HYALURONAN BINDING BY CD44 ON T CELLS

by

NINA MIHARU MAESHIMA

B.Sc., The University of British Columbia, 2001

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Microbiology and Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

August, 2010

© Nina Miharu Maeshima, 2010
ABSTRACT

CD44 is a transmembrane protein that binds to hyaluronan, a component of the extracellular and pericellular matrices. Hyaluronan supports cell migration and proliferation during embryonic development, wound repair, as well as tumourigenesis. Hyaluronan binding to CD44 can also regulate leukocyte migration and adhesion. On naïve CD4 and CD8 T cells, CD44 is in its inactive form, but it is upregulated and induced to bind hyaluronan upon T cell activation. High CD44 expression is used as a marker for effector and memory T cells, and recent evidence has implicated CD44 in the formation of CD4 but not CD8 memory T cells. However, it is not clear if effector T cells bind hyaluronan and unknown if memory T cells bind hyaluronan. Hyaluronan binding has additionally been shown to mark a subset of the most suppressive regulatory CD4 T cells and here hyaluronan promotes FoxP3 expression, but the significance of why only a subset of these cells binds hyaluronan is unclear. Thus, the current understanding of when and on which cells hyaluronan binding is induced during an immune response, as well as its function on T cells, is incomplete.

The first aim of this work was thus to determine when CD4 and CD8 T cells bind hyaluronan. T cells were activated in vitro with PMA and ionomycin and in vivo during an immune response to *Listeria monocytogenes*. Hyaluronan binding was assessed over a time course and found to occur on the mostly highly proliferative, activated T cells, as well as on a subset of memory T cells. The second aim of this work was to determine the consequences of binding hyaluronan. Hyaluronan binding on activated T cells was found to enhance their adhesion to fibronectin and inhibited both chemokinesis and chemokine-induced migration. Furthermore, hyaluronan induced chemokine-independent polarization of CD44, but inhibited CD44 co-polarization with phosphorylated ERM proteins. Together, the data suggests that
hyaluronan binding is induced on highly proliferative T cells and may function as a stop signal for migration.
# TABLE OF CONTENTS

ABSTRACT........................................................................................................................................... ii  
TABLE OF CONTENTS................................................................................................................ iv  
LIST OF FIGURES ................................................................................................................... viii  
LIST OF ABBREVIATIONS........................................................................................................... x  
ACKNOWLEDGEMENTS......................................................................................................... xii  
STATEMENT OF COLLABORATION........................................................................................... xiii

CHAPTER 1: INTRODUCTION ........................................................................................................... 1  
1.1 The immune system ............................................................................................................. 1  
1.2 T cells in an immune response ........................................................................................... 2  
1.3 CD4 and CD8 T cells ........................................................................................................... 2  
1.4 T cell activation ................................................................................................................... 4  
1.4.1 T cell proliferation and IL-2 ............................................................................................ 6  
1.4.2 Effector T cell responses ................................................................................................. 8  
1.4.3 Contraction of the immune response ............................................................................ 8  
1.4.4 Regulatory T cells .......................................................................................................... 10  
1.4.5 Memory T cells ............................................................................................................. 10  
1.4.6 Effector and memory T cells: models of differentiation .............................................. 13  
1.4.7 Rechallenge and secondary immune responses ............................................................ 15  
1.5. T cell homing and recruitment ......................................................................................... 17  
1.5.1 Chemoattractants and their receptors ....................................................................... 18  
1.5.2 Polarization and the polarity complex ......................................................................... 19  
1.6 Adhesion and adhesion molecules ..................................................................................... 21  
1.7 CD44 ................................................................................................................................. 22  
1.7.1 Hyaluronan .................................................................................................................... 23  
1.7.2 CD44 knockout mice .................................................................................................... 23  
1.7.3 The cytoplasmic domain of CD44: interaction with ERM proteins ............................ 25  
1.7.4 Function of CD44 on leukocytes .................................................................................. 27  
1.7.5 Function of CD44 on T cells ........................................................................................ 28  
1.7.6 CD44 binding to hyaluronan on T cells ....................................................................... 29  
1.8 Thesis aims and rationale ................................................................................................... 30
CHAPTER 2: MATERIALS AND METHODS ................................................................. 32
  2.1 Materials .................................................................................................................. 32
    2.1.1 Mice .................................................................................................................... 32
    2.1.2 Antibodies .......................................................................................................... 32
    2.1.3. Reagents used for this study .............................................................................. 34
    2.1.4. Cell lines .......................................................................................................... 34
  2.2 Methods ...................................................................................................................... 35
    2.2.1 Cell transfection and cell line culture conditions ............................................. 35
    2.2.2 Isolation of mouse splenic and LN T cells ......................................................... 36
    2.2.3 Adoptive transfer and bacterial infections ......................................................... 36
    2.2.4 In vitro mouse T cell culture and activation ..................................................... 37
    2.2.5 Generation of activated mouse bone marrow-derived dendritic cells .......... 38
    2.2.6 T cell activation using antigen and antigen presenting cells ............................ 38
    2.2.7 Flow cytometry .................................................................................................... 39
    2.2.8 Cell cycle analysis ............................................................................................... 39
    2.2.9 In vivo BrdU labeling ............................................................................................ 40
    2.2.10 In vitro BrdU assay ............................................................................................ 40
    2.2.11 Intracellular cytokine staining .......................................................................... 40
    2.2.12 Blocking proliferation assay .............................................................................. 41
    2.2.13 Transwell migration assays .............................................................................. 41
    2.2.14 Cell polarization and fluorescent microscopy .................................................. 42
    2.2.15 Adhesion assay to fibronectin ......................................................................... 44
    2.2.16 Western blot ....................................................................................................... 44
    2.2.17 Cytokine analysis by ELISA ............................................................................. 45
    2.2.18 Cell stimulation using plate-bound antibodies ................................................ 45
    2.2.19 Killing assay ...................................................................................................... 46
    2.2.20 Statistical analysis ............................................................................................. 47
CHAPTER 3: HYALURONAN BINDING ON T CELLS ............................................ 48
  3.1 Introduction ............................................................................................................... 48
  3.2 Results ...................................................................................................................... 50
3.2.1 Hyaluronan binding is transiently induced on in vitro activated CD4 and CD8 T cells ............................................................................................................................................... 50
3.2.2 Hyaluronan binding occurs on a subset of CD8<sup>+</sup> OT-I T cells after pathogenic challenge in vivo.................................................................................................................................................... 52
3.2.3 Hyaluronan binding occurs on a subset of CD44<sup>+</sup> CD25<sup>+</sup> T cells ........................................................................................................................................ 54
3.2.4 Hyaluronan binding enriches for cycling T cells and marks the most proliferative T cells ........................................................................................................................................ 56
3.2.5 Hyaluronan does not significantly enhance or inhibit proliferation in vitro on day 2 after activation with PMA and ionomycin ........................................................ 58
3.2.6 Hyaluronan binding occurs on a subset of memory T cells ........................................................................................................................................ 60
3.2.7 Hyaluronan binding can be re-induced if cells are restimulated with anti-CD3 .......... 62
3.2.8 Culture with the cytokines IL-2, IL-7, or IL-15 is sufficient to induce hyaluronan binding on activated T cells .............................................................................................. 64
3.2.9 Hyaluronan binding marks the fastest proliferating memory phenotype cells in vitro 64
3.2.10 Interaction of the extracellular domain of CD44 with hyaluronan does not costimulate for naïve T cell activation ........................................................................................................................................ 67
3.2.11 Neither hyaluronan nor CD44 have a significant effect on CTL-mediated killing in vitro ........................................................................................................................................ 70
3.2.12 There is no effect of CD44 on the generation of a primary immune response or CD8 memory cells following *Listeria monocytogenes* infection in vivo .............................................. 72
3.2.13 There is no effect of CD44 on the generation of a secondary CD8 memory T cell immune response in vivo following *Listeria monocytogenes* re-challenge .............................................. 74
3.2.14 CD44-deficient T cells outcompete WT T cells in memory T cell numbers following *Listeria monocytogenes* infection ........................................................................................................................................ 74
3.3 Discussion ........................................................................................................................................ 78

CHAPTER 4: HYALURONAN BINDING ON ACTIVATED T CELLS INHIBITS MIGRATION AND DISRUPTS THE CO-LOCALIZATION OF CD44 WITH PHOSPHORYLATED ERM PROTEINS ........................................................................................................................................ 81
4.1 Introduction ........................................................................................................................................ 81
4.2 Results ........................................................................................................................................... 83
4.2.1 Cell and CD44 polarization correlate with optimal CXCL12-induced T cell migration ........................................................................................................................................ 83
4.2.2 CD44 is not required for cell polarization and migration to occur ........................................................................................................................................ 85
4.2.3 An inability to localize CD44 to the uropod correlates with poor migration ........................................................................................................................................ 87
4.2.4 Loss of the cytoplasmic domain of CD44 inhibits CD44 re-localization to the uropod and optimal migration................................................................. 90
4.2.5 Hyaluronan inhibits migration of activated day 2 T cells ......................... 90
4.2.6 Hyaluronan enhances activated T cell adhesion to fibronectin ............... 92
4.2.7 Hyaluronan pre-treatment disrupts CD44:pERM co-polarization in response to chemoattractant................................................................. 95
4.2.8 Hyaluronan treatment can signal to transiently dephosphorylate ERM proteins .... 96
4.3 Discussion ........................................................................................................ 98
CHAPTER 5: SUMMARY AND DISCUSSION ................................................................. 103
5.1 Summary of findings......................................................................................... 103
5.2 Discussion and future directions ................................................................. 105
5.3 Perspectives.................................................................................................... 115
REFERENCES ...................................................................................................... 116
APPENDIX: ANIMAL CARE AND BIOSAFETY CERTIFICATES......................... 130
LIST OF FIGURES

Figure 1.1: T cells in an immune response. ................................................................. 3
Figure 1.2: Model for effector and memory T cell differentiation. ......................... 16
Figure 1.3: T cell polarization upon chemokine stimulus........................................ 20
Figure 1.4: CD44 and hyaluronan........................................................................... 24
Figure 3.1: Hyaluronan binding on T cells in vitro. ................................................... 51
Figure 3.2: Hyaluronan binding is induced in vivo. .................................................. 53
Figure 3.3: Hyaluronan binding occurs on a subset of activated T cells. ............... 55
Figure 3.4: Hyaluronan binding enriches for the most proliferative, cycling T cells.. 57
Figure 3.5: Hyaluronan does not promote proliferation. ....................................... 59
Figure 3.6: Hyaluronan binding is found on memory T cells.................................... 61
Figure 3.7: Hyaluronan binding is re-induced upon restimulation. ...................... 63
Figure 3.8: Culture with IL-2, IL-7, or IL-15 is sufficient to induce hyaluronan binding on activated T cells. ................................................................. 65
Figure 3.9: Hyaluronan binding marks the fastest proliferating memory phenotype cells in vitro. ........................................................................................................... 66
Figure 3.10: Hyaluronan is not costimulatory for T cell activation.......................... 69
Figure 3.11: Neither CD44 nor hyaluronan have an effect on CD8 T cell killing ...... 71
Figure 3.12: The loss of CD44 does not affect the generation of effector or memory T cells following a primary challenge with Listeria monocytogenes. ................................................................. 73
Figure 3.13: The loss of CD44 does not affect the generation of effector CD8 T cells following a secondary challenge with Listeria monocytogenes. ................................................................. 75
Figure 3.14: CD44-deficient T cells outcompete WT for the generation of memory CD8 T cells. ........................................................................................................... 77
Figure 4.1: Cell and CD44 polarization correlate with optimal CXCL12-induced T cell migration. ........................................................................................................... 84
Figure 4.2: CD44 is not required for cell polarization and migration. .................... 86
Figure 4.3: CD44 is not required for mouse lymph node T cell migration at various stages of activation................................................................. 88
Figure 4.4: An inability to polarize CD44 correlates with reduced migration. ........... 89
Figure 4.5: The cytoplasmic domain of CD44 is required for its re-localization to the uropod and for optimal migration. ........................................................................... 91
Figure 4.6: Hyaluronan inhibits the migration of HA⁺, but not HA⁻, T cells. ................................. 93
Figure 4.7: Hyaluronan enhances activated T cell adhesion to fibronectin.................................... 94
Figure 4.8: Hyaluronan binding uncouples the CD44:pERM interaction and disrupts CD44:pERM co-polarization in response to chemoattractant. ................................................................. 97
Figure 5.1: Summary of hyaluronan binding during various stages of an immune response.... 104
Figure 5.2: Proposed model from this project. ............................................................................. 114
LIST OF ABBREVIATIONS

7AAD  7-aminoactinomycin D
APC   allophycanin
ATCC  American Type Culture Collection
BHI   brain heart infusion (broth)
BrdU  5-Bromo-2’-deoxyuridine
CCL   chemokine (C-C motif) ligand
CTL   cytotoxic lymphocyte
CXCL  chemokine (CXC motif) ligand
Cy    cyanine
DC    dendritic cell
ELISA enzyme-linked immunosorbent assay
ERM   ezrin/radixin/moesin
FACS  fluorescence-activated cell sorting
FITC  fluorescein isothiocyanate
FL-HA fluoresceinated hyaluronan
GTPase guanosine triphosphatase
HA    hyaluronan
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP   horseradish peroxidase
ICAM-1 intercellular adhesion molecule
IFN   interferon
Ig    immunoglobulin
IL    interleukin
LFA-1 lymphocyte function-associated antigen 1
LM-OVA Listeria monocytogenes expressing OVA antigen
LPS   lipopolysaccharide
MHC   major histocompatibility complex
MIP-1α macrophage inflammatory protein-1 alpha
NK    natural killer (cell)
OVA   ovalbumin
PBS   phosphate-buffered saline
PE     phycoerythrin
pERM   phosphorylated ezrin/radixin/moesin
PKC    protein kinase C
PMA    phorbol 12-myristate 13-acetate
PVDF   polyvinylidene fluoride
S1P    sphingosine-1-phosphate
SDF-1   stromal cell-derived factor-1
TBS    Tris buffered saline
TCR    T cell receptor
Th     T helper
TNF    tumour necrosis factor
T_{reg} regulatory T cell
VLA    very late antigen
ACKNOWLEDGEMENTS

I am grateful to my supervisor, Dr. Pauline Johnson, for her mentorship, support, advice, and perhaps most of all, her enthusiasm for science, during the course of this project. I am also thankful to the members of the Johnson lab, past and present, for their help with experiments, their feedback, and their friendship.

I thank my committee members, Dr. Ninan Abraham, Dr. Marc Horwitz, and Dr. Fumio Takei, for their scientific expertise and constructive comments. I also owe a thank you to Dr. Abraham’s and Dr. Hung-Sia Teh’s labs for their generosity with regards to some of the materials used in this study, as well as for technical advice.

Finally, I am always in debt to my family, for their unconditional support and encouragement.

This work was supported by the Canadian Institute of Health Research/Michael Smith Foundation for Health Research training program in transplantation, a graduate fellowship from the University of British Columbia, and travel funds from the Canadian Society of Immunology.
STATEMENT OF COLLABORATION

Some of the flow cytometry experiments in figure 3.1 and ELISA assays in 3.10A were performed by Grace Poon. Some of the ELISA assays in figure 3.10B were performed by Katharine Kott, who also prepared the dendritic cells used in those experiments. The bone marrow preparations used in figure 3.14 were performed by Manisha Dosanjh, who helped with some of the labeling for flow cytometry in figure 3.14, as well as some of the Jurkat polarization assays in figure 4.2 and 4.5. Jacky Yau, an undergraduate student under my mentorship, performed the AKR polarization assays in figure 4.4, and Aleksi Suo, another undergraduate student, performed some of the BW migration assays in figure 4.1. Darlene Birkenhead and Jennifer Cross helped to generate and genotype the OT-I CD44 knockout mice, Jennifer Cross performed the intravenous injections for one experimental repeat in figure 3.1, and Jacqueline Lai, Grace Poon, Manisha Dosanjh, and Pauline Johnson assisted with the intraperitoneal injections of BrdU.
CHAPTER 1: INTRODUCTION

1.1 The immune system

The immune system is assigned the task of protecting the body from pathogenic challenge by initiating inflammation and clearing the pathogen. In order to do this effectively it must also be able to resolve the inflammation and turn off the immune response once the pathogen is cleared. Such self-regulation is additionally necessary to avoid unwanted reactions to self-antigens or innocuous environmental antigens, as failure to do so is associated with autoimmunity and allergy.

The innate immune system is considered the first line of defense against a pathogen. The cells of the innate system recognize and are activated by signals that alert them to the presence of pathogens like bacteria and viruses. These signals are called pathogen-associated molecular patterns (or PAMPs) and include, for example, lipopolysaccharide (LPS) and double-stranded RNA. Alternatively, innate immune cells can also be activated by damage-associated molecular patterns (DAMPs), for example, uric acid, which alert them to tissue damage or necrotic cell death (Kono and Rock, 2008). Innate immune cells are not specific for a particular antigen, but rather recognize evolutionarily conserved “danger” signals (Gallucci and Matzinger, 2001). The first response to a danger signal is the initiation of inflammation, which recruits immune cells to the site to contain the infection. If this initial response is not sufficient to clear the pathogen, innate immune cells such as dendritic cells present antigenic fragments derived from the pathogen to the cells of the adaptive immune system in order to activate them.

The adaptive immune system consists of T and B cells. These cells express receptors that are antigen-specific: the specificity is generated through gene recombination of the antigen receptor chains in T and B cells and allows the recognition of an almost infinite number of
antigenic determinants (Oltz, 2001). Mice with mutations that prevent antigen-receptor chain
gene rearrangement, for example, severe combined immunodeficiency (SCID) mice, lack T and
B cells, and succumb to infection with bacteria and viruses (Bosma and Carroll, 1991). Besides
antigen specificity, a second key component of adaptive immunity is its ability to respond more
quickly and with greater magnitude to a secondary challenge – memory T and B cells provide the
host with improved protection in the event of future encounters with the same pathogen (Kaech
et al., 2002; McHeyzer-Williams and McHeyzer-Williams, 2005).

1.2 T cells in an immune response

In response to a pathogenic challenge, T cells expressing receptors specific for antigenic
determinants derived from that pathogen are activated in lymphoid organs and undergo rapid
proliferation followed by differentiation into effector and memory T cell subsets (Williams and
Bevan, 2007). Effector T cells are able to migrate throughout the body to the site of
inflammation where they contribute to clearance of the pathogen. Following pathogen clearance,
90-95% of antigen-specific T cells die by apoptosis leaving behind a small population of
memory T cells that persist in the host and provide long-term protection in the event of future
encounters with the same pathogen (Williams and Bevan, 2007). An overview of this process is
shown in figure 1.1.

1.3 CD4 and CD8 T cells

T cells can be separated into two lineages, based on the mutually exclusive expression of
the coreceptors CD4 and CD8. CD8 T cells recognize peptide antigen presented in the context
Figure 1.1: T cells in an immune response.
Naïve antigen-specific T cells (dark pink) are activated and undergo expansion and differentiation into effector T cells. Most of these antigen-specific T cells undergo apoptosis upon resolution of the immune challenge, which leaves behind a small population of memory T cells. Adapted from (Harty and Badovinac, 2008).
of MHC class I and primarily exert cytotoxic functions. Cytotoxic CD8 T cells function in killing cells infected with viruses and intracellular bacteria, and also in killing tumour cells (Harty et al., 2000). CD4 T cells recognize peptide antigen presented by MHC class II molecules and perform mostly helper functions (Castellino and Germain, 2006). There is also a special class of CD4+FoxP3+ T cells called regulatory T cells (T_{reg} cells) which originate in the thymus but migrate to the periphery and can suppress proliferation and cytokine production by self-reactive T cells (Tang and Bluestone, 2008). CD4 T helper cells have been found in some systems to be essential for the generation of an effective secondary immune response by CD8 memory cells (Bevan, 2004). The basis for this is not clear, but possible mechanisms for CD4 cell help in CD8 immune responses include stimulation of dendritic cells through CD40:CD40L interactions, and production of the cytokine IL-2 (Castellino and Germain, 2006).

CD4 T cells can differentiate into various effector lineages, including the T helper (Th) 1 and 2 subsets, as well as Th17 and the inducible regulatory T cells. Th1 T cells produce IFN\(\gamma\) to help infected macrophages clear intracellular bacteria. Th2 T cells, also known as follicular helper T cells, produce IL-4 and are effective in helping B cells to produce antibodies by enabling class switching and affinity maturation. Follicular helper cells are also essential for B cell germinal centre formation. Th17 cells, which produce IL-17 and IL-22, are thought to function in recruiting other cell types to kill extracellular bacteria or fungi. Inducible regulatory T cells express inhibitory cytokines and cell surface receptors (reviewed in (Weaver et al., 2007)).

1.4 T cell activation

In the lymph nodes, naïve T cells engage in transient contacts with dendritic cells (DCs) and scan the surface of DCs for cognate antigen presented by MHC molecules. If a T cell does
not recognize its specific antigen on the DC, the interaction is brief, and the T cell will move on to scan other DCs for antigen. Upon recognition of specific MHC:peptide complexes, the T cell will stop and form a more prolonged contact, leading to the development of the immunological synapse (Mempel et al., 2004; Stoll et al., 2002).

The organization of the immunological synapse is a highly ordered process which involves adhesive interactions at the cell interface between LFA-1 on the T cell and ICAM-1 on the antigen presenting cell and the close interaction of the T cell and antigen presenting cell membranes, which allows TCR/MHC:peptide interactions to occur. CD4 and CD8 co-receptors expressed on the surface of the T cell bind non-polymorphic regions of MHC class II and MHC class I molecules, respectively. Engagement of CD4 and CD8 at the immunological synapse then results in recruitment of Lck to the TCR/CD3 complex, and the subsequent phosphorylation of the TCR-associated CD3 zeta chains on immunoreceptor-based tyrosine activation motifs (ITAMs). This leads to recruitment of the ZAP-70 tyrosine kinase and the activation of further downstream signaling molecules (reviewed in (Fooksman et al.)).

However, the TCR/MHC interaction alone is not sufficient for naïve T cell activation, as TCR stimulation in the absence of a second signal, termed costimulation, causes T cells to undergo anergy, rather than activation (Appleman and Boussiotis, 2003). The costimulatory molecule CD28 on T cells interacts with CD80 or CD86 on dendritic cells and has been proposed to amplify signals through the TCR/CD3 complex by activating a signaling pathway involving phosphatidyl inositol 3-kinase (PI3K) (Rudd et al., 2009).
1.4.1 T cell proliferation and IL-2

Interleukin-2 or IL-2 is a cytokine that can promote activated T cell proliferation and survival as well as $T_{\text{reg}}$ cell development (Malek et al., 2002) and expansion (Tang et al., 2004). IL-2 is produced by CD4 and, to a lesser extent, CD8 T cells (Malek, 2008). Activation through the TCR and the costimulatory molecule CD28 in vitro leads to the activation of transcription of the cytokine IL-2 and the stabilization of its mRNA (Fraser et al., 1991; Lindstein et al., 1989). As T cell activation also leads to upregulation of the high affinity receptor for IL-2, IL-2R$\alpha$ (CD25), on the T cell surface, IL-2 binding to its receptor can signal to drive proliferation and expansion of the activated antigen-specific population (Malek, 2008).

