Novel Biomarkers in DLBCL

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Abstract

Diffuse large B cell lymphoma (DLBCL) is the commonest aggressive lymphoma. Despite the advent of combined chemo-immunotherapy, one third of patients still die from their disease. Prognostication of the disease still relies on a clinical scoring system known as the International Prognostic Index (IPI). This divides patients into risk categories. However marked heterogeneity within IPI sub-categories persist. The IPI is a clinical score based predominantly on estimates of patient fitness and tumour burden, but does not utilize information regarding the biology of the tumour cell or the immune tumour microenvironment (TME), in which the malignant B cells reside. The latter is the focus of this thesis. The anti-CD20 monoclonal antibody rituximab has improved the outcome for patients with DLBCL, however the impact of host genetics on its effectiveness is still unclear. The mechanisms of action for rituximab include antibody dependent cytotoxicity (ADCC) and complement mediated cytotoxicity (CDC). Recent reports suggest genetic polymorphisms in the FCGR3A receptor (expressed on NK-cells and monocytes which mediate ADCC) may be a predictor of event free and overall survival in B-cell lymphoma. Data also implicates the same polymorphism in the susceptibility to rituximab induced late-onset neutropenia (LON). There remains no data on the impact of genetic polymorphisms on either outcome or LON in genes involved in the CDC pathway such as C1qA. One hundred and fifteen DLBCL patients treated with ‘Ru CHOP’ (rituximab/cyclophosphamide/vincristine/doxorubicin/prednisolone) chemotherapeutic therapy were compared with 105 healthy Caucasian controls with regards to FCGR3A q V158F and C1qA q A276G polymorphisms. Event free and overall survival (EFS and OS) and LON incidence were analysed for linkage to either polymorphism. The FCGR3A q V158F but not the C1qA q A276G polymorphism influenced the risk of developing LON. 50% of FCGR3A-158V/V patients experienced LON. In contrast, only 7% V/F and 2% F/F experienced LON. The FCGR3A-158V/V genotype was associated with LON compared to V/F (p=0.028) and F/F genotypes (p=0.005). Although no patients with either LON or FCGR3A-158V homozygosity relapsed compared to 33% FCGR3A-158F/F and 21% non-LON, this did not translate into improved EFS or OS and results are
likely to be influenced by lead-time bias. Polymorphic analysis may be a predictive tool to identify those at high-risk of LON. Larger prospective studies are required to definitively establish if LON or FCGR3A-158V/V genotype influences outcome.

Host immune status has consistently been shown to play an important role in DLBCL. The manipulation of the host immune environment has shown promise in solid and lymphoid tumours and identification of patients benefiting from immune based therapy may have therapeutic relevance in DLBCL. The impact of circulating monocytes and circulating and intratumoural lymphocytes on outcome were assessed in a cohort of 122 patients with DLBCL. All were treated with R-CHOP, and median follow up was 48 months. Lymphocyte and monocyte counts were assessed prior to commencement of therapy, and in addition the majority had data from flow cytometric immunophenotyping on lymphocyte (but not monocyte) subsets available on fresh diagnostic lymphoid tissue.

The circulating lymphocyte to monocyte ratio (LMR) was a significant predictor of outcome with patients with high LMR having an estimated five-year survival of 86% compared to 63% (p=0.01) in patients with low LMR. This finding was independent of the IPI. Low (0,1) and intermediate IPI (2,3) did not predict a significantly different survival from each other in our cohort, with both having excellent outcome (83% estimated 5 year survival in these combined groups). However when the 90 patients in these low risk IPI groups were split by the LMR, there was a significant overall survival advantage (estimated 5 year OS 93% vs. 72%, p=0.01) for patients with a higher LMR. Amongst intratumoural lymphocyte subsets, CD4+T cell infiltration was the most significant predictor of improved outcome. Those with high intratumoural CD4+ T cells had a superior EFS (p=0.009) and OS (p=0.006) compared to those with low values. CD3+T cell infiltration was also associated with improved EFS (P=0.01) and OS (p=0.01), whereas CD8+T cell infiltration did not predict outcome. CD4+T cells were independent of IPI and LMR for both EFS and OS. Importantly when low/intermediate IPI groups were analyzed, a high CD4+T cell infiltration remained a striking predictor of OS (5 year OS 93% vs. 60%, p=0.004). These results confirm the importance of the local immune environment in patients with
DLBCL treated with chemo-immunotherapy, and indicate that peripheral immune subsets are surrogate markers of intratumoural immunity. Based on these findings, it was envisaged that detailed functional and quantitative assessment of blood would enable identification of the optimal diagnostic tissue based TME immune-effector and monocyte/macrophage-checkpoints to assist sub-stratification of conventional prognosticators. Blood from 140 R-CHOP treated DLBCL patients in the NHL21 Australasian Leukaemia and Lymphoma Group trial were prospectively analysed. A circulating immune-effector: monocyte-checkpoint signature segregating interim-PET/CT-positivity was identified. Intratumoural applicability was tested in two independent R-CHOP treated DLBCL cohorts, with cell-of-origin (COO) and international prognostic index (IPI) as co-variates. CD163⁺CD14⁺HLA-DRlo blood monocytes were immunosuppressive. CD8⁺:CD163⁺CD14⁺HLA-DRlo ratios were highest in interim-PET/CT⁻ve patients (P≤0.0001). Digital multiplexed gene expression (DMGE) in 191 DLBCL tissues demonstrated co-clustering of CD8 with immune-checkpoints (CD163/PD1/PDL1/PDL2/TIM3/LAG3, all P<0.001), indicating an adaptive immune-checkpoint response to immune-effector activation. In multivariate analysis of 128 R-CHOP treated DLBCL patients, CD8:CD163 ratios (net anti-tumoral immunity) were prognostic independent of IPI and COO. Combining CD8:CD163 to the germinal centre B-cell marker LMO2 (LMO2/CD8:CD163) strengthened the predictive ability. Results were externally validated in 233 patients, separating good-risk IPI (0-2) into two categories of 85% and 48% (P=0.0003) 4 year survival after R-CHOP. Similarly, poor-risk IPI (3-5) stratified into two survival groupings of 66% and 36% (P=0.0007). In patients with DLBCL, a measure of net anti-tumoral immunity within the TME is a powerful new prognosticator that is independent of IPI and COO. LMO2/CD8:CD163 adds to the predictive ability of IPI.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

(Signed)_____________________________
Acknowledgements

I would firstly like to thank Maher Gandhi for his amazing support throughout this PhD. He has helped me become a much better researcher, medical writer and doctor. His dedication and hard work are an inspiration to everyone in the laboratory. I would also like to thank the support I have received from Griffith University and in particular my supervisors Rod Lea and Lyn Griffiths.

I would like to thank everyone at the Gandhi Lab who helped during my research in particular Jamie Nourse, Frank Vari and Pauline Crooks. They showed great patience and support in helping someone with limited laboratory experience to get through this research. I would also like to thank Frank in particular for his hard work and insights to our co-authored paper.

I would like to thank my funding sources and in particular the Leukaemia Foundation, without whom I would have been unable to perform any of this research. They should be acclaimed for not only work they perform in supporting research but also the tireless work they perform in trying to make life better for patients with haematological malignancy.

I would like to thank my work colleagues at the Princess Alexandra Hospital who have been supportive throughout my research and in particular Devinder Gill who is always supportive of translational research in the unit. I wish to acknowledge Dipti Talaulikar for her help in gathering additional patient samples which have had a huge impact on the quality of my work. I would also like to thank Mark Hertzberg, who as the clinical lead on the ALLG study has been so supportive in trying to get as many samples and clinical data for all our patients. I want to thank all the patients who participate in all our research including NHL21. One hopes that we can do their hard work and trust in us justice, and hopefully contribute in some small way to improving the length and quality of life for patients with lymphoma in the future.

I would like to thank my wife Sharon and three children who have put up with a lot over the last few years. Their understanding in allowing me to spend the last few years being a student again has meant huge sacrifices on their behalf and I will forever be grateful for their support.
Publications Arising from this Thesis (*indicates papers directly forming part of thesis)

*1. Measures of net anti-tumoral immunity add to the predictive power of conventional prognostic factors in diffuse large B cell lymphoma (DLBCL).
Colm Keane*, Frank Vari*, Mark Hertzberg, John Seymour, Rodney Hicks, Devinder Gill, Pauline Crooks, Kimberly Jones, Erica Han, Rod Lea, Lyn Griffiths, Maher Gandhi.
Submitted to Cancer Discovery May 2014
*Co-Authors

*2. CD4(+) tumor infiltrating lymphocytes are prognostic and independent of R-IPI in patients with DLBCL receiving R-CHOP chemo-immunotherapy.
Keane C, Gill D, Vari F, Cross D, Griffiths L, Gandhi M.

Jones K, Nourse JP, Keane C, Bhatnagar A, Gandhi MK.

4. Serum CD163 and TARC are disease response biomarkers in classical Hodgkin lymphoma.

5. High-resolution loss of heterozygosity screening implicates PTPRJ as a potential tumor suppressor gene that affects susceptibility to Non-Hodgkin’s lymphoma.

6. Tumor-specific but not non-specific cell-free circulating DNA can be used to monitor disease response in lymphoma.
Kimberley Jones, Jamie P. Nourse, Colm Keane, Pauline Crooks, David Gottlieb, David S. Ritchie, Devinder Gill and Maher K. Gandhi
American Journal of Haematology

*7. Homozygous FCGR3A-158V alleles predispose to late onset neutropenia after CHOP-R for Diffuse Large B-cell Lymphoma
Colm Keane, Jamie P. Nourse, Pauline Crooks, Do Nguyen-Van, Howard Mutsando, Peter Mollee, Rod A. Lea, Maher K. Gandhi
8. Epstein-Barr virus-positive diffuse large B-cell lymphoma of the elderly expresses EBNA3A with conserved CD8+ T-cell epitopes

Do Nguyen-Van, Colm Keane, Erica Han, Kimberley Jones, Jamie P. Nourse, Frank Vari, Nathan Ross, Pauline Crooks, Olivier Ramuz, Michael Green, Lyn Griffith, Ralf Trappe, Andrew Grigg, Peter Mollee, Maher K. Gandhi

Am J Blood Res 2011;1(2):146-159

Book Chapters

"Rituximab induced Late-onset Neutropenia" for the book 'Rituximab: Pharmacology, Clinical Uses and role in Investigating B cell immunology in Man' published by Novus in 2012

Colm Keane, Jamie Nourse, Maher K. Gandhi

Presentations Arising from this Thesis

1. Net antitumoral immunity and the predictive power of conventional prognosticators in diffuse large B-cell lymphoma

Poster Highlights Session

Merit Award Winner

American Society of Clinical Oncology, Annual Meeting 2014

Colm Keane, Frank Vari, Mark S. Hertzberg, Michael R Green, Erica Han, John Francis Seymour, Rodney J Hicks, Devinder Singh Gill, Pauline Crooks, Clare Gould, Kimberley Jones, Kristen Radford, Lyn Griffiths, Dipti Talaulikar, Sanjiv Jain, Josh Tobin, Maher K. Gandhi

2. Noninvasive monitoring of cellular versus acellular tumor DNA from immunoglobulin genes for DLBCL

Oral Presentation

American Society of Clinical Oncology, Annual Meeting 2014


3. "Utility Of Non-Invasive Monitoring Of Circulating Tumor DNA At Diagnosis, Interim Therapy, and Relapse Of DLBCL Using High-Throughput Sequencing Of Immunoglobulin Genes"

Poster Presentation

American Society of Haematology 2013

Michael R Green, Scott Bratman, Chih Long Liu, Kazuhiro Takahashi, Cynthia Glove*, Colm Keane, Shingo Kihira, Katie Kong, Malek Faham, MD, PhD, Corbelli Karen, David B. Miklos, Ranjana H. Advani, Ronald Levy, Mark S. Hertzberg, Maher K Gandhi, Maximilian Diehn, Ash A. Alizadeh,
4. “CD163+ Identifies A Highly Immunosuppressive Subset Of Monocytic-Myeloid Derived Suppressor Cells (moMDSCs) In Poor-Risk Diffuse Large B-Cell Lymphoma (DLBCL): An ALLG Laboratory Sub-Study Of NHL21.”
Oral Presentation
International Conference on Malignant Lymphoma, Lugano 2013
Colm Keane, Mark Hertzberg, John Seymour, Rodney Hicks, Devinder Gill, Frank Vari, Pauline Crooks, Kimberly Jones, Erica Han, Rod Lea, Lyn Griffiths, Maher Gandhi.

5. Flow Cytometric analysis demonstrates prognostic significance of tumour-infiltrating CD4+T lymphocytes in patients with diffuse large B cell lymphoma receiving R-CHOP chemoimmunotherapy.
Poster Presentation
American Association of Cancer Researchers
Tumour Immunology Meeting 2012
Colm Keane, Frank Vari, Lynn Griffiths, Devinder Gill, Peter Mollee, Rod A. Lea, Maher K. Gandhi

5. Serum CD163 and TARC in Combination As Disease Response Biomarkers in Classical Hodgkin Lymphoma
Oral Presentation
American Society of Haematology 2012

6. Circulating microRNAs as prognostic and disease response biomarkers in patients with high-risk diffuse large b-cell lymphoma (DLBCL): a prospective Australasian leukaemia & lymphoma group study
Poster Presentation
European Haematology Association Annual Meeting 2012
Colm Keane, Mark Hertzberg, John Seymour, Rodney Hicks, Devinder Gill, Frank Vari, Pauline Crooks, Kimberly Jones, Erica Han, Rod Lea, Lyn Griffiths, Maher Gandhi.

7. Tissue Microarray in Patients with DLBCL Receiving R-CHOP Chemo-immunotherapy Shows Survival Benefit for Coexpression of LMO2/BCL6
Poster Presentation
American Society of Haematology 2011
Colm Keane, Linda Shen, Jamie Nourse, Erica Han, Rod Lea, Peter Mollee, Devinder Gill, Maher Gandhi

8. Monocytes are Associated with Impaired T-cell Immunity and Residual Interim- PET/CT Avidity after 4 Cycles of CHOP-R In Patients with High-Risk DLBCL
Poster Presentation
American Society of Haematology 2011
Frank Vari, Mark Hertzberg, Erica Han, John F Seymour, Rodney Hicks, Devinder Gill, Colm Keane, Pauline Crooks, Kristen Radford, Maher K. Gandhi
Oral Presentation
HSANZ 2011
Colm Keane, Linda Shen, Jamie Nourse, Erica Han, Rod Lea, Peter Mollee, Devinder Gill, Maher Gandhi

10. Monocytes are Associated with Impaired T-cell Immunity and Residual Interim- PET/CT Avidity After 4 Cycles of CHOP-R In Patients With High-Risk DLBCL
Oral Presentation
Haematology Society of Australia and New Zealand Annual Meeting 2011
Frank Vari, Mark Hertzberg, Erica Han, John F Seymour, Rodney Hicks, Devinder Gill, Colm Keane, Pauline Crooks, Kristen Radford, Maher K. Gandhi

11. Lymphoma-Specific But Not Non-Specific Cell-Free Circulating DNA Can Be Used to Monitor Disease Response in Lymphoma
Oral Presentation
Haematology Society of Australia and New Zealand Annual Meeting 2011
Kimberley Jones, Jamie P Nourse, Colm Keane, Pauline Crooks, David Gottlieb, David S Ritchie, Devinder Gill, Maher K Gandhi

12. Tissue Microarray in DLBCL patients receiving CHOP-R chemo-immunotherapy shows survival benefit for coexpression of LMO2/BCL6 and poor outcome for EBER-ISH positive patients.
Oral Presentation
International Conference on Malignant Lymphoma, Lugano June 2011
Colm Keane, Linda Shen, Jamie Nourse, Erica Han, Kimberley Jones, Maher Gandhi

13. Homozygous FCGR3A-158V alleles predispose to late onset neutropenia after CHOP-R for Diffuse Large B-cell
Poster Presentation
International Conference on Malignant Lymphoma meeting, Lugano June 2011
Colm Keane, Jamie Nourse, Pauline Crooks, Do Nguyen Van, Howard Mutsando, Maher Gandhi

Poster Presentation
European Haematology Association meeting, London, June 2011
Frank Vari, Mark Hertzberg, Erica Han, Colm Keane, J. Alejandro Lopez, Kristen Radford, John F Seymour, Devinder Gill, Maher K Gandhi.
15. EBV-positive DLBCL of the elderly is a distinct clinico-biological entity with poor outcome in CHOP-R treated patients, with properties likely amenable to anti-EBV targeting. 
Oral Presentation.
The 14th Biennial Conference of the International Association for Research on Epstein - Barr virus & Associated Diseases, Birmingham, September 2010.
Do Nguyen-Van, Colm Keane, Jamie P. Nourse, Erica Han, Nathan Ross, Kimberley Jones, Pauline Crooks, and Maher K. Gandhi.

Oral Presentation
Jamie P Nourse, Pauline Crooks, Colm Keane, Do Nguyen-Van, Sally Mujaj, Nathan Ross, Kimberley Jones, Frank Vari, Erica Han, and Maher K. Gandhi.
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Full Title</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>ALC</td>
<td>Absolute Lymphocyte Count</td>
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<tr>
<td>ALLG</td>
<td>Australasian Leukaemia Lymphoma Group</td>
</tr>
<tr>
<td>AMC</td>
<td>Absolute Monocyte Count</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>ASCT</td>
<td>Autologous stem cell transplant</td>
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<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>BCL6</td>
<td>B-cell CLL/lymphoma 6</td>
</tr>
<tr>
<td>BD</td>
<td>Becton Dickenson</td>
</tr>
<tr>
<td>BMDCs</td>
<td>Bone marrow-derived dendritic cells</td>
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<tr>
<td>BTK</td>
<td>Bruton's tyrosine kinase</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete Blood Count</td>
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<tr>
<td>CCL3</td>
<td>Chemokine (C-C motif) ligand 3</td>
</tr>
<tr>
<td>CCND2</td>
<td>G1/S-Specific Cyclin-D2</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster Differentiation</td>
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<tr>
<td>CDC</td>
<td>Complement dependent cytotoxicity</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>COO</td>
<td>Cell-of-Origin</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
</tr>
<tr>
<td>CTLs</td>
<td>Cytotoxic T lymphocytes</td>
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<tr>
<td>DA-EPOCH-R</td>
<td>Dose-adjusted etoposide / prednisone / vincristine / cyclophosphamide, hydroxyduanorubicin / rituximab</td>
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<tr>
<td>DLBCL</td>
<td>Diffuse Large B cell Lymphoma</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EBV</td>
<td>Epstein Barr Virus</td>
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<td>EFS</td>
<td>Event Free Survival</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>FBC</td>
<td>Full Blood Count</td>
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<td>FCG3A</td>
<td>FCGammaReceptor 3A</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded tissue</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FN1</td>
<td>Fibronectin 1</td>
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<tr>
<td>FOXP1</td>
<td>Forkhead box P1</td>
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<tr>
<td>GA101</td>
<td>Other name for Obinotuzumab</td>
</tr>
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<td>GCB</td>
<td>Germinal Cell B cell like</td>
</tr>
<tr>
<td>GCET1</td>
<td>Germinal center B-cell expressed transcript 1</td>
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<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IL1</td>
<td>Interleukin 1</td>
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<tr>
<td>IL10</td>
<td>Interleukin 10</td>
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<tr>
<td>IL12</td>
<td>Interleukin 12</td>
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<tr>
<td>IL13</td>
<td>Interleukin 13</td>
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<tr>
<td>IL4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPI</td>
<td>International Prognostic Index</td>
</tr>
<tr>
<td>LAG3</td>
<td>Lymphocyte-activation gene 3</td>
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<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LMO2</td>
<td>LIM domain only 2</td>
</tr>
<tr>
<td>LMR</td>
<td>Lymphocyte to Monocyte ratio</td>
</tr>
<tr>
<td>M1</td>
<td>Macrophage Type I</td>
</tr>
<tr>
<td>M2</td>
<td>Macrophage Type II</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>moMDSC</td>
<td>Monocytic Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MUM1</td>
<td>Melanoma associated antigen (mutated) 1</td>
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<tr>
<td>NHL</td>
<td>Non-Hodgkins Lymphoma</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>Non-GCB</td>
<td>Non-Germinal Cell B cell like</td>
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<tr>
<td>Ob-ADCC</td>
<td>Obinotuzumab-Antibody dependent cellular cytotoxicity</td>
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<tr>
<td>OS</td>
<td>Overall Survival</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononucleated cell</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death 1</td>
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<tr>
<td>PD-L1</td>
<td>Programmed cell death ligand 1</td>
</tr>
<tr>
<td>PD-L2</td>
<td>Programmed cell death ligand 2</td>
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<tr>
<td>pDCs</td>
<td>Plasmacytoid derived dendritic cells</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>peg-G-CSF</td>
<td>Pegalated granulocytic colony stimulating factor</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
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<tr>
<td>PRDM1</td>
<td>PR domain containing 1, with ZNF domain</td>
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<tr>
<td>PTLD</td>
<td>Post-Transplant Lymphoproliferative Disease</td>
</tr>
<tr>
<td>R-ADCC</td>
<td>Rituximab-Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>R-CHOP</td>
<td>Cyclophosphamide / doxorubicin / vincristine / prednisone / rituximab</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>SCYA3</td>
<td>Alternate name for CCL3</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor 1</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal transducer and activator of transcription 1</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour Associated Macrophages</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>Th1</td>
<td>T Helper cell I</td>
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<tr>
<td>Th2</td>
<td>T Helper cell II</td>
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<tr>
<td>TILs</td>
<td>Tumour infiltrating lymphocytes</td>
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<tr>
<td>TIM3</td>
<td>T-Cell Membrane Protein 3</td>
</tr>
<tr>
<td>TME</td>
<td>Tumour Microenvironment</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
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<tr>
<td>Z-BEAM</td>
<td>Zevalin- Carmustine, Etoposide, Cytarabine, Melphalan</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
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<tr>
<td>µM</td>
<td>Micromolar</td>
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To be named as an author, a researcher must have made a substantial scholarly contribution to the creative or scholarly work that constitutes the research output, and be able to take public responsibility for at least that part of the work they contributed. Attribution of authorship depends to some extent on the discipline and publisher policies, but in all cases, authorship must be based on substantial contributions in a combination of one or more of:

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Included in this thesis are papers in Chapters 3, 4 and 5 which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details (if published or accepted for publication)/status (if prepared or submitted for publication) for these papers including all authors, are:

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Chapter 3:
Homozygous FCGR3A-158V alleles predispose to late onset neutropenia after CHOP-R for Diffuse Large B-cell Lymphoma
Colm Keane, Jamie P. Nourse, Pauline Crooks, Do Nguyen-Van, Howard Mutsando, Peter Mollee, Rod A. Lea, Maher K. Gandhi

Rituximab induced Late-onset Neutropenia" for the book 'Rituximab: Pharmacology, Clinical Uses and role in Investigating B cell immunology in Man" published by Novus in 2012
Colm Keane, Jamie Nourse, Maher K. Gandhi

Chapter 4:
CD4(+) tumor infiltrating lymphocytes are prognostic and independent of R-IPI in patients with DLBCL receiving R-CHOP chemo-immunotherapy.
Keane C, Gill D, Vari F, Cross D, Griffiths L, Gandhi M.

