparanase (HPSE) and the effect of the treatments on autorosetting was evaluated. Remarkably, HPNSE and HPSE treated erythrocytes showed a greatly impaired ability to autorosette, compared to mock treated erythrocytes (5.5% and 6.2% vs. 51.7% autorosetting, \(P < 0.001\)) (FIG. 3.8B). The residual autorosetting by HPNSE and HPSE treated erythrocytes is most likely due to the remaining HS chains, as indicated by the anti-HS mAb (clone 10E4) exhibiting substantial binding to treated erythrocytes (FIG. 3.8C). It should be emphasised, however, that the 10E4 HS-binding mAb interacts with a HS epitope not easily removed by HPNSE or HPSE treatment and probably binds to HS stubs remaining after digestion (Parish C.R. pers. comm.). Thus, overall these data highlight HS as the key autorosetting ligand on erythrocytes.

Since HS is the autorosetting ligand and heparin is a highly sulfated version of HS, the structural features of HS involved in autorosetting were determined by using chemically modified heparins as inhibitors. Initially, both bovine lung and porcine intestinal mucosa heparin were found to totally inhibit autorosetting at 10 µg/mL, although chemically modified porcine intestinal mucosa heparin was used in all subsequent experiments. Completely desulfated (de-S) heparin was not inhibitory, compared to untreated thymocytes (62.7% vs. 67.8% autorosetting, \(P > 0.05\)), indicating that sulfation is essential for autorosetting receptor recognition, neither the carboxyl groups nor the sugar backbone of heparin/HS directly interacting with the autorosetting receptor. Decarboxylation (de-COO\textsuperscript{−}) and de-2-O-sulfation (de-2S) of heparin appeared to significantly and partially reduce the autorosetting inhibitory activity of heparin.
(12.4% and 19.7% autorosetting, $P < 0.05$ and $P < 0.01$, respectively). $N$-linked desulfation (de-NS) of heparin, which replaced the negatively charged $N$-linked sulfate groups with positively charged amines appeared to be non-inhibitory but re-acetylation of the positively charged $N$-linked amines (de-NS/re-NAc) considerably restored autorosetting inhibition (69% to 23.5% autorosetting, $P < 0.001$). Moreover, autorosetting inhibition by de-2-O-sulfated, de-NS/re-NAc (de-2S, de-NS/re-NAc) heparin was comparable to the precursor compound de-NS/re-NAc heparin (24% and 23.5% autorosetting, respectively), suggesting that the 2-O-sulfate group needs to act cooperatively with $N$-linked sulfates in order to interact with the autorosetting receptor. Similarly, re-$N$-sulfated de-S (de-S/re-NS) heparin did not inhibit autorosetting, suggesting that $N$-linked sulfate groups alone are insufficient to bind to the receptor. Remarkably, removal of 6-O-sulfate groups (de-6S) from heparin completely destroyed the ability of heparin to inhibit autorosetting (FIG. 3.8D). These data suggest that autorosetting is influenced by the net negative charge of the HS ligand, with the 6-O-sulfate group being an essential epitope for autorosetting to occur.

### 3.3.5 Direct evidence that CD8 interacts with HS-like molecules

Based on the data presented in Section 3.3.3 and Section 3.3.4, it was concluded that CD8β and, to a lesser extent, CD8α interact with HS. The CD8-HS interaction was further investigated, either indirectly, by measuring the ability of sulfated polysaccharides that resemble HS (i.e., heparin and DxS) to competitively inhibit anti-CD8 mAbs binding or based on FITC-heparin binding to thymocytes. For comparison, the effect of the sulfated polysaccharides on the binding of an anti-CD45 mAb was examined as CD45 was previously reported to bind heparin/HS (Parish et al., 1988a, Coombe et al., 1994). Likewise, an anti-CD4 mAb was assessed as CD4 was previously shown to carry a polyanion binding site that may interact with sulfated polysaccharides (Parish and Warren, 1991).

The fluorescence histograms in FIG. 3.9A illustrate the shift in fluorescence intensity of the mAbs binding to thymocytes in the presence of the different sulfated polysaccharides at 0.1-100 µg/mL. The inhibition of mAb binding by the sulfated polysaccharides is presented as %control mAb binding in FIG. 3.9B. Remarkably, anti-CD8β mAb binding was almost totally inhibited by DxS$^{500}$ kDa at 1-100 µg/mL, to a lesser extent by heparin and the least by DxS$^{5}$ kDa. Furthermore, a less dramatic but
similar pattern of sulfated polysaccharide inhibition was observed with anti-CD8α mAb binding, whereas anti-CD4 mAb binding was only inhibited by Dxs\(^{500 \ \text{kDa}}\) and anti-CD45 mAb binding was only marginally affected by the presence of the different sulfated polysaccharides.

**FIG. 3.9:** Analysis of anti-CD45, anti-CD4, anti-CD8α and anti-CD8β mAb binding to thymocytes in the presence of the sulfated polysaccharides heparin and dextran sulfate (5 & 500 kDa). (A) Representative fluorescence histograms and (B) graphical presentations of the extent of anti-CD45, anti-CD4, anti-CD8α or anti-CD8β mAb binding to thymocytes in the absence or presence of 0.1-100 µg/mL of the sulfate polysaccharides heparin and Dxs (5 & 500 kDa), as examined by flow cytometry. Thymocytes were incubated with the respective inhibitors (0.1-100 µg/mL) on ice for 30 min, then without removing the inhibitors, fluorescently-tagged mAbs (5 µg/mL) were added and the cells were incubated on ice for another 30 min before being washed and analysed by flow cytometry. Data are mean % control mAb binding ± SD of triplicate samples.
FIG. 3.10: Binding of heparin to thymocytes requires CD8 molecules. Binding of FITC-heparin to (A) thymic or peripheral T cell subsets, with subset labelling as in FIG. 3.4B, (B) CD8β.KO DP and CD8 SP thymocytes, compared to WT thymocytes and (C) CD8α.KO, CD8β.KO and CD4.KO, compared to WT thymocytes. FITC-heparin binding (1 µg/mL) was performed on ice for 60 min before the cells were washed and analysed by flow cytometry. FITC-heparin binding is expressed as mean fold increase in fluorescence relative to a background autofluorescence control ± SD of triplicate samples. NS, not significant. **, P < 0.01. ***, P < 0.001. ****, P < 0.0001, based on Student’s t-test in (B) and 1-Way ANOVA with Tukey’s multiple comparisons test in (C).

Surprisingly, it was found that different lymphocytes populations varied markedly in their ability to bind FITC-heparin. Thus, FITC-heparin binding was highest on DP followed by CD8 SP thymocytes (13.6 and 7.6-fold background, respectively), with binding being much less on DN and CD4 SP thymocytes and peripheral CD4+ and CD8+ T lymphocytes (1.7, 3.6, 1.9 and 3.3-fold background respectively) (FIG. 3.10A). Importantly, consistent with the autorosetting data (FIG. 3.6D), DP and CD8 SP thymocytes from CD8β.KO mice displayed substantially reduced FITC-heparin binding compared to WT DP and CD8 SP thymocytes (12.1 vs. 2.4-fold background, P < 0.0001 and 5.0 vs. 1.8-fold background, P < 0.01, respectively) (FIG. 3.10B), this reduction in FITC-heparin binding being comparable in CD8α.KO and CD8β.KO thymocytes (2.4 vs. 3.4-fold background, P > 0.05) (FIG. 3.10C). Additionally, consistent with autorosetting (FIG. 3.5C), there was comparable binding of FITC-heparin to CD4.KO and WT thymocytes (11.4 vs. 11.5-fold background, P > 0.05) (FIG. 3.10C), indicating that CD4, despite binding polyanions, does not contribute to FITC-heparin binding by thymocytes. Collectively, these data are consistent with CD8
being the dominant heparin and, presumably, HS binding molecule on the surface of DP and CD8 SP thymocytes.

### 3.3.6 Sialylation influences the interaction of CD8 with HS-like molecules

Previous studies have reported a developmentally regulated α2-3 sialylation of the stalk-like region of the CD8β molecule expressed by CD8 SP thymocytes and peripheral CD8⁺ T lymphocytes (Moody et al., 2001, Merry et al., 2003). This modification changes the conformation of the CD8 molecule and consequently impairs the ability of the molecule to bind to MHC-I and act as a coreceptor for TCR signalling (Daniels et al., 2001, Moody et al., 2003). Therefore, it is possible that a similar modification of CD8 is responsible for the weak autorosetting (FIG. 3.4) and FITC-heparin binding (FIG. 3.10A) of peripheral CD8⁺ T lymphocytes. To test this hypothesis, cells were treated with neuraminidase (type II from *V. cholerae*) to enzymatically remove surface α2-3 sialic acid residues and the impact of desialylation on FITC-heparin binding and autorosetting studied. The glycosylation status of the treated lymphocytes was further examined based on the binding of the α2-3 sialic acid specific *Maackia amurensis* lectin II (MAL-II) (Wang and Cummings, 1988) and the Galβ1-3GalNAc-Ser/Thr core 1 O-glycan specific peanut agglutinin (PNA) (Pereira et al., 1976). Thus, the MAL-II and PNA lectins can be used to monitor α2-3 sialic acid removal by neuraminidase, MAL-II detecting sialic acid loss and PNA measuring the exposure of subterminal galactose residues following sialic acid cleavage.

Initially, it was noted that with untreated cells, the level of MAL-II binding was highest on peripheral CD4⁺ and CD8⁺ T lymphocytes, binding being progressively lower on CD8 SP, CD4 SP, DP and DN thymocytes. Conversely, PNA binding was highest on DP thymocytes, with the other lymphocyte subsets exhibiting little or no binding of PNA. Neuraminidase treatment resulted in a dramatically reduced MAL-II binding to all lymphocyte subsets except DN thymocytes and, at the same time, PNA binding to all lymphocytes subsets was greatly elevated, altogether these data being reflective of successful enzymatic desialylation (FIG. 3.11A and B). At the thymic level, neuraminidase treated DP, CD8 SP and CD4 SP thymocytes showed significantly enhanced FITC-heparin binding and autorosetting. Remarkably, neuraminidase-treated peripheral CD8⁺ T lymphocytes displayed the highest improvements in FITC-heparin binding (2.6 to 16.7-fold background, *P* < 0.001) and autorosetting (3.7% to 41%
autorosetting, \( P < 0.001 \)). In contrast, neuraminidase treated peripheral CD4\(^+\) T lymphocytes displayed no significant improvement in autorosetting (3.7 % to 5.6% autorosetting, \( P > 0.05 \)) despite significantly increased FITC-heparin binding (2.6 to 4.9-fold background, \( P < 0.01 \)) (FIG. 3.11C and D). These data are consistent with CD8 sialylation reducing the heparin/HS binding and autorosetting potential of the molecule.

To more directly demonstrate that CD8\(\beta\) sialylation influences heparin/HS recognition or autorosetting advantage was taken of lymphocytes from CD8\(\beta\) deficient mice. Interestingly, MAL-II and PNA binding by CD8\(\beta\).KO DP and CD8 SP thymocytes was significantly lower than by WT cells (\( P < 0.05 \)), whilst comparable lectin binding by both WT and CD8\(\beta\).KO peripheral CD8\(^+\) T lymphocytes was observed. In contrast, following neuraminidase treatment, a significant difference in MAL-II and PNA binding to CD8\(\beta\).KO and WT lymphocytes was only evident with peripheral CD8\(^+\) T lymphocytes (\( P < 0.01 \)) (FIG. 3.12A and B). These data suggest that CD8\(\beta\) can either directly or indirectly influence the sialylation status of developing T cells. Importantly, the enhanced FITC-heparin binding and autorosetting observed following neuraminidase treatment of WT cells was dramatically reduced with CD8\(\beta\).KO DP and CD8 SP thymocytes and peripheral CD8\(^+\) T lymphocytes (FIG. 3.12C and D). Furthermore, the enhanced autorosetting of neuraminidase treated DP and CD8 SP thymocytes and peripheral CD8\(^+\) T lymphocytes was shown to be dependent on recognition of erythrocyte HS as HPNSE and HPSE treatment of erythrocytes dramatically reduced the ability of these cells to autorosette (FIG. 3.13). Collectively, these data demonstrate the importance of developmentally controlled sialylation of CD8\(\beta\) in regulating the CD8-HS interaction.
FIG. 3.11: **Analysis of the influence of the sialylation status of lymphocytes on their heparin binding ability and autorosetting capacity.** Measurement by flow cytometry of lymphocyte desialylation following *V. cholerae* neuraminidase treatment based on the binding of FITC-labelled (A) *Maackia amurensis* lectin II (FITC-MAL-II) (α2-3 sialic acid specific) and (B) peanut agglutinin (FITC-PNA) (Galβ1-3GalNAc-Ser/Thr core 1 O-glycan specific). (C) The effect of desialylation on FITC-heparin binding as detected by flow cytometry. FITC-MAL-II, FITC-PNA and FITC-heparin binding were performed at 1 µg/mL on ice for 60 min, before the cells were washed and examined by flow cytometry. Data in (A-C) are expressed as mean fold increase in fluorescence relative to a background autofluorescence control ± SD of triplicate samples. (D) Comparison of the extent of autorosetting by untreated and neuraminidase-treated T cells. Lymphocytes subset labelling as defined in FIG. 3.4B. Data are mean % autorosetting ± SD of triplicate samples. NS, not significant. **, *P* < 0.01. ***, *P* < 0.001. ****, *P* < 0.0001 (Student’s *t*-test).
FIG. 3.12: Analysis of the contribution of CD8β to the sialylation status of CD8+ T cells and impact of CD8β-deficiency on heparin binding and autorosetting following desialylation. Comparison of the binding of (A) FITC-MAL-II, (B) FITC-PNA and (C) FITC-heparin to untreated and *V. cholerae* neuraminidase-treated WT, CD8β.KO, DP, CD8 SP thymocytes and peripheral CD8+ T cells, and (D) the effect of such treatment on autorosetting. Lymphocyte subsets defined as in FIG. 3.4B and the lectins MAL-II and PNA defined as in FIG. 3.11. Data in (A-C) are expressed as mean fold increase in fluorescence relative to a background autofluoresence control ± SD of triplicate samples. Data in (D) are mean % autorosetting ± SD of triplicate samples. NS, not significant. *, P < 0.05. **, P < 0.01. ***, P < 0.001. ****, P < 0.0001 (Student’s t-test).
FIG. 3.13: **Surface HS is required for erythrocytes to autorosette with neuraminidase-treated T cells.** Autorosetting between different neuraminidase-treated lymphocyte populations as defined in FIG. 3.4 and mock treated control, bacterial heparinase-I (HPNSE) or mammalian heparanase (HPSE)-treated erythrocytes as detected by flow cytometry. HPSE and HPNSE treatments were performed as described in FIG. 3.8C. Data are mean % autorosetting ± SD of triplicate samples. ***,** \( P < 0.001 \) (1-way ANOVA with Dunnett’s multiple comparisons test to untreated control).