The IL-2 receptor is made up of three subunits, CD25 (IL-2R$\alpha$), CD122 (IL-2R$\beta$), and CD132 (common gamma chain). IL-2 initially interacts with CD25, and then this complex binds CD122 and CD132. Interaction of all three receptor subunits is required for formation of the high affinity receptor for IL-2 (Malek, 2008). Entry of T cells into cell cycle in vitro requires sustained signaling through IL-2 receptor, thus, there is a need for cells to produce IL-2 and express IL-2 receptor subunits over a prolonged period of time (Cantrell and Smith, 1984).

The importance of IL-2 in proliferation is a little less clear in vivo, as IL-2- and IL-2 receptor-deficient mice exhibit a severe lymphoproliferative disorder and autoimmune diseases (Contractor et al., 1998; Sadlack et al., 1993; Willerford et al., 1995), which were found to be due to a lack of $T_{\text{reg}}$ cells (Malek et al., 2002). One model that has been used for studying the effect of IL-2 on activated T cell proliferation in response to an immune challenge in vivo involves the adoptive transfer of CD25$^{+/+}$ or CD25$^{-/-}$ T cells expressing a transgenic TCR (OT-I) that is specific for OVA peptide antigen into recipient mice (D'Souza and Lefrancois, 2003). When these mice were challenged with OVA antigen, the CD25-deficient T cells were deficient...
for expansion at later stages of the immune response (day 4 or later) although they initially expanded to similar levels. Thus, these authors concluded that IL-2 is important for the sustained expansion in vivo of activated T cells (D'Souza and Lefrancois, 2003).

IL-2 has also been suggested to have a role in effector T cell survival and death. The number of effector T cells from IL-2-deficient D011.10 mice dramatically declined following transfer into recipient mice compared to wildtype D011.10 effector T cells over a time course of about one month (Dooms et al., 2004). These effector T cells were generated in vitro by stimulation with antigen presenting cells and OVA peptide, and the survival of the IL-2-deficient T cells could be rescued if IL-2 was added to the culture when the cells were activated (Dooms et al., 2004). Not surprisingly, then, a lack of IL-2 receptor on T cells has been shown to inhibit memory T cell development (Dooms et al., 2007). On the other hand, activation-induced cell death is dependent on the presence of IL-2, as previously activated T cells undergo apoptosis at a 2-fold higher frequency upon restimulation in the presence of exogenous IL-2, and apoptosis is inhibited in IL-2-deficient T cells upon restimulation (Refaeli et al., 1998).

A recent study has also shown a role for IL-2 in regulating effector T cell homing (Sinclair et al., 2008). IL-2 activated PI3K can lead to the accumulation of PIP₃, which downregulates KLF2, a transcription factor important for expression of lymphoid homing receptors such as CCR7 and CD62L. Thus, the downregulation of CD62L on activated and effector T cells, which prevents their re-entry into lymph nodes, is likely due to the expression of CD25 on activated T cells and signaling by IL-2 (Sinclair et al., 2008). Thus, while IL-2 is critical for proliferation in vitro, and has been shown to have some effect on proliferation in vivo, it also has a diverse set of alternate functions in vivo (Ma et al., 2006).
1.4.2 Effector T cell responses

Following pathogenic challenge, antigen-specific T cells rapidly expand and differentiate into effector T cells, which leave the lymph nodes, migrate to the site of infection and exert various effector functions. These include cytotoxicity (killing) against cells infected with viruses and bacteria or the production of cytokines like interferon-gamma (IFN-γ), which can activate macrophage-mediated killing, and tumour-necrosis factor alpha (TNF-α), which can signal to initiate apoptosis of infected cells or activate adhesion molecule expression on endothelial cells and promote the recruitment of additional leukocytes (Harty et al., 2000).

Infection with Listeria monocytogenes is a well-characterized model for effector T cell responses in vivo (Pamer, 2004). L. monocytogenes is an intracellular bacterium that infects and resides within macrophages. The normal route of infection is through the gastrointestinal tract, as it is typically a food-borne pathogen (Hamon et al., 2006). Infection through an intravenous route results in bacterial replication in the spleen and liver, with bacterial load peaking at about 3-4 days after infection. The initial response is mediated by monocyte-derived TipDCs and MHC-class-Ib-restricted CD8 T cells, but bacterial clearance requires MHC-class-Ia-restricted CD8 effector T cells, which first appear about 5 days following infection, peak in numbers at about day 7 or 8, and thereafter undergo contraction at around day 9 or 10 (Pamer, 2004).

1.4.3 Contraction of the immune response

Following pathogen clearance, typically 90-95% of the antigen-specific T cell pool is eliminated by death by apoptosis, leaving behind only a small pool of memory cells (Williams and Bevan, 2007). Apoptosis is mediated by the caspase family of proteins, which cleaves proteins essential for cell survival and generates double-stranded breaks in DNA. Caspases can
be activated by both the extrinsic and intrinsic pathways of cell death. The extrinsic pathway of cell death is initiated by interaction of Fas (CD95) on the surfaces of T cells with Fas ligand (CD95L), and leads to formation of the death inducing signaling complex (DISC) and subsequent caspase activation. The intrinsic pathway is thought to occur in response to stress signals, for example, cytokine starvation, which leads to activation of pro-apoptotic molecules such as BIM (Bcl-2 interacting mediator of cell death). BIM can then interact with the anti-apoptotic molecule Bcl-2 (B cell lymphoma-2), which sequesters Bcl-2 away from interaction with BAX (Bcl-2 associated X protein) and BAK (Bcl-2 antagonist/killer), allowing BAX and BAK to oligomerize and initiate mitochondrial membrane permeabilization and release of cytochrome c, and caspase activation (reviewed in (Bouillet and O'Reilly, 2009)).

The signals that program cells for death are incompletely understood. Since contraction often correlates well with clearance of the infection, it would seem reasonable to think that effector cells are able to sense the absence of the pathogen and initiate a program of death. However, when mice were treated with antibiotics to help clear infection with *Listeria monocytogenes* on day 2 following infection, contraction of the CD8 effector T cells still occurred with similar kinetics (Corbin and Harty, 2004). On the other hand, pro-inflammatory signals, such as the cytokines IFN-γ and IL-12, have been shown to regulate contraction. In IFNγ-deficient mice, CD8 T cells do not undergo contraction following infection with attenuated *Listeria monocytogenes* (ActA mutant) although the mice were able to clear the infection as well as their wildtype counterparts (Badovinac et al., 2000).

A possible model for effector cell apoptosis proposes that it is a drop in the levels of cytokines that had supported T cell proliferation and survival that creates “stress” as the remaining T cells compete for survival signals (Bouillet and O'Reilly, 2009). This leads to
activation of BIM and induction of the intrinsic pathway for cell death. There is also evidence for a role for CD95 and the extrinsic pathway for cell death during chronic immune responses (Hughes et al., 2008). Effector T cells are susceptible to activation-induced cell death (AICD) upon restimulation, and this process is dependent on Fas (CD95) and TNF-α (Bouillet and O'Reilly, 2009).

1.4.4 Regulatory T cells

Regulatory T (T<sub>reg</sub>) cells are a crucial means of dampening or inhibiting an immune response. Several types of regulatory T cells have been defined, including the naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells, inducible regulatory T cells, IL-10-producing Tr1 cells, T helper 3 cells, and CD8<sup>+</sup> T suppressor cells (Tang and Bluestone, 2008). Depletion of the CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in mice leads to catastrophic autoimmunity (Kim et al., 2007; Sakaguchi et al., 1995). Naturally occurring T<sub>reg</sub> cells develop in the thymus and express FoxP3, a transcription factor important for their development and regulatory function (Hori et al., 2003). There are several mechanisms by which T<sub>reg</sub> cells are thought to regulate immune responses. T<sub>reg</sub> cells can secrete immune response-dampening cytokines such as IL-10 and TGFβ, and also express inhibitory signaling receptors such as CTLA-4 (Tang and Bluestone, 2008). Second, due to their high surface expression of CD25, the receptor for IL-2, it is thought that T<sub>reg</sub> cells soak up available IL-2 and thereby deprive effector T cells of the cytokine (Pandiyan et al., 2007).

1.4.5 Memory T cells

Although the majority of effector cells undergo contraction and cell death following clearance of the pathogen, a population of memory T cells persists and is maintained in order to
protect the body from subsequent exposure to the same pathogen. A secondary or memory response is distinct from the primary response in that it is faster, greater in magnitude, and can occur in response to lower doses of antigen (Pihlgren et al., 1996; Rogers et al., 2000). Memory T cells are also defined by their ability to undergo proliferation and differentiation into secondary effector and memory subsets upon re-challenge (Jameson and Masopust, 2009). Once established, the memory T cell pool for a particular antigen is maintained at relatively constant numbers by signals for both survival as well as low-level division to counteract cell death. The cytokines interleukin-7 and -15 (IL-7, -15) are known to deliver the signals that promote CD8 memory T cell survival, and proliferation, respectively, in vivo (Ma et al., 2006; Surh and Sprent, 2008).

IL-7 and IL-15 belong to the gamma chain family of cytokines, which also includes IL-2, and is so named because these receptors share a common gamma chain (CD132). The receptor for IL-15 consists of IL-15Rα, CD122 (IL-2Rβ) and CD132. CD122 expression is low on naïve T cells but is upregulated following T cell activation and is often used, along with CD44 and CD127, as a marker for memory T cells (Boyman et al., 2006; Zhang et al., 1998). The receptor for IL-7 consists of CD127 (IL-7Rα) and CD132 and is highly expressed on naïve T cells but downregulated upon T cell activation through IL-2 signaling (Rochman et al., 2009). CD127 is expressed on memory cells, and it has been suggested that effector cells that express high levels of CD127 preferentially survive to become memory cells by outcompeting other cells for survival signals from limiting amounts of IL-7 (Kaech et al., 2003). IL-7 is produced by fibroblastic reticular cells in the T cell zones of lymph nodes and also by stromal cells in the bone marrow (Link et al., 2007; Mazzucchelli et al., 2009).
IL-7 is also thought to be responsible for homeostatic proliferation, which occurs upon conditions of lymphopenia (Surh and Sprent, 2008). Normal T cell homeostasis is achieved through low-level proliferation initiated by IL-7 binding to IL-7R on naïve T cells (Maraskovsky et al., 1996; Tan et al., 2001). When lymphocyte numbers drop below a certain threshold, the excess IL-7 is thought to cause proliferation and expansion to replenish numbers. Lymphopenia-induced proliferation induces an upregulation of CD44 and CD122, and thus alters the phenotype of the T cells (Surh and Sprent, 2008).

CD8 T cells with a memory-like phenotype (MP CD8 T cells, which are CD44<sup>hi</sup>CD122<sup>hi</sup>) can be generated in the absence of antigen stimulation and are thought to arise in lymphopenic hosts in response to homeostatic proliferation (Cho et al., 2000). Significantly, memory phenotype antigen-specific T cells generated by transfer of OVA-specific OT-I transgenic CD8 T cells into sublethally irradiated mice were found to be equally protective against <i>Listeria monocytogenes</i> challenge as antigen-experienced memory T cells generated by transfer of OT-I T cells into mice followed by a primary antigen challenge (Hamilton et al., 2006). Memory phenotype T cells have also been generated in vitro, by culture of activated T cells in high concentrations of IL-15. These cells have a central memory-like phenotype, characterized by the expression of high levels of CD62L, which is typically downregulated upon T cell activation and low on effector cells, opposite to the expression levels of CD44 (Weninger et al., 2001).

Memory CD8 T cells have traditionally been classified into two subsets based on their expression of homing receptors and adhesion molecules (Sallusto et al., 1999). Central memory T cells are marked by expression of the chemokine receptor CCR7, which facilitates homing to lymph nodes in response to CCL19 and CCL21, as well as L-selectin or CD62L, which also promotes homing to lymph nodes. Therefore, central memory T cells typically reside in
secondary lymphoid organs such as the lymph nodes. Effector memory T cells are CCR7lo and CD62Llo and circulate throughout the body. Although effector memory T cells are present in higher numbers immediately following antigen clearance, they are gradually overtaken in numbers by the central memory T cells, likely through increased homeostatic turnover. A second difference between effector and central memory T cells is that while both can produce the effector cytokine IFNγ upon rechallenge, central memory T cells more readily produce IL-2, which could facilitate their increased expansion during a secondary response (Sallusto et al., 2004).

The function and phenotype of CD4 memory T cells, unlike CD8 memory T cells, is less well understood. While CD8 memory T cells are well-maintained throughout the lifetime of the host, CD4 memory T cells were found to progressively decline in numbers following LCMV infection. CD4 memory T cells typically do not express high levels of CD127, although their survival, like CD8 memory T cells, is dependent on IL-7, and only about 30% upregulate CD62L. CD4 memory T cells also do not express high levels of CD122, but do undergo slow turnover in response to IL-15 (Macleod et al., 2010; Stockinger et al., 2006).

1.4.6 Effector and memory T cells: models of differentiation

The differentiation of naïve T cells following activation and expansion into effector and memory T cells has been the subject of much debate in recent years and several models of how this differentiation occurs have been proposed. It has been shown that naïve T cells, upon activation, can generate effector cells, which mediate the primary response, and also yield a population of memory cells that survive contraction and mediate a secondary response upon subsequent re-exposure to the same pathogen. When a single antigen-specific CD8 T cell was
transferred into a naïve host, this cell could yield both effector and memory T cell populations (Stemberger et al., 2007). Early studies proposed a linear differentiation model, whereby naïve T cells were activated and differentiated into effector T cells, and then a small subset of these survived contraction to differentiate into memory T cells (Kaech et al., 2002). Thus, all memory cells had to first progress through an effector cell stage.

In contrast, a breakthrough paper in 2007 by Chang et al. provided evidence for the uneven or asymmetric division of molecules upon the very first division following CD8 T cell activation that resulted in two very different daughter cells (Chang et al., 2007). The proximal daughter cell, or the cell at the immunological synapse formed with the antigen presenting cell, expressed high levels of IFNγ and granzyme B, but low levels of L-selectin or CD62L, and high levels of the activation markers CD25 (IL-2Rα), CD44, and CD69, consistent with an effector cell phenotype. By contrast, the distal daughter cell expressed low levels of IFNγ, granzyme B, CD25, and CD69, medium levels of CD44, and high levels of CD62L and also had high levels of IL-7Rα, consistent with a memory phenotype. More significantly, when these proximal and distal populations were sorted and transferred into naïve recipient mice, both populations were able to respond to an immediate challenge with Listeria presenting a specific antigen (gp30), but only the distal population, which had a memory phenotype, was able to effectively clear the pathogen when the mice were challenged 30 days following T cell transfer. This suggested that effector and memory cell fates were decided upon the very first cell division, and memory T cells did not have to undergo an initial effector phase.

More recently, a study by Bannard et al. has elaborated on this model by demonstrating that CD8 T cells that had differentiated into effector cells that produced the effector molecule granzyme B could still replicate and contribute to a secondary immune response upon
rechallenge, suggesting that effector T cells are not terminally differentiated cell types (Bannard et al., 2009). Additionally, a study by Harrington et al. (Harrington et al., 2008) used a technique to irreversibly mark CD4 IFNγ-producing effector T cells and showed that these cells also survived and contributed to the memory pool. Thus, a possible model that incorporates these data is summarized in figure 1.2, where naïve T cells are activated and differentiate into both effector and memory T cell subsets, but effector T cells are also able to contribute to the memory T cell pool.

1.4.7 Rechallenge and secondary immune responses

Tissue resident memory T cells are thought to act as a first line of defense upon rechallenge with a previously recognized pathogen, and are able to rapidly expand and exert effector functions upon reactivation in the tissues. Recent evidence has demonstrated tissue-specific memory CD8 T cell activation in peripheral tissues by antigen-presenting dendritic cells (Wakim et al., 2008). The pro-inflammatory milieu is thought to recruit additional memory T cells from the circulation. Although the number of antigen-specific memory T cells present in the tissues and recruited from the circulation is not great, their expansion and effector function is likely to contain the pathogen and prevent its spread while dendritic cells migrate to draining lymph nodes to present antigen to, and activate, lymphoid organ-resident memory cells, which can then generate secondary effector T cells. These then exit the lymph nodes and join in the response. Thus, secondary responses are faster than primary responses, and can contain and eliminate higher doses of pathogen than primary responses (reviewed in (Woodland and Kohlmeier, 2009)).
Figure 1.2: Model for effector and memory T cell differentiation.
Upon activation, T cells have been shown to differentiate into distinct daughter cells with effector or memory T cell phenotypic and functional properties. However, effector T cells have also been suggested to contribute to the memory T cell pool. Cytokines important for maintenance or proliferation of naïve (IL-7), activated (IL-2), and memory T cells (IL-7 and IL-15) are also summarized.
1.5. T cell homing and recruitment

Adhesion and migration are highly regulated and important processes during a T cell immune response. Naïve T lymphocytes circulating in the blood have been shown to form transient interactions with the walls of high endothelial venules (HEV) that are dependent on interactions between L-selectin and sialylated ligands such as peripheral node addressins (PNAdS) (Ley et al., 2007). These transient interactions facilitate cell rolling under conditions of blood flow, and increase the likelihood of encountering chemokines such as CCL19 and CCL21, important for entry into lymph nodes, on the vascular lumen. The integrin LFA-1 is activated on the T cell by chemokine-induced inside-out signaling and binds its ligand, ICAM1 on the endothelial cells, which allows the rolling cells to form tight adhesive interactions with the endothelium (Ley et al., 2007). Adhesive interactions involving junctional adhesion molecules (JAMs) on the endothelial cells and integrins on the leukocytes then mediate leukocyte transmigration across the endothelium (Ley et al., 2007). Once in the lymph nodes, T cells migrate along the fibroblastic reticular cell (FRC) network. This is thought to involve guidance by the chemokines CCL19 and 21 which can direct T cells to the T cell areas in the paracortex (Bajenoff et al., 2006; Okada and Cyster, 2007). Here, they search for antigen-bearing dendritic cells. Upon encountering cognate antigen-bearing dendritic cells, T cells are activated and expand by undergoing rapid proliferation. The chemokine CCL3, or MIP-1α, attracts CD8 T cells to dendritic cell:CD4 T cell complexes (Castellino et al., 2006). T cell recruitment to sites of inflammation then require circulating T cells to extravasate from blood vessels into tissues and migrate through tissues along extracellular matrix components to the site of inflammation. This involves the upregulation of receptors for inflammatory chemokines, such as CCR1 and CCR5 on Th1 effector T cells (Sallusto and Mackay, 2004). Thus, the orchestration of naïve T cell
homing into lymph nodes and effector cell recruitment into tissues is critically dependent on being able to regulate migratory and adhesive signals.

1.5.1 Chemoattractants and their receptors

The interaction of chemoattractants with their receptors is crucial for directing immune cells to the appropriate tissues both during resting and inflammatory states. Homeostatic chemokines such as CCL19/21 and CXCL12 are produced under resting conditions and facilitate the normal trafficking of cells through the lymph nodes (Baekkevold et al., 2001; Gunn et al., 1999; Robbiani et al., 2000) and bone marrow (Bleul et al., 1996; Hauser et al., 2002), respectively. Inflammatory chemokines such as CCL5 (RANTES) and CCL3 are upregulated following leukocyte activation (Schrum et al., 1996) and mediate effector CD8 and CD4 Th1 cell recruitment (Crane et al., 2006; Harlin et al., 2009).

Getting the right cells to the right tissues not only requires guidance by the appropriate homeostatic or inflammatory chemokines, it is also dependent on the regulated expression of the appropriate chemokine receptors. The receptor for sphingosine-1-phosphate, which allows circulating naïve T cells to leave the lymph nodes, is highly expressed on naïve T cells but is downregulated upon T cell activation (Matloubian et al., 2004). It is thought that the downregulation of the receptor, S1P1, allows activated T cell retention in the lymph nodes until they have expanded, differentiated into effector T cells, and are ready to get recruited to peripheral sites of inflammation. Chemoattractant binding to its receptor thus serves multiple functions in migration: it induces migration and regulates adhesion, as mentioned above, and also signals to drive polarization (Gomez-Mouton et al., 2001).
1.5.2 Polarization and the polarity complex

In response to a chemoattractant stimulus, T cells undergo dramatic shape changes mediated by rearrangement of their actin cytoskeleton (Vicente-Manzanares and Sanchez-Madrid, 2004). Migrating T cells polarize to form actin-rich lamellipodia at the leading edge, and a trailing edge, or uropod, which is enriched in certain adhesion molecules such as CD43, intercellular adhesion molecules (ICAMs), PSGL-1, and CD44, and with actin-rich linker proteins, ezrin/radixin/moesin (ERM), which link transmembrane receptors to the actin cytoskeleton (Vicente-Manzanares and Sanchez-Madrid, 2004). At the leading edge, the Rho family GTPase Rac induces actin polymerization which generates protrusive forces necessary for migration (Nobes and Hall, 1999). Integrins localize along the base of the midbody of the cell through adhesive interactions with substrate (Smith et al., 2005), and these interactions are thought to generate the traction necessary for migration on a two-dimensional surface (Lammermann and Sixt, 2009). Contractile forces at the trailing edge mediated by Rho signaling support de-adhesion and retraction of the uropod (Alblas et al., 2001), which is thought to allow the cell body to move forward (Lammermann and Sixt, 2009). Thus, polarization allows re-organization of the cell into specialized zones that have distinct functions in migration (figure 1.3A).

T cell polarity is controlled by the Par, Crumbs, and Scribble polarity complexes, which can regulate the localization of adhesion receptors, signaling molecules, and organelles (Krummel and Macara, 2006). The partitioning defective (Par) complex is comprised of Par3, Par6 and PKC-ξ, the Scribble complex includes Scribble, Dlg, and Lgl, and the Crumbs complex consists of Pal1 and PatJ (Krummel and Macara, 2006). These complexes are themselves differentially localized throughout the cell body (figure 1.3B), with Par proteins
Figure 1.3: T cell polarization upon chemokine stimulus.
A. Localization of adhesion molecules, ERM proteins, and chemokine receptors in migrating polarized T cells. B. Localization of polarity complexes.
concentrated at the front of the cell and in the cell midbody, Crumbs proteins localized throughout the cell, and Scribble localized almost exclusively in the uropod (Ludford-Menting, 2005). Sh-RNA mediated knockdown of Scribble prevented uropod formation and the localization of typical uropod constituents like CD44 and ezrin to the uropod (Ludford-Menting, 2005). Furthermore, Scribble knockdown also impaired T cell migration. This suggests that polarization and uropod formation are critical for T cell migration.

Although some studies have addressed the significance of re-localizing certain adhesion receptors and signaling intermediates to the uropod of the cell, their function in migration is still unclear. Previous work has suggested that recruiting ICAM molecules to the uropod could help to recruit other cells, such that a migrating cell pulls other migrating cells along with it through cell-cell adhesion involving LFA-1 and ICAM-1 and -3 (del Pozo et al., 1997). However, blocking other adhesion molecules, such as CD43 and CD44, showed that not all adhesion molecules in the uropod are important in this process (del Pozo et al., 1997). Savage et al. have shown that inhibiting CD43 re-localization to the uropod impairs uropod de-adhesion (Savage et al., 2002), but this did not have an effect on the ability of the cells to migrate. Recently, Mrass et al. observed that a deficiency in CD44 polarization to the uropod is associated with poorer recruitment of killer T cells to a tumour site (Mrass et al., 2008).

