Investigating the role of tumour-associated macrophages in a mouse model of B-cell Non-Hodgkin Lymphoma
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Investigating the role of tumour-associated macrophages in a mouse model of B-cell Non-Hodgkin Lymphoma

A thesis submitted for the degree of Doctor of Philosophy

at the University of London

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Abstract

A large number of infiltrating tumour-associated macrophages (TAM) is associated with a poor prognosis in many diverse cancers. In B-cell Non-Hodgkin Lymphomas (B-NHL), the situation is less clear with conflicting reports on the clinical significance of the total number of macrophages. Comprehensive gene expression analysis of unsorted diagnostic biopsy specimens in the two most common B-NHL, Follicular Lymphoma (FL) and Diffuse Large B-cell Lymphoma (DLBCL), implies differential expression of macrophage genes between good and bad prognosis cases. The hypothesis being tested in this thesis is that macrophages play a fundamental role in the progression of B-NHL. This functional significance may not be fully reflected simply by counting the numbers of macrophages, but rather by revealing the functional roles of TAM in lymphoma.

In this thesis we critically review the clinical and laboratory evidence relating to lymphoma-macrophage interactions, before detailing our own laboratory investigations. The laboratory goal of this project is to establish proof-of-concept, principally using mouse models, that TAM are partners in a lymphoma-supporting microenvironment. A large number of diagnostic human biopsies of DLBCL were interrogated for evidence of correlations between macrophage numbers, phenotype, and tumour proliferation. Our subsequent experimental strategy was to manipulate the numbers and phenotype of TAM in a transgenic Myc-driven mature B-lymphoma transplanted to immune-competent hosts. The use of macrophage ablation with toxic liposomes, and with a transgenic host possessing drug-inducible macrophage-specific cell death, explored the impact of gross macrophage depletion in lymphoma. Adoptive transfer experiments investigated the impact of supplementary macrophages of different phenotypes. The administration of a colony-stimulating factor-1 receptor (CSF-1R) inhibitor was used to block the accumulation of TAM in growing lymphoma, and act as a pre-clinical model for this promising class of therapeutic agents. We detail the first reported use of CSF-1R inhibition in a model of B-NHL, and discuss the efficacy and consequences of this strategy. CSF-1R inhibition reduces circulating monocyte and TAM numbers and diminishes lymphoma growth.
Dedication

My family.
My wife, Rôla.
My friends.
My patients, past, present and future.
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# Table of Contents

1 Macrophage Biology. ................................................................. 22
  1.1 Macrophage development. .................................................. 22
    1.1.1 The traditional paradigm of macrophage development......... 22
    1.1.2 Monocyte subsets have distinct effector functions in both mice and humans... 25
    1.1.3 Monocyte subsets may represent a developmental series................ 28
    1.1.4 Local extra-medullary proliferation contributes to renewal and expansion of resident tissue macrophages.......................... 29
  1.2 Macrophages have vital roles in embryology and homeostasis........ 31
  1.3 Macrophages participate in cancer-related inflammation, demonstrating opposing functions in different settings and diseases.............................................. 32
  1.4 Phenotypic diversity underlies the broad spectrum or roles played by macrophages in cancer............................................................. 34
    1.4.1 M1 macrophage phenotype............................................. 34
    1.4.2 M2 macrophage phenotype............................................. 35
  1.5 Criticism of the M1-M2 model of macrophage phenotype................ 38
  1.6 Macrophages exhibit phenotypic plasticity that may be harnessed therapeutically in cancer......................................................... 39
  1.7 Tumour cells recruit and activate monocytes towards a pro-tumoural phenotype.. 41
  1.8 Links between malignant B-cell apoptosis, macrophage recruitment, activation and anti-tumour immune responses............................................ 43

2 Macrophages in the tumour microenvironment.................................. 44
  2.1 Angiogenesis........................................................................ 44
  2.2 Invasion and metastasis.......................................................... 46
  2.3 Immune-suppression................................................................ 47
  2.4 Stimulation of tumour cell proliferation.................................... 48
  2.5 Summary. ............................................................................. 50

3 Critical roles for the CSF-1R......................................................... 51
  3.1 CSF-1R ligation promotes the formation, survival, maturation and proliferation of monocytes and macrophages........................................ 51
  3.2 The CSF-1R is a conceptually attractive therapeutic target in cancer........ 53
  3.3 Exploring CSF-1R inhibition..................................................... 55
  3.4 Blocking the intracellular tyrosine kinase domain may be the most effective strategy to inhibit the CSF-1R......................................................... 58
  3.5 CSF-1R inhibition in lymphoma.................................................. 59

4 B-cell Non-Hodgkin Lymphomas..................................................... 60
  4.1 Background to B-NHL............................................................... 60
    4.1.1 Staging of B-NHL............................................................ 60
    4.1.2 The Ann Arbor system for staging B-NHL................. 61
    4.1.3 B-NHL arise by dysregulated expression of oncogenes during aberrant maturation of B-lymphocytes.................................................. 61
    4.1.4 B-NHL and their relationship to TAM............................. 63
  4.2 Diffuse large B-cell lymphoma.................................................. 64
    4.2.1 Prognostication in DLBCL............................................... 64
    4.2.2 Gene expression profiling reveals biological heterogeneity and highlights roles for the microenvironment in DLBCL................................. 65
    4.2.3 Tumour-associated macrophages in DLBCL...................... 66
    4.2.4 Counting tumour-associated macrophages in DLBCL........... 68
  4.3 Follicular lymphoma............................................................... 71
    4.3.1 Clinical features of FL................................................... 72
    4.3.2 Therapy for FL............................................................. 73
    4.3.3 Prognostication in FL – the follicular lymphoma international prognostic index... 75
4.3.4 Gene expression profiling in FL reveals the prognostic influence of the cellular microenvironment. ................................................................. 77
4.3.5 Counting tumour-associated macrophages in FL............................................. 80
4.3.6 Counting tumour-associated macrophages in relation to therapy in FL............. 84
4.4 Burkitt lymphoma ..................................................................................... 86
4.4.1 Biology of BL in relation to tumour-associated macrophages. ......................... 89
4.5 Lessons from T-cell lymphomas ................................................................. 91
4.6 Macrophages and rituximab ....................................................................... 95
4.6.1 Macrophages cooperate with rituximab and other anti-CD20 monoclonal antibodies in malignant B-cell killing. ........................................ 95
4.6.2 Rituximab and the mechanisms of action of anti-CD20 monoclonal antibodies.... 98
4.6.3 Malignant-cell Fc receptors also play a crucial role in rituximab action ........... 101
5 Current clinical priorities in B-NHL ............................................................. 102
6 Targeting macrophages in cancer ............................................................... 104
6.1 Liposomes and other nanoparticles ............................................................ 105
6.2 Systemic macrophage apoptosis in MaFIA mice ........................................... 108
6.3 Cellular adoptive transfer ......................................................................... 110
7 Experimental rationale ............................................................................... 112
8 Experimental approach ............................................................................... 114
8.1 Demonstrating functional interplay between macrophages and malignant lymphocytes in mouse models ..................................................... 114
8.1.1 Mouse models of B-NHL .......................................................................... 115
8.2 Background – the Eµ-myc transgenic mouse ................................................ 117
8.3 The Eµ-myc/bcl2 mouse – a chemoresistant model ........................................ 119
8.4 Eµ-myc/bcl-2, chemoresistance and macrophages ........................................ 121
8.5 Eµ-myc/bcl-2, apoptosis, senescence and macrophages .................................. 122
8.6 Experimental strategy .............................................................................. 124
9 Materials and methods ............................................................................... 125
9.1 Immunohistochemistry of human tissues ................................................... 125
9.1.1 Interrogating the human tissue bank ......................................................... 125
9.1.2 Principles of immunohistochemistry ....................................................... 126
9.1.3 Practical aspects of immunohistochemistry ............................................ 127
9.1.4 Image analysis using the Ariol system .................................................... 130
9.1.5 Tissue microarrays ................................................................................ 131
9.2 Liposomal cladronate and liposomal PBS ................................................... 131
9.3 Eµ-myc/bcl-2 tumour cells ......................................................................... 131
9.4 Collection of tumour conditioned media .................................................... 132
9.5 Cell viability ............................................................................................. 132
9.6 Mice ........................................................................................................ 132
9.7 Injections .................................................................................................. 133
9.8 Oral gavage .............................................................................................. 133
9.9 Assessment of in vitro cytotoxicity ............................................................ 133
9.9.1 Experimental protocol for CytoTox-ONE™ Homogeneous Membrane Integrity Assay. ........................................................................... 134
9.10 Preparation of stored Eµ-myc/bcl-2 lymphoma cells for in vivo use ............... 136
9.11 Cytokine analysis ..................................................................................... 136
9.11.1 Detailed experimental protocol ............................................................. 137
9.12 Lymphoma progression assessment ............................................................ 139
9.13 Quantification of liver cross-sectional tumour area ..................................... 139
9.14 Preparation of FFPE sections of bone and bone marrow.............................. 140
9.15 Embedding of tissues .............................................................................. 140
9.16 Blood sampling by cardiac puncture ........................................................ 141
9.17 Sacrifice and organ removal.................................................................141
9.18 Preparation of MaFIA dimerizer AP20187........................................142
9.19 Formation of single cell suspensions for flow cytometry..................142
9.20 Flow cytometry antibody staining......................................................142
9.21 Analysis by flow cytometry.................................................................143
9.22 Collection of mouse peripheral blood for morphological review........143
9.23 Staining of mouse peripheral blood for morphological review.............143
9.24 Assessment of tumour proliferation by BrdU incorporation..............144

9.24.1 Immunofluorescent staining of blood and lymph nodes for assessment of BrdU by flow cytometry.................................................................145
9.25 Extraction of RNA with Qiagen kit......................................................146
9.26 Counting macrophages in mouse tissue sections..................................147
9.27 Cyclophosphamide..............................................................................147
9.28 RNA analysis......................................................................................147
9.29 RNA integrity....................................................................................147
9.30 Reverse transcription of RNA to yield cDNA......................................148
9.31 Quantitative RT-PCR........................................................................148
9.32 Mouse bone marrow derived macrophages (BMDM)..........................150
9.33 Preparation of the small molecule CSF-1R tyrosine kinase inhibitor......151

10 Statistical considerations.................................................................152

11 Protocols and results........................................................................155
11.1 Interrogating diagnostic human biopsies of DLBCL...........................155
11.2 Investigating macrophage–lymphoma interactions in a living system – the Eu-myc/bcl-2 mouse lymphoma.................................................................161

11.2.1 Characterisation of Eu-myc/bcl-2 lymphoma..................................161
11.3 Effects of intravenous liposomal clodronate on normal healthy C57BL/6 mice...164
11.4 The impact on lymphoma progression of macrophage depletion with liposomal clodronate.................................................................173

11.4.1 Liposomal clodronate versus Eu-myc/bcl-2 cells in vitro......................173
11.4.2 Effects of liposomal clodronate mediated macrophage depletion on the progression of transplanted Eu-myc/bcl-2 lymphoma.................................174
11.5 Investigating macrophage depletion and lymphoma progression in the MaFIA mouse model.................................................................194

11.5.1 Investigating the effects of the MaFIA dimerizer AP20187 on Eu-myc/bcl-2 lymphoma cells in vitro.................................................................194
11.5.2 Investigating the effects of macrophage depletion on Eu-myc/bcl-2 lymphoma progression in the MaFIA mouse.................................................................195
11.6 Adoptive transfer of bone marrow derived macrophages......................200

11.6.1 Assessment of purity of bone marrow derived macrophages and in vitro phenotype manipulation.................................................................200
11.6.2 Adoptive transfer of bone marrow derived macrophages......................204
11.7 Investigating CSF-1R inhibition in lymphoma........................................213

11.7.1 Investigating the in vitro effects of the CSF-1R inhibitor on Eu-myc/bcl-2 cells.213
11.7.2 Investigating the in vivo effects of CSF-1R inhibitor at 50 mg/kg for 5 days in mice with lymphoma.................................................................214
11.7.3 Investigating the in vivo effects of CSF-1R inhibitor at 100 mg/kg for 10 days in mice with lymphoma.................................................................220
11.7.4 Investigating the consequences of CSF-1R inhibition in transplantable Eu-myc/bcl2 lymphoma.................................................................228
11.8 Combination with chemotherapy.........................................................244

11.8.1 Macrophage depletion following cyclophosphamide.......................244
11.8.2 Macrophage depletion prior to cyclophosphamide..........................249

12 Discussion.........................................................................................254
12.1 Critical interpretation of data................................................................. 254
  12.1.1 Interrogation of human DLBCL biopsies............................................. 254
  12.1.2 Interpretation of Liposomal Clodronate data...................................... 255
  12.1.3 Interpretation of MaFIA mouse data............................................... 257
  12.1.4 Interpretation of adoptive transfer data......................................... 260
  12.1.5 Interpretation of CSF-1R inhibitor data.......................................... 262
  12.1.6 Interpretation of gene expression data......................................... 266
  12.1.7 Interpretation of flow cytometry data........................................... 267
  12.1.8 Interpretation of plasma cytokine data......................................... 270
  12.1.9 Interpretation of data from combination with chemotherapy................ 272
12.2 Discussion of methods........................................................................... 273
  12.2.1 Measuring tumour burden............................................................... 273
  12.2.2 Criticisms of the transplantable Eμ-myc/bcl-2 lymphoma model............ 274
12.3 Consideration of the role of IL-6................................................................ 276

13 Summary. ................................................................................................. 278
13.1 Implications for future research............................................................. 279
13.2 Concluding statement.............................................................................. 284
References...................................................................................................... 285
Buffers and Solutions..................................................................................... 324
Publications and abstracts arising from this work ........................................... 326
List of Figures

Figure 1 - The traditional and simplified scheme of macrophage development..........................22
Figure 2 - Divergent monocyte and granulocyte differentiation pathways in the bone
marrow. .........................................................................................................................24
Figure 3 - Monocyte subsets in mice. .......................................................................................25
Figure 4 - Features of M1 phenotype macrophages, activated by bacterial LPS..................35
Figure 5 - Features of M2 phenotype macrophages. .................................................................35
Figure 6 - The CSF-1R, its ligands and downstream events. ....................................................52
Figure 7 - Monocyte subsets as a potential developmental series under the control of CSF-
1R ligation. ......................................................................................................................53
Figure 8 - CSF-1R inhibition might induce a maturation block and relative reduction in
"patrolling" monocytes and TAM ......................................................................................57
Figure 9 - CSF-1R inhibition strategies. ..................................................................................58
Figure 10 - Approaches to manipulate macrophages in a mouse model of lymphoma... 113
Figure 11 - A tumour-associated macrophage in follicular lymphoma (original
magnification (OM) x 60). ...............................................................................................126
Figure 12 - Principles of indirect polymer immunoperoxidase IHC .......................................127
Figure 13 - FFPE section of liver stained with H&E with overlying graticule to
demonstrate method of quantification of cross-sectional area of lymphoma (OM x
2.5) ...............................................................................................................................140
Figure 14 - Preparation of phenotype-modulated bone marrow derived macrophages. 150
Figure 15 - Example data demonstrating limitations of Mann-Whitney analysis. ............153
Figure 16 - Representative sections of DLBCL TMA stained with CD68 (top) and Ki67
(bottom). .......................................................................................................................156
Figure 17 - Distribution of scores for CD68 and CD163 staining by area (above) and Ki67
staining by percentage of positive nuclei (below). .......................................................157
Figure 18 – Comparison of areas of CD68 and CD163 staining across the whole TMA, with
each patient biopsy average score represented by a dot for each marker, joined by a
straight line (n=136). ..............................................................................................158
Figure 19 - Correlations between CD68 and CD163 staining (above) and each macrophage
marker and Ki67 (below). A regression line is depicted in red. .................................159
Figure 20 – Gating of live, CD19 positive cells from whole fresh lymph nodes. The CD19
positive portion was selected, and assessed below for expression of surface
immunoglobulin..............................................162
Figure 21 - Histograms depicting surface IgM and IgD expression of live, CD19 positive
cells from the fresh lymph nodes of mice with lymphoma. ........................................162
Figure 22 - Mutually exclusive expression of CSF-1R and B220 in fresh lymph nodes of
mice with lymphoma. .................................................................................................163
Figure 23 - Protocol investigating the effects of liposomal clodronate in healthy mice. 164
Figure 24 - Body weight of mice treated with iv LC (n=5) or untreated (n=4). Plot shows
mean values with standard error of the mean (SEM) for each group at each time
point. ......................................................................................................................165
Figure 25 - Liver weights. .....................................................................................................165
Figure 26 - Lymph node volumes. .........................................................................................166
Figure 27 - Spleen volumes. ................................................................................................166
Figure 28 - Percentage of live cells in the spleen. .................................................................167
Figure 29 - Percentage of live cells in lymph nodes. ............................................................167
Figure 30 - Flow cytometric measurement of macrophages in lymph nodes of healthy
mice with no manipulation (n=4) or iv LC (n=5), percentage of all node cells. ............168
Figure 31 - Percentage of CD3 positive T-lymphocytes in lymph nodes of normal healthy
mice (n=4) or those receiving iv LC (n=5). ..................................................................169
Figure 32 - Percentage of CD19 positive and B220 positive B-lymphocytes in lymph nodes
of normal healthy mice (n=4) or those receiving iv LC (n=5). .......................................169
Figure 33 - Percentage of CD3 positive T-lymphocytes in the spleen of normal healthy
mice (n=4) or mice with iv LC (n=5). ........................................................................170
Figure 34 - Percentage of CD19 positive and B220 positive B-lymphocytes in the spleen of normal healthy mice (n=4) or mice with iv LC (n=5). ........................................ 170
Figure 35 - Spleen stained with F4/80 and haematoxylin (OM x 40). ......................... 171
Figure 37 - Lymph Node stained with F4/80 and haematoxylin (OM x 40). .............. 172
Figure 38 - Bone marrow stained with F4/80 and haematoxylin (OM x 20). .......... 172
Figure 39 - Eμ-myc/bcl2 cells in vitro exposed to LC........................................ 173
Figure 40 - Protocol investigating liposomal clodronate in mice with lymphoma ...... 174
Figure 41 - Body weight of mice, untreated (n=4) or transplanted with 2×10^5 Eμ-myc/bcl-2 lymphoma cells, then given iv LC (Lymphoma + LC, n=5) or liposomal PBS (Lymphoma + LP, n=5) or no further injections (Lymphoma only, n=5). .......... 175
Figure 42 – Macroscopic appearance of partially dissected mice on day 12 following iv injection with Eμ-myc/bcl-2 lymphoma cells demonstrating smaller spleen and lymph nodes in the mice treated with LC.................................................... 176
Figure 43 - Spleen volumes...................................................................................... 177
Figure 44 - Lymph node volumes............................................................................. 177
Figure 45 - Liver weights........................................................................................... 177
Figure 46 - FFPE section of liver 10 days after iv lymphoma, stained with H&E (OM x 20). ........................................................................................................... 178
Figure 47 - FFPE section of liver, 10 days after iv lymphoma stained with F4/80 (OM x 40) ............................................................................................................... 178
Figure 48 - Area of liver infiltrated by lymphoma in FFPE sections stained with H&E.
Number of infiltrated squares was counted for 5 randomly selected LPF per section, one section per mouse and 5 mice per group.................................................. 179
Figure 49 - Normal mouse liver stained with H&E, FFPE section............................ 180
Figure 50 - Liver 10 days following iv lymphoma, FFPE section. ......................... 180
Figure 51 - Liver 10 days following iv lymphoma and intensive iv LC, FFPE section... 180
Figure 52 - Percentage of live cells in the spleen, measured by flow cytometry (n=5 per group). “Untreated” refers to unmanipulated normal healthy mice. .......... 181
Figure 53 - Normal spleen stained with H&E............................................................ 182
Figure 54 - Spleen stained with H&E day 12 after iv injection of lymphoma........... 182
Figure 55 - Spleen stained with H&E day 12 after iv injection of lymphoma and intensive regime of iv LC ........................................................... 182
Figure 56 - Normal bone marrow............................................................................ 183
Figure 57 - Bone marrow day 12 following iv lymphoma. ..................................... 183
Figure 58 - Bone marrow day 12 following iv lymphoma and intensive iv LC........ 183
Figure 59 - Bone marrow macrophage frequency, measured by counting of FFPE sections. 5 HPF per mouse, 4 mice per group....................................................... 184
Figure 60 - Measurement by flow cytometry of blood monocytes (n=6 per group)... 185
Figure 61 - Blood granulocytes (n=6 per group). ...................................................... 185
Figure 62 – Nodal macrophage frequency by flow cytometry (n=5 per group). ........ 186
Figure 63 - Nodal macrophage population (n=5 per group). .................................... 186
Figure 64 - Splenic macrophage frequency by flow cytometry (n=5 per group) ........ 187
Figure 65 - Protocol investigating dose effects of liposomal clodronate in mice with lymphoma. ......................................................................................................... 188
Figure 66 - Lymph node volumes............................................................................ 189
Figure 67 - Spleen volumes...................................................................................... 189
Figure 68 - Liver weights........................................................................................... 190
Figure 69 - Liver macrophage frequency, measured by counting of FFPE sections. 5 HPF per mouse, 5 mice per group....................................................... 190
Figure 70 - Liver area infiltrated by lymphoma, measured in FFPE tissue sections. 5 LPF per mouse, 5 mice per group .......................................................... 191
Figure 71 - Protocol investigating the survival impact of liposomal clodronate in mice with lymphoma. ............................................................................................... 192
Figure 72 - Time to reach UK Home Office limits of survival (n=12 per group). ....... 193
Figure 73 - Eμ-myc/bcl2 cells in vitro exposed to MaFIA dimerizer AP20187 ............ 194
Figure 74 - Protocol investigating lymphoma progression in MaFIA mice. .............. 195
Figure 107 – Lymph node macrophage frequency measured in FFPE sections stained with F4/80. Data represents 5x HPF per mouse and 4 mice per group. ................................. 217
Figure 108 – Liver area infiltrated with lymphoma in FFPE tissue sections. Data represents 5x HPF per mouse and 4 mice per group. ................................. 217
Figure 109 - Fold changes in gene expression at day 10 in lymph nodes of mice with lymphoma given 50 mg/kg twice daily CSF-1Ri for 5 days (n=5), compared to vehicle controls (n=5). .................................................. 219
Figure 110 - Protocol investigating prolonged higher dose CSF-1R inhibition in mice with lymphoma ................................................................. 220
Figure 111 - Lymph node weights. ........................................................................ 222
Figure 112 - Total body weight at sacrifice .............................................................. 222
Figure 113 - Lymph node weight corrected for total body weight ....................... 223
Figure 114 - Lymph node frequency of CD19 positive cells ................................... 223
Figure 115 - Lymphoma burden expressed as a function of lymph node weight and frequency of CD19 positive cells. ........................................ 224
Figure 116 - Lymphoma burden expressed as a function of lymph node weight corrected for total body weight, and frequency of CD19 positive cells. ................................. 224
Figure 117 - Lymph node CD11b+Ly6G- macrophages frequency of all lymph node cells, measured by flow cytometry (vehicle n=8, CSF-1Ri n = 7). ....................... 225
Figure 118 - Frequency of F4/80 positive macrophages in lymph nodes, by flow cytometry, percentage of CD19- cells (vehicle n=8, CSF-1Ri n = 7). ....................... 225
Figure 119 - Macrophage burden expressed as a function of lymph node weight and frequency of F4/80 positive cells (vehicle n=8, CSF-1Ri n = 7). ....................... 226
Figure 120 - Macrophage burden, expressed as a function of lymph node weight and frequency of CD11b+Ly6G- macrophages by flow cytometry. ......................... 226
Figure 121 - Macrophage burden expressed as a function of node weight corrected for body weight, and frequency of CD11b+Ly6G- macrophages by flow cytometry. .... 227
Figure 122 - Flow cytometry plot of live-gated blood cells stained for B220 and CSF-1R. Data represents pooled samples from 4 mice per group. ......................... 229
Figure 122 - Flow cytometry plot of live-gated lymph node cells stained for B220 and CSF-1R. Data represents pooled samples from 4 mice per group. ......................... 248
Figure 123 - Flow cytometry plot of live-gated blood cells stained for B220 and CSF-1R. Data represents pooled samples from 5 mice per group. ......................... 231
Figure 124 - Frequency of blood CD3+ cells by flow cytometry (n=8 per group). .... 232
Figure 125 - Frequency of blood CD19+ cells by flow cytometry (n=8 per group). .... 232
Figure 126 - Frequency of granulocytes and monocytes in the blood by flow cytometry, expressed as a percentage of CD19- live cells (n=8 per group). ....................... 233
Figure 127 - Histogram displaying relative expression of F4/80 by blood monocytes and granulocytes, representative data ........................................... 234
Figure 128 - Selection of blood CD19-CD11b+Ly6G- monocytes (black box) for expression of Ly6C (red box) or not (orange box), representative data. ......................... 234
Figure 129 – “Patrolling” monocyte frequency as a percentage of CD19-CD11b+Ly6G- cells in the blood (n=8 per group). ........................................... 235
Figure 130 – “Inflammatory” monocyte frequency as a percentage of CD19-CD11b+Ly6G- cells in the blood (n=8 per group). ........................................... 235
Figure 131 – Lymph node frequency of proliferating lymphoma cells expressed as percentage of CD19+ cells incorporating BrdU (n=7 per group). ................. 236
Figure 132 - Blood frequency of proliferating lymphoma cells expressed as percentage of CD19+ cells incorporating BrdU (n=7 per group). ......................... 237
Figure 133 - Plasma CSF-1 levels in normal healthy MaFIA mice (n=6), MaFIA with advanced lymphoma (n=4) and MaFIA with lymphoma and macrophage depleting dimerizer injections (n=3). .................................................. 239
Figure 134 - Plasma IL-6 concentrations in C57BL/6 mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6). ................. 240
Figure 135 - Plasma IL-10 concentrations in C57BL/6 mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6). ................. 240
Figure 136 - Plasma CXCL1 concentrations in mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).........................241
Figure 137 - Plasma IL-12p70 concentrations in C57BL/6 mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6)....241
Figure 138 - Plasma TNF-α concentrations in mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).........................242
Figure 139 - Plasma IL-1β concentrations in mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).........................242
Figure 140 - Plasma IFN-γ concentrations in C57BL/6 mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).............243
Figure 141 - Protocol investigating chemotherapy followed by CSF-1R inhibition in mice with lymphoma. ...........................................................................................................244
Figure 142 - Representative blood smears stained with modified Wright’s stain (OM x 2.5)..................................................................................................................................246
Figure 143 - Average body weights of mice in each group (n=10 per group).............247
Figure 144 - Time taken to reach Home Office guidelines limits of survival (n=10 per group). .................................................................................................................................248
Figure 145 - Protocol investigating cyclophosphamide after macrophage depletion in mice with lymphoma. ...........................................................................................................249
Figure 146 - Lymph node weights of mice exposed to macrophage depletion or vehicle prior to cyclophosphamide...............................................................................................250
Figure 147 - Lymph node cell counts. .................................................................................250
Figure 148 - Frequency of lymph node CD19 positive cells by flow cytometry (Vehicle n=7, CSF-1Ri n=7, LC n=8). No statistically significant differences detected.............251
Figure 149 - Lymphoma burden in lymph nodes expressed as function of lymph node total cell count (by Trypan-blue exclusion assay) and frequency of CD19 positive cells. ...............................................................................................................................251
Figure 150 - Lymphoma burden in lymph nodes expressed as function of lymph node weight and frequency of CD19 positive cells. .................................................................................252
Figure 151 - Frequency of proliferating CD19+ cells expressed as percentage of CD19+ cells incorporating BrdU (Vehicle n=7, CSF-1Ri n=7, LC n=8).................................253
Figure 152 - Frequency of CD19-CD11b+ cells in whole lymph nodes by flow cytometry (Vehicle n=7, CSF-1Ri n=7, LC n=8)..............................................................253
Figure 153 - CSF-1R ligation in monocyte production in the bone marrow..................263
Figure 154 - Summary of mechanisms proposed by Gilbert and Hemann......................276
List of Tables

Table 1 - Common chromosomal translocations in B-NHL .................................................61
Table 2 - Adverse prognostic variables of the FLIPI scoring system. .................................76
Table 3 - Relationship of FLIPI score to survival. .................................................................76
Table 4 - Summary of available data in relation to immunohistochemical enumeration of
macrophages in DLBCL ........................................................................................................96
Table 5 - Summary of published data in relation to immunohistochemical enumeration of
macrophages in FL. .............................................................................................................97
Table 6 - Antibodies used for TMA IHC. ...............................................................................129
Table 7 - Sensitivity of cytokine detection in MSD pro-inflammatory 7-plex kit. ............137
Table 8 - Statistical outcomes of data analysis. ...................................................................159
Table 9 - Genes selected for measurement by qRT-PCR....................................................218
Table 10 - Data from a representative experiment of prolonged CSF-1R inhibition in
lymphoma .........................................................................................................................221
Table 11 - Measured cytokines and their lower limits of detection (LLD). ......................238
Table 12 - Flow cytometry antibodies ...............................................................................325
List of Abbreviations

ABC  Activated B-cell
ADCC  Antibody dependent cellular cytotoxicity
AIDS  Acquired immune deficiency syndrome
APRIL  A proliferation inducing ligand
ATM  Ataxia telangiectasia mutated
B-NHL  B-cell non Hodgkin lymphoma
BAFF  B-cell activating factor of the TNF family
BCL2  B-cell lymphoma 2
BCR  B-cell receptor
bFGF  Basic fibroblast growth factor
BL  Burkitt lymphoma
BLAME  B-lymphocyte activator macrophage expressed
BMDM  Bone marrow derived macrophages
BrdU  Bromodeoxyuridine
C57BL/6  C57 black 6 mouse strain
CAC  Colitis-associated carcinogenesis
CCL  Chemokine ligand
CCR  Chemokine receptor
CD-  Cluster of differentiation
CDC  Complement dependent cytotoxicity
CDP  Common DC precursor
CEBPα  CCAAT/enhancer-binding protein alpha
CHOP  Cyclophosphamide, doxorubicin, vincristine, prednisolone
CLL  Chronic lymphocytic leukaemia
CMP  Common myeloid progenitors
CNS  Central nervous system
CORAL  Collaborative trial in relapsed aggressive lymphoma
COX  Cyclooxygenase
CR  Complete remission
CSF-1  Colony-stimulating factor-1
CSF-1R  CSF-1 receptor
CT  Cycle threshold
CTL  Cytotoxic T-lymphocyte
DAPI  4’,6-diamidino-2-phenylindole
DBA  Dilute brown non-agouti mouse strain
DC   Dendritic cell
DFS  Disease free survival
DLBCL Diffuse large B-cell lymphoma
DLI  Donor lymphocyte infusion
DMEM Dulbecco’s modified eagle medium
DMSO Dimethyl sulphoxide
DNA  Deoxyribonucleic acid
EBV  Epstein Barr virus
ECM  Extracellular matrix
EFS  Event free survival
EGF  Epidermal growth factor
EGFP Enhanced green fluorescent protein
EGR  Early growth response
ETS  E-twenty six
FAB  Fragment antigen-binding
FACS Fluorescence-activated cell sorting
FCS  Foetal calf serum
FcγR Immunoglobulin G fragment C receptor
FDC  Follicular dendritic cell
FDG-PET Fluorodeoxyglucose-positron emission tomography
FFPE Formalin fixed paraffin embedded
FGR Gardner-Rasheed feline sarcoma viral oncogene homologue
FKBP FK506-binding protein
FL   Follicular lymphoma
FL-MSC Follicular lymphoma-mesenchymal stromal cell
FLIPI Follicular lymphoma international prognostic index
FOXPI Forkhead box protein
GC   Germinal centre
GCB  Germinal centre B-cell
GILT Gamma interferon induced lysosomal transferase
GM-CSF Granulocyte-macrophage-colony stimulating factor
GMP  Granulocyte macrophage progenitor cells
GPI  Glycosylphosphatidylinositol
GvHD Graft versus host disease
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>GvL</td>
<td>Graft versus leukaemia/lymphoma effect</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HD-ASCT</td>
<td>High dose therapy with autologous stem cell rescue/therapy</td>
</tr>
<tr>
<td>HD-MSC</td>
<td>Healthy donor-mesenchymal stromal cell</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HP</td>
<td>Helicobacter pylori</td>
</tr>
<tr>
<td>HSCT</td>
<td>Haematopoietic stem cell transplant</td>
</tr>
<tr>
<td>HSPC</td>
<td>Haematopoietic stem and progenitor cell</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGH</td>
<td>Immunoglobulin heavy chain</td>
</tr>
<tr>
<td>IKKβ</td>
<td>IkappaB kinase beta</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin-</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IPI</td>
<td>International prognostic index</td>
</tr>
<tr>
<td>IR1/2</td>
<td>Immune response 1 or 2</td>
</tr>
<tr>
<td>iv</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus associated kinase</td>
</tr>
<tr>
<td>L-MTP-PE</td>
<td>Liposome-encapsulated muramyl tripeptide phosphatidylethanolamine</td>
</tr>
<tr>
<td>LC</td>
<td>Liposomal clodronate</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
</tr>
<tr>
<td>LLD</td>
<td>Lower limit of detection</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Ly6G/C</td>
<td>Lymphocyte antigen 6 complex, locus G/C</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MaFIA</td>
<td>Macrophage Fas-induced apoptosis</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa associated lymphoid tissue</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------------------------------------</td>
</tr>
<tr>
<td>MCL</td>
<td>Mantle cell lymphoma</td>
</tr>
<tr>
<td>mCSF-1</td>
<td>Membrane bound CSF-1</td>
</tr>
<tr>
<td>MDP</td>
<td>Macrophage dendritic cell precursor</td>
</tr>
<tr>
<td>MDP</td>
<td>Monocyte-macrophage/DC progenitor</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MP</td>
<td>Myeloid progenitor</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>MRC</td>
<td>Medical research council</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
</tr>
<tr>
<td>MSF</td>
<td>Migration-stimulating factor</td>
</tr>
<tr>
<td>MUM-1</td>
<td>Multiple myeloma oncogene-1</td>
</tr>
<tr>
<td>MYD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-beta</td>
</tr>
<tr>
<td>NHL</td>
<td>Non Hodgkin lymphoma</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitrous oxide</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature tissue embedding compound</td>
</tr>
<tr>
<td>OM</td>
<td>Original magnification</td>
</tr>
<tr>
<td>op/op</td>
<td>Osteopetrotic</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNSL</td>
<td>Primary central nervous system lymphoma</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PPI</td>
<td>Proton pump inhibitor</td>
</tr>
<tr>
<td>ProMACE-</td>
<td>methotrexate, doxorubicin, cyclophosphamide, etoposide</td>
</tr>
<tr>
<td>MOPP</td>
<td>mechlorethamine, vincristine, procarbazine, prednisolone</td>
</tr>
<tr>
<td>PyMT</td>
<td>Polyoma middle T oncprotein</td>
</tr>
</tbody>
</table>
qRT-PCR  Quantitative realtime-polymerase chain reaction
R-CHOP  Rituximab-CHOP
RNA     Ribonucleic acid
ROS     Reactive oxygen species
SEM     Standard error of the mean
SHM     Somatic hypermutation
SHPS-1  SH2 domain-containing protein tyrosine phosphatase substrate 1
siRNA   Small inhibitory RNA
SPARC   Secreted protein, acidic and rich in cysteine
SRA     Scavenger receptor class A
STAT    Signal transducer and activator of transcription
TAM     Tumour-associated macrophage
TCM     Tumour conditioned media
tFL     Transformed FL
TGF-β   Transforming growth factor-beta
TLR     Toll-like receptor
TNF-α   Tumour necrosis factor-alpha
TNFRSF6 Tumour necrosis factor receptor superfamily member 6
Treg    Regulatory T-cell
VEGF    Vascular endothelial growth factor
WHO     World health organisation
ΔLNGFR  Low affinity nerve growth factor receptor
1 Macrophage Biology.

1.1 Macrophage development.

1.1.1 The traditional paradigm of macrophage development.

The traditional and simplified paradigm of macrophage development is that haematopoietic stem and progenitor cells (HSPC) within the bone marrow give rise, via a common myeloid precursor cell, to monocytes (Fogg et al., 2006, Gordon and Taylor, 2005). Monocytes are released from the bone marrow and circulate throughout the blood and tissues of the lymphoid system. Monocytes migrate across endothelial barriers to enter tissues within which they differentiate into macrophages or dendritic cells (DC). A gradient of chemo-attractive cytokines regulates this migration and differentiation, and so enables steady-state turnover of resident tissue macrophages, as well as rapid accumulation of macrophages in response to inflammation in the context of tissue damage, infection or malignancy.

![Diagram of macrophage development](image)

Figure 1 - The traditional and simplified scheme of macrophage development.

The development of monocytes and macrophages is controlled principally by the cytokine colony stimulating factor-1 (CSF-1) and the recently discovered interleukin-34 (IL-34) (Lin et al., 2008), both acting on the CSF-1 receptor (CSF-
CSF-1R is expressed on early multipotent cells, mononuclear phagocyte precursors, monocytes, and macrophages (Sasmono et al., 2003, Dai et al., 2002, Cecchini et al., 1994, Wiktor-Jedrzejczak and Gordon, 1996, Kondo et al., 2000). CSF-1 has roles in both the development and function of monocytes and macrophages, as revealed by investigation of op/op mice, spontaneous mutants that lack a functional Csf-1 gene (Wiktor-Jedrzejczak and Gordon, 1996). The more severely compromised phenotype seen in CSF-1R deficient mice versus op/op CSF-1 deficient mice, strongly suggests that both CSF-1 and IL-34 contribute to monocyte and macrophage development and function (Dai et al., 2002).

CSF-1R ligation contributes to several stages of monocyte lineage differentiation, as depicted in the figure overleaf, and the precise points that are critical is debated (Lenzo et al., 2012). Certainly, generation of common myeloid progenitors (CMP) from HSPC in the bone marrow is promoted by CSF-1R ligation, and depends upon the activity of PU.1, an E-twenty six (ETS) family transcription factor (Dakic et al., 2005, Iwasaki et al., 2005). CMP mature into granulocyte macrophage progenitor cells (GMP). GMP represent a critical stage at which pursuit of either a granulocytic or monocytic maturation is determined. The fate of such bipotent GMP cells depends upon the relative activities of different transcription factors. CCAAT/enhancer-binding protein alpha (CEBP\(\alpha\)) is responsible for directing granulocytic differentiation by activating the zinc-finger protein Gfi-1. Monocyte differentiation via a bone marrow resident macrophage dendritic cell precursor (MDP) stage is directed by PU.1, antagonising the actions of CEBP\(\alpha\) (Zhang et al., 1997).
Figure 2 – Divergent monocyte and granulocyte differentiation pathways in the bone marrow. CSF-1R ligation promotes a monocyte fate at several stages, in cooperation with PU.1 transcription factor.

CSF-1 dependent macrophage differentiation critically depends upon PU.1 activation of downstream early growth response (EGR) transcription. The critical nature of PU.1 action in CSF-1R ligation induced monocyte differentiation is underlined by the fact that experimentally induced CSF-1R expression cannot rescue monocyte-macrophage differentiation in PU.1 deficient cells (DeKoter et al., 1998). Cells at the MDP stage lose Ly6G expression are committed to a monocyte or dendritic cell fate and have no granulocytic potential (Fogg et al., 2006, Massberg et al., 2007, Jiang and Schwarz, 2010). Granulocytes maintain Ly6G expression but only express CSF-1R at very low levels (Sasmono et al., 2007, Geissmann et al., 2008). Among CD11b positive cells in the blood it is only granulocytes that express Ly6G (Auffray et al., 2009). Monocytes are defined by CD11b expression and the absence of Ly6G expression, with variable expression of Ly6C, a GPI-anchored molecule also expressed by Ly6G positive granulocytes and subsets of NK cells (Auffray et al., 2009). In the following section we will discuss how variable expression of Ly6C appears to define functional subsets of monocytes in mice.
1.1.2 Monocyte subsets have distinct effector functions in both mice and humans.

There is now a greater appreciation that monocytes are important immune effector cells in their own right, rather than simply being macrophage precursors. Further, monocyte subsets have been identified that appear to be of distinct functional significance, and also developmental significance in terms of their potential maturation to resident tissue macrophages. In mice, two functional subsets of monocytes can be distinguished on the basis of surface expression of the Ly6C antigen, as depicted below in figure 3.

**Figure 3 - Monocyte subsets in mice.**

Ly6C+ monocytes are short-lived and termed “inflammatory” as they derive macrophages and DC in a variety of animal models of infection (Geissmann et al., 2003). These “inflammatory” monocytes produce high levels of tumour necrosis factor-alpha (TNF-α), reactive oxygen species (ROS) and nitrous oxide (NO), and are thus specialised to participate in acute inflammatory responses. Ly6C- monocytes can be distinguished by higher expression of the chemokine receptor CX3CR1 and the integrin LFA-1, and reduced expression of the chemokine receptor CCR2 and L-selectin (Wiktor-Jedrzejczak and Gordon, 1996). *In vivo* studies reveal that they are functionally distinct from Ly6C+ inflammatory monocytes, possessing a prolonged half-life and a long-range crawling activity exhibited along the luminal surface of vascular endothelium.
This behaviour has given rise to the term “patrolling” monocytes, and it is this subset that appears to be recruited to sites of tissue damage and gives rise to resident tissue macrophages (Nahrendorf et al., 2007). An elegant example of this phenomenon has been observed in a myocardial ischaemia model, in which Ly6C- patrolling monocytes are recruited at a delayed phase after tissue injury, and increase their production of vascular endothelial growth factor (VEGF). Further evidence of the “wound healing” phenotype of “patrolling” monocytes has been gained by gene expression profiling of such cells in a model of Listeria monocytogenes infection, demonstrating similarities with the transcription profile of macrophages involved in wound healing (Steinman et al., 1974, Auffray et al., 2007). The fact that DC can be generated in vitro by exposure of human and mouse monocytes to GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994, Geissmann et al., 2008) highlights the broad developmental potential of monocytes in vivo, that they are not committed to a macrophage fate.

There is evidence for functional homologues of mouse monocyte subsets in humans. Cros et al. performed characterisation of human monocytes by surface marker expression, gene expression pattern and functional transfer studies, and were able to further elucidate distinct monocyte populations based upon earlier observations (Ziegler-Heitbrock, 1996) that surface expression patterns of CD14 and CD16 varied among human monocyte functional groups. They demonstrated that CD14+CD16- (classical) and CD14+CD16+ (intermediate) monocytes resembled Ly6C+ mouse “inflammatory” monocytes, and that a minority population (7%) of CD14dimCD16+ (non-classical) monocytes behaved in a manner similar to “patrolling” mouse monocytes, had attenuated inflammatory cytokine responses to lipopolysaccharide (LPS), toll-like receptor (TLR) 4 or TLR2 agonists, and were unable to present antigens to T-cells. Despite exhibiting a broadly anti-inflammatory phenotype in their steady state, CD14dim CD16+ monocytes could be stimulated to elicit a pro-inflammatory repertoire of TNF-α, IL-1 and chemokine ligand 3 (CCL3) in response to viruses and nucleic acids. A pathway involving TLR7/8 ligation, myeloid differentiation primary response gene 88 (MYD88) and mitogen-activated protein kinase kinase (MEK) activation is responsible for this phenomenon, closely paralleling the behaviour of “patrolling” monocytes in the mouse, which can also be
stimulated by TLR7 agonists. Experimental evidence of some functional equivalence with mouse monocyte subsets, whilst intriguing, leaves many crucial questions as yet incompletely answered. The significance of human monocyte subsets in disease, particularly in cancer, is poorly understood, and the potential for therapeutic benefit from manipulating the balance of human monocyte subsets remains to be demonstrated.
1.1.3 Monocyte subsets may represent a developmental series.