### 3.3.7 Autorosetting inhibition by serum derived HRG

Autorosetting inhibition by sera from various mammalian species (Charreire and Bach, 1975, Kolb, 1977, Ghanta et al., 1978, Hsu et al., 1980) has been shown previously to be ascribed to HRG (Rylatt et al., 1981, Sia et al., 1982). Here, it was confirmed that purified human HRG strongly inhibited mouse thymocytes autorosetting (IC\(_{50}\) = 2.53 µg/mL) (FIG. 3.14A). Based on earlier studies it would be expected that HRG deficient serum would not inhibit autorosetting, hence, the ability of HRG.KO (Tsuchida-Straeten et al., 2005) and WT mouse sera to inhibit autorosetting was evaluated. Western blotting and ELISA analyses confirmed the absence of detectable HRG in HRG.KO sera (FIG. 3.14B). Surprisingly, autorosetting inhibition by the HRG.KO sera was found to be considerably more efficient than the WT sera, i.e., autorosetting was reduced by 50% by HRG.KO sera diluted 1/512 compared with a 1/64 dilution of WT sera (~8 fold difference) (FIG. 3.14C). Despite Coomassie blue staining of SDS-PAGE gels showing no obvious difference in total protein constituents of WT and HRG.KO sera (FIG. 3.14D, *top panel*), it is possible that in the absence of HRG, the levels of another serum factor that inhibits autorosetting has been elevated.
FIG. 3.14: Autorosetting inhibition by histidine-rich glycoprotein (HRG) and by a compensatory HRG-like serum protein. (A) Autorosetting inhibition of mouse thymocytes by different concentrations (6.4 ng/ml - 20 µg/mL) of purified human serum HRG (hHRG). (B) Detection of HRG in WT and HRG.KO mouse sera in three different mice by Western blotting (top panel) and ELISA (bottom panel) using polyclonal rabbit anti-hHRG. Recombinant mouse HRG (rec. mHRG) and hHRG are used as controls in the Western blot. (C) Autorosetting inhibition by WT and HRG.KO mouse sera. (D) Analysis of total protein content (top panel) by Coomassie Blue staining and detection by Western blotting (middle panel) and ELISA (bottom panel) of HRG in untreated and Co²⁺-bead binding protein preparations from WT and HRG.KO mouse sera. (E) Autorosetting inhibition by Co²⁺-bead depleted WT and HRG.KO mouse sera. Data in (A), (C) and (E) are mean % autorosetting ± SD of three replicates. ELISA data in (B) and (D) are mean absorbance ± SD of three replicates. In (E), NS, not significant. ***, P < 0.001 (1-Way ANOVA with Tukey’s multiple comparisons test).
Due to its histidine-rich domain, HRG is readily depleted from plasma by Co\(^{2+}\)-affinity chromatography (Patel et al., 2013). Indeed, the absence of detectable HRG in WT mouse sera by Western blotting and ELISA and the detection of HRG in the Co\(^{2+}\)-beads binding fraction of WT sera by Western blotting (FIG. 3.14D middle and bottom panels) confirms that Co\(^{2+}\)-affinity chromatography completely depletes HRG from mouse serum. Furthermore, Co\(^{2+}\)-bead-depleted WT serum was unable to inhibit autorosetting but unexpectedly, Co\(^{2+}\)-bead-depleted HRG.KO serum also lacked autorosetting inhibitory activity (FIG. 3.14E). SDS-PAGE analysis of the Co\(^{2+}\)-bead-binding fractions from WT and HRG.KO sera revealed seven identical Coomassie blue staining protein bands of 15 kDa to >150kDa, despite an extra band (i.e., HRG) being predicted to be present in the WT serum fraction (FIG. 3.14D, top panel). Detailed 2D-SDS-PAGE and mass spectrometry studies need to be performed to resolve this issue.

3.4 Discussion

In this Chapter a flow cytometry-based autorosetting assay was developed to investigate the molecular basis of murine autorosetting. Using the assay the main findings were: (i) autorosetting cells are primarily DP and to a lesser extent CD8 SP thymocytes, whereas autorosetting by peripheral T lymphocytes is negligible, (ii) autorosetting is mediated by the CD8αβ heterodimer on thymocytes, with CD8β making a major contribution to erythrocyte binding, (iii) CD8 interacts with highly sulfated HS on the erythrocytes surface, this interaction specifically requiring the presence of 6-O-sulfate groups, (iv) sialylation of CD8β on peripheral CD8\(^{+}\) T lymphocytes results in the masking of the HS binding site on CD8 molecules, a process that can be reversed by neuraminidase treatment and, finally, (v) it was confirmed that HRG in serum inhibits autorosetting although other, biochemically similar, molecules in plasma appear to be inhibitory.

Consistent with previous reports (Charreire and Bach, 1975, Kolb, 1977, Sia and Parish, 1980a, Sia and Parish, 1980b), the data shown in FIG. 3.4 confirmed that the thymus is the primary depot for autorosetting lymphocytes, 60-80% of DP and 20% of CD8 SP thymocytes autorosetting, whereas few peripheral CD8\(^{+}\) T lymphocytes (i.e., less than 5%) autorosetted. At face value, it is conceivable that autorosetting is developmentally controlled, being enabled at a certain point during DP thymocyte maturation, but eventually being disabled in CD8 SP thymocytes and severely impeded in peripheral
lymphocytes. Although previous reports claimed that up to 20% of peripheral lymphocytes autorosetted, only 6% of peripheral lymphocytes autorosetted in this study, a discrepancy that is likely to be due to the technical advantages of the flow cytometry-based autorosetting assay. Firstly, Hoechst-33342 labelling allowed the analysis of autorosetting to be entirely on viable lymphocytes, eliminating the artificial binding of erythrocytes by dead/dying cells which could have occurred in earlier studies. Secondly, the shear force of the flow cytometer sheath fluid would disperse weakly formed autorosettes. It should also be noted that the subset-specific analysis of autorosetting in the current assay eliminated the dilution effect of non-rosetting populations, presumably encountered in earlier studies. Thus, the data presented in this Chapter were generated by a highly objective assay that only counted stable autorosettes formed by viable lymphocytes and, consequently, was ideally placed to facilitate the identification of the autorosetting receptor on thymocytes and its ligand on erythrocytes.

Earlier studies suggesting that autorosetting is MHC-I restricted (Primi et al., 1979, Charreire and Bach, 1975, Sia and Parish, 1980a), which indirectly implicated CD8 in autorosetting as this molecule binds MHC-I. Indeed, based on the ability of anti-CD8 mAbs to inhibit autorosetting and the failure of CD8-deficient thymocytes to autorosette (FIG. 3.6), it was established that the CD8αβ heterodimer is the autorosetting receptor, with CD8β being the key autorosetting component of the heterodimer. Although partial inhibition of autorosetting by an anti-TCRβ mAb (FIG. 3.5) would suggest that TCRβ participates in autorosetting, this results is probably due to steric hindrance as the TCR complex is located proximal to the CD8αβ molecule (Devine et al., 2006). Unfortunately, the study of autorosetting by TCR-deficient thymocytes is not feasible as TCR-deficiency results in aberrant thymocytes development (Mombaerts et al., 1992). Moreover, MHC-I molecules on erythrocytes are not the main autorosetting ligand interacting with the CD8αβ heterodimer on thymocytes as their absence in KO mice did not affect autorosetting (FIG. 3.7). A possible explanation for this discrepancy is that the high shear forces in the flow cytometry assay used in this thesis to measure autorosetting eliminated the contribution of weak TCR-MHC-I interactions to the formation of autorosettes.

Consistent with an earlier report (Parish et al., 1984), it was confirmed that the sulfated polysaccharides heparin and Dxs completely inhibited autorosetting (FIG. 3.8A). These
data suggested that analogous molecules on the erythrocyte surface, namely HS, could function as an autorosetting ligand, a role ultimately confirmed by the failure of HPNSE and HPSE treated erythrocytes to autorosette (FIG. 3.8C). In addition, inhibition of the binding of anti-CD8 mAbs to lymphocytes by heparin and Dxs (FIG. 3.9) suggested that HS may interact with the CD8αβ heterodimer, particularly CD8β. Indeed, the impaired ability of CD8 deficient thymocytes to bind FITC-heparin was consistent with the CD8αβ heterodimer directly binding heparin/HS (FIG. 3.10B and C). Furthermore, this HS interaction is 6-O-sulfate dependent and is optimal when maximum negative charges are available on the heparin/HS chains (FIG. 3.8D).

CD8β is developmentally α2-3 sialylated (FIG. 3.11A and B), the ST3Gal-1 sialyltransferase being upregulated in CD8 SP thymocytes and peripheral CD8+ T lymphocytes, with this modification being previously reported to alter the conformation of the CD8αβ heterodimer (Moody et al., 2003). Additional to the blocking effect on MHC-I binding (Daniels et al., 2001), results presented in this Chapter indicate that α2-3 sialylation of CD8αβ impedes the interaction of CD8 on thymocytes and peripheral CD8+ T lymphocytes with HS. Thus, biochemical desialylation of CD8 by neuraminidase treatment strikingly augmented thymocyte and restored peripheral CD8+ T lymphocyte autorosetting (FIG. 3.11C and D). Hence, α2-3 sialylation not only limits the ability of CD8 molecules on peripheral CD8+ T lymphocytes to bind heparin/HS, as desialylation of DP thymocytes also enhances heparin/HS binding by CD8 as well as autorosetting. Moreover, data shown in FIG. 3.13 unequivocally show that autorosetting by neuraminidase treated peripheral CD8+ T lymphocytes is HS-dependent.

The data shown in FIG. 3.14A confirmed that serum-derived HRG strongly inhibits autorosetting, consistent with earlier reports from the Parish Laboratory (Rylatt et al., 1981, Sia et al., 1982). Although not tested in this study, HRG is likely to block autorosetting by binding to erythrocyte HS (Jones et al., 2004), thus preventing it from binding to CD8β. Whilst HRG is the major serum component that is responsible for autorossetting inhibition by WT serum, the enhanced ability of HRG deficient serum to inhibit autorossetting suggests the dominance of a substitute inhibitor in the HRG.KO mice (FIG. 3.14C). Western blotting revealed that HRG was absent from HRG.KO sera but Co2+-affinity chromatography, which depleted HRG from WT serum, also removed the unknown autorosetting inhibitor from HRG.KO serum. Thus, it is possible that the
autorosetting inhibitor in HRG.KO serum is an isoform of HRG not recognised by the generic anti-HRG polyclonal Ab used for Western blotting, being produced from an artifactual transcript encoded by the altered HRG gene in HRG.KO mice. Further work is required to resolve this issue.

In summary, using a new flow cytometry-based autorosetting assay, the molecular mechanisms that facilitate and control murine autorosetting have been characterised. The CD8αβ heterodimer, but mainly CD8β, on DP thymocytes binds directly to highly negatively charged HS chains, carrying 6-O-sulfate groups, on erythrocytes. During development in the thymus, and in the periphery, α2-3 sialylation of CD8β impedes the ability of CD8 to bind HS, an inhibitory effect that is reversed by enzymatic desialylation. Thus, together with circulating HRG, the ability of CD8 to interact with HS is constantly and tightly suppressed in peripheral CD8⁺ T lymphocytes.
Chapter Four

Functional Significance of Murine Autorosetting

4.1 Abstract

In Chapter 3 it was shown that murine autorosetting is mediated by CD8 on DP thymocytes interacting with HS on erythrocytes. Earlier studies by the Parish Laboratory discovered that certain sulfated polysaccharides, particularly DxS\textsuperscript{500 kDa}, that resemble HS and inhibit autorosetting, can induce a sustained Ca\textsuperscript{2+} flux in a subpopulation of immature murine thymocytes corresponding to DP thymocytes. In this Chapter it was initially confirmed that DxS\textsuperscript{500 kDa} induces a Ca\textsuperscript{2+} flux in DP thymocytes, but not in peripheral T lymphocytes, that is dependent on the uptake of extracellular Ca\textsuperscript{2+}. Furthermore, the extent of the DxS\textsuperscript{500 kDa}-induced Ca\textsuperscript{2+} flux was severely impaired in CD8\textbeta.KO DP thymocytes, this effect being more evident in CD8\textalpha.KO DP thymocytes, where both CD8\textbeta and CD8\textalpha molecules were absent. It was also shown that neuraminidase treatment markedly restored the DxS\textsuperscript{500 kDa}-induced Ca\textsuperscript{2+} flux in peripheral CD8\textsuperscript{+} T lymphocytes. Also, using mutant mouse strains it was discovered that the DxS\textsuperscript{500 kDa}-induced Ca\textsuperscript{2+} flux was independent of TCR-induced proximal
signalling, being unaffected by Zap70-deficiency, but was substantially impaired in Slp76-deficient DP thymocytes. Finally, DxS\textsuperscript{500 kDa} was able to markedly enhance thymocytes activation by an immobilised anti-CD3 mAb, as measured by CD69 upregulation. Collectively, these data suggest that the CD8-HS interaction observed with thymocytes autorosetting, induces a Ca\textsuperscript{2+} flux in DP thymocytes that lowers the threshold required for TCR-mediated activation of DP thymocytes.

4.2 Introduction

In developing thymocytes proper ligation of the TCR by self-peptide/MHC leads to the initiation of downstream signalling events, including a substantial rise in cytoplasmic Ca\textsuperscript{2+}, which may activate relevant transcription factors that are then translocated to the nucleus to either suppress or activate genes controlling cellular activation, proliferation, and cytokine production (Gascoigne and Palmer, 2011, Fu et al., 2014). The binding of CD8/CD4 to the non-cognate regions of MHC-I and MHC-II, respectively, may aid the process by increasing the avidity of TCR-ligand binding and fine-tuning downstream signalling (Li et al., 2013). In particular, CD8\(\beta\) has been shown to position the CD8\(\alpha\)\(\beta\) heterodimer proximal to the TCR-CD3 complex, consequently, enhancing peptide/MHC-I binding and, intracellularly, promoting the formation of a signalling platform involving Lck and LAT (Bosselut et al., 2000, Arcaro et al., 2001).

The release of Ca\textsuperscript{2+} from intracellular stores and the influx of extracellular Ca\textsuperscript{2+} via an array of plasma membrane Ca\textsuperscript{2+} channels contributes to the rise in cytoplasmic Ca\textsuperscript{2+} in developing thymocytes. Following TCR activation, phosphorylated Slp76 regulates ITK-mediated phosphorylation of PLC-\(\gamma\)1, which then converts PIP\(_2\) to IP\(_3\) and DAG. IP\(_3\) then binds to IP\(_3\) receptors on endoplasmic reticulum (ER) to trigger the release of ER-stored Ca\textsuperscript{2+}. In parallel, the binding of Ca\textsuperscript{2+} to STIM1 on ER membranes changes the conformation of STIM1, which enables its interaction with CRAC/ORAI1 channels and trigger the uptake of extracellular Ca\textsuperscript{2+} across the plasma membrane (Feske et al., 2012). Furthermore, plasma membrane voltage-gated Na\textsuperscript{+} channels (VGSC) (Lo et al., 2012), purinergic P2X channels (Freedman et al., 1999) and voltage-dependent Ca\textsuperscript{2+} channels (Ca,1.4) (Omilusik et al., 2011) have been linked with the influx of extracellular Ca\textsuperscript{2+} in stimulated thymocytes. In the cytoplasm, Ca\textsuperscript{2+} may also activate Ca\textsuperscript{2+}-calmodulin dependent pathways, notably calcineurin, a Ca\textsuperscript{2+}-dependent
phosphatase which dephosphorylates phosphorylated-NFAT. Dephosphorylated NFAT is then translocated to the nucleus to participate in gene transcription (Adachi et al., 2000).

The Parish laboratory has shown previously that certain sulfated polysaccharides that inhibit autorosetting, particularly Dxs^{500\text{ kDa}}, can induce a sustained Ca^{2+} flux in subpopulations of cortisone-sensitive thymocytes, but not in cortisone-resistant thymocytes and peripheral lymphocytes. Adding Ca^{2+}-chelating EGTA to the extracellular medium totally abolished the Dxs^{500\text{ kDa}}-induced Ca^{2+} flux, implying that the Ca^{2+} rise is totally dependent on the uptake of extracellular Ca^{2+}, thus, implicit indicating that the response is unlikely to require the release of Ca^{2+} from intracellular stores. Also, inhibiting VGSC has no effect on the Dxs^{500\text{ kDa}}-induced Ca^{2+} flux, thus, eliminating these ion channels from playing a role in the response (Tellam and Parish, 1987, Weston et al., 1991).

Since cortisone-sensitive thymocytes are DP thymocytes, the same thymocyte subpopulation that exhibits CD8-dependent autorosetting, an attractive hypothesis was that Dxs^{500\text{ kDa}} may be inducing a Ca^{2+} flux via the CD8 molecule. Therefore, this Chapter describes experiments to test this hypothesis and investigate the functional significance of the sulfated polysaccharide-induced Ca^{2+} flux in lymphocytes.