1.6 Adhesion and adhesion molecules

Adhesion molecules are surface proteins that mediate cell-cell and cell-substrate adhesive interactions and have been grouped into families based on their structural similarities (reviewed in (Aplin et al., 1998)). The immunoglobulin superfamily is a large group of molecules which have one or multiple copies of an immunoglobulin domain. Members of this family include CD4
and CD8, which are important in T cell activation, as well as ICAM-1 and -2, VCAM-1 and PECAM-1, which are important in trafficking. Cadherins are membrane proteins that localize to cell-cell adherence junctions and share in common an amino-terminal extracellular domain consisting of tandem repeats of a cadherin-specific motif. Members of the integrin family are heterodimeric receptors consisting of alpha and beta chains and bind extracellular matrix proteins, such as fibronectin and collagen, or transmembrane counter-receptors on other cells, and can exist in both low and high affinity binding states. In some cases, integrin adhesion can support migration, as migration is enhanced on two-dimensional surfaces coated with integrin substrates such as fibronectin (Vuori and Ruoslahti, 1993). Recent evidence looking at three-dimensional migration through the tissues, however, showed that integrin adhesion is unnecessary for migration, since knocking out all possible integrin heterodimers did not impair T cell or dendritic cell migration through 3D collagen gels, although it did impair 2D migration on immobilized ICAM-1 or fibronectin substrates (Lammermann et al., 2008). The selectin family proteins have a lectin domain that binds sialylated or sulfated glycans on other cells. CD44 is a member of the Link module superfamily which binds hyaluronan. The Link module possesses some structural similarity to the C-type lectins (Johnson et al., 2000; Teriete et al., 2004).

1.7 CD44

CD44 is a transmembrane cell adhesion molecule that is widely expressed on multiple cell types, including leukocytes, endothelial cells, epithelial cells, and fibroblasts, and has been implicated in cellular processes involving cell adhesion and migration, including leukocyte trafficking, wound healing, angiogenesis and tumor metastasis (Ponta et al., 2003). CD44 is encoded by a single gene, but can exist in multiple forms due to alternative splicing of ten exons.
The most common form is the smallest form, CD44s (standard) or CD44H (hematopoietic), which has a molecular mass of about 80 kilodaltons and consists of an amino terminal hyaluronan binding domain, a 23-amino acid transmembrane domain, and a 72-amino acid cytoplasmic domain (figure 1.4A). CD44 is best characterized as a receptor for the extracellular matrix component, hyaluronan. However, CD44 has also been shown to bind to other adhesion molecules, such as L- and E-selectin, and also binds components of the extracellular matrix, including fibronectin, collagen, and vitronectin (Ponta et al., 2003).

1.7.1 Hyaluronan

The extracellular domain of CD44 has a Link domain which binds hyaluronan, a component of the extracellular and pericellular matrices of cells. Hyaluronan is a glycosaminoglycan made up of repeating units of the disaccharide (1-4) D-glucuronic acid-β (1-3)-N-acetyl-D-glucosamine (figure 1.4B). Hyaluronan can exist at various sizes due to the action of hyaluronidases which can cleave hyaluronan into small fragments. While high molecular weight (>10^3 kDa) hyaluronan has been suggested to have anti-inflammatory properties, low molecular weight hyaluronan fragments (<500 kDa) are pro-inflammatory (Jiang et al., 2007; Turley et al., 2002). Hyaluronan binding by CD44 is regulated by the expression levels of CD44, isoform usage, and post-translational modifications such as sulfation, sialylation, and glycosylation (reviewed in (Ruffell and Johnson, 2009)).

1.7.2 CD44 knockout mice

CD44 knockout mice develop normally and for the most part CD44 does not appear to have any function during homeostasis, with the exception of a slight defect in homing to lymph
Figure 1.4: CD44 and hyaluronan.
A. The extracellular domain of CD44 interacts with hyaluronan, and the cytoplasmic domain of CD44 interacts with ERM proteins, which link CD44 to the actin cytoskeleton. B. Hyaluronan is composed of repeating disaccharide units (adapted from (Jiang et al., 2007)).
nodes and thymus (Protin et al., 1999) in the CD44 knockout. However, there are significant
differences between the wildtype and knockout in their immune response to a challenge.
Granulomas in the livers of CD44 knockout mice were significantly larger and more numerous
compared to those in wildtype mice after injection of heat-killed Corynebacterium parvum, an
intracellular parasite (Schmits et al., 1997). In an experimental model of collagen-induced
arthritis (CIA), CD44 knockout mice on a DBA/1 background are protected from arthritis
development compared to their wildtype counterparts, likely due to decreased recruitment to
inflamed joints (Stoop et al., 2002; Stoop et al., 2001). On the other hand, upon bleomycin-
induced lung injury, CD44 knockout mice had increased inflammation and impaired clearance of
apoptotic neutrophils and hyaluronan fragments compared to wildtype mice (Teder et al., 2002).
Thus, studies using knockout mice have shown both pro- and anti-inflammatory effects of CD44.
Significantly, CD44 expression and its ability to bind its ligand hyaluronan are upregulated upon
activatory or pro-inflammatory stimuli in several cell types, including T cells, B cells, monocytes,
and dendritic cells, suggesting that the function of CD44 is likely to be more apparent upon
immune challenge.

1.7.3 The cytoplasmic domain of CD44: interaction with ERM proteins

CD44 has been shown to signal to the actin cytoskeleton and mediate cell shape changes
(Wong et al., 2008) but does not contain any actin binding motifs. Instead, CD44 has been
shown to bind ERM proteins, which comprise a family of cytoskeletal adaptor proteins that link
transmembrane receptors to the actin cytoskeleton (figure 1.4). The ERM family members, ezrin,
radixin, and moesin share a high degree of sequence identity (70-85%), although their expression
patterns in various cell types and their ability to interact with certain partner proteins are reported to be distinct (Ilani et al., 2007; Schwartz-Albiez et al., 1995). Ezrin, but not moesin, has been shown to interact with ZAP-70 and CD95 (Ilani et al., 2007; Parlato et al., 2000). T cells predominantly express ezrin and moesin, with moesin highly expressed in naïve T cells, and moesin and ezrin expressed at roughly equal levels in activated T cells (Ilani et al., 2007).

The N-terminus of ERM proteins consists of a band 4.1 ERM homology (FERM) domain, which mediates association with transmembrane receptors, including CD44, as well as CD43, PSGL-1, and ICAMs. The C-terminus has an F-actin binding domain (Ivetic and Ridley, 2004). ERM proteins are regulated by phosphorylation of a C-terminal threonine residue, Thr 567 in ezrin, 558 in moesin, and 564 in radixin (Ivetic and Ridley, 2004). In their active or phosphorylated state, ERM proteins associate with both membrane receptors and F-actin. In response to TCR or chemokine stimulus, ERM proteins are rapidly and transiently dephosphorylated (Brown et al., 2003; Faure et al., 2004). Dephosphorylation of the C-terminal threonine residue results in an intramolecular interaction of the N- and C-termini, masking both the F-actin binding site, and the membrane protein binding site (Ivetic and Ridley, 2004). The CD44:ERM interaction is also regulated by serine phosphorylation of CD44. One study showed that PKC activation led to phosphorylation of the juxtamembrane serine 291 residue, which inhibited CD44 binding to ezrin (Legg et al., 2002).

The actin-binding function of ezrin is critical for cell migration, as expression of ezrin mutated for key residues in the C-terminal actin binding domain inhibited NIH3T3 transfectant cell migration (Saleh et al., 2009). There is also evidence that phosphorylated ERM proteins can induce cell polarization. Overexpression of a phosphomimetic mutant of ezrin (T567D) in human T cells (Li et al., 2007) or in a mouse EL4 T cell line (Lee et al., 2004) could cap ezrin
and CD44 in the absence of a chemoattractant stimulus (Li et al., 2007), increased the size of the uropod (Lee et al., 2004) and increased migration in response to the chemoattractant SDF-1 (Lee et al., 2004; Li et al., 2007), whereas a phosphorylation-negative mutant (T567A) did not polarize and did not have an effect on migration (Lee et al., 2004; Li et al., 2007). Thus, ERM protein phosphorylation and polarization to the uropod are important in migration, although it is not clear what causes ERM proteins to go to the uropod.

Previous evidence indicated that a constitutive phospho-mimetic ERM mutant can induce CD44 re-localization (Lee et al., 2004), and interaction with ERM proteins is important in recruitment of PSGL-1 (Serrador et al., 2002) and CD43 (Savage et al., 2002) to the uropod. However, the recent finding that CD44-deficient T cells poorly polarized ERM proteins (Mrass et al., 2008) was surprising and suggested that ERM protein localization in the uropod is dependent on CD44. This would point to a critical role for CD44 in T cell polarization and migration.

1.7.4 Function of CD44 on leukocytes

Several functions have been attributed to CD44 and hyaluronan binding in various leukocyte cell types. In neutrophils, loss of CD44 leads to reduced random and directed (fMLP) migration, and a reduced ability to polarize in response to fMLP (Alstergren et al., 2004). In this study, migration of WT (CD44 positive) neutrophils was inhibited on an immobilized hyaluronan substratum, suggesting that while CD44 has a positive role in migration, hyaluronan has an opposing, negative effect (Alstergren et al., 2004). In natural killer (NK) cells, the absence of CD44 and LFA-1 on NK cells significantly inhibits conjugate formation with target cells and target cell killing. Here, treatment with hyaluronidase to digest hyaluronan on the
surface of target cells also inhibits killing, suggesting an important role for hyaluronan in target cell recognition, binding, and cytotoxicity by NK cells (Matsumoto et al., 1998).

1.7.5 Function of CD44 on T cells

Early studies for a role for CD44 on T cells pointed to a possible function as a costimulatory molecule. Co-ligation of CD44 using crosslinking monoclonal antibodies augments IL-2 production and T cell proliferation induced by crosslinking CD3 (Foger et al., 2000). A positively-charged peptide that inhibits hyaluronan binding was shown to inhibit IL-2 and IFN\(\gamma\) production and T cell proliferation upon co-culture of D011.10 transgenic T cells with OVA-presenting dendritic cells (Mummert et al., 2002). Others have also suggested hyaluronan can costimulate allogeneic T cell activation by enhancing activation of dendritic cells in a CD44-independent manner, although the presence of CD44 on the T cells was important for optimal activation (Do et al., 2004).

CD44 knockout effector CD8 T cells were recently shown to be defective for recruitment to tumour sites and thus CD44 knockout mice have increased tumour numbers and size compared to wildtype mice (Mrass et al., 2008). This effect occurred independently of hyaluronan, as the effector cells used did not bind hyaluronan. Additionally, an earlier study has shown that hyaluronidase treatment inhibited effector T cell recruitment to peripheral sites of inflammation (DeGrendele et al., 1997a).

Recent work has shown a deficiency in the generation of CD44 knockout memory CD4 T cells in the lung in response to influenza virus (Baaten et al., 2010). In vitro-generated CD44 knockout Th1 cells also had poorer survival upon transfer into recipient mice compared to their wildtype counterparts, suggesting that CD44 has an important function in survival of effector
cells and the generation of memory. In fact, CD44 knockout T cells had lower levels of phosphorylated Akt compared to wildtype T cells (Baaten et al., 2010). Administration of a hyaluronan blocking anti-CD44 antibody, KM201, in mice, had a similar effect to inhibit memory T cell development (Baaten et al., 2010), however a direct role for hyaluronan in memory T cell development or persistence has not been established. Curiously, the effect of knocking out CD44 only affected the survival of T helper type (Th) 1, but not Th2, Th17, or CD8 T cells (Baaten et al., 2010).

1.7.6 CD44 binding to hyaluronan on T cells

On naive mouse T cells, CD44 is present as the inactive, non-hyaluronan binding form, but can be induced to bind hyaluronan upon activation through the TCR (Ariel et al., 2000; DeGrendele et al., 1997b; Firan et al., 2006; Lesley et al., 1994). On human peripheral T cells, adhesion to immobilized hyaluronan can be induced upon exposure to pro-inflammatory cytokines such as TNF-α and IL-2 (Ariel et al., 2000). Lesley et al. showed that a subpopulation of CD4 and CD8 T cells could be induced to bind hyaluronan in vivo 7-10 days following an allogeneic challenge (Lesley et al., 1994). Here, hyaluronan binding marked a population of T cells with cytotoxic ability, although treatment of these cells ex vivo with hyaluronan did not have an effect on cytotoxicity (Lesley et al., 1994). Bonder et al. demonstrated low-level hyaluronan binding induced on Th1 and Th2 CD4 helper T cells upon in vitro antigen stimulation and differentiation with IL-12 and IL-4, respectively (Bonder et al., 2006). Here, interaction with immobilized hyaluronan facilitated cell rolling. This supported a previously published role for hyaluronan binding in T cell extravasation to inflammatory sites (DeGrendele et al., 1997a). Hyaluronan binding was also induced on a subset of CD4⁺CD25⁺ regulatory T
cells upon activation in vitro (Firan et al., 2006), and the ability to bind hyaluronan marked cells with the greatest suppressor activity. Others have since demonstrated that hyaluronan treatment functions to enhance FoxP3 expression and the production of the cytokines IL-10 and IL-2 (Bollyky et al., 2009). However, hyaluronan binding was also induced on a subset of non-regulatory CD4 T cells (Firan et al., 2006). Taken together, the existing evidence suggests that hyaluronan binding can be induced on T cells with distinct immunological functions and yet only marks a subpopulation of all of these cell types. It is still unclear whether effector and memory cells, which express high levels of CD44, are able to bind hyaluronan.

1.8 Thesis aims and rationale

CD44 has been used as a marker for activated effector and memory T cells (Dutton et al., 1998). During the course of this work, recent developments have emerged demonstrating that hyaluronan can induce death in activated T cells restimulated in the presence of IL-2, conditions known to cause activation-induced cell death (Ruffell and Johnson, 2008). On the other hand, there has also been work showing that CD44 and perhaps hyaluronan might affect the survival of effector T cells and the generation of memory T cells (Baaten et al., 2010). These developments underline a need to first identify and characterize the populations of both CD4 and CD8 T cells which bind hyaluronan during an immune response. Once this is done, the consequences of acquiring the ability to bind hyaluronan can be determined.

The first aim of this project will thus be to characterize the T cells that bind hyaluronan during an immune response. To date, it is not clear whether CD44 on effector and memory T cells binds hyaluronan. Lesley et al. identified a subpopulation of CD8 T cells with cytotoxic activity that binds hyaluronan following allogeneic challenge in vivo (Lesley et al., 1994), but
antigen-specific T cells in a study by Mrass et al. (Mrass et al., 2008) were found to have downregulated their HA binding. Furthermore, while certain functions have been attributed to hyaluronan binding in specific T cell types, for example, in promoting survival of regulatory T cells (Bollyky et al., 2009), there is no explanation for what hyaluronan binding does on the other cell types on which it is induced, for example, the non-regulatory CD4 T cells identified in the study by Firan et al. (Firan et al., 2006). Thus, it seems likely there are other downstream consequences for binding hyaluronan.

The second aim of this project will be to determine the effect of CD44 and hyaluronan binding on T cells during an immune response. Wildtype and CD44 knockout mice will be compared for their ability to mount a primary and secondary immune response to a pathogenic challenge using *Listeria monocytogenes* as the infectious agent. To follow up these studies, the effect of CD44 and hyaluronan on naïve and effector T cells in vitro will be determined using IL-2 production and cytotoxicity, respectively, as physiological readouts.
CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Mice

C57BL/6 mice were obtained from Jackson, maintained through homozygous matings and housed in Wesbrook Animal Unit at the University of British Columbia. Congenic Boy/J mice expressing CD45.1 antigen were also obtained from Jackson Laboratory. CD44\textsuperscript{-/-} mice (Schmits et al., 1997) backcrossed onto the C57BL/6 background for 6 generations were housed and maintained in the same facility. OT-I transgenic mice expressing TCR specific for OVA\textsubscript{257-264} in the context of H2-K\textsuperscript{b} (Hogquist et al., 1994) and OT-II mice expressing TCR specific for OVA\textsubscript{323-339} in the context of IA\textsuperscript{b} (Barnden et al., 1998) are available from Jackson and were also maintained in this facility. OT-I were mated to CD44\textsuperscript{-/-} mice to generate OT-I CD44\textsuperscript{-/-} mice. Mice used for experiments were between 6-20 weeks of age. All animal experiments were conducted in accordance with protocols approved by the University Animal Care Committee and Canadian Council of Animal Care guidelines.

2.1.2 Antibodies

Antibodies specific for mouse antigens were used for flow cytometry: CD4 clone GK1.5, CD8\textalpha clone 53-6.7, CD11c clone N418, CD25 clone PC61.5, CD40 clone HM40-3, CD45.1 clone A20, CD45.2 clone 104, CD62L clone MEL-14, CD69 clone H1.2F3, CD80 clone 16-10A1, CD86 clone GL1, CD122 clone 5H4, CD127 clone A7R34, CD3 clone 145-2C11, MHC class II I-A/I-E clone M5/114.15.2, interferon gamma clone XMG1.2, and interleukin-2 clone...
JES6-5H4 (all from eBioscience, conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), PECy5, PECy7, allophycocyanin (APC), APCCy7, or Pacific blue), CXCR4 clone G19 (Santa Cruz Biotechnology), human Ki67 clone B56 conjugated to PE (BD Biosciences), and CD44 clone IM7 was obtained from ATCC and conjugated according to the manufacturer’s instructions to Alexa 647 or Pacific blue (Molecular Probes). Antibody against 5-Bromo-2’-deoxyuridine, BrdU clone MOPC-21 conjugated to Alexa647 (Molecular Probes) and used for flow cytometry to measure proliferation. Isotype control antibodies used for intracellular cytokine staining were rat IgG1 (for IFNγ, eBioscience) and rat IgG2b (for IL-2, eBioscience).

Biotin-conjugated antibodies used for T cell isolation by negative selection were against B220 antigen clone RA3 6B2, CD11b (Mac-1) clone M1/70, Ter119 clone Ter119, CD4 clone GK1.5, and CD8 clone 53-6.7 (BD Biosciences or the BRC antibody facility).

Purified antibodies used for T cell stimulation were against CD3ε clone 145-2C11 (ATCC) CD44 clone IM7 (ATCC) and CD28 clone 37.51 (BD Pharmingen). A rat IgG antibody was used as an isotype control for IM7.

Antibodies used for microscopy include: anti-phospho-ezrin/radixin/moesin (pERM) rabbit polyclonal (Cell Signaling) and rabbit monoclonal (Cell Signaling), anti-phospho-moesin (Santa Cruz Biotechnology) which cross reacts with anti-phospho-ezrin and anti-phospho-radixin, anti-CD44 clone IM7 (ATCC), goat anti-rat IgG conjugated to Alexa 488 or Alexa 568 (Molecular Probes), donkey anti-goat IgG conjugated to Alexa 488 (Molecular Probes), goat anti-rabbit IgG conjugated to Alexa 488 or Alexa 568 (Molecular Probes).

Antibodies used for Western blotting include: anti-phospho-ezrin/radixin/moesin (pERM) rabbit polyclonal (Cell Signaling), anti-ezrin/radixin/moesin (ERM) rabbit polyclonal (Cell Signaling), and goat anti-rabbit:horseradish peroxidase (Jackson Immunoresearch Laboratories).
2.1.3. Reagents used for this study

Flourescein-tagged hyaluronan (FL-HA) was prepared according to (de Belder and Wik, 1975)) using rooster comb hyaluronic acid sodium salt (hyaluronan), which has an expected molecular weight of 1-4x10^3 kDa, from Sigma Aldrich, Hyaluronidase from Streptomyces hyaluronlyticus sp. from Calbiochem, OVA peptide (SIINFEKL) 257-264 and OVA peptide (ISQAVHAAHAEINEAGR) 323-339 from Genscript, PE conjugated dimeric mouse H2-K^b:Ig fusion protein from BD Biosciences, 5-Bromo-2'-deoxyuridine (BrdU) was from Calbiochem, 7-aminoactinomycin D (7AAD) from Sigma, ribonuclease A (RNase A) from Pharmacia, deoxyribonuclease (DNase) I (bovine pancrease) from Roche, recombinant mouse Interleukin (IL)-2 from RnD Systems, mouse IL-7 from RnD Systems, recombinant mouse IL-15 from RnD Systems, Phorbol 12-myristate 13-acetate or PMA from Sigma Aldrich, Ionomycin from Calbiochem, recombinant mouse CXCL12 from Peprotech, recombinant mouse CCL3 from RnD Systems, recombinant mouse CCL5 from RnD Systems, Sphingosine-1-phosphate (S1P) D-erythro from BIOMOL international, Lympholyte M from Cedarlane, Carboxy SNARF-1 from Molecular Probes, Brefeldin A from Penicillium brefeldianum from Sigma Aldrich, mitomycin C (Roche), 1,4-Diazabicyclo-(2,2,2)-octane in PBS (DABCO) from Sigma Aldrich, human plasma fibronectin from Sigma Aldrich.

2.1.4. Cell lines

AKR.1.G.1.OVAR.1.26 cells (hereafter referred to as AKR cells) are a CD44-negative mouse T lymphoma cell line available from the American Type Culture Collection (ATCC) and transfectants positive for a cytoplasmic domain deletion mutant of CD44 expressing only the first 2 amino acids of the cytoplasmic domain (CD44cy2) were obtained from B. Hyman. EL4 is
a mouse leukaemic T cell line that is available from ATCC. BW5147.G.1.4.OUAR.1 and the CD45 deficient variant (BW5147 (T200-a) 5.2) are an AKR/J-derived T thymoma cell line and were transfected with CD3 (Ng et al., 1997) and are hereafter referred to as BW⁺ cells and BW⁻ cells, respectively. Jurkat T cells (clone E6.1) are a human acute T cell leukemia line that is CD44-negative and CD45 positive (ATCC).

2.2 Methods

2.2.1 Cell transfection and cell line culture conditions

Mouse AKR T cells expressing full-length CD44 constructs expressed in pBCMGSneo vector (AKR CD44wt) or vector alone (pBCMGSneo) were generated as described in (Brown et al., 2005). Positive transfectants were grown in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% horse serum (HyClone), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma), as well as 1 mg/ml of active G418 (Invitrogen) to select for transfected cells. The human Jurkat T cell line E6.1 (ATCC) was transfected with full-length mouse CD44 (CD44wt) or a mutant of CD44 with only the first 6 amino acids of the cytoplasmic domain generated by Lesley et al. (Lesley et al., 1992) or vector alone (pBCMGSneo) from (Karasuyama and Melchers, 1988), and positive transfectants were selected for high levels of CD44 expression. Positive Jurkat transfectants were grown in RPMI 1640 media (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine, 1 mM sodium pyruvate, and positive transfectants were selected using 1mg/ml of active G418. BW5147 and EL4 T cell lines were grown in DMEM supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine, and 1 mM sodium pyruvate.
2.2.2 **Isolation of mouse splenic and LN T cells**

Mouse CD4 and CD8 T cells were isolated from splenocytes or lymph nodes cells of 6- to 20-wk-old C57BL/6 and CD44 knockout mice. Briefly, single cell suspensions were treated with a solution of 0.83% ammonium chloride to lyse red blood cells then resuspended in PBS containing 2 mM EDTA and 0.5% BSA and labeled first with biotinylated antibodies against B220 (4 µg/10^8 spleen cells), CD11b (4 µg/10^8 spleen cells), and Ter-119 (0.5 µg/10^8 spleen cells) (BRC antibody facility), then with (80 µl/10^8 spleen cells) anti-biotin magnetic beads (Miltenyi). This mixture was then run through MACS LS columns (Miltenyi) according to the manufacturer’s instructions, and the negative fraction was collected and found to typically contain 90-95% CD3 positive T cells. For purification of OT-I transgenic CD8 positive T cells, or OT-II transgenic CD4 positive T cells, biotinylated antibodies to CD4 or CD8 (10 µg/10^8 cells), respectively, were additionally used to label the cells prior to adding the beads.