Not only is evidence emerging of monocytes with distinct functions, but also that these subsets may well have very different fates as either inflammatory or wound healing macrophages. Moreover, “inflammatory” and “patrolling” monocytes may, in certain circumstances, represent not just a functional classification, but also an important developmental series in relation to each other. Evidence for this being a potential maturation series comes from adoptive transfer experiments. Bone marrow Ly6C+ monocytes are able to move between the bone marrow and blood compartments, and eventually lose Ly6C expression (Varol et al., 2007, Yrlid et al., 2006). As will be discussed later in much greater detail, experiments with CSF-1R inhibition demonstrate that this strategy can block the maturation of Ly6C+ monocytes into Ly6C- monocytes, and so reduce the accumulation of mature resident tissue macrophages.

However, contrary to this view, neither antibody mediated depletion of Ly6C+ monocytes nor a genetic defect (Feinberg et al., 2007, Alder et al., 2008, Scatizzi et al., 2006, Mildner et al., 2007) in Ly6C positivity (Kruppel-Like Factor 4 knockout in haematopoietic cells) appears to abrogate the ability to form Ly6C- monocytes. It remains unclear as to whether this phenomenon represents an absence of, or a redundancy in, the Ly6C+ to Ly6C- monocyte maturation pathway. The developmental origin of resident tissue macrophages also appears to be more complex and diverse than was hitherto appreciated, seemingly varying between different tissue-specific resident macrophage populations. This insufficiently understood topic is an area of active research.
1.1.4 Local extra-medullary proliferation contributes to renewal and expansion of resident tissue macrophages.

Evidence is accumulating that in certain circumstances resident tissue macrophages are able to proliferate in situ, and so obviate the need for monocyte recruitment and maturation to replenish or supplement their numbers. Constitutive egress and circulation in the blood of bone marrow resident HSPC (Wright et al., 2001) has been shown to enable accumulation of tissue-resident innate immune myeloid cells through local proliferation of HSPC in response to TLR agonists (Massberg et al., 2007). Specifically in relation to macrophages, this phenomenon has been described in the mouse brain (Mildner et al., 2007, Ajami et al., 2007) and epidermis (Chorro et al., 2009), tissues with limited potential for rapid trafficking of monocytes within which the postulated contribution of this proliferation is to populate tissue with macrophages during organogenesis (Davies et al., 2011) and then at a much lower rate to maintain tissue macrophage populations during normal homeostasis. A more recent study describes rapid bursts of proliferation of resident macrophages during Th2-type inflammation induced by parasitic infections (Jenkins et al., 2011). Davies et al. observed not only a proliferative burst of resident peritoneal macrophages during an episode of acute-resolving infection, but also that the accompanying peripheral monocytosis made only a negligible contribution to the peritoneal resident tissue macrophage accumulation. The authors theorized that self-sustenance by local proliferation of mature tissue macrophages “might represent a general paradigm and may not simply be restricted to tissue that has limited or controlled leukocyte access.” This certainly challenges the traditional paradigm of tissue macrophage numbers increasing during inflammatory processes due to recruitment of bone marrow generated monocytes. Further, that Jenkins et al. observed resident macrophage proliferation during Th2-type inflammation and under the control of IL-4 raises the prospect that the accumulation of tumour-associated macrophages (TAM) in a growing tumour, in which similar conditions prevail, might also be partly due to local macrophage proliferation.

Human macrophage proliferation has been observed in the setting of renal pathology (Isbel et al., 2001, Yang et al., 1998). Although evidence of proliferation of physiological resident macrophages in humans has not yet been
published, we do know that human monocytes can proliferate (Cheung and Hamilton, 1992) and that they regulate cell cycle genes under the control of CSF-1 (Martinez et al., 2006). The significance of local macrophage proliferation to the accumulation of human TAM is unclear, although it is interesting to speculate that this process might also be governed by CSF-1, and so be susceptible to CSF-1R inhibition.
1.2 Macrophages have vital roles in embryology and homeostasis.

Macrophages are involved in wound healing, tissue repair, iron metabolism, bone remodelling, graft-versus-host reactions, acute and chronic inflammation and cancer-related inflammation. These roles are mainly achieved by performing the cellular processes of phagocytosis, cytokine production and antigen presentation to variable extents at different times in different tissues. Aside from their roles in innate immunity, macrophages function as regulator and effector cells in both humoral and cell-mediated immune responses (Martinez et al., 2006). Upon phagocytosis, macrophages degrade proteins and process antigens for presentation by major histocompatibility (MHC) molecules (Hume, 2008c), so T-cells can recognize the presented substances as ‘foreign’. When activated in an immune response, macrophages can acquire microbicidal and tumouricidal activities involving reactive-oxygen species and reactive nitrogen metabolites.

Macrophages have the potential to orchestrate the tissue microenvironment, contributing to very many aspects of physiological homeostasis. During embryonic development, macrophages play an important role in scavenging dying cells and clearing areas of apoptosis, and thereby contribute to organogenesis (Hume, 2008a).

Under normal physiologic conditions, host tissues exist in a homeostatic balance with cells of the innate and adaptive immune systems. Acute and transient insults usually produce an acute inflammatory response, which resolves with healing of normal host tissues. Malignant cells have the potential to elicit an unresolved chronic inflammatory response.
1.3 Macrophages participate in cancer-related inflammation, demonstrating opposing functions in different settings and diseases.

A persistent population of malignant cells acts as a chronic inflammatory stimulus, exciting an ongoing and unresolved immune response, in which the normal homeostatic balance is not regained. Solid tumours consist of neoplastic cells, non-malignant stromal cells and migratory haematopoietic cells. In 1863 Virchow noted leukocytes in neoplastic tissues and made a connection between inflammation and cancer (Balkwill and Mantovani, 2001). Deidier observed a positive correlation between infection and remission of malignant disease in the eighteenth century (Garay et al., 2007). We now appreciate that there are indeed common pathways of inflammation shared by responses to infection and to malignancy.

Complex interactions between the different cell types in the malignant microenvironment regulate inflammation, tissue remodelling, tumour growth, progression, metastasis and angiogenesis (Sica and Bronte, 2007, Murdoch et al., 2008). The tumour microenvironment consists of an inflammatory component, and activation of the innate immune system appears to contribute to the progression of cancer (Sica and Bronte, 2007, Mantovani et al., 2008b). Plasticity of macrophage function is well described, and evidence suggests that TAM are involved in complex chemical cross talk with tumour cells and other cells of the tumour microenvironment (Mantovani et al., 1992, Hagemann et al., 2004, Sica et al., 2000a). This interplay has the potential to modulate macrophage phenotype, which may in turn affect the ability of the tumour to thrive.

In several carcinomas (breast, prostate, endometrium, bladder, kidney, and oesophagus) an abundance of TAM is associated with a poor prognosis (Tsutsui et al., 2005, Lissbrant et al., 2000, Ohno et al., 2004, Hanada et al., 2000, Hamada et al., 2002, Koide et al., 2004). There is overwhelming evidence for macrophages as obligate partners in cancer progression; not only from association studies, but also from experimental cancer models showing that ablation of macrophage function, or inhibition of their infiltration, inhibits growth and metastasis (Lin et al., 2001). Such compelling evidence is incomplete in relation to B-NHL and is considered later. An abundance of macrophages in
colon and gastric carcinomas, and melanoma is associated with improved clinical outcome (Forssell et al., 2007, Ohno et al., 2003, Piras et al., 2005). As such, it is important not to assume a pro-tumoural role of macrophages in B-NHL. Rather, it is necessary to study the functional consequences of manipulating interactions between macrophages and malignant B-cells in the laboratory.
1.4 Phenotypic diversity underlies the broad spectrum or roles played by macrophages in cancer.

Macrophages display enormous variation in phenotype, with specialised populations seen in different tissues. This variation in phenotype of resident tissue macrophages with a common origin, which can to a certain extent be distinguished by the expression of cell surface markers, provides supporting evidence for the plasticity of macrophage phenotype. Well-described specialised resident tissue macrophages include Kupffer cells (liver), osteoclasts (bone), microglia (brain), histiocytes (connective tissue) and alveolar macrophages (lung). The anatomical and temporal variation in macrophage phenotype is influenced by activation signals from the surrounding microenvironment and can be strongly regulated by bacterial lipopolysaccharide (LPS) and the products of T-lymphocytes, natural killer (NK) cells and tumour cells, in particular interferon-gamma (IFN-γ) and a cytokine network involving IL-4, IL-10, IL-12, and IL-13, as well as tissue oxygen tension and pH. Macrophage activation states have been described in terms of M1 or M2 phenotype, emulating the Th1/Th2 paradigm, and in vitro data has established that peripheral blood monocyte-derived macrophages can be polarised into M1 or M2 phenotypes. This has been extensively validated as an in vivo phenomenon for M1 macrophages, which are activated by IFN-γ or by bacterial cell wall derived LPS, and predominate during acute inflammatory responses.

1.4.1 M1 macrophage phenotype.

M1 macrophages produce large quantities of pro-inflammatory cytokines, express high levels of MHC molecules, and are implicated in the killing of pathogens and tumour cells (Gordon and Taylor, 2005). It was already apparent in the early 1960s (Bennett et al., 1963, Old and Boyse, 1964) that peritoneal-harvested macrophages from mice could be cytotoxic to tumour cells, and that this was augmented by exposure to certain stimulating agents such as starch, and by opsonising tumour-specific anti-sera. Macrophages harvested from the peritoneal cavity following the injection of inflammatory stimulants were found to have increased cytoplasm volumes and increased rapid spreading ability (North, 1969) hinting at phenotypic plasticity. In 1971 (Alexander and Evans, 1971) it was recognised that exposure to LPS (and also to DNA) rendered macrophages cytotoxic, so describing what is now known as the M1 or
"classically activated" macrophage phenotype.

1.4.2 M2 macrophage phenotype.

M2 macrophages behave in a “wound-healing” manner, and can also be described as “alternatively activated” (Gordon, 2003). M2 macrophages moderate the inflammatory response and can promote angiogenesis and tissue re-modelling (Murdoch et al., 2008, Mantovani et al., 2008a). Stimulation with IL-4 (Becker and Daniel, 1990, Stein et al., 1992) from CD4+ T-cells (De Nardo et al., 2009b), IL-13 for example from NK T-cells (Sinha et al., 2005), transforming growth factor-β (Flavell et al., 2010) (TGF-β), IL-10 (Sica et al., 2000b, Wong et al., 2010); immune-complexes, glucocorticoid hormones, and agonists of certain TLR or the IL-1R, drives macrophages towards an M2 phenotype (Mantovani et al., 2004b).

A window on the true complexity of in vivo conditions and subsequent macrophage phenotypes is provided by literature describing multiple subsets of M2 macrophages (Martinez et al., 2008, Biswas and Mantovani, 2010) and
specialised subsets seen in vascular disease (Gleissner et al., 2010). The
tumour microenvironment appears to ‘educate’ TAM towards a tumour-
promoting M2 phenotype (Pollard, 2004), but the mechanisms underlying this
phenomenon are not fully understood. Isolation of TAM from experimental
tumours, and determination of their gene expression, provides further evidence
that TAM develop an immunoregulatory phenotype (Biswas and Lopes de
Faria, 2006, Ojalvo et al., 2009, Pucci et al., 2009). TAM show considerable
variability, yet mainly display an IL-10\textsubscript{high}, IL-12\textsubscript{low} phenotype with expression of
mannose receptor (MR/CD206) and scavenger receptor class A (SRA/CD204)
(Mantovani et al., 2002), and approximate an M2 phenotype. Increased
expression of MR appears to be a feature of M2 macrophages that is
particularly common in TAM, and may have important roles in the progression
of some types of tumour. MR is able to mediate phagocytosis in an actin-
dependent manner (Aderem and Underhill, 1999, Kruskal et al., 1992), and so
contribute to clearance of apoptotic tumour cells without exciting a
proinflammatory cytokine response. Similarly, MR participates in the removal of
lysosomal hydroxylases and neutrophil derived myeloperoxidase, so limiting
tissue damage and moderating elements of the acute inflammatory response
(Shepherd and Hoidal, 1990, Stahl et al., 1980). High levels of secreted IL-10
have been found in follicular lymphoma (FL), diffuse large B-cell lymphoma
(DLBCL) and Burkitt lymphoma (BL) (Ogden et al., 2005, Voorzanger et al.,
1996). IL-10 in the tumour microenvironment seems to direct aspects of the M2
phenotype through inhibition of macrophage nuclear factor-kappa B (NF-\kappa B)
signalling, leading to reduced production of IL-12 and attenuated anti-tumoural
activity (Sica et al., 2000b), and IL-10 is itself subsequently produced as a
hallmark of the M2-like TAM phenotype.

The impact of macrophages may not simply be a reflection of their phenotype.
The activation phenotype of TAM must be seen within the context of the whole
tumour microenvironment. There is compelling \textit{in vitro} evidence that not only is
activation of TAM required to promote tumour growth, but that defects in the
regulation of such activated macrophages might also be necessary. By
observing immune synapses formed between natural killer (NK) T-cells and
macrophages, Nedvetzki and colleagues (Nedvetzki et al., 2007) showed that
macrophagelytic synapses developed between these cell types if the
macrophages were in a state of activation. This indicates a surveillance role for NK T-cells in eradicating activated macrophages and reflects important interactions between immune cell populations. Defects in this auto-regulation of activated macrophage numbers may contribute to tumour progression. Moreover, as a therapeutic approach, NK T-cells might be directed to eradicate TAM. As such, the functional significance of macrophage activation is likely to depend on many factors in addition to macrophage phenotype alone, in particular the function of other immune cells. This project aims to focus on the role of TAM in lymphoma, but will also consider the direction and composition of the overall immune response to this disease, and how this relates to features of the macrophage population.
1.5 Criticism of the M1-M2 model of macrophage phenotype.

Whilst classification systems based upon function are useful when trying to understand macrophage biology, they are to a certain extent artificial distinctions, with discrete phenotypic subsets probably not existing as separate populations in complex and dynamic multi-cellular *in vivo* systems. Attempts to simplify macrophage phenotype further by interrogating populations for the presence of absence of M1 or M2 markers are fraught with the potential for making misleading assumptions, given that individual markers do not appear to correlate well with each other when analysed across large data sets, or in response to different stimuli (Gordon and Martinez, 2010, Mabbott et al., 2010). Multiple diverse cytokines and adhesive interactions with surrounding cells and the extracellular matrix influence macrophage phenotype. The specific composition of the activating signal leads to a variety of macrophage functions, which differ significantly. Macrophage phenotype appears to be very stimulus specific, and not stereotyped or binary in response to different stimuli, as might be erroneously assumed to be the case if misinterpreting the M1/M2 schema. This schema remains, nonetheless, a useful tool for descriptive purposes and laboratory testing of broad principles of macrophage phenotype.
1.6 Macrophages exhibit phenotypic plasticity that may be harnessed therapeutically in cancer.

Macrophage plasticity of phenotype appears to extend to the ability, once activated, to switch phenotypes under certain conditions (Biswa and Mantovani, 2010). Arnold et al. found in mice that CX3CR1<sub>low</sub> Ly6C+ inflammatory monocytes recruited after skeletal muscle injury switched to a CX3CR1<sub>high</sub> Ly6C- anti-inflammatory macrophage phenotype on entering muscle tissue, postulated to support myogenesis (Arnold et al., 2007). Monocytes and macrophages were tracked first by ablating existing phagocytes with injection of toxic liposomes, followed by labelling newly formed monocyte/macrophages with fluorescent latex beads. In vitro studies showed that inflammatory macrophages began secreting TGF-β1 after ingestion of myocyte debris, and that inhibition of phagocytosis with cytochalasin D or recombinant Annexin V prevented the phenotypic switch. The specific components of the myocyte debris responsible for this phenomenon were not identified. Additionally, experiments with tissue-specific conditional gene knockout (Hagemann et al., 2008) and also with small molecule inhibitors (Fong et al., 2008) have demonstrated that inhibition of macrophage NF-κB activity has the potential to switch M2 to M1 phenotypes, providing both a proof-of-concept for the plasticity of mature activated macrophage phenotype, and also a possible therapeutic strategy. A tantalising glimpse of what might be possible in humans has been provided by pre-clinical studies with human cells, investigating the therapeutic use of IFN-γ to reverse the immune-suppressive phenotype of TAM (Duluc et al., 2009). TAM isolated using CD14+ magnetic beads from ovarian cancer ascites were of a M2-like phenotype. Subsequent publications identified that high levels of IL-6 and leukaemia-inhibitory factor (LIF) in ovarian cancer ascites primed monocytes to consume the high levels of CSF-1 also present and so mature in to M2-like TAM (Duluc et al., 2007, Jeannin et al., 2011). When exposed to IFN-γ they recovered a M1-like phenotype with IL-10<sub>low</sub> IL-12<sub>high</sub> CD86+ expression, able to induce enhanced proliferation of CD4+ lymphocytes and enhanced cytotoxic function of CD8+ T-cells, with reduced production of VEGF and matrix metalloproteinase 9 (MMP9). Peripheral blood mononuclear cell (PBMC) fraction derived monocytes from healthy donors cultured in ovarian cancer ascites with the addition of...
supplementary IFN-γ tended to an M1 rather than M2 TAM phenotype. Consistent with this is the observation from a phase III clinical trial of first-line chemotherapy for ovarian cancer with cisplatin and cyclophosphamide showing that the addition of seven sub-cutaneous injections of IFN-γ during each of a maximum of six 28-day cycles was associated with prolonged progression-free survival (Windbichler et al., 2000).
1.7 Tumour cells recruit and activate monocytes towards a pro-tumoural phenotype.

Owing to plasticity of function, resident tissue macrophages are able to contribute to tumour initiation and propagation by switching to a pro-tumoural M2-like phenotype. However, in a growing tumour, it appears as though the bulk of the TAM population derives from immature monocytes recruited from the circulation, which subsequently undergo maturation to specialised macrophages within the tumour microenvironment. This phenomenon has been observed and extensively studied in animal models of cancer such as the polyoma middle T oncoprotein (PyMT) induced mouse mammary tumour (Lin et al., 2006, Lin et al., 2001, Pollard, 2009, Wyckoff et al., 2007) and strikingly in orthotopic tumours in the transgenic ‘MacGreen’ model, in which cells of the monocyte/macrophage lineage express an enhanced green fluorescent protein (Sasmono et al., 2003). Similar patterns have been detected in human endometrial and breast cancers (Lewis and Pollard, 2006, Smith et al., 1995). Macrophages are recruited in the presence of CSF-1 (De Nardo et al., 2009a, Hamilton, 2008, Lin et al., 2001) synthesized by tumour cells. In humans, the greatest concentration of CSF-1 protein expression and of macrophages is found at the tumour invasion edge (Lin et al., 2001, Scholl et al., 1994, Smith et al., 1995), supporting the importance of this relationship in humans.

Intimate cooperation between macrophages and tumour cells has been observed in a mouse model of breast cancer, such that tumour-derived CSF-1 induces macrophage migration and secretion of epidermal growth factor (EGF), which in turn activates tumour cell migration (Wyckoff et al., 2004). This mechanism neatly links macrophage recruitment to tumour spread. In this case, as in the majority of cancers and cancer models studied, the CSF-1 receptor is restricted to monocyte/macrophages, and the EGF receptor to tumour cells. A similar reciprocal interaction has been described between human monocytes and human pancreatic cancer cells in vitro (Solinas et al., 2010). Exposure of PBMC-derived monocytes to tumour conditioned media (TCM) from 16 cancer cell lines representing ovarian, colon and pancreatic cancers, showed that TCM from two of the pancreatic cancer cell lines induced differentiation of monocytes to macrophages of an M2-like phenotype. Ligation of the CSF-1R with CSF-1 was critical to this process, as demonstrated by blocking with anti-CSF-1R.
antibodies. In addition to up-regulating several genes implicated in the organisation of the extra-cellular matrix (ECM), induction of migration-stimulating factor (MSF) in TCM-exposed monocytes was found at a gene expression and protein level, and was found to promote increased tumour cell migration across a transwell chamber membrane. Elevated MSF levels have previously been reported in patients with breast cancer (Picardo et al., 1991), but not in healthy individuals or non-malignant adult cells (Schor et al., 1988), and administration of labelled anti-MSF antibodies in a mouse xenograft of oesophageal cancer showed localisation to tumour vasculature and produced a reduction in tumour mass (Hu et al., 2009).
1.8 Links between malignant B-cell apoptosis, macrophage recruitment, activation and anti-tumour immune responses.

CSF-1 is certainly not the only monocyte chemo-attractant. CCL2 is well described as a chemo-attractant for monocytes and macrophages, in health, inflammation and in cancer (Mantovani et al., 2004b, Mantovani, 1994, Loberg et al., 2006, Mantovani et al., 2004a, Loberg et al., 2007). CCL2 levels are raised in the blood of patients with non-haematological cancers (Bingle et al., 2002, Mantovani et al., 1992) and this cytokine has more recently been implicated in an amplification loop with IL-6 resulting in increased survival and acquisition of pro-tumoural features of human monocytes and macrophages (Roca et al., 2009a). Human PBMC derived monocytes in vitro were treated with CCL2 and IL-6, and it was found that each was able to induce increased production of the other. Both were able to improve monocyte survival by up regulating the production of anti-apoptotic molecules including BCL2 and BCXL, inhibit elements of the caspase cascade, and up-regulate expression of CD14 and MR, features of M2-like macrophages.

The rapid removal of apoptotic cells by macrophage phagocytosis prevents potentially detrimental inflammatory reactions during physiological processes (Savill et al., 1993). Macrophages use CD14 to bind apoptotic cells prior to initiating phagocytosis (Devitt et al., 1998), and so in cancer-related inflammation, CD14 expression is a key element in suppressing anti-tumour immune responses that might otherwise be sparked by a failure of apoptosis. As previously discussed, MR is strongly implicated in the immune-suppressive phenotype of TAM. IL-6 is abundant in the tumour microenvironment and contributes to tumour cell proliferation and survival (Cavarretta et al., 2007, Tricot, 2000). Combined, these data suggest that CCL2 and/or IL-6 from tumour cells or other elements of the tumour microenvironment might drive the survival and alternative activation of TAM which in turn promote tumour proliferation through further IL-6 production, promote further TAM accumulation through CCL2 production, and limit anti-tumour immunity.
2 Macrophages in the tumour microenvironment.

In this chapter we will broadly outline the major roles ascribed to TAM in supporting the survival, growth and spread of tumours. In a later chapter we critically review the literature specifically relating to such roles for TAM in B-NHL.

2.1 Angiogenesis.

The majority of cancers and cancer models show increased tissue vascular density during transformation to the malignant state. Multiple cell types contribute to this “angiogenic switch” (Hanahan et al., 1996) of which macrophages are a major component (Zumsteg and Christofori, 2009). It appears that hypoxia plays a role in directing macrophages towards a pro-angiogenic phenotype, and so promoting tumour progression. Studies from our own research group have found a relationship between the location of macrophages expressing CD163, an M2 phenotype marker (Mantovani et al., 2004b), and neo-angiogenic vascular sprouts in human biopsies of poor prognosis Follicular lymphoma (FL) (Clear et al., 2010).

Functional studies in mouse models of cancer provide further evidence for a link between macrophages and the “angiogenic switch.” Macrophage depletion is observed in CSF-1 constitutive genetic knockout mice, in which PyMT induced mammary tumours show vastly attenuated angiogenic switch (Lin et al., 2006). There is evidence from this model that VEGF production by TAM may be a key element in the “angiogenic switch.” Using transgenic VEGF-A op/op PyMT mice, VEGF over-expression at a benign stage in such macrophage-depleted mice produces increased angiogenesis and accelerates the transition to outright malignancy (Lin et al., 2007). Moreover, myeloid cell-specific ablation of the VEGF-A gene retards the “angiogenic switch” in mouse mammary tumour models (Stockmann et al., 2008).

Hypoxia is a typical feature of growing tumours and, in addition to being positively correlated with the extent of TAM infiltration, promotes a pro-tumoural TAM phenotype and thus VEGF production. Hypoxia-inducible factor-1α (HIF-1α) has a key role in controlling this response. Hypoxia stabilizes the α-subunit, preventing hydroxylation and subsequent degradation via the proteasome. Persistent α-subunits attach β-subunits in the nucleus before binding hypoxic
response elements of oxygen-sensitive genes, altering their expression (Semenza, 2002). In this way, hypoxia stimulates TAM to secrete pro-angiogenic cytokines and enzymes, immunosuppressive agents and mitogens (Lewis and Murdoch, 2005). *In vitro* work has identified a distinct hypoxia induced pro-angiogenic human macrophage phenotype, with upregulation of VEGF, basic fibroblast growth factor (bFGF), CXCL8, cyclooxygenase-2 (COX2), hepatocyte growth factor (HGF), VEGFR1, tissue factor and MMP12 (White et al., 2004, Burke et al., 2003). In human breast cancer, TAM express VEGF almost exclusively in hypoxic areas, and upregulate VEGF on migrating to hypoxic regions (Lewis et al., 2000).

Tumour cells expressing membrane bound CSF-1 (mCSF-1) appear capable of eliciting anti-tumour immune responses from macrophages in several non-haematological cancer models (Graf et al., 1999, Jadus et al., 2003, Dan et al., 2001, Williams et al., 2001a) but conversely a tumour growth-promoting effect in studies of lymphoma cell lines *in vitro* and *in vivo* (Wang et al., 2008). In these studies, the lymphoma cell lines Namalwa and Ramos were stably transfected to express mCSF-1, and showed increased *in vitro* proliferation when co-cultured with macrophages. Injected subcutaneously in irradiated BALB/c mice, these cell lines grew larger tumours than non-mCSF-1 over-expressing controls, with more TAM and increased blood vessel density. This indicates that in B-lymphomas, TAM are more plentiful, pro-angiogenic and pro-tumoural in the presence of mCSF-1.
2.2 Invasion and metastasis.

Tumour cell migration, particularly in epithelial tumours, requires proteolytic destruction of the surrounding matrix to facilitate the escape of tumour cells from the confines of the basement membrane. Common to all spreading tumours is the need for subsequent proteolysis of surrounding dense tissue stroma. Macrophages are potent producers of many proteases, including cathepsins, MMPs, and serine proteases (Egeblad and Werb, 2002). Increased production of MMPs is a feature of the M2 macrophage phenotype and is typical of TAM (Coussens et al., 2000). Macrophage-specific depletion of cathepsins or urokinase plasminogen activator results in reduced tumour cell invasion and inhibition of metastasis in mouse mammary tumour models (Gocheva et al., Vasiljeva et al., 2006, Almholt et al., 2005). In a model of invasive colorectal cancer, immature myeloid cells producing both MMP9 and MMP2 were observed to accumulate at the tumour invasion front (Kitamura and Taketo, 2007). Breast cancer cell-lines show increased invasiveness on co-culture with macrophages, due to TNF-α induced up-regulation of MMPs (Hagemann et al., 2004). As discussed earlier in more detail, M2-polarised macrophage-derived MSF is a potent stimulant of pancreatic cancer cell line migration in vitro (Solinas et al., 2010).
2.3 Immune-suppression.

Macrophages are central to many immune responses and under certain circumstances, particularly during bacterial infection, are potent antigen presenting cells. However, within the tumour microenvironment, it appears that TAM adopt an immunoregulatory role, suppressing anti-tumour immune responses. Macrophages can inhibit cytotoxic T-cell responses through several mechanisms. For example, macrophages produce IL-10 that in turn induces monocytes to express the co-stimulatory molecule programmed death ligand-1 and suppresses cytotoxic T-cell responses (Kuang et al., 2009). In human ovarian cancers, macrophages produce CCL22, a chemokine that influences the influx of regulatory T-cells (Tregs) that suppress cytotoxic T-cell responses. The abundance of Tregs in ovarian cancer predicts poor survival (Curiel et al., 2004). In mammary tumour xenografts, a recruited macrophage population suppressed immune responses through synthesis of prostaglandin-E2 and transforming growth factor-beta (TGF-β) (Torroella-Kouri et al., 2009). In human B-NHL and Hodgkin Lymphoma it appears that the role of Tregs might be different from that in ovarian cancer, with several studies describing an association between high numbers of infiltrating Tregs and a favourable prognosis (Tzankov et al., 2008, Lee et al., 2006).
2.4 Stimulation of tumour cell proliferation.

M2 macrophages secrete a variety of growth factors that can stimulate the differentiation and proliferation of other cells. Macrophage-elicited platelet-derived growth factor (PDGF) and TGF-β1 stimulate the differentiation of fibroblasts and myofibroblasts. It seems plausible that these and other growth factors might also promote the differentiation and proliferation of malignant cells, as has been postulated by Mantovani (Mantovani et al., 2004b).

IL-6 is a growth signal that blocks apoptosis and is one of the effector signals of activated NF-κB in the promotion of neoplasia. IL-6 can be produced by activated macrophages, and signals through STAT3, which is in turn activated in many different cancers (Haura et al., 2005). This transcription factor regulates genes that mediate cell proliferation (such as Cyclin-D1 and c-Myc), promotes angiogenesis (through regulation of VEGF) and suppresses apoptosis (Bcl-xL) (Aggarwal et al., 2006).

Studies involving deletion of IKKβ from enterocytes or myeloid cells reveal distinct pathways through which NF-κB contributes to colitis-associated carcinogenesis (CAC) (Greten et al., 2004). In a mouse model of CAC induced by the carcinogen azoxymethane, in the context of dextran sulfate sodium salt-stimulated inflammation, deletion of IKKβ in enterocytes resulted in many fewer tumours compared to wild-type mice. This is thought to be a direct result of more apoptosis in pre-neoplastic enterocytes. IKKβ deletion in myeloid cells, however, also led to a decrease in tumour size and this mechanism is thought to depend on decreased IL-6 production leading to slower tumour growth. IL-6 receptor antagonism in the mouse model of CAC slowed tumour growth markedly but did not change the number of individual tumours that arose (Becker et al., 2004). These findings suggest that IL-6 has its most profound effect on tumour promotion and progression rather than initiation. In the context of lymphoma, Gilbert and Hemann describe a role for stromal derived IL-6 in sustaining malignant B-cells (by inducing anti-apoptotic BCXL) within a chemoresistant niche in a mouse model of B-lymphoma (Gilbert and Hemann, 2010).

In their work, IL-6 knockout mice with lymphoma had prolonged survival following chemotherapy than wild type controls.
A proliferation inducing ligand (APRIL) is a cytokine of the TNF family (Hahne et al., 1998, Kalled et al., 2005, Kelly et al., 2000) that promotes the survival and proliferation of neoplastic B-lymphocytes (Planelles et al., 2004, Burjanadze et al., 2009, Planelles et al., 2008, Roosnek et al., 2009). Munari et al. reported that APRIL is produced almost exclusively by an M2-like subset of macrophages (CD68+CD163+) in primary samples of human gastric MALT lymphoma, and was not detectable from tissue macrophages in areas of normal healthy gastric mucosa (Munari et al., 2011). In vitro studies demonstrated that autologous PBMC derived macrophages produced APRIL when directly stimulated with either Helicobacter pylori (HP) or with patient-derived HP-specific T-cells. HP eradication therapy leading to remission of gastric MALT lymphoma was associated with a reduction in APRIL producing macrophages.
2.5 Summary.

The phenotype and role of TAM is not entirely consistent amongst cancers, and there is a need for detailed study of the specific features of TAM in B-lymphoid malignancies. Moreover, specialised macrophage subpopulations within each tumour appear to have very different roles in supporting tumour progression, and might represent attractive new therapeutic targets (Qian and Pollard, 2010). Even in the setting of cancer, the majority of monocytes and macrophages in the body remain involved in critical physiological roles. For this reason, identifying and specifically targeting macrophage subpopulations is likely to be crucial in any attempt at macrophage-directed anti-cancer therapy.

There is a wealth of recent evidence strongly indicating a pivotal role for the CSF-1R in monocyte development and the accumulation of resident tissue macrophages and TAM. As will be critically reviewed in the following chapters, CSF-1R inhibition might offer the opportunity to target the accumulation of TAM in a growing tumour, without impacting significantly on the ability of the host to mount vital acute inflammatory responses.
3 Critical roles for the CSF-1R.

3.1 CSF-1R ligation promotes the formation, survival, maturation and proliferation of monocytes and macrophages.

The majority of mononuclear phagocytes express the macrophage colony-stimulating factor receptor, CSF-1R, a type III integral membrane protein tyrosine kinase also known as CD115, or in humans, M-CSF. Ligation of the CSF-1R results in non-covalent receptor dimerization, and phosphorylation of intracellular tyrosine kinase residues. These act as docking sites for a variety of signalling molecules, activating downstream pathways (Jacquel et al., 2009). Phosphatidylinositol 3-kinase (PI3K) / protein kinase B (AKT) and mitogen activated protein kinase (MAPK) pathways are critically involved in CSF-1R ligation dependent monocyte to macrophage maturation (Gobert Gosse et al., 2005). mRNA encoding the CSF-1R is expressed in such a highly restricted manner in the monocyte-macrophage lineage in mouse and man (Ovchinnikov et al., 2010, Bonifer and Hume, 2008) that the promoter region has been used to generate CSF-1R-EGFP transgenic mice to enable *in vivo* imaging of this cell type (Sasmono et al., 2003). Circulating levels of CSF-1 rise with pregnancy (Pollard et al., 1987, Bartocci et al., 1986), infections, cancer, and chronic inflammation (Chitu and Stanley, 2006, Sweet and Hume, 2003, Hamilton, 2008). CSF-1 controls the formation, proliferation, differentiation, adaptation and survival of mononuclear phagocytes (Chitu and Stanley, 2006, Sweet and Hume, 2003). *In vitro* studies have confirmed that GM-CSF, IL-3 and IFN-γ act with CSF-1 to further promote the proliferation and maturation of macrophages from bone marrow cells. In mice, macrophages undergo cell death in the absence of CSF-1 (Irvine et al., 2006, Williams et al., 1990).

A second ligand for the CSF-1R, IL-34, was recently described (Lin et al., 2008) and a partial redundancy of function of either ligand may explain the extreme derangement of phenotype of mice with CSF-1R mutations compared to the *op/op* mutated mice with isolated CSF-1 deficiency in which total circulating monocyte numbers are maintained (Dai et al., 2002). Studies of the impact of CSF-1 deficiency in these mice is limited, however, as macrophage deficiency is present during embryogenesis, resulting in gross developmental
abnormalities of the central nervous system, pancreas, mammary glands and reproductive organs (Chitu and Stanley, 2006, Dai et al., 2002). Although binding the same receptor, the finding that some anti-CSF-1R monoclonal antibodies block the binding of CSF-1 but not of IL-34 strongly indicates that the two ligands bind at different sites on the CSF-1R (Chihara et al., 2010).

![Diagram of CSF-1R, its ligands, and downstream events](image)

**Figure 6 - The CSF-1R, its ligands and downstream events.**
3.2 The CSF-1R is a conceptually attractive therapeutic target in cancer.

Transcriptional profiling of human monocytes and macrophages *in vitro* has revealed that CSF-1 induces monocyte to macrophage differentiation. *In vitro* work with mouse and human cells indicates that when CSF-1 is the sole activating stimulus for monocyte-derived macrophages, then macrophages are polarised away from an antigen presenting phenotype and towards immunosuppression (Hume, 2008b, Hume, 2008c) and promoting tumour cell migration (Solinas et al., 2010), and hence are M2-like (Martinez et al., 2006, Svensson et al., 2011). However, CSF-1 stimulated macrophages are not of a pure M2 phenotype, and respond to IL-4 supplementation with further immunosuppressive changes in gene expression (Gordon and Martinez, 2010, Martinez et al., 2006, Svensson et al., 2011, Rehli et al., 2005). A potential role for CSF-1R ligation in directing monocyte subset differentiation and maturation towards immune-suppressive macrophages is outlined in the figure below. As we will detail in the following chapter, CSF-1R ligation is implicated in the maturation of Ly6C+ monocytes to Ly6C- monocytes, and so in the formation of TAM.

![Diagram showing the differentiation and maturation of monocytes under the control of CSF-1R ligation.](image)

*Figure 7 - Monocyte subsets as a potential developmental series under the control of CSF-1R ligation.*
CSF-1 supplementation in mice produces an increase in numbers of mature resident tissue macrophages (Hume et al., 1988, Munn et al., 1990), and in non-human primates produces an increase in the anti-inflammatory CD16 positive (non-classical) monocytes (Munn et al., 1990). Increased CSF-1 serum levels have been found in several malignancies, including breast carcinoma, ovarian cancer, and endometrial carcinoma, cancers in which high macrophage numbers are associated with poor prognosis (Kacinski, 1997). The expression of epithelial CSF-1 and CSF1-R in metastases of ovarian carcinoma was shown to be associated with poor clinical outcome (Chambers et al., 1997). In several other tumour types, a CSF-1 response gene signature is observed in the primary tumour and corresponding metastases, correlating with increased angiogenesis (Espinosa et al., 2011), higher tumour grade and worse prognosis (Webster et al., 2010, Sharma et al., 2010, Espinosa et al., 2009). As such, CSF-1 and its receptor present rational targets for cancer therapies aiming to ablate or modify TAM. A detailed understanding of the physiological and pathological roles of the CSF-1/CSF-1R axis is required to predict the consequences of therapeutic interference.

The CSF1-R is itself expressed by some tumour cells (Pollard, 2009, Condeelis and Pollard, 2006, Wyckoff et al., 2007, Steidl et al., 2011), raising the possibility that CSF-1R inhibition in these cancers may produce some direct anti-tumour effects. It remains to be seen whether CSF-1R expressed by malignant cells is fully functional and sensitive to ligation with CSF-1 or IL-34, and whether inhibition of CSF-1R on malignant cells has any therapeutic benefits.
3.3 Exploring CSF-1R inhibition.

As outlined earlier, Hume and co-workers have developed the ‘MacGreen’ mouse, in which an enhanced green fluorescent protein (EGFP) reporter gene is driven by the Csf-1r promoter (Sasmono et al., 2003). This model enables visualisation of the participation of CSF-1R positive cells in tumour progression and metastasis (Wyckoff et al., 2007) and enables assessment of the number, phenotype and fate of CSF-1R positive cells upon administration of an anti-CSF-1R antibody (MacDonald et al., 2010). This work sheds further light on the consequences of inhibition of CSF-1R, confirming that signalling through the CSF-1R is required for maturation of mononuclear phagocytes into resident tissue macrophages. The administration of a novel CSF-1R blocking antibody selectively reduced the CSF-1R+Ly6C- ‘patrolling’ monocyte precursors of resident tissue macrophages. CSF-1R+Ly6C+ ‘inflammatory’ monocytes were correspondingly increased, supporting other evidence for their position as developmental precursors of ‘patrolling’ monocytes and ultimately mature tissue macrophages (Sunderkotter et al., 2004, Tacke et al., 2006).

CSF-1R blockade had no effect on CSF-1R+Ly6C+ inflammatory monocyte recruitment in LPS-induced lung inflammation, wound healing, peritonitis and severe acute graft-versus-host disease. However, CSF-1R blockade reduced the numbers of TAM in syngeneic tumour models of mesothelioma and lung carcinoma, although this had no impact on tumour growth. This work indicated that CSF-1R ligation and subsequent maturation to a CD11b+Ly6C- status is a necessary step in TAM development from immature monocytes.

These data indicate that CSF-1R signalling is required only for the maturation of Ly6C- resident-type monocytes and tissue macrophages from Ly6C+ precursors, and may not be absolutely required for monocyte production itself, or for effective function of inflammatory monocytes. Alternatively, it may be that CSF-1R inhibition in this model was partial, with sufficient residual activity to permit monocyte production but not maturation to TAM. In a study of graft-versus host disease (GvHD) following allogeneic bone marrow transplantation modelled in mice, treatment with CSF-1 expanded the numbers of suppressive macrophages, which correlated with a reduction in severity of GvHD (Hashimoto et al., 2011) thus one could hypothesize that the reverse effect might be seen with CSF-1R inhibition. Indeed, MacDonald et al. demonstrated
this using anti-CSF1R monoclonal antibody (MacDonald et al., 2010) with a reduction in accumulated macrophages and increased severity of acute GvHD. Growth of transplantable tumours in op/op CSF-1 deficient mice is markedly impaired (Nowicki et al., 1996). Moreover, experimental CSF-1 blockade via antisense oligonucleotides and siRNA, or inhibiting CSF-1R receptor signalling, significantly suppresses tumour growth (Aharinejad et al., 2004, Kubota et al., 2009, Priceman et al., 2010). Likewise, as mentioned earlier, spontaneous mammary cancer development in the PyMT model is altered in an op/op CSF-1 null background, with no effect on tumour growth but a delay in the development to invasive, metastatic carcinomas (Lin et al., 2001). Prolonged use of the anti-CSF-1R antibody M279 in an established tumour selectively reduced numbers of tissue macrophages, with no significant effect on the ability to mount an inflammatory monocytosis in response to a variety of triggers (MacDonald et al., 2010). The depletion of resident macrophages was notable despite the fact that tumours were established prior to starting treatment. There was, however, no discernable effect on tumour growth.

Taken together, current evidence from genetic models, treatment with CSF-1 and treatment with CSF-1R inhibitors indicates that the non-redundant function of CSF-1R signalling is to promote the differentiation of monocytes from bone marrow precursors, and possibly to direct monocyte maturation towards the formation of resident tissue macrophages (Hume and Macdonald, 2012). By extrapolation, one might postulate that CSF-1R inhibition could selectively reduce TAM numbers in a mouse model of lymphoma, while not significantly attenuating the ability to generate neutrophilia and a Ly6C+ monocytosis and so mount successful acute inflammatory and anti-infective immune responses. Moreover, the contrasting dynamics of TAM accumulation and physiological macrophage turnover suggest that a brief period of CSF-1R inhibition-induced attenuation of monocyte production, survival, trafficking and maturation might preferentially reduce TAM accumulation without significantly reducing physiological tissue macrophage numbers. TAM accumulate rapidly in growing tumours, yet turnover of physiological resident tissue macrophages is relatively slow, in terms of several weeks rather than days.
Figure 8 - CSF-1R inhibition might induce a maturation block and relative reduction in "patrolling" monocytes and TAM.