Chapter 5:
The immunobiological score: a robust 3-gene assay that segregates the international prognostic index into disparate survival categories in aggressive B-cell lymphoma
Colm Keane*, Frank Vari*, Mark Hertzberg, John Seymour, Rodney Hicks, Devinder Gill, Pauline Crooks, Kimberly Jones, Erica Han, Rod Lea, Lyn Griffiths, Maher Gandhi.
*Joint Authorship
Submitted to Cancer Discovery May 2014

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Name of Student

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Supervisor: Name of Supervisor
(tures should be included for each paper).
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CHAPTER 1

Introduction
Introduction

Brief Aim 1
Examine the influence of C1qA-A276G and FCGR3A-V158F polymorphisms on survival in patients with DLBCL treated with R-CHOP chemo-immunotherapy. I will also investigate if these polymorphisms influence the risk of rituximab induced late onset neutropenia.

Brief Aim 2
Examine if absolute lymphocyte and monocytes and their interactions predict outcome in patients with DLBCL treated with standard R-CHOP chemo-immunotherapy.
Investigate if the level of T-cell infiltration in DLBCL tumours, as assessed by flow cytometry, predicts outcome in patients with DLBCL.

Brief Aim 3
Using a novel investigative platform with digital bar-coding to obtain highly accurate gene expression data on paraffin based tissue, an extensive investigation of 191 DLBCL patient samples will be performed examining the role of immune effectors and checkpoints with regards to expression and outcomes in patients treated with standard chemo-immunotherapy.

1.1 Background
Diffuse large B cell lymphoma is a malignancy of B-lymphocytes. DLBCL is the most common aggressive subtype and accounts for approximately 40% of all patients with non-Hodgkin’s lymphoma.[1] The median age of presentation is usually in the 7th decade but it is not uncommon in younger adults and can occur in children. The incidence of this disease has risen rapidly over the last 4 decades but has now plateaued.[2] The reasons for this are unclear, however it is likely that better diagnostics and improved life expectancy likely contribute to this increase. In addition there has been increased levels of immune-impairment seen in this time period, secondary to the rise of HIV in the 1980s, but also due to the increasing levels of immune suppression used to treat autoimmune conditions or protect tissue and bone marrow grafts in patients undergoing transplantation.[3-5] Primary and acquired
impaired immunity are well-recognised strong risk factors for the development of B cell lymphoma. However at present these reasons seem insufficient to account for the marked increase in lymphomas, and there may be additional environmental factors of importance.[6, 7]

Clinically patients with DLBCL can present with fatigue, painless adenopathy or symptoms related to tumour bulk such as abdominal pain. It can occur as a primary lesion in a nodal or extranodal site (e.g. bone, liver, lung etc.). Primary central nervous system DLBCL, testicular DLBCL, primary DLBCL of the bone or of the skin though less common, are well described. Patients can also present with so called “B –Symptoms” such as night sweats, weight loss >5% or unexplained fevers. Paraneoplastic syndromes such as hypercalcaemia can also occur. Biopsy specimens from patients with DLBCL show a diffuse proliferation of large centroblast like cells that completely disrupt normal lymph node architecture but the predominant cell can also be immunoblastic or anaplastic.[1] By flow cytometry the malignant B cells typically express the surface markers CD19/20/22/79A with another 50% of tumours also expressing CD10.[8] The proliferation marker Ki-67 is usually high in DLBCL varying from 40-90%. Tumours expressing greater than 90% should raise suspicion of a myc gene translocation which if present is generally classified as a tumour intermediate between DLBCL and Burkitt Lymphoma and is associated with poorer outcome.[9] In general a myc gene rearrangement is found in approximately 10% of diagnosed DLBCL and is associated with poor outcome.[10-12] Clonally rearranged immunoglobulin heavy and light chains are usually detected at diagnosis. Both BCL6 and BCL2 rearrangements are commonly found in DLBCL.[1] There has been remarkable progress made in this disease over the last decade with the addition of the anti-CD20 monoclonal antibody rituximab to standard chemotherapy.[13] Prior to the introduction of rituximab, 5-year survival was approximately 45-50%.[14, 15] The five-year overall survival is now between 55-75%. Despite these improvements, one third of patients will still die from their disease.[16, 17]

DLBCL is staged using the Ann Arbor classification with Cotswold’s modification. In 1993, a seminal paper was published that attempted to identify the most important prognostic factors in order to derive a score that might help to predict survival.[18] This scoring system is called the International Prognostic Index (IPI) which predicts survival accurately in patients with DLBCL. A patient is scored according to Age>60,
stage >II, LDH level>normal, Eastern Cooperative Group performance status >1 and number of extra-nodal sites >1. A patient receives a score of one if any of the prognostic features is positive and zero if absent. The score thus ranges from zero to five, with five having poor survival and patients with no factors present having excellent outcome approaching five-year survival of 95%. Despite its usefulness, heterogeneity of outcome within IPI sub-groups persists. For example, although 30% of patients are known to relapse within 2 years, the IPI fails to accurately identify which patients these will be. For example poor risk IPI (IPI 3-5) categories still have cure rates of greater than 55%.[18, 19] Despite the improved outcome in DLBCL with the introduction of rituximab, the IPI is still highly predictive of outcome but it now splits patients into 3 rather than 5 groups, as was the case prior to its introduction to front-line therapy. An IPI score of zero predicts excellent outcome, IPI score of 1 or 2 is associated with good outcome but patients scoring >3 have poor outcome. [19] Given the IPI’s relative inability to predict very poor outcome, recently some of the parameters of the IPI have been expanded to provide better stratification.[20] Importantly, the IPI tells us little about potential therapeutic targets or mechanisms of disease resistance. Despite the relative accuracy of the IPI in predicting outcome, most patients still receive 6-8 cycles of standard chemo-immunotherapy irrespective of their IPI score. There is thus, a pressing need to identify the significant clinical and pathological prognostic factors that may better guide therapy for patients who are unlikely to benefit from standard therapy. This would allow physicians to not only investigate new therapies for these poor risk patients, but would also allow confidence to identify patients with excellent outcome with current standard therapy who do not require alternative strategies.

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<tr>
<th>IPI Factors (Score 1 for each factor present)</th>
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<tr>
<td>Stage&gt;2</td>
</tr>
<tr>
<td>LDH&gt;N</td>
</tr>
<tr>
<td>Extranodal sites&gt;1</td>
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<tr>
<td>Performance Status (ECOG&gt;2)</td>
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<td>Age&gt;60</td>
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Table 1. IPI Factors
1.2 Molecular Classification of DLBCL

It has been known for some time that there are distinct molecular subgroups of DLBCL. Alizadeh and colleagues in their seminal paper from 2000, showed three distinct molecular groupings in DLBCL. These were classified as germinal centre, activated B cell and a third group termed unclassifiable that lay in between these two groups.[21] What he showed, and what has been shown consistently since then is that dividing patients into two groupings, germinal centre-like B cell (GCB) and non-GCB could separate patients into two distinct groups with very different outcome after standard chemotherapy. [21-23] The prognosis of the unclassified grouping seems to lie in-between these two groups but it is generally included in a poor risk group incorporating the non-GCB subtype.[24, 25]

These gene expression findings have been backed up by similar findings from other groups who found that even low IPI scoring patients do relatively poorly if they are in a poor gene expression subclass such as non-GCB type.[26] The non-GCB subtype seems to have higher proliferation with a reduced immune reaction against it, and have a signature very different from normal lymph node signals. However, even in studies showing cell of origin as predictive of outcome the survival differences in patients treated with chemo-immunotherapy is modest between GCB and non-GCB with a 15-35% improvement in outcome for GCB patients. Prognosis based on COO based on gene expression from frozen tissue appears most accurate.[20, 27, 28] The data, although not conclusive, indicates that rituximab is particularly beneficial in the non-GCB group rather than the GCB classification.[28] However not all gene expression data published at this time is consistent or confirms that GCB and non-GCB subtypes are prognostic.[29] This shows the problems with performing analysis on different platforms, using different analysis methods and heterogeneous patient cohorts.[30] Because of these varied results, Lossos et al. set out to identify genes found in all the three previously described gene expression array studies performed at that time to find common genes that might allow development of a robust small number of genes to predict outcome and be more user friendly in the diagnostic/prognostic setting.[31] This group tested all 36 prognostic genes from gene expression studies and also any genes described as being prognostic from a number of other studies using real time-PCR in their patients’ samples. They found six genes that added additional prognostic value to the IPI. The six genes were LMO2, BCL6, FN1, CCND2, BCL2 and SYCA3. Of note two of these genes were associated with
the GCB phenotype (LMO2, BCL6) while FN1 is a normal lymph node signature. The three genes (CCND2, BCL2 and SYCA3) associated with poor prognosis are all associated with the non-GCB (ABC) subtype. While this data has subsequently been replicated by this group it required frozen tissue and it has not been widely applied. However in 2008 the authors published data confirming prognostic usefulness in paraffin based tissue samples from an R-CHOP tested cohort.[32] However these methods are still relatively complex for routine clinical use and have not translated into general clinical practice.

One of the challenges for applying these and associated classifications is the failure of any of these poor prognostic groups to benefit from alternate therapy to the current standard (R-CHOP). However a number of studies in recent years have shown that there may well be some therapeutic strategies that might specifically target tumours of the ABC subtype. Targeted therapies have been developed based on gene expression data such as Bortezomib or dose modified etoposide, doxorubicin, vincristine, prednisolone, cyclophosphamide and rituximab that may be more effective against the phenotype with a poor outcome (non-GCB).[33, 34]. Newer strategies such as the use of BTK inhibitors seem to preferentially target ABC DLBCL and current clinical trials are awaited to assess their use in DLBCL.[35, 36] However it is imperative that new technologies are validated and tested to ensure accurate classification so that the right patients receive the correct drug.

1.3 Clinical utility of Molecular Classifications
Because of the cost of DNA microarray technology, it is not routinely used in lymphoma diagnosis. It also requires frozen tissue, which is very difficult to acquire, as this requires the diagnosis to be known before biopsy, (unless procedures are in place at an Institute to snap freeze all potential new lymphoma diagnosis) and special storage facilities are required. It is only large tumour banks that have this tissue available.[30] Immunohistochemistry on formalin fixed paraffin embedded tissue (FFPET) is the standard test for most pathology departments for the diagnosis and investigation of malignancy and it is still the gold standard for diagnosis of diffuse large cell lymphoma. An attempt was made by Hans et al. in 2004 to establish an immunohistochemical algorithm that might approximate gene expression findings.[24] This algorithm used the immunohistochemical markers CD10, MUM1,
BCL6 in a sequential arrangement to divide patients into GC and non-GC subtypes and these groupings did have significantly different outcome. As would be expected BCL6 and CD10 conferred good outcome whereas MUM1 conferred poor outcome as single markers. The 5-year OS rate for GC patients was 76% compared to 34% for the non-GCB rates. This is similar to previous described cDNA analysis. They also had cDNA data on a number of patient samples and these showed reasonable correlation with IHC with the algorithm sensitive in picking up 74% of GCB subtype and 87% of non-GCB subtype. This shows that there are still significant issues with this algorithm with approximately one fifth of patients being misclassified. This is perhaps not surprising since it is unlikely that 3 proteins would provide similar results to 36 genes, nor does this algorithm identify the unclassifiable cases that are identified by gene expression profiling. In addition, a number of large studies have subsequently shown no survival difference between immunohistochemical-defined cell of origin including a sub-study of the large RICOVER-60 trial, which had full data on over 500 patients treated with R-CHOP.[37-39] However other research has shown that the GCB /non-GCB prognostic divide is still relevant in the rituximab era.[40, 41]. The Choi algorithm, which uses all the Hans antibodies but includes two additional antibodies to identify GCB and non-GCB, has also been found to be effective at predicting outcome based on cell of origin. [25] The additional antibodies are GCET1 and FOXP1. This study also had gene expression data and the IHC had a 91% concordance with it. A number of studies have confirmed the accuracy of this Choi algorithm compared to the other described algorithms but there are still large studies finding no prognostic impact for it. [39, 42] This heterogeneity certainly restricts the clinical use of these classifications.

There is also concern about reproducibility of IHC staining between different labs with the Lunenburg Consortium suggesting that a number of antibodies used in the Hans algorithm gave variable results between labs, with BCL6 (a nuclear staining antibody) in particular demonstrating marked variability.[43, 44] In most algorithms BCL6 positivity is a ‘deciding’ antibody for classification. For example, in the Hans classification, CD10 negative DLBCL biopsies are classified as non-GCB if BCL6 negative. Therefore inter-laboratory variability with this antibody is a particular concern. BCL6 is a germinal centre marker that also appears in a number of gene expression and IHC models that predict outcome with expression of BCL6 usually associated with improved outcome. In addition the importance of prognostic markers
such as BCL6 may have changed with the improvement in outcome seen with rituximab. In numerous pre-rituximab studies it has been shown to be associated with good prognosis. The addition of rituximab to standard chemotherapy appears to have improved outcome in BCL6 negative patients in particular, so that the prognostic significance of BCL6 expression in patients treated with rituximab now appears less clear.

In contrast is BCL2, which is an ABC marker. Prior to introduction of rituximab this IHC marker always predicted poor outcome however the utilization of rituximab seems to have overcome the poor prognosis associated with this protein.[45, 46]

There is currently no substitute for gene expression for defining cell of origin but there is still a paucity of gene expression data in the rituximab era. For routine use immunohistochemical methods for classification would still appear to be the most practical, with newer algorithms such as those described by Choi and Meyer likely to replace the Han’s classifications.

New technologies such as digital gene multiplex hybridization (DGME) hold promise for more clinically applicable use of cell of origin classifications. One such platform is NanoString nCounter ® that accurately assess gene expression from FFPE. This technology has promise for defining important subgroups based on cell of origin and requires minimal technical and bioinformatics expertise and therefore is transferrable to the routine diagnostic laboratory.[47]

### 1.4 FCG3A and C1qA polymorphisms and their impact in DLBCL

The anti-CD20 monoclonal antibody rituximab is a standard component of front-line DLBCL chemo-immunotherapy, typically as part of ‘R-CHOP’. It has resulted in a marked improvement in response and survival.[16] The importance of host genetics on the mode of action of rituximab in DLBCL is unclear.

Two principle mechanisms of action for rituximab are postulated. The first is antibody dependent cellular cytotoxicity (ADCC) whereby rituximab binds to FCGammaReceptor (FCGR) bearing Natural Killer (NK) cells, resulting in destruction of CD20⁺ normal and malignant B-cells by the reticulo-endothelial system.[48-51] The other is direct lysis via complement dependent cytotoxicity (CDC).[52-56] There is also data to support direct apoptosis of malignant cells on exposure to rituximab.[57-59]
Rituximab is a chimeric murine-human monoclonal antibody directed against the CD20 antigen. Its broad efficacy and attractive toxicity profile has resulted in its use for the treatment of a variety of malignant and autoimmune disorders. It is generally well tolerated with most of its side effects occurring during the first infusion, typically brief fever and rigors.\[60, 61\] Since rituximab targets CD20, expression of which is restricted to benign and malignant B-cells, it is not associated with the acute myelosuppression that is commonly seen following cytotoxic agents. Intriguingly however, it is associated with a phenomenon termed ‘late-onset neutropenia’ (LON), an idiosyncratic and relatively rare but well-recognized late complication of rituximab containing therapy.\[62\] LON is defined as neutropenia occurring after neutrophil recovery, at least 4 weeks from last therapy, and in the absence of other causes. It is therefore distinct from the occasional episodes of neutropenia that have been reported during the administration of rituximab monotherapy (neutropenia that developed within 30 days after completion of treatment). The latter has been postulated as due to the accelerated destruction of neutrophils caused by binding of rituximab–antigen complexes to neutrophil Fc receptors.\[63\] LON is relatively benign phenomenon with spontaneous neutrophil recovery being the norm, however severe neutropenic sepsis has occurred.

Due to the lack of controlled studies, the incidence of LON has yet to be adequately defined. Genentech, the manufacturer of rituximab, in post-marketing surveillance, declared an overall rate of 0.02% in more than 300,000 patients.\[62\] However, given that many cases may not have been reported via this mechanism, this figure is almost certainly an under-estimation. The majority of patients followed up post therapy have a routine blood test only every 3 months and unless the patient has a fever related to neutropenia, any LON occurring in this period will not be detected. LON has been reported in association with rituximab use in a variety of pathologies, particularly lymphoproliferative disorders but also stem cell transplantation and autoimmune diseases.\[64-68\] In these reports, LON was attributed to rituximab, but the results are difficult to interpret because of variable use of concomitant treatments and differing rituximab regimens. There is also inconsistency in neutropenic cut-off points. Furthermore, incidence of LON may vary between individual disorders treated with rituximab, and disease specific estimates might provide a more accurate assessment.
FCGR3A is a low-affinity receptor capable of binding the FC portion of complexed but not monomeric IgG. A polymorphism, alternatively encoding for a valine (V) or phenylalanine (F), has been identified. FCGR3A-V158 has a higher binding affinity for IgG than FCGR3A-F158.\[69\] There is evidence that this polymorphism is important in the treatment of colon and breast cancers with monoclonal antibodies such as cetuximab and trastuzumab respectively.\[70, 71\] Data on the impact of the FCGR3A-V158F polymorphism in DLBCL treated with R-CHOP is conflicting.\[72, 73\] There are also reports that the polymorphism may contribute to the development of late-onset neutropenia (LON).\[74, 75\] Although not designed for survival analysis, it is notable that in previous case series lymphoma patients with LON have strikingly low incidences of disease progression. It has therefore been proposed that development of LON is a measure of good outcome, perhaps reflecting enhanced potency of rituximab.\[76\] However lead-time bias needs to be considered carefully when interpreting such data. It is important to note that many patients with poor outcome in DLBCL are either refractory to initial therapy or relapse shortly after completing therapy, and thus may not develop LON. It is likely that the neutropenia caused by rituximab will be masked by salvage therapy or progressive disease.

Binding of C1q to the Fc portion of immune complexes activates CDC through initiation of the complement cascade. C1q is encoded by C1qA, whose sole coding polymorphism is at position 276, coding for adenine (C1qA-A276) or guanine (C1qA-G276). C1qA-A276 results in lower C1q protein levels than the C1qA-G276 polymorphism. Breast cancer patients heterozygous or homozygous for the C1qA-G276 genotype have a higher rate of metastasis.\[77\] In a study of 133 patients with follicular lymphoma treated with single agent rituximab, possession of the C1qA-A276 allele was associated with increased response rates and prolonged response duration, even after adjusting for FCGR3A-V158F polymorphisms.\[78\] The role of C1qA polymorphisms in DLBCL has not yet been evaluated.

**Immune Parameters in DLBCL**

**1.5 Tumour Infiltrating Lymphocytes**

There remains a critical need for identification of simple reproducible prognostic factors in DLBCL that are capable of identifying the approximately one-third of patients that will go on to have refractory disease or relapse early. In addition such
factors would be inherently more valuable if effective therapeutic modalities could be instigated to negate the adverse outcome associated with these factors. Recently, in addition to new therapies that target tumours directly, a number of therapies have emerged that alter host immune status in order to improve tumour surveillance.[79-81].