4.3 Results

4.3.1 Induction of a Ca^{2+} flux in lymphocytes by Dxs^{500\text{ kDa}}

Consistent with earlier reports (Tellam and Parish, 1987, Weston et al., 1991) the data presented in this thesis confirmed that Dxs^{500\text{ kDa}} can induce a sustained Ca^{2+} flux in thymocytes. Although heparin has been previously shown to induce a weak Ca^{2+} flux in thymocytes, here the same effect was not observed, whereas Dxs^{5\text{ kDa}} consistently failed to induce a Ca^{2+} flux in thymocytes (FIG. 4.1Ai), despite these two sulfated polysaccharides inhibiting autorosetting (FIG. 3.8A). The addition of 2 mM EGTA to the assay buffer to chelate extracellular Ca^{2+} totally abolished the Dxs^{500\text{ kDa}}-induced Ca^{2+} flux, thus, confirming that extracellular Ca^{2+} was essential for the Ca^{2+} flux observed in Dxs^{500\text{ kDa}}-stimulated thymocytes (FIG. 4.1Aii). Furthermore, titration of...
FIG. 4.1: High but not low molecular weight sulfated polysaccharides are able to induce a Ca\(^{2+}\) flux in thymocytes that is dependent on the uptake of extracellular Ca\(^{2+}\). (A) Comparison of the ability of 100 µg/mL of heparin, DxS\(^5\)kDa and DxS\(^{500}\)kDa to trigger a Ca\(^{2+}\) flux in thymocytes in HEPES-buffered saline (pH 7.4) (HBS) in (i) the absence or (ii) the presence of EGTA (2 mM). (B) Titration of DxS\(^{500}\)kDa to determine the minimal concentration required to trigger a Ca\(^{2+}\) flux in thymocytes. (C) Effect of pre-treatment of thymocytes with (i) heparin or (ii) DxS\(^5\)kDa (100 µg/mL) on induction of a Ca\(^{2+}\) flux by DxS\(^{500}\)kDa (1 µg/mL). Ca\(^{2+}\) fluxes in Indo-1-loaded thymocytes were determined for 22 min by flow cytometry, including a 2 min pre-stimulation baseline for each treatment. Arrows indicate the time the sulfated polysaccharides were added. Data are expressed as a ratio of fluorescence (GMFI) of Ca\(^{2+}\)-bound Indo-1 (425 nm) vs. fluorescence of unbound Indo-1 (510 nm).
FIG. 4.2: The sulfated polysaccharide DxS\textsuperscript{500 kDa} specifically induces a Ca\textsuperscript{2+} flux in subpopulations of thymocytes. Analysis of DxS\textsuperscript{500 kDa}-induced Ca\textsuperscript{2+} flux in (A) thymic and (B) peripheral T cell subsets, with the Ca\textsuperscript{2+} flux in different Indo-1-loaded lymphocytes populations being monitored for 22 min by flow cytometry, including a 2 min pre-stimulation baseline. Data are expressed as a ratio of fluorescence (GMFI) of Ca\textsuperscript{2+}-bound Indo-1 (425 nm) vs. fluorescence of unbound Indo-1 (510 nm). Arrows indicate the time DxS\textsuperscript{500 kDa} was added. (C) Percentage of different thymocyte subsets exhibiting a Ca\textsuperscript{2+} flux (upper values in each panel) vs. percentage failing to produce a Ca\textsuperscript{2+} flux (lower values in each panel) when unstimulated or stimulated with DxS\textsuperscript{500 kDa} (1 µg/mL) for 20 min, when the Ca\textsuperscript{2+} flux had plateaued. Binding of Ca\textsuperscript{2+} by Indo-1 results in an increase in 425 nm fluorescence and a decrease in 510 nm fluorescence that is Ca\textsuperscript{2+} concentration-dependent. ‘Total’ refers to response of total lymphocytes population, with lymphocyte subsets as in FIG. 3.4B.
the DxS\(^{500}\) kDa concentration showed that 0.1 µg/mL was sufficient to induce a substantial Ca\(^{2+}\) flux (FIG. 4.1B). Interestingly, it was also found that pre-treatment of thymocytes with 100 µg/mL of either heparin or DxS\(^{5}\) kDa substantially reduced the DxS\(^{500}\) kDa-induced Ca\(^{2+}\) flux (FIG. 4.1C), therefore, suggesting that heparin and DxS (5 and 500 kDa) bind to the same receptor despite differences in their ability to induce a Ca\(^{2+}\) flux.

Previously, it has been shown that cortisone-sensitive thymocytes are the main lymphocyte subset displaying a substantial Ca\(^{2+}\) flux following DxS\(^{500}\) kDa stimulation, whereas cortisone-resistant thymocytes and splenocytes are non-responsive to DxS\(^{500}\) kDa stimulation (Tellam and Parish, 1987, Weston et al., 1991). Indeed, here it was confirmed that DP thymocytes and to a lesser extent, CD8 SP and CD4 SP thymocytes, were the DxS\(^{500}\) kDa-responsive subsets, whereas DN thymocytes and peripheral CD4\(^+\) and CD8\(^-\) T lymphocytes consistently failed to show any detectable Ca\(^{2+}\) flux following DxS\(^{500}\) kDa stimulation (FIG. 4.2A and B). To complement these data, it was found that 62.8% of total thymocytes, in particular, 72.2% of DP, 32% of CD8 SP and 25.9% of CD4 SP thymocytes showed a noticeable Ca\(^{2+}\) flux following DxS\(^{500}\) kDa stimulation (FIG. 4.2C). Collectively, these data implied that only subpopulations of immature thymocytes could be stimulated by DxS\(^{500}\) kDa to produce an increase in intracellular Ca\(^{2+}\).

### 4.3.2 The molecular basis of the DxS\(^{500}\) kDa-induced Ca\(^{2+}\) flux in thymocytes

Since heparin and DxS (5 and 500 kDa) bind to the CD8αβ heterodimer and block autorosetting (FIG. 3.8, 3.9 and 3.10), and heparin and DxS\(^^{5}\) kDa partially blocked the Ca\(^{2+}\) response induced by DxS\(^{500}\) kDa (FIG. 4.1C), it was hypothesised that the CD8αβ heterodimer is the receptor on thymocytes responsible for the DxS\(^{500}\) kDa-induced Ca\(^{2+}\) flux. To test this hypothesis, the extent of the DxS\(^{500}\) kDa-induced Ca\(^{2+}\) flux in CD8.KO thymocytes was evaluated. Indeed, it was found that CD8α.KO thymocytes, which completely lacked the CD8αβ heterodimer, displayed an almost completely impaired DxS\(^{500}\) kDa-induced Ca\(^{2+}\) flux, the level of impairment being closely matched by CD8β.KO thymocytes (FIG. 4.3Ai). Similarly, examination of different thymocyte subpopulations also showed that CD8β made a major contribution to the Ca\(^{2+}\) flux induced by DxS\(^{500}\) kDa in DP and CD8 SP thymocytes (FIG. 4.3B). Collectively, these data indicate that CD8β makes a major contribution to the response, but is aided by
CD8α. This conclusion was confirmed by the observation that the Ca\(^{2+}\) flux of CD8β.KO thymocytes that were pre-treated with an anti-CD8α mAb was comparable to that of CD8α.KO thymocytes (FIG. 4.3Ai lower panel). The proportion of the different thymocytes populations producing a Ca\(^{2+}\) flux in response to Dxs\(^{500\text{ kDa}}\) further supports this finding (FIG. 4.3C). Thus, compared to 64% of WT thymocytes, only 25.4% of CD8β.KO thymocytes gave a Dxs\(^{500\text{ kDa}}\)-induced Ca\(^{2+}\) response, this responsive population being further reduced to 15.9% in the presence of an anti-CD8α mAb, although not quite as low as with CD8α.KO thymocytes (10.4%).

In contrast, the magnitude of the Dxs\(^{500\text{ kDa}}\)-induced Ca\(^{2+}\) flux in WT and CD4.KO thymocytes was comparable (FIG. 4.3Aii). At face value, this implies that CD4 does not contribute to the response. Intriguingly, however, Dxs\(^{500\text{ kDa}}\) induced a marginal Ca\(^{2+}\) flux in CD4 SP thymocytes (FIG. 4.2A and C) that was seemingly unaffected by CD8β-deficiency (FIG. 4.3B), thus implying the presence of a CD8-independent response to Dxs\(^{500\text{ kDa}}\).

Since the Dxs\(^{500\text{ kDa}}\)-induced Ca\(^{2+}\) flux in thymocytes is predominantly CD8-dependent, it appeared likely that the response may involve components of the TCR-signalling pathway. To test this possibility, the extent of the Dxs\(^{500\text{ kDa}}\)-induced Ca\(^{2+}\) flux in DP thymocytes from N-ethyl-N-nitrosourea (ENU)-generated mutant mice, which lacked the major signal transduction proteins Zap70 (Siggs et al., 2007) and Slp76 (Siggs et al., 2015), with Zap70 acting upstream of Slp76 (Fu et al., 2014), were evaluated. Initially, it was found that there were marked differences in the percentage of DN, DP, CD4 SP and CD8 SP thymocytes in WT, Zap70\(^{-/-}\) and Slp76\(^{-/-}\) mice. In particular, the development of Zap70\(^{-/-}\) thymocytes appeared to be arrested at the DP stage, whereas Slp76\(^{-/-}\) thymocytes showed impaired progression from the DN to the DP stage (FIG. 4.4A), consistent with earlier reports claiming that Zap70 and Slp76 deficiencies lead to aberrant T lymphocyte development (Siggs et al., 2007, Siggs et al., 2015). However, despite CD8 being comparably expressed on WT, Zap70\(^{-/-}\) and Slp76\(^{-/-}\) DP thymocytes, the Dxs\(^{500\text{ kDa}}\)-induced Ca\(^{2+}\) flux was comparable in WT and Zap70\(^{-/-}\) DP thymocytes, but markedly impaired in Slp76\(^{-/-}\) DP thymocytes (FIG. 4.4B). Indeed, this finding was complemented by the observation that 67.5% of WT, 62% of Zap70\(^{-/-}\) and 35.5% of Slp76\(^{-/-}\) DP thymocytes showed a substantial Ca\(^{2+}\) flux following Dxs\(^{500\text{ kDa}}\) stimulation.
FIG. 4.3: Contribution of CD8 molecules to the DxS\(^{500}\) kDa-induced Ca\(^{2+}\) flux in thymocytes. 

(A) DxS\(^{500}\) kDa-induced Ca\(^{2+}\) flux in (i) WT, CD8\(\alpha\).KO and CD8\(\beta\).KO thymocytes (top panel), with the effect of the addition of an anti-CD8\(\alpha\) mAb (5 \(\mu\)g/mL) on the DxS\(^{500}\) kDa-induced Ca\(^{2+}\) flux in CD8\(\beta\).KO thymocytes shown (bottom panel) and in (ii) WT and CD4\(^{-/}\) (ENU) thymocytes. In (i) note the \(y\)-axis of the bottom panel has been expanded. 

(B) Effect of CD8\(\beta\)-deficiency on the DxS\(^{500}\) kDa-induced Ca\(^{2+}\) flux in different thymocyte subsets, with the Ca\(^{2+}\) flux being monitored for 22 min by flow cytometry, including a 2 min pre-stimulation baseline. Data are expressed as a ratio of fluorescence (GMFI) of Ca\(^{2+}\)-bound Indo-1 (425 nm) vs. fluorescence of unbound Indo-1 (510 nm). Arrows indicate the time DxS\(^{500}\) kDa was added. 

(C) Percentage of WT, CD8\(\alpha\).KO and CD8\(\beta\).KO thymocytes, the latter in the absence or presence of an anti-CD8\(\alpha\) mAb (5 \(\mu\)g/mL), exhibiting a Ca\(^{2+}\) flux (upper values in each panel) vs. percentage failing to produce a Ca\(^{2+}\) flux (lower values in each panel) when unstimulated or stimulated with DxS\(^{500}\) kDa (1 \(\mu\)g/mL) for 20 min (i.e., when the Ca\(^{2+}\) flux had plateaued). Binding of Ca\(^{2+}\) by Indo-1 results in an increase in 425 nm fluorescence and a decrease in 510 nm fluorescence that is Ca\(^{2+}\) concentration-dependent.
Collectively, these data suggest that DxS<sup>500 kDa</sup> induces a Ca<sup>2+</sup> flux in DP thymocytes via an intracellular signalling pathway that involves Slp76, but the upstream Zap70, which is associated with the initiation of TCR signalling, plays little or no role in the process.

**FIG. 4.4: Zap70 but not Slp76 is dispensable for the DxS<sup>500 kDa</sup>-induced Ca<sup>2+</sup> flux in thymocytes.** Analysis of (A) the distribution of thymocyte subsets and (B) DxS<sup>500 kDa</sup>-induced Ca<sup>2+</sup> flux in WT, Zap70<sup>−/−</sup> and Slp76<sup>−/−</sup> DP thymocytes, with the Ca<sup>2+</sup> flux in different Indo-1-loaded thymocytes being monitored for 22 min by flow cytometry, including a 2 min pre-stimulation baseline. Data in (B) are expressed as a ratio of fluorescence (GMFI) of Ca<sup>2+</sup>-bound Indo-1 (425 nm) vs. fluorescence of unbound Indo-1 (510 nm). Arrow indicates the time DxS<sup>500 kDa</sup> was added. (C) Percentage of WT, Zap70<sup>−/−</sup> and Slp76<sup>−/−</sup> DP thymocytes exhibiting a Ca<sup>2+</sup> flux (upper values in each panel) vs. percentage failing to produce a Ca<sup>2+</sup> flux (lower values in each panel) when unstimulated or stimulated with DxS<sup>500 kDa</sup> (1 µg/mL) for 20 min (i.e., when the Ca<sup>2+</sup> flux had plateaued). Binding of Ca<sup>2+</sup> by Indo-1 results in an increase in 425 nm fluorescence and a decrease in 510 nm fluorescence that is [Ca<sup>2+</sup>]-dependent.

Since O-sialylation of CD8<sub>β</sub> on CD8<sup>+</sup> SP thymocytes and peripheral CD8<sup>+</sup> lymphocytes impedes the ability of CD8 to interact with sulfated polysaccharides (FIG. 3.12), a likely possibility is that O-sialylation also impairs the DxS<sup>500 kDa</sup>-induced Ca<sup>2+</sup> flux in these cells. Thus, it was predicted that neuraminidase treatment would restore the DxS<sup>500 kDa</sup>-induced Ca<sup>2+</sup> flux in peripheral CD8<sup>+</sup> T lymphocytes. Indeed, neuraminidase
treated peripheral CD8$^+$ T lymphocytes showed a substantial Ca$^{2+}$-flux following DxS$^{500\,\text{kDa}}$ stimulation, whereas mock-treated control cells did not respond to DxS$^{500\,\text{kDa}}$ (FIG. 4.5). Also, neuraminidase-treated CD8$\beta$.KO peripheral CD8$^+$ T lymphocytes were non-responsive to DxS$^{500\,\text{kDa}}$, thus demonstrating that the DxS$^{500\,\text{kDa}}$-induced Ca$^{2+}$ flux in peripheral CD8$^+$ T lymphocytes, like with thymocytes, is highly CD8$\beta$-dependent (FIG. 4.5).

**FIG. 4.5:** Neuraminidase treatment restores the CD8$\beta$-dependent DxS$^{500\,\text{kDa}}$-induced Ca$^{2+}$ flux in peripheral CD8$^+$ T cells. Analysis of the DxS$^{500\,\text{kDa}}$-induced Ca$^{2+}$ flux in untreated and *V. cholerae* neuraminidase treated WT and CD8$\beta$.KO peripheral CD8$^+$ T cells, with the Ca$^{2+}$ flux in different Indo-1-loaded peripheral CD8$^+$ cells being monitored for 22 min by flow cytometry, including a 2 min pre-stimulation baseline. Data are expressed as a ratio of fluorescence (GMFI) of Ca$^{2+}$-bound Indo-1 (425 nm) vs. fluorescence of unbound Indo-1 (510 nm). Arrows indicate the time DxS$^{500\,\text{kDa}}$ (1 µg/ml) was added.