MacDonald et al. reported a several-fold increase in CSF-1R expression by Ly6C- compared to Ly6C+ monocytes (MacDonald et al., 2010). This, and subsequent differential effects on monocyte subsets of CSF-1R inhibition were not reproduced in similar work using a different sub-class of IgG anti-CSF-1R antibody (Lenzo et al., 2012). This is an area of active research and warrants investigation in any new studies with a CSF-1R inhibitor.
3.4 Blocking the intracellular tyrosine kinase domain may be the most effective strategy to inhibit the CSF-1R.

CSF-1 is cleared from the circulation by receptor-mediated endocytosis (Bartocci et al., 1987). CSF-1R blockade by monoclonal antibodies causes a massive elevation in circulating CSF-1 concentration, as is also observed in CSF-1R knockout mice (Dai et al., 2002). As the antibody concentration subsides, there is an enhanced CSF-1 signal and rebound monocyte production. This may also occur if CSF-1R bearing cells are killed, for example by toxic liposomes or macrophage directed toxic transgenes (Hume, 2011). As such, intermittent or brief CSF-1R inhibition with antibody might cause a paradoxical increase in monocyte production. This does not occur with the administration of small molecule CSF-1R kinase inhibitors (unless they are cytotoxic) (Irvine et al., 2006). A better understanding of the physiological roles of the CSF-1/CSF-1R axis has been obtained by studies using selective inhibitors of CSF-1R kinase activity (Conway et al., 2005, Conway et al., 2008, Manthey et al., 2009), and drug-inducible CSF-1R dependent cell suicide (Burnett et al., 2004). These are both strategies that we have explored in a mouse model of lymphoma.

![Diagram of CSF-1R inhibition strategies](image)

Figure 9 - CSF-1R inhibition strategies.
3.5 CSF-1 R inhibition in lymphoma.

A case report provides some support for pursuing CSF-1R inhibition in lymphoma, detailing elevated plasma CSF-1 levels with CSF-1 positive-staining abnormal lymphocytes in a splenectomy specimen from a patient with DLBCL in whom the plasma CSF-1 levels and peripheral blood monocyte percentage fell after chemotherapy (Nakayama-Ichiyama et al., 2010). In the model of lymphoma we have used (to be described later) the major disease sites, as determined by lymphoma bulk, appear to be lymph nodes and spleen. In the physiological steady state, Hume and co-workers found that in healthy mice, the majority of splenic and nodal macrophages were unaffected by anti-CSF-1R antibody treatment, and so may not be derived from CSF-1R dependent monocytes. However, various macrophage subpopulations exist within these tissues, and splenic marginal zone macrophages and nodal sub capsular sinus macrophages do express the csf-1r-EGFP transgene at high levels, and were depleted in antibody-treated animals in keeping with findings from previous work in which the development of splenic marginal zone macrophages was dependent upon CSF-1 (Takahashi et al., 1994). Moreover, the accumulation of TAM was not investigated, which may well be CSF-1R dependent. Further, one must take in to account the potential reduced efficacy of anti-CSF-1R antibodies, as used in this work, compared to the potentially more potent small molecule inhibitors of the intracellular tyrosine kinase domain of the CSF-1R that are not compromised in their action by disrupting receptor mediated endocytosis.

We obtained compound AZ268 from AstraZeneca; a novel, orally administered, small molecule CSF-1R tyrosine kinase inhibitor that is not yet commercially available and is under ongoing investigation. We studied its effects on a mouse model of lymphoma in C57BL/6 mice. We were particularly interested in the effects on different monocyte and macrophage populations in the blood and lymph nodes, and the impact on lymphoma progression.

Having introduced macrophage biology we will now discuss NHL, before outlining our approach to studying the role of macrophages in NHL.
4 B-cell Non-Hodgkin Lymphomas.

4.1 Background to B-NHL.

B-NHL is characterised by abnormal clonal accumulations of B-lymphocytes. The stage of development at which B-cell maturation is arrested varies, as does the presence, nature and balance of abnormalities in proliferation and apoptosis. This was reflected in the early classification of B-NHL into disease entities largely on the basis of dynamics, as well as macroscopic and microscopic features of malignant cells and their infiltration of tissues and organs (National Cancer Institute, 1982). The wide variation in clinical behaviour of B-NHL is a consequence of the multitude of genetic and epigenetic abnormalities underlying these conditions. As we expand our knowledge and understanding of these underlying abnormalities and the critical molecular events consequent on them, the classification of these diseases is developing to become more relevant to pathophysiology rather than gross clinical features. This is reflected in the latest document from the world health organisation (WHO) updating the classification of lymphoid malignancies (Campo et al., 2011). The population age-adjusted incidence of lymphoma diagnoses rose from the 1970s to the 1990s, and appears to be stabilising more recently, with NHL now being the 7th most common malignant diagnosis in the USA (Jemal et al., 2010). More widespread and accurate cancer diagnosis and reporting, and earlier diagnosis of asymptomatic indolent forms and stages of lymphoma has contributed to this rise, although there is evidence for a genuine underlying increase that is only partly explained by the rise of HIV-related lymphomas (Friedberg and Fisher, 2008, Hartge et al., 1994).

4.1.1 Staging of B-NHL.

The extent of disease is determined by imaging of enlarged lymph nodes and extranodal sites, and by bone marrow biopsy. Routine sampling of cerebrospinal fluid is undertaken in lymphomas that are commonly disseminated at diagnosis; including Burkitt lymphoma (BL) and high risk diffuse large B-cell lymphoma (DLBCL). Conventional imaging by computed tomography may be supplemented by metabolic scanning, such as fluorodeoxyglucose-positron emission tomography (FDG-PET), and tissue biopsy to confirm the presence of
B-NHL in enlarged nodes or extranodal sites. FL, DLBCL and BL are usually staged using the Ann Arbor system.

4.1.2 The Ann Arbor system for staging B-NHL. 
This staging system, first developed in 1971 principally for Hodgkin lymphoma (Carbone et al., 1971), depends upon both the location and extent of lymphoma, as well as the presence of specific systemic symptoms attributable to it. Stage I indicates only one affected region, Stage II two separate regions both above or both below the diaphragm, and Stage III involving sites above and below the diaphragm. Stage IV indicates infiltration of sites outside of lymph nodes or spleen, commonly the bone marrow, abdominal or thoracic viscera. The Ann Arbor stage at diagnosis is incorporated into prognostic scoring algorithms in FL and DLBCL.

4.1.3 B-NHL arise by dysregulated expression of oncogenes during aberrant maturation of B-lymphocytes. 
B-NHL are derived from either committed lymphoid progenitor cells or from more mature B-lymphocytes that acquire chromosomal translocations through errors in the immunoglobulin (IG) gene remodelling processes during normal B-cell differentiation, usually involving immunoglobulin heavy chain (IGH) genes juxtaposed to a variety of oncogenes (Willis and Dyer, 2000).

<table>
<thead>
<tr>
<th>Histology</th>
<th>Translocation</th>
<th>Upregulated Gene</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL</td>
<td>t(14;18)(q32;q21)</td>
<td><strong>BCL2</strong></td>
<td>70-95</td>
</tr>
<tr>
<td></td>
<td>t(3;14)(q27;q32)</td>
<td><strong>BCL6</strong></td>
<td>10-15</td>
</tr>
<tr>
<td>DLBCL</td>
<td>t(3;14)(q27;q32)</td>
<td><strong>BCL6</strong></td>
<td>30-35</td>
</tr>
<tr>
<td></td>
<td>t(14;18)(q32;q21)</td>
<td><strong>BCL2</strong></td>
<td>20-30</td>
</tr>
<tr>
<td></td>
<td>t(8;14)(q24;q32)</td>
<td><strong>MYC</strong></td>
<td>10</td>
</tr>
<tr>
<td>BL</td>
<td>t(8;14)(q24;q32)</td>
<td><strong>MYC</strong></td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>t(2;8)(q12;q24)</td>
<td><strong>MYC</strong></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>t(8;22)(q24;q11)</td>
<td><strong>MYC</strong></td>
<td>22</td>
</tr>
</tbody>
</table>

Table 1 - Common chromosomal translocations in B-NHL (Tomita, 2011).
In B-NHL, the majority of oncogenes targeted by translocations are regulators of proliferation, apoptosis, development and differentiation. In FL, the characteristic chromosomal translocation, t(14;18), establishes a premalignant cell that must acquire secondary mutations to be capable of generating malignant lymphoma. FL cells invariably show somatic hypermutation (SHM) of both \textit{IGH} alleles, suggesting that the immortalized B-cells with \textit{BCL2} over-expression must have pursued an ongoing differentiation pathway, with transit through the germinal centre. SHM can also target \textit{BCL6}, \textit{MYC} and other oncogenes in germinal centre-derived B-NHL (Shen et al., 1998, Pasqualucci et al., 2001).

DLBCL and BL are derived from more mature germinal centre-derived lymphocytes and although there is evidence for secondary mutations in most cases, lymphocytes with \textit{MYC} rearrangements that deregulate self-renewal pathways may not require such events, and are able to act as lymphoma-originating cells able to drive malignancy. \textit{MYC} is a transcription factor that is mutated and deregulated in a large proportion of B-cell lymphomas of different subgroups through various genetic mechanisms (Haluska et al., 1986, Neri et al., 1988). Unlike other lymphoma oncogenes, forced expression of \textit{Myc} in lymphoid cells in mice is sufficient to generate B-cell lymphoma (Adams et al., 1985), yet translocations juxtaposing \textit{MYC} and \textit{IG} genes can be detected in non-malignant conditions including HIV in humans and normal B-cell differentiation in mice (Muller et al., 1995, Roschke et al., 1997). \textit{Myc} activation is a necessary step for lymphoma to develop in \textit{Bcl2} transgenic mice (Strasser et al., 1990).

In this thesis we shall consider the two most common B-NHL; FL and DLBCL, in greatest detail. In addition we will discuss BL, owing to its molecular and dynamic parallels with the chosen mouse model of lymphoma, and other lymphomas and lymphoma models in so far as there is relevant literature to inform discussion. We shall introduce the basic features that distinguish each lymphoma, before critically appraising the literature in relation to evidence for the roles of TAM in each disease.
4.1.4 B-NHL and their relationship to TAM.

Most mature B-NHL are incurable. This is despite most cases showing significant malignant-cell death when initially treated with cytotoxic chemotherapy. There is growing evidence that crosstalk between malignant B-cells and surrounding stromal and immune cells contributes to B-NHL survival, growth, and resistance to chemotherapy. One could speculate that if a lymphoma stem cell population exists (Martinez-Climent et al., 2010), then it too might be subject to similar support from its microenvironment. It is conceptually very attractive to explore combining traditional cytotoxic approaches with novel strategies to disrupt the permissive signals provided by elements of the lymphoma microenvironment (Burger et al., 2009). An early study comparing CD68+ macrophage infiltration between normal lymphoid tissue, FL, mantle cell lymphoma (MCL), DLBCL and BL found that grade of tumour and proliferation rate by Ki67 expression showed a positive correlation with macrophage density (Hermann et al., 1998). The more aggressive and proliferative diseases of BL and DLBCL contained significantly more macrophages than the more indolent FL, MCL and normal lymphoid tissue. The authors speculated that a functional relationship might exist between the extent of macrophage infiltration and the biologic grade of lymphoma. Our own laboratory work will consider this question.
4.2 Diffuse large B-cell lymphoma.

DLBCL is the most common of all adult lymphomas, accounting for 28% of all lymphoid malignancies and 40% of B-NHL (Morton et al., 2006). DLBCL is approximately 50% more common in men than women, and has a peak incidence in the 60-70 year age group (Hartge and Devesa, 1992). DLBCL has an aggressive natural history, with an untreated median survival of less than one year (Fisher et al., 2004). Combination chemotherapy containing anthracyclines, such as cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP) is potentially curative (Fisher et al., 1993). Addition of the chimaeric (mouse-human) anti-CD20 monoclonal antibody (mAb) rituximab to this regime improves overall survival by 10-15% (Coiffier et al., 2002).

4.2.1 Prognostication in DLBCL.

The international prognostic index (IPI) predicts survival based on 5 factors; age, disease stage, serum lactate dehydrogenase (LDH), performance status and number of extra-nodal sites of disease (International NHL Prognostic Factors Project, 1993). Owing perhaps to the biological heterogeneity even within the risk groups, the IPI score does not influence decisions regarding initial therapy. In an attempt to understand the complex and heterogeneous biology of DLBCL better, comprehensive genome-wide expression profiling exercises have investigated how prognosis relates to the molecular features of the disease at diagnosis (Alizadeh et al., 2000, Shipp et al., 2002, Rosenwald et al., 2002).
4.2.2 Gene expression profiling reveals biological heterogeneity and highlights roles for the microenvironment in DLBCL.

Two biologically and clinically distinct molecular groups have been identified (Rosenwald et al., 2002, Alizadeh et al., 2000). Germinal centre B-cell (GCB) subtype probably arises from normal GCB-cells and has an improved prognosis with CHOP chemotherapy compared with the activated B-cell (ABC) subtype (Wright et al., 2003) which may arise from post-GC B-cells that have experienced a block during plasma cell differentiation (Lenz et al., 2008). Supervised analysis has enabled the construction of models to predict outcomes in DLBCL, based on the expression of subsets of highly discriminatory genes (Shipp et al., 2002, Rosenwald et al., 2002). Shipp et al. identified a 13-gene predictive model, and Rosenwald et al. a 17-gene predictive model. There was no overlap of the genes in these two models, which were derived using different microarray platforms and different statistical algorithms. Immunohistochemical surrogates of the gene expression profile-derived ABC/GCB schema might provide more practical clinical applications. The classic Hans algorithm (Hans et al., 2004) uses staining for BCL6, CD10 and MUM-1. Newer, expanded versions of this appear to provide greater accuracy when predicting individual patient outcomes (Meyer et al., 2011b, Choi et al., 2009). Of note, a recent trial of rituximab plus chemotherapy demonstrated the validity of the ABC/GCB schema by gene expression profiling, yet this has not been matched by concordance with immunohistochemical surrogate algorithms (Gutierrez-Garcia et al., 2011).
4.2.3 Tumour-associated macrophages in DLBCL.

In DLBCL, variable representation of macrophage gene expression has been discovered amongst different subsets of the disease (Monti et al., 2005). Whole genome arrays and multiple clustering statistical methods describe 3 distinct subsets of DLBCL: Oxidative Phosphorylation, B-Cell Receptor/Proliferation, and Host Response. Although there were no differences in 5 year overall survival (OS) among the subsets, as distinct biological entities they may have importance not only in identifying pathogenetic mechanisms, but also identifying and exploiting subset specific rational targets. The Host Response group was enriched for several genes of critical importance in monocyte/macrophage development and function, including CD14, CD163, B-lymphocyte activator macrophage expressed (BLAME), APRIL and CSF-1R. These biopsies also had increased expression of T/NK receptor and activation pathway components, the complement cascade, inflammatory mediators, CD2+/CD3+ cells and S100+/gamma interferon induced lysosomal transferase positive (GILT+) CD1a-/CD123- DCs. This study highlights the heterogeneity not just between different B-cell lymphomas, but also within them, and that with relevance to this thesis, TAM may have different and even opposite roles in different lymphoma subtypes, as well as at different times, locations within tumours, and in response to different therapies.

Lenz et al. studied the gene expression signatures of pre-treatment biopsies of a training group of 181 patients with DLBCL treated with CHOP (or similar) chemotherapy, and applied their findings in a validation group of 233 patients treated with rituximab plus CHOP (Lenz et al., 2008). They looked for survival-associated gene expression signatures, refined by hierarchical clustering and grouping of co-ordinately expressed genes and identified the two signatures most predictive of favourable and unfavourable prognosis. By sorting the malignant from the non-malignant components of the tumour on the basis of CD19 expression, they established that the non-malignant components were responsible for the two signatures, termed Stromal-1 and Stromal-2. The Stromal-1 signature predicted a favourable prognosis and contained genes associated with extra-cellular matrix (ECM) deposition and organisation as well as infiltration with mononuclear phagocytes. The Stromal-2 signature predicted an unfavourable prognosis and contained genes associated with angiogenesis.
This work highlights the discriminating nature of different stromal responses. With both the Stromal-1 and Stromal-2 signatures being feasibly linked to TAM function, it also supports the concept that TAM populations might be performing different and prognostically opposing functions in different tumours.

Other gene expression profiling enterprises have yielded reproducible prognostic signatures, notably the work of Lossos et al., that developed a predictive model based upon the expression of only 6 genes from formalin fixed paraffin embedded (FFPE) samples (Lossos et al., 2004). They examined expression using quantitative real time polymerase chain reactions (qRT-PCR) of 36 candidate genes drawn from earlier genome-wide association studies from other groups, correlating this information with clinical features and outcomes in 66 diagnostic biopsies from the pre-rituximab era. This model was applied to the data set from Lenz et al. and found to ‘perform well’, distinguishing risk groups ‘nearly as well’ as the model of Lenz et al., but with the added advantage of being logistically and financially more feasible as a potential routine clinical tool (Alizadeh et al., 2009). Nonetheless, despite its limitations as a potential routine clinical tool, as a hypothesis forming paper, the work of Lenz et al. is of great value, illuminating complex biological subtypes of DLBCL and suggesting important interactions with the microenvironment.
4.2.4 Counting tumour-associated macrophages in DLBCL.

CD68 is considered a pan-macrophage marker in humans. It is a glycoprotein found in lysosomes, phagosomes and neutrophil granules (Pulford et al., 1989, Pulford et al., 1990, Saito et al., 1991), and so although highly expressed in macrophages, may be detectable at lower concentrations in a variety of cells containing these organelles, such as mast cells and neutrophils, but also in some malignant cells (Gloghini et al., 1995, Lau et al., 2004).

Several studies report enumeration of CD68+ macrophages in diagnostic biopsies of previously untreated DLBCL. Hasselblom et al. counted CD68+ macrophages in samples from 176 patients, none of whom received rituximab, some received radiotherapy, and all received anthracycline based therapy (Hasselblom et al., 2008). No correlations were found between macrophage numbers and OS, progression free survival (PFS), proliferation as assessed by Ki67 staining, ABC versus GCB subtype, or any clinical factors, other than age. The only statistically significant correlation was that patients over the age of 60 years had more CD68+ macrophages than younger patients.

Abundant expression of secreted protein, acidic and rich in cysteine (SPARC) in the microenvironment is reported to correlate with favourable prognosis in some patients with DLBCL (Meyer et al., 2011a). SPARC is a glycoprotein that can modulate the ECM, integrin activity, adhesion, growth factor signalling and apoptosis (Bradshaw et al., 2003, Raines et al., 1992, Sangaletti et al., 2003, Weaver et al., 2008). SPARC can be expressed in variety of cells, including a subset of tissue-injury associated macrophages (Lenz et al., 2008, Reed et al., 1993). Meyer et al. investigated SPARC and CD68 staining by immunohistochemistry in pre-treatment biopsies of 262 patients with DLBCL, subsequently administered rituximab plus CHOP (or similar) chemotherapy. CD68 staining alone was not predictive of any survival measure. The presence of any SPARC positive cells in the microenvironment correlated with longer OS, and those with high numbers of SPARC staining cells had longer event free survival (EFS) than those with fewer. On subgroup analysis, this was found to be almost entirely due to a strong prognostic effect in ABC-type DLBCL, with no effect seen in GCB-type DLBCL. Moreover, SPARC gene expression was represented within the Stromal-1 signature of favourable prognosis DLBCL (Lenz et al., 2008), reinforcing its association with good risk disease. Combined,
these data suggest that TAM with different phenotypes are present in different biological subtypes of DLBCL with different biology, and might be associated with contrasting roles. The identification of TAM-specific features associated with pro- or anti-tumour phenotypes might ultimately aid the selection of patients who would benefit most from novel therapies targeted against TAM.

The number of macrophages was enumerated in 112 patients with newly diagnosed DLBCL, subsequently treated without rituximab, to examine the prognostic significance when biopsies were assigned to a high or low macrophage group, using CD68 and a single cut-off value (Cai et al., 2011). They found no difference in the CR rate, and the median OS was not reached for the low CD68 group.

From our own research group, Coutinho et al. report that a high number of CD68 positive macrophages correlates with a good prognosis in terms of OS, PFS and disease-free survival (DFS) in diagnostic biopsies of 218 patients, 67% of whom did not receive rituximab (Coutinho et al., 2011). Numbers of CD163 positive cells did not have any correlation with clinical outcomes, and subgroup analysis of rituximab treated versus non-rituximab treated patients is awaited.

The 2 year OS of 101 cases of newly diagnosed DLBCL treated subsequently with a rituximab containing regimen was assessed examining the total numbers of macrophages, using CD68, and also the numbers of macrophages on which a marker of M1 phenotype (HLA-DR) or M2 phenotype (CD163) was also present (Wada et al., 2012). The presence of a large total number of CD68 positive macrophages was associated with a worse outcome than a low number of CD68 positive macrophages. The presence of a large number of M2 macrophages correlated with a worse overall survival than those with a low number of M2 macrophages. There was no such prognostic difference between high and low numbers of M1 macrophages, and the investigators found that the number of M1 macrophages was similar to the number of M2 macrophages in each individual biopsy. We suggest that this study was perhaps ambitious in hypothesizing that the in vitro phenomenon of pure M1 and M2 phenotypes might be sufficiently faithfully replicated within a human tumour, and particularly that biologically distinct phenotypes could be distinguished using only a single
marker in each case. Given the expected immune-suppressive milieu of the tumour microenvironment, one might expect to be unable to detect any macrophages of a truly M1-like phenotype.

A smaller study of 43 patient samples of untreated primary central nervous system lymphoma (PCNSL) investigated not only macrophage phenotype, but also its links with the activation state of the malignant cells, as measured by STAT3 staining (Komohara et al., 2011). This was on the basis of earlier studies demonstrating the significance to tumour progression of malignant cell STAT3 activation in DLBCL (Stewart et al., 2009, Ding et al., 2008) and other cancers (Cheng et al., 2003, Yu et al., 2007) and its correlation with the number of tumour-infiltrating M2 phenotype macrophages in ovarian cancer (Takaishi et al., 2010). Interrogating FFPE samples with the M2 macrophage phenotype markers CD163 and SRA/CD204, they report that increased PCNSL malignant cell STAT3 correlates with a denser infiltration with CD163 positive (but not SRA/CD204 positive) macrophages. In vitro co-culture without direct cell-cell contact demonstrated an up-regulation of STAT3 in lymphoma cell lines in the presence of healthy donor PBMC-derived macrophages that had been matured with GM-CSF and polarised with tumour-conditioned media. No correlation with prognosis was seen with quantification of staining for CD68, CD163, SRA/CD204, or STAT3. However, this study is of interest in identifying a possible mechanism and bi-directional relationship whereby secreted substances present in tumour-conditioned media are able to direct macrophage phenotype that subsequently impacts upon tumour cell activation state.

Co-culture of monocytes promotes the survival of normal B-cells and primary human DLBCL B-cells. DLBCL cells also show increased proliferation with monocytes, further enhanced by supplementary B-cell activating factor of the TNF family (BAFF) or IL-12 (Mueller et al., 2007). This process is reinforced by the fact that macrophages can activate B-cells through the B-cell receptor (BCR), stimulating their release of CCL5, a leukocyte chemo-attractant included in the Host Response signature of DLBCL (Monti et al., 2005). Mueller et al. speculate that blocking CCL5 might provide a therapeutic strategy to attenuate monocyte/macrophage recruitment in DLBCL, and so reduce malignant B-cell survival and proliferation in this disease.
4.3 Follicular lymphoma.

FL is a malignancy of germinal centre B-lymphocytes that accumulate to varying extents in the lymphoreticular, haematopoietic and extranodal compartments. Affected lymph nodes contain a mixture of small regular lymphoid cells (centrocytes) and larger more irregular lymphoid cells (centroblasts), within a variably conserved lymph node follicular architecture. This conservation of normal lymph node architecture, with attendant T-cells and FDC meshwork, hints at the importance of supporting non-malignant cells to the survival of FL, corroborated by the need for CD40L stimulation to maintain in vitro culture of primary FL (Ghia et al., 1998). FL is graded from I to III depending on the number of centroblasts seen per high power light microscope field.

Malignant B-lymphocytes of FL express characteristic cell surface markers including the pan-B-cell markers CD19, CD20, CD22, as well as monoclonal light chains, CD10 (in 60%), but not CD5. The chromosomal translocation (14;18) is identified in 70-95% of FL (Rowley, 1988, Horsman et al., 1995) and 20-30% of DLBCL (Weiss et al., 1987, Lipford et al., 1987), and causes rearrangement and over-expression of the anti-apoptotic protein BCL2, such that in contrast to non-malignant B-cells without high affinity for antigen, they do not undergo apoptosis but rather proliferate in germinal centres. In FL, BCL2 expression can be readily identified in histological sections, and is thought to contribute to the pathophysiology of the accumulating malignant clone.

With an annual incidence of about 3 new cases per 100 000 of the English population, FL contributes 2% to all malignancies diagnosed in the UK each year. The median age at diagnosis is 60 years, with an equal sex distribution. The worldwide incidence of all types of NHL, including FL, has been increasing at a rate of between 1-4% each year since the 1970's, resulting in almost a doubling in overall incidence in this time. Such analyses have also confirmed a striking geographical variation in FL, being more common in Western nations (Muller et al., 2005). The incidence of FL is particularly low in China and Japan. In the USA, the incidence is 2-3 times higher in Caucasians than in African Americans, suggesting that genetic factors may be significant, in addition to environmental modifiers, which remain unidentified.
4.3.1 Clinical features of FL.

FL presents most commonly with palpable lymph node enlargement in the neck, axillae or groins. Rarely, cases are observed to spontaneously regress (Gattiker et al., 1980, Krikorian et al., 1980, Horning and Rosenberg, 1984, Kumar et al., 2004), but sustained spontaneous remissions are extremely rare, and the vast majority eventually develop progressive lymph node enlargement. Untreated, FL usually progresses to infiltrate the bone marrow, resulting in compromised haematopoiesis of all cell lines. The natural history of this disease concludes with eventual death from bone marrow failure; infection, bleeding and consequences of anaemia, or less commonly from the mass effect of enlarged malignant lymph nodes.

For each affected individual, FL typically behaves in a variably indolent fashion, with months or years of clinically imperceptible change or very gradual asymptomatic progression, interrupted by episodes of obvious clinical progression. Approximately 2% of patients with FL each year experience clinical “transformation” to a more aggressive and rapidly progressive disease termed transformed FL (tFL), which is histologically identical to DLBCL. tFL has a very poor prognosis (Acker et al., 1983) and is commonly a terminal event. Data collected prospectively over 25 years in our centre, relating to 325 diagnoses of FL demonstrated a 28% risk of transformation to histologically proven tFL/DLBCL over a 10-year period. These individuals had only a 1.2 yr median survival from transformation (Montoto et al., 2007).
4.3.2 Therapy for FL.

Localised disease, though relatively rare, can be cured with radiotherapy (Chen et al., 1979, Paryani et al., 1983, Vaughan Hudson et al., 1994, Wilder et al., 2001, Mac Manus and Hoppe, 1996). Widespread FL is almost always incurable with conventional chemotherapeutic approaches, yet is described as an indolent malignancy, with a median survival of about 12-14 years from diagnosis (Hoppe et al., 1981, Fisher et al., 2005). The clinical course of FL is highly variable. This presents major clinical challenges. Some patients may never require treatment, even after more than 20 years, whilst others succumb to aggressive disease in less than 2 years. It remains difficult to predict accurately individual patient survival from diagnosis.

Until the introduction of combined chemo-immunotherapy incorporating the monoclonal anti-CD20 antibody rituximab, evidence suggested that medical intervention had no significant impact on overall survival in FL (Hoppe et al., 1981, Brice et al., 1997, Ardesha et al., 2003, Young et al., 1988). Chemotherapy was reserved for symptomatic or bulky disease to induce regression to a once-more asymptomatic stage. The goal of therapy was to maintain the best quality of life and treat only when symptoms dictated. Any alteration to this approach required demonstration of improved survival with early therapy, or identification of criteria that defined patients at sufficiently high risk to merit early therapy (Gribben, 2007). The advent of rituximab challenges this approach. There is now a robust body of evidence indicating that rituximab in combination with chemotherapy improves the proportion of patients achieving a complete remission, prolongs PFS and OS (Marcus et al., 2005, Forstpointner et al., 2004, Hiddemann et al., 2005, Schulz et al., 2007). There is also evidence that single agent rituximab given as long-term maintenance therapy prolongs PFS in first remission (Salles et al., 2011) and after remission re-induction for relapsed FL (van Oers et al., 2010).

The aim of current management is to achieve and maintain the best possible response to therapy for the longest period, and to prolong survival. First-line treatment options for stage III or IV follicular lymphoma include single-agent or combination chemotherapy regimens based on alkylating agents, with or without steroids, and in combination with rituximab. There is no consensus as to
the best timing or combination of therapy in FL, and patients should always be considered for entry into clinical trials. 

There is compelling evidence for an effective immune response to FL in patients who successfully undergo allogeneic haematopoietic stem cell transplantation (HSCT). A graft-versus-lymphoma (GvL) effect has been demonstrated in vivo, with remissions occurring in response to donor lymphocyte infusions (DLI) in patients with relapsed FL following allogeneic HSCT (Morris et al., 2004). Allogeneic HSCT incurs a significant morbidity and mortality, and appropriate patient selection and timing of transplantation is critical in this indolent malignancy. However, FL remains incurable by both conventional and novel therapies, and young patients will likely die of their disease far short of the population average life expectancy. tFL in particular, has a poor prognosis despite the benefits to some selected patients of high dose chemotherapy supported by autologous HSC rescue (Foran et al., 1998, Kasamon et al., 2011, Eide et al., 2011, Bastion et al., 1997, Chen et al., 2001, Williams et al., 2001b, Oliansky et al., 2010). Ongoing studies are addressing whether there is a role for allogeneic HSCT in young patients and those with rapidly progressive, relapsed or refractory disease (Toze et al., 2004).
4.3.3 Prognostication in FL – the follicular lymphoma international prognostic index.

The most rigorously validated and universally adopted prognostic scoring index available is the FL international prognostic index (FLIPI). This is a 5-factor index based on clinical characteristics and routine blood test results at diagnosis, and was established by retrospective analysis of 4167 patients diagnosed between 1985 and 1992 and treated without rituximab (Solal-Celigny et al., 2004). Age, stage, number of nodal sites, haemoglobin concentration, and serum LDH, defines 3 prognostic groups. In patients with a good prognosis (0-1 adverse factor), the 10-year OS is 71%, falling to 36% with 3 or more adverse factors. Subsequent work has established the continued validity of the FLIPI score to patients treated with rituximab (Buske et al., 2006). Although prognostically useful, so far the clinical impact of the FLIPI score has been very limited, informing tactical decisions but not directing the optimal specific therapy for each patient group. The FLIPI has benefit in communicating risk to patients, and in comparing populations in clinical trials and laboratory analysis of patient samples.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Adverse Factor</th>
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<tbody>
<tr>
<td>Age</td>
<td>&gt; 60 years</td>
</tr>
<tr>
<td>Stage</td>
<td>&gt; 2</td>
</tr>
<tr>
<td>Affected nodal sites</td>
<td>&gt; 4</td>
</tr>
<tr>
<td>Lactate</td>
<td>&gt; Upper limit of normal</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>normal</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>&lt; 12mg/dL</td>
</tr>
</tbody>
</table>

Table 2 - Adverse prognostic variables of the FLIPI scoring system.

<table>
<thead>
<tr>
<th>Prognosis</th>
<th>No. of adverse factors</th>
<th>10-year survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good</td>
<td>0 or 1</td>
<td>71</td>
</tr>
<tr>
<td>Intermediate</td>
<td>2</td>
<td>51</td>
</tr>
<tr>
<td>Poor</td>
<td>3 or more</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 3 - Relationship of FLIPI score to survival.
4.3.4 Gene expression profiling in FL reveals the prognostic influence of the cellular microenvironment.

A seminal study strongly implicated a critical role for the tumour microenvironment in the prognosis of FL (Dave et al., 2004). Diagnostic lymph node biopsies were collected from 191 patients diagnosed with FL at a variety of international centres including our own and divided into a training set and a test set, balanced for institution and length of survival. RNA gene expression was determined using Affymetrix U133A and U133B human whole genome microarrays. A complex statistical approach was used to determine survival associated gene expression signatures, in this way avoiding the analytical limitations of multiple comparisons of large individual gene expression data sets. In the training set, the Cox model was used to determine genes associated with survival, and these genes were arranged by hierarchical clustering with respect to their prognostic association. Genes that had correlated expression were grouped into survival-associated gene-expression signatures.

Each individual biopsy in the training set was then assigned a “signature average” relating to its own gene expression. The whole training set was interrogated to find associations between survival and expression of different combinations of multiple survival signatures. A binary model of one good prognosis signature and one poor prognosis signature was found to be highly predictive of survival in the training set. The two signatures were respectively termed “immune response 1” (IR1) and “immune response 2” (IR2). This model was then positively validated in the test set of diagnostic biopsy specimens.

In an attempt to derive functional information from the statistical associations, the authors attributed cells of origin to genes in each signature. This was pursued by FACS of cell suspensions obtained from four fresh biopsy specimens, into CD19 positive (malignant) and CD19 negative (non-malignant) populations, and each subpopulation profiled for gene expression. Further analysis of the non-malignant cell population was attempted by comparison of gene expression in the FL biopsies with gene expression of monocytes purified from peripheral blood lymphopheresis samples from normal donors, magnetic sorted for CD14, a monocyte/macrophage marker, and a receptor for LPS.
Of note, the IR1 signature included genes encoding T-cell markers and genes highly expressed in macrophages, and IR2 included genes preferentially expressed in macrophages and DC. Interestingly, the IR1 and IR2 signature averages, and expression of individual component genes, were higher in the CD19 negative, non-malignant cells than the CD19 positive malignant cells. This suggested that gene expression of the non-malignant cells contributed more to prognostic discrimination at diagnosis, than did gene expression in the malignant B-lymphocytes. The authors speculated that the nature of the infiltrating immune cells was the predominant feature of the tumour that predicted length of survival, and that the relative contribution of immune cell gene expression, rather than absolute levels, was critical. With hindsight, this would appear to have been a highly prescient suggestion, given the inconsistent findings of subsequent studies simply attempting to relate absolute numbers of infiltrating macrophages to prognosis in FL. They suggested that an understanding of the nature of “trophic signals” provided by the microenvironment in FL might provide new targets for therapy. In this respect, their work provides an appropriate rationale for our own studies, attempting to elucidate the nature and significance of TAM in B-NHL. It is interesting to note that genes ascribed to monocytes and macrophages were present in both the IR1 and IR2 signatures, perhaps providing further clues that simply the abundance of these cells is not likely to be prognostic, but rather that determining their differing gene expression and phenotypes might be more illuminating.

Several criticisms have since been raised regarding this work. In particular, it has been suggested that more extensive positive selection of immune cell subtypes would have yielded crucial missing information (Naresh, 2005). Additionally, the same authors contend that the identification of gene expression signatures was of limited use compared to the identification of smaller sets of highly discriminatory genes and their associated molecules that might translate into useful clinical applications in diagnostic immunohistochemistry. Whilst acknowledging the validity of such statements, we feel that the over-riding strength of Dave’s work is in hypothesis generation, and so directing future studies. The experimental and statistical design does not appear to have been formulated to answer questions about the specific roles of individual genes or even cellular subtypes beyond discriminating the
contributions of CD19 positive or negative or CD14 positive populations. Other criticisms of the techniques employed by Dave et al. are perhaps more valid. It is now recognised that a minority population of malignant B-lymphocytes in FL express CD19 at very low levels or not at all (Masir et al., 2006). Conceivably this might diminish the accuracy of conclusions drawn from using CD19 selection to separate malignant from non-malignant cells. Furthermore, the assessment of gene expression in fresh biopsy specimens was limited to only four samples, from which conclusions were drawn regarding the whole data set. Additionally, attribution of genes expressed in FL biopsies to monocyte/macrophages was based on the assumption that this directly paralleled gene expression in circulating monocytes collected from normal healthy donors. Our knowledge of monocyte diversity, macrophage phenotypic plasticity, and the particular activation status observed in tumour-associated macrophages, informs that such assumptions are unlikely to be entirely valid.

Tibshirani re-examined the statistical findings, and suggested that the results were rather fragile (Tibshirani, 2005). On swapping the training and test sets and reapplying the same formulae, they found that the same model did not emerge, and indeed that by these means, no such model could be significantly associated with survival. Dave and colleagues disputed the appropriateness of the statistical methods employed by Tibishirani, and defended their original methods, model and findings. Regardless of who is correct, the work of Dave and colleagues heralded much work investigating correlations between the numbers of non-malignant cells and prognosis in FL. Our own project aims to build on this, and contribute to the nascent field investigating functional interactions in the lymphoma microenvironment.
4.3.5 Counting tumour-associated macrophages in FL.
Several independent groups have counted macrophages in specimens of FL, and searched for correlations with clinical features and outcomes. There is no clear consensus regarding the prognostic importance of numbers of macrophages in FL. There are wide variations between studies regarding patient selection, immunohistochemical staining protocols, and cell counting methods.

Farinha and colleagues investigated 99 young patients with FL, all below the age of 61 years (median age 44yrs), treated uniformly, and without rituximab (Farinha et al., 2005). Therapy consisted of 6-agent combination chemotherapy plus steroids, followed by involved region radiotherapy. The median OS in this group was 16.3 years. The vast majority of patients, 87 in total, were classified as having few macrophages by CD68 immunohistochemistry, with less than 15 positive cells per high power field, and had a median OS of 16.3 years. 12 patients had more than 15 positive cells per high power field, with a median OS of 5.0 years (p=<0.01). This statistically significant p-value suggested that a large number of macrophages correlated with a poor prognosis in FL. A subsequent study assessed 60 frozen FL lymph nodes for macrophage numbers by IHC and the expression of 35 candidate “indicator” genes of immune response by qRT-PCR. A high number of CD68+ macrophages correlated with a shorter OS, as did high levels of CCR1 gene expression, a marker of monocyte activation (Byers et al., 2008).

Wahlin and colleagues quantified infiltrating immune cells in pre-treatment biopsies in 70 FL patients at the extremes of survival, using both immunostaining and flow cytometry (Wahlin et al., 2010). An abundance of interfollicular CD68+ macrophages correlated with poor prognosis. An abundance of CD4+ cells also correlated with poor outcome (especially when follicular). Staining with programmed death-1 (particularly follicular) and CD8+ cells correlated with good prognosis (particularly when interfollicular). Follicular FOXP3+ cells correlated with good prognosis. This study supports the conceptually attractive proposition that the overall composition, balance and location of immune infiltrating cells are critical. The authors urged caution in interpreting the results relating to macrophage numbers and prognosis owing to the fact that this was not a primary objective in their study hypothesis, and that
the patient cohort was heterogeneous in respect to therapy, with 30% receiving rituximab at some point.

Alvaro and colleagues interrogated 211 diagnostic biopsies of an older patient group than Farinha (Alvaro et al., 2006b). These patients were not treated with exactly the same regimens, and 5 received rituximab. They found that CD68+ cells were predominantly located in the interfollicular regions, and that an abundance of such macrophages was associated with features of good prognosis, including normal bone marrow at diagnosis, and earlier stage disease (I or II), but was not significantly, independently correlated with OS. Interestingly, their study focused on the discovery of different immune-cell signatures by immunohistochemistry. They identified an “immunosurveillance pattern” rich in T-lymphocytes and macrophages associated with “favourable clinical behaviour”, and an “immune-escape pattern” rich in CD57+ T-cells, associated with a higher frequency of adverse manifestations such as bone marrow infiltration by lymphoma. The authors state that their work does not provide conclusive evidence regarding such associations, rather a further guide toward future research questions.

In our own centre, no correlation was found between numbers or location of CD68+ cells and survival amongst a large number of diagnostic biopsies from patients who never received rituximab (Lee et al., 2006). Similarly, Glas and colleagues studied biopsies of patients with FL who never received rituximab. Using the same cut-off criteria as Farinha, they found that CD68+ cells (and regulatory T-cells) were not differentially present with respect to risk of transformation and survival (Glas et al., 2007).

Alvaro and colleagues, studying the same samples as discussed above (Alvaro et al., 2006b) attempted to derive information regarding the activation status of macrophages in FL (Alvaro et al., 2006a). Double immunofluorescence with CD68 and signal transducer and activator of transcription-1 (STAT-1) was performed. STAT-1 staining co-localised exclusively with CD68 and was prognostic of shorter OS. The authors stated that patients with STAT-1 expression and low infiltration of macrophages have reduced OS and more frequent progression than those without STAT-1 expression. As such, it would appear as though the presence of a low number of macrophages, but with a
high proportion expressing STAT-1, correlated with a poor prognosis. However, STAT-1 expression alone was not prognostic for survival, but became significant on multivariate analysis with the FLIPI score. These findings might support the notion of macrophage activation status and phenotype being more important than numbers alone, but remain to be independently validated in a separate data set.

Work from our group has provided evidence for a functional association between TAM and angiogenesis within poor prognosis FL (Clear et al., 2010). In an attempt to gather functional information from FFPE sections of FL, the proximity of macrophages to tiny neovascular angiogenic sprouts was investigated. There was evidence for both a greater angiogenic activity in the poor prognosis group, and also a correlation between increased angiogenic sprouting and abundant CD163+ macrophages in the interfollicular regions. It has been suggested that CD163 might be a marker of macrophage activation towards a pro-tumoural M2 phenotype, particularly as evidenced by whole genome transcriptome analysis of M2 polarised human macrophages (Martinez et al., 2006).

Recent elegant co-culture experiments with human tissue highlight interactions between mesenchymal stromal cells (MSC), monocytes, macrophages and malignant cells in FL (Guilloton et al., 2012). MSC produce anti-inflammatory mediators that direct an IL-10 high macrophage phenotype (Cutler et al., 2010, Nemeth et al., 2009, Choi et al., 2011). MSC from patients with FL (FL-MSC) had distinct gene expression profiles than from age-matched healthy donors (HD-MSC), up-regulating CCL2. CCL2 is known to act as a monocyte chemo-attractant, and may also have roles in driving the M2 macrophage phenotype (Roca et al., 2009b, Qian et al., 2011, Shi et al., 2011). HD-MSC were found to up-regulate CCL2 on co-culture with malignant B-lymphocytes derived from FL infiltrated lymph nodes. CCL2 administration had no direct effect on FL B-lymphocyte survival in vitro, but that their survival was promoted by FL-MSC alone, macrophages alone and synergistically by FL-MSC and macrophages. FL-MSC directed co-cultured monocytes towards a pro-angiogenic and LPS-unresponsive phenotype with increased IL-10 release, less TNF-α, more IL-6 and more VEGF. Functional studies indicated that FL-MSC secreted more CCL2 and recruited monocytes more efficiently than HD-MSC, a difference that was abolished by the depletion of CCL2. As such, a tripartite relationship was
revealed such that MSC recruit and modulate monocytes to create a favourable niche for the survival of malignant B-lymphocytes.