2.2.3 **Adoptive transfer and bacterial infections**

5x10^4 OT-I or OT-I CD44 knockout T cells (or 10^5 of a 1:1 mixture of the two) purified from pooled peripheral lymph nodes and spleens were adoptively transferred intravenously into non-irradiated congenic Boy/J recipients. One day later, recipient mice were infected intravenously with 3x10^3 colony-forming units (CFU) of a recombinant strain of *L. monocytogenes* that expresses a secreted form of ovalbumin (OVA) antigen (Pope et al., 2001). For secondary challenges, mice on day 30 after the initial challenge were infected intravenously with 1.5-4.5x10^3 CFU *L. monocytogenes* expressing OVA. Bacteria used for injection were grown in brain-heart infusion (BHI) broth to mid-log growth phase then diluted in PBS. Bacterial counts were verified by plating diluted culture on BHI agar plates. Mice were
sacrificed for analysis at the indicated time points. For IL-15-mediated expansion in vivo, 1 µg of recombinant mouse IL-15 diluted in 200 µl PBS was injected intravenously in the mouse on day 30 and cells were allowed to expand over 7 days (adapted from (Schluns et al., 2004)).

2.2.4 In vitro mouse T cell culture and activation

Isolated ex vivo T cells were cultured in RPMI complete media containing RPMI 1640 supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 55 µM 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin. Five million cells were plated in 5 ml in 6-well tissue-culture coated plates (Costar) or 60 mm tissue culture dishes (Corning). Cells were activated with 1-2.5 ng/ml PMA and 0.5 µg/ml ionomycin for the indicated time points. On day 2 after activation, and every other day thereafter, cell culture media was supplemented with a final concentration of 20 U/ml recombinant IL-2. For T cell cultures to test the effect of hyaluronan, activated T cells on day 1 after stimulation were spun down, washed one time with X Vivo 15 serum free medium, which does not contain hyaluronan (Lonza) then resuspended in serum free media supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES and 55 µM 2-mercaptoethanol at 10^6 cells/ml with 2.5 ng/ml PMA and 0.5 µg/ml ionomycin added back, in the presence or absence of 50 or 500 ng/ml rooster comb hyaluronan or 0.5-1 U/ml hyaluronidase. For restimulation experiments, cells were plated at 10^6/ml in wells of a 96-well plate (Corning) pre-coated with 5 µg/ml anti-CD3 (2C11). To generate memory phenotype T cells, cells were recultured six days after activation in fresh RPMI complete media containing 100 U/ml IL-2, or 10 ng/ml mouse recombinant IL-7 or 10 ng/ml mouse recombinant IL-15.
2.2.5 Generation of activated mouse bone marrow-derived dendritic cells

Dendritic cells were generated as described previously (Cross et al., 2008). Briefly, mouse bone marrow was harvested from femurs and tibias of C57BL/6 mice and plated at 2x10^5/ml in 100 mm Petri dishes (Falcon) in RPMI 1640 media supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, non-essential amino acids, and 55 µM 2-mercaptoethanol (all from Invitrogen) plus 4% (v/v) GM-CSF containing J558L supernatant (Stockinger et al., 1996). On day 3 of culture, an equal volume of medium containing GM-CSF was added to the plates. On day 6 of culture, half of the culture was removed and replaced with fresh medium and GM-CSF. On day 7 of culture, cells were stimulated with 100 ng/ml UltraPure LPS (0111:B4; Sigma-Aldrich) overnight, then dendritic cells were purified by positive selection on day 8 using anti-CD11c MACS magnetic microbeads (Miltenyi) and LS columns (Miltenyi). Purity assessed was greater than 95%.

2.2.6 T cell activation using antigen and antigen presenting cells

OT-II CD4 T cells were activated by a 2:1 T:DC co-culture with activated dendritic cells loaded with 1 µM OVA (ISQAVHAAHAEINEAGR) peptide for 2 days, the point at which they were found to produce IL-2 and express high levels of the activation markers CD69, CD25, and CD44. OT-I CD8 T cells were activated by a 1:6 (T:splenocyte) co-culture with splenocytes treated with 50 µg/ml mitomycin C and loaded with 1uM OVA (SIINFEKL) peptide for 3 days in the presence of 20 U/ml recombinant mouse IL-2. On day 3, CD8 T cells had the ability to kill OVA-loaded target cells.
2.2.7 Flow cytometry

Cells were resuspended in PBS containing 0.5% BSA and stained on ice for 20 minutes with antibodies against the following surface mouse antigens, conjugated to the appropriate fluorophores: anti-CD8α, anti-CD4, anti-CD62L, anti-CD69, anti-CD25, or anti-CD122. Anti-CD44 antibodies were purified from tissue culture supernatant (IM7, from ATCC), purified, and conjugated to Alexa dyes according to the manufacturer’s instructions (Invitrogen). Staining for OVA-specific CD8 T cells was done using phycoerythin-conjugated MHC-I dimers pre-loaded with SIINFEKL peptide overnight at 37°C according to the manufacturer’s instructions.

Hyaluronan binding was measured by incubating cells with 2.5 µg/ml fluorescein-conjugated hyaluronan on ice for 20 minutes in PBS with 0.5% BSA, unless otherwise indicated. Flow cytometry was performed on a FACScan or LSRII (BD Biosciences) machine and analyzed using FlowJo software (Treestar).

2.2.8 Cell cycle analysis

Cells were spun down in 5ml polypropylene tubes with caps (Falcon) then stained for hyaluronan binding. They were then washed and resuspended in 0.2 ml PBS and ice-cold 75% ethanol was added drop by drop to a total of about 2ml. The cells were resuspended by inverting the tubes several times then incubated overnight at -20°C. The tubes were spun down, washed one time with PBS, then resuspended in 0.5 mg/ml ribonuclease A. After 10 minutes of incubation at 37°C, cells were stained with 7-aminoactinomycin D at a final concentration of 0.05 mg/ml. The cells were incubated for 30 minutes at room temperature, then DNA content was analyzed by flow cytometry. The 7AAD signal was detected in channel 3 on a linear scale.
2.2.9 *In vivo BrdU labeling*

Mice were injected intra-peritoneally with 2 mg BrdU 1 day prior to sacrificing. To detect BrdU uptake, cells were first fixed following surface staining by addition of 95% ethanol drop by drop to a total of about 2 ml, then washed and resuspended in 1% paraformaldehyde (Canemco). Cells were washed two times and incubated in 100 µl/10⁶ cells of 50 Kunitz U/ml deoxyribonuclease I (Roche) in 4.2 mM MgCl₂ and 0.15 M NaCl, pH 5, at room temperature for 20 minutes, then stained with 2 µg/10⁶ cells of anti-BrdU antibody conjugated to Alexa647 for 30 minutes at room temperature. Following two washes in PBS, cells were analyzed by flow cytometry to detect the percentage of proliferating cells.

2.2.10 *In vitro BrdU assay*

Cells were pulsed with BrdU at a final concentration of 10 µM for 4-8 hours at 37°C prior to detection as described above.

2.2.11 *Intracellular cytokine staining*

Spleen cell preparations were plated at 10⁶/ml in RPMI complete media then activated with 2.5 ng/ml PMA and 0.5 µg/ml ionomycin, or alternatively with OVA SIINFEKL peptide at 1 µM, in the presence of 5 µg/ml brefeldin A for 4-5 hours at 37°C. After this incubation, cells were treated with Fc receptor block (2.4G2 TCS) for 15 minutes on ice, then stained for surface markers for 20 minutes on ice. Cells were then washed, fixed with 1% v/v paraformaldehyde in PBS for 10 minutes at room temperature, and permeabilized with 0.5 ml saponin buffer (PBS with 0.1% saponin and 1% BSA) for 30 minutes at room temperature. IL-2 or IFNγ were detected with phycoerythrin-conjugated antibodies after staining for 30 minutes at room
temperature. Isotype-matched control antibodies conjugated to phycoerythrin were used as negative controls.

2.2.12 Blocking proliferation assay

Freshly isolated T cells were stained with 5 μM Carboxy-1 SNARF (Molecular Probes) in PBS without calcium or magnesium for 10 min at 37°C, then quenched with an equal volume of FCS. Cells were washed two times then resuspended at 10^6/ml and activated with PMA and ionomycin in the presence or absence of 2.5 μg of anti-CD25 blocking antibody for 2 days at 37°C. On day 2, cells were incubated with FL-HA for 20 minutes on ice then analyzed by flow cytometry.

2.2.13 Transwell migration assays

Transwell chemotaxis assays, except the assays in figure 4.6, were performed using 24-well plates and 6.5 mm diameter polycarbonate transwell inserts with 5 μm pores (Costar). 5x10^5 cells were resuspended in 100 μl chemotaxis medium (RPMI 1640/0.5% BSA/10 mM HEPES pH 7.2 for Jurkat T cells, DMEM/0.5% BSA/10 mM HEPES for AKR T cells, and RPMI1640/0.5% fatty acid free BSA/10 mM HEPES for mouse T cells) and serum starved at 37°C for 30 minutes. The cells were then added to each insert, and inserts were placed in wells containing 600 μl of chemoattractant at the indicated concentrations, or 600 μl of buffer alone. After 3 hours of incubation at 37°C and 5% CO2, the cells that had migrated into the wells were collected and counted with a FACScan (averaged 3 counts of 30 seconds each). % migration was calculated as the number of cells that had migrated divided by the number of cells in 100% migration control wells, for which 5x10^5 cells were directly added to 600 μl of buffer in the well,
then multiplied by 100. AKR and Jurkat T cell transfectants were analyzed by flow cytometry to ensure similar levels of CD44 (in CD44^+ cells) and CXCR4 expression (the receptor for CXCL12). For migration of unactivated T cells to recombinant mouse CXCL12, whole lymph node cell preparations were used in the assay, since incubation on ice, which is necessary for T cell purification, resulted in poorer migration. T cell migration was quantitated by collecting the cells from transwell plates after the 3 hour migration assay, blocking with 2.4G2 tissue culture supernatant for 15 minutes at 4°C, then labeling T cells with anti-CD3, anti-CD4, and anti-CD8 antibodies after the assay. For migration of day 2 activated T cells in response to CCL3, 2.5x10^5 activated d2 T cells were resuspended in 0.5 ml chemotaxis medium (RPMI1640/0.5% BSA/10 mM HEPES) and serum starved for 20 minutes before pre-treating or not with FL-HA at 2.5 µg/ml for an additional 20 minutes at 37°C prior to the migration assay. Cells were then added to 10.5 mm inserts with 8 µm pores to facilitate migration (Falcon). Inserts were placed in wells containing 1 ml of chemoattractant at the indicated concentration or buffer alone. After 3 hours at 37°C, the numbers of FL-HA positive and negative cells that had migrated into the bottom wells were collected and counted as before, and % migration was calculated as the number of positive or negative cells that had migrated divided by the number of positive or negative cells in 100% migration control wells, then multiplied by 100. For migration in the absence of hyaluronan, cells were not pre-treated with hyaluronan, but were instead labeled with FL-HA after the migration assay and quantitated as before.

2.2.14 Cell polarization and fluorescent microscopy

For Jurkat and AKR T cell polarization assays, cells were resuspended in chemotaxis medium (RPMI 1640 or DMEM/0.5% BSA/10 mM HEPES) and plated at 2x10^5 cells/180
µl/well in 96-well flat-bottom tissue culture plates (Falcon) for 30 minutes. 20 µl of recombinant mouse CXCL12 at 2 µg/ml (final concentration = 200 ng/ml) was pipetted down the side of the well, and cells were incubated at 37°C 5% CO₂ for 30 minutes to one hour, then fixed with 4% paraformaldehyde (final) for 10 minutes at room temperature. Polarization assays for activated T cells were done by pre-treating the cells with 2.5 µg/ml FL-HA in chemotaxis media then plating as above and incubating at 37°C for 20 minutes. 20 µl of recombinant mouse CCL3 at 100 ng/ml (final concentration = 10 ng/ml) was pipetted down the side of the well, and cells were incubated at 37°C 5% CO₂ for 30 minutes to polarize. For detection of CD44, cells were washed and labeled with purified IM7 at 5 µg/ml in 3% BSA/PBS for 1.5 hours, then washed and labeled with goat anti-rat IgG antibody conjugated to Alexa 568 at 40 µg/ml in 3% BSA/PBS for 2 hours, at room temperature. For detection of pERM proteins, the cells were fixed, washed, and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at RT, then incubated with 1% BSA at RT for 30 minutes to prevent non-specific binding. Cells were incubated for 1 hour with anti-pERM antibody in 3% BSA/PBS, then washed and incubated with corresponding Alexa 488 conjugated chicken anti-rabbit secondary antibody for 1 hour. Cells were then washed three times and mounted in 90% glycerol/2.5% 1,4-Diazabicyclo-(2,2,2)-octane in PBS (DABCO). Control labeling without the primary antibody was included to ensure no cross-reactivity occurred between the secondary antibodies. Images of cells were captured with a Zeiss Axiophot fluorescent microscope using a 60x oil immersion objective. Typically, 50-100 cells from random fields were collected and analyzed. Fluorescent images were processed in ImageJ (NIH). For the analysis of polarization in figure 4.8, cells were analyzed by measuring the fluorescent intensity of CD44, FL-HA, or pERM on two opposite ends of the cell, and a ratio of greater than 2 (or less than 0.5) was considered polarized. Cells where CD44/FL-HA and
pERM were polarized in this manner to the same part of the cell were considered to have co-localized these signals.

2.2.15 Adhesion assay to fibronectin

Cells on day 2 after activation were stained with 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes) at 10 µM for 30 minutes, washed, resuspended in Hanks Balanced Salt Solution (HBSS), then pre-treated with rooster comb hyaluronan at 0.05 µg/ml for 20 minutes. They were then added to wells pre-coated with 20 µg/ml fibronectin in PBS and incubated at 37°C. After 30 minutes, cells were removed by 2 sequential washes using HBSS, and adherent cells were quantified using a SpectraMax plate reader (Molecular Devices) with a filter to detect wavelengths at 530nm. Data was acquired using SoftMax Pro software (Molecular Devices). % adhesion is equal to the signal received from wells after 2-3 washes divided by the signal from wells without any washing (100% controls), multiplied by 100.

2.2.16 Western blot

Day 2 activated T cells were resuspended in chemotaxis media (RPMI/0.5% BSA/10 µM HEPES) with or without (time 0) hyaluronan at 2.5 µg/ml and incubated in eppendorf tubes at 37°C. At the indicated time points, the cells were pelleted and lysed with 20 µl 3x reducing sample buffer. Lysates were then boiled for 5 minutes and loaded on a 7.5% SDS-polyacrylamide gel and transferred to a PVDF membrane (Immobilon P, Millipore).

To detect phosphorylated ERM, and total ERM proteins, PVDF membranes were incubated with 1/1000 anti-phospho ERM antibody in 5% skim milk/TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% v/v Tween 20) for 1 hour at room temperature. After a brief wash
with TBST, the blot was incubated with 1/2500 anti-rabbit IgG-HRP. The blot was then washed several times with TBST for 15 minutes and then developed using ECL (Amersham Pharmacia Biotechnology). Membranes were stripped with 50 mM glycine, pH 2.5, 150 mM NaCl, 0.1% v/v Nonidet P-40 for 30 minutes at room temperature, washed several times with TBST before re-probing with 1/1000 anti-ERM in 5% skim milk/TBST and 1/2500 anti-rabbit IgG-HRP.

2.2.17 Cytokine analysis by ELISA

Culture supernatants were collected on the indicated days and IL-2 secretion was analyzed by ELISA IL-2 kit (eBioscience) according to the manufacturer’s instructions.

2.2.18 Cell stimulation using plate-bound antibodies

2x10^5 lymph node or spleen mouse T cells were plated in 200 µl RPMI complete media in wells of a 96-well non-tissue culture (Corning) plate coated overnight at 4°C with antibodies against CD3 (1 µg/ml) with or without anti-CD28 (5 µg/ml), anti-CD44 IM7 (1 µg/ml) or soluble hyaluronan (0.5 or 5 µg/ml). Cells were stimulated for 2 days at 37°C, then the plate was spun down at 1200 rpm for 5 minutes and supernatants were collected for analysis by ELISA for IL-2. Although even minor contamination of non-T cells on day 2 was unlikely, cells were blocked with 2.4G2 TCS for 15 minutes on ice, then labeled with antibodies against CD69, CD44, and CD25 to measure levels of activation markers, as well as FL-HA, to determine % HA binding.
2.2.19 Killing assay

Preparation of killer T cells: CD8 killer T cells were generated by co-culturing purified OT-I CD8 T cells pooled from the spleen and lymph nodes of OT-I or OT-I CD44 knockout transgenic mice with mitomycin C-treated C57BL/6 splenocytes pre-loaded with 1 µg/ml OVA (SIINFEKL) peptide for 3 hours at 37°C (at a 6:1 splenocyte:T ratio). Recombinant IL-2 was added to the media at 20 U/ml and the cells were co-cultured for 3 days. On day 3, CD8 T cells were found to have antigen-specific cytotoxic activity against EL4 T lymphoma target cells pre-loaded with 1 µM SIINFEKL peptide.

Preparation of target cells: EL4 T lymphoma target cells were stained with 1 µM Cell tracker orange dye for 30 minutes at 37°C then quenched with 10% FCS in RPMI for 30 minutes. Cells were resuspended in complete RPMI media containing 1 µM SIINFEKL peptide and incubated for 2 hours at 37°C. Cells that were not loaded with peptide were used as a negative control for the assay. Target cells were then treated or not with a titration (0.05 to 50 µg/ml) hyaluronan for 40 minutes at 37°C. Cells were then washed with complete media, resuspended at 5x10⁵/ml, and 0.1 ml (5x10⁴ cells) were pipetted into wells of a 96-well round bottom tissue culture plate (Costar).

Killing: 0.1 ml of T cells were added to the wells to give effector:target ratios of 10, 3, 1, and 0.1. The assay was carried out at 37°C for 4 hours, then the plate was spun down at 1200 rpm for 5 minutes at 4°C to pellet the cells. 0.1 ml of 7AAD diluted to 1 µg/ml was added to each well to resuspend the cells and the plate was incubated on ice for 10 minutes. Cells were then transferred to FACS tubes containing 0.2ml PBS and analyzed by FACS. Target cells were gated for cell tracker orange staining and % dead cells gated for a positive 7AAD signal.
Spontaneous death was defined as the % dead in the wells that had only target cells and no effector cells. % killing was equal to % dead minus % spontaneous death.

2.2.20 Statistical analysis

Statistical analysis was performed using a two-tailed student’s t test. P < 0.05 was considered statistically significant (* represents p<0.05, ** represents p<0.01, and *** p<0.001).
CHAPTER 3: HYALURONAN BINDING ON T CELLS

3.1 Introduction

CD44 expression is highly upregulated on T cells following TCR activation and CD44 is used as a marker for effector and memory T cells (Dutton et al., 1998). The ability of CD44 to bind hyaluronan is tightly regulated and is induced following activation through the TCR (Ariel et al., 2000; DeGrendele et al., 1997b; Firan et al., 2006; Lesley et al., 1994). Hyaluronan fragments generated by hyaluronidase cleavage or reactive oxygen species are enriched at sites of inflammation and can activate macrophages and induce the expression of pro-inflammatory mediators (Pure and Cuff, 2001; Turley et al., 2002). CD44 has been implicated in multiple T cell functions, including costimulation (Foger et al., 2000; Mummert et al., 2002), effector CD4 T cell survival and memory cell generation (Baaten et al., 2010), cell death (Baaten et al., 2010; McKallip et al., 2002) and effector T cell recruitment to tumour sites (Mrass et al., 2008).

However, how CD44 is mediating these effects, and what its role is on the different stages of T cells, are unclear. It is also unclear why hyaluronan binding is only induced on a subset of T cells. Hyaluronan has been shown in other cell types to mediate adhesion to target cells and facilitate killing by NK cells (Matsumoto et al., 1998), regulates proliferation in smooth muscle cells and fibroblasts (Kothapalli et al., 2008; Kothapalli et al., 2007), and enhances cytokine production in NK cells (Sague et al., 2004). Similar roles for hyaluronan might also exist for T cells and can be tested.

It is of critical importance to determine when and which cells bind hyaluronan during an immune response. Determining the significance of why only a subset of activated T cells acquire the ability to bind hyaluronan, and what these cells are, is an important step toward determining its function on T cells. Thus, the first aim of this chapter will be to determine the hyaluronan
binding status of T cells at various stages of activation and differentiation upon in vitro stimulation and following a pathogen challenge in vivo.

Then, the second aim will assess the role of CD44 and hyaluronan on T cells at various stages of an immune response. Although crosslinking CD44 antibodies can enhance IL-2 production by anti-CD3 stimulation of naïve T cells (Foger et al., 2000), a better model is the natural ligand, hyaluronan. Although hyaluronan is reported to stimulate dendritic cells and induce the upregulation of costimulatory molecules such as CD80 and CD86, this effect occurred independently of CD44 (Do et al., 2004). A positively-charged peptide that prevents hyaluronan binding, Pep-1, inhibited IL-2 production by T cells co-cultured with dendritic cells and antigen (Mummert et al., 2002). However, hyaluronan binding is induced upon T cell activation and does not occur until 7 days after allogeneic challenge in vivo (Lesley et al., 1994). It thus seems unlikely that activation-inducible hyaluronan binding on T cells functions in costimulation of naïve T cells. Since hyaluronan binding has already been shown to mark a population of effector cells with cytolytic activity in vivo, it is more likely that hyaluronan functions on effector T cells. Antigen-specific cytotoxic CD8 T cells will be generated from wildtype and CD44 knockout mice, then the effect of hyaluronan on effector T cell killing will be determined using target cells pre-treated or not with hyaluronan. Additionally, a lack of CD44 severely compromised the development of memory CD4 T cells, and a role for hyaluronan was implied by the fact that a blocking anti-CD44 antibody had a similar effect (Baaten et al., 2010). It is possible hyaluronan is involved in effector T cell survival and memory T cell development, although it is not known whether these cells bind hyaluronan.
3.2 Results

3.2.1 Hyaluronan binding is transiently induced on in vitro activated CD4 and CD8 T cells

In order to characterize when T cells bind hyaluronan, two in vitro models of T cell activation were established. First, mouse CD4 and CD8 T cells were purified from spleens of C57BL/6 and CD44 knockout (CD44−/−) mice, then activated using PMA and ionomycin. A time course analysis showed that hyaluronan binding was induced transiently on CD4 T cells by day 1 after activation, increased by day 2, then declined and returned to almost baseline levels by day 4 (figure 3.1A). Similar results were obtained for CD8 T cells. Hyaluronan binding did not occur on T cells from CD44−/− mice at any time point, and the level of hyaluronan binding paralleled the increase and decrease in CD44 expression (mean fluorescence intensity, MFI) over the same time course. The expression of others markers of activation, such as CD25 (IL-2Rα) and CD69 was also examined. The peak of CD44 expression and hyaluronan binding was similar to that of CD69 and CD25, the expression of which was regulated normally in the CD44−/− T cells (figure 3.1A). Hyaluronan binding was induced on a subset of about 20-30% of both CD4 and CD8 T cells on day 2 following stimulation with PMA and ionomycin (figure 3.1B).