### 4.3.3 Contribution of sulfated polysaccharide recognition to thymocyte activation

Based on the ability of DxS$^{500\,\text{kDa}}$ to induce a substantial Ca$^{2+}$ flux in DP thymocytes, it was hypothesised that this Ca$^{2+}$ flux could lower the threshold required to activate T cells, with expression of cell surface CD69 on thymocytes being used as a marker of recent activation (Swat et al., 1993, Yamashita et al., 1993). Initial studies revealed that DxS$^{500\,\text{kDa}}$ at 10 and 100 µg/mL induced a significant increase in the percentage of CD69$^+$ thymocytes after 4 hr incubation, but heparin and DxS$^{5\,\text{kDa}}$ had no effect on CD69 expression (FIG. 4.6A). This result mirrors the ability of these sulfated polysaccharides to induce a Ca$^{2+}$ flux in thymocytes (FIG. 4.1A). Furthermore, although DxS$^{500\,\text{kDa}}$ could induce increased CD69 expression by CD8$\beta$.KO thymocytes, this was evident only at the highest concentration of DxS$^{500\,\text{kDa}}$ used (100 µg/mL) (FIG.
4.6B), indicating that CD8β makes a major contribution to the induction of CD69 expression by Dxs^{500\,kDa}.

**FIG. 4.6:** Dxs^{500\,kDa} enhances anti-CD3ε mAb-mediated thymocyte activation in a CD8β-dependent manner. (A) CD69-expressing thymocytes (%) after 4 hr incubation at 37°C alone or in the presence of 1-100 µg/mL of the sulfated polysaccharides heparin and Dxs (5 and 500 kDa). (B) CD69-expression by WT and CD8β.KO thymocytes (%) following 4 hr incubation at 37°C alone or in the presence of Dxs^{500\,kDa} (1-100 µg/mL). In (A) and (B), ***, P < 0.001. ****, P < 0.0001 (1-way ANOVA with Dunnett’s multiple comparisons test to unstimulated control). (C) CD69-expressing thymocytes (%) following 4 hr incubation at 37°C alone or in the presence of an immobilised anti-CD3ε mAb (0.4, 2 and 10 µg/mL) with or without Dxs^{500\,kDa} (1-100 µg/mL). **, P < 0.01. ***, P < 0.001 (1-way ANOVA with Dunnett’s multiple comparisons test to without anti-CD3ε mAb stimulation). (D) CD69-expressing WT and CD8β.KO thymocytes (%) after 4 hr incubation at 37°C in the presence of an immobilised anti-CD3ε mAb (10 µg/mL) and with or without Dxs^{500\,kDa} (1-100 µg/mL). NS, not significant. ****, P < 0.0001 (2-way ANOVA with Bonferroni multiple comparisons test). Data are expressed as mean %CD69-expressing thymocytes ± SD of triplicate samples, as measured by flow cytometry.
The ability of DxS$^{500\text{ kDa}}$ to independently activate thymocytes was consistent with the hypothesis that DxS$^{500\text{ kDa}}$ stimulation may synergise with and lower the threshold for an anti-CD3 mAb (i.e., TCR signal) to activate thymocytes. To test this hypothesis, the percentage of thymocytes expressing CD69 following 4 hr incubation at 37°C with an immobilised anti-CD3ε (145-2C11) mAb, in the absence or presence of DxS$^{500\text{ kDa}}$, was measured by flow cytometry. Initially, it was found that the immobilised anti-CD3ε mAb at all concentrations used (0.4, 2 and 10 µg/mL) produced no change in the percentage of CD69$^+$ thymocytes above background levels. This suggested that stimulation with an immobilised anti-CD3ε mAb, without the addition of DxS$^{500\text{ kDa}}$, is insufficient to activate thymocytes after short-term culture (i.e., 4 hr). However, whilst incubation with 1 µg/mL of DxS$^{500\text{ kDa}}$ alone consistently failed to activate thymocytes, co-incubation with 10 µg/mL of immobilised anti-CD3ε mAb resulted in a small but significant increase in the percentage of CD69$^+$ thymocytes (9.23% vs. 11.6% CD69$^+$ cells, $P < 0.01$). The ability of DxS$^{500\text{ kDa}}$ to enhance thymocyte activation by immobilised anti-CD3ε mAb was more evident in the presence of increasing concentrations of DxS$^{500\text{ kDa}}$, with thymocytes co-incubated with 10 and 100 µg/mL of DxS$^{500\text{ kDa}}$ showing significantly enhanced CD69 expression at all three concentrations of immobilised anti-CD3ε used (FIG. 4.6C). Furthermore, the ability of DxS$^{500\text{ kDa}}$ to enhance anti-CD3ε mAb-mediated activation was significantly impaired in CD8β.KO thymocytes (FIG. 4.6D). Collectively, these data demonstrate that DxS$^{500\text{ kDa}}$ can synergise with and lower the activation threshold required for TCR-mediated activation of thymocytes, this process being CD8β dependent.

4.4 Discussion

DxS$^{500\text{ kDa}}$ has been previously shown to induce a substantial and sustained Ca$^{2+}$ flux in subpopulations of immature thymocytes (Weston et al., 1991) but how this occurs and its functional implications are not known. In this Chapter the main findings were: (i) it was confirmed that DxS$^{500\text{ kDa}}$ induces a sustained Ca$^{2+}$ flux in subpopulations of DP thymocytes and to a lesser extent in CD8 SP and CD4 SP thymocytes, whereas peripheral CD4$^+$ and CD8$^+$ T lymphocytes are non-responsive to DxS$^{500\text{ kDa}}$ stimulation, (ii) the DxS$^{500\text{ kDa}}$ triggered Ca$^{2+}$ flux is dependent on the CD8αβ heterodimer with CD8β playing a major role in the process but being aided by CD8α, (iii) sialylation of CD8β completely inhibits the DxS$^{500\text{ kDa}}$-induced Ca$^{2+}$ flux in peripheral CD8$^+$ T
lymphocytes, an inhibition that can be reversed by neuraminidase treatment, (iv) the CD8-DxS<sup>500 kDa</sup> interaction triggers the uptake of extracellular Ca<sup>2+</sup> by DP thymocytes via a signalling pathway that is independent of TCR associated Zap70 but requires downstream Slp76 and, finally, (v) ligation of the CD8αβ heterodimer by DxS<sup>500 kDa</sup> lowers the threshold required for anti-CD3ε mAb-mediated activation of thymocytes.

Consistent with earlier reports (Tellam and Parish, 1987, Weston et al., 1991) DxS<sup>500 kDa</sup> induced a sustained Ca<sup>2+</sup> flux (FIG. 4.1A) in subpopulations of DP thymocytes and to a lesser extent, in CD8 SP and CD4 SP thymocytes, whereas peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes were non-responsive to DxS<sup>500 kDa</sup> (FIG. 4.2). Similar to autorosetting, it appears that the sensitivity of thymocytes to the DxS<sup>500 kDa</sup>-induced Ca<sup>2+</sup>-flux is developmentally controlled and, although not directly examined in this thesis, probably represents the subpopulation of DP thymocytes that is capable of autorosetting. It has been shown previously that heparin and DxS<sub>5 kDa</sub> induced smaller Ca<sup>2+</sup> rise in thymocytes (Tellam and Parish, 1987) however, here the response was negligible (FIG. 4.1A). This discrepancy may be due to the subtle differences in the batches of heparin and DxS<sub>kDa</sub> used in the two studies. In contrast, heparin and DxS<sub>5 kDa</sub> inhibited the ability of DxS<sup>500 kDa</sup> to induce a Ca<sup>2+</sup> flux (FIG. 4.1C) indicating that heparin and DxS (5 and 500 kDa) bind to the same receptor on DP thymocytes. The ability of DxS<sup>500 kDa</sup> to substantially inhibit the binding of an anti-CD8β mAb and, to a lesser extent, an anti-CD8α mAb to thymocytes (FIG. 3.9), coupled with the almost complete lack of a DxS<sup>500 kDa</sup>-induced Ca<sup>2+</sup> flux in CD8.KO thymocytes (FIG. 4.3Ai), strongly suggest that the CD8αβ heterodimer is the key DxS<sup>500 kDa</sup> receptor involved in triggering a Ca<sup>2+</sup> flux. Also, consistent with autorosetting, sialylation of CD8β on CD8 SP thymocytes and peripheral CD8<sup>+</sup> T lymphocytes deterred the interaction of CD8 with sulfated polysaccharides (FIG. 3.11 and 3.12) and, hence, explains why these cells were non-responsive to DxS<sup>500 kDa</sup> stimulation. Indeed, this inhibition was reversed by neuraminidase treatment (FIG. 4.5). Finally, the residual Ca<sup>2+</sup> flux in CD8.KO thymocytes (FIG. 4.3Aii) and CD4 SP thymocytes (FIG. 4.3B) suggests the existence of a CD8-independent response to DxS<sup>500 kDa</sup> and, although not tested, possibly mediated by the binding of DxS<sup>500 kDa</sup> to other DxS/HS-binding cell surface receptors, including CD45, CD90 (Parish et al., 1988a), LFA-1 and α4β1/VLA-4 (Vermot-Desroches et al., 1991, Schlesinger et al., 2009, Bendas and Borsig, 2012)
In developing thymocytes, proper engagement of the TCR by self-peptide/MHC-I complexes, together with the ligation of CD8 by a non-cognate region of MHC-I, leads to the initiation of intracellular signalling cascades including a rise in cytoplasmic Ca\(^{2+}\) (Fu et al., 2014). Earlier studies have demonstrated that Zap70 (Williams et al., 1999) and Slp76 (Kumar et al., 2002) are crucial for Ca\(^{2+}\) mobilisation during thymocytes development. The full extent of intracellular signalling events activated by the CD8-DxS\(^{500\, \text{kDa}}\) interaction is not known, but the data shown in this Chapter indicate that the induction of the Ca\(^{2+}\) flux requires the adapter protein Slp76, whereas Zap70, which is the key transducer of TCR signalling but upstream of Slp76, apparently plays no role in the process (FIG. 4.4). It is most unlikely that DxS\(^{500\, \text{kDa}}\) induces the release of intracellularly stored Ca\(^{2+}\) as Zap70 did not contribute to the response and, chelation of extracellular Ca\(^{2+}\) by EGTA resulted in no intracellular Ca\(^{2+}\) rise, thus, unequivocally confirming that the DxS\(^{500\, \text{kDa}}\)-induced Ca\(^{2+}\) flux is totally due to the uptake of extracellular Ca\(^{2+}\) (FIG. 4.1Aii). The involvement of Slp76, in particular, in the uptake of extracellular Ca\(^{2+}\) is surprising as this has not been reported before and, as VGSC do not contribute to the response (Tellam and Parish, 1987), further work is required to delineate the intracellular signalling events involved and the mechanism of intracellular Ca\(^{2+}\) entry induced by DxS\(^{500\, \text{kDa}}\).

In the absence of TCR stimulation, based on CD69 expression thymocytes were substantially activated after 4 hr incubated with high concentrations (100 µg/mL) of DxS\(^{500\, \text{kDa}}\) with CD8\(\beta\) and, although not tested directly, CD8\(\alpha\) presumably making a contribution to the process (FIG. 4.6A and B). Whilst TCR stimulation by an immobilised anti-CD3\(\varepsilon\) mAb failed to activate thymocytes after 4 hr incubation, co-incubation with DxS\(^{500\, \text{kDa}}\) markedly augmented thymocytes activation above that evident with DxS\(^{500\, \text{kDa}}\) alone. Importantly, thymocytes stimulated with increasing DxS\(^{500\, \text{kDa}}\) concentrations required lower concentrations of immobilised anti-CD3\(\varepsilon\) mAb to achieve comparable levels of CD69 expression, with the augmenting effects of DxS\(^{500\, \text{kDa}}\) being CD8-dependent (FIG. 4.6C and D). Collectively, these data demonstrate that DxS\(^{500\, \text{kDa}}\) lowers the threshold required for TCR-mediated activation of thymocytes, particularly at early time points (i.e., 4 hr) after initiation of anti-CD3 mAb activation. It should be noted that if anti-CD3 mAb induced CD69 expression on thymocytes was examined after 24 hr incubation, all concentrations of immobilised
anti-CD3 mAb induced a high proportion of CD69\(^+\) thymocytes and DxS\(^{500\text{kDa}}\) had little or no enhancing effect (data not shown).

In summary, this Chapter describes the likely functional implications of the CD8-HS mediated autorosetting phenomenon observed with DP thymocytes. DxS\(^{500\text{kDa}}\) represents a HS mimetic that binds to CD8 on DP thymocytes and generates a Zap70-independent, Slp76-dependent signalling events that induce a sustained influx of extracellular Ca\(^{2+}\) in these cells. Sialylation of CD8\(\beta\) on peripheral CD8\(^+\) T lymphocytes impedes the CD8-HS interaction and, consequently, the associated intracellular Ca\(^{2+}\) flux, but this inhibitory effect can be reversed by enzymatic desialylation. Thus, the CD8-HS interaction detected by autorosetting appears to trigger intracellular signalling events that lower the TCR-mediated activation threshold in DP thymocytes. Future investigations are needed to further examine the intracellular events triggered in thymocytes by the CD8-HS interaction, as well as the ability of this interaction to fine-tune antigen-induced thymocyte activation. However, of more immediate importance is the identification of the cells in the thymus that ‘rosette’ with thymocytes and, presumably, provide the HS ligand that triggers a Ca\(^{2+}\) flux in developing thymocytes. Experiments described in the next Chapter (Chapter 5) attempt to address this issue.
5.1 Abstract

The thymus provides unique microenvironments which enable T cell precursors to become functional and self-tolerant MHC-restricted TCRαβ T lymphocytes. Immature thymocytes migrate through distinct thymic niches and interact with stromal cells by scanning self-antigens in the context of MHC while progressively undergoing TCR rearrangements. In the cortex only TCR clones with low to intermediate affinity for self-peptide/MHC complexes on cTEC surfaces are positively selected to survive and develop into SP thymocytes, whereas strongly reactive TCR clones are negatively selected upon interacting with self-peptide/MHC complexes on cortical DC. The surviving TCR clones migrate to the medulla and face a second wave of negative selection against tissue-restricted antigens expressed by mTEC and DC. The data shown in this Chapter demonstrate that highly sulfated HS expressed on the surface of cTEC participates in the heterotypic interaction between cTEC and thymocytes, thus allowing the two cell types to form rosettes in a similar way to autorosetting (Chapter 3), this
process being CD8αβ-dependent and being augmented by the presence of MHC molecules on cTEC. Furthermore, the functional relevance of the CD8-HS interaction in cTEC-thymocyte rosetting is evident from the induction of a sustained Ca\(^{2+}\) influx in the rosetting thymocytes, consistent with the findings in Chapter 4. Collectively, these data indicate that the CD8-HS interaction facilitates the adhesion of DP thymocytes to cTEC and initiates a Ca\(^{2+}\) flux in the adherent thymocytes that lowers the threshold required for positive selection of DP thymocytes.

5.2 Introduction

Multipotent lymphoid progenitor cells from the bone marrow migrate to the thymus via the circulation to undergo intrathymic developmental processes involving tightly regulated interactions with thymic stromal cells, finally producing functional MHC-restricted CD4\(^+\) or CD8\(^+\) TCRαβ T lymphocytes (Anderson and Jenkinson, 2001). In the thymus CD4\(^-\)CD8\(^-\) (DN) thymocytes arrive at the corticomedullary junction and whilst migrating to the subcapsular zone (Lind et al., 2001) differentiate from the DN1 stage into the DN2 and DN3 stages (Godfrey et al., 1993). DN3 thymocytes are screened for productive TCRβ chain expression (β-selection) and then undergo proliferative expansion and differentiate into CD4\(^+\)CD8\(^-\) DP (CD3\(^+\)TCRβ\(^{\text{b0-Pre-TCRα^+}}\)) thymocytes (Dudley et al., 1994, Hoffman et al., 1996, Kreslavsky et al., 2012). DP thymocytes then migrate inwards to the inner cortex and undergo TCRα chain rearrangements (Petrie et al., 1993), whilst scanning self-peptides in the context of MHC on cortical thymic epithelial cells (cTEC) (Lorenz and Allen, 1989, Anderson et al., 1994) and dendritic cells (DC) (Gallegos and Bevan, 2004, Ladi et al., 2008). DP thymocytes expressing TCRs with low to intermediate affinity for self-peptide/MHC complexes on cTEC are positively selected and develop towards the CD8 SP (MHC-I-restricted) or CD4 SP (MHC-II-restricted) lineages. In contrast, TCR clones strongly reacting with self-peptide/MHC complexes on DC are deleted (negative selection) (McCaughtry et al., 2008, Baba et al., 2009), whereas TCR clones that have little or no affinity for self-peptide/MHC complexes are lost due to ‘death by neglect’, a process that results in the deletion of useless TCR clones (Hernandez et al., 2010). The TCRα\(^{\text{hi}}\) CD4 SP and CD8 SP thymocytes migrate to the thymic medulla where they are screened against AIRE-regulated (Liston et al., 2003) tissue-restricted antigens (TRA) (Derbinski et al., 2001) presented by medullary TEC (mTEC) or by thymic DCs that have acquired the
mTEC-derived TRA (Gallegos and Bevan, 2004, Hubert et al., 2011). Strongly reactive TCR clones are either clonally deleted or, in the case of CD4 SP thymocytes, may differentiate into CD4\(^+\)FoxP3\(^+\)CD25\(^+\) natural regulatory T lymphocytes (Itoh et al., 1999, Jordan et al., 2001).