A recent publication illuminates a mechanism through which monocytes and T-cells cooperate via IL-15 and CD40L to promote the growth of human normal and FL B-cell populations (Epron et al., 2012). Cells of the monocyte/macrophage/DC lineage are known to be the major producers of IL-15 and do so maximally in the presence of the Th1 cytokine IFN-γ (Rochman et al., 2009). Soluble IL-15 alone has no direct effect on B-cell survival or proliferation in vitro (Do and Min, 2009). Epron et al. found that IL-15 was over-expressed in purified TAM from FL biopsies, and that STAT5 was over-expressed in FL malignant B-cells. In vitro, CD40L signalling triggered an up-regulation of STAT5 expression in FL B-cells, and favoured SRC-dependent phosphorylation of STAT5 in response to IL-15. Monocytes and lymphoid derived macrophages could trans-present IL-15 to normal B-cells, and in cooperation with T-cell derived CD40L, favour B-cell proliferation. This work suggests a role for Th1 activated monocytes and macrophages in lymphomagenesis.
4.3.6 Counting tumour-associated macrophages in relation to therapy in FL.

Evidence has accumulated to suggest strongly that the direction and magnitude of correlation between macrophage numbers at diagnosis and outcomes might be dependent upon the subsequent therapies administered. In particular, exposure to, or lack of exposure to, rituximab, appears to be relevant within several studies, such that exposure to rituximab might circumvent an adverse prognostic influence of macrophages.

Taskinen and colleagues counted CD68+ macrophages in 141 patients with FL using the same criteria as Farinha (Taskinen et al., 2007). 96 received rituximab initially or at relapse, 45 did not. In those not receiving rituximab, a high number of macrophages predicted shorter PFS (17 months versus 41 months). However, following rituximab therapy, many macrophages correlated with prolonged PFS (57 months versus 17 months).

In a smaller study, Kelley and colleagues found by sub-set analysis of 69 patients treated at the outset for a diagnosis of FL (rather than managed expectantly), that high numbers of CD68+ cells correlated with shorter OS in those not receiving rituximab (Kelley et al., 2007). This association was lost in those receiving rituximab.

Canioni and colleagues counted CD68+ cells in 194 patients with FL enrolled to a trial assessing the addition of rituximab to a combination regimen of chemotherapy and interferon-α (Canioni et al., 2008). In those not receiving rituximab, low numbers of intra- or interfollicular macrophages correlated with longer event free survival (EFS). This association was critically dependent on the counting thresholds used, and was entirely absent in those treated with rituximab. It should be noted that follow up was relatively short (42.9 months), with no differences in OS discovered in this time.

A retrospective analysis was reported of the prognostic value of total, intrafollicular and extrafollicular CD68 TAM numbers in patients treated in 3 separate trials conducted by the South Western oncology group (SWOG), 2 of which exposed patients to rituximab (Sweetenham et al., 2010). All had long follow-up. In the first trial 103 patients received ProMACE-MOPP (Methotrexate, doxorubicin, cyclophosphamide, etoposide - Mechlorethamine, vincristine, prednisolone, procarbazine) and were randomized to receive no further therapy.
or IFN-γ. In the second study, patients received R-CHOP and 30 were eligible for this analysis, and in the third, 47 were eligible from a study using 131I Tositumimab plus CHOP chemotherapy. The numbers of infiltrating macrophages had no correlation with OS in any of the studies, nor did the numbers of Tregs.

A relatively small study of 50 patients with previously untreated FL who were to begin therapy, found that an abundance of intrafollicular CD68+ macrophages heralded a shorter OS and 3 year PFS than low numbers of intrafollicular CD68+ macrophages (Andjelic et al., 2012). 38 of these patients were not treated with rituximab, and subgroup analysis revealed that the prognostic significance of CD68+ macrophages held for this group, but not for the 12 patients treated with rituximab. It was not stated whether such small numbers in the rituximab treated group provided sufficient statistical power to demonstrate a reasonably expected difference.
4.4 Burkitt lymphoma.

There are 3 clinical variants of BL with similar histology; endemic, sporadic, and AIDS-associated. Epstein-Barr virus (EBV) appears to be an important co-factor in the development of BL (Rowe et al., 2009), possibly blocking apoptosis (Kelly et al., 2009a, Vereide and Sugden, 2011). It is usually present in endemic cases, variably in AIDS-associated, and infrequently in sporadic cases in Europe and North America (Jaffe and Pittaluga, 2011).

The malignant cells in BL are medium-sized with uncleaved, round nuclei with multiple nucleoli and deeply basophilic, vacuolated cytoplasm. BL has a mature B-cell phenotype with surface IgM expression and is a highly aggressive disease with frequent involvement of extranodal sites and occasionally a leukaemic phase. BL cells express BCL-6 and CD10, reflecting a GC B-cell origin. In addition, they have detectable surface IgM, CD19, CD20, CD22 and CD79a. Ki67 staining is almost, if not entirely, universal amongst malignant B-cells in BL, giving a doubling time of approximately 25 hours. BL cells are negative for CD5, CD23 and TdT (Blum et al., 2004).

Taking all three groups together and worldwide, BL is most common among children, and in the developing world, as endemic BL. This disease is commonest in equatorial Africa between ages 4 and 7 years where it constitutes 70% of all childhood lymphoma diagnoses (Parkin and International Agency for Research on Cancer., 1998) and manifests with frequent involvement of the jaw and kidneys (Blum et al., 2004). In wealthy nations, the standard of care for children with BL is dose-intensive multi-drug chemotherapy with central nervous system (CNS) prophylaxis, giving long-term survival to 60-90% of patients (Reiter et al., 1999, Cairo et al., 2007, Patte et al., 2007). AIDS-associated BL, unlike other HIV-related NHL, frequently occurs with CD4 counts above 200 cells/µl (Davi et al., 1998) and has a similar clinical presentation to sporadic BL.

The majority of adult patients with BL in the developed world are aged over 40 years, are HIV negative and have sporadic BL. Usually having large abdominal tumour masses, they account for about 3% of all adult lymphomas in England and Wales. Adults with sporadic or AIDS-related BL typically present with large abdominal nodal masses with extranodal spread commonly detected in the
bowl, although other organs can be infiltrated (Blum et al., 2004). Bulky and rapidly growing disease results in a typical biochemical picture of raised serum LDH and uric acid concentrations. Bone marrow involvement is detected in 30-38% and CNS infiltration in 13-17% of adults with sporadic BL (Mead et al., 2002, McMaster et al., 1991, Soussain et al., 1995). BL can be staged using the Ann-Arbor system.

There have been no randomized trials to inform choice of therapeutic regime in adults with BL. It is fairly clear from comparisons of different treatment series that the underlying principles should be prompt therapy with intensive courses of chemotherapy including fractionated alkylating agents and cell-cycle specific agents that cross the blood-brain barrier (Linch, 2012). The outcomes from 14 treatment series have recently been analysed (Kelly et al., 2009b). All patients received dose-intensive multi-drug regimes, yet OS was found to be worse in this group than in younger patients, but had improved with the passage of time, with 6 out of 7 studies published since 2000 giving 2 year OS of over 60%. One of these series incorporated rituximab (Thomas et al., 2006), and reported a 3 year OS of 89%. A more recent retrospective analysis of 23 adults with non-HIV related B-NHL with proliferation indices >95% (including 14 with typical sporadic BL) reports 83% OS at a median follow up of 34 months after treatment with rituximab plus intensive combination chemotherapy with CNS penetration (Mohamedbhai et al., 2011). Despite these improved figures with rituximab, there remains a need for additional novel therapies for those patients who do not achieve a complete remission (CR), or who relapse, as there is currently no satisfactory salvage strategy, and the majority of these patients will die of BL.

The largest published series of allogeneic HSC transplants for BL reports 71 patients from the European group for blood and marrow transplantation (EBMT) registry data aged 4 to 48 years treated either in first CR or at relapse (Peniket et al., 2003). Only 20% of these patients had chemoresistant disease at transplant. OS correlated with chemosensitivity, and not with the presence of acute GvHD, and matched patients undergoing high dose therapy with autologous stem cell rescue had a similar relapse rate and superior OS. A single case report describes only transient regression of relapsed BL with the onset of GvHD following withdrawal of immunosuppression and administration
of IFN-α after allogeneic HSC transplant (Grigg and Seymour, 2002). Together, these data do not provide convincing evidence for a strong GvL effect in BL.
4.4.1 Biology of BL in relation to tumour-associated macrophages.

Diagnosis of BL is complicated by cases of typical immunophenotype but variant morphology, cases of typical morphology without evidence of \textit{MYC} rearrangement, and cases of DLBCL with \textit{MYC} rearrangement. Gene expression profiling has revealed that BL has a signature that is clearly distinct from DLBCL, is similar in adults and children, and a small proportion of intermediate cases exist with signatures showing features of both BL and DLBCL (Dave et al., 2006, Hummel et al., 2006). This has not yet been adopted as a routine diagnostic tool.

BL is characterised by high rates of \textit{MYC}-driven proliferation (Milner et al., 1993) and apoptosis (Evan et al., 1992, Shi et al., 1992) with abundant TAM producing a “starry sky” appearance to histological sections, within which can be found apoptotic bodies containing tumour cell debris (Gregory et al., 1991). Macrophage phagocytosis of apoptotic cell debris serves to regulate anti-lymphoma immune responses (Savill et al., 2002) by swiftly removing tumour antigens without exciting an acute inflammatory response and so failing to generate adaptive immune responses against apoptotic cell-derived antigens (Albert et al., 1998, Ronchetti et al., 1999). Moreover, the mechanisms underlying macrophage clearance of apoptotic cell debris activate anti-inflammatory wound healing processes within macrophages, likely to further promote tumour growth and repel anti-tumour immunity (Ogden et al., 2005).

With relevance to our project, macrophage ablation might, therefore, result in failure to clear apoptotic tumour cell debris, attenuate the anti-inflammatory macrophage phenotype and allow a more robust anti-tumour immune response to mount.

CX3CL1 released from apoptotic lymphocytes is able to attract monocytes and macrophages expressing CX3CR1 (Truman et al., 2008). CX3CR1 deficiency reduces macrophage recruitment to the germinal centres of lymphoid follicles. Induction of apoptosis with UV radiation enhances the chemo-attraction of macrophages \textit{in vitro}, as does exposure to cell-free supernatants of cultured malignant BL cells.

It has been shown that macrophages support BL growth \textit{in vitro} (Levens et al., 2000). More recent work sheds light on some of the responsible mechanisms.
TAM depletion in lymphoma might conceivably contribute to reduced tumour progression through the removal of lymphoma cell survival factors such as BAFF, and decreased macrophage-phagocytosis and removal of apoptotic cells resulting in enhanced anti-lymphoma immunity. BAFF is known to promote the survival of normal and malignant B-cells *in vitro* (Schneider et al., 1999, Moore et al., 1999, Novak et al., 2002, Novak et al., 2004, He et al., 2004). Tumour and macrophage derived IL-10 appears a crucial cytokine in this regard, with IL-10 activated macrophages *in vitro* demonstrating enhanced ability to engulf apoptotic cells and increased production of BAFF, which is further augmented by co-culture with malignant B-cells of BL (Ogden et al., 2005). Ogden et al. describe that the microenvironment in BL is rich in IL-10. Enhancement of BL cell survival by IL-10 activated TAM is mediated by macrophage-derived BAFF, and diminished by blocking BAFF or its receptor. Whilst blockade of BAFF, APRIL and IL-10 diminished the growth of BL populations *in vitro*, residual growth suggests roles for other pathways not identified by this work.

In a later chapter we shall review further evidence for links between malignant B-cell apoptosis and TAM when discussing our use of a *Myc*-driven mouse model of lymphoma.
4.5 Lessons from T-cell lymphomas.

In this section we shall critically review several publications relating to the role of TAM in mouse models of T-cell lymphoma. Whilst not relating specifically to mature B-NHL, they provide interesting and informative pointers as to the interactions between TAM, malignant lymphocytes, and other partners in the lymphoma microenvironment.

Some of the very earliest evidence for the potential anti-lymphoma effects of TAM derives from work with T-cell lymphoma cell-lines and mouse models. L5178Y, a T-lymphoblastic lymphoma (Fischer, 1958) arising after carcinogenic methylcholanthrene exposure of DBA/2 inbred mice, was found to be sensitive to macrophage ingestion in vitro. Peritoneal macrophages, harvested after injection with the irritant agent thioglycollate, phagocytosed L5178Y cells more than control macrophages (Evans, 1971). More recent in vitro studies revealed that direct cell contact with L5178Y cells primes naïve macrophages to be sensitive to subsequent stimulation by LPS to a tumour cell cytotoxic phenotype (Buhtoiarov et al., 2007). This phenomenon is not seen with a B-cell lymphoma line (Daudi) and appears dependent on CD40:CD40L interactions.

The in vivo growth of transplanted EL4 T-cell lymphoma was retarded in mice deficient for the M2 phenotype marker SRA, compared to wild type controls. This correlated with increased expression of the M1 macrophage phenotype-associated genes NO and IFN-γ in SRA deficient tumour tissues (Komohara et al., 2009) and increased production of these proteins by in vitro cultured macrophages from SRA deficient mice, suggesting important roles for SRA in modulating macrophage phenotype, and that lymphoma growth might be sensitive to these changes. Attempts to understand the true nature of the lymphoma microenvironment require an appreciation of the complex interactions between different immune cell populations. Interactions between Tregs and TAM are likely to be complex and bidirectional regardless of which cell type (if either of these) is the prime orchestrator of lymphoma-related immune responses, therapeutic perturbations of which would be likely to have significant impacts on the entire tumour microenvironment. It has been proposed that Tregs are able to suppress monocyte and macrophage activation and cytokine production whilst up regulating their expression of inhibitory
molecules (Mahajan et al., 2006, Taams et al., 2005, Kryczek et al., 2006, Tiemessen et al., 2007). Treg depletion is known to induce macrophage accumulation in secondary lymphoid organs (Kim et al., 2007). A virally induced T-cell lymphoma in mice (RMA-S) has been studied from the perspective of the interactions between Tregs, IFN-γ and TAM in a multicellular immune response to lymphoma (Galani et al., 2010). Depletion of Tregs with anti-CD25 mAb led to tumour rejection, dependent on T-cells, NK cells and IFN-γ. This phenomenon was also abrogated by neutralizing IFN-γ. IFN-γ was produced by T-cells and NK cells in the absence of Tregs. This correlated with an increase in TAM numbers of an M1-like phenotype, overexpressing MHCII and producing high levels of pro-inflammatory cytokines, and inhibiting tumour proliferation. This supported their hypothesis that in an unmanipulated situation, Tregs prevent the accumulation and anti-tumour activities of TAM. This work did not outline in detail the phenotype of the TAM present in the unmanipulated Treg non-depleted scenario, although co-culture with RMA-S cells of identical numbers of TAM from the Treg deplete setting produced far more suppression of tumour proliferation than TAM from the unmanipulated setting, suggesting that in unmanipulated lymphoma, TAM might cooperate with Tregs in suppressing anti-tumour immune responses.

Laboratory studies with Dalton’s lymphoma, a T-cell lymphoma manifesting with prominent ascites, indicates anti-lymphoma effects of M1-like macrophages, and the fact that macrophage plasticity allows this phenotype to be achieved in vivo, in this case with the intraperitoneal (ip) administration of a proton pump inhibitor (PPI), pantoprazole (Vishvakarma and Singh, 2010). This intervention led to enhanced TAM recruitment, with more features of an M1-like phenotype, compared to controls given ip PBS, with increased production of nitrous oxide, IL-1, TNF-α and IL-2R. The macrophages recruited following PPI administration had different properties ex vivo than control macrophages; with increased phagocytosis of yeast cells, and decreased survival of co-cultured lymphoma cells. Adoptive transfer of TAM from PPI treated mice retarded the growth of lymphoma and prolonged survival of host mice. Macrophages from healthy mice did not adopt these M1-like features when exposed to PPI either in vivo or in vitro. PPI treated lymphoma cells produced less IL-4, IL-10 and TGF-β, and more IFN-γ. Together, these data suggest an indirect effect whereby PPI
administration alters lymphoma cell biology, impacting on macrophage phenotype, and thus feeding back as an effective anti-lymphoma reaction. Features of tumour cell biology impact on macrophage phenotype, which in turn may influence tumour growth and survival, yet this itself may be a simplified interpretation. The true in vivo interactions involving a multitude of other immune and stromal cells are likely to be labyrinthine in their complexity.

There is evidence for a potential cooperative anti-tumour partnership between macrophages and cytotoxic T-cells, coming from a mouse study using EG7 T-lymphoma cells (Asano et al., 2011). Fluorescently labelled, x-irradiated, killed tumour cells were injected to the footpads of mice, and fluorescence was observed to accumulate within CD11b+F4/80+MHC+CD169+ macrophages. Inoculation with dead tumour cells induced tumour antigen specific CD8 T-cell activation that was severely impaired in mice depleted of CD169+ macrophages, using the CD169 diphtheria toxin receptor (CD169-DTR) transgenic mouse. Mice were protected from subsequent injection with live EG7 tumour cells if they had undergone prior dead EG7 cell immunization, unless they were CD169 depleted at the time of immunization, in which case the tumour established and grew. This work showed a critical role for CD169+ macrophages in anti-tumour immunity, by cross-presenting dead cell associated antigens to CD8+ T-cells. CD169 is the sialic acid binding receptor, sialoadhesin, which is expressed by activated macrophages in states of chronic inflammation including malignancy, chronic viral infections, and autoimmune disease (Hartnell et al., 2001). Expression of CD169 by TAM appears therefore to indicate a favourable anti-lymphoma immune endeavour, optimization of which might be an effective therapeutic strategy.

Other studies point to the variably tumoricidal relationship between macrophages and T-cells in lymphomas, serving to highlight how the complex balance of the dynamic multi-cellular and cytokine-rich tumour microenvironment can impact on macrophage phenotype and so favour or suppress tumour growth and survival. Vicetti Miguel et al. described how cytotoxic T-lymphocyte (CTL) induction of NO production by TAM is critical for tumour elimination in a mouse model of T-cell lymphoma (Vicetti Miguel et al., 2010). Tumours transferred to and growing in the skin were sensitive to the transfer of tumour antigen specific CTL, whereas those developing in the
relatively immune privileged site of the anterior chamber of the eye appeared resistant. Examining the CD11b+ populations in each site, the authors found that CD11b+Ly6G-F4/80+ cells were the dominant population in the skin, and that NO production by these cells following CTL injection was critical for tumour regression. In the eye, the majority population of CD11b+ cells were Ly6G-F4/80- cells and failed to produce tumouricidal NO. When NOS2+ activated TAM were injected to the eye, they still failed to elicit tumouricidal activity, suggesting perhaps an over-riding influence of the surrounding milieu that inhibited the enzymatic activity of the potentially tumouricidal macrophages. This work suggests a role for CTL in the induction of tumouricidal activity in TAM for the effective elimination of lymphomas, and highlights that such a relationship may also be influenced by other, as yet poorly understood and in the case of this study unidentified, factors in the tumour environment. One might reasonably speculate that the CD11b+Ly6G-F480- population dominant within the eye might be myeloid cells of an immune suppressive phenotype, so attenuating tumouricidal immune activity, although this was not explored further in the paper.
4.6 Macrophages and rituximab.

4.6.1 Macrophages cooperate with rituximab and other anti-CD20 monoclonal antibodies in malignant B-cell killing.

In DLBCL and FL, gene expression profiling implicates different groups of macrophage genes in different biological subtypes of disease (Monti et al., 2005, Lenz et al., 2008, Dave et al., 2004). As summarised in tables 4 and 5, the literature does not provide evidence for an entirely consistent and reproducible association between overall TAM numbers and PFS, EFS or OS in FL or DLBCL. As outlined previously, there is evidence suggesting that macrophage phenotype is related to the biology and response to therapy in FL, DLBCL and BL (Wada et al., 2012, Meyer et al., 2011a, Komohara et al., 2011, Alvaro et al., 2006a, Clear et al., 2010, Ogden et al., 2005). Particularly in FL, there is evidence that a large number of TAM has opposing prognostic associations depending upon the therapy subsequently administered. Specifically, several publications suggest that a large number of TAM confers a poor prognosis in patients with FL treated without rituximab, and a good prognosis in those treated with rituximab (Taskinen et al., 2007, Kelley et al., 2007, Canioni et al., 2008, Andjelic et al., 2012), although one large study did not find this pattern (Sweetenham et al., 2010). It is intriguing to speculate that macrophages might confer a favourable prognosis when rituximab is administered in B-NHL through cooperating in rituximab-induced killing of malignant B-lymphocytes. Most evidence for this phenomenon comes from observations in FL, and studies with another indolent B-cell malignancy, chronic lymphocytic leukaemia (CLL).
<table>
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<td></td>
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<tr>
<td>Meyer</td>
<td>262</td>
<td>Yes</td>
<td>Nil</td>
<td>CD68+/SPARC+ good prognosis</td>
</tr>
<tr>
<td>(2011)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Komohara</td>
<td>43</td>
<td>Not stated</td>
<td>Nil</td>
<td>CD163 $\propto$ tumour STAT3</td>
</tr>
<tr>
<td>(2011)</td>
<td></td>
<td></td>
<td></td>
<td>CD68+/CD163+ Nil</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CD68+/CD204+ Nil</td>
</tr>
<tr>
<td>Cai</td>
<td>112</td>
<td>No</td>
<td>Nil</td>
<td>None</td>
</tr>
<tr>
<td>(2011)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coutinho</td>
<td>218</td>
<td>67% No</td>
<td>Good prognosis</td>
<td>CD163 Nil</td>
</tr>
<tr>
<td>(2011)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wada</td>
<td>101</td>
<td>Yes</td>
<td>Bad prognosis</td>
<td>CD68+/CD163+ bad prognosis</td>
</tr>
<tr>
<td>(2012)</td>
<td></td>
<td></td>
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<td></td>
</tr>
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Table 4 - Summary of available data in relation to immunohistochemical enumeration of macrophages in DLBCL.
<table>
<thead>
<tr>
<th>Author</th>
<th>Number of samples</th>
<th>Treated with rituximab</th>
<th>Relative prognostic impact of many CD68+ cells</th>
<th>Phenotypic data</th>
</tr>
</thead>
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<tr>
<td>Farinha (2005)</td>
<td>99</td>
<td>No</td>
<td>Bad prognosis</td>
<td>None</td>
</tr>
<tr>
<td>Alvaro (2006)</td>
<td>211</td>
<td>98% No</td>
<td>Nil</td>
<td>Few CD68+ with high proportion STAT1+ = bad prognosis</td>
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<td>Lee (2006)</td>
<td>59</td>
<td>No</td>
<td>Nil</td>
<td>None</td>
</tr>
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<td>Glas (2007)</td>
<td>58</td>
<td>No</td>
<td>Nil</td>
<td>None</td>
</tr>
<tr>
<td>Taskinen (2007)</td>
<td>141</td>
<td>32% No</td>
<td>Bad prognosis</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68% Yes</td>
<td>Good prognosis</td>
<td></td>
</tr>
<tr>
<td>Kelley (2007)</td>
<td>69</td>
<td>51% No</td>
<td>Bad prognosis*</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>49% Yes</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>Canioni (2008)</td>
<td>194</td>
<td>No</td>
<td>Bad prognosis</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>Byers (2008)</td>
<td>60</td>
<td>No</td>
<td>Bad prognosis</td>
<td>None</td>
</tr>
<tr>
<td>Wahlin (2010)</td>
<td>70</td>
<td>70% No</td>
<td>Bad prognosis*</td>
<td>None</td>
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<td>Sweetenham (2010)</td>
<td>180</td>
<td>57% No</td>
<td>Nil</td>
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<td></td>
<td></td>
<td>53% Yes**</td>
<td>Nil</td>
<td></td>
</tr>
<tr>
<td>Andjelic (2012)</td>
<td>50</td>
<td>76% No</td>
<td>Bad prognosis</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>24% Yes</td>
<td>Nil</td>
<td></td>
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</tbody>
</table>

Table 5 - Summary of published data in relation to immunohistochemical enumeration of macrophages in FL.

* = data refers to interfollicular CD68+ cells

** = 17% rituximab, 36% $^{131}$Tositumamab (total 53%)
4.6.2 Rituximab and the mechanisms of action of anti-CD20 monoclonal antibodies.

Rituximab in combination with chemotherapy has improved response rates, PFS and OS in FL, DLBCL, BL and MCL. Rituximab is thought to exert its effects partly through direct lymphoma killing, and partly through immune mediated mechanisms, in which activatory immunoglobulin G fragment C receptor (FcγR) expressing macrophages play a prominent role (Beers et al., Glennie et al., 2007). Relapse and resistance to rituximab are prominent clinical challenges in B-NHL and so there is great interest in developing new anti-CD20 antibodies with even better efficacy in even more patients. There are three major mechanisms by which rituximab operates:

i) Direct cell death: mAb binding induces growth inhibition and apoptosis.

ii) Complement-dependent cytotoxicity (CDC).

iii) Antibody-dependent cellular cytotoxicity (ADCC).

ADCC is of greatest relevance when considering the potential role of TAM-rituximab interactions in B-NHL, as this mechanism crucially involves the cooperation of FcγR expressing macrophages and NK-cells. Interaction between the Fc portion of bound anti-CD20 mAbs and the FcγR of macrophages or NK cells precipitates cell-killing. As such, the efficacy of ADCC in this instance might be influenced by engineered modifications to the Fc region of anti-CD20 antibodies, and host variations in the conformation or number of FcγR, and indeed the number of macrophages/NK cells present in the tumour microenvironment to display the FcγR and effect B-cell killing.

Moreover, there are different classes of anti-CD20 mAb with different properties. The binding epitope of a given anti-CD20 mAb to B-cell surface CD20 dictates these functional differences, giving a classification as type I or type II mAb (Glennie et al., 2007, Chan et al., 2003, Cragg and Glennie, 2004). Type I mAbs such as rituximab are potent inducers of CDC with low level induction of direct cell death. Type II mAb are potent inducers of direct cell death and poor inducers of CDC (Cragg and Glennie, 2004). Also, Type II mAb have been found to persist for longer in complex with CD20 at the B-cell surface.
(Beers et al., 2010), putatively exposing for longer the Fc region to immune effector cells capable of performing ADCC.

In support of macrophages having a crucial role in rituximab’s mechanism of action in B-NHL, macrophage ablation with toxic liposomes abolished the therapeutic benefit of a mAb against CD20 in a mouse model of lymphoma (Minard-Colin et al., 2008). A fascinating insight has been provided by in vitro work with malignant B-lymphocytes, demonstrating that M2-like macrophages phagocytose rituximab-opsonised malignant B-cells more efficiently than M1-like macrophages, leading the authors to suggest that M2-like TAM actually switch from a tumour promoting to a tumour-inhibiting function with the addition of rituximab (Leidi et al., 2009).

The phenotype of TAM appears to impact on their ability to bind and phagocytose malignant B-cells that have been opsonised with rituximab (Leidi et al., 2009). Human macrophages matured from PBMC with CSF-1 showed two to three times the phagocytic activity of those matured with GM-CSF. Binding and phagocytosis was further enhanced by supplementary IL-10, and attenuated by IL-4. This is consistent with earlier reports that phagocytosis of apoptotic cells is part of the wound healing macrophage phenotype (Mantovani et al., 2004b, Lewis and Pollard, 2006) and that CSF-1 or IL-10 are potent inducers of phagocytic activity in macrophages (Akagawa, 2002, Verreck et al., 2004, Xu et al., 2006, Ogden et al., 2005) and IL-4 a suppressor of phagocytic activity (Mantovani et al., 2004b, Martinez et al., 2006). Phagocytosis in these assays was correlated with expression of the three Fcγ receptors CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγR1), and was completely inhibited by excess human immunoglobulin, demonstrating a critical role for FcγR in this phenomenon. Polymorphisms in the activatory FcγRIIIα/CD16a have been described as correlating with response to rituximab (Cartron et al., 2002, Weng and Levy, 2003, Farag et al., 2004). Two variants at position 158 of human FcγRIIIα exist; a common low-affinity form 158F and less common higher affinity form 158V which is present in a homozygous state in approximately 15% of patients (Cartron et al., 2004), which in the case of FL correlates with better response rates and PFS when treated with rituximab-containing regimens (Cartron et al., 2002, Weng and Levy, 2003). Therapeutically, there is interest in increasing the affinity of mAb interactions with innate immune effector cell
FcγRIIIa. One strategy might be to alter key structural elements of anti-CD20 mAb through glycoengineering and defucosylation (Mossner et al., 2010).

One prominent example of this approach is the development of GA101, the first Fc-engineered type II humanized IgG1 anti-CD20 mAb. GA101 has improved direct and immune effector cell mediated cytotoxicity with increased affinity of binding to FcγRIII on macrophages and NK cells. It has demonstrated improved anti-tumour activity in human lymphoma xenografts, with greater B-cell depletion in lymphoid tissues in non-human primates, and is now being administered to patients through several phase I-III clinical trials in B-NHL and B-CLL (Sehn et al., 2012, Salles et al., 2012).

It is not simply the presence of FcγR on macrophages (and NK cells) that is important, but of course their interactions with bound mAb, and the degree of involvement of inhibitory FcR, both on the target cells and effector cells (Clynes et al., 2000, Uchida et al., 2004, Nimmerjahn and Ravetch, 2007, Beers et al., 2008).
4.6.3 Malignant-cell Fc receptors also play a crucial role in rituximab action.

Important mechanistic insights into the roles of TAM in anti-CD20 mAb therapy of B-cell malignancies have perhaps begun to explain both individual rituximab resistance, and the differential sensitivity of B-cell lymphomas and leukaemias to anti-CD20 mAb. In 48 primary CLL samples, the presence of the inhibitory FcγRIIb on normal and malignant B-cells was found to promote rituximab internalisation, reduce subsequent macrophage phagocytosis, and reduce its clinical efficacy (Lim et al., 2011). This built on previous work with mice showing that internalization of anti-CD20 monoclonal antibodies by target B-cells reduced their phagocytosis by macrophages (Beers et al., 2010). In human CLL studies, maximal phagocytosis of rituximab was restored in vitro by blocking FcγRII on CLL B-cells with AT10 fragment antigen-binding (Fab) fragments. FcγRII expression levels between different B-cell lymphoma/leukaemia diseases correlates with their observed responsiveness to rituximab therapy, with FL and DLBCL expressing less FcγRII and being more sensitive to rituximab than CLL and MCL. These observations strengthen the case that TAM function is of critical importance in B-lymphoma treated with rituximab or other anti-CD20 mAb.
5 Current clinical priorities in B-NHL.

The ultimate ambition remains to cure all B-NHL patients, with the minimum treatment related toxicity. Realistically, this is likely to be achieved in a stepwise fashion, and by identifying, and addressing in turn, key clinical priorities:

1) Prognostication:

Can we identify more accurately those patients in whom early aggressive or novel therapies are warranted, and those who can be safely observed without therapy, or treated with conventional regimens, without missing a “window-of-opportunity” to modulate the natural history of the disease?

2) Promoting regression:

Temporary “spontaneous” regression of FL is commonly observed, often lasting many months, and very rarely a long-term complete remission occurs without any therapy. One might reasonably speculate that this represents an effective host immune response against FL. A similar, but allogeneic, tumouricidal immune response is seen with successful allogeneic haematopoietic stem cell transplantation. Exploiting immune phenomena could provide an effective, prolonged, and well-tolerated means of controlling FL in particular.

3) Preventing progression of indolent B-NHL:

Can we indefinitely prolong the “plateau phase” of FL during which the disease is present but seemingly dormant?

4) Effective treatment of transformation and chemoresistance:

It is a typical feature of FL that initial chemosensitivity is progressively lost with each relapse. Death usually occurs after several relapses, following the advent of chemoresistance. When this is accompanied by transformation to a highly proliferative and clinically aggressive lymphoma, then survival is usually brief, even if high dose chemotherapy is used. An urgent challenge is to develop novel effective therapies to overcome chemoresistance and successfully treat transformed FL. Similar challenges are presented by the more aggressive B-NHL, in
those patients who experience primary refractoriness to therapy, or relapse with chemoresistant disease.

5) High efficacy, low toxicity:

Traditional approaches to relapse, chemoresistance or transformation have relied on increasing the doses of combination chemotherapy. If highly myelosuppressive doses are used, this may be combined with autologous haematopoietic stem cell rescue. However, with an ageing population, and prolonged survival in this disease since the advent of rituximab, then effective therapies must be tolerable to many patients of advanced age, and so also to those with common medical co-morbidities, such as heart disease, airways disease, renal disease and the complications of diabetes mellitus.

In the following chapter we shall consider the available strategies to target or manipulate macrophage populations in vivo, both in the laboratory and potentially in the clinic. This will be with the aim of establishing the nature of lymphoma-macrophage interactions, and the feasibility and impact of therapeutic intervention. An improved understanding of the innate immune response to lymphoma may well help address the priorities we have highlighted in this chapter. Improved biomarkers and novel therapeutic targets for immunotherapy have the potential to improve the management of B-NHL.
6 Targeting macrophages in cancer.

The preceding chapters outline the extensive research undertaken to describe and better understand the roles of TAM, and the molecular mechanisms that contribute to these roles. Successfully translating this knowledge into effective clinical applications is an enormous challenge. Even in the setting of an individual with malignant disease, very many of the macrophages in the body are likely to be performing beneficial, physiological functions. Furthermore, within and surrounding tumours themselves, different macrophage populations are described with various phenotypes, only some of which may be considered maladaptive. The plasticity of macrophage phenotype adds a further layer of complexity, such that the challenge includes not only targeting the right macrophages in the right place, but also at the right time. Whilst certain functional characteristics of TAM appear common in different tumours, distinct differences are well described, and so optimal approaches are likely to differ depending upon the specific cancer diagnosis. Targeting macrophages in the laboratory has been achieved in a variety of ways, each of which have major limitations when considered as therapy in humans, yet have enabled a greater understanding of TAM biology. The objectives of any laboratory or clinical approach to target TAM should be to remove pro-tumoural influences, introduce, maximise and maintain tumouricidal effects, whilst preserving physiological macrophage functions in normal tissues.
6.1 Liposomes and other nanoparticles.

In the 1980s, a “macrophage suicide” technique was developed, whereby cytotoxic liposome-encapsulated bisphosphonates were delivered into cells, exploiting the preferential engulfment of nanoparticles by phagocytes. Intracellular accumulation of bisphosphonate leads to apoptosis, primarily of monocytes and tissue macrophages (van Rooijen et al., 1996). The most effective bisphosphonate for this purpose is clodronate (dichloromethylene bisphosphonate) (Van Rooijen and Sanders, 1996). Liposomal clodronate (LC) has been extensively used to try and demonstrate proof-of-concept in mouse models of cancer (Zeisberger et al., 2006, Gazzaniga et al., 2007, Hiraoka et al., 2008, Miselis et al., 2008), to establish a functional relationship between the presence of TAM and tumour progression. LC has been evaluated as therapy in dogs with a spontaneous histiocytic neoplasm (Hafeman et al., 2010). Two of five dogs demonstrated tumour regression with an intravenous schedule of 0.5 ml/kg over 60 minutes, repeated once or twice, at 2 weekly intervals. The authors believe that direct tumour cell killing was at least partly responsible for the clinical response observed in this situation, rather than only that mediated by macrophage depletion. Nonetheless, this work showed that such a short course of LC was feasible and well tolerated in larger mammals. Encouraging results have recently been published with a mouse model whereby macrophage ablation with LC given prior to radiotherapy inhibits relapse of a melanoma model (Meng et al., 2010). Identification of such critical therapeutic windows for macrophage ablation, perhaps to augment responses to chemotherapy or radiotherapy, offers the prospect of optimising anti-tumour effects while minimising detrimental effects to physiological processes. Consequently, there have been calls for urgent clinical trials of LC in humans (Meng et al., 2010, Hafeman et al., 2010, Steidl et al., 2010). However, this relatively unselective macrophage ablation technique destroys many physiological tissue macrophages, and in its unrefined form is likely to be very limited in its human application.

Systemic delivery of un-encapsulated bisphosphonates has been common clinical practice for many years, intended to inhibit osteoclast-mediated bone resorption in the setting of osteoporosis, multiple myeloma and cancer metastasising to bone. Intriguingly, recent data strongly suggest that the effects
of systemic bisphosphonates go beyond simply reducing bone resorption or metastasis, also appearing to modulate overall cancer progression. In a clinical trial of 1803 pre-menopausal women with oestrogen-responsive early breast cancer, the addition of the intravenous bisphosphonate zoledronic acid, improved disease free-survival (Gnant et al., 2009). In addition to fewer bone metastases there were reductions in loco-regional recurrence, distant recurrence and disease in the contralateral breast, implying anti-tumour effects extending beyond the bone microenvironment. Similarly, the Medical Research Council Myeloma IX Study of 1960 patients reports a survival benefit from zoledronic acid, distinct from that associated with prevention of skeletal-related events, again implying anti-cancer properties by mechanisms that remain unclear (Morgan et al., 2010). The contribution of macrophages to this effect remains to be established as direct anti-cancer cell effects may also operate. \textit{In vivo} work in a mouse model of mesothelioma has indicated that systemic zoledronic acid might impair myeloid cell differentiation to tumour-associated macrophages (Veltman et al., 2010), and further work is needed to clarify and confirm this mechanism of action.

\textit{In vitro} studies with lung cancer cell lines and mouse alveolar macrophages suggested that empty endotoxin-free poly(isobutylnicyanoacrylate) nanoparticles are able to induce tumouricidal activity in macrophages with an associated increase in Th1 cytokines in the co-culture supernatant (Al-Hallak et al., 2010). Silica nanoparticles, under investigation as gene delivery vectors to the CNS, induce inflammatory and potentially tumouricidal phenotypic changes in rat brain resident macrophages (Choi et al., 2010) increasing the production of reactive oxygen and nitrogen species, and down-regulating TNF-\alpha gene expression. Nanoparticles with additional functionality can be engineered with surface ligands to enhance binding to target cells, be they cancer cells or TAM. An alternative approach has been to try to exploit the tumour homing capacity of macrophages as a method of targeting delivery of anti-cancer therapeutics to notoriously hard to reach hypoxic areas of tumours (Holden et al., 2010). Such work has demonstrated the technical feasibility of immobilising nanoparticles containing such agents that would otherwise be toxic to macrophages if directly exposed, on the surface of chemically modified macrophages. It is hoped that with further development of this technique, engineered macrophage-
nanoparticle hybrids might be adoptively transferred and migrate to the centre of tumours, driven by hypoxic stimuli.

Building on the concept of delivering drugs to macrophages through liposomes has been the development of mifamurtide (Mepact®, Takeda). Mifamurtide is liposome-encapsulated muramyl tripeptide phosphatidylethanolamine (L-MTP-PE). It is a liposome packaged synthetic glycopeptide, which also occurs naturally as a component of bacterial cell walls, and is capable of activating macrophages to an inflammatory M1-like potentially tumouricidal phenotype, with production of IL-1, IL-2, IL-6, IL-12 and TNF-α (Nardin et al., 2006). This imaginative strategy has proven an effective anti-cancer therapy in osteosarcoma. Intravenous delivery results in accumulation of mifurmatide particles in the pulmonary vasculature, exactly the site most at risk of micro metastasis and relapse in this disease. In an adjuvant setting with multi-drug chemotherapy, Mifermatide reduces mortality by 70-78% and has been approved for use by the Food and Drug Administration, European Medicines Agency and the National Institute for Health and Clinical Excellence.
6.2 Systemic macrophage apoptosis in MaFIA mice.

MaFIA (Macrophage Fas-induced apoptosis) mice are a transgenic strain (C57BL/6J-Tg (Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6) 2Bck/J) developed to facilitate inducible in vivo macrophage depletion (Burnett et al., 2004). This model enables laboratory investigation of the consequences of systemic macrophage depletion. We have used this model to investigate macrophage depletion in a transplantable mouse lymphoma. The CSF-1R promoter is used to express a drug-inducible suicide gene, that when activated by a specific synthetic compound, leads to macrophage-lineage specific Fas-mediated apoptosis via tumour necrosis factor receptor superfamily member 6 (TNFRSF6, also known as apoptosis-mediating surface antigen FAS). This model was developed following work to create a suicide construct with the cytoplasmic domain of FAS lined to two copies of FK506-binding protein (FKBP) (Spencer et al., 1996). The modified transgene insert in this case contains a mutant human FK506 binding protein 1A, which preferentially binds the dimerization drug AP20187. AP20187 was developed by Ariad Pharmaceuticals to be otherwise inactive, and to not affect endogenous FKBP. Administration of the drug causes cross-linking of Fas-receptors, leading to activation of the caspase-8 pathway and apoptosis of cycling and resting cells (Ashkenazi and Dixit, 1998). The MaFIA transgene also contains an otherwise non-functional membrane insertion unit of the low affinity nerve growth factor receptor (\(\Delta\)LNGFR) (Thomis et al., 2001). Owing to the absence of the promoter gene in relevant HSPC, monocyte production is recoverable and the effect is reversible once dimerizer administration is halted. The 11.1 kb MaFIA transgene was introduced into C57BL/6 donor eggs and the resultant male mice bred with C57BL/6 mice until pure breeds of both sexes were obtained. Owing to the presence of normal numbers and subsets of monocytes, macrophages and DC during organogenesis, MaFIA mice have no developmental abnormalities. In this way, Burnett et al. used genes coding for the CSF-1R promoter, EGFP, and the \(\Delta\)LNGFR-FKBP-Fas suicide construct to generate this model of systemic, drug-inducible macrophage cell death, in which macrophages express EGFP, and cell death occurs independently of cell cycle dynamics.
Data on the impact of intraperitoneal administration of dimerizer to healthy MaFIA mice indicates widespread and majority, but sub-total, macrophage depletion with variations between different tissues (Burnett et al., 2004). Daily ip injections of 10 mg/kg for 5 consecutive days to MaFIA mice (‘treated mice’) was compared to an identical schedule in wild type mice, and vehicle control injections in MaFIA mice. Macrophage depletion was evident in ‘treated mice’ from 24 hours after the third injection. 24 hours after the last of the 5 injections, EGFP positive cells were quantified by flow cytometry. Compared to vehicle control treated MaFIA mice, ‘treated mice’ had reductions of over 90% in the bone marrow and peritoneum, and over 70% in the blood, spleen, lungs and thymus. No significant reductions were seen in lymph nodes or brain. Macrophage numbers started to rise in the bone marrow and spleen, 7 days after the last injection.

‘Treated mice’ lost 18.1% (+/- 6.7%) of their total body weight. The authors postulated that this was due to macrophage loss, as weight loss was not seen in any of the control groups. ‘Treated mice’ also displayed splenomegaly, lymph node enlargement and thymic atrophy. Prominent extramedullary haematopoiesis was evident in the spleen, yet lymph node architecture appeared normal, although expanded in overall cell number. The failure to achieve macrophage depletion in lymph nodes was hypothesised to be due to poor tissue penetration of the ip administered dimerizer.
6.3 Cellular adoptive transfer.

