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Non-redundant and critical roles for leukotriene B4 receptors BLT1 and BLT2 in mouse models of inflammatory arthritis.

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NON-REDUNDANT AND CRITICAL ROLES FOR LEUKOTRIENE B_4 RECEPTORS BLT1 AND BLT2 IN MOUSE MODELS OF INFLAMMATORY ARTHRITIS

by

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DEDICATION

This work is dedicated to my family who has helped me through my scholastic endeavors. Also, this is dedicated to the individuals in my laboratory without whom this work would not be possible.

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I would like to thank my mentor, Dr. Haribabu Bodduluri who helped to mold and guide me through this process. Getting to the end-point of these studies took a lot of effort, which he was always willing to give. I would like to thank Dr. Deming Sun who helped me with the generation of monoclonal antibodies. This tool has benefited us greatly and will be useful in ongoing studies. I would like to thank Dr. Michael Tseng who was critical in the atherosclerosis experiments our laboratory carried out, though those data do not appear in this thesis. Finally, I would like to thank the Microbiology and Immunology department and the James Graham Brown Cancer for providing the opportunity and facilities with which to work.

ABSTRACT

NON-REDUNDANT AND CRITICAL ROLES FOR LEUKOTRIENE B_4 RECEPTORS, BLT1 AND BLT2, IN MOUSE MODELS OF INFLAMMATORY ARTHRITIS

Steven Mathis

April 4, 2007

Inflammation is now recognized as an important factor in several age-related diseases such as arthritis, atherosclerosis, multiple sclerosis and diabetes. In each case, sub-clinical chronic inflammation occurs over years and leads to progressive destruction of the tissue until the symptoms become clinically apparent. Eicosanoids such as prostaglandins and leukotrienes play an important role in inflammation. Among these, leukotriene B_4 (LTB_4) has had a long history of being associated with numerous inflammatory diseases. A high affinity receptor for LTB_4, LTB_4 receptor 1 (BLT1) has been well-characterized in human and murine tissues. While the molecular mechanisms remain unclear, its importance in diverse inflammatory diseases was demonstrated in mice lacking BLT1. More recently, a second highly conserved low affinity LTB_4 receptor, LTB_4 receptor 2 (BLT2) was identified, but its functional significance remains completely unknown. Both BLT1 and BLT2 are seven-transmembrane G protein coupled receptors transducing signals through heterotrimeric G-proteins.

Chapter II of this thesis describes the generation and characterization of monoclonal antibodies to BLT1. Immunization of BLT1-deficient mice with 300.19, a murine pre-B cell line expressing high levels of either human or murine BLT1 allowed isolation of highly species specific anti-BLT1 antibodies. Using an approach involving a series of human/murine BLT1 chimeric receptors the monoclonal antibody binding sites were mapped to extracellular loop-2. Extensive characterization of BLT1 in murine

primary cells revealed high levels of BLT1 in neutrophils and eosinophils and a previously unsuspected regulation of BLT1 in macrophages. While bone marrow derived macrophages expressed high levels of BLT1 its expression in peripheral tissue macrophages was significantly reduced suggesting a potential novel mechanism, and a role for BLT1 in inflammation-induced egress of bone marrow derived cells. The antibodies also allowed demonstration of normal BLT1 expression in the recently generated BLT2 deficient mice. Future studies could explore the therapeutic potential of these antibodies.

In Chapter III, we investigated the role of BLT1 and BLT2 in inflammatory arthritis in mouse models. Collagen induced arthritis (CIA) is a model where mice are immunized with chicken collagen type II in complete freund's adjuvant and leads to a polyarthritis in the distal joints. BLT1 deficient mice on the DBA/1 background were completely protected from the development of CIA which confirms the previous observations with BLT1 antagonists and BLT1 deficient mice on the C57Bl/6 background.

Arthritis was also induced by transfer of K/BxN serum to naïve mice. K/BxN is a T cell receptor transgenic mouse (KRN) crossed with a NOD mouse. These mice spontaneously develop arthritis due to auto-antibody production against glucose-6-phosphate isomerase, a ubiquitous enzyme. In this model, we report for the first time that BLT2 deficient mice are completely protected from inflammatory arthritis. Histopathologic examination revealed a massive inflammatory cell influx in wild-type mice that was completely absent in BLT2 deficient mice. Further analysis of these mice using bone marrow transplantation studies demonstrated development of arthritis requires

BLT2 expression on bone marrow-derived cells. Wild-type mice which received bone marrow from BLT2 -/- mice showed little sign of disease on histological analysis, while BLT2-/- mice receiving wild-type bone marrow showed intense inflammation leading to severe destruction in cartilage and bone. When BLT1/BLT2 double deficient mice were transplanted with a mixture of BLT1 deficient and BLT2 deficient bone marrow, a gain of clinical disease was observed. All these data demonstrate that BLT2 has a critical role in arthritis and it is non-redundant with the role played by BLT1. Further study is needed to determine the function of BLT2 and in which cell types it is important.

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CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

A. Biosynthesis of Leukotrienes

Leukotrienes have been linked to inflammation for decades. The importance of these mediators was realized in 1980 when the slow-reacting substance of anaphylaxis was discovered to consist predominantly of LTC_4 with a lower content of LTD_4 and LTE_4 (1). In the same year, it was established that LTB_4 serves as a potent chemoattractant for neutrophils (2). LTB_4 is a derivative of arachidonic acid (AA), found in cell membranes. Upon activation of a cell, cytosolic phospholipase A2 releases AA from the membrane where it is acted upon by 5-lipoxygenase (5-LO) in concert with 5-lipoxygenase activating protein (FLAP) to produce leukotriene A_4 (LTA_4). LTA_4 can either be converted to leukotriene C_4 through the addition of glutathione (GSH) by LTC_4 synthase, or alternatively, can be converted to LTB_4 by LTA_4 hydrolase (LTA_4H). A scheme of leukotriene biosynthesis is shown in Figure 1. LTA_4 is exclusively made by leukocytes and is exported to endothelial cells to be converted into LTB_4 or LTC_4 where LTA_4 hydrolase and LTC_4 synthase are present, which is an interesting phenomenon termed transcellular synthesis(3).