In the second in vitro model, transgenic T cells isolated from OT-II (CD4) and OT-I (CD8) mice were activated by co-culture with OVA peptide-loaded dendritic cells or mitomycin C-treated splenocytes, respectively. Again, hyaluronan binding was induced on a subset of both CD4 and CD8 T cells following stimulation with OVA peptide antigen for 2 or 3 days, when CD44 expression was also found to be upregulated (figure 3.1C).
Figure 3.1: Hyaluronan binding on T cells in vitro.

A. Splenic T cells from C57BL/6 and CD44−/− mice activated with PMA and ionomycin and analyzed on days 0-4 by flow cytometry for levels of FL-HA binding and activation markers. MFI = mean fluorescence intensity. Data for CD4 T cells is shown, and is an average of 4 mice from 2 independent experiments of 3. Error bars indicate standard deviation. B. FL-HA binding on C57BL/6 (black lines) and CD44−/− (gray lines) splenic T cells activated with PMA and ionomycin on day 0 and day 2. C. FL-HA binding and CD44 expression levels on activated (day 2 or 3) or unactivated (day 0) OT-II CD4 and OT-I CD8 T cells. FL-HA binding was assessed as in B, gray lines indicate unstained controls for OT-II CD4 T cells and CD44−/− negative control T cells for OT-I CD8 T cells. The right panels show an overlay of CD44 levels from day 0 (gray lines) and day 2 or 3 after activation (black lines). Data is representative of 2-3 experiments. In all cases, cells were labeled using FL-HA at 2.5 µg/ml.
As shown in figure 3.1C, hyaluronan binding was found on a higher percentage of OT-I CD8 T cells (about 60%) than was found on CD4 T cells (typically around 10 to 20%) following antigen stimulation. Hyaluronan binding on the CD4 T cells did not increase if cells were stimulated for a shorter (1 day) or longer (3 days) duration of time, and increasing the concentration of peptide used (5 µM instead of 1 µM) also did not increase the percentage of hyaluronan binding cells. Since there was no difference in hyaluronan binding on CD4 and CD8 T cells activated with PMA and ionomycin (figure 3.1B), it is unlikely there is an intrinsic difference between CD4 and CD8 T cells to inducibly bind hyaluronan. However, it was found that titration of PMA up to 2.5 ng/ml with 0.5 µg/ml ionomycin increased the percentage of hyaluronan binding cells up to approximately 70% on day 2 after activation (see figure 3.3). This suggests that the strength of stimulus can positively regulate the frequency of cells that are induced to bind hyaluronan, and would support the OVA257-264 peptide/MHC I interaction with the OT-I T cell receptor being a stronger interaction than the OVA323-339/MHC II interaction with the OT-II T cell receptor. In subsequent experiments in vitro, this higher concentration of PMA (2.5 ng/ml instead of 1 ng/ml) was used to maximize the hyaluronan binding population.

3.2.2 Hyaluronan binding occurs on a subset of CD8+ OT-I T cells after pathogenic challenge in vivo

Hyaluronan binding was also induced on antigen-specific T cells during an immune response in vivo (figure 3.2). CD8+ T cells (5 x 10^4) from OT-I or OT-I CD44−/− mice (CD45.2+) were adoptively transferred into congenic Boy/J (CD45.1+) recipients. These mice were then infected them with 3000 colony forming units (CFU) of Listeria monocytogenes engineered to express OVA (LM-OVA). OT-I or OT-I CD44+/− T cells were identified by gating on
Figure 3.2: Hyaluronan binding is induced in vivo.
A. CD8⁺CD45.2⁺ T cells were analyzed on day 5, 7, and 10 after infection by labeling with 2.5 µg/ml FL-HA. The black lines represent FL-HA binding by OT-I T cells and the gray lines OT-I CD44⁻ T cells. B. Time course of FL-HA binding and CD44 levels on CD8⁺CD45.2⁺ OT-I T cells. An average of 2 mice from one representative experiment of 3 is shown, with error bars to indicate standard deviation.
CD8⁺CD45.2⁺ cells on day 5, 7, and 10 of the immune response (figure 3.2A). Hyaluronan binding occurred on a subset of about 20% of OT-I (solid lines), but not OT-I CD44⁻/⁻ (dotted lines), CD8 T cells on day 7 after infection and this declined to about 10% by day 10. This transient increase again coincided with CD44 expression (figure 3.2B), although in vivo, the levels of hyaluronan binding and CD44 expression did not decline to previous unstimulated levels, which will be addressed later in the chapter. Taken together, this indicates that CD44-dependent hyaluronan binding is transiently induced on a subset of activated CD4 and CD8 T cells in vitro and in vivo following TCR stimulation, and peaks when the cells are maximally expressing CD44.

3.2.3 Hyaluronan binding occurs on a subset of CD44⁺hi CD25⁺ T cells

In order to gain insight into why only a subset of T cells was binding hyaluronan, and ascertain clues as to the function of hyaluronan, T cells on day 1 and 2 after activation in vitro and on day 7 following LM-OVA infection in vivo were co-labeled with FL-HA and antibodies against the activation markers CD44, CD25, and CD69. By day 1, all of the cells were positive for CD44, CD25, and CD69, but only a subset of these cells bound hyaluronan (figure 3.3A). By day 2, a greater percentage of the cells bound hyaluronan. The hyaluronan binding cells could not be distinguished based on CD25 and CD69 expression, but did correlate with the cells expressing the highest levels of CD44. In vivo, hyaluronan binding cells were positive for CD44 and CD25, but low for CD69 expression (figure 3.3B).
Figure 3.3: Hyaluronan binding occurs on a subset of activated T cells. 
A. In vitro activated splenic T cells from C57BL/6 mice were co-labeled with FL-HA and antibodies against CD44, CD25, and CD69 and analyzed by flow cytometry on day 1 and 2. Data is representative of activated T cells from 4 mice in two independent experiments. B. In vivo activated CD8^+CD45.2^+ OT-I T cells, 7 days after LM-OVA infection, were labeled with FL-HA and antibodies against CD44, CD25, and CD69. Data is from one representative experiment of 3.
3.2.4 Hyaluronan binding enriches for cycling T cells and marks the most proliferative T cells

Since hyaluronan binding is only found on a subset of highly activated T cells, it was possible that hyaluronan binding marked cells at a particular phase of the cell cycle. To address this, cells were co-labeled with FL-HA and 7-aminoactinomycin D (7AAD) on day 2 after activation, then analyzed for DNA content to determine what fraction of the HA binding (HA⁺) and HA non-binding (HA⁻) cells were in G0/G1, S and G2/M phases of the cell cycle (figure 3.4). As shown, cells were 98% HA-negative on day zero prior to activation and mostly in G0/G1 phase (figure 3.4A). Interestingly, analysis of the very small number of hyaluronan binding cells present on day 0 (unactivated T cells) indicated that approximately half of them were in the S or G2/M phases of cell cycle. Following 2 days of activation, approximately 50% of the cells entered the S or G2/M phase of the cell cycle and approximately 70% of the cells bound hyaluronan. Hyaluronan binding does not delineate a particular phase of the cell cycle, as there are hyaluronan binding cells in every phase of the cycle. However, a greater percentage of the HA⁺ population was in the replicative phases of the cell cycle (S/G2/M) and there was a lower percentage in the resting phase (G0/G1) compared to the HA⁻ population (figure 3.4B).

Since there were more hyaluronan binding cells in cycle, this suggested that hyaluronan binding may promote cell cycle progression and thus proliferation. To determine the rate of proliferation, day 2 in vitro activated T cells were incubated with 10 μM BrdU for 4 hours, then labeled with FL-HA and BrdU uptake was analyzed by flow cytometry. The HA⁺ population had consistently taken up more BrdU than the HA⁻ cells (figure 3.4B), suggesting that hyaluronan binding marks a subpopulation of the fastest proliferating T cells. The proliferation of HA⁺ cells was also evaluated in vivo in adoptively transferred OT-I CD8⁺ T cells after pathogenic challenge with LM-OVA. BrdU was injected 6 days after infection and HA⁺ and
Figure 3.4: Hyaluronan binding enriches for the most proliferative, cycling T cells.

A. Naïve (d0) and day 2 activated T cells (d2) were co-labeled with FL-HA and 7AAD to label DNA content, and analyzed by flow cytometry. FL-HA binding is shown in the first panel, with percentages in positive and negative gates indicated. Cells in HA+ and HA- gates were then analyzed for 7AAD on a linear scale. Gates drawn indicate cells in G0/G1, S and G2/M phases of the cell cycle. 

B. Summary of the results from 3 independent experiments, with error bars to indicate standard deviation. *** p<0.001.

C. % BrdU incorporation of HA+ and HA- cells on day 2 after activation in vitro (top) and on adoptively transferred OT-I T cells, 7 days after infection with LM-OVA (bottom). FL-HA binding gates are shown in the first panel. Data in graphs is an average of 4 (in vitro) or 3 (in vivo) independent experiments with error bars to indicate standard deviation. **p<0.01
HA\(^{-}\)CD8\(^{+}\)CD45.2\(^{+}\) T cells were analyzed on day 7. In vivo activated HA\(^{+}\) T cells were also significantly more proliferative than their HA\(^{-}\) counterparts (figure 3.4C), which supports the in vitro data that HA\(^{+}\) T cells proliferate at a faster rate.

3.2.5 Hyaluronan does not significantly enhance or inhibit proliferation in vitro on day 2 after activation with PMA and ionomycin

In order to determine whether hyaluronan was causing the increased proliferation of the hyaluronan binding T cells, or whether hyaluronan binding was merely a consequence of faster proliferation, activated T cells were cultured in the presence or absence of hyaluronan and proliferation was measured by flow cytometry for BrdU uptake on day 2 after activation. One problem that may obscure any effect of hyaluronan is the fact that fetal calf serum and blood plasma contain hyaluronan (Engstrom-Laurent et al., 1985; Laurent and Fraser, 1992), and secondly, T cells express hyaluronan synthase (HAS) and have been shown to synthesize and secrete their own hyaluronan (Mahaffey and Mummert, 2007; Mummert et al., 2002). Thus, T cells activated for one day were recultured in serum free media to eliminate fetal calf serum as a source of hyaluronan. The addition of exogenous hyaluronan up to 500 ng/ml had no significant effect on the proliferation rate of the HA\(^{+}\) cells one day later, and HA\(^{+}\) T cells still proliferated faster than HA\(^{-}\) T cells in serum free media (figure 3.5A). There was a small decrease in the proliferation of HA\(^{+}\) cells upon the addition of hyaluronan, but this was not significant and occurred to some extent with the HA\(^{-}\) cells as well. The addition of hyaluronan had no effect on the percentage of cells in the different phases of the cell cycle (figure 3.5B) and hyaluronidase, which was added to remove any endogenously synthesized hyaluronan, also had no effect. Also consistent with hyaluronan not causing the increased proliferation was the fact that CD44\(^{-}\) day 2
Figure 3.5: Hyaluronan does not promote proliferation.
A. % BrdU positive cells in the HA⁺ and HA⁻ subsets of day 2 activated T cells after incubation from day 1 in serum free media (SFM) with (+) or without (-) 50 ng/ml hyaluronan. Data is an average of 3 independent experiments. B. % of HA⁺ cells on day 2 in G₀/G₁, S, and G₂/M subsets that were incubated with 50 ng/ml hyaluronan or not (SFM). HA’ase indicates cells that were recultured in SFM in the presence of 0.5-1 U/ml hyaluronidase. Data is an average of T cells from 3 mice in one experiment. C. % BrdU uptake in CD44⁺⁺⁺ (C57BL/6) or CD44⁻⁻⁻ T cells on day 2 in the presence (+) or absence (-) of 50 ng/ml hyaluronan, data is an average of 4 experiments. D. IL-2 in culture supernatants of activated day 2 cells recultured in serum free media with or without the indicated amounts of hyaluronan on day 1. Data is an average of 3 experiments. For all, error bars indicate standard deviation. E. Proliferation was assessed by SNARF labeling and FL-HA binding of day 2 activated T cells in the presence and absence of a blocking CD25 mAb. Data is from one experiment of two.
activated T cells proliferated similarly to CD44 positive cells and again, the addition of hyaluronan had no significant effect (figure 3.5C). Furthermore, the amount of IL-2 produced by in vitro activated day 2 T cells is not affected by the addition of hyaluronan on day 1 (figure 3.5D). Thus, although hyaluronan binding marked the fastest proliferating cells, there is no evidence for hyaluronan to enhance the proliferation rate of day 2 activated T cells. Conversely, reducing IL-2 mediated activated T cell proliferation by an IL-2 receptor (CD25) blocking monoclonal antibody reduced hyaluronan binding (figure 3.5E), implying that T cell proliferation is required for the induction of hyaluronan binding.

3.2.6 Hyaluronan binding occurs on a subset of memory T cells

Although CD44 is known to be highly expressed on memory T cells (Dutton et al., 1998), its ability to bind hyaluronan on these cells is unknown. Given the above findings, it was of interest to determine if memory T cells bound hyaluronan and if so, whether this also marked the most proliferative cells. While there are no FL-HA binding T cells in the spleen or lymph nodes of naïve mice (figure 3.6A shows data from spleen, similar results were obtained for lymph nodes) there was a small subset (~6%) of hyaluronan binding cells in the CD8 positive population in the bone marrow. The hyaluronan binding population contained two populations, both memory phenotype populations; one CD44^{hi} and CD122^{hi}, and one CD44^{vhi} and CD122^{int}, whereas the non-hyaluronan binding population contained both memory and non-memory phenotype cells (figure 3.6A). As mentioned in the introduction, previous work by others has shown a small population of memory phenotype cells in the bone marrow (Mazo et al., 2005), and showed that the bone marrow is a major homing site for central memory T cells. Others have also reported that bone marrow is the preferred site for proliferation of memory T cells.
Figure 3.6: Hyaluronan binding is found on memory T cells.
A. Spleen (SPL) and bone marrow (BM) CD8 T cells from C57BL/6 (WT) and CD44-/-(CD44k/o) mice were co-labeled with CD44 and FL-HA and analyzed on day 0. HA⁺ and HA⁻ subsets in BM were further analyzed for CD44 and CD122 expression. B. Mice were also challenged with 3x10³ CFU LM-OVA then sacrificed on day 30 after infection, and spleen and bone marrow CD8 T cells were analyzed for FL-HA binding, CD44 and CD122 expression. C. CD8 T cells from OT-I mice were adoptively transferred into C57BL/6 mice and one day later were challenged with LM-OVA, then sacrificed on day 30 for analysis of CD8⁺ OVA-specific (OVAsp) T cells in the spleen and bone marrow (spleen is shown). Graph indicates FL-HA binding on spleen and bone marrow CD8⁺ OVA-specific T cells on day 30, average of 3-5 mice from 3 experiments, with error bars to indicate standard deviation.
(Becker et al., 2005). Therefore, subsequent analyses of memory T cell populations in vivo were performed using cell suspensions from both spleen and bone marrow.

Thirty days after infection with LM-OVA, there was a small population (2%) of hyaluronan binding cells in the spleen and an increased population (15%) of hyaluronan binding cells in the bone marrow (figure 3.6B). The hyaluronan binding population in both the spleen and bone marrow had a CD44hiCD122hi memory phenotype. Again both memory phenotype and other cells were present in the non-hyaluronan binding fraction, indicating that hyaluronan binding marks a subset of memory phenotype cells.

To determine whether hyaluronan binding was marking a population of antigen-specific memory T cells, CD45.2+ OT-I CD8 T cells were transferred into congenic CD45.1+ Boy/J recipients and these mice were infected with LM-OVA. On day 30 after infection with LM-OVA, OVA peptide antigen-specific T cells were identified by gating on CD8+ T cells that bound OVA257-264 (SIINFEKL) peptide-loaded MHC I dimers (figure 3.6C). Approximately 40% of antigen-specific CD8 T cells in the spleen and bone marrow bound hyaluronan.

3.2.7 Hyaluronan binding can be re-induced if cells are restimulated with anti-CD3

In vitro, hyaluronan binding is transient and by day 6 following T cell activation, there are almost no hyaluronan binding cells (figure 3.7). However, hyaluronan binding can be re-induced on cells that had previously stopped binding hyaluronan upon restimulation with immobilized anti-CD3 antibody on day 6. This occurs in both CD4 and CD8 T cells, and occurs in parallel with the upregulation of CD44 and CD25, and the transient upregulation of CD69. This indicates that hyaluronan binding is re-induced with other activation markers upon TCR stimulation (figure 3.7).
Figure 3.7: Hyaluronan binding is re-induced upon restimulation.
T cells from CD44+/+ (C57BL/6) and CD44−/− (CD44k/o) mice on day 6 following activation with PMA and ionomycin were restimulated with immobilized anti-CD3 (2C11, 5 µg/ml) antibody. Cells were co-labeled with FL-HA and antibodies against CD44, CD25, and CD69 and analyzed by flow cytometry on day 0 (unrestimulated = 6 + 0), 1 (6 + 1), and 2 (6 + 2) following restimulation. An average of 2 mice is shown from one representative experiment of 3. Error bars indicate standard deviation.
3.2.8 Culture with the cytokines IL-2, IL-7, or IL-15 is sufficient to induce hyaluronan binding on activated T cells

IL-15 has previously been shown to induce the development of T cells with a memory phenotype in vitro (Weninger et al., 2001), and IL-2 and IL-7 in vivo (Dooms et al., 2007). Thus day 6 cells were cultured in the presence of the cytokines IL-2, IL-7, or IL-15. By day 10, approximately 40-60% of the T cells had a memory phenotype, consisting of high CD44 and CD122 expression, and a significant proportion of these (~25-35%) bound hyaluronan (Fig. 3.8A and data not shown). Culturing the cells in these gamma chain cytokines induced hyaluronan binding in 10-20% of both CD4 and CD8 T cells and this correlated with high levels of CD44 expression (Fig. 3.8B).

3.2.9 Hyaluronan binding marks the fastest proliferating memory phenotype cells in vitro

To determine if hyaluronan binding marked the most proliferative memory phenotype cells, these cells were examined for cell cycle status and proliferation rate. The HA+ cells cultured in either IL-7 or IL-15 had a significantly higher percentage of cells in the replicative phases of the cell cycle, (S/G2/M), whereas HA− cells had significantly more cells in the resting phase of the cell cycle, or G0/G1 (Fig. 3.9A). In addition, HA+ cells proliferated significantly faster in response to IL-7 and IL-15 (Fig. 3.9B). This demonstrates that, as for activated T cells, HA+ memory phenotype cells are the most rapidly dividing cells.
Figure 3.8: Culture with IL-2, IL-7, or IL-15 is sufficient to induce hyaluronan binding on activated T cells.
A. T cells on day 6 after activation with PMA and ionomycin were re-cultured in IL-2, IL-7, or IL-15 until day 10, then labeled with FL-HA and antibodies against CD4 and CD8. HA⁺ and HA⁻ subsets in both CD4⁺ and CD8⁺ populations were then analyzed for the expression of CD44 and CD122 by flow cytometry. Top, IL-7 cultured CD8 T cells, bottom, IL-15 cultured CD8 T cells. B. Graph summarizing FL-HA binding and CD44 levels (MFI) in all cultures. None = cultured in media only from day 6 to 10. Data is an average of 3-4 independent experiments, with error bars to indicate SD.
Figure 3.9: Hyaluronan binding marks the fastest proliferating memory phenotype cells in vitro.
A. Day 10 HA$^+$ and HA$^-$ T cells in IL-7 (top) or IL-15 (bottom) were analyzed for DNA content by staining with 7AAD. % of cells in G$_0$/G$_1$, S, and G$_2$/M is indicated. Data shown is an average of 4 independent experiments, with error bars to indicate SD. B. Day 10 IL-7 and IL-15 cultured HA$^+$ and HA$^-$ T cells analyzed for BrdU uptake. Data shown is an average of T cells from 3 mice in one representative experiment of 4, with error bars to indicate SD. Differences are significant across all experiments, * = $p < 0.05$, ** = $p < 0.01$. 
3.2.10 Interaction of the extracellular domain of CD44 with hyaluronan does not costimulate for naïve T cell activation

Having characterized hyaluronan binding cells as a subset of the most proliferative, cycling, activated CD4 and CD8 T cells in vitro and in vivo, and found a subset of hyaluronan binding cells on memory-phenotype and memory cells, the next step was to determine the effect of hyaluronan in T cell activation, as well as in effector and memory responses. As mentioned in the introduction, there has been some indication of a role for CD44 in costimulation (Foger et al., 2000; Mummert et al., 2002) but it was difficult to envision a role for hyaluronan, as naïve T cells do not bind hyaluronan. Freshly isolated C57BL/6 spleen T cells were first stimulated with a suboptimal dose of 1 µg/ml immobilized anti-CD3 alone, or in the presence of 0.5 (HAlo) or 5 µg/ml hyaluronan (HAhi), or IM7 (anti-CD44) antibody for 2 days. Anti-CD28 antibodies were used as a positive control for costimulation, and rat IgG1 antibody (rIgG) was used as an isotype control for CD44. As shown in figure 3.10A, hyaluronan is not costimulatory for T cell activation, as it did not enhance IL-2 production or the upregulation of activation markers CD25 and CD69 compared to anti-CD3 stimulation alone. Anti-CD28 antibodies were partially costimulatory, as they enhanced activation marker expression, but did not enhance IL-2, whereas CD28 was fully costimulatory and enhanced the expression of all three indicators (figure 3.10A).

It is possible that the hyaluronan needs to be presented on dendritic cells in order to perform a costimulatory function. Others have reported that dendritic cells express hyaluronan synthase, which is upregulated upon TLR stimulation, and have shown that a hyaluronan blocking peptide inhibited IL-2 production induced following co-culture of transgenic T cells with antigen-presenting dendritic cells (Mummert et al., 2002). Bone marrow-derived dendritic cells from wildtype C57BL/6 mice were generated in vitro as previously described (Cross et al.,
2008), and stimulated overnight with 100 ng/ml LPS. These dendritic cells were found to
upregulate hyaluronan binding upon LPS stimulation, which means they should be able to bind
and present hyaluronan to T cells during co-culture. Thus, co-cultures were set up using OT-II
transgenic CD4 T cells, which recognize OVA\textsubscript{323-339} peptide, and wildtype dendritic cells pre-
loaded with 1 µM OVA\textsubscript{323-339} peptide, in the presence or absence of 0.05 or 0.5 µg/ml hyaluronan.
Alternatively, the dendritic cells were pre-treated with 0.5 U/ml hyaluronidase to get rid of
hyaluronan that might already be expressed on their surface, and an additional 0.5 U/ml
hyaluronidase was added to the co-culture. CD44-deficient dendritic cells, which do not bind
hyaluronan by FACS, were used as a negative control, and also to determine whether CD44 on
dendritic cells, in the absence of hyaluronan, had any effect (independent of hyaluronan) on the
ability of the dendritic cells to activate naïve T cells. After 2 days of co-culture, culture
supernatants were measured for IL-2 production by ELISA.