Given that some of a growing TAM population is probably recruited from circulating monocytes, it is reasonable to suppose that supplementary populations of injected monocyte/macrophages might preferentially accumulate within tumours. Without manipulation, these cells might contribute to the TAM population and so encourage tumour growth. Manipulation of monocyte/macrophages either in vitro or subsequently in vivo might provide the potential to deliver classically activated macrophages with anti-cancer properties. This is a strategy we employ in a mouse model of lymphoma in later chapters. Alternatively, or in addition, these macrophages might be used as vectors to express genes within the tumour, such as for inflammatory cytokines, or pro-drug conversion genes to optimise local delivery of active chemotherapeutics (Griffiths et al., 2000).

Studies in mice have demonstrated the therapeutic potential of macrophage adoptive transfer. In 1974, Isaiah Fidler and colleagues studied the ability of syngeneic macrophages to inhibit pulmonary metastases in C57BL/6 mice bearing a progressively growing B16 melanoma (Fidler, 1974). Their work demonstrated that peritoneal macrophages, harvested after thioglycollate-induced inflammation, reduced the number of pulmonary metastases. This activity was dependent upon the macrophages being specifically treated in vitro and then injected intravenously. Whilst unable to describe the macrophage phenotype in language with which we are now familiar, it was clear that the tumour-cytotoxic activity of the macrophages was influenced by their prior in vitro exposure to sensitising agents during maturation. Those exposed to rat lymphocytes sensitised to tumour in vivo in addition to tumour supernatants were highly active in preventing pulmonary metastases, compared to those exposed only to tumour supernatants, which had no effect. Later work described that the subsequent tissue distribution in a normal host mouse of transferred macrophages was determined by the method used to elicit and activate them from the donor peritoneum (Wiltrout et al., 1983). It is much clearer now that macrophage subtype and phenotype is likely to be crucial in successfully populating and influencing growing tumours.

Similarly successful adoptive transfer of macrophages with tumour regression has been repeated and refined in other mouse models of cancer (Hagemann et
Human studies in the late 1980s demonstrated that macrophage adoptive transfer is feasible and safe in patients with cancer (Lacerna et al., 1988, Stevenson et al., 1987). Greater than $3 \times 10^9$ autologous monocyte-derived macrophages, sensitised \textit{in vitro} with IFN-\gamma or LPS, or \textit{in vivo} with GM-CSF, can be safely infused by iv, ip or intrapleural routes. Systemically delivered macrophages are first retained in the lungs before pooling in the liver and spleen, concentrating at sites of metastases (Andreesen et al., 1998). Macrophages delivered ip remain within this cavity for more than 7 days and accumulate at sites of major tumour growth. In one study of patients with ovarian or gastric cancer, ip macrophages significantly reduced the volume of malignant ascites in 3 out of 7 patients, but without impacting on tumour mass (Andreesen et al., 1990). Unfortunately these results proved to be exceptional, with the majority of early human \textit{in vivo} studies showing little or no measurable benefit in a variety of cancers (Lacerna et al., 1988, Faradji et al., 1991b, Faradji et al., 1991a, Lopez et al., 1992, Eymard et al., 1996, Hennemann et al., 1995). One reason for this failure of effect, despite successful transfer, might be an overwhelming ability of the tumour to manipulate the phenotype of transferred macrophages, so down-regulating their tumouricidal and pro-inflammatory functions. This might be overcome by transfecting macrophages with critical pro-inflammatory cytokines. \textit{In vitro} and \textit{in vivo} studies in mice support the feasibility of this approach with respect to IFN-\alpha, IL-4, IL-6, TNF-\alpha, also showing increased tumouricidal activity (Nishihara et al., 1995). Immunotherapy approaches using adoptive transfer of \textit{ex vivo} IFN-\gamma activated macrophages shows that they can be manufactured on a large scale, cryopreserved and safely administered to patients with cancer (Baron-Bodo et al., 2005), and in concert with rituximab in B-CLL participate in ADCC killing mediated by Fc\gammaRI on macrophages (Lefebvre et al., 2006).
7 Experimental rationale.

This project is hypothesis driven, namely, that TAM form part of a lymphoma-supporting microenvironment, and might themselves be rational and effective targets in these diseases. Of course, it would be simplistic to postulate that macrophages might act as sole and prime orchestrators of the tumour microenvironment. Nonetheless, manipulation of their number or phenotype might critically alter the overall balance of cancer-related immunity, and so be sufficient to significantly alter tumour progression.

We interrogated human diagnostic biopsies of DLBCL to investigate correlations between macrophage numbers, phenotype and lymphoma growth rates. We then aimed to establish proof-of-concept for a cooperative relationship between macrophages and lymphoma by performing varied and sometimes opposing manipulations of macrophage populations in mice harbouring a growing population of malignant mature B-cells. Our experimental rationale was to inject a known and consistent number of lymphoma cells in to the venous circulation of mice of the same age and sex. Using techniques that will be detailed later, we manipulated macrophages in these mice, measuring the impact on lymphoma progression. We ablated macrophages in two different ways, also looking to see if there is a dose-response relationship between macrophage ablation and lymphoma progression. We then supplemented macrophages, and investigated whether supplementary macrophages of different phenotypes had different impacts. Subsequently, we investigated the effects of attempting to preferentially reduce the number of TAM by inhibiting the CSF-1R. We also investigated the impact of macrophage depletion on responses to chemotherapy.
Figure 10 - Approaches to manipulate macrophages in a mouse model of lymphoma.

1. Macrophage Ablation
   a. Toxic Liposomes
   b. Transgenic inducible macrophage depletion

2. Adoptive transfer of phenotype-modulated macrophages (M1, M2, TAM)

3. Attempt to specifically reduce numbers of TAM (CSF-1 receptor inhibitor)
8 Experimental approach.

8.1 Demonstrating functional interplay between macrophages and malignant lymphocytes in mouse models.

Our primary purpose of using a mouse model of lymphoma is to investigate the functional relationships between lymphoma and macrophages. Direct interactions between lymphoma cells and macrophages in isolation can readily be studied using \textit{in vitro} co-culture techniques. Lymphomas consist of malignant lymphocytes interspersed with stromal cells and infiltrating immune cells, of which macrophages form a minority population. Mouse models allow a more faithful reproduction of the complex multicellular interactions occurring in nature.

By manipulating quantitative and qualitative aspects of the macrophage population, mouse models allow us to observe the effects on other cellular populations, as well as on the total lymphoma mass. In this way, we have been able to test our hypothesis that TAM form part of a complex lymphoma-supporting microenvironment. As such, not only might features of the TAM population (number and phenotype) correlate with outcomes, but also might present novel therapeutic targets in this disease.
8.1.1 Mouse models of B-NHL.

Several mouse models have been developed to simulate different aspects of the various lymphoid malignancies. Human lymphoma xenografts have the advantage of utilizing the true human malignant cells, yet are far from suitable for our purposes, as they require a significantly immune-suppressed host. In a variety of genetically engineered models, mice eventually develop lymphoid malignancies. These models are particularly valuable for studying the role of specific introduced genetic lesions in lymphoid tumourigenesis. The variable frequency, timing, and lineage of malignancy in these transgenic models would create significant challenges for standardisation of experimental conditions. Furthermore, introduced genetic lesions affecting lymphoid populations necessarily alter the immune microenvironment of transgenic mice. We wanted to work with a B-lymphoid malignancy that could be readily transplanted to immune-competent hosts, enabling standardised in vivo study of the interactions between macrophages and malignant B-cells.

A proposal for the classification of lymphoid neoplasms in mice has been compiled to aid communication about mouse models of human lymphoid diseases (Morse et al., 2002). This work attempts to pair mouse lymphoma models with human equivalent counterparts, based upon features of histology, cell surface or cytoplasmic antigens, and genetic lesions. The authors found it very difficult to make the case that any diseases in the two species were true homologues. “Follicular lymphoma” models that have been developed thus far tend to consist of mixed centroblasts and centrocytes rather than almost entirely centrocytes, and arise predominantly in the spleen rather than lymph nodes, thus not replicating the characteristic follicular architecture of human FL. Raffergerst and colleagues discovered that an established MHC class II transgenic mouse line that had been developed as a model for autoimmunity (Ito et al., 1996), eventually developed spontaneous lymphoid tumours (Raffegerst et al., 2009). This chimaeric HLA-DR4-H2-E (DR4) homozygous transgenic mouse develops very diverse haematological cancers, equally distributed in frequency between T- and B-cell lymphomas. A disease with features of FL develops after 18 months in under half of mice over-expressing BCL2 under the control of Vav, the panhaematopoietic promoter (Egle et al.,
2004). The authors were not successful in transplanting this lymphoma even to immune-compromised hosts.

The observation that DLBCL can derive from germinal centre B-cells with dysregulated expression of \( BCL6 \) led to the development of transgenic mice with constitutive \( BCL6 \) expression in B-cells, under the control of the immunoglobulin \( I\mu \) promoter (Cattoretti et al., 2005). This model produces mature B-lymphomas in the majority of mice by 20 months, yet unfortunately cannot be transplanted successfully to immune-competent hosts (oral communication from Professor R. Dalla-Favera). Other potentially suitable models of DLBCL include one with dysregulated \( Tcl1 \) expression (Hoyer et al., 2002) that is reported as being transplantable, and another with deletion of the pro-apoptotic molecule BAD (Ranger et al., 2003). We chose to work with a lymphoma derived from the \( E\mu\)-myc/bcl-2 mouse. The principal advantages included the very extensive characterisation and successful ongoing use (Gilbert and Hemann, 2010) of this model in the literature over the past 26 years, its ease and reproducibility of transplantation to normal hosts, and local laboratory experience.
8.2 Background – the Eµ-myc transgenic mouse.

The Eµ-myc lymphoma was developed on the basis of work in the early 1980’s (Brinster et al., 1983, Storb et al., 1984, Grosschedl et al., 1984, Rusconi and Kohler, 1985) and has since been extensively characterised (Adams et al., 1985, Leder et al., 1986, Harris et al., 1988). Eµ-myc tumours are considered to be a good model of human NHL (Adams et al., 1985) with the highly proliferative nature drawing parallels with BL (Gilbert and Hemann, 2010, Cory, 1986). The Eµ-myc model of B-NHL has been extensively used to characterise in vivo responses to anticancer drugs (Schmitt et al., 2000, Schmitt et al., 2002).

In this model, transgenic mice harbour the Myc proto-oncogene coupled to the immunoglobulin µ enhancer, resulting in tissue-specific constitutive Myc overexpression in the lymphocyte lineage. Dysregulated MYC expression drives many human cancers including BL, and some cases of aggressive DLBCL and possibly transformed FL, in which the acquisition of quantitative or qualitative abnormalities in the expression of MYC have been reported to occur at transformation (Yano et al., 1992, Lossos et al., 2002) with one large series reporting increased expression of MYC in 14 of 20 paired samples of FL at transformation to tFL (Davies et al., 2007). Eµ-myc mice develop a polyclonal B-cell hyperplasia progressing to a monoclonal lymphoma, and die within a few months of birth. Tumour histology reveals effacement of normal lymph node architecture, with monotonous appearing intermediate sized lymphocytes with round to oval nuclei, small indistinct nucleoli and a rim of basophilic cytoplasm. Numerous apoptotic bodies are present, giving a “starry-sky” appearance (Minard-Colin et al., 2008).

Phenotypically, Eµ-myc mice with lymphoma display massive lymph node enlargement, splenic enlargement, and infiltration of malignant lymphocytes in the liver, bone marrow and blood. As such, these mice develop a disseminated lymphoma with associated leukaemia. As first described, isolated tumour cells were all monoclonal B-lymphoblasts (Adams et al., 1985), but theoretically the Eµ-enhancer is active at several stages of B-cell maturation. The stage of B-cell maturation of the dominant clone varies between individual mice, and in some cases over time. Some display characteristics of pre-B cells (B220+, CD19+
IgM-), others mature B-cells (B220+, CD19+, IgM+, CD5-), and some have been documented to progress from pre-B to B-cell phenotype.

Isolated Eµ-myc malignant B-cells are capable of reproducing disseminated lymphoma in genetically matched, non-transgenic recipient mice, such that the reconstituted malignancy is histopathologically indistinguishable from the primary lymphomas (Schmitt et al., 1999, Bearss et al., 2000). For the purposes of our work, this offers the advantage of being able to transplant lymphoma into immune competent hosts. We believe that used in this way we are able to orthotopically model aspects of tumour growth and dissemination in a whole organism multicellular environment. However, with the intravenous transplant of established tumour populations, this model is unlikely to provide useful information regarding the roles of monocytes and macrophages in de novo lymphomagenesis. As such, our studies focus on the role of lymphoma-associated macrophages in cancer-induced inflammation, rather than their potential role in inflammation-induced cancer formation. There are well-documented links between specific chronic infections, chronic inflammation, and subsequent development of lymphomas, epitomised by Helicobacter pylori-associated gastric mucosa associated lymphomas (Isaacson, 1993, Wotherspoon et al., 1991, Nakamura et al., 1997). Indeed, there is compelling evidence for the recruitment and action of macrophages and other myeloid cells being of critical importance in animal models of tumourigenesis. In a colitis induced carcinogenesis model, blocking the actions of the chemokine CCL2 reduced macrophage recruitment and tumourigenesis (Popivanova et al., 2009). In gastric carcinogenesis, macrophage recruitment under the control of bacterial infection and prostaglandin E2 induced CCL2 expression was found to be a non-redundant step. We considered these aspects beyond the scope of this thesis.
8.3 The Eµ-myc/bcl2 mouse – a chemoresistant model.

There is an urgent clinical need to identify improved strategies to treat aggressive MYC driven lymphomas in humans, and more specifically in their common chemoresistant phase which is often associated with defective apoptosis regulated by the BCL2 protein family.

Whilst the majority of B-NHL diagnoses are not MYC driven, nor do they over-express BCL2, those that harbour either or both mutations often represent the very worst prognosis groups with conventional therapies. Bento et al. presented hypothesis-forming data supporting this view from a small number of diagnostic patient biopsies of DLBCL. MYC and BCL2 alterations at diagnosis appear to identify a high-risk population for whom conventional therapy is inadequate (Bento et al., 2011, Aukema et al., 2011). Cuccuini et al. (Cuccuini et al., 2011) investigated the presence of MYC and BCL2 rearrangements by break apart FISH in 156 of 396 analysable patient samples from the collaborative trial in relapsed aggressive lymphoma (CORAL) study. This study recruited patients with relapsed and/or refractory DLBCL. 24 patients (16%) had MYC rearrangements of which 21 (87.5%) also had BCL2 rearrangements. The outcome of MYC-rearranged patients was significantly worse regardless of which chemotherapy regime they received, in terms of both PFS and OS. It is also well documented that cases of t-FL, although not commonly having translocations over-expressing MYC, represent a highly proliferative BCL2 over-expressing lymphoma with very poor outcomes to conventional chemotherapy, even at high doses. As such, it is for these groups of poor prognosis patients that novel therapies must most urgently be developed and must demonstrate efficacy in pre-clinical models if they are to be of maximal clinical utility. The Eµ-myc/bcl2 lymphoma models several aspects of these poor prognosis groups and would appear a rational choice of model in which to investigate novel therapeutic approaches.

The BCL2 gene is normally expressed in pre-B cells, is quiescent in resting B-cells, is expressed in proliferating B cells, and down-regulated in differentiated B-cells. Lymphomas with the t(14;18) display inappropriately elevated levels of BCL2-Ig fusion RNA for their mature B-cell stage of development (Graninger et al., 1987, Seto et al., 1988). This situation can be mirrored by the manipulation
of the Eµ-myc lymphoma. Defined cancer genotypes can be created \textit{ex vivo}
using a mouse stem cell-virus based retroviral vector to transduce a gene of
interest into Eµ-myc lymphomas (Schmitt et al., 2000). In this case, insertion of
the \textit{Bcl2} gene leads to over-expression of BCL2 by malignant B-lymphocytes in
the Eµ-myc/bcl2 mouse.
8.4 $E_{\mu}$-myc/bcl-2, chemoresistance and macrophages.

The validity of the $E_{\mu}$-myc/bcl-2 model has been established by therapeutic studies, with $Bcl2$ insertion and over-expression conferring chemo-resistance to a wide range of agents. Specifically, when compared to parental $E_{\mu}$-myc lymphomas, $E_{\mu}$-myc/bcl-2 is resistant to treatment with the alkylating agents cyclophosphamide (Mason et al., 2008) and maphosphamide, the topoisomerase II inhibitor adriamycin, the microtubule inhibitor docetaxel (Schmitt et al., 2000), and the HDAC inhibitor vorinostat (Lindemann et al., 2007). Furthermore, $E_{\mu}$-myc/bcl-2 mice have been used successfully as a model for testing BCL2 antagonists (Mason et al., 2008).

We hypothesised that macrophage depletion might improve the response to genotoxic chemotherapy in lymphoma. Two studies exemplify different ways in which this might occur. Gilbert and Hemann found that microenvironmental signals, including IL-6, helped create a chemo-resistant niche in which lymphoma cells were able to survive genotoxic stress and subsequently generate systemic relapse (Gilbert and Hemann, 2010). Given that TAM are abundant in the tumour microenvironment and can produce IL-6, this work supports the concept of prior-ablation of TAM to maximise the effect of genotoxic chemotherapy. Ahn et al., using a xenograft of human hypopharyngeal carcinoma, demonstrated that tumour re-growth following radiation therapy was highly dependant on subsequent macrophage-induced angiogenesis (Ahn et al., 2010). Tumour re-growth was attenuated by inhibiting macrophage repopulation of irradiated tumours using an antibody against macrophage-1 antigen (CD11b/CD18). This study suggests that macrophage depletion following cytotoxic therapy might slow or prevent relapse. We investigated the impact of macrophage depletion both prior to and following chemotherapy, and used the nitrogen mustard derivative and pro-drug cyclophosphamide (Arnold et al., 1958), one of the key components of therapy for FL, DLBCL and BL (Bishop et al., 2000). Advanced $E_{\mu}$-myc/bcl-2 lymphomas respond to 200-300 mg/kg single ip doses of cyclophosphamide, but always relapse (Mason et al., 2008), unlike $E_{\mu}$-myc lymphoma in which long-term survival can be induced (Schmitt and Lowe, 2001). This partial response provides a scenario in which a measurable benefit might be observed should macrophage ablation improve responses.
8.5 $E_{\mu}$-myc/bcl-2, apoptosis, senescence and macrophages.

Similar to $E_{\mu}$-myc transgenic mice, $E_{\mu}$-myc/bcl-2 tumours have a lymphomyeloid progenitor phenotype (Strasser et al., 1990, Strasser et al., 1996), such that it is vital that the lineage and maturation stage of any given population is fully characterised. $E_{\mu}$-myc/bcl-2 mice die with enlarged spleens, livers and lymph nodes, and lymphoblasts in the blood and bone marrow (Mason et al., 2008).

There is further evidence to suggest that a Myc driven mouse model of lymphoma might yield relevant and interesting insights specifically in relation to lymphoma-macrophage interactions. A recent review, focusing on Myc driven cancers, discusses the links between oncogene action, tumour cell apoptosis, immune-cell response and senescence induction as a mechanism of potential tumour suppression (Lee et al., 2011). Myc stimulates aberrant proliferation and cell growth, and as a countermeasure to this uncontrolled expansion, evokes apoptosis, seemingly as a safeguard. Equally, autophagy or senescence have been identified as cell-autonomous safeguard strategies in such circumstances (Schmitt, 2003). In the relatively apoptosis resistant $E_{\mu}$-myc/bcl2 mouse, Schmitt et al. found that inactivation of senescence (by genomic deletion of the senescence related histone methyltransferase suv39h1) promoted B-lymphoma development.

The mechanism identified is that Myc-induced apoptosis attracts and activates macrophages which produce TGF-$\beta$ that can induce terminal cell-cycle arrest (senescence) in Myc-driven lymphoma cells without causing a pro-inflammatory, and, therefore, potentially pro-tumourigenic cytokine response (Reimann et al., 2010). Genetic inactivation of senescence accelerated Myc-driven lymphomagenesis, as did neutralization of TGF-$\beta$ action. When exogenous TGF-$\beta$ was administered to lymphoma cells in vitro there was a dose dependent slowing of proliferation until growth stopped and features of senescence appeared, except in the suv39h1 knockout mice. Lymphoma cells themselves did not secrete TGF-$\beta$. This was found to be macrophage derived, in response to apoptosis. If apoptosis is blocked, there are fewer macrophages and less senescence. Liposomal clodronate macrophage depletion further reduced senescence. One can reasonably postulate that macrophage depletion
in the premalignant stage might promote lymphomagenesis in situations of *Myc* and *Bcl2* overexpression. An adequate macrophage response in this model may therefore have a role to play in the suppression of lymphomagenesis, despite possibly having lymphoma growth promoting effects in established malignancy. Qualifying this theory, the authors themselves state that the cells and cytokines delaying lymphomagenesis via senescence do not necessarily continue to operate as “tumour constraints during later steps of cancer progression.” They recognise the evidence that both TAM and TGF-β have potentially pro-tumoural effects in established malignancies. We used lymphoma cells from the same *Myc* and *Bcl2* over-expressing transgenic mice. By transplanting cells of an established mature B-cell lymphoma, we modelled the interactions between macrophages and established lymphoma, in contrast to the work described above that focused on lymphomagenesis in the transgenic mice.
8.6 Experimental strategy.

We chose to propagate and repeatedly use a batch of malignant cells isolated from the lymph nodes of a single Eµ-myc/bcl-2 mouse. This enabled us to characterise the lineage and maturation stage of the tumour cells, and keep this constant throughout our experiments. Using the lymphoma cells, rather than the transgenic animals, allows better investigation of the effects of microenvironmental manipulation. The transgenic animals would potentially over-express Myc and Bcl2 in non-malignant lymphocytes, thus affecting the immune response to lymphoma. Transplantation of the lymphoma into C57BL/6 mice enabled me to observe interactions with wild-type macrophages. Moreover, the ability to transplant allows me to introduce lymphoma in other mice, genetically manipulated in such ways as to specifically alter their immune response. This enables investigation of critical pathways of macrophage-lymphoma interaction in, for example, tissue specific gene knockout mice.

Transplantation of lymphoma by intravenous tail vein injection effectively constitutes an orthotopic tumour model, with early widespread haematogenous dissemination of malignant lymphocytes. Preliminary studies in our laboratory (Bossard M, unpublished data) suggested that the first signs of deterioration in overall condition would reliably occur from day 12 following the injection of $2 \times 10^5$ Eµ-myc/bcl-2 cells. At sacrifice, transplanted mice exhibited widespread enlarged lymph nodes, and enlarged spleen and liver. Given our wish to avoid causing distress to the animals and work well within UK Home Office regulations regarding maximum allowable tumour volumes, we therefore planned elective sacrifice by day 12 post-injection.
9 Materials and methods.

9.1 Immunohistochemistry of human tissues.

9.1.1 Interrogating the human tissue bank.
We were interested to explore whether there was any correlation between macrophage numbers and lymphoma growth rates at diagnosis. We felt this might provide a straightforward indication as to whether the growth dynamic of B-NHL is linked to the presence of macrophages. This approach was intended to shed light on the biology of B-NHL in the absence of the variable confounding effects of different treatment regimes, and provide a further basis for investigating the dynamic impact of macrophage ablation in a mouse model. To achieve this we interrogated a large number of diagnostic biopsies representing treatment-naïve DLBCL, and used the expected natural variation in tumour proliferation rates in this disease to act as an “experiment in nature,” allowing comparison of this variable with macrophage numbers. BL samples would have been unsuitable for this purpose owing to their lack of variation with almost universal proliferation rates of 98% and above. FL samples would be expected to show variation in proliferation, but over a narrower range than DLBCL.

Principles and methods of assessing proliferation.
Ki67 staining was used as a measure of tumour cell proliferation. Ki67 protein can be detected by the Ki67 antibody during all active phases of the cell cycle and not during the G0 resting phase (Gerdes et al., 1983). It is thought that the expression of no other protein is as tightly regulated in relation to proliferation as Ki67 (Scholzen and Gerdes, 2000). As such, measurement of the proportion of cells in a population expressing Ki67 enables the calculation of a proliferation fraction, or Ki67 labeling index, for any given tissue. This technique was optimised for NHL, and correlated very closely with classification in to high or low grade NHL as determined principally by cellular morphology as part of the Kiel classification of malignant lymphomas (Gerdes et al., 1984, Lennert, 1975).

Macrophage assessment.
Macrophage quantification of CD68 staining was less straightforward than with nuclear Ki67 staining. CD68 is a membrane/cytoplasmic stain. Owing to the large size and branching polydendritic shape of macrophages, thin tissue
sections contain multiple fragments of CD68 staining cytoplasm, extending between different sections. As such, it is impossible to determine which fragments belong to which individual cells, and so it is difficult to reliably count numbers of macrophages. In the figure below is a FFPE section of FL stained with anti-CD68 KP1 (brown) and haematoxylin (blue). A single large macrophage displays characteristic cytoplasmic staining with CD68, and long, branching cytoplasmic processes.

![Image of a macrophage](image)

Figure 11 - A tumour-associated macrophage in follicular lymphoma (original magnification (OM) x 60).

However, working on the reasonable assumption that macrophage size and shape is the same in biopsies from the same tissue of the same diagnosis in different patients, then the area of a tissue section occupied by brown-staining CD68 positive cellular material will be proportional to the number of macrophages in that section, and allow comparison between biopsies on any given tissue microarray (TMA) slide. This technique was optimised on a TMA of Hodgkin lymphoma in my host laboratory and the technique and findings recently published (Greaves et al., 2012).

9.1.2 Principles of immunohistochemistry.

Immunohistochemistry (IHC) is the process of detecting proteins in tissues using the inherent avidity of antibody binding to specific antigens. Bound
antibodies are then visualised courtesy of prior conjugation to, or subsequent specific binding of, coloured or fluorescent dyes.

We used an indirect enhanced polymer immunoperoxidase technique that is outlined in the figure below. An unlabelled primary antibody is selected with specificity for the target antigen. Tissue is exposed to the primary antibody and unbound antibody washed off after a period of incubation. Tissue is then exposed to a secondary antibody with species and IgG isotype specificity for the primary antibody. Multiple secondary antibodies bind the primary antibody, providing signal amplification. Further amplification is provided by the secondary antibody being conjugated to a polymer molecule, itself holding multiple molecules of the enzyme reporter, horseradish peroxidase (HRP). A chromogenic substrate, diaminobenzidine (DAB) is then exposed to the tissue. This binds to HRP molecules on the secondary antibody-polymer-HRP complex and is converted to a coloured substance, detectable by brightfield microscopy.

Figure 12 - Principles of indirect polymer immunoperoxidase IHC.

In our work the specific polymer reagent used was a proprietary compound manufactured by BioGenex and provided as a component of the SuperSensitive™Polymer-HRP IHC Detection System.

9.1.3 Practical aspects of immunohistochemistry.

Sample preparation, staining, and image capture was performed by A.Clear. I designed experiments in terms of choice of disease and markers to investigate, and performed subsequent data analysis.
Dako Autostainer.
For efficiency and to standardise the protocol, all slides for any given experiment were stained at one time using an automated system. This consisted of a slide rack, with a robotic arm holding a nozzle to dispense common reagents from bulk reservoirs and specialised reagents as directed from a rack. Dako Autostainer Plus software facilitated bespoke programming of staining protocols.

Protocol for immunohistochemical staining using Dako Autostainer.

Removal of paraffin and dehydration.
1. Place TMA slides overnight at 60°C.
2. Remove paraffin using xylene in two stages: suspend slides in xylene for 5 min then transfer to second xylene pot.
3. Transfer to industrial methylated spirits (IMS) for 2 min.
4. Transfer to hydrogen peroxide (2 ml reagent grade) in 100 ml IMS for 2 min. Transfer to a second pot of hydrogen peroxide and IMS for 2 min.
5. Transfer to a fresh IMS pot for 2 min (final dehydration step).

Heat induced epitope retrieval.
An epitope retrieval process was necessary as formalin fixatives cause protein cross-linking, resulting in compromised binding of certain antigens to their complementary antibodies. A variety of antigen retrieval methods can break the protein cross-links formed by formalin fixation and so better expose antigenic sites. Optimal conditions for antigen retrieval were determined for each antigen using whole tissue sections exposed to no antigen retrieval step, or an enzymatic protocol, or immersion in a heated solution. A heat induced epitope retrieval method produced the best results with the three antigen/antibody combinations of interest.

Use of a domestic pressure cooker prevents changes in the buffer solute concentrations resulting from evaporation, and owing to positive pressure, allows temperatures of above 100°C without boiling (which can otherwise physically disrupt specimen integrity).
6. Heat 3000 ml of antigen unmasking solution in a pressure cooker on low heat setting (100-110°C).
7. As soon as the solution is just beginning to boil, fully immerse slides in plastic racks.
8. Seal pressure cooker lid and increase the heat to high setting (120-
130˚C) and leave for 10-15 min.

9. Remove from heat and cool pressure cooker under cold running tap
water for 5-10 min.

10. Retrieve slides and rinse in wash buffer.

11. Ensure slides remain wet with wash buffer at all times during subsequent
staining process.

**Antigen staining.**

The following antibodies were optimised and used at the stated dilutions.

<table>
<thead>
<tr>
<th>Antigen target</th>
<th>Antibody Clone</th>
<th>Manufacturer</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td>KP1</td>
<td>Dako</td>
<td>1:8000</td>
</tr>
<tr>
<td>CD163</td>
<td>10D6</td>
<td>Leica</td>
<td>1:3000</td>
</tr>
<tr>
<td>Ki67</td>
<td>MIB-1</td>
<td>Dako</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 6 - Antibodies used for TMA IHC.

12. Mark slides using hydrophobic marker pen around the edge of the array
field: this is to retain liquid on the slide preventing drying, and to ensure
exposure of the whole area of interest to dispensed solutions.

13. Input directions using Autostainer control software, specifying number of
slides to be stained, reagents and incubation times, rinse steps, non-
specific protein block steps (using casein-based commercial protein
compound), primary antibodies to be used at optimal dilutions (in
BSA/azide), polymer-conjugated secondary antibody, and chromogenic
substrate (DAB).

14. Start the Autostainer. The process takes 2-3 hours including incubation
and wash steps.

15. Replace all slides in plastic racks ready for counterstaining and and
mounting.

**Counterstaining, rehydration and mounting.**

Counterstaining was performed with haematoxylin to provide an outline of
cellular and tissue architecture, and highlight the presence of those cells not
marked by the primary and secondary antibodies. Haematoxylin is a blue/purple
dye that binds histone proteins, and so highlights nuclei.

16. Rinse in tap water for 5 min.
17. Suspend in haematoxylin solution for 5 min.
18. Rinse for 2 min in tap water
19. Perform five rapid dips in to acid alcohol solution.
20. Wash immediately in tap water for 2 min.
21. Rehydrate tissue using three 2 min suspensions in IMS.
22. Remove residue and clarify tissue by suspending in two xylene baths, then suspend in final bath prior to mounting.
23. Using distyrene, plasticizer and xylene (DPX) mountant apply cover slip removing trapped air bubbles. Air dry.

9.1.4 Image analysis using the Ariol system.

TMA slides were scanned using an Olympus BX 61 microscope with an automated platform. Chosen parameters were measured across all intact cores by automated image analysis system (Ariol) as previously described (Kleiner et al., 2009). This platform allows the standardised and rapid enumeration of a huge number of events from multiple images. The Ariol system was trained by an experienced operator (AC) to discriminate stained from unstained cells by contrasts in aspects of colour and shape. A pixel mask of positive events was constructed and refined in terms of shape and size, then tested to ensure its sensitivity and specificity, before being applied to all TMA sections.

Different staining patterns require different training of Ariol. Ki67 is a nuclear stain and so the system is trained to count positive staining and non-staining nuclei, readily providing the number and proportion of positive nuclei per section. CD68 and CD163 are predominantly membrane and cytoplasmic stains. Macrophages are relatively large branching cells that commonly extend cytoplasmic processes across sequential tissue sections. As such, Ariol can be trained to measure the area of positive and negative staining per section, and with this proportional area of macrophage cytoplasm, so derive a proxy measure of macrophage number. Provided that macrophage volume and shape is, overall, consistent between patients, then this provides a reasonable relative measure of macrophage infiltration.

Each case was entered into the subsequent analysis only if there were two or three high quality cores representing a total of 2-3 mm². A mean count per 1 mm² core for lymphoid markers and mean percentage area for CD68 or CD163 was then calculated. From the original cohort there was further attrition of cases based on these quality criteria.
9.1.5 Tissue microarrays.
A TMA of 218 diagnostic biopsies of DLBCL was constructed from high quality FFPE tissue blocks collected at our centre between 1977 and 2009 (Coutinho et al., 2011). The TMA included tissue from 128 males and 90 females, with a median age of 55 (18-94) years; 23.2% high-intermediate /high risk IPI; with a median follow up of 3.1 years. 33% of patients were treated with rituximab containing regimes. All patients had provided informed consent for excess biopsy tissue to be used for research purposes in accordance with the Declaration of Helsinki, and ethical approval was obtained from the North East London Research Board (05/Q0605/140).
Use of this TMA enabled rapid, efficient and standardised staining and assessment of large numbers of biopsies on a small number of glass microscope slides. The TMA was constructed following previously described principles (Kononen et al., 1998). 1 mm² cores were taken from 3 separate regions of biopsy material per block, having been marked by an expert histopathologist (MC) from sections stained with H&E. Acceptable regions were of high cellularity, containing malignant cells, without more than 10% by area of fibrosis or other cause of acellularity. Cores were arranged vertically in to a recipient paraffin block, sectioned and transferred onto glass slides. Control cores of tonsil were used to represent normal lymphoid tissue, and cores of myocardium or intenstine were placed as orientation markers. Subsequent quality control review by expert histopathologists excluded any sections that were damaged or of less than 90% cellularity.

9.2 Liposomal clodronate and liposomal PBS.
We obtained liposome preparations from Dr Nico van Rooijen, Department of Molecular Cell Biology, Vrije Universiteit, Amsterdam. Liposomes contained either clodronate at 7 mg/ml, or simply PBS as a vehicle control, which were manufactured as described (Van Rooijen and Sanders, 1996). Mice were injected into a lateral tail vein with 200 µl of LC or vehicle control, unless otherwise specified.

9.3 Eµ-myc/bcl-2 tumour cells.
Eµ-myc/bcl-2 cells were a kind gift of Leonie A Cluse, Cancer Immunology Program, The Peter MacCallum Cancer Institute, Trescothick Research
Laboratories, East Melbourne, Australia. Eµ-myc/bcl-2 lymphomas had been engineered by retroviral transduction of freshly isolated Eµ-myc lymphoma cells as previously described (Schmitt et al., 2000, Lindemann et al., 2007). The tumour line was propagated by injection to normal healthy C57BL/6 mice. Brachial lymph nodes were harvested at day 10, mashed, and stored in solution with dimethyl sulphoxide (DMSO) under liquid nitrogen at a concentration of 3 x 10^7 in 1 ml. For subsequent injection a single vial was thawed, washed twice in media, counted, resuspended in ice cold PBS, and injected immediately at room temperature (to avoid culture artefacts). For in vitro studies, tumour cells were maintained by incubating at 37°C in 5% CO₂ at a starting concentration of 1 x 10^6 cells per ml in a volume of 2 ml in 6-well plates.

**9.4 Collection of tumour conditioned media.**

TCM was collected and stored following a published schedule (Solinas et al., 2010). Lymphoma cells were cultured in vitro to 90% confluence, assessed visually. Cells and media were transferred to a tube, an aliquot counted using trypan blue exclusion, and spun at 450 Xg for 4 min in an Eppendorf 5810R centrifuge. Supernatant was aspirated and discarded, and the cell pellet gently re-suspended in fresh warmed media. Cells in fresh media were plated in 6-well plate at identical cell density as before and incubated at 37°C in 5% CO₂ for 24 hours. Cells and media were collected and spun at 450 Xg for 4 min. Supernatant was collected, filtered through a 0.2 µm filter and stored at -20°C.

**9.5 Cell viability.**

Cell viability was determined by trypan blue exclusion assay using a Beckman Coulter Vi-CELL™ XR cell viability analyser.

**9.6 Mice.**

Wild-type female C57BL/6 mice were purchased from Charles River and Harlan Laboratories. Male and female homozygote transgenic MaFIA mice were purchased from The Jackson Laboratory and paired for breeding. For all experimental protocols, mice were age matched between groups. For experiments with MaFIA mice, groups were balanced for biological sex.

All mice were housed at a maximum of 6 per cage in a temperature-controlled pathogen-free animal facility. Water and food were freely available. Regulated
procedures on living animals were conducted within the scope of my personal licence (PIL 70/2249) awarded after satisfactory completion of accredited training and under the project licence of Professor F. Balkwill in accordance with the Home Office Animals (Scientific Procedures) Act 1986.

9.7 Injections.

Iv and ip injections were performed using a 1 ml capacity syringe and 25-gauge needle (BD Microlance). Iv injections were made into the lateral tail veins.

9.8 Oral gavage.

Gavage was performed with 20-gauge x 25 mm curved metal gavage needles (VetTech Solutions Ltd).

9.9 Assessment of in vitro cytotoxicity.

We administered three different agents to mice in order to specifically manipulate their macrophage populations, and then observe the impact on lymphoma progression. Namely, these were toxic liposomes, a dimerizer agent to trigger macrophage depletion in the MaFIA mouse, and a CSF-1R inhibitor. We wanted to assess whether any of these agents were directly toxic to Eµ-myc/bcl-2 lymphoma cells. For this purpose we cultured Eµ-myc/bcl-2 lymphoma cells in vitro and exposed identical cell numbers to a wide range of concentrations of these agents, separately, for the same length of time, and then measured the number of non-viable cells using the proxy measure of LDH release into the media (Korzeniewski and Callewaert, 1983, Decker and Lohmann-Matthes, 1988). Using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega Cat No G7890), we quantified LDH release through its conversion of administered resazurin to a fluorescent product, resofurin in the presence of excess supplemental diaphorase, pyruvate and nicotinamide adenine dinucleotide (NAD+). Generation of fluorescent resofurin is proportional to LDH release. Use of appropriate controls is absolutely critical to the reliable interpretation of results from this fluorometric technique. LDH is present in animal serum used in tissue culture media, and is elicited by human lymphomas. As such, acellular complete media and untreated lymphoma controls are required, in addition to a 100% cell lysis control. This is generated by the addition of a 9% solution of the detergent Triton® X-100 in water to
untreated E\textsubscript{\mu}-myc/bcl-2 lymphoma cells at the end-point of the assay. Concentrations of agents were calculated to span a very wide range encompassing the very peaks of concentrations that might be experienced \textit{in vivo}.

\textbf{9.9.1 Experimental protocol for CytoTox-ONE\textsuperscript{™} Homogeneous Membrane Integrity Assay.}

A 96-well plate was prepared with 5 replicates of each condition. The conditions studied were as below. The entire experiment was repeated once to confirm the results.

A) E\textsubscript{\mu}-myc/bcl-2 lymphoma culture media alone.

B) E\textsubscript{\mu}-myc/bcl-2 lymphoma cells with E\textsubscript{\mu}-myc/bcl-2 lymphoma culture media.

C) E\textsubscript{\mu}-myc/bcl-2 lymphoma cells with E\textsubscript{\mu}-myc/bcl-2 lymphoma culture media to which 100% Cell Lysis Solution was added (maximum LDH release control).

D) E\textsubscript{\mu}-myc/bcl-2 lymphoma cells with E\textsubscript{\mu}-myc/bcl-2 lymphoma culture media and 4 different concentrations, separately, of LC, CSF-1R inhibitor or MaFIA dimerizer.

\textbf{9.9.1.1 Experimental sequence.}

1) E\textsubscript{\mu}-myc/bcl-2 lymphoma cells were plated at their optimal growth density of 1 \texttimes 10\textsuperscript{6} per ml, giving 5 \texttimes 10\textsuperscript{4} cells per well in 50 \textmu l of media.

2) Agents or control media were added in 50 \textmu l to make each well to 100 \textmu l. There were 4 different concentrations of each agent starting in the mM range and reducing on a standard base 10 logarithmic scale.

3) The plate was covered and incubated for 18 hours at 37°C in 5% CO\textsubscript{2}.

4) The plate was removed to room temperature to equilibrate for 20 min. LDH enzymatic activity is influenced by temperature.

5) Substrate Mix and Assay Buffer were equilibrated to room temperature at 22°C.

6) 11 ml Assay Buffer was added to and gently mixed with each vial of Substrate Mix, protecting from bright light.
7) 2 µl of 100% Cell Lysis Solution was added to the maximum LDH release control wells.

8) 100 µl Reagent Mix was added to each well and the plate agitated for 30 sec. Resazurin dye in the Reagent Mix is light sensitive as is the resorufin product, so light exposure of the plate was minimized.

9) The plate was incubated at 22°C in the dark for 10 min.

10) 50 µl Stop Solution was added to each well in the same sequence as Reagent Mix to standardise incubation times with active reagent. The plate was covered, kept out of bright light, and read with minimal delay.

11) The plate was analysed using a POLARstar Optima (BMG Labtech) multidetection microplate reader with excitation at 560 nm and emission 590 nm.
9.9.1.2 Calculation of percentage cytotoxicity.

The percentage cytotoxicity of each experimental condition was calculated using the following formula, incorporating units of fluorescence (FU) for each measured well.

\[
\frac{100 \times (\text{FU of well} - \text{Mean FU of culture medium only})}{\text{Mean FU of maximum LDH release control} - \text{Mean FU of culture medium only}}
\]

9.10 Preparation of stored Eµ-myc/bcl-2 lymphoma cells for in vivo use.

Stored Eµ-myc/bcl-2 lymphoma cells were thawed at room temperature, washed twice and suspended in ice-cold PBS at a concentration of \(2 \times 10^6\) cells/ml. Mice were injected iv with 100 µl volume containing \(2 \times 10^5\) Eµ-myc/bcl-2 lymphoma cells.

9.11 Cytokine analysis.

Circulating cytokine levels were measured from healthy mice, mice with lymphoma, and those subjected to macrophage manipulations. Specifically, changes were assessed in circulating levels of CSF-1 and inflammatory cytokines. Given the small volumes of plasma available from sacrificed mice (approximately 500 µl per mouse), a multiplexed cytokine assay kit was used in addition to a specific CSF-1 assay kit, both purchased from Meso Scale Diagnostics®. Sample cytokines are measured using a sandwich immunoassay. Each well of a 96-well plate contains multiple spots, each with separate pre-coated specific capture antibodies bound to a base-plate that can act as an electrode. The sample is added and binds to the capture antibody. A detection antibody is then added and will bind any sample antigen held by the capture antibody. The detection antibody is linked to SULFO-TAG™ (ruthenium (II) tris-bipyridine-(4-methylsulfonate) NHS ester) molecules. After incubation and washing steps, a read buffer is added and the plate loaded to a MSD
SECTOR™ Imager 6000 reader instrument. Here, a voltage is applied across the base plate electrode, causing any bound SULF-TAG molecules to emit light at an intensity proportional to the amount of bound sample. Multiple excitation cycles provide signal amplification, adding to the sensitivity of this technique.