In addition to the TCR signals (Fu et al., 2014), cTEC are thought to express unique cell surface molecules that can provide ‘accessory signals’ that aid MHC-restricted positive selection and maturation of DP thymocytes into CD8 SP and CD4 SP thymocytes (Anderson et al., 1994). Moreover, cTEC can tightly interact with thymocytes, consequently forming rosettes (Oliveira-dos-Santos et al., 1997, Oliveira-dos-Santos et al., 1998), the most extreme example of this interaction being thymic nurse cells (TNC) that interact with large numbers of thymocytes (Wekerle and Ketelsen, 1980, Wekerle et al., 1980, Kyewski et al., 1982, Li et al., 1992). Earlier reports demonstrated that cTEC express high levels of cell surface heparan sulfate (HS) that is rich in 6-O-sulfates within the highly sulfated regions of the molecules. These researchers also suggested that this HS serves as a cell adhesion ligand that contributes to the heterotypic interaction between cTEC and thymocytes (Britz and Hart, 1983, Werneck et al., 1999, Werneck et al., 2000).

In Chapter 3 of this thesis it was shown that erythrocyte surface HS, with similar structural characteristics to the HS on cTEC surfaces as described above, can interact predominantly with CD8\(\beta\) and, to lesser extent, with CD8\(\alpha\) on DP thymocytes, thus mediating autorosetting. The data presented in Chapter 4 demonstrates the functional consequences of the CD8-HS interaction, HS mimetics that bind CD8 inducing a sustained Ca\(^{2+}\) flux in DP thymocytes that lowers the threshold for TCR-mediated thymocyte activation. Hence, it was postulated that the CD8-HS interaction contributes to the heterotypic interaction (rosetting) between cTEC and thymocytes, the interaction inducing a sustained Ca\(^{2+}\) flux in bound thymocytes independent of TCR-MHC recognition. This Chapter describes experiments that attempted to test this hypothesis.
5.3 Results

5.3.1 Identification of HS-expressing cells in the murine thymus

Earlier studies have demonstrated that TEC express high level of cell surface HS (Britz and Hart, 1983) with 6-O-sulfate groups being abundant in the highly sulfated, heparin-like regions of the HS molecules (Werneck et al., 1999). Here, the expression of HS in murine thymus was examined in situ via immunohistochemistry of deparaffinised thymic sections labelled with an anti-HS (clone 10E4) mAb. Indeed, HS was weakly but ubiquitously expressed throughout the cortex and was less evident in the medulla. Strikingly, HS appeared to intensely label ‘rosette-like structures’ that were found exclusively in the thymic cortex. These preformed rosettes were approximately 40 µm in diameter and consisted of a central HS+ staining cells containing and surrounded by multiple (>20 cells/rosette), small (<10 µm) HS− cells that were presumably thymocytes (FIG. 5.1). At face value, these rosettes resemble TEC-rosettes (Oliveira-dos-Santos et al., 1997, Oliveira-dos-Santos et al., 1998) and TNC (Wekerle and Ketelsen, 1980, Wekerle et al., 1980, Nakagawa et al., 2012).

The possibility that the HS-expressing cells within rosettes (FIG. 5.1) were cTEC was examined by flow cytometry. Pooled thymic fragments were enzymatically digested with collagenase/dispsase to release the stromal cells before being subjected to unit gravity sedimentation on a cushion of FCS for 1 hr, the large preformed rosettes pelleting at the bottom of the tube (Kyewski et al., 1982, Oliveira-dos-Santos et al., 1997). Light microscopy analysis revealed large rosettes averaging ~20-40 µm in diameter alongside smaller non-rosetting cells (i.e., 10-20 µm in diameter) (FIG. 5.2Ai). The published literature defines TEC as EpCAM+CD45− cells (Gray et al., 2002, McLelland et al., 2011, Wong et al., 2014). In this study, however, exclusion of the CD45+ population from EpCAM+ cells was deliberately omitted to ensure that CD45+ thymocyte-containing rosettes were not depleted. Indeed, the sedimented cells were found to be a mixed cell population, a substantial proportion being EpCAM+ TEC (32.5%), which could be subdivided into cTEC (i.e., Ly51interUEA-1lo cells) and mTEC (i.e., Ly51bUEA-1hi cells) (51% and 21.4% of EpCAM+ cells, respectively) (FIG. 5.2B top panel). Moreover, cTEC could be further subdivided into Ly51inter and Ly51hi (i.e., cTEChi) populations (42.5% and 8.5% of TEC, respectively).
FIG. 5.1: **Immunohistochemical detection of cells expressing high levels of heparan sulfate (HS) in the cortex of the mouse thymus.** Thymic sections (deparaffinised) from a 5 week old C57BL/6 mouse were incubated with an anti-HS mAb (clone 10E4) or an isotype control (mouse IgM), bound mAbs being detected by a HRP-conjugated secondary Ab and subsequent development with the AEC substrate with one cell being enlarged (*inset panel*, scale bar: 20 µm). Arrows indicate the location of high HS-expressing cells in the thymic cortex and not in the thymic medulla. Thymic sections were counterstained with haematoxylin. Scale bars: 250 µm (top panel) and 50 µm (bottom panel).

Next, the expression of cell surface HS by sedimented preformed rosettes and thymic stromal cells was evaluated by flow cytometry using the anti-HS (10E4) mAb. Thymic stromal cells were manually enriched via magnetic-activated cell sorting (MACS) of anti-EpCAM mAb labelled cells from enzymatically-digested thymic fragments, as described above. Light microscopy revealed smaller preformed rosettes present alongside non-rosetting cells in the MACS-enriched cell suspensions (FIG 5.2Aii). The alacrity of TEC to form multicellular complexes with thymocytes most likely reflects the unique role of TEC in intrathymic thymocyte development (Oliveira-dos-Santos et al., 1998, Hare et al., 2003, Hendrix et al., 2010, Nakagawa et al., 2012). Thus, the exclusion of CD45+ T lymphocytes from MACS-enriched EpCAM+ TEC preparation was deliberately avoided here and in subsequent experiments so to include functioning TEC, namely those rosetting with exogenous thymocytes *in vitro.*
FIG. 5.2: Identification of different subsets of thymic stromal cells. Thymic fragments from 4 thymuses of 3 week old mice were repetitively digested with collagenase/dispase. The heavier and denser pre-formed rosetting cells were sedimented at unit gravity on a FCS layer for 1 hour. Alternatively, EpCAM-expressing stromal cells were enriched via positive selection by magnetic-activated cell sorting (MACS) after the cells were labelled with biotinylated anti-EpCAM mAb linked to Streptavidin-Microbeads. (A) Bright field images illustrate thymic stromal cells that were enriched by either unit (i) gravity sedimentation or (ii) by MACS. Arrows indicate the presence of pre-formed multicellular conjugates. (B) Characterisation of enriched thymic stromal cells by flow cytometry. After discriminating viable cells from dead cells and cellular debris (P1), PE-Cy7-EpCAM labelled stromal cells were gated (P2) and separated into different subsets based on FITC-UEA-1 lectin binding and Ly51 cell surface expression (P3-P6). TEC = thymic epithelial cells. Subsets: non-TEC (P3) = Ly51<sup>lo</sup>UEA-1<sup>hi</sup>; medullary-TEC, mTEC (P4) = Ly51<sup>lo</sup>UEA-1<sup>hi</sup>; cortical-TEC, cTEC (P5) = Ly51<sup>int</sup>UEA-1<sup>lo</sup>; cTEC<sup>hi</sup> (P6) = Ly51<sup>hi</sup>UEA-1<sup>lo</sup>. Percentage of cells in different gates indicated in dot plots.
Indeed, 98% of EpCAM⁺ TEC were successfully enriched via MACS using this approach, and were subdivided into cTEC, mTEC and non-TEC (i.e., Ly51’UEA-1’) populations representing 61%, 3% and 31.6% of the EpCAM⁺ cells, respectively (FIG. 5.2B lower panel). Strikingly, the 10E4 mAb strongly labelled cell surface HS on both the sedimented (FIG. 5.3A) and the MACS-enriched (FIG. 5.3B) mTEC and cTEC populations. Furthermore, the sedimented preformed rosettes present in the cTEC⁺ subpopulation, which most likely correspond to TNC, expressed the highest HS levels, whereas the non-TEC population (FIG. 5.2B lower panel) expressed little cell surface HS (FIG. 5.3C).

**FIG. 5.3:** Heparan sulfate is highly expressed by thymic stromal cells. Representative fluorescence histograms depicting the background autofluorescence (grey histogram) and the binding of the anti-HS (10E4) mAb (open histogram) to thymic stromal cells enriched by (A) 1 g sedimentation and (B) MACS as in FIG. 5.2. (C) Comparison of the expression of HS on the surface of various thymic stromal cell subsets, indicated by GMFI of anti-HS mAb-labelled cells minus the respective GMFI background autofluorescence in (A) and (B). Thymic stromal cells subset labelling as defined in FIG. 5.2B. Data in (C) are mean GMFI ± SD of triplicate samples.
Chapter 5

5.3.2 The molecular basis of cTEC-thymocyte rosetting

In Chapter 3 it was demonstrated that HS on the surface of erythrocytes facilitates autorosetting with DP thymocytes. Based on our findings that HS expression is evident in the thymic cortex where preformed thymic rosettes were prominent (FIG. 5.1), that cell surface HS is abundantly expressed by cTEC (FIG. 5.3) and that DP thymocytes, the main autorosetting population (FIG. 3.4), inhabit the thymic cortex alongside cTEC, it was hypothesised that cTEC can rosette with thymocytes ex vivo via the CD8-HS interaction. To test this hypothesis, the flow cytometry based autorosetting assay described in Chapter 3 was adapted to monitor rosetting between MACS-enriched thymic stromal cells and thymocytes in vitro.

MACS-enriched stromal cells pre-labelled with fluorescently-conjugated anti-EpCAM and anti-Ly51 mAbs and lectin UEA-1 to delineate the different TEC subsets (FIG. 5.2B), were mixed with CPD$^{670}$ or CTV-labelled thymocytes (FIG. 5.4A), rosetting with TEC being depicted by the TEC acquiring the fluorescence profiles (CPD$^{670+}$ or CTV$^+$) of the labelled thymocyte, the rosetting threshold being the binding of ≥3 thymocytes per stromal cell. The cell mixtures were immediately analysed to determine %coincidence values or induced to rosette by brief centrifugation (200 g for 1 min) and incubation for 1 hr on ice before %uncorrected rosetting values were measured by flow cytometry (FIG. 5.4B). The true %rosetting values were then calculated by subtracting %coincidence (%C) from %uncorrected rosetting values (%R$_{raw}$). As predicted, cTEC, which express high levels of cell surface HS displayed a much higher level of rosetting with thymocytes (79.2%) than non-TEC (9.9%) that carry little cell surface HS (FIG. 5.4C).

In Chapter 3 it was shown that CD8 binds to HS to mediate autorosetting, whereas an earlier study implicated CD4 molecules in TEC-thymocyte rosetting in vivo (Oliveiras-dos-Santos et al., 1998). Thus, CD8α.KO, CD8β.KO, CD4.KO, and CD4/CD8β.KO thymocytes were tested for their ability to rosette with MACS-enriched cTEC, both in terms of the percentage of cTEC rosetting with thymocytes and the number of thymocytes bound per cTEC. Compared to WT thymocytes, rosetting by CD8β.KO thymocytes was partially and significantly reduced, whereas CD8α.KO thymocytes were the weakest to rosette with cTEC, thus confirming that CD8α and CD8β markedly
FIG. 5.4: **Fluorometric assay for detection of thymocytes rosetting with thymic stromal cells.** Equal volumes (50 µL) of MACS-enriched stromal cells (5x10^6 cells/mL) and CPD_{670}-labelled thymocytes (5x10^7 cells/mL) were mixed and either immediately analysed by flow cytometry to measure ‘coincidence’ or induced to rosette by brief centrifugation (200 g, 1 min) and incubation on ice for 60 min, prior to evaluation by flow cytometry, i.e., stromal cells acquiring CPD_{670} fluorescence. (A) Gating of rosetting thymic stromal cells. After discriminating viable cells from dead and cellular debris (P1), PE-Cy7-EpCAM labelled stromal cells were identified (P2) and subdivided into non-TEC (P3) and cTEC (P4) populations based on binding of the UEA-1 lectin and Ly-51 cell surface expression, as defined in FIG. 5.2B. (B) Representative fluorescence histograms depicting rosetting (open black histograms) and coincidence (grey histograms) for non-TEC and cTEC. Fluorescence histogram (open red) of CPD_{670} thymocytes alone is depicted. Gate includes thymic stromal cells associating with ≥3 thymocytes. True rosetting values represent %R_{raw} - %C, i.e., non-TEC-thymocyte rosetting (15.3% - 0.2%) = 15.1%; cTEC-thymocyte rosetting (82.7% - 3.5%) = 79.2% in example shown. (C) Percentage (%) of rosetting non-TEC and cTEC, with data being mean % rosetting ± SD of triplicate samples.
FIG. 5.5: Thymocyte CD4 and CD8 molecules, cTEC MHC and sulfated polysaccharides contribute to rosetting between cTEC and syngeneic thymocytes. All data presented as either percentage of cTEC rosetting with thymocytes (left y-axis) or the number of thymocytes bound per cTEC (right y-axis). 

(A) Comparison of the ability of WT, CD4.KO, CD8β.KO, CD4/CD8β.KO and CD8α.KO thymocytes to rosette with WT cTEC. 

(B) Comparison of the ability of the sulfated polysaccharides heparin and DxS (5 and 500 kDa, 100 µg/mL, pre-incubated for 30 min) to inhibit rosetting between cTEC and thymocytes. NS, not significant. **, *P* < 0.01. ***, *P* < 0.001 (1-Way ANOVA with Tukey’s multiple comparisons test). 

(C) Comparison of the ability of WT and MHC-I/II.KO cTEC to rosette with WT thymocytes, in the absence or presence of heparin (100 µg/mL). NS, not significant. **, *P* < 0.01. ***, *P* < 0.001 (1-Way ANOVA with Dunnett’s multiple comparisons test for within strains and 2-Way ANOVA with Bonferroni multiple comparisons test between WT and MHC-I/II.KO strains). 