Tumor infiltration by non-malignant T-cells has been demonstrated in multiple lymphoma histologies and a variety of solid-organ non-haematopoietic tumours. There is heterogeneity with regards to prognostic importance of specific T cell subset in B cell lymphomas with CD4 T-cells, CD8 T-cells and CD4 T-regulatory cells all appearing to be of importance in predicting survival.[82-86] Even within subsets of CD4 such as T_{h1} and T_{h2} subtypes, there is heterogeneous data with regards to outcome. Not only is there heterogeneity within the subsets and number of these cells, but the location of these T cells in different tumours appears important such as whether the tumour is infiltrated at the centre of the tumour, tumour edges or tumour stroma all giving variable results.[87, 88] Key studies have shown that culture and re-infusion of tumour infiltrating lymphocytes (removed from the tumour site of patients) from melanoma tumours induces effective response rates in approximately 50% of patients with advanced disease indicating importance of these cells in cancer.[89-91]

Despite the findings in solid tumours, the data in DLBCL is surprisingly sparse with regards to immune cell infiltration. Immune parameters have been shown to be predictive of outcome in DLBCL.[26, 92-97] Iatrogenic immunosuppression given to prevent rejection post organ transplantation or acquired immunosuppression induced by HIV infection leads to high rates of lymphoma development. [98] The risk of lymphoma development is directly related to the intensity of immune-suppression with transplant recipients that require intense immune suppression, such as those undergoing small bowel and multi-organ transplants, having the highest rates of lymphoma development.[4, 99] Renal transplant patients have generally modest immune suppression but represent the largest number of PTLD patients seen, as this would be the most common transplantation procedure performed. Simply removing immune suppression can lead to complete remission in some cases despite morphology consistent with DLBCL.[100]

In an attempt to identify simple clinical markers of the immune microenvironment, two recent studies have used circulating absolute lymphocyte counts (ALC) and
absolute monocyte counts (AMC) as surrogate markers of host immune-effectors and immune-checkpoints to successfully predict outcome.[101, 102] These parameters are an inexpensive simple assessment of immune status that are routinely performed, and that are available to all treating physicians. While these markers assess circulating immunity in DLBCL, little is known about how reflective they are of immune cells within the tumour microenvironment (TME) and in particular the tumour infiltrating lymphocytes (TILs) in DLBCL. Prior to the introduction of rituximab, it had been shown that low levels of CD4+ T cells in the diagnostic biopsy as assessed by flow cytometric immunophenotyping was associated with inferior outcome.[82, 83] Recent studies have confirmed the importance of T cell activation in the tumour microenvironment with the T cell activation marker CD137 predicting outcome in a large DLBCL cohort.[95] This is of particular interest given an agonist antibody to CD137 may lead to improved immune responses against lymphoma.[95, 103] High levels of CD4+ T cells are associated with improved outcome in many malignancies.[84] This is however tempered by the fact that CD4 T cells are heterogeneous and are composed of subsets with variable functions such as T_H1, T_H2 and Tregs. There is no clear evidence about which CD4 subset contributes to improved outcome and certainly this needs to be addressed in prospective trials. The T_reg subset are identified as CD4^+FOXP3^+, (or CD4^+CD25^hiCD127^−). They are associated with poor outcome in epithelial cancers but paradoxically appear to be associated with improved outcome in some B cell lymphomas and within subsets of epithelial cancers.[85, 86, 104] One explanation is that increased numbers of T_reg reflects a relatively intact host immune system. Alternatively it has also been postulated that T_reg have a direct negative effect on proliferation of B cells.[105] In many studies immunohistochemical staining with FOXP3 is used to define a T_reg subset and in two reported DLBCL cohorts, higher levels of FOXP3 lymphocytes are associated with improved outcome.[106, 107] The second potentially important subset of CD4+T cells of interest is T helper cells. T helper cells can be divided into two subgroups. T_H1 subtype (which express the transcription factor T-bet, and secrete interferon-γ and TNF-α) is universally associated with good outcome in malignancy as they assist immunological clearance of tumours. The T_H2 subtype (which express the transcription factor GATA-3, and secrete IL-4, IL-5, IL-10 and IL-13) is associated with immunosuppression, and is
generally associated with inferior outcome.[84, 108] There is very limited data on the role of these various CD4 subsets in DLBCL. Interestingly there is evidence obtained prior to the introduction of rituximab that circulating lymphocytes of patients with DLBCL before treatment are skewed to a T_{h2} phenotype and revert to a T_{h1} phenotype with successful treatment.[109]

A number of recent animal models in B cell lymphoma have shown that CD4^{+} T cells are key cells in creating an anti-tumour microenvironment.[110] T_{h1} cells in particular stimulate and up-regulate antigen presentation and tumour clearance.[108] Improved antigen presentation has been shown to be a key survival determinant in patients with DLBCL.[37, 96, 111, 112] These animal models have shown that cytokines derived from T_{h1} cells such as Interferon gamma, IL1 and TNF alpha are implicated in stimulating macrophage mediated malignant B cell clearance. Mouse models have also shown that PD-1 which is present on many CD4 T cells may eventually down regulate CD4^{+} T cell tumour surveillance leading to relapse.[110] PD-1 has been found expressed in 27% of DLBCL tumours, but when one looks at the tumour microenvironment 38% of PD1 non-expressing DLBCL have PD1 positive histiocytes surrounding the tumours.[113] It is possible that PD-1 could be responsible for down-regulating CD4^{+} T cells in a large number of DLBCLs. This is of particular interest as two highly successful trials targeting PD-1 in advanced cancers have recently been described in solid tumours.[79, 80] To date two trials using an antibody to PD-1 have been described in B cell lymphomas.[114, 115] CT-011 (anti-PD1) directly increases the numbers of CD4^{+} T cells post autograft for relapsed disease with survival higher than historical controls.[115] In another study using Ipilimumab (an anti-CTLA-4 antibody) in a range of advanced relapsed and refractory lymphomas the antibody produced an ongoing complete remission in one of the three patients with DLBCL.[116] Recent publication of results of blocking PD-1 in DLBCL and follicular lymphoma show the feasibility and potential excellent response rates using immune checkpoint blockade in B cell lymphoma however larger studies are required to confirm these published findings.[117, 118] The study in follicular lymphoma seems to indicate enhanced T and NK cell function as a result of effective treatment with the anti-PD1 antibody.

Many of these molecules target immune checkpoints that are present in healthy immune responses to restrict excessive tissue damage and contain the immune response to where it is needed most. In some malignancies these checkpoints are
expressed at high levels within the tumour milieu and by tumour cells as an adaption to escape immune attack. [119]

1.6 CD8 cells in DLBCL

CD8 T cells are the end effector cells for the immune system and are directly cytotoxic to cells. CD8 cells with specificity for EBV epitopes have been shown in numerous studies to elicit responses in EBV positive lymphomas. [120-122] One of the commonest mutations found in DLBCL relates to loss of the key MHC I related protein beta-2-microglobulin with a recent study showing this molecule mutated in 29% of DLBCL cases. [123] This study also identified deletions in CD58 that can also affect CD8 recognition of antigen in 21% of cases, with many cases showing mutations in both genes. In addition alternate mechanisms of aberrant expression of MHC Class I and CD58 were frequent, indicating that cumulatively antigen presentation to CD8 cells is defective in >60% of patients with DLBCL. Prior IHC based studies had also shown a direct correlation between absence of immune recognition proteins such as HLA Class I and II molecules and CD80/CD86 that was associated with inferior outcome as well as reduced levels of CD4 and CD8 lymphoma infiltration in chemotherapy treated patients [124] However the data is sparse with regards to the impact of CD8 infiltration and outcome in DLBCL in the chemo-immunotherapy era. Early studies (pre rituximab) showed that poor outcome was associated with high levels of activated CD8 cells in the TME. The authors postulated that this led to killing of HLA-Class I expressing malignant B-cells allowing progression of tumours lacking HLA expression. [125] In particular large numbers of testicular and CNS lymphomas have loss of HLA expression but high numbers of CD8 T cells and have more aggressive disease. [126] However this data is conflicting with other studies showing high levels of CD8 infiltration in tumours with high levels of CD40 postulated to lead to consequent enhanced autologous tumour clearance and improved outcome. [127] It should also be noted that there is a subtype of DLBCL associated with extensive CD8 and to a lesser extent CD4 infiltration and relatively sparse CD20 malignant B cell population. These tumours appear to behave more aggressively with small studies indicating that the CD8 T-cells infiltrating these tumours are anergic and have reduced cytotoxic activity. [120] In contrast, data in an indolent B cell lymphoma termed follicular lymphoma shows enhanced outcome when tumours infiltrated by CD8 T cells were treated with single agent...
rituximab[128, 129] DLBCL is an aggressive B cell lymphoma in which rituximab is only administered in combination with anthracycline based combination chemotherapy such as R-CHOP. It is unknown whether CD8 T cell infiltration is a predictor of outcome of patients treated with R-CHOP in DLBCL.

1.7 Tumour associated Macrophages/Monocytes

Much data regarding the role of tumour-associated macrophages was described in Hodgkin Lymphoma. A seminal paper by Stiedl et al. looked at two groups of Hodgkin Lymphoma patients with differing outcomes using gene expression, and found that those genes associated with the tumour stroma/environment were strongly associated with poor outcome.[130] CD68 enumeration by immunohistochemistry seemed to act as a surrogate of the adverse macrophage gene expression signature. However not all studies have been able to replicate the IHC findings. [131, 132] A seminal paper from Lenz et al demonstrated that gene expression related to tumour microenvironment was predictive of outcome in DLBCL.[27] Poor outcome was defined by a particular stromal signature associated with angiogenesis. A second stromal signature was associated with improved outcome, and this was associated with monocytic and histiocytic infiltration with expression of CD68 noted to be elevated in this subgroup. In this study and in a subsequent analysis the immunohistochemical stain for SPARC acted as a basic surrogate of this complex gene expression signature.[133] Thus increased immune cell infiltration was associated with improved outcome, however in this study T cell infiltration was not prognostic and a strong MHC II immune signature was only prognostic in the cohort of patients not treated with rituximab.

There is added complexity to the importance of tumour-associated macrophages in DLBCL given the improved survival and standard adoption of rituximab in combination chemotherapy for the disease. ADCC is likely one of the main effector mechanisms causing rituximab induced death of tumour cells.[134] Macrophages are a main contributor to this mode of cell death (along with NK cells). One of the key reasons for the heterogeneous results with regard to macrophages in DLBCL may relate to overreliance on CD68 staining to classify macrophages. This stain is not specific to macrophages and other stromal cells can stain positively.[135] In addition there are two main subtypes of macrophages found in tumour microenvironments. These are categorized as type I and type II or M1 or M2 subtype.[136] These two
subtypes have distinct functions and macrophage sub-types are best viewed as a continuum, with M1 and M2 as opposite ends of a spectrum. The M1 subtype is associated with a pro-inflammatory response and might be considered potentially beneficial in tumour clearance. The M2 subtype is anti-inflammatory. There is strong evidence that many tumors manipulate their local environment to create an immune suppressing microenvironment that contains high levels of M2 macrophages to circumvent the natural immune response. In addition it is possible that the initial immune effector response against a tumour is strong with little immune suppression, but in the majority of cases this response is eventually counter-balanced by increased immune checkpoints/suppressors that down-regulate immune-effectors so as to prevent damage of normal tissue. Alternatively, or in addition, it is possible that tumours manipulate their local environment to counter effective immune responses by up-regulating immune checkpoints or down-regulating immune recognition mechanisms. Thus a large M1 macrophage response or a strong T cell response would be rendered ineffective if there is a large M2 macrophage response to counter it. CD68 alone is unable to distinguish between M1 and M2 macrophages. A combination of 2/3 markers will provide more information regarding macrophage status although this is inconsistently performed.[136] The combination of CD68$^{hi}$/HLA-DR$^{hi}$ positive cells favours an M1 phenotype whereas a CD68$^{hi}$/CD163$^{hi}$ phenotype favours an M2 phenotype.[137, 138] HLA-DR is associated with antigen recognition and is associated with an active immune response. CD163 is a heme scavenger molecule, and high expression is specific for M2 macrophages.[137, 139] However there is no definitive cut-off to distinguish between these two subtypes using these markers, and given that there is a spectrum of macrophages from M1 to M2, it would be extremely difficult to classify macrophages in the middle of these two subtypes. In addition it is felt that macrophages can reverse function and transition from M2 to M1 phenotype and vice versa depending on local cytokine signaling.[3] The cytokines produced by the subtypes of macrophages are very distinctive and represent the most accurate assessment of status but is relatively difficult to measure unless one has specific cytokine arrays and fresh cells. The M1 phenotype is associated with IL1, IL6, IL12 and TNF-alpha whereas M2 is associated with IL4, IL10, IL13 and TGF-beta production.[4, 10] These cytokines appear to mimic those associated with CD4 T cells where CD4 $^{T_h1}$ are associated with immune activation and the CD4 $^{T_h2}$ subtype appears similar to the M2 type macrophage.
Early studies in DLBCL seem to indicate that the M2 macrophage as detected by CD163 is associated with inferior outcome and that an increased ratio of CD68 to CD163 may indicate improved outcome in patients receiving chemo-immunotherapy.\cite{140} A small study from Japan showed that specific M1/M2 subtyping using CD68/HLA-DR and CD163/CD68 was also prognostic in DLBCL.\cite{141} However these findings are not always consistent and do not appear to be comparable to findings prior to the introduction of rituximab.\cite{142, 143} A large study of R-CHOP treated patients found no prognostic impact for expression of CD68 in 262 patients, however a macrophage marker consistent with a stromal 1 signature (SPARC) as described in Lenz et. al did predict outcome but had no correlation with CD68.\cite{92, 133} These findings indicate that CD68 is not specific enough for assessment of macrophage subtype nor survival outcome, as there is likely to be significant prognostic differences between tumours infiltrated by an M1 or M2 phenotype of macrophage, that cannot be identified using a single immunohistochemical stain.\cite{133, 143}

Whilst using CD163 as a more specific marker of the M2 Macrophage using immunohistochemistry has been widely published, there is relatively little known about the role that cells expressing CD163 play in the circulation. In agreement with tissue expression of CD163, it is now felt that circulating cells bearing CD163 contribute to an immune-suppressive environment in many cancers. A number of studies indicate a direct inhibition of lymphocytes by CD163. For example, in a published study from my research group in Hodgkin Lymphoma, high circulating levels of soluble CD163 (sCD163) were associated with significantly lower lymphocyte count at diagnosis.\cite{144, 145} It is felt that increased levels of these markers may indicate systemic immune suppression triggered by the tumour cells. There is no data of sCD163 in DLBCL. An initial study in melanoma used an ELISA based assay to measure sCD163 levels in over 200 patients with early stage disease.\cite{146} High levels of serum sCD163 were associated with inferior outcome though not necessarily disease specific survival. Interestingly, in this study levels of CD163 in patients were not consistently higher than healthy control participants. In Hodgkin Lymphoma, circulating sCD163 is markedly elevated at diagnosis compared to controls and acts as a disease response biomarker.\cite{145}
1.8 Myeloid Derived Suppressor Cells
Recent studies in NHL indicate that particular subsets of monocytes may have a phenotype consistent with that of an M2 macrophage. Lin et al described a myeloid suppressor subset that was associated with immune dysfunction and suppression that seemed to be associated with inferior outcome in a heterogeneous B-NHL population.[147] These cells that have been described in a number of malignancies are characterised by a phenotype of high CD14+ and low HLA-DR expression.[148, 149] Circulating monocytes infiltrate tissue such as lymph nodes to become macrophages so their phenotype may be critical in determining the tumour microenvironment. Whilst there is data supporting inferior outcome in DLBCL relating to high CD163 levels as discussed, this is not always consistent. There is no data investigating the relative interactions of CD163 in tissue or the circulation with immune effectors such as CD4 and CD8 in DLBCL. CD163 is only one of many molecules that may counter effective immune responses.

In addition there is minimal data on the relative impact on expression of key immune checkpoints in DLBCL. As described, targeting the immune checkpoints CTLA4, PD-1, PDL-1 and PDL-2 have all shown promise in the treatment of melanoma, renal cancer, lung cancer and there is emerging data on the targeting of these pathways in lymphoma.[84, 150, 151] In DLBCL responses have been seen in patients treated with an anti-CTLA4 antibody and adjuvant use of an anti-PD1 antibody post stem autograft in relapsed patients appears to improve outcome.[116, 118, 152] These therapies are relatively well tolerated and early evidence suggests excellent activity and safety in combining these agents with anti-CTLA4 and anti-PD1 therapy combined therapy giving remarkable responses in metastatic melanoma.[153] In addition, targeting of checkpoints such as LAG3 and TIM3 will soon enter clinical trials.[150] There are many other molecules of interest in this field which have yet to be fully investigated.[154] Little is known on how tumour infiltration by cells of the immune system might dictate response not only to current standard therapy but also the new wave of immune modifying agents.

1.9 Digital multiplex gene expression (DMGE) by nanoString nCounter.
NanoString is a new novel technology first described in 2009.[155] It captures and measures mRNA transcripts without any amplification or enzymatic steps. Each gene is detected by a probe with a particular color based barcoding system which then
attaches to a capture probe which allows the reporter probe to be immobilized. Excess probes and capture are washed off and a digital reader calculates the relative expression of the mRNA transcript based on the colour code of the reporter probe. Given this unique mechanism the bioinformatics load is relatively mild and data can easily be generated via a simple analysis program provided by the company. The correlation between runs is highly reproducible but more importantly data from matched frozen and paraffin tissue samples shows exceptionally high correlation which has now been replicated in a number of published studies.[156-158] The quality of data from paraffin tissues opens up much larger banks of lymphoma samples from retrospective databases as good quality gene expression data no longer requires snap frozen tissue. Indeed an early study in DLBCL has shown that NanoString will likely be highly sensitive at accurately determining cell of origin in DLBCL.[159] Early results from a small series of aggressive large cells lymphomas indicated that Nanostring technology could be effective at distinguishing between standard DLBCL and aggressive subtypes more closely related to Burkitt Lymphoma, however this study was small.[160] To date these two relatively small studies are the only research using digital gene barcoding in DLBCL. Nanostring is now an accepted diagnostic platform approved by the FDA for assessment of prognosis in Breast Cancer patients with recent approval of the PAM50 gene signature.[161]

References:


CHAPTER 2

Research Design and Methods
Research Design and Methods

Introduction

Many of the research design and methods are described in the published chapters making up this PhD. However in the following pages, I have tried to expand some of the important methods in more detail. Published articles give a basic review of methods so I felt it appropriate to include more detailed explanations in this section.

2.1 Clinical Sample Accrual

My research questions required well annotated patient cohorts in addition to tissue availability to produce meaningful results. I will describe the cohorts used in each of my research questions below as there was minor changes in exact numbers of patients used for each of these questions based on available tissue and clinical information at the time of analysis.

Firstly, I acquired a clinical database from Dr. Peter Mollee at Princess Alexandra Hospital which included all patients diagnosed with DLBCL between 2003-2009. Using this database I was able to identify approximately 200 patients with DLBCL. I personally continued this database and researched and obtained additional samples occurring between 2009-2011 and other patients missing from the database but treated at PA hospital. This time period was chosen as it reflected the introduction of rituximab in Queensland. However not all patients in this time period received rituximab as it only was initially available to patients over the age of 60 until early 2004 at which stage government funding allowed all patients with DLBCL to be treated with rituximab in association with their standard chemotherapy.

A formal request was then made to Pathology Queensland for access to biopsy specimens related to these patients. Approval for tissue collection was provided by the Ethics Committee of the Princess Alexandra Hospital. Unfortunately, biopsy specimens are only kept on-site for 6-9 months after which they are stored at a commercial contractor by Pathology Queensland. Due to significant costs in contracting this commercial company to find relevant biopsies for my study ($50 per specimen), Pathology Queensland allowed me to get special access to the offsite commercial storage facility to obtain these samples. I spent
12-15 hours reviewing boxes of archived FFPE tissue in order to obtain DLBCL specimens.

Due to Pathology Queensland and legal guidelines, I was only allowed to obtain material in samples with significant amounts of tissue as all specimens must be kept for 10 years in case of requirement for review and sufficient tissue must remain for re-analysis. In addition, large number of patients with DLBCL are diagnosed without ever having an excisional biopsy, rather these patients have core biopsies which are generally so small that is highly unlikely there will be sufficient tissue for research processing. These factors had significant impact on the number of patient samples obtained. Unsurprisingly, this is also reflected in the NHL21 cohort in which at the time of analysis I received approximately 50% of tissue samples compared to almost 87% of patients in whom we have received blood samples on. Additional samples have been received but these were not available during the time period of my laboratory analysis for this thesis. My initial ideal requirements were to obtain 3x30 micron slices. Processing was performed at Pathology Queensland, PAH and at the Pathology Department at Queensland Institute of Medical Research. The tissue slices were used to extract RNA and DNA required for PCR and gene expression based analysis. The restrictions imposed by small diagnostic biopsies and institutional and legal requirements has significant impacts on amount of tissue available for research studies. My research was significantly helped when it became possible to access further tissue samples from another Australian retrospective cohort based at Canberra, courtesy of Dr Dipti Talaulikar. In combination with current tissue processed, I have developed a database of 195 samples from patients with DLBCL (63 retrospective from PAH, 65 retrospective from Canberra, 67 samples from the prospective NHL21 cohort) all with tissue available and good clinical data. However, at this stage there is no survival data available for the NHL21 cohort with a first survival analysis due to be performed in mid-2015.

2.2 Patient Cohorts

Retrospective Cohort for Chapter 3

One hundred and fifteen patients were identified. R-CHOP consisted of an intravenous infusion of cyclophosphamide 750 mg/m^2, adriamycin 50 mg/m^2,
vincristine 1.4 mg/m$^2$ (capped at 2 mg), oral administration of 100 mg prednisone on days 1 to 5 (CHOP), and Rituximab 375 mg/m$^2$ at day 1 before CHOP chemotherapy began. Patients with stage I/II disease typically received 4 courses of chemoc immunotherapy followed by involved field radiotherapy (30c 40 Gy), while patients with advanced stage disease received 6 to 8 cycles of chemoc immunotherapy followed by radiotherapy to bulky sites. In patients receiving <8 cycles of combination therapy, further rituximab was administered as monotherapy so that in total patients received 8 doses. Maintenance rituximab after completion of Rc CHOP therapy was not administered. DNA extracted from formalin fixed paraffin embedded tissue (FFPET) was of sufficient quality to perform PCR analysis in 90 patients for FCGR3A2 V158F polymorphisms and 81 patients for C1qA2 A276G polymorphism. One hundred and five consenting healthy adult volunteers served as controls. Controls specifically denied haematological or autoimmune disorders of any kind.