In addition to an individual kit to assay for mouse CSF-1, we used a mouse pro-inflammatory 7-plex assay ultra-sensitive kit (Cat No. K11012C-2). The cytokines included, and their lower levels of detection (LLD) are detailed in the table below.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LLD (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>0.38</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.75</td>
</tr>
<tr>
<td>IL-6</td>
<td>4.5</td>
</tr>
<tr>
<td>IL-10</td>
<td>11</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>35</td>
</tr>
<tr>
<td>CXCL1</td>
<td>3.3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 7 - Sensitivity of cytokine detection in MSD pro-inflammatory 7-plex kit.

9.11.1 Detailed experimental protocol.

1) Reagent preparation.

All reagents were brought to room temperature except the Calibrator stock, which was thawed on ice.

In order that an 8-point standard curve could be constructed, 4-fold serial dilutions of Calibrator were made from a first standard point solution of 10 µl of Mouse Proinflammatory 7-plex Calibrator Blend in 990 µl of Diluent 4. The final standard was Diluent 4 alone. This gave a range for the standard curve from 0 pg/ml to 10000 pg/ml.
The Detection Antibody Blend 50X stock solution was diluted 60 µl in to 2.94 ml of Diluent 5.

Equal volumes of 4X Read Buffer T and deionized water were mixed.

2) Assay protocol.

25 µl of Diluent 4 was dispensed to each well of the pre-coated plate. This was sealed with an adhesive film and agitated on a Dynatech microshaker plate-shaker for 30 min at room temperature.

25 µl of sample plasma or Calibrator standard was dispensed to individual wells in duplicate. The plate was sealed and agitated as before at room temperature, for 2 hours.

The plate was washed 3 times with PBS plus 0.05% Tween-20. 25 µl of 1X Detection Antibody Solution was dispensed to each well, and the late sealed and agitated as before at room temperature for 2 hours.

The plate was washed 3 times as before. 150 µl of 2X Read Buffer T was dispensed using careful reverse pipetting to each well and the plate. This was to ensure no fluid bubbles were formed that might interfere with the subsequent plate reading process.

3) Plate reading using MSD SECTOR™ Imager 6000.

This imager uses a charge-coupled device (CCD) camera. This digital imaging technology allows high quality images to be constructed from emitted light by converting incoming photons into readable electric charges.
9.12 Lymphoma progression assessment.

Whenever feasible, independent observers performed tissue quantification blinded to the therapy received by each group. Initial assessments of lymphoma mass consisted of measurement of the long and perpendicular axes of spleens and lymph nodes using 0-200 mm electronic digital callipers with 0.01 mm resolution (VWR).

Organ volume was derived from the formula:

\[
\text{Volume} = 0.52 \times (\text{short axis}^2 \times \text{long axis})
\]

It became apparent that this method was potentially fragile, with heavy reliance on observer judgment of the correct placing of the calliper arms, particularly with smaller nodes. To abolish this variability, and establish a more robust measure with no possibility of observer bias, we used organ weights alone as a proxy measure of lymphoma mass. Dissected lymph nodes and spleens were cleaned of adherent fat and connective tissues, and weighed on highly sensitive scales.

To validate using organ weight as a proxy for lymphoma mass it was necessary to establish the relationship between organ weight and cross-sectional area of tissue infiltrated by lymphoma in histological sections.

9.13 Quantification of liver cross-sectional tumour area.

FFPE sections of liver were cut and stained with H&E. As is typical with intravenously transplanted lymphomas in mice, tumour cells appeared to congregate around hepatic sinusoids and veins. This is visible in the image overleaf as dark-staining areas that at higher magnification are revealed to be composed of cells with a high nuclear to cytoplasmic ratio. Using a low magnification lens, multiple images were photographed at random locations, obtained without looking through the viewfinder. The images were imported to Adobe® Photoshop® software and overlaid with a graticule. The number of whole and half squares occupied by lymphoma was counted for each image and expressed as units of area, corresponding to cm² per low power field. The figure below indicates the principle of this approach. In practice, a finer graticule with more sub-divisions was used to enable more accurate quantification. An independent scientist, blinded to experimental groups and the aims of the project, repeated this process.
Figure 13 - FFPE section of liver stained with H&E with overlying graticule to demonstrate method of quantification of cross-sectional area of lymphoma (OM x 2.5).

9.14 Preparation of FFPE sections of bone and bone marrow.

Femurs of sacrificed experimental mice were dissected, cleaned of soft tissues, and submerged in 4% formaldehyde for 24 hours at room temperature. Bones were then removed, washed in slow running tap water for 5 min before being placed in 10% formic acid for 24 hours and rotated slowly at room temperature. Decalcification was confirmed by the ability to bend a femur using forceps and minimal force. Decalcified bones were washed under slow running tap water for 30 min prior to routine paraffin embedding.

9.15 Embedding of tissues.

1) A mould was filled with molten paraffin wax

2) The tissue cassette was opened the tissue transferred to the mould with warmed forceps.

3) Gentle pressure was applied to embed the tissue evenly in the mould.

4) The mould was chilled on a cold plate with the tissue cassette base placed on top.
5) More molten paraffin was added to completely fill the mould and cassette base

6) Once solid, the block with attached labelled cassette base was removed from the mould.

9.16 Blood sampling by cardiac puncture.

Blood was harvested by terminal cardiac puncture using a 1 ml syringe containing a drop of unfractionated heparin, and a 25-gauge needle (BD Microlance) under inhalational general anaesthesia with isoflurane and oxygen. Mice were placed supine exposing their ventrum, the needle inserted immediately caudal to the xiphoid process and advanced towards the left shoulder with continuous gentle suction until blood was freely aspirated. At completion, the needle was withdrawn and sacrifice performed by cervical dislocation. Blood was then stored on wet ice, spun in an Eppendorf Microcentrifuge (Cat. No. 5415D) at 1600 Xg for 2 min to separate the plasma from the cellular fraction. Plasma was frozen on dry ice and stored at -80°C. The cellular pellet was resuspended, and processed for analysis by flow cytometry.

9.17 Sacrifice and organ removal.

Mice were sacrificed by cervical dislocation. Organs were immediately weighed and then placed in appropriate storage depending upon subsequent intended use:

- **FACS/ flow cytometry** – Ice-cold PBS
- **FFPE sections** – 4% Formaldehyde
- **Frozen sections** – OCT on dry ice
- **RNA extraction** – 2 ml micro-centrifuge tube on dry ice
9.18 Preparation of MaFIA dimerizer AP20187.

1) Lyophilized AP20187 was dissolved in 100% ethanol at a concentration of 13.75 mg/ml
2) An injection solution was prepared in sterile water consisting of 10% PEG-400 and 1.7% TWEEN.
3) AP20187 in 100% ethanol was diluted with injection solution in a ratio of 1:25 by volume.
4) The final product in sterile water for injection consisted of:
   - 0.55 mg/ml AP20187
   - 4% ethanol
   - 10% polyethylene glycol (PEG-400)
   - 1.7% TWEEN

This product was administered by ip injection within 30 min of preparation, with the volume injected adjusted by weight to deliver 10 mg AP20187 / kg per dose.

9.19 Formation of single cell suspensions for flow cytometry.

Organs in ice-cold Dulbecco’s modified eagle medium (DMEM) were macerated through 40 μm nylon cell strainers (BD falcon Ref 352340) using a 5 ml syringe plunger, spun and resuspended in red blood cell lysis buffer (BD Pharm Lyse™ 1x concentration), spun, washed and nucleated cells stained for analysis by flow cytometry.

9.20 Flow cytometry antibody staining.

Cells of interest were suspended at 1 x 10^7 per ml and 80 μl pipetted to each well in a 96-well plate with round-bottomed wells. The plate was spun at 950 Xg for 2 minutes using an Eppendorf 5810R centrifuge and the supernatant discarded. The pellet was re-suspended in 100 μl flow cytometry buffer and spun again at 950 Xg for 2 minutes, discarding the supernatant. The washed cell pellet was resuspended in 50 μl of CD16/CD32 Fc receptor block (diluted 1:100 in flow cytometry buffer) to minimise non-specific binding to Fc receptor expressing cells. The plate was incubated at 4°C for 15 minutes before adding flow cytometry antibodies diluted at 1:100 in 50 μl of flow cytometry buffer. The plate was incubated at 4°C for 30 minutes in the dark before spinning at 950 Xg at 4°C for 5 minutes and discarding the supernatant. Two further washes with
flow cytometry buffer were performed, prior to resuspending the pellet in 50 μl
2% formaldehyde to fix the stained cells. A table of flow cytometry antibodies,
their manufacturer and optimal dilution is provided as an appendix.

9.21 Analysis by flow cytometry.
Samples were read using the BD FACSCalibur™ and the BD Fortessa™
platforms. Data was analysed using FlowJo 8.8.6 software. Accurate analysis of
fluorochrome detection was achieved with unstained and isotype controls, and
compensation performed with positively staining cellular samples and
compensation beads.

9.22 Collection of mouse peripheral blood for morphological
review.
A mouse was held within a restraining tube allowing its tail to protrude. The tail
was held proximally, cleaned with an alcohol swab and sprayed with ethyl
chloride to provide local anaesthesia, then 1-2 mm of the tip swiftly removed
with a scalpel blade. Drops of blood were collected on individual glass slides
and spread to allow subsequent clear visualisation of cellular morphology.
Pressure was applied with clean gauze until haemostasis was achieved, and
this was secured by painting the tip with a silver nitrate pencil.

The project licence dictates that no mouse can be bled on more than 3
occasions in its entire lifetime. Each bleed must be separated by at least 7 days
from the next, and the total loss of blood must constitute less than 15% of
circulating volume. In a protocol containing 10 mice per group, bleeding each
mouse every 10 days on a staggered rota would allow a single representative
blood smear from each group on every day for 30 days.

9.23 Staining of mouse peripheral blood for morphological review.
Each blood smear was dried, fixed and stained using the following rapid manual
dip protocol. We chose to use a modified Wright's stain (WS16 Sigma
Aldrich®). This is a Romanowsky-type stain containing methylene blue, azure B
and eosin Y dyes, allowing excellent discrimination between nucleus and
cytoplasm in the interpretation of white blood cell morphology (Wright, 1902). It
also contains methanol, acting as a solvent and fixative.
1) Slides were dried horizontally in still air at room temperature. Staining proceeded within one hour of drying.

2) 50 ml modified Wright's stain was placed in a Coplin jar.

3) 50 ml deionised water was placed in another Coplin jar.

4) Each slide was placed feather-edge down in modified Wright stain for 15 seconds.

5) Each stained slide was then placed feather-edge down in deionised water for 30 seconds.

6) Each slide was rinsed by gently passing 5-10 ml of deionised water over its surface using a 5 ml plastic transfer pipette.

7) Slides were dried horizontally in still air at room temperature before evaluation.

9.24 Assessment of tumour proliferation by BrdU incorporation.

We wished to assess the proliferative rate of Eµ-myc/bcl-2 lymphoma cells in different in vivo conditions. As a proxy measure, we determined the proportion of malignant cells entering and progressing through the S phase of the cell cycle. We used flow cytometry to measure cellular immunofluorescent staining of bromodeoxyuridine (BrdU) that had been incorporated in vivo. BrdU is a thymidine analogue, and is incorporated into newly synthesised DNA (Eidinoff et al., 1959, Hakala, 1959). Bolus ip injection of weight adjusted doses of BrdU allowed robust comparison between individual mice and groups. Equally important was ensuring identical time-intervals between injection of BrdU and storage at 4°C of harvested tissue from sacrificed mice.

This was carried out using the BD Pharmingen™ APC BrdU Flow Kit (Catalog No. 552598). The dose per mouse was 0.02 mg per kg of a 10 mg/ml solution of BrdU in sterile PBS, delivered in a single ip injection (Rocha et al., 1990).
9.24.1 Immunofluorescent staining of blood and lymph nodes for assessment of BrdU by flow cytometry.

Red cell lysis was performed with phamlyse RBC lysis buffer as previously described. Lymph node single cell suspensions were formed as previously described.

1) Staining cell-surface antigens.

$1 \times 10^6$ cells were resuspended in $50 \mu l$ staining buffer (PBS with 3% FCS) in individual 1.2 ml Linbro® Liquisystem polypropylene tubes (MP Biomedicals, Cat No. 61226C2).

Fluorescent antibodies for all cell-surface antibodies were added in 50 $\mu l$ of staining buffer, mixed well and incubated on ice in the dark for 15 min.

1 ml of staining buffer was added to each tube and spun at 300 Xg for 5 min, discarding the supernatant.

2) Fixation and permeabilisation.

The cell pellet was resuspended in 100 $\mu l$ of BD Cytofix/Cytoperm Buffer and incubated on ice in the dark for 15 min. 1 ml of BD Perm/Wash Buffer was added to each tube, spun at 300 Xg for 5 min and the supernatant discarded.

Cells were resuspended in 100 $\mu l$ of BD Cytoperm Plus Buffer, incubated for 10 min in the dark on ice, then washed with 1 ml BD Perm/Wash Buffer, and spun as before.

The cell pellet was resuspended in 100 $\mu l$ of BD Cytofix/Cytoperm Buffer, incubated in the dark for 5 min on ice, washed once more with 1 ml BD Perm/Wash Buffer and spun as before.

3) DNase treatment to expose incorporated BrdU.

Cells were resuspended in 100 $\mu l$ of DNase 300 $\mu g/ml$ in PBS and incubated for 60 min at 37°C in the dark, before washing once more with 1 ml of BD Perm/Wash Buffer and spinning as before.
4) Staining of BrdU and other intracellular antigens with fluorescent antibodies.

Cells were resuspended in 50 µl of BD Perm/Wash Buffer containing anti-BrdU APC at a concentration of 1:50 then incubated at 22°C in the dark for 20 min before washing in 1 ml BD Perm/Wash Buffer and spinning as before. The fully stained and fixed cells were then resuspended in 1 ml of staining buffer and analysed by flow cytometry.

9.25 Extraction of RNA with Qiagen kit.

RNA was extracted according to tailored protocols from the RNeasy® Mini and Micro Handbooks. Snap-frozen lymph nodes or spleen tissue were placed in gentleMACS™ M Tubes (Miltenyi Biotec) with 0.5 ml of Buffer RLT (Qiagen) and homogenized for total RNA isolation using the gentleMACS Dissociator on the RNA-02 program for frozen tissues. The resultant foamy homogenate was spun at 2000 Xg for 1 minute in an Eppendorf 5810R centrifuge and 350 µl aspirated into a QIAshredder spin column placed in a 2 ml collection tube and spun for 2 minutes at 16000 Xg in an Eppendorf Microcentrifuge 5415D. The supernatant was transferred to a fresh 2 ml centrifuge tube. 350 µl of 70% Ethanol was added and thoroughly mixed by pipetting.

700 µl of each sample was transferred to an RNeasy spin column placed in a 2 ml collection tube and spun for 15 seconds at 16000 Xg. The flow through was discarded. On-column DNase digestion was then performed, as follows. 350 µl of Buffer RW1 was added to each sample and spun for 15 seconds at 16000 Xg. The flow through was discarded and 80 µl of DNase solution in Buffer RDD was added directly to the RNeasy spin column and left at room temperature for 15 minutes.

350 µl Buffer RW1 was added to the RNeasy spin column and spun for 15 seconds at 16000 Xg, with the flow through being discarded. 500 µl Buffer RPE was added to the RNeasy spin column and spun for 15 seconds at 16000 Xg, and the flow through discarded. This step was repeated but spinning for 2 minutes to dry the RNeasy spin column membrane. The column was then carefully removed and placed in a fresh collection tube.
40 µl of RNase-free water was added directly to the column and spun for 1 minute at 16000 Xg to elute the RNA. The flow through was aspirated and pipetted once more onto the column membrane before repeating this spin cycle. The flow through was collected and stored at -80°C.

**9.26 Counting macrophages in mouse tissue sections.**

This was performed manually rather than by using the automated Ariol system. Macrophages marked by the presence of brown stain were included in the count if they were clearly distinguishable as a discrete cell with a nucleus surrounded by positively staining cytoplasm and membrane. Positively staining fragments without a nucleus were not counted. High magnification images were taken from multiple areas of each section, selected randomly without looking through the microscope. A graticule was superimposed over each image using Adobe Photoshop software, to facilitate counting. Counting was performed by me, and then repeated by independent scientists, blinded to the purpose of the experiments and the treatment groups. The data included in this thesis derives from the independent observers.

**9.27 Cyclophosphamide.**

Cyclophosphamide was provided as 500 mg of white powder. It was reconstituted with 25 ml of sterile 0.9% saline, giving a 6 mg/ml concentration. This was given to mice with lymphoma as a single bolus ip injection, as detailed for each protocol.

**9.28 RNA analysis.**

Purity and quantity was analysed using the ND1000 Spectrophotometer, reading absorbance at 260/280 nm (NanoDrop, Wilmington, DE, US).

**9.29 RNA integrity.**

9.30 Reverse transcription of RNA to yield cDNA.

For each sample of RNA, 2 µl of RNA was added to a 2 ml centrifuge tube with 10 µl DEPC H$_2$O and 2 µl of Random Primers. Each tube was heated at 75°C for 5 minutes before cooling on wet ice for 5 minutes.

To each centrifuge tube was added:

- 5 µl M-MLVT RT reaction buffer
- 5 µl Mixed Nucleotide Pool
- 1 µl M-MLV RT enzyme
- 0.6 µl RNasin® RNase inhibitor (Promega)

After 10 minutes at room temperature, each tube was incubated at 40°C for 50 minutes. Subsequently, 75 µl of DEPC H$_2$O was added to each sample, which was stored at -20°C.

9.31 Quantitative RT-PCR.

Analyses were performed using the ABI Prism 7700 Sequence Detection System Instrument and software (PE Applied Biosystems). RT-PCR was performed using sample cDNA (FAM) and internal control 18sRNA (VIC) and specific TaqMan® probes.

qRT-PCR was carried out with the TaqMan® Universal PCR Master Mix (PE Applied Biosystems) in a 96 well plate with optical film (ABI). Each well contained the following reagents:

- 10 µl TaqMan® Universal PCR Master Mix
- 6 µl DEPC H$_2$O
- 2 µl Sample cDNA
- 1 µl Probe/primer set of gene of interest
- 1 µl Primers and probes for 18s RNA

The thermal cycling conditions, for a total of 60 cycles, were:

- 50°C 2 min
- 95°C 10 min
- 95°C 15 sec
60°C 1 min

Samples were tested in triplicate. Target mRNA was normalised (ΔCT) to 18s RNA by subtracting the cycle threshold (CT) of the 18s RNA sample from the cycle threshold (CT) of each sample.

The expression level of each target gene in the intervention group samples was determined relative to the initial experimental controls (Livak and Schmittgen, 2001):

\[ \Delta\Delta CT = \Delta CT \text{ intervention} - \Delta CT \text{ control group} \]

This was expressed as a fold change in gene expression using the formula:

\[ \text{Fold Change} = 2^{-\Delta CT} \]
9.32 Mouse bone marrow derived macrophages (BMDM).

Mouse BMDM were prepared under sterile conditions by flushing bone marrow from the centre of femora and tibiae of healthy 6-12 week old female C57BL/6 mice using a 5 ml syringe filled with ice cold DMEM and a 25 gauge needle (BD Microlance). A single cell suspension was created by maceration with the back of a syringe plunger through a 40 µm cell strainer, with cells spun and resuspended at 1 x 10⁶/ml in DMEM with 10% FCS and 1 µl of recombinant CSF-1 (Miltenyi Biotec) per 1 ml of media at a final concentration of 10 ng/ml, then incubated at 37°C with 5% CO₂, and re-stimulated with CSF-1 at day 3.

In vitro polarisation of macrophage phenotype was performed by overnight stimulation on day 6 by replacing with fresh media supplemented with LPS 1 µg/ml and IFN-γ 20 ng/ml to derive classically activated M1 macrophages, IL-4 20 ng/ml to derive alternatively activated M2 macrophages, and replacing with TCM for TAM polarised macrophages.

Adherent macrophages were removed by incubation at 37°C for 30 minutes with 10 ml cell dissociation buffer (Gibco, Invitrogen), followed by gentle physical removal with a cell scraper (BD Falcon), washing and resuspension in appropriate media.

Figure 14 - Preparation of phenotype-modulated bone marrow derived macrophages.

To identify adoptively transferred macrophages, a red fluorescent dye (PKH26, Sigma-Aldrich) was incorporated into mouse BMDM in vitro as described by the
manufacturer (Product Information, PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labelling). Post mortem frozen tissue sections were visualized by fluorescent microscopy to identify labelled macrophages. BMDM purity was assessed by flow cytometry.

9.33 Preparation of the small molecule CSF-1R tyrosine kinase inhibitor.

The compound AZ268 was provided by AstraZeneca as a yellow powder. Diluent consisted of 0.1% Tween-80 and 0.5% methylcellulose in sterile water. Diluent was prepared by adding 100 µl of Tween-80 to 100 ml sterile water and placing on a stir plate. 0.5 g methylcellulose powder was added slowly and stirred overnight, before sterile filtering. The compound powder was added to this diluent to produce a final concentration of 20 mg/ml, and sterile filtered and injected within 2 hours of preparation. To deliver 100 mg/kg to 20g mice, 100 µl was administered to each mouse by oral gavage.
10 Statistical considerations.

Statistical advice was kindly provided by Dr Donna Neuberg, Senior Lecturer on Biostatistics, Dana-Farber Cancer Institute, Massachusetts, USA, who is a long-term collaborator of J. Gribben and receives funding from his Programme Grant. This included tuition in the use of power calculations to determine minimum numbers of mice required to achieve meaningful results for in vivo experiments. This input also facilitated the appropriate use of statistical tests and representation of data. The majority of mouse data showing weights or volumes of tissues and organs involved fairly small numbers of individual data points, relatively tightly clustered. As such, greatest clarity was achieved by representing the data with individual points for each measurement, and a line at the median, without crowding the figure with error bars. Our primary interest was to determine differences between groups. The most appropriate test for analysing differences between the groups was to perform a rank test. For this purpose we chose the Mann-Whitney test (Mann and Whitney, 1947). This is a non-parametric significance test requiring that observations in each group are independent of each other (not paired samples), responses are continuous measurements and that we are testing a null hypothesis that the independent groups are equally distributed. It does not require identical numbers in each group, unlike the otherwise similar Wilcoxon rank-sum test. The values from all the experimental samples, regardless of whether in the treatment or control group, are ranked according to the measured variable. This creates a single ranked series. The relative distribution of the rank values between the groups being compared is used to calculate the likelihood of a difference between the groups being due to chance alone.

Particularly in relation to comparison of the weights of lymph nodes, many of the protocols produced very tightly clustered data points within each group, and obvious differences between groups. As such, a variety of different statistical tests would each have demonstrated statistical significance. A rank test was chosen as the most appropriate method of answering the question of whether differences existed between groups. It is prudent to consider the limitations of the p-values obtained from such tests. With small numbers of mice and clear differences in data values between two groups, the p-values from rank tests will not distinguish large magnitudes of difference in one experiment from small
magnitudes of difference in another, provided the rank positions of data points between groups are identical from one experiment to the next. This is demonstrated with example data below. There is clearly a greater magnitude difference in values between group A and group B, than between group A and group C. Although this can be appreciated from inspecting the data points on the dot plot, it is not reflected in the p-value for Mann-Whitney analysis. This is because the difference between the sums of the rank positions is the same in both instances. As such, several of the p-values were identical between our different experiments.

Figure 15 - Example data demonstrating limitations of Mann-Whitney analysis.

We believe that the Mann-Whitney analysis was appropriate for these experiments, and that the focus of future work should be establishing if there is biological significance for the observed statistically significant phenomena.

The Mann-Whitney test is considered to be more robust than the Student’s t-test in situations such as here using small numbers of animals, in which the presence of a single extreme data point might otherwise result in the spurious attribution of significance. Furthermore, such a rank test is more appropriate in answering the question of whether a difference exists following an intervention, and in what direction. At this point, the magnitude of difference seen when manipulating macrophages in our model is perhaps less relevant until it has been established that a difference does indeed exist.

**Unless otherwise specified, the following conditions apply to our data:**

Dot Plots: Each dot represents a data point for an individual mouse. When depicting lymph node volume or weight, each dot is a cumulative value for 4 lymph nodes per mouse; paired bilateral inguinal and brachial nodes. A single horizontal bar is at the median value. The p-value derives from Mann-Whitney
analysis.

Bar Charts: Each bar represents combined data from several mice from one specified experimental group. The upper limit of the solid bar represents the mean value, and the up-going vertical arm with horizontal bar, the standard error of the mean (SEM). The p-value derives from Mann-Whitney analysis. Statistical significance corresponds to a p-value of less than 0.05.
11 Protocols and results.

We interrogated human biopsies of DLBCL to investigate whether there was correlative evidence supporting a relationship between macrophage numbers and lymphoma growth.

11.1 Interrogating diagnostic human biopsies of DLBCL.

We reviewed the diagnoses of the 218 cases included on the aggressive lymphoma TMA, as recorded in the clinical and histological databases at our centre. Given that the impact of TAM might be different in different types of lymphoma, we chose to exclude those aggressive B-NHL that are variants of DLBCL:

- Primary mediastinal B-cell lymphoma 19 cases
- T-cell/histiocyte rich B-cell lymphoma 16 cases
- HIV-related lymphoma 6 cases
- Primary CNS lymphoma 2 cases
- Lymphoplasmacytic lymphoma 1 case

Additionally, 29 cases were excluded as technically inadequate. There remained 145 DLBCL with at least some data on staining for CD68, CD163 or Ki67. Of these, 62 were female and 83 male. According to the Hans algorithm, 95 were ABC phenotype, and 47 were GCB phenotype. 3 cases could not be assigned to either group. The median age at diagnosis was 60 years (18-88 years). In each analysis were small numbers of cases without complete data from at least 2 cores for the markers of interest so these cases were excluded from analysis.

Example sections are shown overleaf of cores on the DLBCL TMA stained with CD68, and separately with Ki67. These demonstrate high and low scores for both markers.
Figure 16 - Representative sections of DLBCL TMA stained with CD68 (top) and Ki67 (bottom).
In the following plots, each dot represents a score for that marker in an individual patient. This figure is an average of at least 2 cores from each patient biopsy.

Figure 17 - Distribution of scores for CD68 and CD163 staining by area (above) and Ki67 staining by percentage of positive nuclei (below).
We plotted the percentage area of CD68 and CD163 staining for paired samples, the data for each individual biopsy linked by a straight line, as shown in the figure below. These data derive from sequential sections of the same patient cores for each paired set. Although there are individual exceptions, in the majority of cases the percentage area staining for CD163 was less than the percentage area staining for CD68, and this was statistically significant using a two-tailed Mann-Whitney rank analysis.

Figure 18 – Comparison of areas of CD68 and CD163 staining across the whole TMA, with each patient biopsy average score represented by a dot for each marker, joined by a straight line (n=136).
We investigated correlations between the 3 different markers in the following dot plots.

![Dot plots showing correlations between CD68 and CD163 staining, and each macrophage marker and Ki67. A regression line is depicted in red.]

Figure 19 - Correlations between CD68 and CD163 staining (above) and each macrophage marker and Ki67 (below). A regression line is depicted in red.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Number of cases</th>
<th>Probability of non-zero slope of regression line</th>
<th>Spearman rank coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD163 / CD68</td>
<td>136</td>
<td>p = &lt;0.0001</td>
<td>0.6485</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = &lt;0.0001</td>
</tr>
<tr>
<td>CD68 / Ki67</td>
<td>130</td>
<td>p = 0.0036</td>
<td>0.3155</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.0003</td>
</tr>
<tr>
<td>CD163 / Ki67</td>
<td>129</td>
<td>p = 0.0195</td>
<td>0.2287</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p = 0.0094</td>
</tr>
</tbody>
</table>

Table 8. - Statistical outcomes of data analysis.
CD163 and CD68 staining areas were very closely and positively correlated. Both CD68 and CD163 areas were independently positively correlated with the percentage of Ki67 positive nuclei. Overall, tissues with more abundant macrophages exhibited higher proliferation fractions. There were individual cases that did not appear fit this pattern, but the correlation over the whole group was statistically significant.

When analysed separately, GCB and ABC phenotypes of DLBCL maintained the same relationships as in the whole data set (data not shown).

These data were consistent with published data that tissue sections of more proliferative lymphoma subtypes had more frequent macrophages (Hermann et al., 1998). Our data supplements this by demonstrating a clear correlation within a lymphoma subtype, among a much larger number of cases of DLBCL, and by indicating that TAM in DLBCL have features of alternative activation by virtue of expression of CD163. As such, this work further supported the need to investigate the nature of TAM-lymphoma interactions in a living system.
11.2 Investigating macrophage-lymphoma interactions in a living system – the E\textsubscript{\mu}-myc/bcl-2 mouse lymphoma.

11.2.1 Characterisation of E\textsubscript{\mu}-myc/bcl-2 lymphoma.

Experimental protocol:
The original E\textsubscript{\mu}-myc/bcl-2 transgenic mice exhibit variability in the stage of B-cell maturity at which malignant clones are generated. As such, it was important to determine the exact phenotype of the dominant malignant population in any given batch of cells derived from these mice. For this reason, and for subsequent quantification of B-cell populations, we thawed an aliquot of E\textsubscript{\mu}-myc/bcl-2 cells stored in liquid nitrogen, and incubated them for 3 passages over 12 days. Cells were mixed with panels of flow cytometry antibodies at a concentration of 1:50 and fixed in 2% formaldehyde. Controls included unstained cells, isotype controls, and positive controls for each fluorochrome. This enabled purity of the cell population to be assessed. We attempted to corroborate these in vitro findings by flow cytometry of enlarged lymph nodes from C57BL/6 mice 10 days following iv transplant of 2 x 10\textsuperscript{5} E\textsubscript{\mu}-myc/bcl-2 cells.

Data:
After 3 passages over 12 days, E\textsubscript{\mu}-myc/bcl-2 cells exhibited 84% viability (by Trypan blue dye-exclusion) at a concentration of 3.5 x 10\textsuperscript{6} /ml. The vast majority of cells (>85%) were of one phenotype, indicating that this was the dominant population. These cells displayed the immunophenotype CD19+, B220+, IgM+, IgD+ consistent with a mature B-cell phenotype. A minority population had weak or absent expression of surface IgD. All cells were negative for T-cell markers including CD3, CD8 and CD5, and were negative for CSF-1R. Flow cytometry of fresh lymph nodes harvested from mice with advanced lymphoma confirmed these findings, as shown overleaf.
Figure 20 – Gating of live, CD19 positive cells from whole fresh lymph nodes. The CD19 positive portion was selected, and assessed below for expression of surface immunoglobulin.

Figure 21 - Histograms depicting surface IgM and IgD expression of live, CD19 positive cells from the fresh lymph nodes of mice with lymphoma.
There was almost totally mutually exclusive expression of CSF-1R and B220, in the lymph nodes of mice with lymphoma, strongly indicating that malignant B-cells do not express CSF-1R in this model. The low frequency of dual positive cells may represent a subset of DC.

Figure 22 - Mutually exclusive expression of CSF-1R and B220 in fresh lymph nodes of mice with lymphoma. 64.4% = B220+/CSF-1R-, 6.7% = CSF-1R+/B220-, 0.18% = B220+/CSF-1R+, 28.7% = B220-/CSF-1R-.

We were satisfied with these data and so propagated and used the same population of Eµ-myc/bcl-2 lymphoma cells in all subsequent experiments.
11.3 Effects of intravenous liposomal clodronate on normal healthy C57BL/6 mice.

We planned to investigate the effects of macrophage ablation on the progression of lymphoma, using liposomal clodronate (LC). This initial experiment investigated the effects of an intensive regime of LC on normal healthy mice without lymphoma.

Experimental protocol:

Healthy mice without lymphoma were repeatedly injected with 200 µl of LC on days 1, 3, 6 and 10 and sacrificed on day 12.

Figure 23 - Protocol investigating the effects of liposomal clodronate in healthy mice.
Data:

Mice given iv LC had significantly less overall weight gain than untreated mice after 11 days of the above schedule.

Figure 24 - Body weight of mice treated with iv LC (n=5) or untreated (n=4). Plot shows mean values with standard error of the mean (SEM) for each group at each time point.

As demonstrated in the following two figures, there was no statistically significant difference observed in the weights of livers, or volume of lymph nodes.

Figure 25 - Liver weights.
Figure 26 - Lymph node volumes.

However, spleens were significantly smaller in mice treated with iv LC.

Figure 27 - Spleen volumes.
Flow cytometry analysis of spleen and lymph nodes demonstrated a reduced proportion of live cells in the spleens of mice receiving LC, but no difference in the lymph nodes. This is shown in the following two figures. The percentage of live cells was measured by displaying the characteristics of single cell suspensions in terms of forward scatter and side-scatter, and constructing a gate to exclude cells with features of being non-viable.

![Figure 28 - Percentage of live cells in the spleen.](image1)

![Figure 29 - Percentage of live cells in lymph nodes.](image2)
As shown below, mice receiving LC had fewer F4/80 staining macrophages in the lymph nodes than un-manipulated mice, as measured by flow cytometry.

Figure 30 - Flow cytometric measurement of macrophages in lymph nodes of healthy mice with no manipulation (n=4) or iv LC (n=5), percentage of all node cells.
There was no difference in the percentage of B-lymphocytes and T-lymphocytes in the lymph nodes or spleens of treated mice and un-manipulated controls.

![Figure 31](image1.png)

**Figure 31** - Percentage of CD3 positive T-lymphocytes in lymph nodes of normal healthy mice (n=4) or those receiving iv LC (n=5).

![Figure 32](image2.png)

**Figure 32** - Percentage of CD19 positive and B220 positive B-lymphocytes in lymph nodes of normal healthy mice (n=4) or those receiving iv LC (n=5).
Figure 33 - Percentage of CD3 positive T-lymphocytes in the spleen of normal healthy mice (n=4) or mice with iv LC (n=5).

Figure 34 - Percentage of CD19 positive and B220 positive B-lymphocytes in the spleen of normal healthy mice (n=4) or mice with iv LC (n=5).
Immunohistochemical staining of FFPE sections of spleen, liver, lymph node, and bone marrow clearly demonstrated macrophage depletion in the organs of mice treated with liposomal clodronate.

**Figure 35 - Spleen stained with F4/80 and haematoxylin (OM x 40).**

**Figure 36 – Liver stained with F4/80 and haematoxylin (OM x 40).**
In summary, an intensive regime of iv LC depletes macrophages from the bone marrow, lymph nodes, liver, and spleen of otherwise healthy female C57BL/6 mice. This is associated with a substantial degree of cell death within the spleen, and impairment of general wellbeing as evidenced by retardation of normal weight gain over 15 days. B-lymphocyte and T-lymphocyte numbers appear unaffected by this intervention.

Having confirmed macrophage depletion with this regime of LC in healthy mice, we planned to investigate its effects in mice with transplanted Eµ-myc/bcl-2 lymphoma. Firstly we conducted *in vitro* experiments that would reveal whether or not LC was directly toxic to Eµ-myc/bcl-2 cells.
11.4 The impact on lymphoma progression of macrophage depletion with liposomal clodronate.

11.4.1 Liposomal clodronate versus E\textsubscript{\textmu}-myc/bcl-2 cells \textit{in vitro}.

Experimental protocol:
E\textsubscript{\textmu}-myc/bcl-2 lymphoma cells were exposed to LC at varying concentrations in a 96 well plate, and cytotoxicity measured as previously described. Controls were media alone, lymphoma cells with no LC, lymphoma cells with a range of concentrations of liposomal PBS, and a positive control of fully lysed lymphoma cells. In this assay, fluorescence is proportional to cell injury and death.

Data:
E\textsubscript{\textmu}-myc/bcl-2 lymphoma cells cultured \textit{in vitro} were not killed by direct exposure to a wide range of concentrations of LC.

![Graph showing fluorescence vs. concentration of clodronate](image)

**Figure 39 - E\textsubscript{\textmu}-myc/bcl2 cells \textit{in vitro} exposed to LC.**

Having established that LC was not directly toxic to E\textsubscript{\textmu}-myc/bcl-2 cells, we were able to investigate its effects on E\textsubscript{\textmu}-myc/bcl-2 growth \textit{in vivo}, with the reasonable assumption that any impact would be indirect, possibly owing to macrophage depletion.
11.4.2 Effects of liposomal clodronate mediated macrophage depletion on the progression of transplanted Eµ-myc/bcl-2 lymphoma.

11.4.2.1 An intensive regime of liposomal clodronate reduces lymph node volume.

Macrophage depletion with LC was commenced prior to lymphoma transplantation, and repeated throughout the experiment, in an attempt to maximise its impact.

Experimental protocol:

Mice received either iv LC or a vehicle control of liposomal PBS on days -1, 3, 6 and 10, in addition to injection of Eµ-myc/bcl-2 lymphoma cells at day 0. Age-matched mice with transplanted lymphoma acted as an untreated control group.

Figure 40 - Protocol investigating liposomal clodronate in mice with lymphoma.
Data:

By day 12, a reduced total body weight relative to normal healthy mice was seen in all groups that had been injected with lymphoma. Similarly, all mice injected with lymphoma displayed early signs of ill health by Day 12, with slightly reduced spontaneous activity levels, a hunched posture and shallower, more rapid breathing than the normal controls.

Figure 41 - Body weight of mice, untreated (n=4) or transplanted with $2 \times 10^5$ Emu-myc/bcl-2 lymphoma cells, then given iv LC (Lymphoma + LC, n=5) or liposomal PBS (Lymphoma + LP, n=5) or no further injections (Lymphoma only, n=5).
At necropsy it was clear that gross differences were present, and consistent, between the groups. Those injected with lymphoma and no further treatment, or treatment with liposomal PBS, had massive splenomegaly extending to the pelvis, massive hepatomegaly, and widespread lymph node enlargement in all areas. Inguinal, brachial and cervical nodes were highly conspicuous, with intra-abdominal and intra-thoracic node enlargement evident on further dissection. Those mice injected with lymphoma who received LC had spleens and livers of approximately normal size. This group did display widespread lymph node enlargement, but clearly to a lesser degree than those with lymphoma not treated with LC.

**Figure 42** – Macroscopic appearance of partially dissected mice on day 12 following iv injection with Eµ-myc/bcl-2 lymphoma cells demonstrating smaller spleen and lymph nodes in the mice treated with LC.
Figure 43 - Spleen volumes.

Figure 44 - Lymph node volumes.

Figure 45 - Liver weights.
In the liver, lymphoma infiltration, as shown in the figure below when stained with H&E, consisted of conspicuous confluent areas of small to medium sized dark-blue staining cells with a high nuclear to cytoplasmic ratio, congregating around hepatic sinusoids and veins.

Figure 46 - FFPE section of liver 10 days after iv lymphoma, stained with H&E (OM x 20).

F4/80 staining revealed macrophages within areas of lymphoma infiltration.

Figure 47 - FFPE section of liver, 10 days after iv lymphoma stained with F4/80 (OM x 40).
Livers of mice treated with LC clearly showed a reduced cross sectional area of lymphoma, suggesting that reduced organ size was contributed to by reduced lymphoma volume, and could not simply be attributed to a loss of macrophages. The reduction in cross-sectional area of lymphoma was quantified using a graticule overlying low magnification images of FFPE sections, as detailed in my methods, and represented in the bar chart below.

Figure 48 - Area of liver infiltrated by lymphoma in FFPE sections stained with H&E. Number of infiltrated squares was counted for 5 randomly selected LPF per section, one section per mouse and 5 mice per group. Units = cm² per unmanipulated LPF image using Adobe® Photoshop® software.
Figure 49 - Normal mouse liver stained with H&E, FFPE section.

Figure 50 - Liver 10 days following iv lymphoma, FFPE section.

Figure 51 - Liver 10 days following iv lymphoma and intensive iv LC, FFPE section.
Lymphoma transplantation produced complete effacement of normal splenic architecture by malignant lymphocytes. Macrophage depletion with LC produced dramatic reductions in spleen size. Normal splenic architecture was not restored, with high magnification images demonstrating widespread areas of necrosis and small islands of remaining lymphocytes. Splenic necrosis with LC was supported by data from flow cytometry, depicted below.

Figure 52 - Percentage of live cells in the spleen, measured by flow cytometry (n=5 per group). “Untreated” refers to unmanipulated normal healthy mice.
Figure 53 - Normal spleen stained with H&E.

Figure 54 – Spleen stained with H&E day 12 after iv injection of lymphoma.

Figure 55 - Spleen stained with H&E day 12 after iv injection of lymphoma and intensive regime of iv LC.
Bone marrow sections from mice with transplanted lymphoma demonstrated increased overall cellularity. F4/80 staining highlighted plentiful macrophages.

Figure 56 - Normal bone marrow.

Figure 57 - Bone marrow day 12 following iv lymphoma.

Figure 58 - Bone marrow day 12 following iv lymphoma and intensive iv LC.
An intensive regime of LC induced almost total macrophage depletion, associated with obviously decreased overall bone marrow cellularity.

Figure 59 - Bone marrow macrophage frequency, measured by counting of FFPE sections. 5 HPF per mouse, 4 mice per group.
Flow cytometry of blood demonstrated monocyte depletion in mice with lymphoma given LC, compared to vehicle and lymphoma only controls. The figures below show that this was associated with an increase in circulating granulocytes.

![Figure 60](image1.png)

**Figure 60** - Measurement by flow cytometry of blood monocytes (n=6 per group).

![Figure 61](image2.png)

**Figure 61** - Blood granulocytes (n=6 per group).
LC did not reduce the macrophage frequency in lymph node samples to a statistically significant degree.

Figure 62 – Nodal macrophage frequency by flow cytometry (n=5 per group).

However, an overall reduced number of macrophages in lymph nodes of mice with lymphoma given LC is revealed when the macrophage frequency is factored by lymph node volume.

Figure 63 - Nodal macrophage population (n=5 per group).
Splenic macrophage frequency was reduced by LC.

**Figure 64 - Splenic macrophage frequency by flow cytometry (n=5 per group).**

In summary, LC depletes circulating monocytes and bone marrow and tissue macrophages in all organs studied. In mice injected with E\(_{\mu}\)-myc/bcl-2 lymphoma, this correlates with significant, consistent and reproducible reductions in overall lymphoma mass.

To further test whether LC-mediated macrophage depletion might be responsible for attenuated lymphoma growth in this model, we investigated in the following protocol whether this phenomenon operated with a dose effect.
11.4.2.2 The effects of liposomal clodronate on lymph node volume operate with a dose-response relationship.

Experimental protocol:

Healthy female C57BL/6 mice were transplanted with lymphoma on day 0. One group received only a single injection of LC, on Day 6. The extent of macrophage depletion, and lymphoma progression, was compared, in the same experiment, with mice receiving the previous intensive schedule, including LC prior to lymphoma transplantation, and also with those receiving no LC.

Figure 65 - Protocol investigating dose effects of liposomal clodronate in mice with lymphoma.
Data:

Lymph node volumes of mice receiving a single dose of LC occupied an intermediate position between untreated mice, and those receiving an intensive schedule of LC.