As shown in figure 3.10B, wildtype and CD44-deficient dendritic cells induced equal
amounts of IL-2 from naïve T cells after 2 days of stimulation with OVA peptide. Incubating the
cocultures in the presence of 0.5 U/ml of hyaluronidase, to eliminate any endogenous
hyaluronan produced by the dendritic cells or the T cells, also did not have an effect on IL-2
production (figure 3.10B). Finally, titrating in 0.05 or 0.5 µg/ml of exogenous hyaluronan also
did not enhance IL-2 production (figure 3.10B).

These results are expected, since naïve T cells do not bind hyaluronan, and even when
hyaluronan binding is induced following T cell activation, it is only found on about 10% of T
cells following antigen stimulation in vitro by day 2 (figure 3.1). Since more hyaluronan
binding cells are generated with PMA and ionomycin after 1 day of stimulation (about 20%),
yaluronan was added to activated T cell cultures on day 1 after stimulation and IL-2 production
Figure 3.10: Hyaluronan is not costimulatory for T cell activation.

A. Freshly isolated splenic T cells from C57BL/6 mice were stimulated with antibody against CD3, and antibodies against CD44 or CD28, or hyaluronan, for 2 days. Isotype control is indicated as rIgG. Cells were analyzed for expression of CD25 and CD69, as well as for IL-2 production. MFI = mean fluorescence intensity. Data is from one representative experiment of 3. Significant differences compared to anti-CD3 alone are indicated, * p < 0.05, ** p < 0.01, *** p < 0.001, over 3 experiments. B. IL-2 in the supernatants of OT-II CD4 T cells co-cultured with LPS-activated bone marrow-derived DCs from C57BL/6 or CD44−/− (CD44k/o) mice pulsed with 0 or 1 μM OVA323-339 peptide. Middle graph, 0.5 U/ml hyaluronidase was added to the co-cultures. Bottom graph, 0.05 or 0.5 μg/ml hyaluronan was added to co-cultures. Data is an average of T cells from 3 mice in one representative experiment of 2-3 experiments. C. T cells activated with PMA and ionomycin for 1 day were recultured in serum free media in the presence or absence of 0.5 or 5 μg/ml hyaluronan, then the concentration of IL-2 in the culture supernatant was determined on day 2. Data is an average of 2 independent experiments. Error bars indicate standard deviation.
was measured by ELISA on day 2. As shown in figure 3.10C, there was again no significant effect of adding hyaluronan on IL-2 production by activated T cells. Collectively, these data argue against a costimulatory role for hyaluronan for naïve T cell activation, although the effect of CD44 is less clear, since crosslinking antibodies can be partially costimulatory.

3.2.11 Neither hyaluronan nor CD44 have a significant effect on CTL-mediated killing in vitro

Since hyaluronan is not induced until T cells are activated with antigen for 2-3 days, it seemed more reasonable to consider a function for hyaluronan binding on activated T cells rather than on naïve T cell activation. Furthermore, since there are reports that CD44 and hyaluronan have a role in NK cell-mediated killing (Matsumoto et al., 1998), it was possible that one function of hyaluronan binding on activated CD8 T cells could be in killing.

To test if this is the case, antigen-specific CD8 effector T cells were generated by co-culturing OVA\textsubscript{257-264}-loaded mitomycin C-treated splenocytes, as antigen presenting cells, with CD8 T cells from OT-I transgenic mice, in the presence of 20 U/ml IL-2 for 3 days. After 3 days, the activated CD8 T cells were found to have killing activity against H-2\textsuperscript{b} positive EL4 thymoma target cells, and were furthermore confirmed to bind hyaluronan at reasonably high levels (about 50%, figure 3.1). EL4 target cells, which can also bind hyaluronan (figure 3.11A) were pre-loaded with 1 µM OVA\textsubscript{257-264} peptide and treated or not with hyaluronan at 2.5 µg/ml. Killing was carried out by co-culturing effector cells with target cells at 1:1, 3:1 and 10:1 effector:target ratios for 4 hours at 37°C. As shown in figure 3.11, there was no difference in the ability of wildtype or CD44-deficient effector T cells to kill target cells in the absence of hyaluronan (figure 3.11B), and additionally no effect of treating target cells with hyaluronan on CD44-positive CD8 T cell killing (figure 3.11C). Thus, although effector CD8 T cells with
Figure 3.11: Neither CD44 nor hyaluronan have an effect on CD8 T cell killing.
A. EL4 cells were labeled with 2.5 µg/ml FL-HA and analyzed by flow cytometry for the ability to bind hyaluronan. Gray line, unlabeled EL4 cells. B. OT-I or OT-I CD44<sup>−/−</sup> (OT-I CD44<sup>k/o</sup>) CD8 effector T cells were incubated with OVA<sub>257-264</sub> peptide-loaded EL4 target cells at the indicated effector:target ratios and as described in Methods to induce killing. After 4 hours, target cell death was analyzed by flow cytometry. C. EL4 target cells were labeled with hyaluronan at 0 (OT-I) or 2.5 µg/ml (OT-I + HA). Data is from one representative experiment of 2, and error bars indicate standard deviation between triplicate wells.
cytotoxic potential bind hyaluronan at fairly high levels, this does not have any effect on their ability to kill target cells treated with hyaluronan.

3.2.12 There is no effect of CD44 on the generation of a primary immune response or CD8 memory cells following Listeria monocytogenes infection in vivo

Since previous work has shown a role for CD44-mediated hyaluronan binding on T cells to facilitate activation-induced cell death in vitro (Ruffell and Johnson, 2008) it was possible that hyaluronan binding on activated T cells was regulating immune contraction or alternatively the survival and development of memory cells. Thus, wildtype and CD44-deficient mice were infected with LM-OVA and the generation and contraction of the primary response was assessed by measuring numbers of IFN-\(\gamma\) positive CD8 T cells over a time course (figure 3.12A). The time points selected on day 5, 7, and 10 are based on previous studies that show that the beginning of the CD8 T cell response occurs around day 5, peaks at around day 7, and declines by day 10 following intravenous administration LM-OVA (Pamer, 2004).

As shown in figure 3.12A, CD44-deficient mice show similar kinetics of induction and contraction of IFN-\(\gamma\) positive CD8 T cells as wildtype mice. Furthermore, when the numbers of antigen-specific and IFN-\(\gamma\) positive CD8 T cells were compared 30 days after infection, there is again no significant difference between wildtype and CD44-deficient mice, suggesting that there is also no effect of CD44 on the generation of antigen-specific memory cells (figure 3.12B). Taken together, for this L. monocytogenes challenge in vivo, there is no effect of CD44 on the generation of a primary CD8 T cell response, nor an effect on the generation of antigen-specific memory T cells.
Figure 3.12: The loss of CD44 does not affect the generation of effector or memory T cells following a primary challenge with *Listeria monocytogenes*.

A. C57BL/6 and CD44−/− (CD44k/o) mice were challenged with 3x10³ CFU LM-OVA and mice were sacrificed on day 5, 7, and 10 for analysis by measuring the number of IFNγ-producing CD8 T cells in the spleen by flow cytometry. Data is an average of 2 mice in one experiment. B. Memory cells in the spleen were quantitated on day 30 after challenge. The number of IFNγ-producing CD8 T cells, as well as the frequency of antigen-specific (OVAsp) CD8 T cells, in the spleen were compared. Data is an average of 2-5 mice, with error bars to indicate standard deviation.
3.2.13 There is no effect of CD44 on the generation of a secondary CD8 memory T cell immune response in vivo following Listeria monocytogenes re-challenge

Since CD44 is highly expressed on memory T cells, and since memory cells bind hyaluronan, it is possible that the function of hyaluronan binding could become apparent during a secondary response. The kinetics of a secondary response were also compared by re-challenging previously challenged C57BL/6 and CD44-deficient mice on day 30 with 1.5-4.5x10⁴ CFU of LM-OVA. The number of OVA-specific CD8 T cells was compared in the spleen 3 and 7 days after re-challenge with LM-OVA. As shown in figure 3.13, there was no significant difference in the number of OVA-specific CD8 T cells between C57BL/6 and CD44-deficient mice on day 3 or day 7 after re-challenge. Thus, CD44 does not affect secondary CD8 T cell responses upon re-challenge.

3.2.14 CD44-deficient T cells outcompete WT T cells in memory T cell numbers following Listeria monocytogenes infection

In the *Listeria* infection model used in this study, hyaluronan binding was transiently induced during the effector phase of the response and declined by day 10 following infection; yet, a large population of CD8 memory T cells bound hyaluronan. This suggests that either the cells that had downregulated hyaluronan binding were re-induced to bind hyaluronan, or that the small population of hyaluronan binding cells present on day 10 had an increased propensity to survive and contributed more to the memory pool than the non-hyaluronan binding subset. If this is the case, hyaluronan binding to CD44 might contribute toward the survival of a subset of effector CD8 T cells and promote their development into memory CD8 T cells. This would parallel the findings reported by Baaten *et al.* for CD4 memory T cells. The effect of CD44 in this study
Figure 3.13: The loss of CD44 does not affect the generation of effector CD8 T cells following a secondary challenge with *Listeria monocytogenes.*

A. C57BL/6 and CD44<sup>−/−</sup> (CD44k/o) mice on day 30 after a primary challenge with LM-OVA were re-challenged with 1.5-4.5x10<sup>3</sup> CFU LM-OVA and mice were sacrificed on day 3 and 7 for analysis. The frequency of antigen-specific (OVAsp) CD8 T cells in the spleen was quantitated. Data is an average of 4 mice from 2 independent experiments, with error bars to indicate standard deviation.
was found when the cells were put in direct competition by co-transfer into the same mouse. Thus, a competitive assay was set up for this model as well, whereby equal numbers of wildtype and CD44-deficient CD45.2+ OT-I T cells were transferred into naïve congenic CD45.1+ Boy/J recipients and one day later mice were infected with LM-OVA, as before. Thirty days later, mice were sacrificed, and the antigen-specific CD8 T cell pool was analyzed for expression of CD44. As shown in figure 3.14A, whole spleen and bone marrow populations were sequentially gated for CD8 expression (left panel), CD45.2 expression (middle panel), and binding to OVA257-264 peptide-loaded MHC I dimers (right panel).

In both spleen and bone marrow populations, there was a greater ratio of CD44−/− OVA-specific CD8−/CD45.2+ T cells than wildtype T cells, by a ratio of about 2:1 on day 30 (figure 3.14B). This was consistent across five experiments, and the average of all experiments is shown in figure 3.14B. Mice were also injected with 1 µg recombinant IL-15 intravenously on day 30 in order to allow for IL-15-mediated expansion of memory populations. One week later, these mice were sacrificed and the antigen-specific CD8 populations were again analyzed. Similar to the results on day 30, CD44-deficient T cells formed 70% of the population in the spleen, and 65% of the population in the bone marrow (figure 3.14C). Thus, the CD44-deficient T cells have a competitive advantage for populating the antigen-specific pool of CD8 T cells on day 30 following infection, and there is no difference following 7 days of expansion in IL-15, suggesting there is no effect of CD44 on expansion. This is a surprising result, as it suggests that CD44 and possibly hyaluronan might inhibit either the generation or survival of CD8 memory T cells, contrary to the findings published for CD4 Th1 cells (Baaten et al., 2010).
Figure 3.14: CD44-deficient T cells outcompete WT for the generation of memory CD8 T cells.
CD45.2+ OT-I and OT-I CD44−/− CD8 T cells were mixed at a 1:1 ratio and co-transferred into CD45.1+ Boy/J recipient mice, then the mice were infected 1 day later with LM-OVA. On day 30, mice were sacrificed and spleens (SPL) and bone marrow (BM) were harvested. A. Gating scheme to identify transferred cells: CD8+CD45.2+CD45.1+ OVA-specific (OVAsp) cells were analyzed. Spleens from control mice that received only OT-I or OT-I CD44−/− cells were analyzed. B. The cells from A were analyzed for CD44 expression and FL-HA binding, and the average distribution of CD44-positive (WT) and CD44-deficient (CD44k/o) T cells over 5 experiments is summarized below. C. Mice were injected with 1 µg IL-15 intravenously on day 30 then sacrificed on 7 days later, and analyzed as described in B. Error bars indicate standard deviation.
3.3 Discussion

In this chapter, it was found that hyaluronan binding is transiently induced on a subpopulation of the most proliferative activated CD4 and CD8 T cells in vitro and in vivo. Hyaluronan binding can be re-induced upon restimulation in vitro, and is also found to mark a subpopulation of memory (in vivo) and memory-like (in vitro) T cells. The hyaluronan binding memory-like cells proliferated better in IL-7 and IL-15 than the non-hyaluronan binding subset in vitro. Collectively, the data suggests that one unifying characteristic of hyaluronan binding on T cells is a cycling, proliferative phenotype. However, while hyaluronan binding can be reduced by inhibiting IL-2 induced proliferation, there was no evidence that it functions to stimulate proliferation. There was no effect of adding exogenous hyaluronan, or of depriving the cell of endogenous hyaluronan by treatment with hyaluronidase, on activated (day 2) T cell proliferation in vitro.

In T cells, treatment with the inhibitor 4-methylumbelliferone, which depletes cellular UDP-glucuronic acid, thereby inhibiting hyaluronan synthesis (Kultti et al., 2009), significantly inhibited T cell proliferation and IL-2 production, while not inhibiting other aspects of T cell activation, such as CD69 upregulation (Mahaffey and Mummert, 2007). Here, the authors proposed that the cell was using endogenous hyaluronan in an autocrine manner to costimulate for increased IL-2 production and proliferation. However, this effect was CD44-independent, as it was not blocked by treatment with anti-CD44 antibodies (Mahaffey and Mummert, 2007). The authors suggested that hyaluronan could bind to non-CD44 receptors on T cells, but this seems unlikely, as CD44-deficient T cells in this study were not found to bind hyaluronan at detectable levels by flow cytometry.
Previous work in smooth muscle cells has suggested positive and negative roles for hyaluronan in cell proliferation through differential regulation of cyclin D1 expression. In smooth muscle cells, high molecular weight hyaluronan inhibited proliferation (Cuff et al., 2001) by inhibiting Rac1 signaling, which blocked cyclin D1 expression and entry into S phase of the cell cycle (Kothapalli et al., 2008; Kothapalli et al., 2007). A similar effect was found for fibroblasts (Kothapalli et al., 2007). On the other hand, low molecular weight hyaluronan promoted proliferation and induced expression of cyclin D1 by activating the Map kinase (Erk) pathway (Kothapalli et al., 2008). These studies demonstrated that hyaluronan can have two opposing roles in regulating proliferation, depending on which signaling pathway hyaluronan activates upstream of cyclin D1.

In the present study, rooster comb hyaluronan, which consists of high molecular mass hyaluronan (>10^6 Daltons), was added to activated T cell cultures to determine the effect of hyaluronan on proliferation. However, it is possible that some lower molecular weight fragments were also present. Indeed, activated T cells upregulate hyaluronidase expression (HYAL 2) (Mummert et al., 2002), which can cleave hyaluronan to fragments of about 20 kilodaltons. Thus, it is conceivable that there is a mixture of different sizes of hyaluronan present, the net effect of which could obscure any effect on proliferation. While this could explain why exogenously added hyaluronan had no significant effect, it does not explain the more rapid proliferation observed in the hyaluronan binding subset.

While there was no evidence for hyaluronan or CD44 to function as a costimulatory signal in T cell activation, or modulate a primary or secondary immune response to *Listeria monocytogenes* (LM-OVA) in vivo in the absence of competition, there was a role for CD44 in the generation of memory T cells when CD44^{+/+} and CD44^{-/-} cells were put into the same mouse,
and thus could compete for growth or survival signals necessary for the generation of memory T cells. However, the unexpected result from this analysis showed a competitive disadvantage of the C57BL/6 compared to the CD44-deficient CD8 T cells.

This result could be due to poorer proliferation, decreased survival or increased cell death in CD44-positive T cells. In some models, CD44 and hyaluronan have been shown to mediate activation-induced cell death (McKallip et al., 2002; Ruffell and Johnson, 2008). Therefore, following activation and expansion, the CD8 T cells from wildtype OT-I mice might contract to a greater extent than T cells from CD44-deficient mice and generate fewer memory T cells. However, if CD44 binding to hyaluronan was a signal for cell death in this model, it is difficult to explain why there wasn’t a difference in memory T cell generation in C57BL/6 and CD44−/− mice in a non-competitive situation.

The fact that there is no difference in memory T cell generation in wildtype compared to CD44-deficient mice, but there is a difference when CD44+/+ and CD44−/− memory T cells develop in the same mouse, suggests that CD44 and/or hyaluronan might be affecting the ability of the cells to compete for limiting resources that facilitate memory T cell survival or proliferation. Given the fact that cytokines like IL-7 and IL-15, which are important for memory T cell survival and proliferation, induce hyaluronan binding, one would have expected a positive role for hyaluronan in memory T cell generation or maintenance. Indeed, during the development of CD4 memory T cells following influenza challenge, CD44 had a positive effect, and hyaluronan was implicated in mediating this effect (Baaten et al., 2010). Baaten et al. reported that they did not see the same positive effect of CD44 on CD8 effector T cell survival that they saw for CD4 Th1 T cells (Baaten et al., 2010). Thus, there is a need to do more work to uncover the mechanism for how hyaluronan and/or CD44 can mediate these disparate effects.
CHAPTER 4: HYALURONAN BINDING ON ACTIVATED T CELLS INHIBITS MIGRATION AND DISRUPTS THE CO-LOCALIZATION OF CD44 WITH PHOSPHORYLATED ERM PROTEINS

4.1 Introduction

The difference in the numbers of CD44<sup>+/+</sup> and CD44<sup>-/-</sup> memory T cells found in chapter 3 could be attributable to differences in homing or retention in niches that facilitate memory T cell development, perhaps through increased survival or proliferation. This hypothesis would suggest a role for hyaluronan binding to CD44 in T cell migration and adhesion. Therefore, it was of interest to determine the effect of CD44 in T cell migration at various stages of the immune response, but particularly on T cells that bind hyaluronan.

In response to a chemoattractant stimulus, T cells normally adopt a motile phenotype which involves a shape change from round to elongated, and extend a tail-like extension or uropod at the back (away from the direction of the chemoattractant), where adhesion molecules such as CD44, CD43, and ICAM-2 are localized, together with ERM proteins (Vicente-Manzanares and Sanchez-Madrid, 2004). Although CD44 has been used as a marker for the back of a polarized or migrating cell (Vicente-Manzanares and Sanchez-Madrid, 2004), there has been no evidence to date for a function for CD44 at the back of the cell, and hence the significance of re-localizing CD44 is unclear. The first aim for this chapter was to determine whether a defect in the ability to polarize CD44 affects the ability of T cells to migrate.

The second aim of this chapter is to determine the effect of hyaluronan on CD44 polarization and T cell migration. CD44 crosslinking antibodies, as well as the addition of hyaluronan, have been shown to induce migration of a human T cell line in the absence of
chemoattractant through 3-D collagen matrices (Fanning et al., 2005). In this study, the authors demonstrate that engagement of CD44 with antibody or hyaluronan induces cytoskeletal rearrangement and cell polarization. Previous studies using crosslinking CD44 antibodies have also demonstrated an effect of CD44 on cell spreading that is dependent on actin cytoskeletal rearrangements (Foger et al., 2001), requires Rac1, and is negatively regulated by CD45 (Wong et al., 2008). This suggests that on T cells activated to bind hyaluronan, hyaluronan can actively signal to the actin cytoskeleton to regulate T cell polarization. Thus, activated T cells on day 2 after stimulation with PMA and ionomycin were assessed for their ability to migrate in response to a relevant chemoattractant, CCL3 (MIP-1α), and polarize CD44, in the presence and absence of a hyaluronan stimulus. Hyaluronan was predicted to enhance migration through the induction of T cell polarization.
4.2 Results

4.2.1 Cell and CD44 polarization correlate with optimal CXCL12-induced T cell migration

Previous evidence had shown a negative regulatory role for CD45 in actin-mediated polarized T cell spreading on immobilized anti-CD44 antibody (Li et al., 2001; Wong et al., 2008), suggesting that the use of T cells expressing or not expressing CD45 may provide a useful model for studying the relationship between polarization and migration. However, it was not known if these cells could polarize in response to a chemoattractant, or migrate. Thus, BW5147 T cell lines that expressed CD45 (BW+) or lacked CD45 (BW−) were treated with a chemoattractant, CXCL12 (stromal cell-derived factor-1) for 30 minutes, and then the number of cells that became polarized were quantitated. The concentration of CXCL12 used was 200 ng/ml, as this was predetermined to be the optimal concentration for the cells to migrate. A polarized cell was defined as a cell that was elongated or had a pod whereas an unpolarized cell was defined as a round cell. As shown in figure 4.1A, the ability to polarize and undergo shape changes in response to CXCL12 was severely compromised in BW5147 T cells lacking CD45. Although there was about 9% BW− cell polarization in the absence of chemoattractant, there was no significant increase in polarization in response to 200 ng/ml CXCL12 (figure 4.1B). In contrast, cells expressing CD45 (BW+) had slightly lower levels (6%) of basal polarization but showed an almost 4-fold increase in polarization (22%) in response to CXCL12. The cells were also labeled using antibodies against CD44 and the percentage of cells that polarized CD44 to one end of the cell was quantitated. Using this measure, it was found that BW− cells were also
Figure 4.1: Cell and CD44 polarization correlate with optimal CXCL12-induced T cell migration.
A. BW T cells lacking CD45 (BW⁻) and expressing CD45 (BW⁺) were treated with CXCL12, then fixed and labeled with the CD44 mAb IM7. B. % polarized equals the % of cells with a polarized shape (elongated or with a pod). % CD44 polarized equals the % of cells where CD44 is polarized to one end of the cell. Graphs are a summary of 3 experiments with error bars to indicate standard deviation. C. Transwell migration of BW⁺ and BW⁻ cells in response to CXCL12. Data shown is one representative experiment of 3 independent experiments. * p < 0.05, *** p < 0.001.
compromised in their ability to polarize CD44 in response to CXCL12 (figure 4.1B). Thus, cells lacking CD45 were deficient for initiating shape changes and re-localizing CD44 in response to CXCL12 stimulation. To determine if this correlated with decreased migration, the migration of BW+ and BW- cells towards CXCL12 was compared in a transwell chemotaxis assay. The BW+ cells were induced to migrate in response to 100 and 200 ng/ml CXCL12, but the BW- cells migrated very poorly at both concentrations of chemoattractant (figure 4.1C). Therefore, an inability to polarize correlated with an inability to migrate.