**Retrospective Cohort for Chapter 4**

Analysis was restricted to DLBCL patients treated with R-CHOP between 1st January 2003 – 1st January 2010 at the Princess Alexandra Hospital, Brisbane. Inclusion criteria were age ≥18 years, histologically confirmed de novo DLBCL (grade IIIB follicular lymphoma and transformed follicular lymphoma were excluded) with full clinical annotation including Rc IPI scores, CBC and survival data. All patients were assigned to Rc CHOP chemoc immunotherapy, as per standard institutional practice. Therapy was delivered as per cohort 1 described above. HIV positive patients or those postc transplant were excluded.

This retrospective cohort included patients treated at the Princess Alexandra Hospital. Tissue was not required for assessment of absolute lymphocyte and monocyte count and 122 patients were available for this assessment. Flow cytometry data was available for 75 of these patients.

**Prospective NHL21 Cohort for Chapter 5**

My prospective cohort consists of patients treated on the ALLG NHL21 trial. This was a planned prospective laboratory subc study sponsored by the ALLG within the NHL21 clinical trial (Clinical Trials Identifier: ACTRN12609001077257). Clinical parameters were source verified and disease stage and IPI were central reviewed. Patients gave written informed consent to
provide blood samples for laboratory analysis. Where possible, tissue slices for RNA/DNA and TMA blocks were mandated to be supplied to our lab if sufficient tissue was available. In addition, thirty milliliters of blood were collected at pre-therapy and day 21 after cycle four of R-CHOP (post-cycle 4). Peripheral blood was also taken from healthy laboratory volunteers without prior diagnoses of malignant, hematological or autoimmune disorders who also provided written informed consent. The study was approved by all participating Hospital/Research Institute Ethics Committees and performed in accordance with the Declaration of Helsinki. Induction chemotherapy consisted of four cycles of R-CHOP administered every 14 days (R-CHOP-14), supported with pegylated granulocyte colony stimulating factor (peg-G-CSF). Post-cycle 4, chemo-immunotherapy was delayed a week and an interim-PET/CT scan performed between days 17-20. R-CHOP consisted of: day 1 rituximab 375 mg/m$^2$ intravenous (IV) infusion, cyclophosphamide 750 mg/m$^2$ IV, doxorubicin 50 mg/m$^2$ IV, vincristine 1.4 mg/m$^2$ (maximum 2 mg) IV, and prednisone 100 mg/day orally for 5 days. Following interim-PET/CT, patients received risk-stratified therapy.

![Diagram of ALLG NHL21 Study](image)

**Figure 2. Schema for ALLG NHL21 Study**
2.3 PET/CT analysis

Prec approved imaging centres performed dualc modality PET/CT, with centralised review at the Peter MacCallum Cancer Centre, Melbourne. All interim scans were reviewed centrally alongside diagnostic prec therapy scans (blinded to the local PET/CT assessment), to verify residual abnormal FDG uptake at sites of previously identified activity. Metabolic response was categorised using the ‘International Harmonisation Project in Lymphoma’ guidelines, on which Professor Rodney Hicks was a member of the working party. Patients with a complete metabolic response were classified as interimc PET/CTc ve, and those remaining PET FDGc avid interimc PET/CT+ve.

General Methods

2.4 RNA/DNA extraction

Nucleic acid extraction was performed using an Ambion Recover All Total Nucleic acid kit as per manufacturer’s instructions. In total between retrospective and prospective cohorts 195 FFPE samples had RNA and DNA extraction performed. This does not include samples were extraction failed. FFPE samples have extensive protein and protein nucleic acid cross linking due to the fixation process which reduces yields and quality of extraction compared to freshly prepared tissue. Nucleic acid modification causes fragmentation of RNA likely due to formaldehyde used in fixation process. In the majority of cases simultaneous DNA and RNA extractions were performed with an approximate 50/50 split of sample to each individual DNA/RNA pathway after initial deparaffisation processing steps were performed. DNA was used in PCRs for the FCG3A and C1Q work. In general good extractions were obtained, but as expected DNA yields tended to be higher due to relative age of specimens and increased level of RNA degradation. DNA/RNA quantity and quality were assessed using Nanodrop. Nanodrop uses spectrophotometric measurement to assess quality/purity. The A260/A280 ratio was measured with majority of specimens being within or close to target ranges of 1.8-2.1 for RNA and 1.7-1.9 for DNA when 1.5ul volumes are measured.
2.5 FCG3A polymorphism PCR

DNA was extracted from FFPE tissue (patients) or buccal scrapes (controls) using standard procedures with analysis performed in batches. **FCGR3A-V/F158** genotyping was performed using allele-specific PCR based on a previously described protocol. This consisted of the following 20 µl reaction (made up to this level with nuclease free water): 0.2 µmol/l each primer (common forward: 5'-TCCAAAGCCACACTCAAAGT-3', F-allele-specific reverse:5'GCGGGCAGGGCGGGCAGGCGGTGATGTTCACAGTCTCGTAAAGA CACATTTTACTCCAGA-3' and V-allele-specific reverse: 5'- TGAAGACACATTTTTACTCCATCc 3'), 0.4 µl Accuprime Taq DNA polymerase (Invitrogen), 1× Accuprime buffer I, 1 mmol/l MgCl and 10 ng genomic DNA. Reactions were cycled using 94°C for 2 min followed by 35 cycles of 94°C for 15 s and 60°C for 15 s. Bands were visualized on agarose gels with the F-allele resulting in an 118 bp and the V-allele in a 73 bp band.

![Figure 3. Electrophoresis for FCG3A polymorphism](image)

2.6 C1QA Polymorphism PCR

For **C1QA** polymorphism general procedures such as DNA extraction were performed as above for FCG3A. **C1QA-A/G276** genotyping was performed using allele-specific PCR based on a previously described protocol. This consisted of the following 20 µl reaction (made up with nuclease free water: 0.4 µmol/l each primer C1qA -F1 5'-GGGGGAACCTGGGCCCTCTGG-3 ,
C1qAc  R1  5 CGGCCGAGTGGTCTGGTACGGc  3, 0.2 µl AmpliTaq Gold DNA polymerase (Invitrogen), 2 µl 10× PCR buffer I, 1.2 µl 25 mmol/l MgCl, 0.4 µl 10mM dNTPs and 50 ng genomic DNA. Reactions were cycled using 95°C for 10 min followed by 40 cycles of 94°C for 15 s and 60°C for 30 s. Bands were visualized on 12% acrylamide/TBE gels with the G-allele resulting in an 170 bp and the A-allele in a 189 bp band. With this PCR there was significant difficulties in getting good bands with the FFPE extracted DNA which was improved by adding 50 ng DNA to each PCR reaction, whereas for fresh buccal swabs only 10 ng was required.

![Electrophoresis for C1QA polymorphism](image)

**Figure 4. Electrophoresis for C1QA polymorphism**

### 2.7 Flow cytometric analysis of retrospective samples

The following protocol was the standard protocol used at the diagnostic flow laboratory at the Princess Alexandra Hospital. Mononuclear cell preparations were made using diagnostic fresh tissue by mechanical disruption in HANKS containing 2% bovine calf serum. Aggregates and large particles were removed by filtration through nylon gauze. A 100 ul aliquot of cells were stained with the appropriate concentration of antibody for ten minutes in the dark at room temperature. Red cells were removed using ammonium chloride lysis buffer and the cells fixed in FACS fixative.

Mononuclear cells were stained with a panel of antibodies against T-, and B-cell markers, including CD3 (APC BD Clone SK7), CD4 (FITC BD Clone SK3), CD5 (CD5
PE BD Clone L17F12), CD8 (PE BD Clone SK1), CD19 (APC BD Clone SJ25C1), CD20 (APC BD Clone L27) and CD45 (PerCP BD Anti-HLe-1 Clone 2D1). Flow cytometric data was acquired on the FACS Calibur 4 colour bench-top analyser, (Becton Dickenson, New Jersey, USA) and analysed using BD CellQuest Pro Software. A minimum of 10,000 cells were analysed per tube. An isotype control was used to set the control levels of fluorescence for each fluorochrome.

Figure 5. (A) Gating of Lymphocytes (Nodal Biopsy) (B) CD4 Gating in high expressing patient (>20%)

2.8 NHL21 Blood processing
The received tubes of blood were spun at 1180 rpm with no brake to separate plasma. Plasma was collected into 10 to 50 ml tubes and spun at 3000 rpm for 10 mins to pellet platelets. Plasma aliquots of 1ml was then placed into Nunc cryovials and stored at c 80°C. The remaining blood was transferred into 50ml tubes with a maximum of 15 mls of blood diluted up to 35mls with RPMI. These samples were mixed well. Ficoll of 10 ml volumes was then gently added as an underlay with care taken not to disturb the interface. These tubes were then centrifuged at 1500 rpm for 20 minutes. The Buffy coat was then harvested into 10 ml tubes and pelleted after centrifugation at 1180 rpm. The supernatant was then aspirated and removed from each tube. Each pellet was then resuspended into a single tube with a small volume of RPMI and then made up to the 10 ml mark with further RPMI. Further centrifugation was performed to derive a final
pellet. The PBMC pellet was resuspended in 10ml of RPMI and 20% fetal calf serum with DMSO as a cryoprotectant. Sample were placed in controlled Mr.Frosty™ containers for gradual freezing over 2/3 days prior to transfer to liquid nitrogen.

2.9 Flow Cytometry NHL21 Study
Between 1 x10⁵ and 5X10⁵ PBMC were diluted in 100 ul of PBS + 2% foetal bovine serum and added to a well in a 96 well U bottom tissue culture plate. A pre-determined amount of the fluorophore labelled antibody was added according to the phenotype of the cells being stained. The tray was placed in a refrigerator for 15c – 30 minutes. At the completion of the incubation a further 100 ul of cool PBS was added to each well. The plate was centrifuged for 5 min at 400 g. The supernatant was carefully removed and replaced with 200 ul of cool PBS. The plate was centrifuged for 5 min at 400 g. The supernatant was carefully removed and replaced with 200 ul of cool PBS + 2% FBS. The supernatant was transferred to a FACS tube and a further 200ul of PBS +2% FBS was added to each. The tube was either refrigerated or placed on ice and subject to analysis within 4 hours of staining. The flow cytometry data was acquired using a BD LSRII flow cytometer controlled by FACSDiva software(BD, Australia). Data analysis was performed on compensated data using FlowJo 4.2 or 9.x software (Tree Star, USA).
2.10 Effector lymphocyte assays

The antiCD20-ADCC assay measures killing of a carboxyfluorescein succinimidyl ester (CFSE)-labeled CD20-positive DLBCL cell-line (SU-DHL4) incubated for 4-6 hours +/- PBMC and +/- rituximab/obinutuzumab. CHL cell-lines were used as CD20-ve control targets. Subtraction of the number of target cells lysed by addition of rituximab/obinutuzumab alone from the total number lysed by antiCD20 mAb with PBMC, enabled enumeration of antiCD20-ADCC mediated killing. CD107ab de-granulation of CD56+ and CD14+ cells, and CD137 activation of CD56+ was measured by flow cytometry.

For Tc cell proliferation, CFSE stained mononuclear cells were seeded in 96c well roundc bottom plates at 2c 5x10^5 cells/well in RPMI 1640 with 10% FBS and 1xP/S, with 10 u/ml interleukinc 2. Antic CD3/CD28/CD137 beads (Invitrogen) at a 1:10 bead: Cell ratios were added to stimulate polyclonal expansion. Cells were cultured for 96c 120 hours and assessed by flow cytometry.

Monocytes were depleted using an immunomagnetic CD14 selection procedure (EasySep, Stemcell Technologies) as per manufacturer recommendations achieving >90% monocyte depletion.
2.11 Enzyme linked immuno-absorbent assays (ELISA)

Plasma arginase I (BioVendor) and CD163 (R&D Systems) were quantified by ELISA according to the manufacturer’s instructions (at 1:20 CD163 dilution). I have summarized the protocol for CD163 assay below.

Basically, 100 ul of assay diluent was added to each ELISA well. 50 ul of standard control or sample were added to each well and incubated for two hours at room temperature. Each well was then aspirated and washed repeated three times for a total of 4 washes. 200 ul of CD163 conjugate was then added and the wells incubated for a further 2 hours. The wash steps were repeated once more. After this wash, 200 ul of substrate solution was added, incubated for 30 minutes and protected from light. 50 ul of stop solution was added was added to each well. The optical density of the wells were read at 450 nm using a microplate reader. Samples were run in duplicate with results and data extrapolated from a standard curve.

2.12 Digital multiplex gene expression by NanoString nCounter.

Nucleic acid was extracted from tumour biopsies using RecoverAll total nucleic acid extraction kit for FFPE (Life Technologies, Carlsbad, CA, USA). Using the nCounter platform (NanoString Technologies, Seattle, WA, USA) gene expression profiling was performed on 191 DLBCL samples in total. An initial pilot project was performed on 83 DLBCL samples and this was extended to 191 DLBCL samples based on significant results garnered from this study. For an initial pilot project, the minimum number of genes that could be tested on each sample was one hundred. This allowed me to assess important genes in DLBCL. Given my earlier work I was most interested in including immune based genes, but I also included cell of origin genes based on the Wright algorithm, and a number of genes found on next generation sequencing to be mutated in DLBCL. At that time, there was no physical machine in Australia, and to my knowledge this is the first large Nanostring project performed in Australia, and the largest study of DLBCL using this technology anywhere. All samples were sent to Nanostring in Seattle, USA for testing. All analysis was performed by myself with self-learning of the Nanostring provided NCounter software program. The success of this project has
now led to my laboratory becoming the first in Queensland to acquire this technology.

Hybridisations were carried out according to the NanoString Gene Expression Assay Manual. Five microliters of each RNA sample (100 ng) was mixed with 20 μl of nCounter Reporter probes in hybridisation buffer and 5 μl of nCounter Capture probes for a total reaction volume of 30 μl. The hybridisations incubated at 65°C for approximately 16–20 hours. For this digital gene expression, two separate runs were performed requiring the production of two identical code sets. Run 1 was an initial pilot study of 97 samples expanded to 191 patient samples with run 2. Expression counts were normalised between code sets based on relative differences between duplicate samples in both runs which allowing us to develop a correction factor for each individual gene between the runs.

Raw data (RCC files) was imported and analyzed in the NanoString® data analysis tool nSolver. For normalization, gene expression data was internally controlled to the mean of the positive control probes to account for interassay variability. Gene normalisation was then performed using the geometric mean of four housekeeper gene to account for factors that affect RNA quality and quantity (PGK1, GAPDH, PGAM1, OAZ1) Housekeeping genes were selected as previously described and as per manufacturer recommendation. After this normalisation procedure 12 of 97 samples from run 1 and 11 of 191 samples from run 2 did not pass QC and were excluded from further analysis.

2.13 Statistics and analysis

I performed the statistical analysis for the majority of the above work. Of note my co-author Frank Vari performed statistical analysis for flow cytometry data related to moMDSC research in this currently submitted paper. I used Graphpad Prism for simple Mann-Whitney, Student –T tests and Kaplan Meier survival testing. I performed multivariate survival analysis using SPSS software. Nanostring data was analysed firstly using nSolver analysis and then data was transferred to Prism based analysis. All my data is stored in excel based spreadsheets. Categorical data was compared using Fisher’s exact test or Chi squared test as appropriate. For non-parametric variables and analysis of factors
influencing outcome, Mann-Whitney U test were applied. Event free survival (EFS) was measured from time of diagnostic biopsy to the date of disease progression, relapse or death as a result of any cause, or the date of last follow up. Overall survival was measured from diagnosis to date of last follow up or death. Survival analysis was performed using Kaplan Meier curves and the log rank test. Multivariate analysis was performed using Cox regression. All tests were two sided at the threshold of $P=0.05$. All statistical analysis were prepared using Graphpad Prism 6 (Graphpad, La Jolla, CA) or SPSS (Statistical Package for the Social Sciences, IBM, NY, USA) version 13.0 for Windows.
CHAPTER 3

1. Homozygous FCGR3A-158V alleles predispose to late onset neutropenia after CHOP-R for Diffuse Large B-cell Lymphoma

Colm Keane, Jamie P. Nourse, Pauline Crooks, Do Nguyen-Van, Howard Mutsando, Peter Mollee, Rod A. Lea, Maher K. Gandhi
Intern Med J. 2011 Sep 1

2. “Rituximab induced Late-onset Neutropenia” for the book 'Rituximab: Pharmacology, Clinical Uses and role in Investigating B cell immunology in Man" published by Novus in 2012

Colm Keane, Jamie Nourse, Maher K. Gandhi
Homozygous FCGR3A-158V alleles predispose to late onset neutropenia after CHOP-R for Diffuse Large B-cell Lymphoma

Colm Keane, Jamie P. Nourse, Pauline Crooks, Do Nguyen-Van, Howard Mutsando, Peter Mollee, Rod A. Lea, Maher K. Gandhi


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Rituximab induced Late-onset Neutropenia” for the book 'Rituximab: Pharmacology, Clinical Uses and role in Investigating B cell immunology in Man” published by Novus in 2012

Colm Keane, Jamie Nourse, Maher K. Gandhi

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CD4(+) tumor infiltrating lymphocytes are prognostic and independent of R-IPI in patients with DLBCL receiving R-CHOP chemo-immunotherapy.
Keane C, Gill D, Vari F, Cross D, Griffiths L, Gandhi M.
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Measures of net anti-tumoral immunity add to the predictive power of conventional prognostic factors in diffuse large B cell lymphoma (DLBCL).

Colm Keane*, Frank Vari*, Mark Hertzberg, John Seymour, Rodney Hicks, Devinder Gill, Pauline Crooks, Kimberly Jones, Erica Han, Rod Lea, Lyn Griffiths, Maher Gandhi.

Submitted to Cancer Discovery May 2014

*Co-Authors
The immunobiological score: a robust 3-gene assay that segregates the international prognostic index into disparate survival categories in aggressive B-cell lymphoma

Colm Keane*, Frank Vari*, Mark Hertzberg, John Seymour, Rodney Hicks, Devinder Gill, Pauline Crooks, Kimberly Jones, Erica Han, Rod Lea, Lyn Griffiths, Maher Gandhi.

*Joint Authorship
Submitted to Cancer Discovery May 2014

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The immunobiological score: a robust 3-gene assay that segregates the international prognostic index into disparate survival categories in aggressive B-cell lymphoma

Frank Vari*, 1,2,3,4 Colm Keane*, 5 Mark Hertzberg, 6 Michael R. Green, 1,2 Erica Han, 7 John F. Seymour, 7 Rodney J. Hicks, 4 Devinder Gill, 1,2 Pauline Crooks, 1,2 Clare Gould, 1,2 Kimberley Jones, 8 Kristen J. Radford, 4 Lyn R. Griffiths, 9,10 Dipti Talaulikar, 10 Sanjiv Jain, 9 Josh Tobin, 1,2,3 Maher K. Gandhi.

1 Experimental Haematology, School of Medicine, Translational Research Institute, University of Queensland, Australia; 2 Queensland Institute of Medical Research, Brisbane, Queensland, Australia; 3 Princess Alexandra Hospital, Brisbane, Queensland, Australia; 4 Genomics Research Centre, Griffith University, Queensland, Australia. 5 Department of Haematology, Westmead Hospital, Sydney, New South Wales, Australia; 6 Division of Oncology, School of Medicine, Stanford University, Stanford, California, USA. 7 Peter MacCallum Cancer Centre and University of Melbourne, Melbourne, Victoria, Australia; 8 Mater Medical Research Institute, Translational Research Institute, Brisbane, Queensland, Australia; 9 Canberra Hospital, Canberra, Australian Central Territory; 10 Australian National University Medical School, Australian Central Territory.

*These authors contributed equally to the manuscript.

Running title. LMO2 +/- CD8:CD163 in DLBCL

Keywords: lymphoma; LMO2; CD8; CD163; gene expression
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**Conflict of Interest**

Roche provided funding towards the NHL21 clinical trial but not the laboratory study.

**Word count: 5520**

**Figures 7, Tables 0. Supplemental Figures 3; Supplemental Tables 3.**
Abstract
Diffuse large B-cell lymphoma (DLBCL) is a common and aggressive lymphoma with approximately 30% mortality. Risk-stratification requires prognosticators to identify poor outcome patients in whom investigational therapeutic intervention is justified. Circulating lymphocyte:monocyte ratios are prognostic, implicating them as surrogate immune-effectors and monocyte/macrophage-checkpoint within the tumor microenvironment. Blood from 140 ‘R-CHOP’ chemo-immunotherapy treated DLBCL patients from an Australasian Leukaemia and Lymphoma Group trial was analysed. Detailed functional and quantitative assessment enabled identification of the optimal immune-effector and monocyte/macrophage-checkpoint molecules to interrogate within the tissue. CD163 identified a highly immunosuppressive subset of CD14+HLA-DRlo monocytoid-myeloid-derived-suppressor cells ‘moMDSC’. Ratios of various immune-effectors to CD163hi moMDSC were used as a measure of total antitumoral immunity: i.e. the net balance between the antagonistic forces of immune-effectors and monocyte/macrophage-checkpoints. All ratios were higher in early R-CHOP responders compared to delayed responders, with CD8:CD163hi moMDSC the most discriminatory. To test for intratumoral applicability, genes were quantified by digital hybridization in an independent cohort of 128 R-CHOP treated DLBCL patients. Co-clustering of CD8 with CD163 was observed, consistent with an adaptive immune-checkpoint response to immune-effector activation. CD8:CD163 ratios were prognostic independent of cell-of-origin and international prognostic index (IPI). An immunobiological score combining CD8:CD163 to the germinal-centre marker LMO2 strengthened the predictive ability, identifying 24% at risk of very poor outcome. It separated low-risk IPI into 91% and 44%, and high-risk IPI into 76% and 26% 4-year survivals. Results were externally validated in 233 patients. The
immunobiological score is a powerful new 3-gene assay that segregates IPI into markedly disparate survival categories.