![Lymph node volumes](image)

**Figure 66 - Lymph node volumes.**

This intermediate effect on organ size was clearer for spleens.

![Spleen volumes](image)

**Figure 67 - Spleen volumes.**
Liver weights were significantly reduced following a single injection of LC, apparently to the same degree as following an intensive regime of LC.

Figure 68 - Liver weights.

Liver macrophage numbers were reduced by a single dose of iv LC.

Figure 69 - Liver macrophage frequency, measured by counting of FFPE sections. 5 HPF per mouse, 5 mice per group.
Consistent with reductions in liver weight, there was a reduction in cross-sectional lymphoma area to the same degree as in mice receiving the intensive regime of iv LC.

![Liver area infiltrated by lymphoma, measured in FFPE tissue sections. 5 LPF per mouse, 5 mice per group. Units = cm² per unmanipulated LPF image using Adobe® Photoshop® software.](image)

A single injection of LC, delivered 5 days after transplantation of lymphoma, was effective in depleting tissue macrophages and retarding lymphoma progression. Both effects were, overall, less than observed with intensive LC, strongly indicating a dose response between LC and extent of macrophage depletion and lymphoma retardation. These data also add to this body of work by indicating that LC-mediated macrophage depletion impacts on Eµ-myc/bcl-2 lymphoma that has become established in vivo. In the following protocol we investigated whether the apparent attenuation of lymphoma growth with this strategy translated into a survival benefit.
11.4.2.3  Liposomal clodronate treatment does not measurably prolong “survival” in this model.

Experimental protocol:

![Experimental protocol diagram]

Figure 71 - Protocol investigating the survival impact of liposomal clodronate in mice with lymphoma.

Mice injected with lymphoma with or without an intensive LC injection schedule were assessed, at twelve hourly intervals, for signs of illness. Individual mice were sacrificed as soon as they experienced 20% weight loss from the start of the experiment, or decreased spontaneous activity, or any other signs of distress. UK Home Office guidelines were strictly adhered to regarding humane end points in animal experiments, and were enforced by an independent observer, blinded to the experimental design and treatment groups.
Data:

No difference in time to end point was observed between those mice that received intensive LC and those that received no LC.

![Graph showing time to reach UK Home Office limits of survival (n=12 per group).](image)

**Figure 72 – Time to reach UK Home Office limits of survival (n=12 per group).**

We will assess the implications of these data in the discussion. We proceeded to use an alternative, well-validated model to further investigate the impact on macrophage depletion on the progression of Eµ-myc/bcl-2 lymphoma. By using a different approach to achieve macrophage depletion we determined to test whether the effects we have reported with LC-mediated macrophage depletion might be widely applicable rather than idiosyncratic.
11.5 Investigating macrophage depletion and lymphoma progression in the MaFIA mouse model.

We conducted *in vitro* experiments that would reveal whether or not AP20187, the dimerizer agent triggering macrophage depletion in MaFIA mice, was directly toxic to Eµ-myc/bcl-2 lymphoma cells.

11.5.1 Investigating the effects of the MaFIA dimerizer AP20187 on Eµ-myc/bcl-2 lymphoma cells *in vitro*.

**Experimental protocol:**
Eµ-myc/bcl-2 lymphoma cells were exposed to AP20187 at varying concentrations in a 96 well plate, and cytotoxicity measured as previously described. Controls were media alone, lymphoma cells with no AP20187, and a positive control of fully lysed lymphoma cells.

**Data:**
Eµ-myc/bcl-2 lymphoma cells cultured *in vitro* did not appear to be killed by prolonged direct exposure to AP20187 until concentrations of over 1 mM were reached.

![Graph showing cytotoxicity](image)

**Figure 73 - Eµ-myc/bcl2 cells in vitro exposed to MaFIA dimerizer AP20187.**

These data indicate that AP20187 is not directly toxic to Eµ-myc/bcl-2 cells at the concentrations that would be achieved *in vivo*. Product literature states that a complete dose response profile can be obtained with a concentration range of 0.01-100 nM in *vitro*, corresponding to *in vivo* doses in mice of 0.005-10 mg/kg (Clontech, 2012).
11.5.2 Investigating the effects of macrophage depletion on Eµ-myc/bcl-2 lymphoma progression in the MaFIA mouse.

In the light of the in vitro data, we investigated the impact of macrophage depletion in MaFIA mice on the progression of transplanted Eµ-myc/bcl-2 lymphoma.

**Experimental protocol:**

10-week-old MaFIA mice were injected with lymphoma, and then received 5 consecutive once-daily ip injections of either AP20187 dimerizer or its vehicle control, as outlined below.

![Diagram showing experimental protocol](image)

**Figure 74 - Protocol investigating lymphoma progression in MaFIA mice.**

**Data:**

MaFIA mice with lymphoma treated with ip injections of dimerizer had lymph nodes of less weight at sacrifice on day 9 than did sex matched control MaFIA mice with lymphoma given vehicle injections ip for the same schedule. Data were collected from 2 separate experiments with identical protocols.
Macrophage ablation resulted in lower lymph node weights at sacrifice.

Figure 75 - Lymph node weights.

Lymph node CD19+ percentage was unchanged by macrophage ablation.

Figure 76 - Lymph node CD19+ frequency by flow cytometry (vehicle n=10, dimerizer n=8).
The CD19- population was selected and the frequency of CD11b+Ly6G- cells measured, as a reliable indicator of tissue macrophage numbers. There was a substantial and significant reduction in lymph node macrophages.

Figure 77 - Whole fresh lymph node single cell suspensions assessed for macrophages by selecting the CD19- population, then the CD11b+ Ly6G- population.

Figure 78 – Lymph node macrophage ablation in MaFIA mice with lymphoma (vehicle n=10, dimerizer n=8).
Blood was stained and analysed by flow cytometry to assess the expression of Ly6G and Ly6C among the CD19- CD11b+ population, using the following gating schedule.

Figure 79 - CD19+ CD11b+ cells were selected as shown.

Figure 80 - Monocyte subsets were assessed by Ly6G and Ly6C expression.
There was a reduction in the overall number of circulating monocytes and of Ly6C- “patrolling” monocytes, and a rise in Ly6C+ “inflammatory” monocytes.

Figure 81 - Overall blood monocytes (top chart) expressed as a percentage of all CD19-CD11b+ cells. Monocyte subsets (middle and lower chart) expressed also as a percentage of all CD19-CD11b+ cells (vehicle n=10, dimerizer n=8).

These data from the MaFIA model are in agreement with those from using LC; namely that macrophage depletion results in reduced lymphoma growth. To further investigate whether this phenomenon represented a causal relationship, and one that might crucially depend on macrophage phenotype, we performed experiments assessing the impact of supplementing macrophages in the transplantable Eμ-myc/bcl-2 lymphoma.
11.6 Adoptive transfer of bone marrow derived macrophages.

11.6.1 Assessment of purity of bone marrow derived macrophages and *in vitro* phenotype manipulation.

BMDM were prepared from the hind legs of 5 healthy female C57BL/6 mice. 3.58 x 10^8 bone marrow cells generated a total of 1.71 x 10^8 viable macrophages by day 7. BMDM viability was 91%. BMDM purity and the impact of *in vitro* phenotype modulation were assessed by flow cytometry.

BMDM were of high purity, with almost all cells being CD11b+ and Ly6G-. The only exception to this was a conspicuous small minority population of M1 polarised BMDM that expressed Ly6G.

![Flow cytometry plots showing CD11b vs Ly6G for M0, M1, and TAM phenotypes.](image)

**Figure 82** - Percentage of all cultured bone marrow cells that were CD11b+Ly6G- BMDM on Day7: M0 = 98.1%, M1 = 96.4%, TAM = 98.8%.
BMDM universally expressed CSF-1R and F4/80. There was a slightly higher level of F4/80 expression in M1 polarised BMDM than other phenotypes.

Figure 83 - Histograms of CSF-1R and F4/80 expression. X-axis depicts the percentage of maximum cells expressing marker. Unstained BMDM are in red.

There was slightly increased expression of MR in TAM than in M1 BMDM.

Figure 84 - Mannose receptor staining of BMDM.
TAM had decreased intracellular staining of IFN-γ. M1 BMDM demonstrated increased surface expression of MHCII.

Figure 85 - Histograms of BMDM staining by flow cytometry. X-axis depicts percentage of maximum cell staining.

In M0 BMDM and TAM, an LPS boost 6 hours before harvest increased the expression of MHCII, an M1 phenotype marker. Absence of such an increase in M1 BMDM suggested they were already maximally polarised to this phenotype.

Figure 86 - Flow cytometry histograms illustrating the impact on MHCII expression of an LPS boost 6 hr prior to BMDM harvest.
These data show that our protocol generated BMDM of very high purity and that *in vitro* polarised BMDM displayed features typical of their intended phenotypes. The following *in vivo* protocols investigated the impact on lymphoma growth of adoptive transfer of different phenotypes and doses of BMDM. If macrophages do support the growth of lymphoma, then AT of TAM or M2 BMDM might promote lymphoma growth, and AT of M1 polarised BMDM might attenuate it.
11.6.2 Adoptive transfer of bone marrow derived macrophages.
Adoptive transfer experimental protocol 1:
This protocol aimed to establish the tolerability of AT, whether BMDM reached target tissues, and whether there was a gross effect on lymphoma progression of M1 or M2 BMDM.

![Diagram](image_url)

**Figure 87 - Protocol investigating adoptive transfer of macrophages in mice with lymphoma.**

As described in our methods, samples of BMDM were stained *in vitro* with a red fluorescent dye prior to AT, to enable visual confirmation of their infiltration of target tissues.

**Data:**

Iv adoptive transfer of syngeneic BMDM was well tolerated, with no immediate adverse reactions observed. Red fluorescent cells were seen in frozen tissue sections of lymph node and spleen, as shown overleaf.
Figure 88 - Immunofluorescent images of 4 µm tissue sections from mice transplanted with Eµ-myc/bcl-2 lymphoma and adoptively transferred red fluorescent bone marrow-derived macrophages, counterstained with DAPI.
AT of M1 polarised BMDM was associated with reduced lymph node volume compared to untreated controls. AT of M2 polarised BMDM was not associated with any statistically significant change in lymph node volume.

Figure 89 - Lymph node volumes.

There was no significant difference in liver cross-sectional area of lymphoma.

Figure 90 - Liver area infiltrated by lymphoma, measured in FFPE tissue sections. 5 LPF per mouse, 5 mice per group (lymphoma n=6, M1 n=5, M2 n=5). Units = cm² per unmanipulated LPF image using Adobe® Photoshop® software.
Adoptive transfer experimental protocol 2:

Having seen no effect of M2 BMDM we subsequently explored whether macrophage depletion prior to AT might increase their impact, as they might then constitute a higher proportion of the lymphoma-populating macrophages. Partial macrophage depletion with 100 \( \mu l \) of LC 48 hours before AT of \( 1 \times 10^6 \) M2 BMDM was conducted as outlined in the figure below.

Figure 91 - Protocol investigating adoptive transfer of M2 macrophages following liposomal clodronate.
Data:
LC macrophage depletion prior to AT of M2 BMDM did not produce a statistically significant difference in lymphoma progression, in terms of lymph node volume or cross-sectional area of lymphoma in liver sections.

Figure 92 - Lymph node volume.

Figure 93 - Liver area infiltrated by lymphoma, measured in FFPE tissue sections (lymphoma n=6, M2 n=5, LC + M2 n=5). Units = cm² per unmanipulated LPF image using Adobe® Photoshop® software.
Adoptive transfer experimental protocol 3:

The dose of M2 polarised BMDM was increased 10-fold to $1 \times 10^7$ cells, delivered over 2 consequetive days.

Figure 94 - Protocol investigating adoptive transfer of an increased dose of M2 macrophages following liposomal clodronate.

Data:

An increased dose of M2 polarised BMDM did not result in any difference in lymph node weights compared to controls.

Figure 95 - Lymph node weights.
Adoptive transfer experimental protocol 4:

On the basis that potentially pro-tumoural TAM in this model might not strictly conform to an IL-4 stimulated M2 phenotype, we assessed the impact of AT of Eμ-myc/bcl-2 specific TAM. BMDM were polarised with TCM from Eμ-myc/bcl-2 lymphoma cells to generate a TAM BMDM as previously described. AT of $1 \times 10^7$ TAM BMDM was compared to M1 BMDM and lymphoma-only control mice.

![Protocol schematic](image)

Figure 96 - Protocol investigating adoptive transfer of M1 macrophages and TAM in mice with lymphoma.

Data:

AT of $1 \times 10^7$ TAM BMDM resulted in significantly greater lymph node weights than those receiving $1 \times 10^7$ M1 polarised BMDM. This was a consistent and statistically significant phenomenon. These differences remained when lymph node weight was corrected for total body weight, and when it was multiplied by the percentage of lymph node CD19 positive cells as determined by flow cytometry, to give an alternative and perhaps even more accurate reflection of lymphoma burden.
Figure 97 - Lymph node weights.

![Lymph node weights graph]

Figure 98 - Lymph node CD19+ cell frequency, measured by flow cytometry (lymphoma n=8, M1 n=8, TAM n=7).

![Lymph node CD19+ frequency graph]
Figure 99 - Lymphoma burden displayed as a function of lymph node weight and CD19+ percentage (lymphoma n=8, M1 n=8, TAM n=7).

Figure 100 - Lymphoma burden as a function of lymph node weight and percentage of CD19+ cells, normalised to untreated lymphoma at 100%. Horizontal dashed line represents mean value of untreated mice with lymphoma.

These data suggest an impact of AT on the progression of lymphoma, and that phenotype can influence the direction of this effect. The magnitudes of impact are small but consistent and statistically significant. Following this we were keen to use transplanted Eμ-myc/bcl-2 lymphoma as a pre-clinical model to investigate the feasibility and efficacy of a novel therapeutic agent that might reduce TAM populations.
11.7 Investigating CSF-1R inhibition in lymphoma.

11.7.1 Investigating the *in vitro* effects of AZ268 CSF-1R inhibitor on $E_\mu$-myc/bcl-2 cells.

Unpublished data provided by AstraZeneca show that AZ268, a small molecule CSF-1R tyrosine kinase inhibitor, reduces circulating monocyte numbers in healthy mice with a dose response such that 100 mg/kg gives approximately a 50% reduction. In an orthotopic xenograft of a breast cancer cell line (MDA-MB-231), the CSF-1R inhibitor (CSF-1Ri) given twice daily at 75 mg/kg for 18-20 days reduced tissue macrophages by 94%. This was associated with tumour growth inhibition, and 100 mg/kg produced 32% tumour retardation. Other tumour xenograft models were less responsive, even though TAM reduction was seen. There is no published data concerning CSF-1R inhibition in lymphoma models. We explored whether the CSF-1Ri might reduce TAM populations and attenuate lymphoma growth in the transplantable $E_\mu$-myc/bcl-2 lymphoma.

**Experimental protocol:**

$E_\mu$-myc/bcl-2 lymphoma cells were exposed to CSF-1R inhibitor (referred to in figures as CSF-1Ri) at varying concentrations in a 96 well plate, and cytotoxicity measured as previously described. Controls were media alone, lymphoma cells with no CSF-1R inhibitor, and a positive control of fully lysed lymphoma cells.

**Data:**

$E_\mu$-myc/bcl-2 lymphoma cells cultured *in vitro* were not killed by direct prolonged exposure to a wide range of concentrations of CSF-1Ri.

![Figure 101 - $E_\mu$-myc/bcl2 cells *in vitro* exposed to CSF-1R inhibitor.](image)
11.7.2 Investigating the \textit{in vivo} effects of CSF-1R inhibitor at 50 mg/kg for 5 days in mice with lymphoma.

As the CSF-1Ri had no direct effect on the survival of E\textsubscript{\mu}-myc/bcl-2 lymphoma cells \textit{in vitro}, we proceeded to administer it to mice with lymphoma, with the reasonable expectation that any subsequent impact on lymphoma growth might be indirectly mediated, possibly by macrophage depletion, should this occur.

**Experimental protocol:**

Initial experiments used a 50 mg/kg dose of CSF-1Ri, given twice daily by oral gavage for 5 consecutive days following the establishment of transplanted E\textsubscript{\mu}-myc/bcl-2 lymphoma.

![Protocol investigating low dose CSF-1R inhibition in mice with lymphoma.](image)

**Figure 102 - Protocol investigating low dose CSF-1R inhibition in mice with lymphoma.**
Treatment was well tolerated. There was a statistically significant reduction in lymph node and liver weights in mice with lymphoma treated with CSF-1Ri, compared to vehicle and untreated control mice with lymphoma. Administration of vehicle by twice daily oral gavage resulted in a reduced lymph node weight compared to untreated controls.

Figure 103 - Lymph node weights.

Figure 104 - Liver weights.
FFPE sections of lymph node were stained with the macrophage marker F4/80 and haematoxylin.

Figure 105 - Lymph node stained with F4/80 and haematoxylin on day 10 following iv lymphoma and 2x daily oral gavage of control vehicle (OM x 40).

Figure 106 - Lymph node stained with F4/80 and haematoxylin on day 10 following iv lymphoma and 2x daily oral gavage of CSF-1R inhibitor (OM x 40).
There was a quantifiable reduction in the number of macrophages in tissue sections of lymph nodes of CSF-1R inhibited mice with lymphoma.

Figure 107 – Lymph node macrophage frequency measured in FFPE sections stained with F4/80. Data represents 5x HPF per mouse and 4 mice per group.

There was a reduced cross-sectional area of lymphoma infiltrating the liver.

Figure 108 – Liver area infiltrated with lymphoma in FFPE tissue sections. Data represents 5x HPF per mouse and 4 mice per group. Units = cm² per unmanipulated LPF image using Adobe® Photoshop® software.

In summary, we observed a reduction in macrophage numbers and the mass of lymphoma bearing organs with CSF-1Ri compared to untreated or vehicle treated mice. The vehicle alone produced a reduction in lymph node weight,
with no significant effect on macrophage numbers, liver weight, or cross-sectional lymphoma area.

**Expression of inflammatory genes in whole lymph nodes.**

RNA was extracted from whole, unsorted lymph nodes of mice treated with CSF-1Ri and vehicle treated controls. qRT-PCR was performed for a panel of genes chosen to reflect different infiltrating immune cell populations and the cytokines typically associated with inflammatory responses.

<table>
<thead>
<tr>
<th>Gene group by cell/function</th>
<th>Specific genes assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocyte/macrophage</td>
<td>F4/80, CSF-1R, CD14</td>
</tr>
<tr>
<td>DC</td>
<td>CD208</td>
</tr>
<tr>
<td>T-cell</td>
<td>CD2, CD8a</td>
</tr>
<tr>
<td>Treg</td>
<td>FOXP3</td>
</tr>
<tr>
<td>NK cell</td>
<td>NK1.1</td>
</tr>
<tr>
<td>Th1 cytokines</td>
<td>IL-12b</td>
</tr>
<tr>
<td>Th2 cytokines</td>
<td>IL-10, IL-6</td>
</tr>
<tr>
<td>Chemokine receptors</td>
<td>CXCR1, CCR3, CCR1</td>
</tr>
</tbody>
</table>

*Table 9 - Genes selected for measurement by qRT-PCR.*
Data:

There was reduced gene expression, of CSF-1R and F4/80, and increased expression of CD14 and FOXP3 within the lymph nodes, with CSF-1R inhibition compared to vehicle. All other alterations in gene expression were less than 2-fold changes.

Figure 109 - Fold changes in gene expression at day 10 in lymph nodes of mice with lymphoma given 50 mg/kg twice daily CSF-1Ri for 5 days (n=5), compared to vehicle controls (n=5).

These data suggest that AZ268 CSF-1Ri is well tolerated and produces a degree of macrophage depletion in lymph nodes. Gene expression data are consistent with these findings, with a reduction in the macrophage specific genes F4/80 and CSF-1R. Subsequently we investigated the impact of delivering a higher dose of AZ268 CSF-1Ri for a prolonged period of time. The unexpected, small, but statistically significant reduction in lymph node weight with gavage of the vehicle will be considered further in the discussion.
11.7.3 Investigating the *in vivo* effects of AZ268 CSF-1R inhibitor at 100 mg/kg for 10 days in mice with lymphoma.

**Experimental protocol:**

In subsequent experiments, the dose of CSF-1R inhibitor was doubled to 100 mg/kg, and the duration of therapy increased, starting with the transplantation of Eµ-myc/bcl-2 lymphoma cells, and with a final dose 1 hour before sacrifice.

![Figure 110 - Protocol investigating prolonged higher dose CSF-1R inhibition in mice with lymphoma.](image)
Data:

The lymph node weight was recorded for each mouse, combining bilateral inguinal and brachial nodes.

<table>
<thead>
<tr>
<th>CSF-1Ri treated</th>
<th>Vehicle control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight</td>
</tr>
<tr>
<td></td>
<td>(g)</td>
</tr>
<tr>
<td>19.99</td>
<td>0.0439</td>
</tr>
<tr>
<td>20.76</td>
<td>0.0578</td>
</tr>
<tr>
<td>14.38</td>
<td>0.0374</td>
</tr>
<tr>
<td>18.03</td>
<td>0.0538</td>
</tr>
<tr>
<td>18.73</td>
<td>0.0572</td>
</tr>
<tr>
<td>18.03</td>
<td>0.0547</td>
</tr>
<tr>
<td>19.25</td>
<td>0.0583</td>
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<td></td>
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</table>

Table 10 - Data from a representative experiment of prolonged CSF-1R inhibition in lymphoma.

There was a substantial reduction in lymph node weight in mice treated with this prolonged higher dose schedule of CSF-1R inhibitor. This was a consistent finding in 4 separate protocols. Data, represented graphically overleaf, is from a representative experiment.
Figure 111 - Lymph node weights.

There was no significant difference in total body weight.

Figure 112 - Total body weight at sacrifice.
A substantial difference was maintained when lymph node weight was corrected for body weight.

Figure 113 - Lymph node weight corrected for total body weight.

The frequency of lymph node CD19 positive B-lymphocytes was identical.

Figure 114 - Lymph node frequency of CD19 positive cells.
Lymphoma burden, expressed in different ways, remained statistically significantly different.

Figure 115 - Lymphoma burden expressed as a function of lymph node weight and frequency of CD19 positive cells.

Figure 116 - Lymphoma burden expressed as function of lymph node weight corrected for total body weight, and frequency of CD19 positive cells.
Lymph node macrophage frequency was reduced in mice with lymphoma receiving CSF-1R inhibition, on the basis of CD11b+Ly6G- cells, and also F4/80 positive cells.

**Figure 117** - Lymph node CD11b+Ly6G- macrophages frequency of all lymph node cells, measured by flow cytometry (vehicle n=8, CSF-1Ri n = 7).

**Figure 118** - Frequency of F4/80 positive macrophages in lymph nodes, by flow cytometry, percentage of CD19- cells (vehicle n=8, CSF-1Ri n = 7).
The overall lymph node macrophage population was markedly reduced, as estimated by multiplying macrophage frequency by measures of lymph node weight.

Figure 119 - Macrophage burden expressed as a function of lymph node weight and frequency of F4/80 positive cells (vehicle n=8, CSF-1Ri n = 7).

Figure 120 - Macrophage burden, expressed as a function of lymph node weight and frequency of CD11b+Ly6G- macrophages by flow cytometry.
There was clearly a statistically significant reduction in TAM and lymphoma burden in mice with lymphoma treated with 10 days of CSF-1R inhibition with AZ268 at 100 mg/kg, compared to vehicle controls. This was a consistent finding in repeated experiments, and appeared to be of greater magnitude than with the previous lower dose and duration schedule.

Blood cells, plasma and lymph nodes were analysed in a series of protocols to elucidate some of the cellular and biochemical consequences of 10 days of CSF-1R inhibition at 100 mg/kg in this model.
11.7.4 Investigating the consequences of CSF-1R inhibition in transplantable Eμ-myc/bl2 lymphoma.

In an attempt to elucidate the consequences of CSF-1R inhibition, and the mechanisms that might be responsible for such consequences, the following investigations were undertaken:

1) Further flow cytometry of blood cells and lymph nodes.

2) Assessment of lymphoma cell proliferation.

3) Measurement of circulating inflammatory cytokines.
11.7.4.1 Flow cytometry of blood cells and lymph nodes.

Multiple protocols were repeated, administering CSF-1R inhibition at 100 mg/kg or vehicle twice daily for 10 days to normal healthy mice, and mice with lymphoma. A variety of multi-colour fluorescent antibody panels were optimised and used to determine changes in the cellular composition of blood and lymph nodes, allowing estimation of malignant B-cell and immune-infiltrating cell populations. I investigated markers in addition to F4/80, whose expression is CSF-1 dependent (Hume et al., 1988) and so may not a reliable indicator of total macrophage numbers following administration of a CSF-1R inhibitor.

**Mice without lymphoma.**

In the blood of normal healthy mice, prolonged CSF-1R inhibition had no substantial effect on the frequency of B220+ cells.

![Flow cytometry plot of live-gated blood cells stained for B220 and CSF-1R. Data represents pooled samples from 4 mice per group.](image)

**Figure 122 -** Flow cytometry plot of live-gated blood cells stained for B220 and CSF-1R. Data represents pooled samples from 4 mice per group.
In the lymph nodes of normal healthy mice, prolonged CSF-1R inhibition resulted in a reduced CSF-1R+ population, but no substantial change in the B220+ population.

Figure 122 - Flow cytometry plot of live-gated lymph node cells stained for B220 and CSF-1R. Data represents pooled samples from 4 mice per group.
Mice with lymphoma.

The blood of mice with lymphoma contained a greatly increased frequency of B220+ cells. This fell slightly with CSF-1R inhibition. The frequency of CSF-1R+ cells also rose substantially with advanced lymphoma, but did not appear to fall significantly with CSF-1R inhibition in this pooled data.

![Flow cytometry plot of live-gated blood cells stained for B220 and CSF-1R](image)

Figure 123 - Flow cytometry plot of live-gated blood cells stained for B220 and CSF-1R. Data represents pooled samples from 5 mice per group.

This is likely to reflect a large increase in the proportion of both malignant B-cells and monocyte/macrophages with the transplantation and progression of lymphoma. We investigated the increase in circulating lymphocytes in later experiments by inspecting the morphology of serial blood films during lymphoma progression. The more subtle possible decrease in circulating malignant B-cells and lack of change in monocyte/macrophages shown by this data from pooled samples warranted further examination with repeated measurement in a larger sample size. The data that follows derives from such experiments.
In mice with lymphoma treated with CSF-1Ri, compared to vehicle control, there was no change in the frequency of CD3+ T-lymphocytes in the blood.

**Figure 124 - Frequency of blood CD3+ cells by flow cytometry (n=8 per group).**

There was a reduced CD19 positive B-lymphocyte frequency in the blood.

**Figure 125 - Frequency of blood CD19+ cells by flow cytometry (n=8 per group).**
There was a clear shift in the relative balance of CD11b+ myelo-monocytic cells. CSF-1R inhibition resulted in an increased population of granulocytes (CD11b+Ly6G+) and a reduced population of monocytes (CD11b+Ly6G-).

Figure 126 - Frequency of granulocytes and monocytes in the blood by flow cytometry, expressed as a percentage of CD19- live cells (n=8 per group).
F4/80 staining confirmed that CD11b+Ly6G- monocytes expressed this monocyte-macrophage surface marker, and CD11b+Ly6G+ cells did not. This finding supported the statement that CSF-1Ri resulted in a true reduction in monocytes in the blood, and is displayed in the figure below.

Figure 127 - Histogram displaying relative expression of F4/80 by blood monocytes and granulocytes, representative data.

The impact of prolonged CSF-1R inhibition on monocyte subsets in the blood was studied using the following flow cytometry gating procedure.

Figure 128 - Selection of blood CD19-CD11b+Ly6G- monocytes (black box) for expression of Ly6C (red box) or not (orange box), representative data.
No significant difference was seen in monocyte subsets in the blood.

Figure 129 – “Patrolling” monocyte frequency as a percentage of CD19-CD11b+Ly6G- cells in the blood (n=8 per group).

Figure 130 – “Inflammatory” monocyte frequency as a percentage of CD19-CD11b+Ly6G- cells in the blood (n=8 per group).
11.7.4.2 Assessment of lymphoma cell proliferation *in vivo*.

Experimental protocol:

The proliferation of lymphoma cells was assessed in mice at the end of the protocol for prolonged CSF-1R inhibition. As described in the methods section, ip injections of BrdU were given to all mice 2 hours before sacrifice. The proportion of cells incorporating BrdU was measured by flow cytometry.

Data:

A significantly smaller percentage of CD19+ B-cells incorporated BrdU in the lymph nodes of CSF-1R inhibited mice with lymphoma than in the lymph nodes of vehicle controls with lymphoma.

![Figure 131 – Lymph node frequency of proliferating lymphoma cells expressed as percentage of CD19+ cells incorporating BrdU (n=7 per group).](image)
There was no such difference detected in the blood.

![Graph showing blood frequency of proliferating lymphoma cells expressed as percentage of CD19+ cells incorporating BrdU (n=7 per group).](image)

**Figure 132** - Blood frequency of proliferating lymphoma cells expressed as percentage of CD19+ cells incorporating BrdU (n=7 per group).

These data show that within the lymph nodes, but not in the blood, CSF-1Ri is associated with decreased B-cell proliferation.

We proceeded to measure changes in the levels of circulating inflammatory cytokines, comparing normal healthy mice to mice with advanced lymphoma, to mice with lymphoma treated with CSF-1Ri. The intention was to reveal aspects of mechanisms that might link CSF-1Ri, reductions in monocyte and macrophage numbers, and reductions in lymphoma burden and B-cell proliferation.
11.7.4.3 Measurement of circulating inflammatory cytokines.

Experimental protocol:

Plasma cytokines were measured in the blood of healthy mice, and mice in both the treatment and control groups at the end of the protocol for prolonged CSF-1R inhibition. The following cytokines were measured, as detailed in the methods section:

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Lower limit of detection (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>0.38</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.75</td>
</tr>
<tr>
<td>IL-6</td>
<td>4.5</td>
</tr>
<tr>
<td>IL-10</td>
<td>11</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>35</td>
</tr>
<tr>
<td>CXCL1</td>
<td>3.3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 11 - Measured cytokines and their lower limits of detection (LLD).

In addition, plasma levels of CSF-1 were measured in healthy MaFIA mice, those with advanced lymphoma, and MaFIA mice with advanced lymphoma treated with macrophage depleting dimerizer injections.
Data:

Plasma CSF-1 levels were higher in mice transplanted with lymphoma 10 days previously, than in healthy mice. Macrophage depletion with dimerizer in MaFIA mice did not have any significant effect on these raised levels.

Figure 133 - Plasma CSF-1 levels in normal healthy MaFIA mice (n=6), MaFIA with advanced lymphoma (n=4) and MaFIA with lymphoma and macrophage depleting dimerizer injections (n=3).
IL-6 levels were undetectable in healthy mice, and rose substantially with lymphoma progression, and fell with CSF-1R inhibition.

Figure 134 - Plasma IL-6 concentrations in C57BL/6 mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).

IL-10 levels rose substantially with lymphoma and were unaffected by CSF-1R inhibition.

Figure 135 - Plasma IL-10 concentrations in C57BL/6 mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).
CXCL1, which is expressed by macrophages, neutrophils and epithelial cells and has neutrophil chemoattractant properties, rose substantially with CSF-1R inhibition in mice with lymphoma.

![Graph showing CXCL1 levels in different conditions](image1)

**Figure 136 -** Plasma CXCL1 concentrations in mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).

IL-12p70 levels did not change with statistical significance.

![Graph showing IL-12p70 levels in different conditions](image2)

**Figure 137 -** Plasma IL-12p70 concentrations in C57BL/6 mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).
TNF-α levels rose with lymphoma and were unaffected by CSF-1R inhibition. Absolute levels were low but above the LLD for the assay.

**Figure 138** - Plasma TNF-α concentrations in mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).

IL-1β readings were mostly below the LLD, although a statistically significant rise was detected with lymphoma, and fall with CSF-1R inhibition.

**Figure 139** - Plasma IL-1β concentrations in mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).
IFN-γ levels rose with CSF-1R inhibition. The absolute levels were low, but above the LLD for the assay.

Figure 140 - Plasma IFN-γ concentrations in C57BL/6 mice with no manipulation (n=5), with lymphoma (n=5) and with lymphoma and CSF-1R inhibition (n=6).

We will interpret these data in the discussion.

In the light of our data indicating a potentially beneficial effect of CSF-1R inhibition with AZ268 in this model of lymphoma, we were interested to explore any additional benefit when used in combination with traditional cytotoxic chemotherapy. The following two protocols investigated the impact of macrophage depletion following chemotherapy, and before chemotherapy.
11.1 Combination with chemotherapy.

11.1.1 Macrophage depletion following cyclophosphamide.

Experimental protocol:
According to the protocol below, C57BL/6 mice with transplanted and established lymphoma were given a single 300 mg/kg ip dose of cyclophosphamide on day 8, followed by CSF1-R inhibitor or vehicle until survival end point was reached. CSF-1R inhibitor 100 mg/kg or vehicle was given twice daily by oral gavage. The aim was to assess impact on time to relapse, and time to reach survival end point as defined by Home Office limits.

![Protocol diagram](image.png)

**Figure 141 - Protocol investigating chemotherapy followed by CSF-1R inhibition in mice with lymphoma.**

Lymphoma progression prior to cyclophosphamide, and in relapse, was assessed subjectively by palpation of inguinal lymph nodes and spleen, and objectively by analysing peripheral blood smears taken from one mouse in each group each day.

**Data:**
All mice became acutely unwell approximately 1 hour after the injection of 300 mg/kg of cyclophosphamide, They developed a hunched posture with reduced spontaneous movement, and so were observed carefully as they appeared to recover fully over the next 2 hours. Consequently, it was decided that in future
protocols the maximum single dose of cyclophosphamide should be reduced to 200 mg/kg.

Blood film analysis, shown overleaf, revealed an increasing number of large irregular lymphoid cells, reaching a peak by day 7, immediately prior to cyclophosphamide. Pancytopenia was evident from day 11 to day 13. A small number of irregular lymphoid cells, as well as some immature myeloid cells, were seen on day 14. The number of large irregular lymphoid cells increased by day 17. Once daily blood film analysis did not reveal any difference in time to leukaemic relapse between the treatment groups. Daily palpation of spleens and inguinal lymph nodes failed to demonstrate any difference in time to macroscopic relapse between the groups.
Figure 142 – Representative blood smears stained with modified Wright's stain (OM x 2.5).
Total body weights of 4 mice dropped by more than 20% during this pancytopaenic phase, and so they were culled. These mice, 2 from the CSF-1R inhibition group and 2 from the vehicle control group, had no organomegaly or lymph node enlargement at this time. On day 15, total body weights rose in all mice except one, which recorded weight gain on day 16. Nucleated blood cell numbers started to rise in the peripheral blood at the same time.

One mouse in the CSF-1R inhibition group became unwell on day 17 and was culled, with evidence of lymphoma relapse. The majority of mice in both groups became unwell and were culled on days 18 and 19, with none surviving beyond day 20.

![Graph showing total body weight changes over days for mice in both groups.](image)

**Figure 143 - Average body weights of mice in each group (n= 10 per group).**
There was no statistically significant difference in time to survival end-point between the groups.

Figure 144 - Time taken to reach Home Office guidelines limits of survival (n=10 per group).
11.1.2 Macrophage depletion prior to cyclophosphamide.

Experimental protocol:

As depicted in the protocol below, mice with lymphoma were subjected to macrophage depletion either with 5 days of oral CSF-1R inhibition or a single 200 µl iv dose of LC on day 3, followed by cyclophosphamide ip on day 5. Control mice received CSF-1R inhibitor vehicle for 5 days followed by cyclophosphamide on day 5. A relapse end-point for the whole protocol was declared when one mouse had easily palpable splenic and lymph node enlargement. At this point, all mice in all groups were sacrificed, and lymphoma progression assessed.

![Protocol investigating cyclophosphamide after macrophage depletion in mice with lymphoma.](image)

Figure 145 - Protocol investigating cyclophosphamide after macrophage depletion in mice with lymphoma.
Data:

All mice in all groups had macroscopic evidence of relapse with splenic and lymph node enlargement. There were no statistically significant differences between the groups with respect to lymph node weight or lymph node cell count.

Figure 146 - Lymph node weights of mice exposed to macrophage depletion or vehicle prior to cyclophosphamide.

Figure 147 - Lymph node cell counts.
Flow cytometry of lymph nodes did not detect any difference between the groups with respect to the size of the CD19 positive population in the lymph nodes, and no difference emerged when factoring this figure by lymph node cell count or weight.

Figure 148 - Frequency of lymph node CD19 positive cells by flow cytometry (Vehicle n=7, CSF-1Ri n=7, LC n=8). No statistically significant differences detected.

Figure 149 – Lymphoma burden in lymph nodes expressed as function of lymph node total cell count (by Trypan-blue exclusion assay) and frequency of CD19 positive cells.
Figure 150 - Lymphoma burden in lymph nodes expressed as function of lymph node weight and frequency of CD19 positive cells.
There was no significant difference in B-cell BrdU incorporation between the groups.

Figure 151 - Frequency of proliferating CD19+ cells expressed as percentage of CD19+ cells incorporating BrdU (Vehicle n=7, CSF-1Ri n=7, LC n=8).

All groups had low percentages of myelo-monocytic cells in the lymph nodes, as measured using CD11b staining. There was a statistically significant, but low magnitude, reduction in CD11b+ cells in the lymph nodes of mice treated with CSF-1R inhibition prior to cyclophosphamide, compared to other groups.

Figure 152 - Frequency of CD19-CD11b+ cells in whole lymph nodes by flow cytometry (Vehicle n=7, CSF-1Ri n=7, LC n=8).

These data will be interpreted in the following discussion.
12 Discussion.

12.1 Critical interpretation of data.

12.1.1 Interrogation of human DLBCL biopsies.

Interrogation of a large number of diagnostic, treatment naïve DLBCL biopsies contributed to existing evidence that TAM in lymphoma might be alternatively activated and are associated with the promotion of lymphoma growth. The tight correlation between the area of tissue staining with the universal human macrophage marker CD68 and the area staining with CD163, a macrophage specific marker of alternative activation and the M2 phenotype, suggests that nearly all macrophages in DLBCL have features of alternative activation in keeping with an M2-like phenotype. It seems reasonable to have made the assumption that macrophage area is directly proportional to macrophage number. Expert histopathologist review (AL) could discern no difference in macrophage size between different patient samples. As such, numbers of CD68 staining macrophages, and numbers of CD163 staining macrophages, both positively correlated with tumour proliferation, as defined by Ki67 staining. This is contrary to earlier published findings of no correlation between CD68 and Ki67 staining in 176 cases of DLBCL (Hasselblom et al., 2008). Referring back to our introduction, CD163 is not only considered a marker of alternative activation in non-lymphoid cancers (Mantovani et al., 2004b). CD163 staining macrophages were associated with increased angiogenesis in FL (Clear et al., 2010) and the promotion of malignant B-cell survival through the secretion of APRIL in gastric MALT lymphoma (Munari et al., 2011), as well as in recent months, with a poor outcome in DLBCL, via unknown mechanisms (Wada et al., 2012). It is therefore entirely plausible, and consistent with our data, that similar processes are occurring in DLBCL, such that CD163 positive macrophages promote the proliferation of DLBCL.

This correlative data reinforced the need to use an animal model in an attempt to reveal whether this might reflect an important functional relationship between TAM and lymphoma. Additionally, such work might give an indication as to the direction and strength of such a relationship, and reveal the molecular and genetic mechanisms responsible. The statistically significantly smaller area represented by CD163 than by CD68 staining in most cores and overall might
represent the fact that CD163 staining macrophages with features of alternative activation are a subset of CD68 positive macrophages. This is biologically plausible, and the acquisition of CD163 expression may represent the acquisition of an alternatively activated phenotype that may be dependant upon maturity or location within the lymphoma microenvironment. A caveat to this is the recognition that although CD163 and CD68 are both membrane bound antigens, it is possible that an unidentified artefact of the IHC process results in reduced areas of CD163 compared to CD68 positivity for any given cell.

It is interesting to speculate how these data relate to the clinical outcomes of the patients included in this TMA by Coutinho et al., who reported in abstract form that abundant CD68+ cells correlated with an improved prognosis following treatment (Coutinho et al., 2011). It may be that more proliferative cases, although more aggressive in their natural history, are more sensitive to cytotoxic chemotherapy. Inconsistent with this suggestion is that the number of CD163+ cells appeared to have no correlation with survival, yet in our analysis correlated with proliferation. A variety of different therapies were used in these patients, some being first treated in the 1970's, and 33% of all patients receiving rituximab. We speculate that subsequent analysis might reveal that the direction and strength of association between macrophage numbers and survival in this cohort is related to the specific treatment received.

### 12.1.2 Interpretation of Liposomal Clodronate data.

Our data indicate that LC depletes monocytes and macrophages in all tissues studied. In the blood, the percentage of CD11b+Ly6G+ granulocytes rises significantly in concert with monocyte depletion. These changes are associated with reduced volume and weight of lymphoma bearing tissues at a set time point. Several findings strongly suggest that this reflects a true reduction in lymphoma at this time point. Firstly, there is a greatly reduced cross-sectional area of lymphoma infiltrating sections of liver. Secondly, lymph nodes appear heavily and diffusely infiltrated with lymphoma in all mice with lymphoma, regardless of whether or not they were exposed to LC. As such, lymph node volume and weight will be related to the number of infiltrating lymphoma cells. Of note, administration of iv LC appeared to cause a greater degree of macrophage depletion and reduction in lymphoma growth in the liver, spleen and bone marrow than in the lymph nodes. It seems reasonable to speculate
that this might reflect respective differences in tissue penetration of liposomes; being far greater in more vascular tissues and less in lymph nodes.

Administration of liposomal PBS \textit{in vivo} neither depletes macrophages, nor is associated with a reduction in the volume or weight of lymphoma bearing tissues, nor a reduction in cross-sectional area of lymphoma in liver or lymph nodes.

\textit{In vitro} LDH release assays indicate that lymphoma cells in isolation are not damaged or killed by even very high concentrations of LC. This supports the notion that LC might mediate attenuation of lymphoma proliferation via indirect means, potentially via macrophage depletion.

An intensive regime of \textit{iv} LC did not prolong the survival of mice with transplanted lymphoma. We postulate several potential reasons for this. These are outlined below:

1) \textbf{The dynamic of the disease and assessment of end-point.}

The transplanted lymphoma is highly proliferative, growing rapidly and causing obvious signs of sickness only 12 days following initial injection. As such, although we observed a consistent and statistically significant reduction in lymphoma growth by day 10 or day 11, this difference may not have been sufficient to prolong survival to an extent that was measurable given our experimental protocol. The end-point was not actually death from lymphoma, rather, and appropriately for animal experiments, signs of any deterioration and suffering, as dictated by UK Home Office regulations. This was assessed every 12 hours, and no difference was discernable. It may be that with a more indolent model of lymphoma, and greater numbers of mice, a “survival” difference might be seen.