Rosetting assays performed as in FIG. 5.4. Number of thymocyte bound per cTEC determined by dividing median fluorescence CPD$^{670^\circ}$ cTEC (gated to include ≥1 thymocyte per cTEC) with GMFI CPD$^{670^\circ}$ of individual thymocyte. Data are mean % cTEC rosetting and median number of thymocyte bound cTEC ± SD of triplicate samples.
FIG. 5.6: Synergistic contributions of MHC and HS on cTEC to thymocyte rosetting capacity. (A) Representative fluorescence histograms depicting number of thymocytes (3 - ≥10) rosetting with WT (black line) and MHC-I/II.KO (red line) cTEC, in the absence (top panel) or presence of heparin (100 µg/mL) (bottom panel). Gate depicts cTEC binding ≥3 thymocytes, with % of WT (black values) or MHC-I/II.KO (red values) cTEC rosetting values indicated. Thymocytes were pre-incubated with heparin (100 µg/mL) on ice for 30 min before being examined for rosetting with cTEC. (B) Percentage of WT (black line) or MHC-I/II.KO (red line) cTEC rosetting with 3-≥10 thymocytes in the absence (Control) or presence of heparin (100 µg/mL). NS, not significant. *, P < 0.05. **, P < 0.01. ***, P < 0.001. ****, P < 0.0001 (2-Way ANOVA with Bonferroni multiple comparisons test between WT and MHC-I/II.KO strains). Data are mean % cTEC rosetting ± SD of triplicate samples.
contribute to thymocytes-cTEC rosetting. Despite CD4.KO thymocytes rosetted normally with cTEC, the extent of rosetting by CD4/CD8β.KO thymocytes was significantly lower than CD8β.KO thymocytes and comparable to the rosetting by CD8α.KO thymocytes (FIG. 5.5A). Collectively, these data indicate that CD8β is a key rosetting receptor on thymocytes, with CD8α and CD4 making relatively minor contributions to the interaction.

As the treatment of MACS-enriched stromal cells with HS-degrading enzymes (i.e., HPSE and HPNSE) resulted in low viability of the treated cells (data not shown), the direct contribution of HS on the cTEC surface to cTEC-thymocyte rosetting could not be assessed. The sulfated polysaccharides heparin and Dxs (5 and 500 kDa) were shown in Chapter 3 to mimic HS by binding to CD8 and inhibiting erythrocyte autorosetting (FIG. 3.8A and FIG. 3.10). Hence, the role of HS in cTEC-thymocyte rosetting was indirectly examined based on rosetting inhibition by these HS mimetics. Thymocytes were pre-treated heparin and Dxs (5 and 500 kDa) at 100 µg/mL before being induced to rosette with MACS-enrich stromal cells. Remarkably, Dxs500 kDa almost completely inhibited cTEC-thymocyte rosetting, whereas heparin and Dxs5 kDa were partially and significantly inhibitory (FIG. 5.5B). These data are consistent with the hypothesis that HS is the key ligand facilitating cTEC-thymocyte rosetting.

The MHC molecules on stromal cells have been previously implicated in the multicellular conjugation between TEC and thymocytes (Bousso et al., 2002, Hare et al., 2003). Also, earlier observations from the Parish Laboratory have demonstrated that MHC can regulate autorosetting (Sia and Parish, 1980a, Sia and Parish, 1980b). Thus, to investigate if MHC molecules on the cTEC surface contribute to cTEC-thymocyte rosetting, the extent of rosetting between WT (i.e., cTECWT) or MHC class I and II-deficient cTEC (i.e., cTECMHC-I/II.KO) and thymocytes was evaluated. It was found that cTECW and cTECMHC-I/II.KO were equally effective at forming rosettes with thymocytes, implying that MHC plays a minor role in cTEC-thymocyte rosetting. In parallel, thymocytes were pre-treated with heparin (100 µg/mL) before being induced to rosette with cTECW or cTECMHC-I/II.KO to evaluate the contribution of MHC to the process when cTEC HS recognition is blocked. Strikingly, in the presence of heparin, the percentage of cTECMHC-I/II.KO rosetting with thymocytes was substantially lower than with cTECW, being 17.3% and 34.5% rosetting, respectively (P < 0.01) (FIG. 5.5Ci).
Furthermore, a detailed cytofluorometric analysis revealed that the absence of MHC on cTEC reduced the number of thymocytes bound per cTEC despite cTEC\textsuperscript{WT} and cTEC\textsuperscript{MHC-I/II.KO} exhibiting a comparable percentage of cTEC rosetting with thymocytes (FIG. 5.5Cii 5.6). Similarly, the presence of heparin reduced the number of thymocytes bound per cTEC, this effect being further enhanced by the absence of MHC on cTEC (FIG. 5.5Cii and 5.6). Collectively, these results corroborate the finding that HS is the key rosetting ligand on cTEC, with the expression of MHC on cTEC stabilising thymocyte binding.

5.3.3 Functional significance of cTEC-thymocyte rosetting

In addition to MHC aiding thymocytes adhesion to TEC, it is also generally believed that MHC is responsible for the Ca\textsuperscript{2+} mobilisation observed in thymocytes interacting with cTEC during positive selection (Hare et al., 2003, Bhakta et al., 2005). However, data from previous studies (Tellam and Parish, 1987, Weston et al., 1991) and recent observations (Chapter 4) indicate that HS mimetics are able to trigger a sustained Ca\textsuperscript{2+} flux in DP thymocytes that is CD8-dependent. Hence, with HS and MHC on the cTEC surface and CD8 molecules on thymocytes contributing to cTEC-thymocyte rosetting, the relative importance of cTEC HS and MHC in the induction of a Ca\textsuperscript{2+} flux in thymocytes bound to cTEC was examined. To answer this question, CTV-labelled thymocytes were pre-loaded with intracellular Ca\textsuperscript{2+} indicator dyes (Fura-Red and Fluo-4) before being induced to rosette with WT or MHC-I/II.KO MACS-enriched stromal cells at 4°C or 37°C, the increase in intracellular Ca\textsuperscript{2+} being detected by elevated Fura-Red/Fluo-4 ratios monitored by flow cytometry.

Initially, it was found that rosetting of thymocytes with cTEC\textsuperscript{WT} and cTEC\textsuperscript{MHC-I/II.KO} occurred rapidly (<5 min) and at comparably high levels at both 4°C and 37°C, thus confirming that cTEC-thymocyte rosetting is primarily an energy-independent process (FIG. 5.7A).

As expected, intracellular Ca\textsuperscript{2+} levels remained low in non-rosetting thymocytes both at 4°C and 37°C, providing the baseline Ca\textsuperscript{2+} level for thymocytes (FIG. 5.7B). Interestingly, rosetting thymocytes displayed a noticeable and sustained increase in their intracellular Ca\textsuperscript{2+} levels following 5, 10 and 30 min incubation at 4°C, a response that was markedly augmented at 37°C.
FIG. 5.7: **Rosetting between thymocytes and cTEC induces a prolonged Ca\(^{2+}\) flux in thymocytes.** (A) Analysis of rosetting between thymocytes and WT and MHC.KO cTEC after co-incubation for 5, 10 and 30 min, at 4°C and 37°C, respectively. Thymocytes (Fluo-4/Fura-Red-loaded, CTV-labelled) and EpCAM\(^{+}\)-enriched thymic stromal cells (4°C or 37°C) were mixed at a 10:1 thymocytes/stromal cell ratio and immediately examined by flow cytometry or induced to rosette, at 4°C or 37°C, respectively. Following 5, 10 and 30 min co-incubation, % thymocytes rosetting with cTEC was quantified by flow cytometry as in FIG. 5.4. Data are mean % cTEC rosetting ± SD of triplicate samples. (B) Analysis of Ca\(^{2+}\) flux in thymocytes rosetting with WT and MHC-I/II.KO cTEC after co-incubation for 5, 10 and 30 min, at 4°C and 37°C, respectively. At each time point, a 30 sec snapshot of Ca\(^{2+}\) flux, depicted by real time GMFI ratios of Ca\(^{2+}\)-bound Fluo-4 (530 nm) over Ca\(^{2+}\)-unbound Fura-Red (695 nm) in rosetting and non-rosetting thymocytes, was measured by flow cytometry. Data are GMFI Fluo-4/Fura-Red ratios ± SD of triplicate samples. (C) Effect of heparin (100 µg/mL) on Ca\(^{2+}\) flux in thymocytes bound to WT and MHC-I/II.KO cTEC after 10 min co-incubation at 37°C. (D) Analysis of Ca\(^{2+}\) flux in WT and CD8α.KO thymocytes bound to WT and MHC.KO cTEC after 10 min co-incubation at 37°C. Data are GMFI Fluo-4/Fura-Red ratios ± SD of triplicate samples. In (C) and (D) *, P < 0.05. **, P < 0.01. ***, P < 0.001. ****, P < 0.0001 (1-Way ANOVA with Tukey multiple comparisons test for within strains and 2-Way ANOVA with Bonferroni multiple comparisons test between WT and MHC-I/II.KO strains).
Furthermore, the overall magnitude and kinetics of the intracellular Ca\(^{2+}\) rise was accentuated in thymocytes rosetting with cTEC\(^{WT}\), compared to cTEC\(^{MHC-I/II.KO}\) (FIG. 5.7B), implying that MHC not only aids rosetting, but substantially enhances Ca\(^{2+}\) mobilisation in rosetting thymocytes.

Since CD8 and HS (FIG. 5.5A and B) are key mediators of cTEC-thymocyte rosetting, their contribution to the Ca\(^{2+}\) flux in thymocytes bound to either cTEC\(^{WT}\) or cTEC\(^{MHC-I/II.KO}\) for 10 min at 37°C was evaluated. The addition of heparin (100 µg/mL) significantly reduced the Ca\(^{2+}\) flux in thymocytes bound to both cTEC\(^{WT}\) and cTEC\(^{MHC-I/II.KO}\), consistent with HS making a contribution to the rosette-mediated Ca\(^{2+}\) flux that is independent of the MHC (FIG. 5.7C). Moreover, the lack of CD8 on thymocytes resulted in these cells giving a markedly reduced Ca\(^{2+}\) flux upon rosetting with both cTEC\(^{WT}\) and cTEC\(^{MHC-I/II.KO}\), indicating that CD8 can act as both a key thymocyte rosetting receptor and a key contributor to the rosette-induced Ca\(^{2+}\) flux, independent of the contribution of the MHC to the Ca\(^{2+}\) flux (FIG. 5.7D). Collectively, these data indicate that the CD8-HS interaction plays a key role in initiating and sustaining the Ca\(^{2+}\) flux observed in thymocytes following their rosetting with cTEC.

5.4 Discussion

In previous Chapters it was shown that the CD8-HS interaction mediates the autorosetting phenomenon observed between DP thymocytes and erythrocytes (Chapter 3) and that the ligation of CD8 by a HS mimetic triggers a sustained Ca\(^{2+}\) flux that lowers the threshold for TCR-mediated activation of DP thymocytes (Chapter 4). However, the physiological relevance of this CD8-HS interaction in the development of thymocytes needed to be clarified. In this Chapter the key findings were: (i) HS is ubiquitously expressed in the thymic cortex, particularly by multicellular complexes resembling TNC, (ii) flow cytometry studies established that cTEC express high levels of cell surface HS and revealed that these cells very efficiently rosette with thymocytes \textit{ex vivo}, (iii) rosetting between cTEC and thymocytes is substantially blocked by HS mimetics and, based on studies with KO mice, is dependent on CD8β and, to lesser extent, CD8α and CD4 expression by thymocytes and on MHC on the surface of cTEC and, finally, (iv) cTEC-thymocyte rosetting triggers a sustained Ca\(^{2+}\) flux in the rosetting thymocytes that is HS, CD8 and MHC-dependent.
Initial \textit{in situ} analysis of murine thymic sections revealed the presence of HS in the cortex and intense labelling of multicellular structures (FIG. 5.1), resembling TNC (Wekerle and Ketelsen, 1980, Wekerle et al., 1980, Kyewski et al., 1982, Li et al., 1992, Oliveira-dos-Santos et al., 1997, Oliveira-dos-Santos et al., 1998, Nakagawa et al., 2012), which have been previously reported to express high levels of highly sulfated cell surface HS (Werneck et al., 2000). Subsequent phenotypic studies of preformed rosettes in thymocyte suspensions sedimented at unit gravity indicated that they predominantly contain cTEC. However, the enrichment of preformed rosettes by the sedimentation method is relatively non-selective, which may explain why only 32% of the sedimanted cells were epithelial (EpCAM$^+$) cells, cTEC being enriched alongside larger and denser non-rosetting cells and also rosettes formed by non-cTEC stromal cells, including mTEC and also non-TEC, such as thymic DC and macrophages (Brelinska et al., 1986, Shortman et al., 1989) (FIG. 5.2). Also, thymocytes released from preformed rosettes could lead to dilution of EpCAM$^+$ cells in the cell suspensions.

Importantly, flow cytometric examination of preformed rosettes by mTEC and cTEC, particularly cTEC$^{hi}$ revealed high expression of cell surface HS (FIG. 5.3C). Presumably, cTEC$^{hi}$ represent the multicellular structures present in the thymic cortex that stain strongly for HS and resemble TNC (FIG. 5.1 \textit{lower left panel}), although this needs further experimental confirmation.

It has been reported previously that the HS produced by cTEC abundantly express 6-O-sulfate groups in its highly sulfated regions (Werneck et al., 1999) and in Chapter 3 it was demonstrated that the 6-O-sulfate groups of heparin are essential for heparin to inhibit CD8-mediated autorosetting (FIG. 3.8D). Such findings are consistent with CD8 on DP thymocytes binding to HS rich in 6-O-sulfate groups on cTEC. Indeed, the data shown in this Chapter reveal a strong link between high levels of surface HS on cTEC (FIG. 5.3) to the ability of cTEC to rosette with thymocytes \textit{ex vivo} ($\sim$80% rosetting), in an energy-independent manner (4°C) (FIG. 5.7A), as opposed to non-TEC, in which the low levels of surface HS is associated with low rosetting values ($<10$% rosetting) (FIG. 5.4C). Unfortunately, the direct contribution of surface HS on cTEC to cTEC-thymocyte rosetting could not be determined, as HPSE and HPNSE treatments appeared detrimental to TEC survival (data not shown). However, the striking ability of HS mimetics to inhibit cTEC-thymocyte rosetting, particularly the total inhibition of rosetting by DxS$^{500}$kDa, indirectly implicates HS as a key player in the process (FIG.
Heparin and DxS\(^{5\text{kDa}}\) were only partially inhibitory probably due to their lower molecular weight resulting in a lower ability to bind multivalently to CD8 molecules on thymocytes than DxS\(^{500\text{kDa}}\) and compete against an array of highly charged HS chains present on cTEC surfaces. Future studies should be aimed at modifying the structure of HS on cTEC by genetic means, especially preventing the 6-O-sulfation of HS chains, and observing the impact that this has on cTEC-thymocyte rosetting.

Whilst earlier reports have shown that MHC molecules expressed at high levels by cTEC (Wekerle and Ketelsen, 1980, Yang et al., 2006) are crucial for cTEC to form multicellular conjugates with thymocytes (Hare et al., 2003), in this Chapter it was demonstrated that HS-mediated rosetting could occur in the absence of MHC molecules (FIG. 5.5Ci). The presence of MHC, however, markedly accentuated the number of thymocyte bound to each cTEC, thus establishing the additive contributions of HS and MHC on cTEC to expanding thymocyte rosetting capacity (FIG. 5.5Cii and 5.6). Thus, it appears likely that MHC stabilises rosettes via peptide-loaded MHC interacting with cognate TCR or non-cognate CD4/CD8 molecules on thymocytes, with cTEC HS securing rosettes by interacting with CD8 on thymocytes. In fact, an attractive hypothesis is that the CD8-HS interaction ensures that DP thymocytes are selectively targeted to cTEC, the subsequent TCR-MHC interactions with cTEC determining T cell fate.

In addition to cTEC MHC recognition by TCR, it is also possible that other thymocyte cell surface receptors could bind HS and contribute to cTEC-CD8\(^{\alpha\text{KO}}\) thymocyte rosetting (FIG. 5.5A). Speculatively, these molecules could be CD2 (Parish et al., 1988b), CD4 (Parish et al., 1990, Parish and Warren, 1991) CD45 and CD90 (Parish et al., 1988a), all four molecules having been shown to react with polyanions resembling HS. Also, the cell adhesion molecules CD44 and LFA-1 have been previously implicated in thymic rosetting in vivo (Oliveira-dos-Santos et al., 1998).