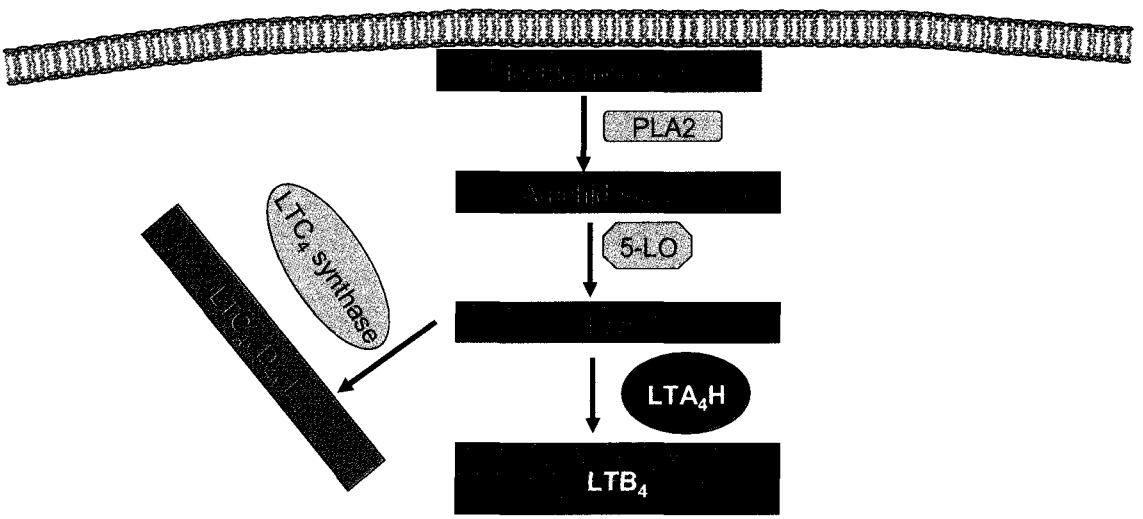
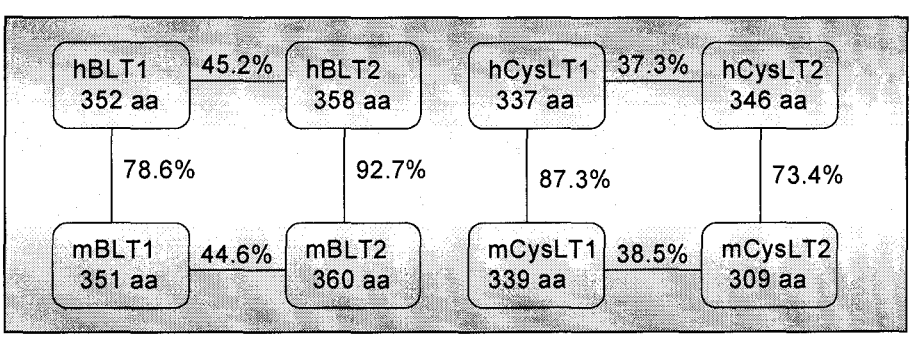
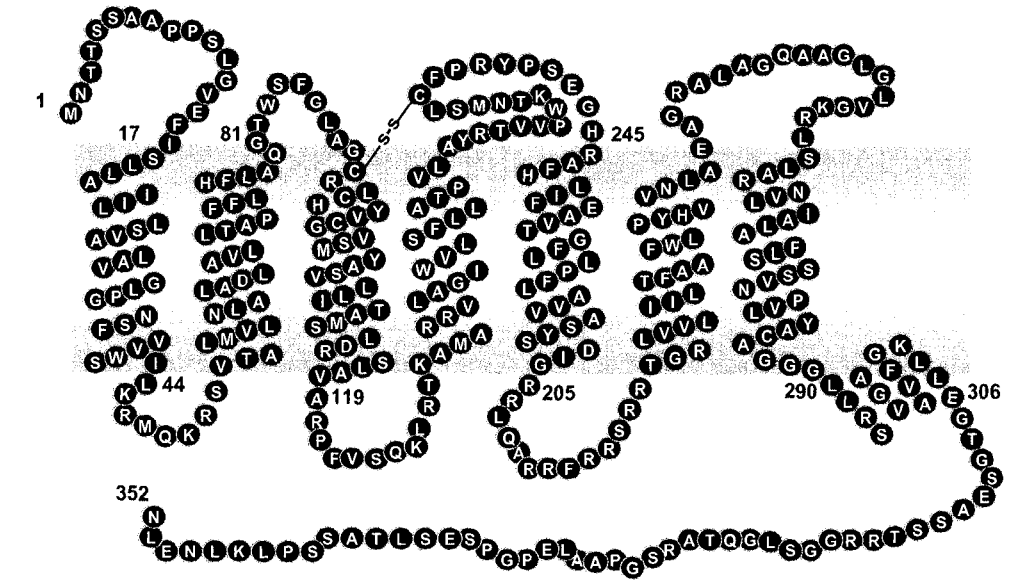


Figure 1: The Arachidonic Acid Pathway. Arachidonic acid is synthesized from phospholipids by phospholipase A2 (PLA2). The conversion of arachidonic acid to leukotriene A_4 (LTA_4) by the enzyme 5-lipoxygenase is the first commited step in leukotriene biosynthesis. LTA_4 is an unstable molecule and is hydrolyzed by LTA_4 hydrolase (LTA_4H) to form Leukotriene B_4 (LTB_4). Alternatively, LTA_4 can be converted to LTC_4 through the addition of glutathione. LTD_4 and LTE_4 are the metabolites of LTC_4.