4.2.2 CD44 is not required for cell polarization and migration to occur

When Jurkat T cells that do not express CD44 were transfected with full-length CD44 (CD44+) or empty vector as a negative control (CD44-) there was no difference in the ability of CD44+ and CD44- cells to initiate shape changes in response to CXCL12 (figure 4.2A). In both cases, CXCL12 induced the re-localization of phosphorylated ERM proteins (pERM) and when expressed, CD44, to the back of the cell (figure 4.2A and B). pERM localized to the back of the cell in both the presence and absence of CD44. Additionally, there was also no effect of CD44 on migration in response to 10 ng/ml CXCL12 (figure 4.2C). Thus in these Jurkat T cells, CD44 is not required for chemokine-induced T cell polarization, migration, or pERM polarization to the back of the cell.

Since these studies were performed in CD44-transfected T cell lines, it was possible that the findings were not representative of real T cells. Therefore, the migration of CD44+ and CD44- ex vivo mouse lymph node T cells was also compared at various stages of activation. Naïve T cell migration to CXCL12 was unaffected by the presence or absence of CD44 (figure 4.3A), similar to what was seen in Jurkat T cells. Upon T cell activation, CD44 is upregulated
Figure 4.2: CD44 is not required for cell polarization and migration.

A. CD44-negative Jurkat T cells transfected with empty vector (CD44−) or CD44 (CD44+) were treated with CXCL12 to polarize cells, then labeled with antibodies against CD44 (IM7, green) and pERM proteins (red). B. % polarized is the percentage of cells that had a polarized shape (elongated or with a pod). % pERM polarized and % CD44 polarized is the percentage of cells where pERM or CD44 were polarized to the pod, or to one end of the cell. Data is an average of 2 independent experiments. Transwell migration of Jurkat transfectants in response to 10 ng/ml CXCL12. Data shown is one representative experiment of 3 independent experiments, with error bars to indicate standard deviation between triplicate wells.
and is highly expressed on effector T cells (Dutton et al., 1998). While these day 2 activated cells no longer migrated to CXCL12 (data not shown), they did migrate towards CCL3 (Castellino et al., 2006). However, there was again no difference in migration between the CD44-positive and CD44-null T cells (figure 4.3B). Finally, T cells on day 5-6 after activation, which behaved like effector cells and were found to produce IFNγ upon restimulation, were examined for migration to chemokines important for leaving the lymph nodes and migrating to sites of inflammation. Day five cells were found to migrate in response to sphingosine-1-phosphate (S1P), which facilitates exit from lymph nodes, and day six cells were found to migrate well to CCL5, which is important for recruiting effector T cells to sites of inflammation (figure 4.3C). Day five and six cells also exhibited a modest level of migration in the absence of chemoattractants (chemokinesis). However, in all cases, CD44-null T cells migrated similarly to CD44 positive T cells, implying that CD44 is not essential for optimal T cell migration.

4.2.3 An inability to localize CD44 to the uropod correlates with poor migration

Despite CD44 not being required for optimal migration, when CD44 is present in the cell, the ability to polarize CD44 correlated well with the ability to migrate (figure 4.1 and 4.2). This was also found to be the case when AKR T cells transfected with CD44 were treated with CXCL12. Both CD44+ and CD44− T cells were polarized in response to CXCL12, and both polarized pERM proteins, yet CD44 was not polarized, and was localized all around the cell (figure 4.4A and B). In these cells, CD44 had an inhibitory effect on CXCL12-induced migration (figure 4.4C). This suggests that the inability of CD44 to re-localize to the back of the cell is inhibitory for T cell migration.
Figure 4.3: CD44 is not required for mouse lymph node T cell migration at various stages of activation.

A. Transwell migration assay using freshly isolated lymph node cells from C57BL/6 or CD44^-/- (CD44k/o) mice in response to CXCL12. Data shown is an average of 3 independent experiments. B. Transwell migration of T cells activated for 2 days with 2.5 ng/ml PMA and 0.5 µg/ml ionomycin in response to CCL3. C. Transwell migration of day 5 effector T cells in response to sphingosine-1-phosphate (S1P) and migration of day 6 effector T cells to CCL5. Data shown for B and S1P migration in C is one representative experiment of 2-3 experiments. Data shown for CCL5 is an average of 2 independent experiments. Error bars indicate standard deviation.
Figure 4.4: An inability to polarize CD44 correlates with reduced migration.
A. CD44-negative AKR T cells were transfected with empty vector (CD44⁻) or mouse CD44 (CD44⁺) and treated with CXCL12 to polarize cells. Cells were labeled with antibody against CD44 (IM7, green) or pERM (red). B. % polarized is the percentage of cells with a polarized shape (elongated or with a pod). % pERM and CD44 polarized indicates cells where CD44 or pERM is polarized to one end of the cell. Graphs are a summary of 2-3 experiments with error bars to indicate standard deviation. C. Transwell migration of AKR transfectants in response to 0 or 10 ng/ml CXCL12. An average of 5 independent experiments is shown, ** p < 0.01.
4.2.4 Loss of the cytoplasmic domain of CD44 inhibits CD44 re-localization to the uropod and optimal migration

In addition to wildtype (full-length) CD44, a mutant of CD44 with the deletion of all but 2 amino acids of the cytoplasmic domain was expressed in AKR T cells at approximately equal levels to wildtype CD44 and was also unable to re-localize to the uropod in response to CXCL12 (figure 4.5A and B). In Jurkat T cells, unlike wildtype CD44, a CD44 mutant with 6 amino acids in the cytoplasmic tail had a reduced ability to re-localize to the uropod in response to CXCL12. There was still some polarization of the CD44 mutant in Jurkat cells, suggesting some residual CD44-polarizing ability in the extra 4 amino acids. In both cell AKR and Jurkat cell transfectants, the cells and pERM were polarized in response to chemokine, but the polarization of the truncated CD44 was impaired (figure 4.5B and C). Interestingly, when the cells were exposed to CXCL12, both the cells expressing the cytoplasmic domain deletion mutants showed significantly reduced migration compared to cell expressing wildtype CD44 (figure 4.5D). This shows that the cytoplasmic domain of CD44 is required for chemokine-induced re-localization to the uropod, which is required for optimal migration.

4.2.5 Hyaluronan inhibits migration of activated day 2 T cells

The data to date supports a model where CD44 is distributed all over the surface of a round, non-migrating T cell, for example, on a naïve circulating T cell. In response to a stimulus such as a chemokine, pERM and CD44 are re-located to the back of a migrating T cell. pERM re-localization occurs independently of CD44, but the cytoplasmic domain of CD44 is required for CD44 to re-localize, suggesting this re-localization is influenced by cellular machinery.
Figure 4.5: The cytoplasmic domain of CD44 is required for its re-localization to the uropod and for optimal migration.

A. Jurkat and AKR T cells expressing CD44 mutants with only 6 (Jurkat = J CD44cy6) or 2 (AKR = A CD44cy2) amino acids in the cytoplasmic domain were polarized with 200 ng/ml CXCL12 and then labeled with antibodies against CD44 (green) and pERM (red).  B. Summary of 2 experiments showing % polarized, % pERM polarized, and % CD44 polarized for Jurkat T cells expressing full-length CD44 (CD44) and mutant (CD44cy6).  C. Similar graphs for AKR T cells, with full-length (CD44) and mutant (CD44cy2).  Data is an average of 2-3 experiments, with error bars to indicate standard deviation.  D. Transwell migration of Jurkat transfectants to CXCL12, data is one representative experiment of 3.  E. Transwell migration of AKR transfectants to CXCL12.  Data is an average of 3 experiments, error bars indicate standard deviation.  * p < 0.05, ** p < 0.01.
such as the actin cytoskeleton. Re-localization of CD44 was important for efficient migration, but it was unknown and of interest to know what effect the interaction of hyaluronan with the extracellular domain would have on the localization of CD44, and therefore on migration.

In order to test this, the migration of activated T cells in the presence and absence of hyaluronan was compared on day 2 after activation, when approximately 50-70% of the cells are induced to bind hyaluronan (see chapter 3). Migration assays were first performed with CCL3 in the absence of the natural ligand, hyaluronan, since the chemotaxis buffer used did not contain serum. As shown in figure 4.6A, hyaluronan positive cells, hyaluronan negative cells and CD44-deficient T cells all showed similar levels of chemokinesis (migration in the absence of chemokine) and CCL3-induced chemotaxis. However, when these cells were first incubated with fluoresceinated hyaluronan for 20 minutes, then subjected to a migration assay, this specifically reduced the migration of the hyaluronan binding population but not the non-binding or CD44-deficient T cells (figure 4.6B).

4.2.6 Hyaluronan enhances activated T cell adhesion to fibronectin

It was also of interest to see whether hyaluronan would affect integrin adhesion on activated hyaluronan binding cells, as CD44 has previously been suggested to affect adhesion of the integrin VLA-4 and cooperate in cell rolling (Nandi et al., 2004). Fibronectin is an extracellular matrix protein secreted by stromal cells and interacts with adhesion molecules such as the integrins α4β1 or VLA-4 and α5β1 or VLA-5 (Leiss et al., 2008; Ruoslahti, 1988). As shown in figure 4.7, the addition of hyaluronan enhanced the adhesion of day 2 activated T cells to immobilized fibronectin, and this effect was CD44-dependent, as there was no significant
Figure 4.6: Hyaluronan inhibits the migration of HA⁺, but not HA⁻, T cells.

A. C57BL/6 and CD44⁻/⁻ (CD44k/o) activated day 2 lymph node T cells were subjected to migration in a transwell migration assay to 0 or 1 ng/ml CCL3. Migrated T cells were removed from the bottom well and labeled with FL-HA to identify HA⁺ and HA⁻ subsets. % migration equals the number of HA⁺, HA⁻ or CD44⁻⁻ T cells migrated divided by the number of each in the 100% migration controls. B. T cells pre-treated with 2.5 µg/ml FL-HA were subjected to migration in a transwell migration assay to 0 or 1 ng/ml CCL3. % migration is equal to the number of HA⁺, HA⁻, CD44⁻⁻ T cells that had migrated divided by the total number of each subset in the 100% migration controls. For both A and B, data indicates the average of 3 replicate wells from one representative experiment of 3, with error bars to indicate standard deviation. ** p < 0.01.
Figure 4.7: Hyaluronan enhances activated T cell adhesion to fibronectin. Splenic T cells from C57BL/6 and CD44−/− (CD44k/o) mice were activated with 2.5 ng/ml PMA and 0.5 µg/ml ionomycin, then labeled with 1 µM CMFDA on day 2, and treated or not with 0.05 µg/ml hyaluronan. Adhesion to wells coated overnight with 20 µg/ml fibronectin was assessed after 30 minutes at 37°C. Data from one representative experiment is shown but data is significant (* p < 0.05) over 3 experiments.
effect of hyaluronan on the adhesion of CD44-deficient T cells. Interestingly, CD44-deficient T cells had enhanced adhesion relative to the CD44-positive T cells in the absence of hyaluronan (figure 4.7). This suggests either that the loss of CD44 is compensated for by increased adhesion through α4 or 5 β1 integrins, or that CD44 has a negative effect on integrin adhesion, in opposition to the positive effect of hyaluronan.

4.2.7 Hyaluronan pre-treatment disrupts CD44:pERM co-polarization in response to chemoattractant

To determine how hyaluronan was affecting migration and specifically whether it was affecting the polarization of CD44, CD44 and pERM re-localization to the uropod in CCL3-treated cells was compared in the presence and absence of hyaluronan. Figure 4.8A shows the normal distribution of CD44 and pERM prior to addition of the chemoattractant. Addition of CCL3 polarized the cell, and induced the polarized localization of both CD44 and pERM to the same part of the cell. However, when FL-HA was added, FL-HA localized to one area of the cell, but did not co-localize with pERM (figure 4.8B). Co-labeling with antibodies against CD44 and pERM revealed that treatment with hyaluronan could induce the redistribution of CD44 but not pERM (figure 4.8C). Quantitative analysis showed that both CCL3 and FL-HA enhanced pERM polarization but this was not additive (figure 4.8D). Furthermore, CCL3 induced colocalization of CD44 and pERM, but treatment with hyaluronan prevented this CCL3-induced colocalization (figure 4.8D). FL-HA induced cell and FL-HA polarization but greatly reduced pERM polarization and there was very little FL-HA:pERM co-polarization (figure 4.8E). Together, the data shows that hyaluronan can act as a polarizing signal, in terms of inducing pod formation (cell shape change) as well as the polarized localization of CD44, but does not induce
pERM co-localization and prevents subsequent CCL3-induced CD44 and pERM co-polarization. This uncoupling of CD44 and pERM upon CCL3 addition provides one explanation of why hyaluronan inhibits CCL3-induced T cell migration.

4.2.8 Hyaluronan treatment can signal to transiently dephosphorylate ERM proteins

ERM proteins are regulated by their phosphorylation status, as unphosphorylated ERM proteins are unable to associate with actin or transmembrane receptors (Ivetic and Ridley, 2004). Both CXCL12 and TCR stimuli have been shown to transiently dephosphorylate ERM proteins (Brown et al., 2003; Faure et al., 2004). In this study, the addition of hyaluronan also induced the rapid and transient dephosphorylation of Thr 567 (ezrin) and Thr558 (moesin) of ERM proteins, recognized by a phosphospecific antibody on day 2 activated T cells (figure 4.8F). An anti-ERM blot is shown as a control to show equal protein levels. This initial dephosphorylation of ERM mimics the effect of the chemoattractant, CCL3 (figure 4.8F) and provides an explanation for the hyaluronan-induced uncoupling of CD44 from pERM.
Figure 4.8: Hyaluronan binding uncouples the CD44:pERM interaction and disrupts CD44:pERM co-polarization in response to chemoattractant.

A. Activated (d2) lymph node T cells were treated or not with CCL3 in the absence of hyaluronan for 30 minutes. Cells were labeled with antibodies against CD44 (green) or pERM proteins (red). B. d2 T cells were treated with 2.5 µg/ml FL-HA then polarized with CCL3 in the presence of FL-HA (green). Cells were labeled with antibody against pERM proteins (red). C. d2 T cells were treated with 2.5 µg/ml unlabeled HA then polarized with CCL3 in the presence of HA. Cells were labeled with antibodies against CD44 (green) and pERM proteins (red). D. % pERM polarization equals the percent of cells with pERM localized to one end of the cell. % CD44 and pERM polarized is equal to the percent of cells where the CD44 and pERM signals are co-localized to one end of the cell. E. Summary of % polarization of each indicator in the legend following treatment of d2 T cell with FL-HA in B. Graphs are an average of two independent experiments. Error bars indicate standard deviation. F. Western blot showing whole-cell lysates of activated d2 T cells treated with 2.5 µg/ml of hyaluronan or 1 ng/ml CCL3 for the indicated time points and probed for pERM or total ERM.
4.3 Discussion

CD44 is one of several adhesion molecules that have been shown to polarize in response to a chemoattractant stimulus and redistribute to the uropod of migrating T cells (Serrador et al., 1999) but the effect of this redistribution, and any role of hyaluronan on T migration, is not clear. Here, CD44 polarization to the uropod of unactivated T cells in response to a chemokine stimulus (CXCL12) correlates with optimal migration to that chemokine while a defect in localizing to the uropod is associated with poor migration. However, the complete loss of CD44 using CD44-deficient mouse T cells or Jurkat T cell lines did not affect migration to chemokine, and CD44 is not needed for cells to polarize in response to chemoattractant stimuli. This suggests that CD44 localized in the body of the cell is inhibitory for migration, and redistributing it to the uropod prevents this inhibition, although it is at present unknown how this might occur.

On the other hand, on activated T cells, an inhibitory effect of CD44 on migration was found to occur through its interaction with hyaluronan. Hyaluronan inhibited the migration of the hyaluronan binding subset of activated T cells both in the absence of chemoattractant, and in response to CCL3, a chemoattractant thought to be important in the lymph nodes for recruiting CD8 T cells to DC:CD4 T cell complexes (Castellino et al., 2006) as well as in recruitment of effector CD4 and CD8 T cells to sites of inflammation (Bromley et al., 2008).

In this study, hyaluronan treatment induced and/or enhanced CD44 polarization in the absence of chemoattractant but blocked chemoattractant-induced polarization, and inhibited pERM co-localization with CD44. This would support an anti-migratory effect of hyaluronan, as the appropriate polarization of the cell is thought to be a necessary pre-requisite for migration. There has been little evidence in the literature to explain how hyaluronan could induce or
mediate T cell polarization. In tumour cells, hyaluronan can signal through CD44 to activate Vav and Rac1 during ovarian tumour cell migration (Bourguignon et al., 2001) and hyaluronan also activates Rac1 in melanoma cells (Kim et al., 2008). In a mouse mammary epithelial cell line, hyaluronan was found to induce lamellipodia formation close to the point at which it was applied using a microinjection needle (Oliferenko et al., 2000). Notably, hyaluronan could induce Rac signaling in these cells, and lamellipodia formation was inhibited in the presence of a dominant negative Rac inhibitor (Oliferenko et al., 2000). This is of interest since Rac1 activation is typically found at the leading edge of migrating or polarized cells (Vicente-Manzanares and Sanchez-Madrid, 2004) and signals to generate actin cytoskeletal protrusions. This could mean that hyaluronan binding on activated T cells results in “frontness” signals occurring where CD44 is localized, which is normally the back of the cell, and result in poor directional migration in response to a chemoattractant.

Hyaluronan treatment caused a transient dephosphorylation of ERM proteins, similar to treatment with CCL3. Chemokine stimulation with CXCL12 has previously been shown in peripheral blood T cells to rapidly but transiently dephosphorylate ERM proteins (Brown et al., 2003). The phosphatase that mediates this process has not been elucidated, but the rapid, transient dephosphorylation of ERM proteins that occurs upon TCR stimulation was dependent on signaling from Vav and Rac1 (Faure et al., 2004), which could thus also be a signaling pathway important in ERM dephosphorylation downstream of hyaluronan, which may activate this pathway.

Previously, hyaluronan binding on BW5147 T cell lines has been shown to upregulate intracellular calcium (Bourguignon et al., 1993). Strong peptide/MHC:TCR interactions have been shown to slow T cell migration, and effect that was accompanied by increases in
intracellular calcium (Skokos et al., 2007). In vitro, calcium ionophores have been shown to inhibit migration (Negulescu et al., 1996). It has been shown that a hyaluronan blocking peptide, Pep-1, inhibited T:DC conjugate formation (Mummert et al., 2002). Thus, hyaluronan could be a stop signal for T cell migration in the lymph nodes, perhaps by signaling to increase intracellular calcium.

Although in this study, hyaluronan was found to inhibit activated T cell migration, a previous study by Fanning et al. showed that hyaluronan supported the migration of a human T cell line through a 3-D collagen gel and, in agreement with this study, promoted the polarization of these cells in the gel (Fanning et al., 2005). Their study differs from this one in that their cells were constitutively able to bind hyaluronan. This is likely an important distinction because the cells they were using were round and unpolarized. As a consequence of having to activate the cells in order to look at hyaluronan binding, many of the cells used in this study were pre-polarized. Thus, if the hyaluronan in their study was polarizing the cells, then in the absence of pre-polarization, or of a chemoattractant signal that could polarize the cells independently of hyaluronan, the polarized phenotype induced by hyaluronan could facilitate migration. On the other hand, when the cells are pre-polarized, or incubated in the presence of a chemoattractant signal, hyaluronan-induced polarization could inhibit migration.

Hyaluronan binding was also found to enhance adhesion to fibronectin, which suggests enhanced adhesion by α4β1 and α5β1 (VLA-4 and VLA-5) integrins. Intravenous administration of hyaluronidase has been shown previously to inhibit activated T cell recruitment into inflammatory sites in the peritoneal cavity (DeGrendele et al., 1997a), although the mechanism for how this occurs has not been established. Previously, Nandi et al. demonstrated that CD44 could form a complex with VLA-4 and that rolling on hyaluronan was slowed by
adding a substrate for VLA-4, VCAM-1, which also increased firm adhesion (Nandi et al., 2004). Thus, there could be an additional effect of CD44 and hyaluronan on activated T cell recruitment into peripheral sites of inflammation perhaps by facilitating extravasation at inflammatory sites. Interestingly, CD44-deficient T cells were found to have increased adhesion to fibronectin compared to CD44-positive T cells. It is possible that in the absence of hyaluronan, (or a chemoattractant stimulus, as none were used for this assay) CD44 is distributed uniformly around the cell and negatively regulates integrin adhesion to fibronectin. Thus, CD44-deficient T cells would be expected to adhere more strongly than CD44-positive T cells. However, in the presence of hyaluronan, CD44 is polarized and perhaps segregated away from integrins, and the inhibitory effect of CD44 is lifted. This would result in similar levels of adhesion by CD44-positive and CD44-deficient T cells.

Together, the data support a model whereby hyaluronan inhibits activated T cell migration, and perhaps additionally has an effect to enhance their adhesion, through the dysregulation of normal CD44 and pERM protein co-localization at the back of the cell. Since in chapter 3, it was found that hyaluronan marked highly proliferative activated T cells, it is possible that hyaluronan binding to CD44 signals to inhibit the migration of proliferating, activated T cells, and retain them at sites of activation. Hyaluronan, similar to CD69, could be a retention signal to allow proliferating cells to differentiate into effector cells prior to leaving the lymph nodes or spleen. Since hyaluronan binding can be re-induced upon restimulation of activated T cells, it could also function to recruit or retain effector T cells at sites of inflammation in the periphery. Future work will address the possible function of hyaluronan on T cell retention, and also try to elucidate a signaling pathway downstream of hyaluronan in T cells, similar to what has been found in tumour cells, in order to better understand how
hyaluronan can regulate T cell polarity in the presence and absence of chemoattractant stimuli, and thus also regulate T cell migration.
CHAPTER 5: SUMMARY AND DISCUSSION

5.1 Summary of findings

The aims of this project were to determine the effect of CD44 and hyaluronan binding on T cells during an immune response, characterize when and which cells bind hyaluronan during the response, and to identify consequences for a cell to bind hyaluronan. Hyaluronan binding was found on a subset of memory T cells on day 30, as well as the most activated antigen-specific T cells on day 7, following challenge with LM-OVA \textit{in vivo}. These results were paralleled \textit{in vitro}, as a subset of the most activated CD4 and CD8 T cells bound hyaluronan on day 2 after stimulation with PMA and ionomycin, or on day 2 or 3 following stimulation with antigen-loaded antigen presenting cells, and on CD44$^+$CD122$^+$ memory phenotype cells on day 10. Here, the hyaluronan binding subsets of both activated (d2) and memory phenotype (d10) T cells were further characterized as being the most proliferative, cycling T cells. It was found that hyaluronan was not important for naïve T cell costimulation or effector T cell killing, and the loss of CD44 did not affect a primary or secondary immune response to LM-OVA, although CD44-deficient CD8 T cells outcompeted wildtype CD8 T cells for the generation of memory cells on day 30 after infection when the cells were injected at a 1:1 ratio into the same mouse. Hyaluronan treatment of the hyaluronan binding subset of activated d2 T cells was found to inhibit migration and enhance adhesion to fibronectin. One signaling consequence of hyaluronan was transient ERM protein dephosphorylation, which resulted in the loss of co-localization of pERM with CD44 in hyaluronan-treated cells. These results are summarized in figure 5.1.
Figure 5.1: Summary of hyaluronan binding during various stages of an immune response.
5.2 Discussion and future directions

The first question from these findings is why hyaluronan binding is only induced on a subset of activated T cells, both in vitro and in vivo, as well as on a subset of memory phenotype day 10 T cells and memory T cells in vivo. This finding agrees with previous evidence that hyaluronan binding only occurs on a subset of regulatory CD4 T cells (Firan et al., 2006) and on a subset of cytotoxic CD8 T cells activated by the in vivo transfer of allogeneic cells into mice (Lesley et al., 1994). It is perhaps most intriguing that hyaluronan binding occurs on proliferating T cells immediately following activation and is induced by culture of effector T cells in high concentrations of gamma chain cytokines (IL-2, IL-7, IL-15), which at these concentrations were all able to stimulate T cell proliferation of resting T cells. This suggests that hyaluronan binding is found on dividing T cells at all stages of (activated, effector, memory) an immune response.