250 words.
Introduction

Diffuse large B-cell lymphoma (DLBCL) is a common and aggressive form of B-cell lymphoma for which approximately only 70% of patients will be cured (1). The majority of those who die from their lymphoma, either display refractoriness to first-line therapy, or relapse within 24 months (2). A number of promising new therapeutic strategies are at various stages of clinical development (3). However, the application of any novel therapeutic strategy requires accurate identification of those patients likely to die despite current therapies. In these patients, prolonged exposure to conventional first-line agents may contribute to the induction of chemo-resistance as well as unnecessary toxicity. Consequently in refractory/early relapsing patients, alternate strategies should be instituted early. However, despite the use of conventional pre-treatment prognosticators such as the international prognostic index (IPI), very considerable heterogeneity of outcome persists (1). Therefore there is a pressing need to develop tools to more accurately predict response to initial therapy.

It has recently been established that patients with low peripheral blood absolute lymphocyte:monocyte ratios (LMR) have inferior outcomes (4-6). It is known that circulating lymphocyte and monocyte subsets each have active roles in DLBCL control (7, 8). For example recent data strongly implicates CD8\(^+\) T-cells in the prevention of murine models of lymphoma (9), and adoptive T-cell therapy has shown clinical benefit for the treatment of immunosuppression related lymphomas (10). Within peripheral blood monocytes, the CD14\(^+\)HLA-DR\(^{lo}\) ‘monocytoid-myeloid-derived-suppressor cells’ (moMDSC) subset is associated with higher rates of disease progression in patients with B-cell lymphoma (11-13).
We have established that circulating immune subsets reflect immunity within the DLBCL tissue tumor microenvironment (TME) (4). A simple, robust and easily standardized tissue biomarker, applicable at diagnosis, that provided additional information to conventional prognosticators, would be a highly useful tool for risk-stratification. With new digital multiplexed gene expression (DMGE) platforms applicable to formalin-fixed, paraffin-embedded tissues (FFPET)(14), simultaneous interrogation of a range of aspects of DLBCL biology, that include the TME, is achievable by the diagnostic laboratory (15, 16).

Our aim was to develop a gene expression score applicable to FFPET that reliably identified those patients with DLBCL at high-risk of treatment failure within 24 months following treatment with conventional ‘R-CHOP’ chemo-immunotherapy. A composite score, incorporating assays of malignant B-cell biology with the balance of the antagonistic forces of immune-effectors and monocyte/macrophage-checkpoints, would likely have more prognostic value than a measure of B-cell biology alone. We rationalized that detailed functional and quantitative assessment of circulating immune-effector and monocyte-checkpoints would enable identification of the optimal immune molecules to incorporate within the composite gene expression score. To develop such a prognostic tool, we utilized sequential blood samples taken as part of an Australasian Leukaemia and Lymphoma Group (ALLG) clinical trial in poor-risk DLBCL. Immune-effector and monocyte/macrophage molecules were identified that were applied to diagnostic tissues. Candidate molecules were quantified by DMGE in DLBCL tissues in an independent Australian R-CHOP cohort. The resultant algorithm was then externally validated in an independent international R-CHOP DLBCL cohort.
Materials and Methods

ALLGNHL21

Details of the study and eligibility criteria are provided with the supplemental methods. Induction chemo-immunotherapy comprised three cycles of R-CHOP administered every 14 days (R-CHOP-14). After a fourth cycle of R-CHOP, chemo-immunotherapy was delayed a week and an interim-PET/CT scan performed between days 17-20. Dual modality PET/CT was performed in pre-approved imaging centres, with centralized review (R.H.) at the Peter MacCallum Cancer Centre, Melbourne. All interim scans were reviewed centrally alongside diagnostic pre-therapy scans (blinded to the local PET/CT assessment), to verify residual abnormal FDG uptake at sites of previously identified involvement. Blood assays were planned prospectively within the ALLGNHL21 clinical trial. Thirty millilitres of blood were collected pre-therapy and on day 21 post-cycle 4 of R-CHOP. Investigators were blinded to PET/CT results. Blood was also taken from 23 healthy participants without prior diagnoses of malignant, hematological or autoimmune disorders. All participants gave written informed consent. The study was approved by responsible Ethics Committees at participating sites and performed in accordance with the Declaration of Helsinki.

Tissue cohorts

Tissue cohort one comprised 128 patients with histologically confirmed DLBCL: 66 from Princess Alexandra Hospital, Queensland; and 62 from Canberra Hospital, Australian Capital Territory. All patients received R-CHOP, and were selected on the basis of tissue and outcome data availability. These tissues were supplemented with 63 FFPET from the ALLG Tissue Bank from whom survival data
was not available for tests of co-clustering (191 FFPET). Only de-novo cases were included. Grade IIIB follicular lymphoma, transformed follicular lymphoma, HIV-positive and post-transplant lymphoproliferative disorder patients were excluded. The external validation tissue cohorts utilized a publicly available data-set (17). Patients were divided into two groups based on treatment regimen (CHOP 181 patients versus R-CHOP 233 patients). Details of RNA quantification of DLBCL samples is provided with supplemental methods.

**Statistical analysis**

Values between groups of data were tested for statistical significance using the 2-tailed paired (e.g. between time-points) or where appropriate non-paired tests. Categorical data were compared using Fisher’s exact test or Chi-squared test as appropriate. Overall survival (OS) was measured from diagnosis to date of last follow-up or death. Survival analysis was performed using Kaplan–Meier curves and the log-rank test. Multivariate analysis was performed using Cox regression. All tests were two sided at the threshold of P=0.05. All analyses were prepared using GraphPad Prism platform (version 6, GraphPad Software, La Jolla California USA) and Statistical Package for the Social Sciences SPSS version 13 (International Business Machines Corporation, New York USA).
Results

Patient characteristics.

Of 161 patients with DLBCL accrued to NHL21, 154 had interim-PET/CT. The 140 patients in whom blood samples were obtained (91%) were included in this study. As anticipated from the inclusion criteria, patients had poorer-risk features (82% stage III-IV, 43% mass ≥7.5cm) than an unselected DLBCL population. There were 23 healthy participants. There was no significant difference in age/sex between healthy participants (median age 49, range 31-68 years, 39% female) and patients (56 years, range 26-70, 34% female). Patient characteristics for NHL21 and the Australian tissue cohort are outlined in supplemental Table S1. Details of the external validation tissue cohorts are as previously published (17).

Monocytes suppress CD8+ and CD4+ T-cell proliferation in poor-risk DLBCL.

Monocytes were increased as a proportion of the mononuclear cells among the DLBCL patients compared to healthy participants (P<0.0001, Figure 1A). The impact of monocytes upon immune-effectors was tested. It has previously been shown that total T-cell proliferation is impaired by monocytes in B-cell lymphomas (13). However as that study did not interrogate individual T-cell subsets, we tested the effect of monocyte depletion in order to assess the differential effect upon CD4+ and CD8+ T-cells. Peripheral blood mononuclear cells (PBMC) from randomly selected patients and healthy participants were tested for T-cell proliferation. Although total CD3+ T-cells were reduced, the proportion of total and CD8+ and CD4+ T-cell subsets in patients that proliferated in response to in-vitro stimulation were not different to healthy participants at both time-points (P=NS). However, monocyte
depletion enhanced T-cell proliferation in pre-therapy samples for total CD3\(^+\), CD4\(^+\), and CD8\(^+\) T-cell subsets, conversely monocyte depletion from healthy participant samples did not (Figure 1B-D).

**Monocytes of poor-risk DLBCL patients suppress rituximab but not obinutuzumab mediated antibody-dependent cell-mediated cytotoxicity.**

AntiCD20 monoclonal antibodies (mAB) have improved outcome in DLBCL (1). However, the impact of circulating monocytes upon antibody-dependent cell-mediated cytotoxicity (ADCC) in B-cell lymphoma patients has not previously been explored. CD107ab degranulation by flow cytometry demonstrated that antiCD20-ADCC was overwhelmingly mediated by NK-cells (as opposed to monocyte mediated ADCC). We assessed the effect of monocyte depletion of PBMC from randomly selected poor-risk DLBCL patients and healthy participants. In healthy participants monocyte depletion did not impact the levels of ADCC mediated by either rituximab or the type-II antiCD20 mAB obinutuzumab (Figure 1E-F, R- and Ob-ADCC, respectively). However, monocyte depletion in patients did enhance R-ADCC (P=0.01), which indicates that any benefit of monocyte mediated antiCD20-ADCC is masked by monocytes mediated immunosuppression. Interestingly, Ob-ADCC was not altered by monocyte depletion (P=NS). Comparing healthy participants and pre-therapy patients, when monocytes were intact, R-ADCC was significantly lower in patients compared to healthy participants (P=0.01) but became equivalent (P=NS) when monocytes were depleted (Figure 1G). Ob-ADCC was equivalent between patients and healthy participants with monocytes intact and depleted (both P=NS).

CD137 (TNFRSF9) is an inducible cell-surface co-stimulatory receptor and immune-effector activation marker. NK-cell activation was measured upon
encountering antiCD20 coated DLBCL cell-lines, by expression of CD137 during R- or Ob- ADCC (Figure 1H). In R-ADCC, NK-cell activation was markedly reduced pre-therapy in monocyte replete poor-risk DLBCL patients compared with healthy participants. However, no difference in NK-cell activation was observed between patients and healthy participants for Ob-ADCC. These results indicate that in poor-risk DLBCL monocytes suppress rituximab- but not obinutuzumab mediated ADCC activity.

**CD163 expression identifies a highly immunosuppressive subset of moMDSC (CD14^+HLA-DR^{lo}) in DLBCL.**

In contrast to healthy participants, PBMC from DLBCL patients collected pre-therapy consisted almost exclusively of CD14^+CD16^- classical monocytes, and had lower levels of HLA-DR (Figure 2A). The CD14^+HLA-DR^{lo} moMDSC subset were ~2-fold elevated (Figure 2B, P<0.0001) compared to healthy participants (representative plot shown in Figure 2C). The absolute number of CD163^+monocytes was also increased in patients (P=0.0054). Absolute moMDSC and CD163^+monocytes values were correlated (Figure 2D, r=0.41, P<0.0001), and pre-therapy CD163^+moMDSC were ~4-fold raised relative to healthy participants (Figure 2E, P=0.001). Arginase production by moMDSC depletes arginine and impairs immune-effector signal transduction and function (18). Consistent with such an effect mediated by the monocytes of patients with DLBCL, arginase activity was elevated compared to healthy participants (Figure 2F, P=0.0005). CD163 mean fluorescent intensity (MFI) on moMDSC showed moderate correlation with arginase activity in patients and healthy participants (Figure 2G, r=0.45, P=0.002).
To characterize CD163\textsuperscript{hi}\textsuperscript{moMDSC} versus CD163\textsuperscript{lo}\textsuperscript{moMDSC}, CD14\textsuperscript{+}HLA-DR\textsuperscript{lo} cells with the highest and lowest (top and bottom thirds) CD163 expression were analysed (Figure 3A-I). A higher proportion of CD163\textsuperscript{hi}\textsuperscript{moMDSC} expressed lymphoid migratory markers CD62L (P<0.0001) and CD11c (P=0.035). CD163\textsuperscript{hi}\textsuperscript{moMDSC} expressed more CD120b (a marker associated with MDSC survival, P=0.001) (19). Cell surface expression of the myeloid specific inhibitory receptor CD33 (P=0.0108), colony stimulating factor-1 (CSF-1R: a monocyte/macrophage trafficking, differentiation and survival factor, P<0.0001), and CD80 (a T-cell regulatory receptor, P=0.0088) were also higher in CD163\textsuperscript{hi}\textsuperscript{moMDSC}. Levels of the monocyte/macrophage marker CD68, the integrin alpha M marker CD11b, and the co-stimulatory molecule CD86 were equivalent.

In aggregate, these results indicate that CD163\textsuperscript{hi}\textsuperscript{moMDSC} have distinct features, including a migratory and regulatory phenotype and elevated arginase activity.

**CD8:CD163\textsuperscript{+}\textsuperscript{moMDSC} ratios are elevated in patients with poor-risk DLBCL that become interim-PET/CT\textsuperscript{-ve} after post-cycle 4 R-CHOP.**

All patients received uniform R-CHOP chemo-immunotherapy for four cycles, after which therapeutic response was assessed with an interim-PET/CT. The rate of interim-PET/CT-positivity for the 140 patients was 29%. Neither individual IPI parameters nor the combined IPI were associated with interim-PET/CT treatment response (each P=NS).

We then tested for associations of immune-effectors and monocyte/macrophage-checkpoints with treatment response. Neither pre-therapy peripheral blood lymphocytes nor any lymphocyte subset were associated with
interim-PET/CT response (P=NS), nor were absolute monocytes (Figure 4A left). It is known that peripheral blood absolute lymphocyte:monocyte ratios (LMR) are predictive of overall survival (4-6), but their association with interim-PET/CT response has not previously been evaluated. There was no difference in LMR between interim-PET/CT^+ve and interim-PET/CT^-ve patients (Figure 4B left). Pre-therapy ratios of total lymphocytes (and NK-cells or CD3^+ T-cells) to monocytes were not associated with interim-PET/CT (Figure 4B) or the individual components of the IPI and the combined IPI score (P=NS).

Next, based on the elevation of immunosuppressive monocyte subsets observed, we evaluated the association of absolute moMDSC and CD163^+moMDSC with treatment response. Interim-PET/CT^+ve patients had ~1.5-fold and ~3-fold higher levels of moMDSC and CD163^+moMDSC than interim-PET/CT^-ve patients (Figure 4A middle and right panels, P<0.001 and P<0.0001 respectively).

Ratios of various immune-effectors with moMDSC and then CD163^+moMDSC ratios were then evaluated for possible association with treatment response. Interestingly, ratios of total lymphocytes (and NK-cells or T-cells) to moMDSC were all ~2-fold elevated in patients that achieved interim-PET/CT negativity (Figure 4C). When CD163^+moMDSC was used as the denominator, ratios to total lymphocytes, NK and CD3^+ T-cells were ~4-fold (P<0.0001), ~6-fold (P=0.0002) and ~6-fold (P<0.0001) elevated respectively in patients that became interim-PET/CT^-ve (Figure 4D). To compare results in specific CD3^+ T-cell subsets, ratios of CD8^+ T-cells and CD4^+ T-cells divided by CD163^+moMDSC were tested for association. Both ratios of CD8^+ T-cell and CD4^+ T-cell to CD163^+moMDSC were higher in interim-PET/CT^-ve patients (each P<0.0001), at ~10-fold and ~4-fold respectively. Plasmacytoid dendritic cells (pDC) are circulating poly-functional innate immune-effectors. Ratios
of pDC to CD163⁺moMDSC were also ~6-fold higher in interim-PET/CT⁻ve patients (P<0.0001).

**Plasma soluble CD163 is associated with lower lymphocytes and higher IPI.**

After metalloproteinase-mediated cleavage, CD163 is released from macrophage or monocyte cell membranes and the extracellular portion of CD163 circulates in blood as a soluble protein (22). Soluble CD163 (sCD163) was measured in healthy participants and pre-therapy and post-cycle 4 plasma of patients with poor-risk DLBCL to further establish its ability to serve as a monocyte/macrophage-checkpoint marker.

Pre-therapy patient sCD163 levels were higher (Figure 5A, P<0.0001) and receiver operator curve analysis was highly discriminatory versus healthy participants (Figure 5B, area under curve 0.94, P<0.0001). Pre-therapy sCD163 levels were associated with lower lymphocytes (P=0.004), advanced stage (P=0.0072), older age (P=0.0143) and higher IPI (P=0.0003), but no other clinical variables (Figure 5C-F). However, pre-therapy sCD163 levels were not significantly different in those becoming interim-PET/CT⁻ve to those remaining interim-PET/CT⁺ve (P=NS). Similarly, post-cycle 4 sCD163 was not associated with interim-PET/CT status (P=NS). Soluble CD163 levels reduced by post-cycle 4 (P<0.0001), but were still raised relative to healthy participants (P<0.0001). Reduction in sCD163 between pre-therapy and post-cycle 4 was similar irrespective of whether the patient became interim-PET/CT⁻ve or remained positive. Finally, tissue CD163 mRNA expression was significantly but only modestly correlated with sCD163 (r=0.4, P=0.018).

**Monocytes are ‘reset’ post-cycle 4 of R-CHOP.**
There is minimal data on the kinetics of circulating immune-effectors and monocyte/macrophage-checkpoints during therapy for DLBCL. By paired analysis, absolute monocyte counts were not reduced post-cycle 4 relative to pre-therapy (P=NS). The MFI of HLA-DR expression on monocytes increased between time-points (P<0.0001). Consistent with this, moMDSC numbers fell between time-points (Figure 6A, P=0.0001) and CD163*moMDSC were lower post-cycle 4 (Figure 6B, P=0.0002). Plasma arginase, a hallmark of moMDSC, was reduced post-cycle 4 relative to pre-therapy (pre: mean 513ng/ml, 56-733 ng/ml, post: 378 ng/ml, 69-709 ng/ml, P=0.004). Gene expression microarray on monocytes isolated from healthy participants and paired pre-therapy-post-cycle 4 monocytes showed distinct clustering of healthy/post-cycle 4 monocytes versus pre-therapy, with up-regulation of antigen presentation and down-regulation of TH2 cytokine and tumor-associated macrophage (TAM) genes in healthy/post-cycle 4 monocytes (Figure 6C). In line with monocytes being ‘reset’ to a healthy profile, there was 2-3 fold up-regulation of STAT1 and associated genes at post-cycle 4.

Monocytes are a source of blood myeloid dendritic cells (BMDCs), which are circulating antigen presenting cells central to the development of innate and adaptive immunity (23). Ex-vivo BMDCs in healthy participants were higher relative to patients at both time-points (both P≤0.004), and increased between time-points (Figure 6D, P=0.0002). In parallel with BMDC, there were fewer pDC in patients compared with healthy participants prior to therapy (P<0.0001). CpG-DNA induced interferon-α production from pDC in patients was reduced relative to healthy participants (Figure 6E, P=0.006), indicating a functional as well as numerical deficit in pDC in DLBCL patients. However pDC increased post-cycle 4 (Figure 6F, P=0.0001) so that they were equivalent to healthy levels.
As expected, chemo-immunotherapy resulted in a marked reduction in lymphocyte subsets post-cycle 4. Ratios of all immune-effectors to moMDSC or CD163⁺moMDSC performed post-cycle 4 were not associated with interim-PET/CT (all P=NS).

**Construction of a tissue-based immune-effector:monocyte/macrophage-checkpoint ratio.**

PBMC assays identified a variety of immune-effectors and monocyte/macrophage-checkpoints that were associated with differential post-cycle 4 treatment response. Of these, CD8 and CD163 were highly discriminatory. To test the applicability of these immune-effectors and monocyte/macrophage-checkpoints as prognosticators within the diagnostic biopsy, FFPET from an independent Australian cohort of 128 patients treated with R-CHOP were utilized. As the median follow-up of the Australian tissue cohort was 3.9 years, the outcome measure chosen was 4-year overall survival. IPI and cell-of-origin (COO) were used as covariates.

NanoString nCounter was used to perform digital multiplex gene expression (DMGE). There was a strong correlation in three paired DLBCL frozen-FFPET (all \( r>0.94, \) \( P<0.0001 \)).

Initially, the predictive ability of multiple immune-effectors was compared. In addition to CD8/CD4/CD56/CD137 (all of which had been shown to be impaired in peripheral blood taken pre-therapy), we tested for TNF\(\alpha\), a cytokine known to be secreted by T-cells, NK-cells and M1 macrophages. Patients were divided as being above or below the median value for the relevant molecule. For all immune-effectors except CD4 and CD56, values above the median segregated patients with superior
survival, compared to those with values below the median (Table 1). CD8, CD137 and TNFα were selected for further evaluation; of these CD8 was marginally the most discriminatory (greatest percent difference in 4-year survival).

As CD163 identified a highly immunosuppressive subset of moMDSC, it was therefore used for further evaluation as a monocyte/macrophage-checkpoint within the lymphomatous tissue (Table 1). It did not stratify patient outcomes on its own. However combining immune-effectors with CD163 as a ratio to measure net anti-tumoral immunity, was highly discriminatory, and a better discriminator than using immune-effectors on alone. For CD8:CD163, those with high ratios (i.e. above the median) had superior survival to those with ratios below the median (P=0.0007). We then tested the remaining immune-effectors as numerators in ratios with CD163 as the denominator. CD137 but not TNFα in ratio with CD163 stratified patients into two distinct groupings (Table 1). However CD8:CD163 remained the best discriminatory ratio with markedly different 4-year survivals for high and low ratios of 89% and 59% respectively (Table 1). This ratio was therefore chosen for additional appraisal as a measure of net anti-tumoral immunity.