In Chapter 4 the functional significance of the CD8-HS interaction was investigated and shown to activate a sustained Ca\(^{2+}\) flux in DP thymocytes, this process being associated with a lowering of the threshold required for TCR mediated activation. In this Chapter, in addition to their substantial role in cTEC-thymocyte rosetting, HS (FIG. 5.7C) and CD8 (FIG. 5.7D) were shown to contribute to the induction of a Ca\(^{2+}\) flux in thymocytes.
bound to cTEC (FIG. 5.7B). Heparin, however, unlike DxS_{500kDa} could only partially block cTEC-thymocyte rosetting (FIG. 5.5B), which may explain why there was still a considerable Ca^{2+} flux in the presence of heparin. Thus, the impact of altering the structure of cTEC HS, as discussed above, is paramount to unequivocally establishing the contribution of cTEC HS to rosette-mediated Ca^{2+} mobilisation. Similarly, whilst CD8 plays a key role in thymocyte adhesion to cTEC, other thymocyte cell surface molecules, as described above, which could bind HS may be responsible for the residual Ca^{2+} flux observed in rosetting CD8α.KO thymocytes.

In summary, the experimental data presented in this Chapter establishes the physiological relevance of the CD8-HS interaction in the heterotypic interaction between cTEC and DP thymocytes in the thymic cortex during the process of MHC-restricted positive selection. Our current *in vitro* rosetting model proposes that CD8β and, to a lesser extent, CD8α on thymocytes interact with HS on cTEC to initiate thymocyte adhesion to cTEC. In parallel, MHC on cTEC stabilises the interaction, presumably via TCR and CD8/CD4 recognition. Both MHC and HS recognition on cTEC independently initiate rises in intracellular Ca^{2+} concentrations in DP thymocytes, the CD8-HS interaction being particularly effective in this regard. Combined with data presented in Chapter 4 demonstrating that the CD8-HS interaction lowers the TCR-mediated activation threshold in thymocytes, future studies should be aimed at confirming that this interaction controls selection of the TCR repertoire in DP thymocytes.
Chapter Six

Final Discussion and Future Directions

This Chapter provides a summary and a general discussion of the key findings described in Chapters 3, 4 and 5 and how this information is likely to fit into current concepts of T lymphocyte development and function, and impact on future research directions.
Chapter 6

6.1 Introduction

HS is a highly acidic linear polyanionic carbohydrate with a very variable structure. It is ubiquitously expressed on cell surfaces and in the ECM/BM of tissues. Synthesised attached to various core proteins to form HSPGs, HS is able to interact with various proteins and exert diverse functions. In Chapter 1 a bioinformatic analysis of mammalian proteins that express a heparin/HS-binding motif and are associated with the immune system identified 235 candidate proteins, a noticeable proportion located intracellularly. This simple analysis suggests HS interacts with many more components of the immune system than previously realised. Numerous studies have also directly shown that HS plays multiple prominent functional roles in the immune system, these include its ability to regulate leukocyte development, leukocyte migration, immune activation and inflammatory processes (Simon Davis and Parish, 2013). This Thesis provides evidence that HS also plays an important role in T cell development in the thymus.

In summary, the key findings of this Thesis are:

a) The CD8 heterodimer on DP thymocytes binds to highly sulfated (particularly 6-O-sulfate rich) HS on cell surfaces, mainly via the CD8β chain of the CD8 molecule. Developmentally linked α2-3 sialylation of CD8 on CD8 SP thymocytes and peripheral CD8+ T lymphocytes inhibits this interaction, with circulating HRG further regulating the CD8-HS interaction in the periphery by masking cell surface HS – Chapter 3.

b) HS-like molecules trigger a sustained, CD8-, Slp76-dependent and Zap70-independent, influx of extracellular Ca²⁺ into DP thymocytes but not other thymic or peripheral T cell populations, this influx being accompanied by a lowering of the anti-CD3-mediated activation threshold of the DP thymocytes. Enzymatic desialylation, however, restores the HS-induced Ca²⁺ flux in peripheral CD8+ T lymphocyte, a population that otherwise is non-responsive to HS – Chapter 4.

c) Collectively, these data indicate that the HS-CD8 interaction facilitates the adhesion of DP thymocytes to cTEC and initiates a Ca²⁺ flux in the adherent thymocytes that lowers the threshold required for positive selection of DP thymocytes. – Chapter 5.
6.2 CD8-HS interaction: important structural consideration

6.2.1 Location of HS binding site on CD8 molecule
To the best of our knowledge, this study is the first to reveal a functional relationship between CD8 molecules and cell surface HS. This is rather an unexpected finding as CD8β is absent from the list of hypothetical heparin/HS-binding proteins identified by a bioinformatic approach (Chapter 1, Simon Davis and Parish, 2013), although HS-binding sites that are dependent on the tertiary structure of proteins rather than linear amino acid sequences would not be detected by this approach (Table. 1.1). However, a lysine-rich and possible ‘HS-binding’ motif is located in the CD8β stalk region and potentially could provide the required electrostatic charge to bind HS, i.e., sequence 146-156, motif: KKTLKKMKKK (mouse) and sequence 146-156: KKSTLKKKRVC (human). The mouse sequence of basic residues would not have been detected by the bioinformatic search that was undertaken in Chapter 1. Also, it should be noted that CD8β is positioned proximal to the TCR and the location of the putative ‘HS-binding’ motif in the stalk region means it is independent of the MHC-Iα3 binding domain of CD8β (Wang et al., 2009, Rettig et al., 2009) and consistent with CD8β being able to bind HS and MHC-I simultaneously. Moreover, CD8α very weakly binds HS, possibly due to the lack of apparent clusters of basic residues to suggest a potential HS-docking site. Hypothetically, basic residues in the stalk of CD8α could contribute to the CD8β HS binding site or, in CD8β deficient mice, form a weak HS binding site in the CD8αα homodimer. Only solving the 3D-structure of a desialylated CD8αβ-HS complex will resolve this intriguing issue.

6.2.2 Sialylation of the CD8 molecule
The stalk region of the CD8β chain on mature T lymphocytes is heavily O-glycosylated at multiple sites and this is thought to control its coreceptor functions (Rudd et al., 2001). The ST3Gal-1 sialyltransferase catalyses the α2-3 sialylation of core 1- O-glycan Galβ1-3GalNAc structures, converting them into Siaα2-3Galβ1-3GalNAc glycoconjugates (Pereira et al., 1976, Kono et al., 1997, Moody et al., 2003). The developmentally controlled α2-3 O-sialylation of CD8β involves the expression of the non-sialylated CD8β glycoform on DP thymocytes and the sialylated form on CD8 SP and peripheral CD8+ T lymphocytes, an expression pattern that strongly correlates with the reduced affinity of CD8αβ for MHC-I molecules (Moody et al., 2001, Moody et al., 2003, Daniels et al., 2001). In this study, the pattern of O-sialylation of developing
Chapter 6

thymocytes matched these earlier reports, based on differential PNA (specific for exposed Galβ1-3GalNAc residues) and MAL-II (specific for α2-3 sialic acid residues) labelling of thymocyte subsets and peripheral CD8⁺ T lymphocytes (FIG. 3.11A and B), with sialylation strongly correlating with decreased HS binding by CD8 SP thymocytes and peripheral CD8⁺ T lymphocytes (FIG. 3.11C and D). Thus, α2-3 sialylation of CD8β confers a developmental switch on the CD8β-HS interaction, probably the negative charge of the attached sialic acid residues interfering with the recognition of the negatively charged HS. Furthermore, ST3Gal-1 is known to catalyse α2-3 sialylation of core 1- O-glycans attached to threonine/serine residues in the stalk region of the CD8β chain i.e., sequence 141-149: TTAPTKKT (mouse) and sequence 140-149: TTAQPTKKST (human), that overlap the predicted HS-binding motif.

In the periphery ST3-Gal-1-catalysed α2-3 O-sialylation of CD8αβ raises the threshold required to activate peripheral CD8⁺ T lymphocytes. During an immune response, however, it is possible that O-glycosialylation is reversed via cessation of ST3Gal-1 function or via sialidase-mediated desialylation of core 1-O-glycans (FIG. 3.11A and B). Such a process would restore the ability of CD8αβ to bind to both MHC-I (Priatel et al., 2000) and HS (FIG. 4.11 and FIG. 3.12), hence lowering the activation threshold required for T cell activation. However, when the immune stimulus declines, ST3Gal-1 catalytic function could be resumed to prevent cell death via apoptosis. In fact, it has been proposed that the resialylation of the CD8αβ heterodimer, combined with other signals, such as IL-7 (Kimura et al., 2013), promotes the survival and differentiation of peripheral CD8⁺ T lymphocytes into memory T cells (Priatel et al., 2000).

Plasma HRG is abundant in the circulation (~100-200 µg/ml) and can strongly bind to HS (Lane et al., 1986, Lijnen and Collen, 1989, Poon et al., 2011). Also, HRG is a negative acute phase protein and, thus, its normal plasma concentration plummets during inflammation and recovers to normal physiological levels as inflammation subsides (Saigo et al., 1990). HRG readily inhibits CD8-mediated autorosetting (FIG. 3.14) by masking HS on erythrocytes suggesting that it has a higher affinity for HS than the CD8αβ molecule. Thus, in addition to the regulatory role of CD8 O-sialylation, HRG may also prevent CD8 interacting with HS, particularly on APCs, particularly in the absence of immune activation. In the event of T cell activation during an infection, however, CD8αβ could be desialylated and HRG levels drop, thus, the ability of the
CD8αβ heterodimer to recognise HS and MHC-I is restored. Conversely, as the immune stimulus subsides, it is likely that increasing concentrations of circulating HRG prevent HS from binding to the ‘HS-binding’ motif of CD8. Thus, a combination of ST3-Gal-1 catalysed α2-3 sialylation of the CD8αβ heterodimer and the masking of cell surface HS on APCs by HRG ensures the tight regulation of CD8-HS interaction in the periphery.

6.2.3 Does CD4 interact with HS?

It is unclear whether CD4 binds HS although it has been reported that CD4 carries a prominent polyanion-binding pocket (Parish and Warren, 1991). Certainly, CD4 deficiency had no effect on the autorosetting of DP thymocytes (FIG. 3.5C) or the binding of FITC-heparin to thymocytes (FIG. 3.10), although any effect of CD4 could be masked by the strong interaction of CD8 with HS. In fact, CD4 appeared to contribute to HS-mediated TEC-thymocytes rosetting in the absence of CD8β (FIG. 5.5A). Thus, it is possible that CD4 binds HS very weakly in a similar manner to the CD8α-HS interaction. A provocative hypothesis is that CD8αβ binds to linear sequences of monosaccharides in HS chains, analogous to an endoglycosidase, whereas CD4 recognises terminal sugars of HS chains, analogous to exoglycosidases (Parish C.R. pers. comm.). If this hypothesis is correct there would be much fewer binding sites for CD4 than for CD8 in cell surface HS or in soluble heparin, which may explain the weaker interaction of CD4 with these molecules. Tetramers of heparin, prepared with heparin biotinylated at the reducing terminus and fluorescent streptavidin, could be used to test this hypothesis, i.e., the tetramer should bind strongly to DP thymocytes from CD8α deficient mice, a binding blocked by anti-CD4 mAbs.

6.3 Implications of CD8-HS interaction: the big perspective

The functional relevance of the CD8-HS interaction goes beyond it being a cell adhesion process that enhances the interaction between thymocytes and cTEC. As hypothesised above, HS is likely to bind strongly to the stalk region of the CD8β chain of the CD8αβ heterodimer to trigger a sustained influx of extracellular Ca^{2+} into DP thymocytes (FIG. 4.1). This activation event is unlikely to occur via the conventional TCR signalling pathway, as the Zap70 molecule was shown to be dispensable whilst downstream Slp76 was required (FIG. 4.4). Intriguingly, the unique signal induced by the CD8-HS interaction synergises with the TCR signal following anti-CD3ε mAb stimulation to lower the anti-CD3ε level required to activate DP thymocytes (FIG. 4.6).
In fact, it was shown that both HS-derived and MHC-derived signals contributed to the steady and sustained Ca\(^{2+}\) influx observed in thymocytes rosetting with cTEC (FIG. 5.7). It is unknown if recognition of HS by CD8 directly enhances TCR signalling pathways or independently promotes the activation of transcription factors that are targeted by TCR signals. Hypothetically, HS may cross-link CD8 to LFA-1 and/or \(\alpha 4\beta 1/VLA-4\) to trigger a Slp-76-dependent ‘outside-in’ signals in T lymphocytes (Cimo et al., 2013, Baker et al., 2009). Whatever the biochemical explanation, it is likely that the CD8-HS interaction plays a crucial role in several phases of TCR\(\alpha\beta\) T lymphocyte development, selection and immune activation (FIG. 6.1).

### 6.3.1 Role of CD8-HS interaction in positive selection

TCR signals delivered by unique thymic stromal cells play a critical role in thymocyte development (FIG. 1.6). This study provided lines of evidence for the involvement of HS in T cell development, particularly at the DP stage, in which the tight interaction with cTEC is imperative for the positive selection of DP thymocytes (FIG. 1.5). It is important that pre-selection DP thymocytes gain a productive TCR signal to avoid death by neglect, and this requires TCR-self-peptide/MHC recognition, alongside CD8/CD4-MHC binding to generate MHC-restriction in TCR\(\alpha\beta\) T lymphocytes (FIG. 1.7) (Van Laethem et al., 2007, Van Laethem et al., 2013). In the event that a pre-selection DP thymocyte expresses a ‘useless’ nascent TCR that fails to recognise self-peptide/MHC on cTEC, the ligation of CD8 by HS is likely to deliver a Ca\(^{2+}\) flux that augments the deletion of this clone by death by neglect (FIG. 6.1A). This outcome is in agreement with the ‘death by instruction’ model proposed by Grebe et al. (2004), albeit via an ill-defined downstream signal induced in thymocytes by anti-CD8\(\alpha\) and anti-CD8\(\beta\) mAbs or by MHC-I mediated CD8 ligation/cross-linking (Grebe et al., 2004). Thus, to survive and be positively selected, TCR on pre-selection DP thymocytes need to recognise with low affinity unique peptides generated in cTEC by the thymoproteosome \(\beta 5t\) (MHC-I restricted peptides) (Sasaki et al., 2015) or cathepsin-L/TSSP (MHC-II restricted peptides) (Nakagawa et al., 1998, Honey et al., 2002, Gommeaux et al., 2009).

In addition, Daniels et al. (2006) showed that CD8 substantially aids the ability of TCRs to bind to low affinity positive selecting self-peptide/MHC-I complexes (Daniels et al., 2006). In this regard, the dual functionality of CD8 to bind simultaneously MHC-I and HS on cTEC (FIG. 6.1B) would increase the probability of low affinity TCRs being
positively selected. Also, it is likely that by working independently of the initial TCR signalling pathway, the HS-induced Ca\(^{2+}\) influx may augment pathways that favour positive selection. For example, the increase in intracellular Ca\(^{2+}\) could sensitise the ERK/AP-1 and Ca\(^{2+}\)/NFAT pathways without involving the robust positive feedback loop arising from membrane localisation of the Grb2-Sos complex (FIG. 1.6) (Daniels et al., 2006), in a similar manner to the contribution of Tespa1 (Wang et al., 2012). Therefore, in the presence of a TCR signal, the CD8-HS interaction may lower the threshold for positive selection of low affinity TCR clones and simultaneously reduce the number of cells dying by neglect. This is evident with the formation of TNCs in the cortex (FIG. 1.5 and FIG. 5.1), with Nakagawa et al. (2012) proposing that TNC formation is due to the persistent interaction between cTEC and thymocytes that are given a ‘second’ chance to rearrange their TCRα chains (Nakagawa et al., 2012). In this ‘facilitated’ selection process, it is not surprising that cTEC adjust weak TCR signals generated by nascent TCRs by offering additional aids such as Ca\(^{2+}\) signalling generated by the CD8-HS interaction, a speculation matched by the noticeably higher cell surface expression of HS on cTEC in TNCs (FIG. 5.1 and FIG. 5.3). It should be noted, however, that TNCs are not prominent in ‘tailored-affinity’ transgenic TCR strains, including the OT-I (OVA-specific) and P14 (LCMV-specific) TCR clones (Nakagawa et al., 2012), perhaps as such homogenous self-sufficient TCR pools are less reliant on HS-induced Ca\(^{2+}\) fluxes to undergo positive selection.