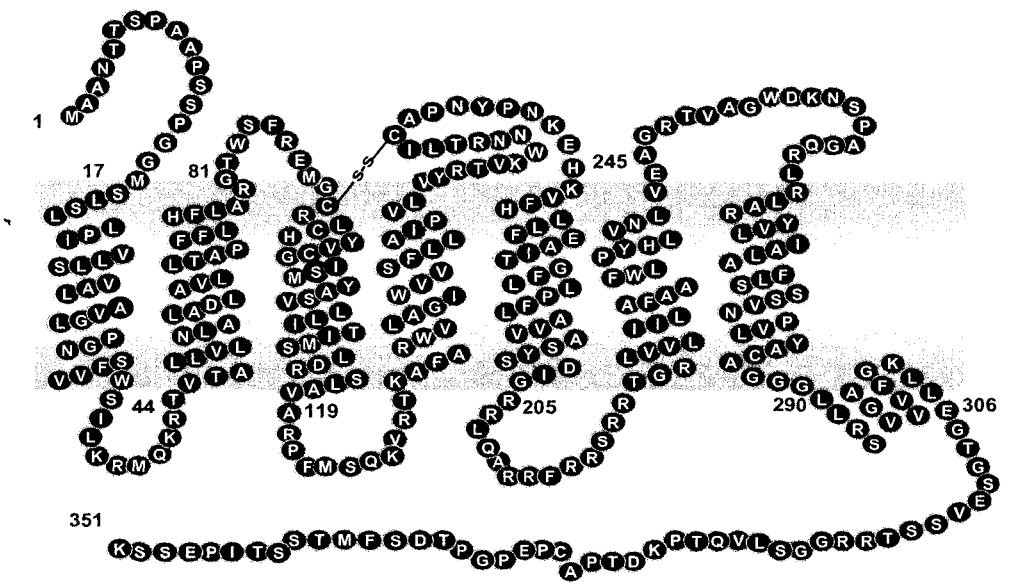
B. Leukotriene Receptors:

Though the role of leukotrienes in inflammatory diseases was known long ago, it took considerable time to clone the receptors for these important mediators. There are two known receptors for LTC_4, (CysLT1 and CysLT2), and for LTB_4 (the high-affinity receptor, BLT1 and the low-affinity receptor, BLT2). The human CysLT1 receptor was cloned in 1999 (4) followed by the cloning of the human CysLT2 receptor in 2000 (5). Human BLT1 was the first to be cloned in 1997 (3), followed quickly by the murine BLT1 in 1998 (6). During the analysis of the promoter region of BLT1, Yokomizo et al. identified an open reading frame (ORF) upstream of the BLT1 gene. This ORF was subsequently identified by this and three other groups as coding for a low-affinity receptor of LTB_4 and termed BLT2 (7-10). The homology of these receptors is shown in Box 1 (11). BLT2 is the most conserved receptor in mice and human. The deduced amino acid sequences of the BLTs are shown in Figure 2 and Figure 3 as a snake diagram depicting extracellular loops, transmembrane regions and intracellular loops. We identified the transmembrane regions of hBLT1 and hBLT2 by molecular dynamic model simulation methods (12). The transmembrane regions of murine BLTs are predicted from hBLTs.