Chang et al. showed the unequal distribution of CD44 on proximal and distal daughter cells following cell division induced by TCR activation (Chang et al., 2007): this suggests that although CD44 expression is upregulated following T cell activation, upon the very first division, half of the divided cells in the population will have higher levels of CD44 than the other. Since hyaluronan binding only occurs on the cells expressing the highest levels of CD44, this could explain why only a subset of T cells are able to bind hyaluronan. In the in vitro models used in this study, hyaluronan binding is induced on a subset of OT-II CD4 and OT-I CD8 T cells stimulated with antigen presenting cells and OVA peptide, and on C57BL/6 T cells stimulated with PMA and ionomycin. Since the latter does not involve interaction with antigen presenting cells, it at first glance seems counterintuitive to suppose that these cells would asymmetrically divide. However, cells activated in this manner were found to undergo polarized shape changes,
and labeling with anti-CD44 antibodies and fluorescence microscopy analysis revealed that a percentage of the cells had a polarized CD44 distribution even in the absence of an exogenous chemokine stimulus (figure 4.8). Thus, CD44 could be unequally distributed upon division in this model even in the absence of an interaction with an antigen presenting cell.

However, this explanation for FL-HA binding on a subset of T cells is compromised by the fact that the highest CD44 expression was found on the proximal daughter cell – also the cell that, upon transfer into naïve recipients, offers good short-term protection but poor long-term protection or memory function (Chang et al., 2007). This would suggest that hyaluronan binding would occur on only the “effector” daughter cells, yet the data from this project shows that memory T cells have a substantial hyaluronan binding subset. This could be explained by the fact that cells that had not bound hyaluronan on day 6 after activation in vitro could be induced to upregulate CD44 expression and bind hyaluronan upon culture in IL-2, IL-7, or IL-15. The “memory” daughter cells could undergo slow turnover in response these cytokines, upregulate their CD44, and acquire hyaluronan binding capability. Alternatively, since it has been shown that effector T cells can contribute to the memory pool (Bannard et al., 2009), it is possible that some of the hyaluronan binding population found on day 30 are in fact CD44hi effector T cells that had survived contraction.

The current literature supports a model where asymmetric division can yield both effector- and memory-fated daughter cells, but the system retains some developmental plasticity, such that effector cells are not necessarily terminally differentiated and can contribute to the memory T cell population (Bannard et al., 2009). Recent evidence shows that the strength of IL-2 signaling on effector T cells is responsible for driving transcriptional programmes that favor
terminal differentiation and death or alternatively survival and acquisition of a memory phenotype (Pipkin et al., 2010).

Memory T cells are a diverse population that have been classified into different groups according to the expression of various markers: for example, central memory and effector memory cells, which differ in their localization to lymphoid tissues and to the peripheral circulation, respectively, are differentiated according to their expression of CD62L and CCR7 (Sallusto et al., 1999). Memory T cell populations generated after infection with Sendai virus could be separated into three separate populations based on their expression of CD27 and CD43, a classification system that differentiated cells based on their proliferative potential and efficacy in mounting a secondary immune response (Hikono et al., 2007). From the results of this study, hyaluronan binding could be used as an additional distinguishing marker which detects the most proliferative memory T cells in response to IL-7 and IL-15, and it is intriguing, although still at the level of speculation, if hyaluronan binding could also differentiate specific types of memory T cells – for example, memory cells that had gone through an initial effector phase.

If hyaluronan binding marks a subset of activated cells that upon asymmetric division are fated to become effector T cells, it is tempting to speculate the role that hyaluronan could have in signaling to enable survival of this subset during contraction. It has previously been shown that IL-7, although necessary for survival, is likely not sufficient for effector T cell survival and memory T cell development, since the transgenic expression of IL-7R does not rescue cells from contraction nor increase the number of memory cells generated (Haring et al., 2008). A role for hyaluronan is supported by Baaten et al. (Baaten et al., 2010), who showed that CD4 memory T cell development can be inhibited by treatment with hyaluronan blocking anti-CD44 antibody (KM201) and promoted by an agonist antibody (IRAWB 14) that enhances hyaluronan binding,
and that Th1 effector T cell survival upon transfer into naïve recipients is dependent on the presence of CD44.

The data from the competition assay used in this study (figure 3.14) suggests that CD44 has a negative effect on the generation of memory T cells. However, since there was no difference in the ability of CD44-deficient and C57BL/6 mice to contract the IFNγ-producing CD8 T cell population following LM-OVA challenge, or in the ability of C57BL/6 and CD44-deficient mice challenged with LM-OVA to generate memory T cells on day 30 (figure 3.12), or any difference in the expansion of IFNγ-producing CD8 T cells on day 3 or 7 after re-challenge with LM-OVA on day 30 (figure 3.13), this argues against a direct role for CD44 or hyaluronan on effector T cell death or survival. Thus, the effect of CD44 and hyaluronan is only apparent when CD44-positive and CD44-deficient T cells are in competition.

In vivo, memory T cells must compete with each other (as well as with naïve T cells) for limiting amounts of IL-7, an important cytokine for memory cell survival (Schluns et al., 2000). IL-7 and IL-15, cytokines important for memory T cell proliferation, are produced by stromal cells in lymphoid tissues such as the thymus (IL-7) and bone marrow (Mazzucchelli et al., 2009; Mrozek et al., 1996). Thus, in vivo, the ability to compete for these IL-7- and IL-15-rich niches is paramount to ensuring optimal survival and proliferation. As mentioned earlier, Becker et al. have shown that bone marrow is a major site of proliferation for memory CD8 T cells (Becker et al., 2005). Homing to bone marrow is dependent on interactions between VLA-4 (α4β1 integrin) and VCAM-1 (Berlin-Rufenach et al., 1999; Koni et al., 2001). Interestingly, Estess et al. have also reported IL-15 inducible hyaluronan binding on endothelial cells in vitro, and intraperitoneal injection of IL-15 facilitated CD44-mediated recruitment of superantigen-stimulated T cells into the peritoneum in vivo (Estess et al., 1999). It is thus possible that a subset of activated T cells
with high CD122 (IL-15Rβ) expression are selected to inducibly bind hyaluronan, which could then function to facilitate their homing to bone marrow, or alternatively their retention in specific niches within the bone marrow that facilitate memory T cell proliferation or survival. In support of this, hyaluronan binding has been reported on CD34+ human stem and progenitor cell lines, and blocking antibodies against CD44 and treatment with hyaluronidase were found to inhibit human stem cell homing to bone marrow in the mouse (Avigdor et al., 2004).

However, the unexpected results from this study were that CD44−/− T cells have a competitive advantage for memory T cell generation relative to CD44+/+ T cells. This could be explained if there are opposing effects of CD44 and hyaluronan on T cell retention in IL-7 or IL-15-rich niches for memory T cells. The results from chapter 4 of this study show that CD44−/− activated T cells adhere more strongly to fibronectin than CD44+/+ T cells, although hyaluronan promotes the adhesion of CD44+/+, but not CD44−/− T cells to fibronectin. In this way, there could be compensation through increased adhesion of other adhesion molecules in the CD44−/− mouse that could mask any differences that are due to hyaluronan. Alternatively, CD44 might have a negative effect on adhesion, which is relieved by hyaluronan-mediated sequestration of CD44 to the uropod.

If there are indeed opposing effects of CD44 and hyaluronan, however, then a comparison of CD44+/+ and CD44−/− T cells is not appropriate for determining the role of hyaluronan in memory T cell generation or turnover. In order to specifically address the effect of hyaluronan, a hyaluronan binding mutant of CD44 needs to be expressed on T cells, co-transferred into naïve recipients, and the generation of memory T cells compared with cells expressing hyaluronan binding-competent-CD44. Mutating arginine 41 in human CD44 to alanine has been shown to inhibit HA binding (Ruffell and Johnson, 2008). This would
eliminate any advantage of increased integrin adhesion in the CD44-deficient T cells, as both the hyaluronan-binding and non-hyaluronan binding forms of CD44 would be expressed on CD44-deficient T cells, and the only difference between the two should be the ability to bind hyaluronan. The corresponding mutant construct of mouse CD44 (R43A) has been generated and this and wildtype CD44 constructs could be retrovirally-transfected into mouse bone marrow from CD44-deficient mice, and used to reconstitute lethally irradiated mice to generate T cells. Then, T cells expressing HA binding competent and HA binding mutants of CD44 can be co-transferred into naïve Boy/J mice, the mice infected with *Listeria monocytogenes*, and the population of memory T cells can be evaluated.

In order to address whether hyaluronan can promote the survival of effector T cells, naïve OT-I CD44−/− T cells can be transfected with retroviral constructs of the R43A mutant and wildtype CD44 and activated in vitro with OVA peptide and antigen presenting cells. On day 3 after activation, when CD8 T cells were found in this study to have acquired effector cytotoxic function (figure 3.11) positive transfectants can be sorted, then a mixture of R43A mutant CD44 and wildtype CD44 expressing cells could be co-transferred into recipient Boy/J mice. A time course of survival of these two subsets could be plotted in order to compare the effect of hyaluronan on survival. The cells could be labeled with different Cell Tracker (Molecular Probes) dyes in order to differentiate them.

The significance of hyaluronan binding on activated (day 2) T cells in inhibiting migration is so far unclear, but here, too, a role in retention is an intriguing possibility. In the lymph nodes, following activation, T cells undergo several rounds of proliferation prior to differentiation into effector T cells and exit from the lymph node (Shiow et al., 2006). In this study, the authors showed a function for the early activation marker CD69 in retention in lymph
nodes, such that expression of CD69 led to its interaction with S1P1, the receptor for sphingosine-1-phosphate, a chemokine important for T cell egress from lymph nodes (Shiow et al., 2006). This interaction prevented S1P1 expression on the cell surface. Thus, while the cells are activated and proliferating, and CD69 expression is high, the cells are retained in lymph nodes because they do not express the receptor needed to leave. When CD69 is downregulated, S1P1 expression goes back up, and the cells are able to migrate out of the lymph nodes and into the periphery (Shiow et al., 2006).

CD44 expression is also upregulated upon activation and hyaluronan binding is transiently induced, but both decline when the cells begin to acquire the ability to migrate to S1P (day 5 as shown in figure 4.3, cells did not migrate on day 2, data not shown), and thus would be ready to leave the lymph nodes. As hyaluronan binding to activated T cells inhibits their migration, this could allow the subset of highly proliferative hyaluronan binding T cells to be retained in lymph nodes. Alternatively, hyaluronan could facilitate retention at sites of inflammation, when effector cells are re-induced to bind hyaluronan upon stimulation with antigen.

In order to test this, an immune response model where T cells are activated in the lymph nodes but migrate out to distinct sites in the tissues is preferred. To this end, influenza virus infection, such as that used in the study by Baaten et al. would be a good model to test. Once again, T cells expressing wildtype CD44 and R43A (no HA binding) mutant CD44 could be generated by bone marrow reconstitution of OT-I CD44 knockout mice, in order to have transgenic T cells, and these could be independently transferred or co-transferred into recipient mice, and the mice challenged intranasally with influenza virus. The potential confounding effect of the CD44−/− T cells having basally higher levels of adhesion than CD44+/+ T cells could
be avoided by comparing hyaluronan binding-competent and -deficient CD44+/+ T cells. An appropriate time course for the infection would have to be established, to determine when activated T cells are expected to proliferate in the lymph nodes, leave the lymph nodes, and get recruited to the lungs. The ratio and numbers of HA+ and HA- cells in the lymph nodes and lungs of co-transferred mice can be measured over time to determine whether there is a difference in the length of time these cells are found in the lymph node prior to migrating to the site of infection in the peripheral tissues. At the same time, the viral load in mice that received HA+ vs. HA- cells independently could be compared, in order to determine the effect that a difference in retention, provided there is one, has on the efficacy of the immune response.

In concordance with this, it would be helpful to know where the hyaluronan binding T cells would “see” hyaluronan, as it is currently not known where in the lymph nodes hyaluronan might be found. Lymph node sections could be processed and stained with a biotinylated hyaluronan binding protein (bovine proteoglycan) which could then be detected with a fluorochrome-conjugated secondary antibody. Lymph node areas are typically demarcated using fluorescent antibodies against CD3 to label T cell zones, and against B220 to label B cell zones. Bone marrow-derived dendritic cells and cultured dendritic cell lines express hyaluronan synthases and have been suggested to present hyaluronan previously (Mummert et al., 2002), so an additional antibody against CD11c would add to the picture.

Overall, I propose the following model, summarized in figure 5.2: naïve T cells are activated and induced to upregulate CD44 and bind hyaluronan on a subset of highly proliferative T cells in the lymph node. Here, hyaluronan binding inhibits their migration and enhances their adhesion, perhaps as a mechanism to promote their retention in the lymph node until they have differentiated into effector cells and are ready to exit into the periphery. As
effector T cells, they can be re-induced to bind hyaluronan at the site of inflammation upon encountering antigen and TCR restimulation. This could function to retain them at the site. On the other hand, memory T cells cycling through cytokine-rich niches (IL-7 or IL-15) could be induced to bind hyaluronan in order to facilitate their retention at sites in the bone marrow, which could favor their enhanced survival and competitiveness for limiting amounts of survival and growth signals.
Figure 5.2: Proposed model from this project.
Effector T cells = $T_{\text{eff}}$. Memory T cells = $T_{\text{mem}}$. Naïve T cells are shown in white, activated T cells are shown in yellow. DC = dendritic cell.
5.3 Perspectives

In this work, I identified a unifying characteristic of hyaluronan binding across multiple T cell types: hyaluronan binding identifies a subset of the most proliferative activated and memory T cells following TCR activation. Some consequences of binding hyaluronan on activated T cells were to inhibit migration, dysregulate cell polarization, and enhance adhesion, and hyaluronan could signal to dephosphorylate ERM proteins. The consequences and significance of hyaluronan binding on a subset of memory T cells still needs to be evaluated, but one promising direction this work could take is in determining the role of hyaluronan binding to CD44 in the survival and development of memory T cells.
REFERENCES


APPENDIX: ANIMAL CARE AND BIOSAFETY CERTIFICATES

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

BREEDING PROGRAMS

Application Number: A08-0936
Investigator or Course Director: Pauline Johnson
Department: Microbiology & Immunology

Animals:
- Mice OT-1 22
- Mice OT-II 22
- Mice Actin-GFP and 485KO-GFP 60
- Mice RAGKO 100
- Mice CD45.2xKbC57Bl6 130
- Mice Roy1 110
- Mice CD45 exon 9 N9 KO 325
- Mice 485RAGKO 110
- Mice OT-44KO 20
- Mice CD44 N7 KO 200
- Mice IL-7R ko 20
- Mice All of named strains 500
- Mice C57Bl6 500

Approval Date: February 2, 2010

Funding Sources:
- Funding Agency: Canadian Institutes of Health Research (CIHR)
  Funding Title: Interactions of CD44 in the immune system
- Funding Agency: Canadian Diabetes Association
  Funding Title: Development of obesity and type II diabetes in DC45 null mice
- Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC)
  Funding Title: Molecular analysis of CD44 on cell adhesion and migration
- Funding Agency: Heart and Stroke Foundation of British Columbia and Yukon
  Funding Title: To investigate the pro- and anti-inflammatory functions on CD44 and hyaluronan
- Funding Agency: Canadian Institutes of Health Research (CIHR)
  Funding Title: Regulation of signaling and dendritic cell function by CD45

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above breeding program.
This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A04-0192

Investigator or Course Director: Pauline Johnson

Department: Microbiology & Immunology

Animals: Mice 60
         Rabbits 2

Start Date: April 1, 2002

Approval Date: July 7, 2006

Funding Sources:

<table>
<thead>
<tr>
<th>Grant Agency</th>
<th>Grant Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural Science Engineering Research Council</td>
<td>CD44 signaling in cell adhesion and cell migration</td>
</tr>
<tr>
<td>Natural Science Engineering Research Council</td>
<td>CD44 signaling in cell adhesion and cell migration</td>
</tr>
</tbody>
</table>

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A07-0292
Investigator or Course Director: Pauline Johnson
Department: Microbiology & Immunology
Animals:

| Mice cd44 knock out 49 |
| Mice OT1/CD44 ko 100   |
| Mice OT 11 30          |
| Mice OT1 46           |
| Mice c57Bl/6 39       |

Start Date: April 1, 2002  Approval Date: September 29, 2009

Funding Sources:

| Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC) |
| Funding Title: Molecular analysis of CD44 on cell adhesion and migration |
| Funding Agency: Natural Sciences and Engineering Research Council of Canada (NSERC) |
| Funding Title: CD44 signaling in cell adhesion and cell migration |

Unfunded title: Molecular analysis of CD44 in cell adhesion and migration

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
ANIMAL CARE CERTIFICATE

Application Number: A07-0344

Investigator or Course Director: Pauline Johnson

Department: Microbiology & Immunology

Animals:

- Mice BoyJ 48
- Mice cd 44 ko 117
- Mice c57Bl/6 105

Start Date: September 1, 2002

Approval Date: November 5, 2009

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)

Funding Title: Interactions of CD44 in the immune system

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093
Biohazard Approval Certificate

PROTOCOL NUMBER: B06-0137

INVESTIGATOR OR COURSE DIRECTOR: Pauline Johnson

DEPARTMENT: Microbiology & Immunology

PROJECT OR COURSE TITLE: Molecular analysis of CD44 in cell adhesion and migration

APPROVAL DATE: August 24, 2009     START DATE: July 27, 2006

APPROVED CONTAINMENT LEVEL: 2 with Containment Level 3 Operating Procedures

FUNDING TITLE: Regulation of Signaling and Dendritic Cell Function by CD45
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

FUNDING TITLE: Molecular analysis of CD44 in cell adhesion and migration
FUNDING AGENCY: Natural Sciences and Engineering Research Council of Canada (NSERC)

FUNDING TITLE: CD44 signaling in Cell Adhesion and Cell Migration
FUNDING AGENCY: Natural Sciences and Engineering Research Council of Canada (NSERC)

FUNDING TITLE: To Investigate the Pro and Anti Inflammatory Functions of CD44 and Hyaluronan
FUNDING AGENCY: Heart and Stroke Foundation of British Columbia and Yukon

FUNDING TITLE: Interactions of CD44 in the immune system
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

UNFUNDED TITLE: Molecular analysis of CD44 in cell adhesion and migration

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the University of British Columbia Policies and Procedures, Biosafety Practices and Public Health Agency of Canada guidelines.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there are no changes. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093
The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111  FAX: 604-822-5093
Biohazard Approval Certificate

PROTOCOL NUMBER: H03-0148

INVESTIGATOR OR COURSE DIRECTOR: Johnson, Pauline

DEPARTMENT: Microbiology & Immunology

PROJECT OR COURSE TITLE: CD44 signalling in cell adhesion and cell migration

APPROVAL DATE: 05-08-22

APPROVED CONTAINMENT LEVEL: 2

FUNDING AGENCY: Natural Science Engineering Research Council

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111  FAX: 604-822-5093
The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: **H06-0137**

INVESTIGATOR OR COURSE DIRECTOR: **Johnson, Pauline**

DEPARTMENT: **Microbiology & Immunology**

PROJECT OR COURSE TITLE: **CD44 signaling in Cell Adhesion and Cell Migration**

APPROVAL DATE: **06-08-16**

APPROVED CONTAINMENT LEVEL: **2**

FUNDING AGENCY: **Natural Science Engineering Research Council**

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111  FAX: 604-822-5093
The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 5190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111  FAX: 604-822-5093
The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111  FAX: 604-822-5093
The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: H06-0137

INVESTIGATOR OR COURSE DIRECTOR: Johnson, Pauline

DEPARTMENT: Microbiology & Immunology

PROJECT OR COURSE TITLE: Molecular analysis of CD44 in cell adhesion and migration

APPROVAL DATE: 08-07-22

APPROVED CONTAINMENT LEVEL: 2 with Level 3 Operational Procedures

FUNDING AGENCY: Natural Sciences and Engineering Research Council of Canada (NSERC)

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111  FAX: 604-822-5093
The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093
The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: H06-0137

INVESTIGATOR OR COURSE DIRECTOR: Johnson, Pauline

DEPARTMENT: Microbiology & Immunology

PROJECT OR COURSE TITLE: Interactions of CD44 in the immune system

APPROVAL DATE: 06-08-16

APPROVED CONTAINMENT LEVEL: 2

FUNDING AGENCY: Canadian Institutes of Health Research

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6100 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111    FAX: 604-822-5093
Biohazard Approval Certificate

PROTOCOL NUMBER: H06-0137

INVESTIGATOR OR COURSE DIRECTOR: Johnson, Pauline

DEPARTMENT: Microbiology & Immunology

PROJECT OR COURSE TITLE: Interactions of CD44 in the immune system

APPROVAL DATE: 08-07-22

APPROVED CONTAINMENT LEVEL: 2 with Level 3 Operational Procedures

FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Westbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above approval date provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111  FAX: 604-822-5093
Biohazard Approval Certificate

PROTOCOL NUMBER: B06-0137

INVESTIGATOR OR COURSE DIRECTOR: Pauline Johnson

DEPARTMENT: Microbiology & Immunology

PROJECT OR COURSE TITLE: Molecular analysis of CD44 in cell adhesion and migration

APPROVAL DATE: August 24, 2009

START DATE: July 27, 2006

APPROVED CONTAINMENT LEVEL: 2 with Containment Level 3 Operating Procedures

FUNDING TITLE: Regulation of Signaling and Dendritic Cell Function by CD45
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

FUNDING TITLE: Molecular analysis of CD44 in cell adhesion and migration
FUNDING AGENCY: Natural Sciences and Engineering Research Council of Canada (NSERC)

FUNDING TITLE: CD44 signaling in Cell Adhesion and Cell Migration
FUNDING AGENCY: Natural Sciences and Engineering Research Council of Canada (NSERC)

FUNDING TITLE: To Investigate the Pro and Anti Inflammatory Functions of CD44 and Hyaluronan
FUNDING AGENCY: Heart and Stroke Foundation of British Columbia and Yukon

FUNDING TITLE: Interactions of CD44 in the immune system
FUNDING AGENCY: Canadian Institutes of Health Research (CIHR)

UNFUNDED TITLE: Molecular analysis of CD44 in cell adhesion and migration

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the University of British Columbia Policies and Procedures, Biosafety Practices and Public Health Agency of Canada guidelines.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there are no changes. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093

144