Interestingly, DMGE in 191 DLBCL tissues found co-clustering of CD8 with CD163 within the TME (r=0.32, P<0.001). To see if similar findings were present in blood, correlations in pre-therapy NHL21 blood samples were performed. Consistent with the tissue findings, CD8+ T-cells and CD163+moMDSC modestly but significantly correlated (r=0.42, P=0.0016). The blood and tissue data combined suggest that an adaptive immune response is present, with the monocyte/macrophage immune-checkpoint CD163+moMDSC activated in response to CD8+ T-cell anti-tumoral immunity.
CD8:CD163 adds to the predictive ability of IPI and COO prognosticators.

In the Australian tissue cohort, IPI as well as a measure of malignant B-cell biology, the COO (stratified as GCB and non-GCB), stratified patients into high and low-risk survival categories as expected (supplemental Figure 1, P=0.002 and P=0.018 and 4-year survivals of 83% versus 61%, and 82% versus 60% respectively). As expected, few deaths occurred after 24 months.

Median CD8:CD163 ratios were then tested for their ability to sub-stratify IPI and COO. Firstly, CD8:CD163 ratios were used to sub-divide patients with low-risk (0-2) and high-risk (3-5) IPI’s (supplemental Figure 2). This showed that survival was superior in those with high CD8:CD163 ratios for high-risk IPI (P=0.03), with a similar but non-significant trend seen for low-risk IPI (P=0.07). Next, CD8:CD163 ratios were used to stratify COO. Patients with high CD8:CD163 ratios had a higher probability of survival than low CD8:CD163 ratio patients in both GCB and non-GCB groupings (P=0.047 and P=0.011). For both IPI and COO, the discriminatory value of CD8:CD163 ratios were most pronounced in those at risk of poorer outcome, i.e. high-risk IPI and non-GCB (supplemental Figure 2B and 2D), compared to low-risk IPI and GCB (supplemental Figure 2A and 2C).

Cox Regression multivariate analysis was consistent with the Kaplan-Meier analysis. This showed that IPI, COO and CD8:CD163 were independently predictive of OS (P=0.046, P=0.038 and P=0.015 respectively), whereas CD137:CD163 was not (P=NS). We then performed Cox Regression in an external validation cohort, comprising 181 patients treated with CHOP chemotherapy-alone and 233 patients with R-CHOP chemo-immunotherapy. This enabled a comparison of the relative importance of CD8:CD163 within the TME, with and without the addition of rituximab. By multivariate analysis IPI, COO and CD8:CD163 remained independently
predictive of OS (P<0.0001, P=0.0006 and P=0.0006 respectively) in R-CHOP treated patients. Interestingly, although CD8:CD163 was prognostic in the CHOP chemotherapy-alone cohort (P=0.0007), only IPI was independently predictive in multivariate analysis.

The 3-gene composite immunobiological score ‘LMO2+/−CD8:CD163’ adds to the predictive ability of IPI and COO in R-CHOP treated patients.

LIM domain only 2 (LMO2) is a germinal-centre B-cell marker associated with improved outcome after R-CHOP (24, 25). In keeping with this patients above the median cut-off for LMO2 typed as GCB in 88% of cases, but only 13% in non-GCB cases. As expected, in the Australian tissue cohort, patients with higher than median expression of the germinal-centre B-cell marker LMO2 had superior outcome to patients with low LMO2 (Figure 7A). The discriminatory value was similar to that of IPI and COO, at 85% (LMO2 high) versus 62% (LMO2 low) 4-year survival (P=0.0046).

We hypothesized that a composite score of LMO2 (as a marker of malignant B-cell biology) and CD8:CD163 as a measure of net anti-tumoral immunity would provide a simple 3-gene prognosticator with increased discriminator value over either parameter alone. A binary score was tested in which high LMO2 and/or low LMO2 with a high CD8:CD163 ratio was designated ‘positive’, against a ‘double-negative’ of low LMO2 and low CD8:CD163. As this measured both malignant B-cell biology and net anti-tumoral immunity, it was termed the ‘immunobiological’ score. This identified two groups with markedly different 4-year survivals of 86% (positive) and 33% (double-negative, P=0.002, Figure 7B). Seventy six percent of patients were positive, and 24% double-negative. Most deaths (>80%) occurred within 24 months.
The new score was compared to a previously proposed two-gene-scoring weighted algorithm that used LMO2 and CD137 (26). Although less effective at identifying a group at risk of a particularly poor outcome than the immunobiological score, the two-gene-score did also effectively stratify patients (4-year survival 89% for above median versus 58% below median, P<0.001).

For the immunobiological score to confer additional value to conventional prognosticators, it was important to ascertain whether it was capable of sub-stratifying IPI and COO. Critically, for each of low-risk and high-risk IPI (Figure 7C-D), and GCB and non-GCB (Figure 6E-F), the composite score very strikingly added to the IPI and COO’s ability to sub-stratify patient survival. With IPI, 4-year survival was 91% and 44% (low-risk IPI, P<0.0001) and 76% and 26% (high-risk IPI, P=0.002), and for COO 4-year survival was 89% and 27% (GCB, P<0.0001) and 79% and 36% (non-GCB, P=0.007).

**External validation of the immunobiological score in R-CHOP treated DLBCL patients.**

The immunobiological score was then applied to the independent Affymetrix tested gene-expression cohort of 181 CHOP treated and 233 R-CHOP treated patients. The score segregated patients into groupings with different survival (Figure 7A-B, P=0.0007 and P<0.0001 respectively). As with the Australian R-CHOP treated tissue cohort, for the Affymetrix R-CHOP cohort the score was additive to IPI and COO (Figure 7D,F,H,J). For IPI, it separated the 79% 4-year survival of low-risk IPI into two categories of 85% and 48% (Figure 7D, P<0.0001). Similarly, the 53% 4-year survival of high-risk IPI was stratified into two groupings of 66% and 36% (Figure 7F, P=0.0007). This confirmed that the immunobiological score had
predictive power and could sub-stratify IPI and COO in an external independent cohort, and that DMGE gave similar findings to Affymetrix tested DLBCL patients.

Interestingly with the CHOP chemotherapy-alone patients, although LMO2+/-.CD8:CD163 had predictive power, this was not additive to non-GCB or low-risk IPI. It did stratify high-risk IPI (P=0.006) patients and there was a non-significant trend with GCB (P=0.056).
Discussion

It is established that in patients with DLBCL treated with CHOP-R chemo-immunotherapy, low peripheral blood absolute lymphocyte:monocyte ratios confer inferior outcomes (4-6, 27). This implicates circulating lymphocytes and monocytes as surrogate markers for further analysis of intratumoral immunity. The present study confirms that detailed functional assessment of blood immune-effector and monocyte/macrophage-checkpoints, permits the rational identification of the optimal immune molecules to incorporate within a lymphoma-tissue based prognostic gene expression score. Furthermore, it demonstrates that the net balance of immune-effectors and monocyte/macrophage-checkpoints are critical to outcome.

DLBCL biopsies are enriched in TAMs (17, 28-31). TAMs are thought to be immunosuppressive ‘M2’ macrophages. TAMs derived from primary tumors are believed to facilitate circulating tumor cell seeding of distant metastases in breast, pancreatic and prostate cancer (32). Although it is known that monocytes can migrate to lymph nodes and differentiate into macrophages, the relationship between moMDSC and TAMs is incompletely understood. Following adoptive transfer of MDSC into tumor-bearing mice, cells with the characteristics of TAMs can be recovered from the tumor microenvironment (33). In other murine models TAMs were shown to express relatively low levels of major histocompatibility class II molecules, and tumor progression is positively correlated with increasing infiltration of the tumor tissues by MHC class IIlo TAMs (34). However data in humans are sparse.

TAMs have high expression of the scavenger receptor CD163, whereas pro-inflammatory M1 macrophages are CD163lo. CD163 is also expressed on ‘classical’ (CD14+CD16−) monocytes. CD163+ monocytes rise with aging and during HIV (35),
and are implicated in the lymphopenia of Hodgkin Lymphoma (36). Tissue CD163 is an adverse prognosticator in melanoma and breast cancer (37, 38). We found that addition of CD163 to conventional moMDSC indicators identified a highly immunosuppressive subset of moMDSCs in DLBCL. MoMDSC and CD163⁺ monocytes values correlated and CD163⁺ moMDSC were enriched within circulating monocytes. The correlation between the M2 TAM marker CD163 and HLA-DRlo on circulating CD14⁺ monocytes suggests a link between circulating moMDSC and M2 TAMs within the malignant lymph node. This is supported by CD163hi moMDSC expressing markers that permit migration into lymphoid tissues. Similar to M2 macrophages (M1 are CD68⁺CD163lo and M2 CD68⁺CD163hi), expression of CD68 was equivalent irrespective of CD163.

Within the circulation, the immune-effector:CD163⁺ moMDSC ratios were lower in those remaining interim-PET/CT⁺ve. Pre-therapy patient sCD163 levels were higher and reduced at post-cycle 4, but unlike our observations in Hodgkin Lymphoma (36), did not become differentially reduced between interim-PET/CT⁺ve/-ve patients. As repeat biopsy was not performed, no definitive conclusion can be made as to whether pre-therapy CD163⁺ moMDSC associate with residual DLBCL versus inflammation within sites of interim-PET/CT-FDG-avidity. Rather the aim was to functionally characterize circulating immune-effector and monocyte/macrophage-checkpoints for further investigation of their expression within the diagnostic FFPET biopsy, with survival as the outcome and IPI and COO as co-variates. New digital hybridization technologies that are specifically applicable to FFPET are now available. In keeping with previous reports, using a DMGE platform we observed strong correlation between gene expression in paired frozen/paraffin samples across
a range of genes (15, 16, 39), indicating that this approach does accurately quantify RNA on FFPET in R-CHOP treated DLBCL patients.

Interestingly, intratumoral CD163 expression alone was not prognostic. However the CD8:CD163 ratio found that those with a lower ratio had inferior outcome. This may explain why results of intratumoral CD163 alone as a prognosticator are inconsistent (40, 41), and emphasises the importance of measuring several markers to more accurately reflect the balance of TME immunity. Similarly, CD8:CD163 ratios as a measure of net anti-tumoral immunity predicted outcome more effectively than CD8 alone. CD8:CD163 was independent of IPI and COO. Results were validated in an external R-CHOP gene-expression cohort. Interestingly, although CD8:CD163 was predictive in a CHOP cohort, this was not independent of conventional prognosticators. This may reflect the relatively increased importance of TAMs in those treated with chemo-immunotherapy versus chemotherapy-alone, and is consistent with our findings that patient monocytes suppress R-ADCC.

Gene expression has identified distinct molecular subtypes of DLBCL, based on the putative cell-of-origin of the malignant B-cell, termed ‘germinal-centre B-cell (GCB)’ and ‘activated B-cell like (ABC)’ (28, 42, 43). LMO2, a GCB marker, alone is also an independent predictor of survival (24, 25). In R-CHOP treated patients, CD8:CD163 ratios were independent of COO, and enhanced the prognostic ability of LMO2. The combination of a marker of B-cell differentiation with net anti-tumoral immunity appears to better distinguish patients (than IPI or COO alone) with markedly different survival. Furthermore LMO2 combined with CD8:CD163 successfully allowed segregation within low and high IPI groups. IPI is influenced by patient fitness, age and tumor burden, which are non-over-lapping with CD8:CD163.
For the R-CHOP Australian tissue cohort, the application of the immunobiological score to low and high-risk IPI segregated patients into groupings with four distinct, well-spaced survival outcomes of 91%, 76%, 44% and 26%. Similar results were seen with the external validation cohort. Although it is known that approximately 30% of patients will remain refractory or relapse following conventional first-line chemo-immunotherapy, upfront identification of this group (as candidates for alternative induction therapy) has remained problematic. A combined clinical and immunobiological score may permit rational selection of patients in whom novel therapies should be tested.

CD137 expression on NK-cells is an important marker of mAB-mediated anti-cancer cell ADCC (44). Interestingly we found that within ex-vivo blood taken from patients enrolled into the NHL21 trial, CD137 up-regulation was reduced in NK-cells relative to healthy participants upon exposure to rituximab coated DLBCL targets. CD137 has also been proposed as a biomarker of tumor-reactive T-cells (45). We observed that within the diagnostic tissue, CD137 gene expression alone had equivalent prognostic ability to CD8. However, CD137:CD163 was less discriminatory than CD8:CD163, and unlike CD8:CD163 was not prognostic by multivariate analysis. A two-gene-score that combined LMO2 with the immune-effector molecule CD137 has previously been recognized to be prognostic (26). Notably, the immunobiological scoring system had markedly greater discriminatory ability than the two-gene-score, and was especially effective in identifying a group at risk of a particularly poor outcome.

These findings have other therapeutic implications. Firstly, monocyte depletion in patients enhanced NK-cell mediated R-ADCC but not Ob-ADCC, indicating that the immunosuppressive effects of monocytes in DLBCL may be
overcome by obinutuzumab (a type II antiCD20 monoclonal antibody). Clinical trials comparing R-CHOP versus Ob-CHOP are ongoing and translational studies in that population could further explore this hypothesis. Another notable finding was the striking co-clustering of CD8 and CD163 within the tissue and circulation. This is in line with emerging data that up-regulation of immune-checkpoints is an adaptive (rather than constitutive) immune-checkpoint response to regulate immune-effector activation (46). The correlations were significant but modest, reflecting the variable success of the host to counter anti-tumoral immunity within the TME. Blockade of other immune-checkpoints is a promising therapeutic approach (47), and strategies that target CD163 may be similarly beneficial (48).

Immune-based strategies are gaining ground in DLBCL (49). The kinetics of immune cells will likely impact the efficacy of these approaches, and may influence dose-scheduling. There is minimal data on circulating moMDSC kinetics in DLBCL, with a small study finding moMDSC returned to normal after therapy (12). We found CD163*moMDSC reduced post-cycle 4, accompanied by an increase in ex-vivo BMDCs and pDCs (i.e. antigen-presenting cells that orchestrate adaptive and innate immunity). Monocytes are an important source of BMDCs in-vivo (23). Reduction in moMDSC may be associated with an enhanced ability of monocytes differentiating into BMDCs. The immunosuppressive profile of monocytes reduced by post-cycle 4, including down-regulation of TH2 cytokines and TAM associated genes, and up-regulation of STAT1 (18, 50). Similarly plasma CD163 reduced by post-cycle 4. As with cHL, plasma CD163 levels associated with stage and reduced lymphocytes (36).

Our data emphasizes the importance of capturing net anti-tumoral immunity within the TME, by measuring the relative balance of immune-effector to
monocyte/macrophage-checkpoints. The immunobiological score is a robust, easily standardized 3-gene assay applicable to FFPET that segregates IPI into markedly disparate survival categories. The data indicates a link between human M2 TAMs and moMDSC, and demonstrate that CD163 identifies a highly immunosuppressive subset of moMDSC in DLBCL. Further investigation of CD163⁺moMDSC as a therapeutic target is warranted, particularly in those with adverse CD8:CD163 ratios.
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References:
Fas ligand-mediated immune surveillance by T cells is essential for the control of spontaneous B cell lymphomas. *Nature medicine.*


34. Wang, B., Li, Q., Qin, L., Zhao, S., Wang, J., and Chen, X. 2011. Transition of tumor-associated macrophages from MHC class II(hi) to
MHC class II(low) mediates tumor progression in mice. *BMC immunology* 12:43.


Figure 1: Depletion of monocytes enhances T-cell proliferation and rituximab but not obinutuzumab mediated ADCC.

A. Monocytes are increased in poor-risk DLBCL patients compared to healthy participants;
B-D. CD3⁺/CD4⁺/and CD8⁺ T-cell proliferation with (depleted) and without (intact) monocyte depletion in healthy participants and pre-therapy patient blood samples;
E. R-ADCC measured in healthy participants (left) and pre-therapy DLBCL patients (right) with and without monocyte depletion;
F. Ob-ADCC measured in healthy participants (left) and pre-therapy DLBCL patients (right) with and without monocyte depletion;
G. Monocytes impair R-ADCC but not Ob-ADCC in pre-therapy DLBCL patients;
H. Reduced activation of NK-cells during R- but not Ob- ADCC in healthy participants and pre-therapy DLBCL patients.

Figure 2: Characterization of CD163⁺moMDSC.

A. Representative flow cytometry plots showing ‘classical’ (CD14⁺CD16⁻), ‘intermediate’ (CD14⁺CD16⁺) and ‘non-classical’ (CD14dimCD16⁺) monocytes in healthy PBMC, with enrichment of CD14⁺HLA-DRlo (moMDSC) in classical monocytes in a pre-therapy DLBCL patient;
B. MoMDSCs are elevated in pre-therapy DLBCL patients compared to healthy participants;
C. HLA-DR expression on CD14+ monocytes is lower at pre-therapy compared to the same participant at post-cycle 4 and to a healthy participant. The CD163 expression on DRlo monocytes (moMDSC) is raised pre-therapy. CD163 is indicated by the shaded histogram, the open histogram is the control;
D. Pre-therapy DLBCL moMDSC and CD163⁺CD14⁺ cells are correlated;

E. CD163⁺moMDSC are elevated in pre-therapy DLBCL patients compared to healthy participants;

F. Arginase activity was elevated compared to healthy participants;

G. CD163 mean fluorescent intensity (MFI) on moMDSC correlated with arginase activity in patients and healthy participants.

**Figure 3**: Cell-surface phenotype of CD163⁺⁺moMDSC versus CD163⁺⁻moMDSC.
Proportion of cell surface markers expressed in CD163⁺⁺ and CD163⁺⁻ moMDSC compared for (A) CD11b, (B) CD11c, (C) CD68, (D) CD120b, (E) CD62L, (F) CD33, (G) CD80 (H) CD86 and (I) CSF-R1.

**Figure 4**: Pre-therapy monocyte subsets are elevated in DLBCL patients remaining interim-PET/CT⁺ve after 4 cycles of R-CHOP chemo-immunotherapy.
A. Pre-therapy absolute (i) monocyte (ii) moMDSC and (iii) CD163⁺⁺moMDSC subset counts in DLBCL patients, grouped by post-cycle 4 interim-PET/CT results.

B. Pre-therapy lymphocyte subset:monocyte ratios in DLBCL patients, grouped by post-cycle 4 interim-PET/CT results. Numerators: panel (i) total lymphocytes; (ii) NK-cells; (iii) T-cells.

C. Pre-therapy lymphocyte:CD14⁺HLA-DR⁺⁺ ratios in DLBCL patients, grouped by post-cycle 4 interim-PET/CT results. Numerators: panel (i) total lymphocytes; (ii) NK-cells; (iii) T-cells.

D. Pre-therapy lymphocyte subset:CD163⁺⁺moMDSC ratios in DLBCL patients, grouped by post-cycle 4 interim-PET/CT results. Numerators: panel (i) total lymphocytes; (ii) NK-cells; (iii) T-cells.
Figure 5: Kinetics of circulating monocytes and dendritic cells in poor-risk DLBCL patients during R-CHOP.
A. Paired moMDSC counts in blood samples taken pre-therapy and post-cycle 4;
B. Paired CD163⁺moMDSC counts in peripheral blood samples taken pre-therapy and post-cycle 4. Arrows denote mean values;
C. Heat map showing gene expression microarray analysis on circulating monocytes isolated from 5 healthy participants and 6 DLBCL patients taken pre-therapy and post-cycle 4, showing up-regulation of antigen-presentation genes, and down-regulation of TAM associated and TH2 cytokine genes at post-cycle 4;
D. Paired BMDC at pre-therapy and post-cycle 4;
E. CpG-DNA induced interferon-α production from pDC in patients and healthy participants;
F. Paired pDC at pre-therapy and post-cycle 4.
Arrows denotes mean values.

Figure 6: A binary composite score of LMO2⁺/⁻CD8:CD163 added to the ability of IPI and COO to stratify patient survival.
Kaplan–Meier estimates of OS are shown. A. Patient tissues were divided by median LMO2 into LMO2 high and low; B. A binary composite score of high LMO2 and/or high CD8:CD163 (‘positive’), versus low LMO2 and low CD8:CD163 (‘double-negative’). C. In low-risk (0-2) IPI patients, composite score positive patients had the higher probability of survival; D. For high-risk IPI, double-negative score patients had the lower probability of survival; E. GCB positive patients had the higher probability of survival than GCB double-negative; F. Non-GCB positive patients had the higher probability of survival than non-GCB double-negative.
**Figure 7: External validation of the immunobiological score in 199 CHOP and 233 R-CHOP treated DLBCL patients.**

The immunobiological score was tested using Kaplan–Meier estimates of survival in a chemotherapy-alone (CHOP) and a chemo-immunotherapy (R-CHOP) treated cohort, in the left and right columns respectively. All CHOP (A) and R-CHOP (B) patients; Low-risk IPI CHOP (C) and R-CHOP (D) patients; High-risk IPI (>2) CHOP (E) and R-CHOP (F) patients; GCB CHOP (G) and R-CHOP (H) patients; non-GCB CHOP (I) and R-CHOP (J) patients.
Supplemental Table S1. Optimization of immune-effector and monocyte/macrophage-checkpoint combinations. Significant P values are in bold.

Supplemental Table S2. Characteristics of the blood and tissue patient cohorts.

Supplemental Table S3. Flow cytometry antibodies used.