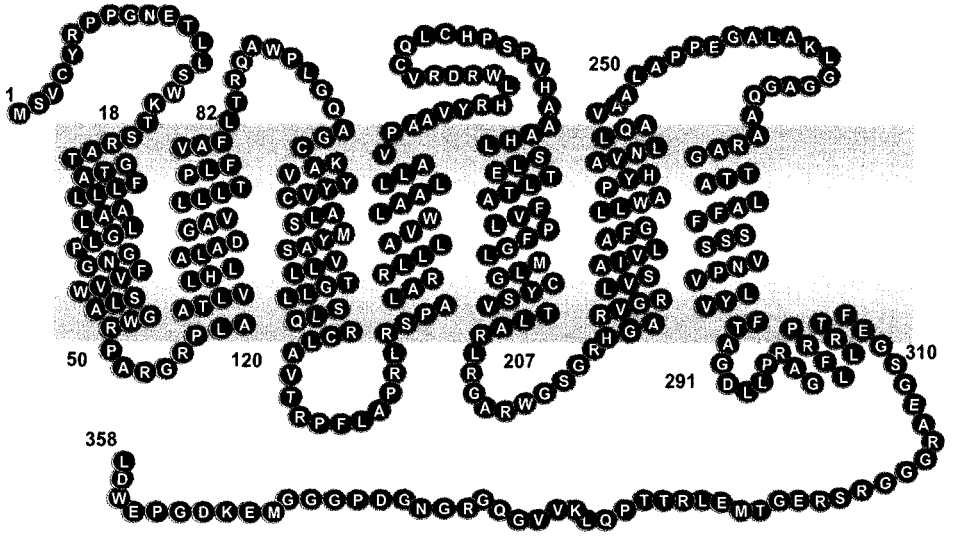


hBLT1

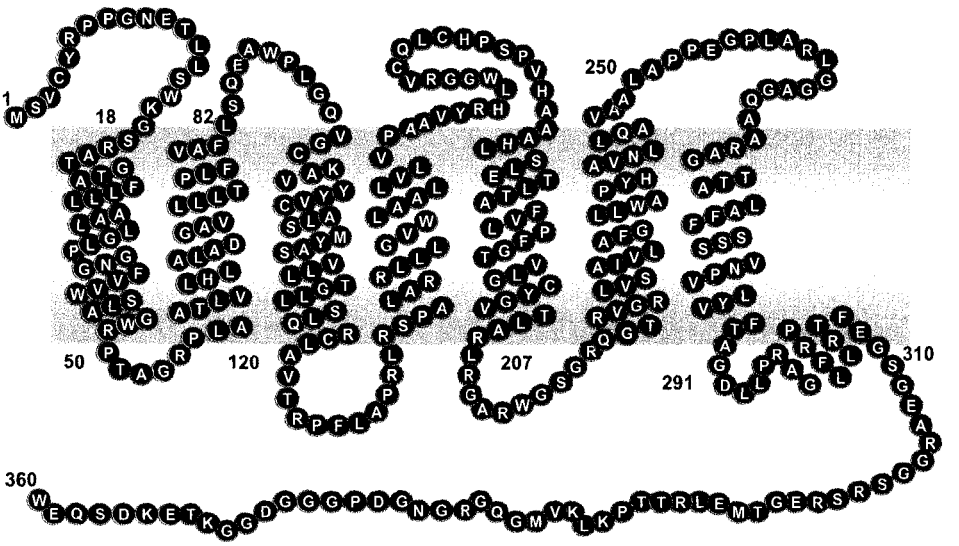


mBLT1

Figure 2: Snake Diagram of Human and Murine BLT1. Snake diagram (2D) are shown for the human BLT1 (top) and murine BLT1 (bottom). The approximate lengths of both the membrane-spanning regions and the extracellular loops were determined using molecular dynamics simulations of BLT1 (Basu et al). Human BLT1 was used as an amino acid template to make murine BLT1. The amino acids that remain the same remain labeled in white text with a black background, while the amino acids that are different are labeled in white text with a red background.



hBLT2



mBLT2

Figure 3: Snake Diagram of Human and Murine BLT2. Snake diagram (2D) shown of the human BLT2 (top) and murine BLT2 (bottom). The approximate lengths of both the membrane-spanning regions and the extracellular loops were determined using molecular dynamics simulations of BLT2. Human BLT2 was used as an amino acid template to generate murine BLT2. The amino acids that remain the same remain labeled in white text with a black background, while the amino acids that are different are labeled in yellow text with a purple background.

C. Expression and Function of BLT1 and BLT2:

Although BLT1 was long known to be a neutrophil chemoattractant receptor, recent studies identified BLT1 expression on macrophages (13), smooth muscle cells (14), endothelial cells (15) , activated T cells (16), mast cells (17), and dendritic cells (18), considerably expanding the potential role of LTB_4. BLT2 has been shown to be expressed widely in humans, with the spleen and peripheral blood leukocytes showing the highest levels of expression (10), while the expression of BLT2 in the mouse has been difficult to ascertain with several laboratories reporting variable results. Iizuka et al. demonstrated expression of murine BLT2 as being highest in the small intestine and lower in the skin by Northern blot. Using in situ hybridization, they were also able to demonstrate the presence of BLT2 in the skin. Upon performing RT-PCR based analysis, they also found BLT2 in the colon and spleen at low levels (19). Another laboratory confirmed the expression of BLT2 in the spleen, but was unable to detect any BLT2 signal in the skin (20). Using RT-PCR, expression of BLT2 was demonstrated in murine mast cells (17). In similar studies, our laboratory as well as another group investigating BLT2 expression were unable to detect BLT2 expression in any of the cell types or tissues tested (21, 22). The reasons for these differences are unclear and must be resolved. Results in this thesis show expression of BLT2 in cells derived from the bone marrow.

The regulation patterns of BLT1 and BLT2 remain elusive. Recently, dexamethasone has been shown to regulate the expression of BLT1 in a number of cell types including human neutrophils, monocytes and T-cells (23, 24). Stankova et al. demonstrated that dexamethasone up-regulates BLT1 in human neutrophils in a time and

concentration dependent manner (24). Dexamethasone and IL-10 were shown to up-regulate BLT1 expression in human monocytes, while IFNγ, TNFα and LPS down-regulated BLT1 (23). Data demonstrating up-regulation of BLT1 on CD8+ T effector cells will be shown in Chapter II. Low levels of BLT1 were discovered in resident peritoneal macrophages in rats (25). However, macrophages elicited by proteosepeptone showed an increase in the level of BLT1 expression in rats. LPS was demonstrated to up-regulate BLT1 in human umbilical cord vascular endothelial cells, while TNFα up-regulated BLT2 (15). BLT1 expression was also up-regulated in smooth muscle cells upon treatment with IL-1β or LPS. When a plasmid over-expressing IKKβ, an inhibitory molecule for NFκB, was used in this assay, the BLT1 up-regulation was blocked, suggesting a requirement of the NF-κB pathway in BLT1 regulation (14). Further research is required to determine how the LTB_4 receptors are regulated using both *in vivo* and *in vitro* models.