**6.3.2 Role of CD8-HS interaction in negative selection**

In a similar manner to positive selection, the CD8-HS interaction is likely to aid the deletion of potentially autoreactive TCR clones by negative selection. SIRPα⁺CD8α⁻ migratory DCs present blood-borne self-antigens from the periphery to DP thymocytes (FIG. 1.5) (Baba et al., 2009, McCaughtry et al., 2008). It should be noted that, compared to the low affinity positive-selecting TCR ligands generated by cTEC, many of the ubiquitous self-antigens presented on other APCs are likely to be recognised with relatively high affinity by nascent TCRs (Klein et al., 2009, Klein et al., 2014). It should be noted that DCs express cell surface HS at high levels, similar to cTEC (unpublished lab observation), that may augment TCR binding to self-peptide/MHC complexes on DCs and promote the recruitment of RasGRP1 to the membrane-bound pLAT complex to accentuate the Ras/ERK cascade (FIG. 1.6) (Daniels et al., 2006, Kortum et al., 2013).
FIG. 6.1. Predicted implications of the CD8-HS interaction for intrathymic MHC-I-restricted TCRαβ CD8⁺ T lymphocyte development and function. (A) Death by neglect. ‘Useless’ TCRs fail to bind self-peptide/MHC-I on cTEC. CD8αβ-HS interaction induces a Ca²⁺ flux that enhances apoptosis of pre-selection DP thymocytes. (B) Positive selection. HS enhances TCR/CD8 binding to low affinity (β5t-generated) self-peptide/MHC-I complexes on cTEC. HS-induced Ca²⁺ influx augments weak TCR signals to lower the threshold required for positive selection. The ion channel that mediates the HS-induced influx of extracellular Ca²⁺ is unknown. (C - D) Negative selection. (C) HS enhances TCR/CD8 binding to ubiquitous self-antigens presented on thymic DCs. HS-induced Ca²⁺ influx augments the TCR and, consequently, lowers the threshold required for deletion of strong self-reactive TCR clones. (D) CD8β on immature CD8 SP thymocyte is modestly α2-3 O-sialylated (indicated by orange star), reducing HS binding to the CD8β stalk region. A partial CD8-HS interaction promotes the ability of high affinity TCRs to bind AIRE-regulated tissue-restricted antigens (TRA) or ubiquitous self-peptides/MHC-I complexes presented by mTEC. HS-induced Ca²⁺ flux augments TCR signal to lower the threshold required to delete TRA-reactive and strongly self-reactive TCR clones. Orange star indicates α2-3 sialic acid. (E – F) Immune activation and anergy. (E) Heavy α2-3 O-sialylation of CD8β on peripheral CD8⁺ (pCD8) T lymphocytes (indicated by red star) fully impairs HS binding to the stalk region. (F) Immune activation triggers the loss of α2-3 sialic acid on CD8β, restoring the ability of the CD8-HS interaction to increase TCR affinity for peptide/MHC-I complexes on APC/DC. HS-induced Ca²⁺ influx augments the strength of the TCR signal to generate productive effector responses (CD28-co-ligated) or anergy (without CD28 ligation).
Final Discussion & Future Directions

A. cTEC

- MHC-I
- HS
- TCR αβ
- CD3
- CD8
- Death by neglect

DP Thymocyte (pre-selection)

B. cTEC

- MHC-I
- HS
- TCR αβ
- CD3
- CD8
- Positive selection

DP Thymocyte (pre-selection)

C. DC

- MHC-I
- HS
- TCR αβ
- CD3
- CD8
- Negative selection

DP Thymocyte

D. mTEC

- MHC-I
- HS
- TRA / Self-peptide
- TCR
- CD3
- CD8
- Negative selection

CD8 SP Thymocyte (immature/Pre-egress)

E. All nucleated cells

- MHC-I
- HS
- TCR
- CD3
- CD8
- Dormant

pCD8 T lymphocytes

F. Peripheral DC

- MHC-I
- HS
- TCR
- CD3
- CD8
- Immune activation

Peripheral DC

Productive response or anergy (without CD28 ligation)
Thus, the CD8-HS interaction could lower the threshold required to delete potentially auto-reactive TCR clones in the first wave of negative selection in the cortex (FIG. 6.1C). The same can be assumed to be taking place in the second wave of negative selection in the medulla as mTEC, like cTEC, express comparable levels of surface HS (FIG. 5.3). In this context, in addition to the presentation of ubiquitous self-antigens, mTEC also present AIRE-regulated tissue-restricted antigens (TRA) to post-selection CD8 SP thymocytes (FIG. 1.5) (Klein et al., 2014). As previously mentioned, CD8β on DP thymocytes after selection and commitment to the SP stage is gradually α2-3 O-sialylated and this partially reduces the binding to HS (FIG. 3.11), thus resulting in a smaller intracellular Ca$^{2+}$ influx being induced by the CD8-HS interaction compared to DP thymocytes (FIG. 4.2). In this scenario, it is likely that a HS-induced Ca$^{2+}$ flux augments the TCR signal and may be the pivotal factor that determines whether borderline auto-reactive TCR clones are to be negatively selected (FIG. 6.1D). Collectively, it can be concluded that by enhancing adhesion between TEC and thymocytes and providing a Ca$^{2+}$ flux, the CD8-HS interaction contributes to the fine-tuning of TCR signals that expand the positively selected TCR repertoire but at the same time increases the efficiency of the filtering process involved in deleting potentially autoreactive TCR clones. Such a process results in a TCR repertoire that is less likely to mediate autoimmunity in the periphery. It is assumed that this process is less important for the CD4 lineage, as in addition to clonal deletion, differential TCR tuning results in the generation of regulatory T cells (Hsieh et al., 2012).

### 6.3.3 Role of CD8-HS interaction in the periphery

As discussed in Section 6.2.2, in the periphery the CD8αβ heterodimer on dormant peripheral CD8$^+$ T lymphocytes is maximally α2-3 O-sialylated to maintain normal cell homeostasis (FIG. 6.1E). High levels of circulating plasma HRG ensure that there are no residual CD8-HS interactions occurring between APC/DC and dormant peripheral CD8$^+$ T lymphocytes, thereby maintaining peripheral tolerance and avoiding the risk of developing autoimmune responses. Following immune activation and an established inflammatory environment the α2-3 sialic acid residues could be lost from the CD8αβ molecules and, in the absence of HRG, CD8αβ molecules could now regain full coreceptor functions. Similarly, it is likely that the CD8-HS interaction induces the uptake of extracellular Ca$^{2+}$ (FIG. 4.5) that promotes the Ca$^{2+}$/NFAT pathway (Macian, 2005) and also augments TCR signalling arising from recognition of foreign
Final Discussion & Future Directions

peptides/MHC-I complexes presented on APC/DC to produce robust peripheral CD8\(^+\) T lymphocyte effector responses. Of course, in the periphery the activation of T cells requires costimulatory signals, such as T cells CD28 interacting with CD80/CD86 expressed by APCs, the absence of costimulation resulting in T cell anergy (FIG. 6.1F). Thus, collectively the CD8-HS interaction may not only be important at the intrathymic lymphopoietic level, but also may contribute to the generation of robust CD8\(^+\) T lymphocyte effector responses and the maintenance of CD8\(^+\) T lymphocyte peripheral tolerance.

6.4 Future research directions

6.4.1 Establishing the structural requirements for the CD8-HS interaction

The studies described in this Thesis provide many lines of evidence supporting the view that there is a direct interaction between HS and the CD8\(\beta\) chain. Nonetheless, the true nature of this interaction will only be resolved when the crystal structure of the CD8-HS interaction has solved, as discussed in Section 6.2.1.

Future work should also be aimed at confirming the structural features of the HS expressed by thymic stromal cells that are recognised by the CD8 molecule. It should be noted that the synthesis of HS chains is a highly complex process involving diverse HS synthesising and modifying enzymes. In fact, earlier studies have shown that germline ablation of genes encoding HS-synthesising and modifying enzymes, such as extose (Ext), N-deacetylase/N-sulfotransferase (Ndst), D-glucuronyl C5-epimerase (Glce), HS 2-O-sulfotransferase (Hs2st) and HS 6-O-sulfotransferase (Hs6st) (this order being reflective of their sequential roles in the synthesis and modification of HS chain) are either embryonically lethal or result in perinatal death (Lin et al., 2000, Ringvall et al., 2000, Li et al., 2003, Stickens et al., 2005, Habuchi et al., 1998, Wilson et al., 2002). This issue, however, can be overcome via tissue-specific ablation using the Cre-LoxP recombination system. In this approach, specific alterations in the HS chains expressed by TEC and DCs can be generated by crossing a mouse strain carrying a floxed allele of the gene encoding a particular HS synthesising/modifying enzyme with a syngeneic mouse strain that carries Cre recombinase under control of the promoter for FoxN1 or Itgax (CD11c) expression, these promoters being specific to TEC and DCs, respectively (Gordon et al., 2007, Caton et al., 2007). The impact of such genetic alterations,
particularly Hs6st-deficiency in TEC and DCs which would result in HS chains lacking 6-O-sulfates, on CD8 binding using the TEC/DC-thymocyte rosetting assay or on the Ca\(^{2+}\) flux in DP thymocytes rosetting with TEC or DCs will be of particular interest. In parallel, these assessments can be done simultaneously with MHC-I and MHC-II deficient HS-variant TEC and DCs to exclude the contribution of MHC molecules to thymocytes rosetting and induction of an intracellular Ca\(^{2+}\) flux. Mouse strains that carry a floxed allele of Ndst, Hs2st and Hs6st are already available as well as appropriate Cre recombinase strains that are required for the proposed experiments (Qu et al., 2011, Wang et al., 2005).

6.4.2 Establishing the role of the CD8-HS interaction in positive and negative selection of DP thymocytes

Several laboratories have established *in vitro* assays to monitor positive and negative selection of DP thymocytes (Daniels et al., 2006). These systems can be used to assess whether the CD8-HS interaction can lower the signalling threshold required for peptide/MHC-I complexes to induce either positive or negative selection of DP thymocytes. Usually TCR transgenic DP thymocytes are used from TAP deficient mice and hence the thymocytes are arrested at the pre-selection DP stage due to the TAP deficiency preventing the peptide loading of endogenous MHC-I molecules on TEC/DCs. Peptide variants that have a range of affinities for the transgenic TCR also may be used to determine whether there are changes in the affinity of the TCR that is positively or negatively selected when pulsed on WT or HS-variant TEC/DCs (Section 6.4.1) are used. To determine DP thymocyte selection a number of parameters can be measured, such as number of surviving DP thymocytes, CD69 up-regulation as a general marker for thymocyte activation (FIG. 4.6) (Hare et al., 1999), TCR downregulation, as a correlative marker for positive versus negative selection (Mariathasan et al., 1998) and CD5 surface expression as a correlate of the signalling capacity of the TCR (Azzam et al., 1998). Also, DP thymocytes from TCR transgenic, TAP-deficient mice that are *Bcl-2* transgenic could be used, as the presence of *Bcl-2* allows negatively selected T cells to survive and be more accurately quantified on the basis of *Bim* expression (Daley et al., 2013).

In addition to cellularity and phenotypic changes as outlined above, the intracellular signalling pathways influenced by the CD8-HS interaction, with respect to positive and
negative selection, require further experimental examinations. In this regard, an unbiased approach to identifying signalling proteins that are phosphorylated in DP thymocytes following HS (DxS) stimulation can be achieved by utilising the Phospho Explorer Antibody Array system (Full Moon, Biosystems) that consists of a microarray with 1318 site-specific antibodies against unique phosphorylation sites in signalling proteins. Thus, this system can be used to assess the phosphorylation status of signalling molecules in DP thymocytes exposed to HS-like ligands, such as DxS, compared to unstimulated and TCR stimulated DP thymocytes. Also, of considerable interest will be the effect of simultaneous DxS and TCR stimulation on signalling pathways, particularly TCR signalling. Eventually, Phosphoflow and Western blotting analysis may be used to confirm the most informative phospho-proteins involved in DxS (HS) signalling and in TCR signalling in the presence of the DxS-induced Ca\(^{2+}\) flux. Appropriate KO mice, if available, can be used to confirm the importance of any identified signalling proteins, particularly those involved in the DxS-induced Ca\(^{2+}\) flux. Finally, an important issue to be resolved regarding the major Ca\(^{2+}\) influx induced by HS/DxS in DP thymocytes is the nature of the Ca\(^{2+}\) channel(s) involved.

### 6.4.3 Establishing the role of the CD8-HS interaction in peripheral CD8\(^{+}\) T lymphocyte effector function

Future studies will examine under what circumstances CD8 molecules become desialylated in the periphery and regain HS and MHC-I binding. Such a process would potentially lower the activation threshold of peripheral CD8\(^{+}\) T lymphocytes, thereby enhancing their capacity to respond to foreign antigens. Theoretically, there are two ways by which CD8 molecules can become desialylated. First, pre-existing CD8 molecules could simply be desialylated by sialidases present in the local, presumably inflammatory, environment. Second, as is the case with DP thymocytes, peripheral CD8\(^{+}\) T lymphocytes could cease producing the ST3Gal-1 sialyltransferase, in this situation all newly synthesised CD8 molecules lacking α2-3 sialic acid residues.

There are four known mammalian sialidases (neuraminidases), NEU1, NEU2, NEU3 and NEU4, (Miyagi and Yamaguchi, 2012) that could potentially desialylate CD8. Scanning the publically available transcriptome databases (e.g., BioGPS), however, it is clear that NEU-2 and -4 have extremely narrow expression profiles and are not detected in the immune system. NEU1 is expressed by immune cells but exclusively by activated
macrophages at very high levels (Seyrantepe et al., 2010). In human DCs NEU3 expression is upregulated following LPS-induced activation although it is unknown if a similar response occurs in murine DCs. Thus, initially the ability of recombinant mouse NEU1 and NEU3 to desialylate CD8 on peripheral T lymphocytes and restore FITC-heparin binding, rosette formation with DCs and Dxs-induced Ca²⁺ fluxing could be examined. These experiments have a very high chance of success as NEU1 and NEU3 have a similar substrate specificity (i.e., high affinity for α2-3 sialic acid) (Miyagi and Yamaguchi, 2012) to the V. cholera neuraminidase used in this Thesis to desialylate CD8. Whether macrophages and DCs expressing high levels of NEU1 (and NEU3?) in inflammatory sites can desialylate CD8 on T lymphocytes infiltrating the site will be of considerable interest. Of course, an alternative possibility is that proliferating CD8⁺ T lymphocytes downregulate expression of the ST3Gal-1 sialyltransferase, as occurs in DP thymocytes. However, a preliminary study provided no evidence of desialylation of CD8 on proliferating OT-I T lymphocytes in vivo, based on FITC-heparin binding (data not shown). An attractive hypothesis, however, is that memory CD8⁺ T lymphocytes downregulate the ST3Gal-1 sialyltransferase to render them more responsive to antigen. Contrary to this hypothesis, it has been suggested that resialylation of CD8αβ promotes CD8⁺ memory T cells (Priatel et al., 2000). Standard in vitro and in vivo methods for generating TCR transgenic (OT-I) memory CD8⁺ T cells (Miyakoda et al., 2012, Berg et al., 2003) could be used to resolve this issue.

6.5 Concluding remarks
Based the future directions outlined in this Chapter it is clear that, although the research reported in this Thesis has identified a new mechanism controlling thymic T cell selection and possibly peripheral CD8⁺ T lymphocyte function, many new questions have been raised that need to be addressed. Also, at the general biological level, the studies reported in this Thesis have provided an elegant example of the importance of HS recognition in a subtle, sophisticated and highly complex biological process, the positive and negative selection of T cells in the thymus. Thus, HS can no longer be considered as a widely expressed polyanion that merely provides a structural scaffold for most tissues and, due to its negative charge, non-specifically binds numerous proteins. The remarkable structural diversity of HS within and between tissues has always implied that HS must be playing a much more specific role in biology. This
study, one hopes, will provide an excellent example of the biological importance of this remarkable family of molecules.
References


137


receptor chains reveals that IL-7 and IL-15 specify CD8 cytotoxic lineage fate in the thymus. J Exp Med, 209, 2263-76.