Supplemental Figure S1: Plasma CD163 is elevated in poor-risk DLBCL.
A. Plasma CD163 is elevated in pre-therapy DLBCL patients compared to post-cycle 4 (paired analysis) and both pre-therapy and post-cycle 4 plasma CD163 are elevated relative to healthy participants.
B. ROC analysis of plasma CD163 levels in pre-therapy DLBCL patients and healthy participants.
C. Plasma CD163 was inversely associated with absolute lymphocyte counts (using a median cut-off for 120/ml).
D. Plasma CD163 was associated with advanced stage.
E. Plasma CD163 was associated with age >60 years.
F. Plasma CD163 was associated with higher IPI.
Panels A, D-F show mean and SEM.

Supplemental Figure S2. IPI and COO stratification in the Australian tissue cohort. A. Low (0-2) and high (3-5) risk IPI; B GCB and non-GCB.
Supplemental Figure S3. CD8:CD163 ratio adds to the predictive ability of clinical and malignant B-cell biology prognosticators.
A. Low-risk IPI; B. High-risk IPI; C. GCB; D. Non-GCB.
Figure 1.

A) % classical monocytes live

B) % CFSElo CD3 T-cell

C) % CFSElo CD4 T-cell

D) % ADCC

E) % ADCC

F) % ADCC

G) % ADCC

H) % CD137 NK-cells
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Supplemental Methods

ALLGNHL21 Clinical study

Eligible patients were aged between 18-70 years with CD20+ DLBCL with IPI 2-5 or IPI 0-1 with bulky tumor (≥7.5 cm), and FDG-PET–positive evaluable disease. Patients with non-bulky IPI 0-1 disease were excluded. Patients with non-bulky IPI 0-1 disease were excluded. Those with previously treated lymphoma, primary central nervous system (CNS) lymphoma, transformed lymphoma or follicular lymphoma grade 3B patients were ineligible. Patients had to have an Eastern Cooperative Oncology Group (ECOG) performance status between 0-3 and be considered suitable for R-CHOP-14, and high-dose chemotherapy with autologous stem-cell rescue. Clinical parameters were source verified and IPI centrally reviewed. All patients had serum creatinine ≤ 150 µmol/L, total bilirubin level ≤ 30 mmol/L, transaminases ≤ 2.5 maximum normal level, neutrophils ≥1.5 x 10⁹/L or platelets ≥ 100 x 10⁹/L unless due to lymphoma. Patients had to be HIV-negative, hepatitis B virus (HBV) surface antigen negative, and/or HBV core antibody negative with HBV surface antibody titre <100iu/ml unless clearly due to prior vaccination. Patients with uncompensated cardiac failure, chronic lung disease with hypoxia, severe psychiatric disease or any history of cancer during the last 5 years (with the exception of non-melanoma skin tumours or in situ cervical carcinoma were excluded.

All interim-PET/CT scans were reviewed centrally alongside diagnostic pre-therapy scans (blinded to the local PET/CT assessment), to verify residual abnormal FDG uptake at sites of previously identified activity. Metabolic response categorisation used a 5-point scale (1). Patients with a complete metabolic response (≤3 on 5-point scale) were classified as interim-PET/CT-ve, whereas those remaining
PET-FDG-avid (>3) were classified as interim-PET/CT positive. R-CHOP was given as follows: day 1 rituximab 375 mg/m$^2$ intravenous (IV) infusion, cyclophosphamide 750 mg/m$^2$ IV, doxorubicin 50 mg/m$^2$ IV, vincristine 1.4 mg/m$^2$ (maximum 2 mg) IV, and prednisone 100 mg/day orally for 5 days. Further therapy was dependent on the outcome of the interim-PET/CT scan and is not the subject of this study (further details are available under Clinical Trials Identifier: ACTRN12609001077257).

**Immuno-phenotyping**

The antibodies used are outlined in supplemental Table S2. The flow cytometry data was acquired using a BD LSRII flow cytometer controlled by FACSDiva software (BD, Australia). Data analysis was performed on compensated data using FlowJo 4.2 or 9.x software (Tree Star, USA). MoMDSC were defined as CD14$^+$ monocytes which were DR$^{lo}$, i.e. with DR expression lower than the median DR expression of B-cells in the PBMC population. Blood myeloid dendritic cells (BMDCs) and plasmacytoid dendritic cells (pDC) were analysed as previously outlined (2). Briefly, among the lineage negative DR$^{hi}$ cells, pDC were CD123$^+$CD11c$^-$ cells while BMDC were CD123$^-$CD11c$^+$. Intratumoral flow cytometry, was performed as previously described (3).

**Functional assays**

T-cell proliferation and antiCD20-ADCC assays were performed with and without monocyte depletion. Monocytes were depleted using an immunomagnetic CD14 selection procedure (EasySep, Stemcell Technologies) as per manufacturer recommendations achieving >90% monocyte depletion.

The antiCD20-ADCC assay measures killing of a carboxyfluorescein succinimidyl ester (CFSE)-labelled CD20-positive DLBCL cell-line (SU-DHL4)
incubated for 4-6 hours +/- PBMC and +/- rituximab/obinutuzumab. Classical Hodgkin Lymphoma (cHL) cell-lines (to confirm specificity for CD20) were used as CD20-ve control targets. The absolute change in targets was measured by flow cytometry. Subtraction of the number of target cells lysed by addition of rituximab/obinutuzumab alone (direct lysis) from the total number lysed by antiCD20 monoclonal antibody with PBMC, enabled enumeration of antiCD20-ADCC mediated killing. CD107ab de-granulation of CD56+ and CD14+ cells was measured by flow cytometry.

For T-cell proliferation, CFSE stained mononuclear cells were seeded in 96-well round-bottom plates at 2-5x10^5 cells/well in RPMI 1640 (Invitrogen) with 10% foetal calf serum (Invitrogen) supplemented with 2 mmol/l L-glutamine and 1×Penicilin/Streptomycin (Invitrogen), termed ‘R-10’, with 10 u/ml interleukin-2. Anti-CD3/CD28/CD137 beads (Invitrogen) at a 1:10 bead:cell ratio was added to stimulate polyclonal expansion. Cells were cultured for 96-120 hours and assessed by flow cytometry.

Isolated monocytes were tested for arginase activity using the Urea assay kit (Abnova Urea Assay ABIN1082256 Taiwan). This measured the metabolite urea, a by-product of arginine degradation.

For pDC function, PBMC in R-10 were cultured at 2 x 10^6 cells/ml in 96-well culture plates and stimulated with type A (CpGA) or type B (CpGB) unmethylated CPG oligodeoxynucleotides (ODN) (5 mM, CpG 2216 and CpG 2016, respectively, InvivoGen, San Diego, USA) for 6-8 hours in a CO₂ incubator. Cells were stained (CD123, CD11c, lineage-cocktail, HLA-DR) for surface staining of pDC, and fixed and permeabilized according to the manufacturer’s instructions (Fix and Perm, BD Bio-sciences). Anti–IFN-alpha (Miltenyi Biotec) was used for intracellular staining.
Enzyme linked immuno-absorbent assays (ELISA)

Plasma levels of arginase I was determined using a human arginase I sandwich ELISA (BioVendor) according to the manufacturer’s instructions. CD163 was quantified using the Quantikine® Human CD163 ELISA kit (R&D Systems) as previously outlined (4).

RNA quantification

For DMGE, RNA was extracted from tumor biopsies using RecoverAll total nucleic acid extraction kit for FFPET (Ambion, Life Technologies, Carlsbad, CA, USA) as per manufacturer’s instructions and stored at -80°C. CD163 mRNA was quantified by real-time RT-PCR as previously described using a Rotorgene 3000 real-time PCR machine (Corbett Research) as previously described (4).

Genes for DMGE using the nCounter platform (NanoString Technologies, Seattle, WA, USA) were chosen to permit COO categorization,(5) and analysis of immune-effectors and immune-checkpoints. Hybridizations were carried out according to the NanoString Gene Expression Assay Manual. Five microliter of each RNA sample (100 ng) was mixed with 20 µl of nCounter Reporter probes in hybridization buffer and 5 µl of nCounter Capture probes for a total reaction volume of 30 µl. The hybridizations incubated at 65°C for approximately 16-20 hours. Two separate runs were performed requiring the production of two identical codesets. Expression counts were normalized between codesets based on relative differences between duplicate samples in both runs which allowing us to develop a correction factor for each individual gene between the runs. Raw data was imported and
analysed in the NanoString® data analysis tool nSolver. For normalization, gene expression data was internally controlled to the mean of the positive control probes to account for inter-assay variability. Gene normalization was then performed using the geometric mean of four housekeeper gene to account for factors that affect RNA quality and quantity (PGK1, GAPDH, PGAM1, OAZ1). Housekeeping genes were selected as previously described and as per manufacturer recommendation (6).

Gene expression data from frozen tissues utilized by Lenz et al. was taken from the NCBI Gene Expression Omnibus database (GEO accession GSE10846) (7). Normalisation was as described in the original published paper. In instances where genes on the Affymetrix platform had multiple expression probes, we chose the single probe that most accurately reflect the actual gene expression as per a recently described scoring method (8).

For blood, RNA was extracted from FACS-sorted CD14⁺ monocytes, and analysed using Illumina Human HT12v4 Bead Array for whole genome expression. The data was extracted and pre-processed using Illumina’s Genome Studio. Analysis was performed using Genespring GX11 (Agilent Technologies) with all data quantile normalised. Clustering was performed using Genesis software (Genomics and Bioinformatics Graz) (9).

## Supplemental Tables

<table>
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<tr>
<th></th>
<th>% 4 year Survival (% difference)</th>
<th>P value</th>
<th>% 4 year Survival (% difference)</th>
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**Table 1. Optimization of immune-effector and monocyte/macrophage-checkpoint combinations.**

Significant P values are in bold.
**Supplemental Table S2. Characteristics of the blood and tissue patient cohorts.**

*Age, sex and IPI were unavailable in 5, 2 and 6 patients respectively.*

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<td>F: 41%</td>
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<td>28%</td>
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</tr>
<tr>
<td>3:</td>
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<td>3:</td>
</tr>
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<td>4,5:</td>
<td>20%</td>
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*Age, sex and IPI were unavailable in 5, 2 and 6 patients respectively.*
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<thead>
<tr>
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<tr>
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<td>APC, PerCP, V500</td>
<td>BD</td>
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<td>Lineage cocktail (CD3,CD14, CD16,CD19, CD20,CD56)</td>
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<td>FITC</td>
<td>BD Pharmingen</td>
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Supplemental Table S3. Flow cytometry antibodies used.
Figure 1 Supplemental.
Figure 2 Supplemental.
Figure 3 Supplemental.
CHAPTER 6

DISCUSSION
Discussion

DLBCL is one of the most common aggressive B cell lymphomas. Despite increased understanding and advances in therapeutic modalities such as the introduction of anti-CD20 monoclonal antibody therapy, up to one third of patients still die from their disease. [1] Prognostic outlook is guided by the IPI, however considerable heterogeneity of outcome exists within IPI groupings. In spite of its accuracy, it is limited by the fact that the poorest scoring patients still have survival rates greater than 50%.[2] In addition the IPI gives no information on the biological prognostic factors that relate to the malignant B cell or the TME. New therapeutic options are emerging, however it is still not clear which patients would most likely benefit from newer regimens. It is important to ascertain immune correlates that might better prognosticate in patients with DLBCL treated with R-CHOP. It is also important to gain insight into how these factors might guide therapy.

Primarily, this research focused on the role of the TME in predicting outcome for patients with DLBCL treated with chemo-immunotherapy. Initially the research focused on the role of host genetics and how this affects immune cells and their interaction with rituximab. It is unclear how these interactions contribute to the effectiveness of the drug and the risk of LON. The research progressed focusing on the immune macroh and microh environment in DLBCL. This initially involved looking at simple measures of immune response in patients using a full blood count differential. This progressed to examining flow cytometric identification of immune cells in tumour biopsies. Finally, I directly analysed circulating immune cells in detail, and applied these findings to the immune TME using DMGE. The DMGE enabled direct digital analysis of immune genes in tumour biopsies in the largest lymphoma dataset to be analysed using this novel technology.

6.1 Immuno-genetic polymorphisms

The likely principle mechanism of action of rituximab is antibody-dependent cytotoxicity whereby rituximab binds to FCG3A receptors on NK Cells and macrophages, resulting in tumour cell lysis by the reticulo-endothelial system.[3-6] Another mechanism of action is complement mediated activation, leading to direct lysis of tumour cells via the complement cascade.[7, 8] Finally, rituximab
has a direct apoptotic effect on lymphoma cells.[9-11] The relative importance of these three mechanisms on the lysis of the malignant B cells in DLBCL remains unclear.

I investigated if polymorphisms in the FCG3A and complement systems could impact on response of patients’ tumour to rituximab. These polymorphisms are not the only factors impacting on rituximab response. It has been speculated that relative levels of CD20 expression are important in responsive lymphomas with high expression conferring good outcome.[12, 13] In addition prior exposure to rituximab can also promote down regulation of CD20 and subsequent resistance to therapy.[14, 15] There is also accumulating evidence for improved response rates in women compared to men in DLBCL treated with chemoh immunotherapy.[16] Male patients and an increased body weight in patients with DLBCL appear to correlate with increased clearance of rituximab. [17, 18] FCG3A is a low affinity receptor capable of binding to complexed but not monomorphic IgG. A polymorphism at position 158 substituting valine for phenylalanine results in stronger binding to IgG. In DLBCL this polymorphism has been shown to predict enhanced responses in a Korean population.[19] Binding of C1q to the Fc portion of immune complexes activates CDC through initiation of the complement cascade. C1q is encoded by C1qA, whose sole coding polymorphism is at position 276, coding for adenine (C1qA-A276) or guanine (C1qA-G276). C1qA-A276 results in lower C1q protein levels than the C1qA-G276 polymorphism. The role of this polymorphism in the complement cascade and its impact on patient outcome is not well understood at present.

At the time of publication, my study was the largest assessment of the impact of FCG3A and C1qA on outcome and incidence of LON as a late side effect. In a uniformly treated DLBCL population, there was no difference in the occurrence of the polymorphisms between healthy controls and lymphoma patients. Only small numbers of patients were homozygous for the strong binding valine FCG3A158 allele (6%). Higher occurrence of valine FCG3A158 allele homozygosity is more frequent in Southh East Asia and may account for a survival benefit seen in some Asian studies in DLBCL. [19] The low frequency of VV homozygotes observed in the Queensland cohort prevents any definitive
conclusions regarding the impact of *FCG3A158* on outcome for patients with DLBCL treated with R-CHOP. The frequency of VV homozygosity that I observed is consistent with other studies. These find rates of 6-15% in Caucasian populations. However a recent German DLBCL study found a rate of 28% for the VV polymorphism. [20] In this study possession of the polymorphism was not predictive of OS but there was a trend towards improved PFS (non-significant) in patients with VV polymorphism. The authors summarise that although *FCG3A-V158* polymorphisms may have a role in predicting outcome in DLBCL treated with chemo-immunotherapy, this cannot be definitively confirmed or excluded without a much larger cohort. They estimate this to be over 2000 patients. The authors postulated that the recent increased intensity of rituximab in 14-day regimens may reduce the influence of *FCG3A* polymorphisms on outcome parameters. In this scenario, the higher concentrations of rituximab that occur as a result of the 14-day administration may negate the disadvantage of a low binding *FCGR3A* allele. The retrospective cohort used in my study had patients treated with both 14 and 21 day R-CHOP regimens, however this data was not collected so definitive conclusions cannot be made with regards to timing of rituximab in this cohort.

Interestingly, although there was no statistical association between *FCG3A-V158* polymorphism and outcome in patients with DLBCL treated with R-CHOP, none of the six patients homozygous for valine have relapsed. Studies have shown that at high concentrations rituximab is equally effective in VV and FF subtypes but that the threshold for effective ADCC in VV patients is four times lower than those for FF patients.[3] The VV polymorphism has been associated with improved response in other settings with a recent large study in rheumatoid arthritis showing improved ongoing response to rituximab and that integration with other RA clinical factors was very predictive of disease response to rituximab.[21, 22]

Other polymorphisms have been described such as *FCG2A* that may impact on rituximab effect, however it appears these are in fact strongly linked to *FCG3A* due to lineage disequilibrium and I did not investigate this further.[23] These relatively consistent findings of differing trends of improved outcome related to
NK and macrophage cell binding has led to the development of new monoclonal antibodies with glycol-engineering to ensure optimal engagement of FCG3A receptors. This has led to the development of GA-101 which is a Type-II antibody undergoing clinical trials with much stronger affinity for FCG receptors and improved responses in early trials.[24, 25]

In spite of the lack of association of survival with regards to the VV polymorphism, I was able to demonstrate a striking association between this allele and the occurrence of the established rituximab side-effect LON. Despite the low occurrence of valine homozygosity, this was significantly associated with the development of LON. We observed a 6% incidence of LON in DLBCL patients treated with R-CHOP chemo-immunotherapy however 50% of patients homozygous for valine developed LON in long term follow up. Previous reports of LON after induction chemo-immunotherapy for B-cell lymphoma have not been restricted to a single histological sub-type or treatment regimen. In addition the neutrophil cut-off used to identify LON has varied between grades 2 to grade 4.[26-28] Once studies are restricted to those using a definition of grade 3/4 neutropenia, then irrespective of ethnicity the incidence of LON (range 7-13%) is of a similar order of magnitude to our series.[29-32] In these, as with our study, incidence may be under-estimated as patients are frequently asymptomatic. [33] The incidence of LON appears to rise markedly in studies that include patients undergoing high-dose therapy with autologous stem cell rescue.[33-35] Put together, it may be that intensity of treatment regimen (use of high-dose over conventional dose chemo-immunotherapy) is the principal determinant of LON incidence rather than lymphoma histology. The incidence of LON in non-malignant states is well described and appears to be lower than levels seen in haematological malignancy.[36-38]

The mechanism behind LON is yet to be established.[33, 38] Notably, bone marrow histology at the time of LON has been variously reported as maturation arrest or myeloid hypoplasia, indicating that several mechanisms may be responsible. Mechanisms postulated include anti-neutrophil antibodies or increased large granular lymphocytes in the absence of B-cells leading to FAS ligand mediated destruction of neutrophils. [39, 40] However this has not been consistently observed. A report of six cases of aggressive lymphoma (including
two with AIDS related lymphoma) treated with DA-EPOCH-R (dose-adjusted etoposide/prednisone/Oncovin[vincristine]/cyclophosphamide, hydroxyduanorubicin / rituximab), implicated perturbations of stromal derived factor-1 (SDF-1) / CXCL12 during B-cell recovery as a potential aetiology.[26] The SDF1 chemokine is important for granulocyte egress from the bone marrow and also in B-cell development. A shift of SDF-1 towards B-cell recovery over granulocyte homeostasis may result in LON due to neutrophil maturation arrest whilst B-cell recovery occurs. Cytokine kinetics were evaluated in detail in a single case of LON associated with granulocytic hypoplasia following fludarabine, cyclophosphamide and rituximab for Waldenstroms macroglobulinaemia. In this patient LON was associated with markedly raised levels of serum B-cell activating factor (BAFF).[41] However, many of the above findings are in small cohorts or single patients and are hypothesis generating only at the current time.

It may be that the pharmacokinetics of rituximab are different in patients homozygous for the FCGR3A-V158 polymorphism. FCGR3A-V158 has a higher binding affinity for IgG1 antibodies (such as rituximab) than FCGR3A-F158.[42] Thus FCGR3A-V158 may result in enhanced clearing of CD20 expressing cells by ADCC by NK cells. FCGR3A transcripts are higher in NK cells from subjects with FCGR3A-V/V158 versus the V/F or F/F genotype and V/V homozygotes have enhanced in-vitro ADCC activity. In-vivo this may result in more profound B-cell depletion.[43] Upon B-cell recovery, heightened stimulation of lymphopoiesis may result in temporary imbalance of cytokines and transient ineffective granulopoiesis.

In contrast with our findings regarding FCGR3A-V158F, there was no significant association between LON and the C1qA-A276G polymorphisms. Furthermore, the combination of C1qA-A276G and FCGR3A-V158F did not appear to expose any linkage disequilibrium and did not appear to influence outcome or development of LON. These findings suggest that C1qA is not implicated in the pathogenesis of LON. We did not observe any difference in EFS or OS related to the C1q-A276G polymorphism. The literature in this area is sparse and conflicting with a recent study showing no effect of this polymorphism in a small follicular lymphoma population.[44] Another study in low-grade lymphoma showed a prolonged
progression free survival in patients receiving single agent rituximab but not in overall survival.[45] A single study in breast cancer using a monoclonal antibody to Herceptin demonstrated reduced metastases associated with this polymorphism, but a more recently published large study of over 2000 breast cancer patients suggests no impact on outcome for this polymorphism in breast cancer patients.[46, 47] There is one recent study described of 129 patients with DLBCL which does show prolonged PFS and OS for patients homozygous for the A allele in a study of a population from Beijing.[48] This study reported almost 30% of patients homozygous for G allele compared to 6% in our population which could make the significance of the A allele more significant in the Chinese population studied. The G allele is associated with worse outcome despite increased and more active complement lysis of cells.[49] It has been postulated that increased complement activity may reduce ADCC and adaptive immunity and that enhanced complement activity rapidly clears tumour cells allowing inadequate time for an effective immune response to develop.[50, 51]

In conclusion, in our uniformly treated group of DLBCL patients who received rituximab, 6% of patients developed LON. The FCGR3A-158 V/V genotype was significantly associated with development of LON. Polymorphic analysis may be a predictive tool to identify those at high-risk of LON. Although no patients with either LON or FCGR3A-158V homozygosity relapsed, neither were associated with improved EFS or OS after R-CHOP. Large prospective studies are required to establish if FCGR3A-V158F polymorphisms have a bearing on response rates in DLBCL, and whether either LON or FCGR3A-V158F polymorphisms are predictors of outcome.