Part of the regulation of BLT1 is due to desensitization by phosphorylation of the receptors and β-arrestin dependent internalization. Conflicting results about the importance of phosphorylation in internalization of the receptors have been published (26, 27). LTB_4-induced internalization of BLT1 was demonstrated to occur in RBL-2H3 cells that express high endogenous levels of GPCR kinase 2 (GRK2), while internalization of BLT1 did not occur in COS-7 cells or HEK-293 cells which do not over-express GRK2. BLT1 internalization was blocked in RBL-2H3 cells when treated with a dominant negative of GRK2. Internalization could be induced in HEK-293 cells by co-expressing with wild-type GRK2. All this data demonstrated that phosphorylation of BLT1 is absolutely required for internalization of the receptor. They went on to

demonstrate that BLT1 did not interact with arrestin either by fluorescence microscopy or by coimmunoprecipitation. Finally, this group demonstrated that the C-tail of BLT1 was required for both GRK2 phosphorylation and internalization(26). In contrast, Jala et al. from our laboratory demonstrated that internalization of BLT1 is phosphorylation independent and β-arrestin dependent (27). BLT1 mutants, which lack phosphorylation sites in the C-tail (14 Ser/Thr are mutated to Ala) internalize through β-arrestin. This mutant showed higher ligand-induced intracellular calcium release indicating a role for receptor phosphorylation in desensitization process. Functional importance of the internalization of BLT1 was demonstrated by Gaudreault et al (28). They showed that the internalization and activation of BLT1 occur through Yes kinase, which was found to be crucial for LTB_4 induced degranulation of neutrophils.

Though BLT1 and BLT2 are GPCRs and bind the same ligand, some differences in G-protein specificity exist between these receptors. In addition, the usage of G-Proteins varies in different cell types. The chemotaxis of CHO cells expressing either human BLT1 or human BLT2 was normal towards LTB_4. The chemotaxis was completely abolished in cells treated with *Bordetella pertussis* toxin (PTX), which blocks $G\alpha_{i/0}$- dependent signaling. LTB_4-induced intracellular calcium release was only partially blocked when cells were treated with PTX, indicating that calcium response was mediated by both PTX-sensitive and PTX-insensitive G proteins in CHO cells (3, 10). In contrast, LTB_4-induced intracellular calcium release in human neutrophils was largely sensitive to PTX treatment (29). The three intracellular loops of BLT1 were shown to be important for the binding of $G\alpha_i$ and G16 proteins in cells over-expressing BLT1 along

with either $G\alpha_i$ or G16 proteins in COS-7 cell lines. A short region in ICL3 was shown to be critical in for the recognition of $G\alpha_i$ protein (30).

In functional assays, BLT2 was shown to be about 100-fold less sensitive to LTB_4 than BLT1. Of interest is the finding that human BLT2 also can bind and display intracellular calcium release to 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE), 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), and 12(S)-hydroperoxyeicosotetraenoic acid (12(S)-HPETE), while BLT1 is very specific for LTB_4 (10) demonstrating that though both are considered LTB_4 receptors, there are possible other functions of BLT2. These two receptors may also be distinguished based on the pharmacological specificity of the antagonists. Most of the antagonists were developed based on inhibition of BLT1 function and, thus, are specific for this receptor. While a few BLT2 antagonists are identified, dual inhibitors are not well characterized at this time. CP105,696 and U75302 are both specific for BLT1, while LY255283 is specific for BLT2 (10, 31).

LTB_4 mediated signal transduction pathways via BLT1 such as chemotaxis (3), degranulation (32), adhesion (33) and prolongation of the cell survival (34) were functions previously attributed to BLT1 in neutrophils. Recent data has started to delineate new roles for BLT1 in neutrophils and in other cell types. For example, smooth muscle cells have been shown to migrate and proliferate in response to LTB_4 through BLT1 (14). Our laboratory has shown the presence of mRNA and LTB_4 mediated chemotaxis via BLT1 in immortalized murine macrophages (35).

The expression of both BLT1 and BLT2 was reported recently in human and murine mast cells (17). This group showed the presence of mRNA for both receptors in murine bone marrow-mast cells (mBMMC) and in human mast cells (hMC). They also

demonstrated LTB_4 induced chemotaxis of these cells. The chemotaxis was blocked by either BLT1 or BLT2 specific inhibitors. It is interesting to note that mBMMCs chemotaxed towards 12-(S)-hydroxyeicosotetraenoic acid, which is a BLT2-specific agonist. The data clearly suggested that both receptors are functional in mast cells. Another new cell type that the LTB_4 receptors have been described in is endothelial cells. BLT1 and BLT2 were shown to be expressed in human umbilical vein endothelial cells (HUVEC) at low levels. Up-regulation of BLT1 (mRNA and protein levels) were seen in vitro when HUVEC cells were treated with LPS, while treatment with TNFα led to an increase in BLT2 mRNA (15). Expression on subsets of T cells has also been demonstrated. It was shown that activating naïve T cells in vitro with anti-CD3 caused up-regulation of BLT1 in Th0, Th1 and Th2 cells and these cells demonstrated chemotaxis toward LTB_4, suggesting a functional role of BLTs in activated T cells. This response was not seen in the mice lacking BLT1 (16) highlighting the fact that LTB_4 mediated signaling occurs via BLT1.

Recent experiments from our laboratory have demonstrated functional expression of BLT1 on both mature and immature dendritic cells (18). It was shown that exposure of immature dendritic cells (iDC) and mature dendritic cells (mDC) to LTB_4 greatly enhanced chemotaxis to the CCR7 ligands CCL19 and CCL21 suggesting a priming effect for LTB_4. In vivo, it was shown that mice in lacking both BLT1 and BLT2 (BLT1/2 KO) mDCs had a deficiency in trafficking to the popliteal lymph nodes when compared to wild-type mDCs at 20 and 48 hours. In contact hypersensitivity (CHS) experiments, it was seen that BLT1/2 KO DCs very poorly initiated a CHS response

when compared to wild-type DCs. These data together show that BLT1 has a direct effect in the control of adaptive immune responses.