2) \textbf{Tissue penetration of LC.}

\textit{Iv} LC will not have penetrated the CNS, being unable to cross an intact blood-brain barrier (Bauer et al., 1995). We have no evidence to suggest that this is a critical site for lymphoma progression in this model, but it is possible that although lymphoma growth was retarded in the lymph nodes, liver and possibly spleen, it may have continued to progress unhindered in the CNS, contributing to the signs of ill health that determined the “survival” end point.
3) Depletion of physiological monocytes and macrophages.

LC depletes physiological monocytes and macrophages, and therefore must impact on important homeostatic processes, and also potentially on any adaptive and beneficial elements of the response to disseminated lymphoma. For instance, phagocytosis of apoptotic bodies, acute responses to pathogens, coordinating innate and adaptive immune responses to pathogens, and iron homeostasis will all be compromised. We speculate that an intensive regime of LC with widespread and profound macrophage depletion contributes to signs of illness, that in conjunction with an albeit more slowly progressing lymphoma, results in the attainment of “survival” end point at the same time as control mice with lymphoma. This does not necessarily argue against our hypothesis that TAM promote lymphoma, but does suggest that an intensive regime of iv LC would not be a safe therapeutic strategy. Nonetheless, experiments with LC were consistent with the theory that macrophages support the growth of lymphoma.

12.1.3 Interpretation of MaFIA mouse data.

Our data from the MaFIA mouse model are broadly supportive of our hypothesis, and of the findings with LC. The LDH release assay found progressive lymphoma cell cytotoxicity in vitro above 1 mM concentration of dimerizer AP20187. This certainly suggests that direct lymphoma cell killing is a consequence of prolonged very high dose AP20187, yet such concentrations are unlikely to be achieved or maintained in vivo, and are far in excess of the nanomolar concentrations required to effect macrophage cell death. According to the data of Burnett et al., our schedule will have begun to deplete macrophages from 24 hours after the third injection of dimerizer, this being day 4 of the in vivo protocol. Monocyte and macrophage re-population of bone marrow and spleen would not be expected to begin until 7 days after the last injection of dimerizer, this being day 12 of the in vivo protocol, and after the day of elective sacrifice. We saw partial monocyte and macrophage depletion in the blood and lymph nodes at sacrifice on day 9.

Monocyte depletion in the blood of MaFIA mice with lymphoma treated with dimerizer was almost entirely due to loss of Ly6C- “patrolling” monocytes, with a relative increase in Ly6C+ “inflammatory” monocytes. This finding might support
the theory that monocyte subsets are a maturation series. If we assume that all monocytes and macrophages were killed in equal proportions by dimerizer induction, then the less mature, shorter lived and so higher turnover, "inflammatory" monocytes might be expected to be the first to repopulate the blood on recovery.

Monocyte and macrophage depletion was associated with a significantly reduced lymph node weight and CD19 positive population by flow cytometry. This is supportive of the hypothesis that TAM promote the growth of lymphoma. By demonstrating this using the MaFIA mouse, we have provided further evidence that the effects seen with LC-mediated macrophage depletion were neither idiosyncratic nor an artefact.

Of note, Burnett et al. found that an identical schedule of dimerizer administration failed to deplete macrophages in the lymph nodes of healthy MaFIA mice, whilst depleting most macrophages in the bone marrow and spleen (Burnett et al., 2004). The partial but consistent and statistically significant macrophage depletion we observed might itself provide insights regarding the accumulation of TAM in growing lymphoma. For this to have occurred, we speculate that either or both of two phenomena may have occurred:

1) Changes in the vascularity of lymph nodes occurred with the dissemination and growth of lymphoma, such that the dimerizer was able to penetrate to a far greater extent than in healthy lymph nodes, thus effecting macrophage cell death.

2) The dimerizer was still unable to penetrate lymph nodes during lymphoma dissemination and spread. Rather, the partial macrophage depletion in lymph nodes during lymphoma progression can be accounted for by the profound loss of monocytes in the bone marrow and spleen, so reducing the circulating pool from which new TAM can be recruited. If this were so, it also suggests that monocyte recruitment plays a significant role in the increase in numbers of TAM seen in lymph nodes with growing lymphoma. By inference, this suggests that local macrophage proliferation can only play a partial role in the increase in TAM numbers in this model.
12.1.4 Interpretation of adoptive transfer data.

Analysis by flow cytometry demonstrated that the protocol we used generated BMDM of very high purity, with almost all cells expressing CD11b, F4/80 and CSF-1R. Phenotype modulation appeared consistent with the literature, with M1 BMDM having relatively higher expression of MHCII (Movahedi et al., 2010) and IFN-\(\gamma\), suggestive of an antigen-presenting and pro-inflammatory phenotype. TAM BMDM had relatively increased expression of MR and decreased expression of IFN-\(\gamma\), consistent with an alternatively activated phenotype.

AT BMDM labelled \textit{in vitro} with a red fluorescent dye were visualised in post-mortem specimens of lymph nodes and spleen. Having been stained \textit{in vitro} and repeatedly washed to prevent injection of dye into recipient mice, these red fluorescent cells likely represent successful adoptive transfer, and not \textit{in vivo} staining of resident macrophages. The main differential explanation for these images is that AT BMDM containing red fluorescent dye reached tissues but did not survive, with their cell debris being phagocytosed by resident tissue macrophages.

\textit{In vivo} AT data suggest that supplemental M1 macrophages slow the growth of lymphoma and M2 macrophages do not. Supplemental BMDM polarised with TCM (TAM polarised) appeared to invigorate the growth of lymphoma \textit{in vivo}.

Together, these findings suggest that:

1) Macrophage phenotype influences lymphoma progression.

2) M1 phenotype attenuates lymphoma progression.

3) M2 phenotype has no discernable gross effect on lymphoma progression.

4) TAM phenotype is pro-tumoural and distinct from either the M1 or M2 phenotype.

This would appear to support the hypothesis that TAM promote lymphoma growth in this model. This may well be the case. These experiments were repeated on several occasions and the differences between the groups are consistent, reproducible and although small magnitude, statistically significant. It is conceptually attractive to embrace such conclusions. However, it is absolutely necessary to consider several caveats when interpreting these data:
1) The proportion of injected BMDM that reach the lymph nodes is small; the majority accumulate within the pulmonary vasculature (Andreesen et al., 1998).

2) The proportion of TAM in lymph nodes that derive from injected BMDM is likely to be small in our experiments.

3) Macrophage phenotype is plastic and may well be modified further *in vivo*.

These three points imply that it might be unlikely that such a relatively small number of AT BMDM could cause a difference in lymphoma growth that would be discernable in such an aggressive model of lymphoma. In other words, there is an issue as to whether it is biologically feasible that such a small number of supplemental macrophages reaching the lymph nodes could have such an impact. We suggest that there are several reasons why they might:

1) BMDM are strongly polarised and so disproportionately influential.

2) BMDM influence lymphoma growth significantly, albeit briefly, and the timing of AT and sacrifice might have been optimal to discern this impact.

3) The lymphoma is disseminated and so BMDM, even lodged in pulmonary capillaries, are able to influence the systemic proliferation of lymphoma and so the growth of lymph nodes, perhaps through secreting high levels of key cytokines. In this way AT BMDM could also act to modulate or recruit other immune effector cells in a pro- or anti-lymphoma response, thus amplifying the effect.
12.1.5 Interpretation of CSF-1R inhibitor data.

CSF-1R inhibition in healthy mice reduced the number of macrophages in lymph nodes and had no effect on the numbers of normal B-cells and T-cells in lymph nodes or blood. We speculate that the slight reduction in macrophages in normal lymph nodes may be multifactorial. The literature reviewed earlier would suggest that CSF-1R inhibition might have the following effects:

1) Reduced formation of CMP and GMP in the bone marrow.
2) Reduced differentiation of MDP from GMP.
3) Compensatory increased differentiation of granulocytes from GMP.
4) Reduced differentiation of GMP to monocytes.
5) Reduced differentiation to patrolling versus inflammatory monocytes.
6) Reduced trafficking of monocytes to tissues.
7) Reduced maturation of monocytes to macrophages.
8) Reduced survival of macrophages.
9) Reduced in situ proliferation of macrophage precursors.
Figure 153 - CSF-1R ligation in monocyte production in the bone marrow.

These same effects could also be responsible for the clear reduction in blood monocytes and lymph node macrophages seen in mice with lymphoma treated with CSF-1R inhibition. The relative importance of these different mechanisms might well be different between situations of health and of lymphoma.

Prolonged use of a higher dose of CSF-1R inhibitor produced a greater degree of blood monocyte and lymph node macrophage depletion and greater relative reduction in lymph node weight, strongly implying a dose effect. The fact that the only populations we found that were depleted by CSF-1R inhibition were monocytes, macrophages and malignant B-lymphocytes, and that a dose response relationship appears to exist, suggests a causal link. CSF-1R inhibition is associated with monocyte and macrophage depletion that is associated with a reduced lymph node weight and reduced lymph node CD19 positive population. Given that the lymphoma cells do not express CSF-1R, nor are directly killed by even very high doses of CSF-1R inhibitor over 18 hours of exposure in vitro, this strongly suggests that CSF-1R inhibition attenuates overall lymphoma growth via intermediary mechanisms. Given that macrophages express CSF-1R, and that the CSF-1RI used has been shown by
the manufacturer to reduce tissue infiltrating macrophage numbers in other models, it seems reasonable to interpret our data as supporting the following statement:

‘CSF-1R inhibition reduces monocyte and TAM numbers in the transplantable \(E_{\mu}\)-myc/bcl2 model of lymphoma, and this is associated with decreased lymphoma growth.’

According to our experiments, CSF-1R inhibition only causes a partial reduction in monocyte and TAM numbers, and a partial reduction in lymphoma burden and proliferation. There are several plausible reasons for this:

1) CSF-1R ligation is only one of the events responsible for the recruitment, maturation, or proliferation of monocytes and TAM. As such, even with maximal CSF-1R inhibition, only partial effects will be seen. As IL-34 also acts at the CSF-1R, and we used a small molecule tyrosine kinase inhibitor, it is unlikely that IL-34 could compensate for CSF-1R inhibition. As mentioned in the introduction, CCL2 can act as a monocyte chemo-attractant, and acts via CCR2 and CCR4, not the CSF-1R. We have not investigated the role of CCL2 and its receptors in this project.

2) Even with maximal specific reduction in TAM numbers, the impact on lymphoma proliferation will be limited. Lymphoma may not be entirely dependent on its microenvironment for survival and proliferation, and certainly is unlikely to be entirely dependent on TAM. Other cells of the immune microenvironment are likely to contribute survival and growth signals, and these other cells may even partially compensate for the reduction in TAM.

3) Only partial CSF-1R inhibition was achieved in our experiments. Twice daily oral dosing is likely to result in peaks and troughs of CSF-1R inhibitor levels in blood and target tissues. This might allow monocyte maturation and trafficking, and possibly even TAM proliferation, to proceed during these troughs.

Effects of CSF-1R inhibition on monocyte subsets, and on granulocyte numbers, are discussed in the following chapters.
The vehicle alone produced a reduction in lymph node weight in mice with lymphoma. This did not correlate with statistically significant lymph node macrophage depletion. This phenomenon was restricted to the lymph nodes, with no reduction in liver weight. There was no reduction in liver cross-sectional area of lymphoma in those receiving the vehicle. When the duration of therapy was increased from 5 to 10 days, this effect on lymph node weight did not appear to increase with a dose effect and became proportionally smaller as the treatment group displayed an even greater reduction in lymph node weight than with the lower dose. We can only speculate as to the cause of this effect of vehicle gavage. Certainly, twice daily gavage appears to be a physically and possibly emotionally traumatic event for the mice, and it seems possible that such episodes of acute stress might result in increased endogenous corticosteroid release, thus reducing lymphoma growth. It is not at all clear, however, how this could be a phenomenon restricted to the lymph nodes. A literature review reveals a sound evidence basis for this theory of gavage-induced stress. The stress induced by oral gavage is considered to be a confounding variable in many studies, and one that can be partially abrogated by pre-coating the gavage needle with sucrose to induce the cooperation of the subjects (Hoggatt et al., 2010). Nonetheless, the firm “scruffing” restraint technique required for safe gavage itself causes a rise in plasma corticosteroid levels in rodents (Dobrakovova and Jurcovicova, 1984), and high viscosity gavage liquids, such as the CSF-1R inhibitor vehicle, containing methylcellulose, also contribute to increased corticosteroid release (Brown et al., 2000).

Our investigations into a biologically plausible mechanism that might link these elements of CSF-1R inhibition, reduction in TAM numbers, and decreased lymphoma growth, are thus far incomplete. However, they do provide some clues and help to shape new hypotheses to guide further work. We will discuss these in the following four sections.
12.1.6 Interpretation of gene expression data.
Gene expression analysis of whole lymph nodes indicated reduced expression of F4/80 and increased expression of CD14 in treated versus untreated mice with lymphoma. CD14 expression is strongest in monocytes and is progressively lost during maturation to lymph node resident macrophages, both in the context of health and in the presence of most lymphomas (Marmey et al., 2006). F4/80 is absent on monocytes, expressed only on mature resident tissue macrophages. This is consistent with our understanding of the expected effects of CSF-1R inhibition in monocyte maturation. As such there might be inhibition of maturation to mature resident macrophages, and so an accumulation of CD14+ monocytes and immature macrophages, and a reduction in F4/80+ mature tissue macrophages. Of potential relevance is that this work was conducted using tissues from an early protocol using only 50 mg/kg CSF-1R inhibitor for only 5 days. As such, it would be interesting to repeat this experiment with prolonged higher dose CSF-1R inhibition.
12.1.7 Interpretation of flow cytometry data.
Prolonged CSF-1R inhibition in mice with advanced lymphoma produced a substantial reduction in the total number of monocytes in the blood when measured using a combination of the surface markers CD11b and Ly6G. There was a substantial increase in the number of circulating CD11b+Ly6G+ granulocytes. F4/80 staining of these two populations, distinguished by Ly6G staining, confirmed differentiation of only the Ly6G- cells towards a macrophage fate. Possible explanations for this shift include:

1) CSF-1R inhibition alters the balance between monocyte and granulocyte differentiation at the bone marrow GMP stage.

2) CSF-1R inhibition reduces the egress of CD11b+Ly6G- monocytes from the bone marrow.

3) CSF-1R inhibition reduces the survival of monocytes in the blood, with no such effect on granulocytes, which do not express CSF-1R.

4) Other changes consequent on CSF-1R inhibition cause a relative prolongation of survival of granulocytes compared to monocytes. Plasma cytokine data support this concept, as increased levels of IFN-γ extend the life of granulocytes by decreasing their apoptosis (Simon et al., 1997, Colotta et al., 1992, Whyte et al., 1997).

There was no discernable shift in the balance between monocyte subsets, however. As such, there was no evidence that CSF-1R inhibition with this drug in this model of lymphoma, had any effect on the relative balance between “inflammatory” and “patrolling” monocyte numbers in the blood, although with reduced overall CD11b+Ly6G- numbers, the absolute numbers of both inflammatory and patrolling monocytes were both reduced by CSF-1R inhibition. CSF-1R inhibition was associated with a reduced number of lymph node TAM in the model we used. A block at any stage in monocyte lineage maturation would reduce the number of monocytes capable of being recruited from the blood and maturing further into tissue resident macrophages. This block could have occurred at several different stages to different extents. Without specifically investigating the bone marrow for numbers of precursors, it is impossible to be certain, however, our data suggest that in particular,
differentiation in to either monocytes or granulocytes at the GMP stage might be exquisitely sensitive to CSF-1R inhibition.

In the blood we found there was a reduction in the malignant B-cell population. However, there was not a reduction in BrdU incorporation amongst this malignant population. It is necessary to pursue this finding further to investigate why this should be so. Several explanations are possible, including:

1) The burden of circulating disease simply reflects overspill from lymph nodes and other tissues such as the bone marrow and spleen. As such, if there is primarily less lymphoma in these tissues, this will simply be reflected in the amount of circulating disease. Supporting this is the observation that total monocyte/macrophage numbers are reduced in the blood yet there is no change in their incorporation of Brdu in this compartment, implying that lymphoma cell biology in the blood is not affected in the same way as in the lymph nodes; it operates in a very different microenvironment.

2) There is a clear shift in the balance in numbers between monocytes and granulocytes in the blood with CSF-1R inhibition. As such, it is plausible that lymphoma within the blood is subject to a very altered microenvironment with CSF-1R inhibition, and its dynamic might also be altered. I have not measured rates of lymphoma cell apoptosis in this model, and it is conceivable that although rates of proliferation might not be altered, rates of apoptosis might be, and so could explain the reduced lymphoma burden in the blood even though proliferation rates are unchanged. The function and impact of the increased number of circulating granulocytes is certainly worthy of future investigation but was considered beyond the scope of this project.

In lymph nodes, there was a clear and substantial reduction in macrophages, as measured using a variety of surface markers including CSF-1R, CD11b, CD11b and Ly6G and F4/80. Unlike in the blood, we did not investigate the relative proportions of CD11b+Ly6G-Ly6C+ and CD11b+Ly6G-Ly6C- cells as this schema applies to circulating monocytes, rather than resident tissue macrophages. There was no percentage change in the malignant B-cell population in lymph nodes, as measured by CD19 or B220 expression. When
combined with lymph node weight, the pattern is consistent and very clear; that there is a reduction in the total number of lymph node resident malignant B-cells in mice subjected to CSF-1R inhibition.
12.1.8 Interpretation of plasma cytokine data.

CSF-1 levels rose with the presence of advanced lymphoma. This is consistent with published data for other malignancies and models, but has not been reported in lymphoma models before. This is also consistent with the hypothesis that lymphoma promotes the generation, survival, trafficking, and maturation of monocytes through producing and secreting CSF-1. Macrophage depletion in MaFIA mice with lymphoma had no effect on these raised levels. We speculate that this might be the net result of opposing factors. Firstly, a reduction in lymphoma mass with macrophage depletion reduces the number of malignant B-cells present and so able to produce CSF-1. Secondly and opposing this, macrophage depletion in MaFIA mice reduces the number of CSF-1R expressing cells and so reduces the rate at which circulating CSF-1 is consumed by ligation with its receptor. We have no data from our own experiments to support this suggestion.

The substantial rise in circulating levels of IL-10 that occurs in advanced lymphoma supports the notion that the lymphoma environment is one dominated by immune-suppression, entirely in keeping with an abundance of alternatively activated TAM.

The rise in CXCL1 levels with CSF-1Ri is of unclear significance. This may reflect its role as a leukocyte chemoattractant, perhaps being produced at increased levels in an attempt to compensate for CSF-1R inhibition. Alternatively, this rise may reflect the increased number of circulating granulocytes that may be producing this ligand.

IL-6 levels change significantly with the presence of advanced lymphoma, and then again with CSF-1R inhibition. IFN-γ levels rise significantly when mice with lymphoma are treated with CSF-1Ri. The plasma concentrations of these two cytokines appear to change with CSF-1R inhibition in a way that might indicate a more favourable immune response. IFN-γ is the archetypal Th1 cytokine, high levels favouring the direction of macrophages towards a pro-inflammatory and potentially tumouricidal M1 phenotype. Levels of IFN-γ rise with CSF-1R inhibition. This could reflect the shift in circulating myelo-monocytic cells with a relative abundance of inflammatory Ly6G+ granulocytes, as well as a reduced acquisition of CSF-1R dependent features of an alternatively activated / M2
phenotype by tissue macrophages. Although the absolute levels of IFN-γ recorded in the plasma were low, they may reflect larger magnitude changes in other critical sites such as the bone marrow or lymph nodes. The rise with progression of lymphoma and then fall in circulating IL-6 levels with CSF-1R inhibition is the most prominent change recorded, and may be of great significance. We discuss the potential roles of IL-6 in a later chapter. Our data do not allow reasonable inference as to whether the fall in IL-6 levels is in any way responsible for the reduced proliferation of lymphoma, or if it simply reflects a decreased number of lymphoma cells, arising through IL-6 independent mechanisms.

One significant limitation of this approach is that plasma cytokine levels are likely to be different from cytokine levels within tissues, such as lymph nodes. It is within such tissues that we hypothesise the most critical TAM-lymphoma interactions occur, and where this relationship might be most vulnerable to local changes in the cytokine milieu.
12.1.9 Interpretation of data from combination with chemotherapy.
Combination of macrophage depletion strategies with cyclophosphamide did not provide evidence for any added benefit of this approach over cyclophosphamide alone. There may be several reasons for this. It might be that the model is too aggressive to allow detection of subtle benefits. It may be that cyclophosphamide was not the ideal choice of agent to combine with macrophage depletion strategies. In fact, in addition to being cytotoxic to dividing malignant cells, cyclophosphamide itself causes macrophage depletion. A principal side effect of this agent is leukopaenia. In guinea pigs, deficiencies in resident tissue macrophages (Hunninghake and Fauci, 1977) and inflammatory macrophage numbers (Winkelstein, 1973) were seen following cyclophosphamide therapy. This was subsequently confirmed in human tissues (Santosuosso et al., 2002). As such, macrophage depletion therapies following cyclophosphamide would need to be sustained beyond the normal period of monocyte/macrophage recovery following chemotherapy to conceivably have additional impact. It would be interesting to study combination with agents that do not cause significant macrophage depletion themselves, so as to determine if any additive or summative benefit could be achieved.
12.2 Discussion of methods.

12.2.1 Measuring tumour burden.

Lymph node dimensions and weight appear to be reasonable proxy measures of lymphoma mass, with histological sections showing diffuse and uniform infiltration with lymphocytes in mice with lymphoma, regardless of therapy. Flow cytometry data is consistent with this, with the proportion of malignant cells unchanged between treatment groups, and so lymph node dimensions/weight being proportional to the overall number of malignant lymphocytes. For the liver and spleen, this is probably less robust. Intensive treatment of normal mice with LC is known to lead to a degree of splenic shrinkage that is out of proportion to simply the loss of macrophages, and has been associated with apoptosis of non-phagocytic lymphocytes, possibly damaged by enzyme release from dying macrophages (van Rooijen et al., 1985). This idiosyncratic effect may explain the dramatically reduced spleen size in mice with lymphoma treated with LC. This reduction in spleen size is out of proportion to the changes in lymph node and liver size. Sections of liver show that despite lymphoma only occupying a minority of the overall liver mass, the cross-sectional area of lymphoma changes with treatment in the same direction as does liver weight and so is in itself a valid measure of changes in hepatic lymphoma mass. In the light of these observations, we were reluctant to consider spleen weights as a measure of overall lymphoma mass, preferring to pay most attention to the most robust measure, lymph node weight, and then quantify B-lymphocytes by flow cytometry.

Flow cytometry for CD19+ cells provides a somewhat indirect but nonetheless accurate relative measure for comparing malignant B-cell populations between experimental groups. In support of this strategy, separation of malignant from non-malignant lymph node cells is commonly performed on the basis of CD19 expression, including in publications in the most highly regarded scientific journals (Dave et al., 2004). Experiments in healthy mice demonstrated that neither LC nor CSF-1R inhibition altered the proportion or total number of normal healthy CD19 expressing B-lymphocytes in lymph nodes or blood. In mice with lymphoma, the proportion and number of CD19 expressing cells is greatly increased. Given that treatment with LC or CSF-1R inhibition do not alter the normal B-cell population, it seems reasonable to assume that differences in
CD19 expressing cells in mice with lymphoma subjected to different therapies are entirely, or almost entirely, consequent on reductions in malignant B-cells.

A further point to reinforce is that flow cytometry measures relative proportions of different cells, often expressed as percentages of gated populations. Aiming to provide a measure of total lymph node tumour burden, it was imperative that the proportion of CD19 positive cells was factored by the lymph node weight or total cell number, so as to derive a measure that truly reflected tumour burden. Similarly, when measuring macrophage numbers, it is important to consider the total number of macrophages present in lymph nodes by also factoring the proportion of cells by the total cell number or lymph node weight.

12.2.2 Criticisms of the transplantable $E_\mu$-myc/bcl-2 lymphoma model.

We believe this model proved to be excellent for the specific purposes of our project. Owing to its highly proliferative biology it was easily transplantable, and enabled rapid collection of data, and repetition of protocols to confirm findings and collect further samples. The model was highly reproducible, with lymphoma progression being consistent within and between protocols. In keeping with expectations, it proved to be chemoresistant. As such, the observed impact of macrophage manipulation is particularly encouraging. We discuss some of the limitations of this model below.

i) Differences between human and mouse macrophages.

Macrophages are an ancient and evolutionarily conserved cell with many functional similarities between mice and humans. TAM expression signatures in mouse models of cancer are represented in human tumour datasets, and can even be of prognostic value (Ojalvo et al., 2009, Zabuawala et al.). Monocyte subsets, as reviewed in the introduction to this thesis, provide an example of comparable functional attributes of equivalent subsets distinguished by different surface markers in mice and humans. The validity of our project relies upon there being a similar comparability between mice and humans with respect to the functional relationship between TAM and malignant B-lymphocytes. Detailed comparisons of mouse and human macrophage genomes indicate overlap as well as particular differences. However, exact duplication of genetic and molecular
events is not as critical to the demonstration of proof-of-principle as the faithful representation of the functional consequences of cellular interactions between these two cell types in man and mouse.

ii) Use of Eµ-myc/bcl-2 lymphoma transplantation.

We identified many advantages to using this lymphoma as a transplant to alternative hosts rather than using the transgenic Eµ-myc mice themselves. The nature of the original transgenic model is that malignant clones can potentially arise at a number of different stages of maturation of lymphoid cells. As such, T-lymphomas as well as B-lymphomas were reported, as well as varieties of pre-B, pro-B and mature B-cell clones. We set out to work with a mature B-cell malignancy and so by characterizing a large mature B-cell population we were able to keep this as a constant between and within experiments, and propagate the population in immune-competent hosts. Critically, we were intent on studying the innate immune response to lymphoma. Within the original Eµ-myc transgenic host, the non-malignant immune cells (in particular the lymphocytes) may not be entirely normal in number and function. Transplantation has allowed me to investigate the interactions between lymphoma and a competent immune system that we were able to manipulate subsequently, and also between lymphoma and genetically altered hosts, such as MaFIA mice.

However, although the Eµ-myc/bcl-2 lymphoma was generated upon a C57BL/6 background, the potential for antigraft immune reactions must be considered. Use of immunocompromised mice might attenuate such reactions, but would prove an even less satisfactory means of studying lymphoma-macrophage interactions. The ideal situation for some of our experiments might be to study spontaneously arising mature B-lymphomas in fully immune competent mice. This is not readily available in sufficient numbers and would not provide the same opportunities for using genetically modified hosts.
12.3 Consideration of the role of IL-6.

A unifying hypothesis that could be generated from this work, and tested by future studies, is that macrophage derived IL-6 drives lymphoma proliferation. Reflecting on the literature, this seems plausible. Becker et al. provided evidence that myeloid cell orchestration of IL-6 production was critical in tumour progression (Becker et al., 2004). Gilbert and Hemann reported that IL-6 promoted the survival of a variant of Eµ-myc lymphoma cells during exposure to doxorubicin *in vivo* and *in vitro*, with IL-6 genetic knockout mice with transplanted lymphoma having prolonged survival (Gilbert and Hemann, 2010). As summarised in the figure below, chemotherapy caused genotoxic stress to thymic endothelial cells which, via Janus associated kinase 2 (JAK2) signalling and p38 MAPK activation caused ataxia telangiectasia-mutated (ATM) independent IL-6 release and so acute induction of anti-apoptotic BcXL in lymphoma cells within this chemoresistant thymic endothelial cell niche.

![Diagram](image)

**Figure 154 - Summary of mechanisms proposed by Gilbert and Hemann.**

Given that we also used a variant of the Eµ-myc lymphoma, the question arises as to how far their findings are relevant to our model. Perhaps of significance is that the different variant Eµ-myc lymphoma we used already constitutively over-expresses anti-apoptotic Bcl2, causing widespread, perhaps non-niche
dependent relative chemo-resistance. We measured high levels of circulating IL-6 in mice with Eµ-myc/bcl-2 lymphoma. Perhaps, in the model we studied, constitutive BCL2-mediated chemoresistance is further augmented by advanced disease and rising IL-6 levels, and this element of chemoresistance might be amenable to therapeutic intervention.

It remains to be demonstrated how much IL-6 is macrophage derived and so potentially providing a mechanism whereby CSF-1R inhibition might reduce IL-6 production by reducing TAM numbers, and so maybe increase constitutive or chemotherapy-induced apoptosis. Additionally, the CSF-1Ri used is a small molecule tyrosine kinase inhibitor, and in line with the work of Gilbert and Hemann, it seems sensible to wonder to what extent it also produces a degree of JAK2/3 inhibition, thus potentially giving a mechanism for reduced IL-6 production, other than simply a reduction in the number of TAM. Looking beyond apoptosis it is necessary to consider what role IL-6 has with regards lymphoma cell proliferation in the model we used. Does IL-6 directly promote the proliferation of Eµ-myc/bcl-2 lymphoma cells? IL-6 certainly appears to promote a tumour permissive immune response (Kortylewski et al., 2005) including an M2 phenotype (Duluc et al., 2007, Jeannin et al., 2011) via STAT3 activation so providing an indirect mechanism for influencing lymphoma progression. In a mouse model of breast cancer, Fra-1, a transcription factor upstream of STAT3, is both overexpressed in TAM and can also stimulate IL-6 production, itself further activating STAT3 (Luo et al., 2010). In the light of the work by Roca et al. it seems logical that future work investigates the impact of macrophage manipulation on CCL2 levels in mice with lymphoma, and whether levels of this chemotactic cytokine are affected by CSF-1R inhibition and macrophage ablation (Roca et al., 2009b).
13 Summary.

The following positive statements can be made regarding the experimental results of this project:

1) In treatment naïve diagnostic specimens of DLBCL, macrophages express CD163, a marker of alternative activation, and their number is positively correlated with the lymphoma proliferation rate.

2) LC depletes macrophages in mice and is associated with less $E_{\mu}$-myc/bcl-2 lymphoma at sacrifice.

3) Activating the macrophage death construct in transgenic MaFIA mice results in macrophage depletion and attenuated $E_{\mu}$-myc/bcl-2 lymphoma growth.

4) AT of M1 macrophages in mice is associated with less $E_{\mu}$-myc/bcl-2 lymphoma at sacrifice than is AT with TAM.

5) Established $E_{\mu}$-myc/bcl-2 lymphoma is associated with a rise in circulating concentrations of IL-10 and IL-6.

6) CSF-1R inhibition in mice causes a reduction in total circulating monocyte numbers, an increase in circulating granulocytes, and a reduction in TAM numbers. This is associated with decreased $E_{\mu}$-myc/bcl-2 lymphoma in lymph nodes and blood at sacrifice. In the blood this is associated with reduced IL-6 and increased IFN-$\gamma$ levels. In lymph nodes it is associated with decreased lymphoma cell proliferation.

Having used CSF-1R inhibition and observed a reduction in monocyte numbers in the blood and a reduced number of TAM, it seems most obvious to infer that this reflects attenuated production and recruitment of monocytes from the blood to tissues. However, this is not necessarily the entire picture, and it might be that CSF-1R inhibition impacts on the already resident TAM by reducing their survival and the in situ proliferation of their precursors.
13.1 Implications for future research.

Several key questions are raised by our data. We will outline those questions below, before exploring how they might be addressed in the laboratory.

1) Not only do macrophages appear to promote lymphoma in this model, but the TAM phenotype appears to be specifically protumoural, as opposed to either the M1 or M2 phenotypes. What is the TAM phenotype in this model? What are the key features of the TAM phenotype that promote lymphoma in this model?

Future work will focus on isolation and comparison of macrophage populations from lymph nodes of normal mice, compared with those from mice injected with lymphoma. Comparison will be made by flow cytometry analysis, to determine macrophage phenotype and subpopulations, as well by RNA extraction and gene expression analysis. Macrophages would ideally be isolated without delay from fresh tissue, selected by as few steps and as little processing as possible, so as to minimise alterations to viability, gene expression and surface markers.

We propose to use FACS to isolate lymph node infiltrating macrophages from normal healthy C57BL/6 mice and from such mice 10 days after the transplantation of Eµ-myc/bcl-2 lymphoma cells. Single cell suspensions will be created from freshly harvested lymph nodes and stained with fluorescent antibodies.

Tissue macrophages will be sorted using the following profile:

1) CD19 negative
2) CD11b positive
3) Ly6G negative

Characterization of these cells will be performed by analyzing the selected population for expression of F4/80 and CFS-1R.

All stages of the process will carried out simultaneously for each group of mice and macrophages, under identical conditions at the same time, from purchasing and housing the mice to sorting the macrophages by FACS. This will be critical in minimising potential artefact differences between the groups. This approach will produce a sufficient number and purity of macrophages that RNA extraction for whole genome expression analysis should be feasible.
2) How is the TAM phenotype acquired? Is it driven by lymphoma cells, and if so, what are the relative contributions of secreted substances versus cell-cell contact? Can key elements in this process be identified, and potentially targeted?

Once key elements of the TAM phenotype have been established on a gene expression level, we will interrogate macrophages in the in vivo setting of transplantable Eμ-myc/bcl-2 lymphoma for their expression as proteins. This will then herald in vitro co-culture of Eμ-myc/bcl-2 lymphoma cells and BMDM to investigate the acquisition of the TAM phenotype, and mechanisms responsible for it. We will perform co-culture with direct cell-cell contact and also using Transwell® (Corning®) chambers to prevent cell-cell contact and allow only diffusible substances. Blocking and stimulation experiments will allow investigation of the role of candidate molecules in the polarization of BMDM towards a pro-tumoural TAM phenotype.

3) The relationship between lymphoma and its microenvironment appears to be bidirectional. In what way do changes to the number of phenotype of macrophages impact on lymphoma biology? More specifically, and perhaps more relevant to the clinic, what changes occur to lymphoma cells with CSF-1R inhibition and the resultant reduction in monocyte and TAM numbers?

Similarly to the approaches outlined in the 2 points above, malignant B-lymphocyte biology will be studied by FACS isolation and gene expression analysis from different in vivo scenarios of Eμ-myc/bcl-2 lymphoma, including untreated and macrophage deplete and CSF-1R inhibited. Co-culture experiments can also be employed to investigate key molecular and genetic events.

4) Why does the consistent reduction in lymphoma mass at sacrifice seen with macrophage depletion not translate into a survival benefit? If this is because the model used is too aggressive to allow us to measure a potential benefit, then what is the impact of macrophage depletion and/or CSF-1R inhibition on more indolent models of lymphoma, that one might speculate are more vulnerable
to changes in the microenvironment than aggressive MYC-driven lymphomas?

An aggressive model of lymphoma has allowed me to perform transplantation of lymphoma to immunocompetent mice, and also to make and repeat these initial observations in a relatively short period of time. With evidence having been collected supporting proof-of-principle, it is attractive to further investigate such strategies in a more indolent model of lymphoma. It is the most common indolent lymphoma, FL, for which there is the largest and most persuasive body of evidence supporting a role for TAM. Relapsing repeatedly and being ultimately incurable, FL is perhaps more likely than DLBCL or BL to present frequent opportunities for the introduction of novel agents in early stage clinical trials.

5) Can the potential benefits of CSF-1R inhibition be translated into the clinic?

There is a limit to how far any mouse model can be used to usefully derive insights into human disease. Having found evidence that CSF-1R expressing TAM and the progression of B-NHL are related in our model, possibly involving IL-6 and rates of lymphoma cell proliferation, we are further interrogating our extensive bank of human tissue for correlative evidence regarding IL-6, the CSF-1R and IFN-γ.

Since the beginning of this project, the number of publications exploring CSF-1R inhibition has greatly increased, with critical reviews by opinion leaders in macrophage biology adding support to the translation of laboratory studies into the clinic (Hume and Macdonald, 2012). An orally available small molecule CSF-1R inhibitor, PLX3397 (Plexxikon) has entered phase 1 clinical trials in metastatic cancers and phase 2 trials in Hodgkin lymphoma. It is reported to have additional inhibitory effects on other type III receptor tyrosine kinase molecules, KIT and FLT3. It will be interesting to follow these trials as they report. It would be fascinating to use this compound in mouse models of B-NHL to provide pre-clinical evidence in support of clinical trials.
6) Is macrophage derived IL-6 a key cytokine driving lymphoma proliferation in this model, as suggested by plasma IL-6 measurements?

As described earlier, IL-6 appears to have many and varied roles in the formation and progression of cancers. In regard to B-cell malignancies, IL-6 is extensively documented to be critical in the formation (Hilbert et al., 1995), survival and progression of multiple myeloma, a plasma cell neoplasm. Further laboratory studies are necessary to elucidate the role for IL-6 in this model. Gene expression analysis of sorted macrophages and lymphoma cells will inform whether the genes for IL-6 or the IL-6 receptor are upregulated in macrophages and lymphoma cells respectively. Supplementing or blocking IL-6, over-expressing or knocking out the IL-6 gene in vitro and in vivo, will be important initial steps. Therapeutic anti IL-6 antibodies and anti IL-6 receptor antibodies have been manufactured and are at various stages of development in inflammatory diseases and cancers, including multiple myeloma. The effects of such antibodies could be studied in relation to TAM-lymphoma interactions in the Eµ-myc/bcl-2 and other mouse models of B-NHL.

7) What cytokine changes occur within lymph nodes with CSF-1R inhibition and the subsequent reduction in TAM numbers?

We have found changes in circulating cytokine levels that provide clues as to important biological mechanisms during lymphoma progression and resulting from CSF-1R inhibition. Focusing on such changes within lymph nodes might provide more relevant information regarding the specific interactions between malignant B-lymphocytes and their immediate microenvironment. We are currently optimising techniques to measure cytokines from mouse lymph nodes.

8) In the light of failing to demonstrate any additional benefit of combining macrophage depletion with cyclophosphamide, can benefit be demonstrated with alternative treatment schedules, or alternative cytotoxic or immunomodulatory agents?

Additive and possibly synergistic impacts might be gained by targeting different aspects of the lymphoma microenvironment simultaneously. Reducing TAM numbers by CSF-1R inhibition might be most efficacious when combined with
other immunomodulatory drugs such as Lenalidomide, perhaps also in addition to traditional cytotoxic chemotherapy. This raises the prospect of improved responses in terms of lymphoma growth, and might translate to survival benefits in this or more indolent models.
13.2 Concluding statement.

In this project we have critically reviewed the relevant literature relating to TAM and lymphoma. We have conducted a series of related laboratory experiments that provide support for a relationship between the presence and function of TAM and the progression of B-cell lymphoma. This thesis includes the first reports of whole organism functional data supporting the strategy of CSF-1R inhibition in the treatment of B-cell lymphoma. Future pre-clinical work in this field will define more precisely the critical cellular, molecular and genetic events responsible for monocyte-macrophage depletion and retardation of lymphoma growth that we have observed with this promising strategy. We speculate that defining the optimal timing and delivery of CSF-1R inhibition in relation to other therapies will be crucial in deriving benefit from this novel approach to the treatment of B-NHL.
References.


gamma reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages. *Int J Cancer*, 125, 367-73.


JADUS, M. R., CHEN, Y., BOLDAJI, M. T., DELGADO, C., SANCHEZ, R., DOUGLASS, T., AL-ATAR, U., SCHULZ, W., LLOYD, C. & WEPSC, H. T. 2003. Human U251MG glioma cells expressing the membrane form of macrophage colony-stimulating factor (mM-CSF) are killed by human monocytes in vitro and are rejected within immunodeficient mice via paraposis that is associated


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SASMON, R. T., EHRNSPERGER, A., CRONAU, S. L., RAVASI, T., KANDANE, R., HICKEY, M. J., COOK, A. D., HIMES, S. R., HAMILTON, J. A. & HUME, D. A. 2007. Mouse neutrophilic granulocytes express mRNA encoding the macrophage colony-stimulating factor receptor (CSF-1R) as well as many


adoptively transferred into the abdominal cavity of patients with peritoneal carcinomatosis. Cancer Res, 47, 6100-3.


Buffers and Solutions

Flow cytometry staining buffer
PBS with 3% foetal calf serum (FCS)

Eµ-myc/bcl-2 lymphoma culture media
400 ml Dulbecco’s modified eagle medium (DMEM)
10% FCS
1% Penicillin-Streptomycin
4 ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
7 µl of 50 mM β-mercaptoethanol
12 ml of 10 mM L-Asparaginase

Vehicle for CSF-1Ri (*requires sterile filtration with 0.22 µm filter)
Sterile water
0.1% Tween® 80
0.5% methylcellulose

Vehicle for AP2087 dimerizer (*requires sterile filtration with 0.22 µm filter)
Sterile water
4% ethanol
10% polyethylene glycol (PEG-400)
1.7% Tween® 80
Flow cytometry antibody reference table

All flow cytometry antibodies were raised in rats and diluted 1:100.

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Table 12 - Flow cytometry antibodies.
Publications and abstracts arising from this work.

Scientific paper
Increased angiogenic sprouting in poor prognosis FL is associated with elevated numbers of CD163+ macrophages within the immediate sprouting micro-environment.

Review articles
HALLAM, S., MONTOTO, S.
Follicular lymphoma: managing an indolent malignancy.
HALLAM, S., ESCORCIO-CORREIA, M., SOPER, R., SCHULTHEISS, A. & HAGEMANN, T.
Activated macrophages in the tumour microenvironment-dancing to the tune of TLR and NF-kappaB.

Book chapter
HALLAM, S. & HAGEMANN, T.
TAM: A moving clinical target.
*Editors: LAWRENCE, T., HAGEMANN, T.*

Oral presentations
HALLAM, S., CANDIDO, J., MANIATI, E., THOMPSON, R., VAN ROOIJEN, N., HAGEMANN, T., GRIBBEN, J.
Depletion of tumour-associated macrophages significantly retards the progression of an aggressive and chemoresistant model of B-cell Non Hodgkin Lymphoma.
*Abstract accepted for Oral Presentation. 16th Congress of the European Hematology Association, 2011*
Exploring the Immune Microenvironment of Diffuse Large B Cell Lymphoma in a Tissue Microarray: Predicting Survival with a Score That Incorporates Macrophages, Cytotoxic and Regulatory T Cells.
*Blood (ASH Annual Meeting Abstracts) 118: 951.2011*

**Poster presentations**

HALLAM, S., CANDIDO, J., COUTINHO, R., MANIATI, E., THOMPSON, R.G., VAN ROOIJEN, N., HAGEMANN, T., GRIIBBEN, J.G.
Manipulating Tumor Associated Macrophages (TAM) in a Mouse Model of B-Cell Non-Hodgkin Lymphoma (NHL).
*Blood (ASH Annual Meeting Abstracts) 118: 1657. 2011*

Manipulating Tumour Associated Macrophages (TAM) in a mouse model of B-cell Non-Hodgkin Lymphoma (NHL).
*European Macrophage and Dendritic cell Society 25th annual meeting, 22-24 September 2011, Brussels. 2011*