6.2 Immune Microenvironment

There is substantial evidence demonstrating the key role of host immunity in DLBCL. Numerous gene expression studies have identified immune signatures that are prognostic in the disease.[52-54] There have also been a number of immunohistochemical studies confirming the importance of the tumour microenvironment in DLBCL.[55-58] However, both gene expression studies and IHC have significant drawbacks. Gene expression is expensive, requiring fresh frozen tissue and is available only in a limited number of centres. While IHC is
relatively cheap there is marked heterogeneity in sample preparation, stains used and interpretations.

I set out to identify immune markers that would be relatively simple to use, inexpensive and available to most diagnostic centres. For this reason I looked at circulating immune cells obtained from full blood counts and also flow immune-phenotyping taken from fresh diagnostic tissue. All lymphoma diagnostic samples at the Princess Alexandra Hospital have a standard B and T cell panel performed to assess cell phenotype at diagnosis. In B cell lymphomas, the information with regards to T cell infiltration is generally ignored as the test is performed for diagnostic information only. I was interested to identify if T cell infiltration might predict survival in patients with DLBCL. In addition I had the ability to look at circulating immune parameters prior to therapy and in particular, absolute lymphocyte and monocyte counts at diagnosis to see if circulating immune status was also important. My study of 122 patients with DLBCL confirms the significance of host immune status in predicting outcome. I have shown for the first time that CD4+ T cell infiltration of fresh tumour (as assessed by flow cytometric immune-phenotyping) is a very strong predictor of OS and EFS when patients with DLBCL are treated with standard chemo-immunotherapy. This was independent of the IPI. The two groups of patients separated by a 20% CD4 cut-off did not differ for any of the single parameters that make up the IPI nor were the groups significantly different in their IPI scores. Interestingly, CD8 TILs were not associated with any outcome endpoints. Importantly the level of CD4+TILs was a significant predictor of outcome in patients with good prognosis disease as assessed by IPI (0, 1, 2, 3). In addition I have confirmed the prognostic significance of the absolute lymphocyte to monocyte ratio with this ratio able to predict outcome in the whole cohort but also predicting poor outcome for patients in good risk IPI categories. Our data does indicate however, that the CD4 TILs are the strongest predictors of outcome in this cohort and were independent of IPI and LMR.

My findings are consistent with two previous studies described prior to the introduction of rituximab.[59, 60] The two smaller studies described, in 55 and 72 patients respectively, demonstrated a benefit for improved outcome in patient samples with higher number of CD4+T lymphocytes prior to the
introduction of rituximab therapy. In the larger study a cut-off of 20% CD4+T cells was also the most predictive of outcome. Interestingly, in agreement with our results, neither of these studies identified CD8+ TILs as being prognostic. Recent studies have confirmed the importance of T cell activation in the tumour microenvironment with the T cell activation marker CD137 predicting outcome in a large DLBCL cohort.[56] This is of particular interest given an agonist antibody to CD137 may lead to improved immune responses against lymphoma.[56, 61] As discussed in the introductory chapter, CD4 T cells are relatively heterogeneous with variable and diverse functions such as enhancing inflammation to overt immunosuppressive effects. In general Th1 cells have anti-tumour effects and Th2 may well have an opposite effect by enhancing immune suppression. [62, 63] T regulatory cells in malignancies are generally associated with inferior outcome, however for unknown reasons at present this may not be the case in B cell lymphomas where high levels of these cells in the TME can be associated with improved outcome. It is felt that increased numbers of T<sub>regs</sub> could reflect a more normal host immune system, while it has also been postulated that T<sub>regs</sub> may have a direct negative effect on proliferation of B cells. [64-67] My study gives no information on the CD4 T cell subsets in the tumour which will be key in future research to more adequately classify these cells. There is still limited data on the role of these various CD4 subsets in DLBCL. Interestingly there is evidence from prior to the introduction of rituximab that circulating lymphocytes of patients with DLBCL before treatment are skewed to a Th2 phenotype and revert to a Th1 phenotype with successful treatment.[68] There is also emerging evidence that T helper cells are key to inducing an anti-tumour effect post vaccination with DLBCL related peptides.[69]Animal models in B cell lymphoma have shown that CD4 T cells are key cells in creating an anti-tumour microenvironment.[70] Th1 cells in particular stimulate and up regulate antigen presentation and tumour clearance.[63] Improved antigen presentation has been shown to be a key survival determinant in patients with DLBCL.[57, 71-73] These animal models have shown that cytokines derived from Th1 cells such as Interferon gamma, IL1 and TNF alpha are the key to stimulating tumour-killing macrophages. It is possible that patients with low CD4+TILs could benefit from interferon therapy. Interferon has been shown to be effective in the treatment of
many lymphomas but its use has been restricted due to short half-life and side effects, however methods of new local tumour delivery systems look promising in lymphomas.[74]

Mouse models have also shown that PD-1 which is present on many CD4 T cells may eventually down regulate CD4+T cell tumour surveillance leading to relapse.[70] PD-1 has been found expressed in 27% of DLBCL tumours, but when one looks at the local tumour microenvironment 38% of PD1 non-expressing DLBCL have PD1 positive histiocytes surrounding the tumours. It is possible that PD-1 could be responsible for down-regulating CD4 T cells in a large number of DLBCLs. This is of particular interest as two highly successful trials targeting PD-1 in advanced cancers have recently been described in solid tumours.[75, 76] To date two trials using an antibody to PD-1 have been described in B cell lymphomas.[77, 78] CT-011 (anti-PD1) directly increases the numbers of CD4+T cells post autograft for relapsed disease with survival higher than historical controls.[78] As of yet it is unclear from these studies what exact role T cells and other immune cells such as macrophages play in eliciting a response with immune checkpoint modulation, and it will be important to determine the influence of PD-1 expression on not only the numbers of TILs but the activity of these key cells in future trials.

With regard to simple immune parameters garnered from the full blood count, I have confirmed the prognostic significance of pre-diagnostic lymphocyte and monocyte counts incorporated into the LMR ratio and also the recently described algorithm from Wilcox et al.[79, 80] I have also shown that a low lymphocyte count on its own is a predictor of poor outcome. This confirms the findings of a number of recent studies that show that a low lymphocyte count at diagnosis and also post therapy is associated with poor outcome.[81-84] There is also emerging data that the AMC may predict outcome in DLBCL.[85] In addition there is evidence that specific subsets of monocytes called monocyte derived myeloid suppressor cells are increased in patients with DLBCL and contribute to marked immune suppression and poor outcome.[67, 85] The importance of these cells are discussed further below.

My data identified the LMR as being a significant prognosticator independent of IPI. In addition LMR could identify poor risk patients in groups of patients with
good risk IPI scores. It is likely that these combined scores reflect the poor outcome associated with systemic immunosuppression. It is likely the combined LMR measures the potential level of immune suppression dictated by the monocyte count and the subsequent effects of this in a low lymphocyte count. Further studies are required to identify the subsets within these two cell types that are contributing to outcome. Interestingly there did not seem to be any correlation between circulating and tumour infiltrating lymphocytes (as assessed from my flow cytometry data). This may reflect the difference in retention and recruitment between nodal tissue and the circulation.[80]

In summary, I have shown that CD4+TILs appear to be very strong predictors of outcome in DLBCL treated with chemo-immunotherapy independent of IPI. Further studies are required to analyse the influence of other local microenvironment factors such as macrophages and histiocytes on CD4 function and numbers. In addition we need to identify the particular CD4 subsets that may contribute to improved survival and thus investigate the specifics of why the lymphocyte to monocyte ratio is prognostic in DLBCL.

6.3 Net Tumoral Immunity

My findings described above confirm that circulating lymphocyte:monocyte ratios are prognostic, implicating them as surrogate immune-effectors and monocyte/macrophage-checkpoints within the tumor microenvironment. I hypothesised that detailed functional and quantitative assessment would enable identification of the optimal immune-effector and monocyte/macrophage-checkpoint molecules to interrogate within the tissue. Blood from 140 ‘R-CHOP’ chemo-immunotherapy treated DLBCL patients from a prospective Australasian Leukaemia and Lymphoma Group trial was analysed. PBMC from the patients enrolled on the NHL21 clinical study were fully characterised so that the lymphocyte and monocyte subsets that may contribute to outcome were identified.

Amongst blood monocyte immune-checkpoint markers, addition of CD163 to conventional moMDSC indicators (CD14+HLA-DRlo) identified a highly immunosuppressive subset of moMDSCs in DLBCL. MoMDSCs and CD163+monocytes values correlated and CD163+moMDSC were enriched within
circulating monocytes. CD163 is a heme scavenger molecule felt to be specific for the immunosuppressive M2 Macrophage in tissue.[86, 87] CD163 is a surrogate of the M2 macrophage and is felt to predict inferior outcome when expressed in tumours in DLBCL and Hodgkin Lymphoma, but also in most solid cancers.[66, 88, 89] Effective treatment of primary tumour leads to reduced levels of these cells.[90] High levels of M2 macrophages around the tumour prevents an effective anti-tumour response. The correlation between the M2 TAM marker CD163 and HLA-DRlo on circulating CD14+ monocytes suggests a link between circulating moMDSC and M2 TAMs within the malignant lymph node. This is supported by CD163hi moMDSC expressing CD62L and CD11c, enabling migration into secondary lymphoid tissues.

MoMDSCs seem capable of causing immune suppression in the circulation but also capable of migration to lymph nodes and exerting local effects in the tumour bed.[91] They are cells in a state of maturation block with an inability to progress to a normal mature myeloid/monocyte cell. Increased levels of these cells in the circulation have been described in a number of cancers.[92, 93] Some of these studies showed a direct link between numbers of moMDSCs/MDSCs and tumour bulk and risk of metastases. MoMDSCs/MDSC related immunosuppression seems to be mediated through multiple mechanisms including arginase, iNOS, peroxynitrite, ROS and H2O2 metabolism.[94-98] These cells also seem to increase the production of other immune suppressive cells such as Tregs via secretion of chemokines such as CCL3. [99]MDSCs can also effect non immune parameters contributing to increased angiogenesis and metastases of tumours.[100] My findings make it likely that moMDSC migration to tissue results in accumulation of M2 macrophages. An M2 macrophage is characterised by positivity for CD68 and CD163 whereas M1 pro-inflammatory macrophage has expression of CD68 and high levels of HLA-DR.

Within the circulation of patients enrolled on the ALLG NHL21 study, the immune-effector: CD163+moMDSC ratios were more informative than the LMR, with ratios lower in those remaining interim-PET/CT+ve. Interim-PET/CT imaging was delayed to day 17-20 post-cycle 4 and scans reviewed centrally. In particular CD4:CD163+moMDSC and CD8: CD163+moMDSC ratios were predicative of early lymphoma clearance as assessed by PET/CT negativity.
However given repeat biopsy was not performed, no definitive conclusion can be made as to whether pre-therapy CD163⁺moMDSC associate with residual DLBCL versus inflammation within sites of interim-PET/CT-FDG-avidity.

My core study findings from the NHL21 study indicate that in DLBCL, the balance of immune-effectors and immune-checkpoints are critical to interim-PET/CT outcome. Given the uncertainty regarding the prognostic significance of interim-PET/CT on overall survival, I went on to validate the markers that I had identified in the peripheral blood in an independent DLBCL cohort. Here, the molecules were applied to the diagnostic tissue, and overall survival was the primary end-point.

128 R-CHOP treated DLBCL patients with full clinical annotation and long-term (median 4 year) survival data derived from two Australian centres (Canberra Hospital and the Princess Alexandra Hospital) were tested. We used the DMGE platform NanoString nCounter™ to permit accurate mRNA quantification on FFPE tissues.[101-103] In keeping with previous reports, we observed strong correlation between gene expression in paired frozen/paraffin samples across a range of genes.

DMGE identified CD8 as the strongest single immune predictor of outcome in DLBCL. [104] CD8 T cells are the end effector cells for the immune system and are directly cytotoxic to tumour cells. Autologous and third-party T cells generated against EBV have shown excellent responses in patients with EBV+ B cell lymphomas in particular.[105-107] One of the commonest mutations found in DLBCL relates to loss of the key MHC I related protein beta-2-microglobulin with a recent study showing this molecule mutated in 29% of DLBCL cases.[108] This study also identified deletions in CD58 which can also affect CD8 recognition of antigen. In addition to gene mutations, antigen presentation is impaired by other mechanisms so that overall 60% of patients with DLBCL have reduced ability to present antigen. Loss of function of the tumor suppressor gene PRDM1 or over-expression of the oncogene BCL6 occurs in a large proportion of DLBCL cases. However, BCL6 is also frequently mutated in activated B cells of healthy individuals. A recent study using PRDM1-deficient and/or BCL6 over-expressing transgenic mice, observed that lymphoma development was delayed and relatively rare. However in the context of polyclonal T cell impairment, the
transgenic mice exhibited a high incidence of rapid-onset aggressive B cell lymphomas.\[109\] The implication is that development of these lymphoma associated mutations is relatively common but that a healthy immune system eradicates cells containing these mutations to prevent overt lymphoma. Prior IHC based studies have also shown that absence of immune recognition proteins such as HLA Class I and II molecules and CD80/CD86 is associated with inferior outcome and reduced levels of CD4 and CD8 tumour infiltration.\[110\] It is interesting that CD8 was not prognostic in my flow cytometry model but it should be noted that flow cytometry reflects the proportion of CD4 and CD8 T cells, not their absolute number. Furthermore, mRNA levels reflect the turnover of the gene signal in a sample. Therefore quantification of CD8 gene expression within the malignant node and CD8 T cell flow cytometry can not be considered strictly comparable.

By DMGE, all markers of T cell infiltration predicted improved outcome. Interestingly, neither intratumoral CD163 nor CD68 expression alone was prognostic. However the CD68:CD163 ratio as an estimate of the relative proportion of macrophage sub-types found that those with a higher proportion of M2 macrophages (low CD68:CD163) had inferior outcome. This may be one explanation why results of intratumoral CD163 alone by IHC as a prognosticator are inconsistent, emphasizing the importance of measuring several markers to more accurately reflect aspects of TME immunity. \[89, 111-113\] Similarly, CD8:CD163 ratios as a measure of net anti-tumoral immunity was more discriminatory in predicting outcome than CD8 alone. Whereas the contribution of immune-effector molecules within the TME has previously been recognized to be prognostic, this is the first report of immune-effector: immune-checkpoint ratios. CD8:CD163 was independent of IPI and COO. Results were validated in an external R-CHOP-like (233 patients) gene-expression cohort.

IPI is influenced by factors such as patient fitness, age and tumour burden, which might reasonably be expected to be non-over-lapping with CD8:CD163. COO reflects the postulated B-cell differentiation stage at which malignant transformation occurred. One interpretation is that CD8:CD163 ratios within the TME are not influenced by differentiation stage of the malignant B-cell. Interestingly, although CD8:CD163 was predictive in the CHOP-like cohort, this
was not independent of conventional prognosticators. This may reflect the relatively increased importance of tumour-associated macrophages within the TME in those treated with immuno-chemotherapy versus chemotherapy alone, and is consistent with our findings that CD163+moMDSC suppress R-ADCC. Although COO did prognosticate, it was an inferior predictor compared to the CD8:CD163 immune signature.[114] Of the 18 genes used to determine COO, only LMO2 as a stand-alone gene was predictive for survival. This confirms findings from previous gene and immunohistochemical studies.[99, 114, 115] In R-CHOP treated patients, CD8:CD163 ratios enhanced the prognostic ability of this GCB marker. Furthermore LMO2 combined with CD8:CD163 successfully allowed segregation within low and high IPI patient groupings. The combination of a marker of B-cell differentiation with net anti-tumoral immunity appears to capture more patients (than predicted by IPI or COO alone) at low and high-risk. This may assist risk-stratification to select patients in whom novel therapies should be tested, and those in whom R-CHOP is sufficient. Patients who have a poor immune ratio and have low LMO2 expression (dual negative) are approximately one quarter of patients. This group of patients do very poorly with R-CHOP and my work allows identification of these patients failing current therapy who need alternate strategies to improve their outcome. It also shows that the other 75% of patients have cure rates approaching 90% with current standard therapy. This differentiation will be very important given that the cost of new emerging therapies will be significant. These compounds will need to be targeted to those patients who will derive most benefit.

These findings have other therapeutic implications. Firstly, monocyte depletion in patients enhanced NK-cell mediated R-ADCC but not Ob-ADCC, indicating that the immunosuppressive effects of monocytes in DLBCL may be overcome by obinutuzumab (a type II antiCD20 monoclonal antibody). Another notable finding was the striking co-clustering of immune-effectors and immune-checkpoints within the tissue and circulation, including CD8-CD163. This is in line with emerging data that up-regulation of immune-checkpoints is an adaptive immune-checkpoint response to immune-effector activation.[116] The correlations were significant but modest, reflecting the variable success of the host to counter anti-tumoral immunity within the TME. However, an alternative
explanation is possible, with the high levels of checkpoints associated with increasing CD8 and other effectors reflecting a healthy immune checkpoint response to prevent local tissue injury in the setting of an effective immune response. Blockade of immune-checkpoints is a promising therapeutic approach, with a variety of antagonistic monoclonal antibodies or small inhibitory molecules in development.[117, 118]

Immune based strategies are gaining ground in DLBCL.[70, 119-121] The kinetics of systemic host immune cells will likely impact the efficacy of these approaches, and may influence dose-scheduling. However there is minimal data on circulating moMDSC kinetics in DLBCL, with a small study finding moMDSC returned to normal once therapy was complete.[122] We found post-cycle 4 that CD163*moMDSC were reduced (accompanied by an increase in numbers of the antigen-presenting cells BMDCs and pDCs). The immunosuppressive profile of monocytes had reduced by post-cycle 4, including down-regulation of TH2 cytokines and TAM associated genes, and up-regulation of STAT1.[91, 109, 123] Similarly elevated plasma CD163 was reduced by post-cycle 4. In line with cHL, higher plasma CD163 levels were associated with advanced stage and reduced lymphocytes.[124]

My data emphasizes the importance of capturing the net anti-tumoral immunity within the DLBCL TME, by measuring the relative balance of immune-effector to tumour-associated macrophages. Addition of LMO2 as a marker of germinal centre B-cells to CD8:CD163 was powerfully prognostic. The work provides a link between M2 TAMs and moMDSC, and demonstrate that CD163 identifies a highly immunosuppressive subset of moMDSC in DLBCL. Further investigation of CD163*moMDSC as a therapeutic target is warranted, particularly in those with adverse CD8:CD163 ratios. Emerging data from mouse models indicate the possibility of switching macrophage phenotype from M2 to M1 and consequent improved immune clearance of the lymphomas using immune modulating drugs such as Pomalidomide.[125] In addition, direct targeting of CD163 using a monoclonal antibody has shown promise as an anti-inflammatory agent and could have the potential for activity in the setting of cancer as a means of targeting the tumour microenvironment.[126]
To my knowledge, this data is the first to establish the importance of the relative balance of immune activation as being key to outcome in DLBCL. Neither intratumoral immune-effectors nor immune-monocyte/macrophage checkpoints should be considered in isolation. A high level of immune effectors does not necessarily predict good outcome, if countered by an equally robust effective immune-monocyte/macrophage-checkpoint response. This data provides a strong rationale for the use of checkpoint inhibitors in the treatment of DLBCL.

6.4 Future directions

A pressing issue from my findings is the identification of patients who could be targeted by prospective immune checkpoint therapy, targeting molecules such as PD-1 and CTLA-4 or by using other immune-modulating agents such as lenalidomide given our findings are based on common, standardised diagnostic tests available to many laboratories. Biomarkers that might predict response to new therapies such as immune checkpoint blockade in lymphoma are urgently required. This will be a complex process. In solid tumours such as melanoma while expression of PDL-1 does predict response to anti-PD1 therapy, up to 10% of patients with no PDL-1/PDL-2 expression in their tumours still responded to anti-PD1 therapy. This indicates the complexity of predicting responses in this new era of immune checkpoint blockade. This needs to be investigated further in prospective studies.

The intratumoral LMO2/CD8:CD163 score was independent of additive to the widely used clinical prognosticator IPI. One future direction would be to develop and prospectively validate an integrated clinical and biological score, that might enhance stratification of DLBCL patients. Importantly, this would allow identification of patients that might benefit from novel agents (preferably given within the context of a clinical trial), and equally importantly predict patients highly likely to be cured with conventional chemo-immunotherapy alone.

Should sufficient NHL21 tissue samples become available (acquisition is ongoing, with approximately 67% of tissues available to date), this cohort will be used to prospectively validate LM02/CD8:CD163. Three year EFS analysis (the clinical studies primary end-point) is be performed in 2015. It will also be possible to
examine if the strong association of circulating immune-effector cells:moMDSC ratios with tumour clearance as assessed by interim PET/CT results in a significant EFS benefit. The confounding factor for both these analyses is that following interim-PET/CT, patients had risk-adapted therapy and hence were no longer uniformly treated.

My intention is also to extend investigation of the immune TME into other lymphomas, including Hodgkin Lymphoma and Follicular Lymphoma, and contrast this with post transplant lymphoproliferative disorder (PTLD). The latter occurs as a consequence of iatrogenic immunosuppression, and CD14+CD163+ monocytes are implicated in reducing the risk of graft rejection.[90].

References:


83. Porrata, L.F., et al., New-onset lymphopenia assessed during routine follow-up is a risk factor for relapse postautologous peripheral blood