D. Role of LTB_4 and its receptors in various inflammatory diseases:

We now have a better understanding of expression patterns of BLT1 and BLT2; however the role of LTB_4 in disease processes is yet to be fully uncovered. LTB_4 is up-regulated in many human diseases and can be found in atherosclerotic plaques (36, 37), rheumatoid arthritis synovium (38), and in exhaled breath condensates in asthma patients (39). Further illustrating the importance of LTB_4 in human disease, recent population studies have shown that certain LTA_4H and FLAP polymorphisms in humans predispose individuals to increased risk of myocardial infarction and stroke (40, 41). This means that regulation of LTB_4 biosynthesis is directly attributable to this increased risk. When samples from chronic obstructive pulmonary disease patients were analyzed, it was found that BLT1 was very highly expressed and could be found in macrophages, CD8+ T cells and neutrophils potentially implicating all three cell types in the disease process (42).

a) Atherosclerosis:

Mouse models of human disease have proven useful in delineating the role of BLT1 in inflammatory diseases. Of these, models of atherosclerosis have demonstrated a direct role of BLT1 in disease progression. Mice deficient in 5-LO were shown to have a 26-fold decrease in lesion development in an atherosclerosis model. In these studies, it was shown that macrophages in the plaques abundantly expressed 5-LO (43). 5-LO deficiency was also shown to protect mice from aortic aneurysm (44). Models utilizing a BLT1 antagonist, CP-105,696, in Ldl -/- and ApoE -/- mice showed a reduced

accumulation of CD11b+ cells in the lesions and limited lesion size (45). Our laboratory was the first to demonstrate that the loss of BLT1 protects mice from the development of atherosclerotic disease. We found a significant decrease in plaque size in the early stages of disease. The plaque analysis of BLT1-/- mice revealed a reduction in macrophage accumulation compared to plaques of BLT1+/+ mice. It was also shown in the same study that LTB_4 up-regulated monocyte chemotactic protein-1, CD36, urokinase-type plasminogen activator, colony stimulating factor-1, and osteopontin (35), all of which already have an established roles in atherogenesis. Up-regulation of MCP-1 by LTB_4 was confirmed in human monocytes (46). This demonstrates that the LTB_4-BLT1 axis provides an amplification loop to attract monocytes and macrophages to the site of plaque formation and also leads to the destabilization of the plaque. Another cell type important in the disease is the smooth muscle cells. The expression of BLT1 on smooth muscle cells as stated before was shown and allowed for the chemotaxis of smooth muscle cells towards LTB_4. This paper demonstrated loss of BLT1 led to a decrease in the thickness of smooth muscle cells in the plaque as well as T cells and macrophages (14). In addition to being a key molecule in plaque progression, recent data have demonstrated that symptomatic plaques express high levels of LTB_4 which correlates with plaque destabilization (47) a phenomenon that was confirmed when it was found that expression of 5-LO and LTA_4H in plaques led to their destabilization (48). Population studies recently have shown that polymorphisms in LTA4H and FLAP predispose the individual to increased risk of myocardial infarct (40, 41).

b) Asthma:

A vast amount of research involving LTB_4 in asthma has been performed owing to the fact that high levels of LTB_4 are present in the airways of asthmatic patients (39). In 2003, the discovery that CD8+ effector T cells express BLT1 was made (49). It was found that CD8+ effector T cells were able to chemotax towards LTB_4, while naïve or central memory T cells were unable. It was also shown that CD4+ Th1 and Th2, but not Th0 T cells expressed BLT1 as they left the lymphoid compartment and entered into tissue (16). It was found in collaboration with our laboratory, that BLT1-/- mice have decreased airway hyper-responsiveness in an ovalbumin-induced airway hyper-responsiveness (AHR) model (50). Reconstitution of allergen sensitized BLT1+/+ T cells fully restored AHR in BLT1-/- mice suggesting a role of BLT1 in AHR. A reduction in goblet cell hyperplasia and interleukin-13 was also seen in BLT1-/- mice in that study. This group performed another model of AHR which is mast cell-dependent. In these studies, mice were sensitized with anti-ovalbumin (OVA) IgE and then challenged with OVA in the airways. This model requires mast cells for the sensitization from IgE and CD8+ cells for the reactive phase of the model. Wild-type mice develop altered airway function, while CD8 -/- mice do not. CD8 -/- mice reconstituted with CD8+ T cells develop AHR, while CD8 -/- mice reconstituted with BLT1-/- CD8+ T cells do not develop AHR (51). This result was confirmed by another laboratory who saw loss of AHR in another strain of mice deficient in BLT1 (52). It was found that only small populations of T cells express BLT1 in healthy individuals. The population increases with Epstein - Barr virus infection and in asymptomatic allergy. The increase in BLT1 expressing T cells was seen only in the airway and not in the blood (53). A

similar result was seen in asthmatic patients where BLT1 expressing CD8+ T cells were found in broncheo-alveolar lavage and in lung tissue, while no BLT1 expressing CD8+ T cells were found in healthy volunteers (54). These results suggest that BLT1 might be a useful target in asthma.

c) Other Inflammatory Diseases:

LTB_4 has also been implicated in many other inflammatory diseases such as inflammatory arthritis, a major focus of studies in this thesis (Chapter III). In addition, LTB_4 was also shown to be important in experimental autoimmune encephalomyelitis (EAE) and peritonitis. It was found that the eosinophil recruitment in EAE is dependent on LTB_4 receptor ligation and further revealed a previously unrecognized role for eosinophils in the pathogenesis of this disease (55). In a mouse model of peritonitis, LTB_4 and BLT1 were found to play important roles in the recruitment and/or retention of leukocytes, particularly eosinophils (56). Using BLT1 deficient mice developed in our laboratory, Liao et al. demonstrated an important role for LTB_4 and BLT1 in auto-immune uveitis (57). Adoptive transfer experiments showed that expression of BLT1 is important on both auto-reactive T cells as well as on innate immune effector cells

The major focus of our laboratory for the last 10 years has been to develop a complete understanding of the structure/function, regulation and biological activities of the LTB_4 receptors. These studies used cell and molecular biological approaches as well as mice deficient in the LTB_4 receptors BLT1 and BLT2 (12, 21, 27, 35, 58-60). The work described in this thesis attempts to further our knowledge of the biology of LTB_4 receptors by developing highly specific monoclonal antibodies for BLT1 (Chapter II) and demonstrating the functional significance of BLT2 (Chapter III).

CHAPTER II

GENERATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST HUMAN AND MURINE BLT1

INTRODUCTION

Evolving technologies have allowed our laboratory and others to demonstrate the presence of BLT1 on cell types that had not previously been known to express BLT1 in the last few years (13-18). We have developed monoclonal antibodies to BLT1, which allowed us to probe for the expression of BLT1 and if any regulation patterns exist. Chapter II will focus on the generation and characterization of these newly described monoclonal antibodies.

The term monoclonal antibody simply means an antibody that has a defined specificity because it arose from one original plasma cell. To produce a monoclonal antibody, a vertebrate animal is immunized with the molecule of interest either purified, conjugated to a carrier protein such as keyhole limpet hemocyanin (KLH), or on cells expressing the molecule. Multiple rounds of immunization lead to the selection and expansion of B cells that recognize the molecule in the spleen or lymph nodes. These

tissues can be taken and the cells extracted and fused with a myeloma cell line using polyethylene glycol (PEG). When grown in culture, the unfused plasma cells have a limited life-time and die. The myeloma cells used are unique in that they lack the hypoxanthine-guanine-phosphoribosyltransferase (HGPRT) gene, which allows them to make purines using hypoxanthine. In medium containing hypoxanthine, aminopterin and thymidine (HAT), unfused myeloma cells do not survive due to the loss of the HGPRT gene. A hybridoma fusion between a plasma cell and a myeloma cell will be immortal and will contain a functional HGPRT gene, therefore, only these fused cells will survive. These cells are diluted so that only one fusion will give rise to a population of cells which make a monoclonal antibody. The resultant monoclonal antibodies are then screened against the antigen by enzyme-linked immunosorbent assays (ELISA) or similar methods or, as in this assay, if the antigen is on a cell surface, flow cytometry can be used.

Monoclonal antibodies are valuable and unique tools to detect and block the antigen functions in biological systems. In research, monoclonal antibodies are used for Western blotting, immunofluorescence, immunohistochemistry, FACS, immunoprecipitation, affinity chromatography and ELISA. Many ELISA kits are available utilizing monoclonal antibodies such as for drug screening and pregnancy tests. Monoclonal antibodies also have gained acceptance as treatments in human disease such as cancer and rheumatoid arthritis and in organ transplantation. In the treatment of cancer, there are two main types of monoclonal antibodies in use. The first are the naked antibodies, which are not conjugated to another molecule. Some naked antibodies such as Rituxan (61) and Campath (62) bind to the surface target and induce the host immune

system to destroy the tumor. Other naked antibodies such as Herceptin (63), Erbitux (64) and Avastin (65) are used to block surface proteins that are necessary for tumor cell survival or growth. The other group of antibodies is the conjugated antibodies which are attached to drugs, toxins or radioactive substances. Only the radiolabeled antibodies Zevalin (66) and Bexxar (67) are currently approved for use in the clinic at this time; the others are still in clinical trials. OKT3, an antibody against the T3 antigen on T cells, is used to combat organ rejection in patients who have received an organ transplant (68). Remicade (69), Humira (70), and Rituxan (71) are currently being used to treat rheumatoid arthritis. Remicade and Humira are both antibodies against TNFα and have been effective in combination with methotrexate in halting the progression of RA. Rituxan targets B cells and has also shown promise in treating RA.

Given all the disease processes mediated by LTB_4 and its receptors, monoclonal antibodies against BLT1 might prove to be useful in treatment of inflammatory diseases. Although monoclonal antibodies have been available for human BLT1 for several years (72, 73), anti-BLT1 antibodies that recognize BLT1 in small animals would be useful to allow studies in mouse models of disease. Due to the potential usefulness of monoclonal antibodies that recognize a common epitope between hBLT1 and mBLT1, we initiated experiments to produce these antibodies utilizing our BLT1-/- mice. The technique that was used had been performed successfully to produce L-selectin antibodies (74) which involves the use of 300.19 mouse pre-B cells. The parental 300.19 cells were transfected with HA-tagged hBLT1 with an expectation that since hBLT1 and mBLT1 are highly homologous, we would get antibodies that were cross-reactive for both. Unfortunately,

immunization with 300.19 cells expressing HA-tagged hBLT1 resulted in the isolation of over 15 antibodies, all of which turned out to be specific for the HA-tag.

A new strategy was adopted, where red fluorescence protein (RFP) was fused at the c-terminal end of BLT1 without HA-tag. The hBLT1-RFP localized to membrane suggesting fused RFP did not alter the folding of BLT1. The expression of hBLT1-RFP was measured by the fluorescence of RFP. The cells expressing stable hBLT1-RFP clonal lines were selected and experiments were repeated as described above. This resulted in the production of over 30 monoclonal antibodies which all turned out to be specific for hBLT1 and showing no reactivity against mBLT1. Therefore, mBLT1 fused to tetramer RFP was expressed and put through the same protocol and this led to the production of 8 mBLT1 antibodies. These antibodies have allowed us to demonstrate the expression of BLT1 on human neutrophils and have allowed us to determine the expression pattern of BLT1 in different mouse tissues. Out of these results came new data demonstrating differential expression of BLT1 on macrophages depending on their activation status and tissue localization.

MATERIALS AND METHODS

Materials

CD3-FITC, B220-PE, NK1.1-PE, GR-1-PE, Siglec-F-PE, CD45.1-PE, CD45.2-FITC, CD11b-FITC, CD16-PE and Streptavidin-conjugated APC were all from BD Pharmingen. GR-1-FITC was from Ebiosciences.

Generation of 300.19 Cells Expressing Murine and Human BLT1

300.19 cells were maintained as a suspension culture in RPMI-1640 medium containing 2 mM L-glutamine, 10% FBS, 1 X β-mercaptoethanol, and 1% penicillin/streptomycin. Transfections were done by electroporation. 20 μg of pDSRED-N1 encoding human BLT1 or mouse BLT1 was electroporated into the cells and cells were sorted by flow cytometric sorting. Single cell clones were made by limiting dilution and the expression of functional receptor was tested by calcium flux in response to LTB_4.

Immunization Protocol for 300.19 cells Expressing BLT1

300.19 cells expressing hBLT1 or mBLT1 were harvested at 1500 rpm for 3 minutes and the cell pellet washed three times with PBS. 50 million cells were resuspended in 1 mL of PBS and injected s.c. on the back of BLT1-/- mice on the Balb/c background. Within a week a large tumor grew at the site of injection and this tumor resolved by two weeks. On day 14, another injection of 50 million cells was given i.p. as a booster and again on day 21. On day 23, a sample of blood was taken and the plasma isolated. The plasma was used to stain 300.19 parental cells and 300.19 cells expressing

BLT1 to check for specific staining for BLT1 over background staining of the parental cells. On day 24, the mice were sacrificed for hybridoma production.

Hybridoma Production

Mice were sacrificed and their spleens were removed. The spleens were placed in a culture dish with 10 mL cDMEM-0 (1% Nonessential Amino Acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 5000 units pen/strep) and wire mesh size 60 (Sigma-Aldrich). The spleens were teased with two 21 gauge needles. The debris was removed by pipetting through the mesh filter several times. The medium was transferred into 50 mL Falcon tubes and the volume was increased to 45 mL with cDMEM. 0.3 mL of FBS was layered under the medium to allow any clumps to settle out for about 3 minutes. The medium was carefully pipetted off from the FBS and placed in a new clean 50 mL tube and spun at 1500 rpm for 5 minutes. The supernatant was discarded and 45 mL of cDMEM-0 was added to resuspend the cells. SP2/0 myeloma cells were taken and placed in several 50 mL tubes and both SP2 and spleen cells were spun at 1500 rpm for 5 minutes. The supernatants were discarded and the spleen cells and SP2/0 were washed twice. The SP2/0 and spleen cells were resuspended separately in 10 mL cDMEM-0 and enumerated. The SP2/0 and spleen cells were mixed at a 1:1 ratio in a 50 mL tube and the tube was filled with cDMEM-0 and then spun at 1500 rpm for 5 minutes. The supernatant was aspirated off and 1 mL of 50% polyethylene glycol (PEG) was added slowly to the pellet over 1 minute drop-wise stirring after each drop. Then, another 1 mL of prewarmed cDMEM was added to the pellet over 1 minute drop-wise stirring after each drop. The addition of medium was repeated once more, and then 7 mL of cDMEM

was added drop-wise over 2-3 minutes. The cells were spun at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in the volume of cDMEM-20/HEPES (10mM HEPES) to give a concentration of 2.5 X 10^6 cells/mL. 100 μL of medium was added to each well of a 96-well plate. On day 2 of the experiment, 100 μL of DMEM-20/HEPES/HAT (1 X HAT) was added to each well. On days 3, 4, 5, 7, 9 and 11, half the volume in the well was removed and another 100 μL of DMEM-20/HEPES/HAT was added to each well. On day 14, half the medium was removed from each well and 100 μL of DMEM-20/HEPES/HT was added. Starting on day 15, any media changes were done with DMEM-20/HEPES.

Screening of Hybridomas

Hybridomas were allowed to grow until they reached approximately 50% confluence. A sample of 10 μL from the supernatant was removed and incubated with a mixture of 5 X 10^5 parental 300.19 cells and 5 X 10^5 300.19 cells expressing HA-tagged hBLT1 in 500 μL of PBS + 1% BSA and incubated for 1 h at 4°C. The cells were washed with PBS + 0.1% BSA and resuspended in 200 μL of PBS + 1% BSA containing 1 μL of goat anti-mouse IgG (Jackson Immunoresearch) and incubated for 45 m at 4°C. The cells were washed once with PBS + 0.1% BSA, resuspended in buffer and analyzed by flow cytometry. Cells were acquired on FACSCalibur (BD Biosciences) and subsequent analysis was done with FlowJo version 4.3 (Treestar, Inc.).

Monoclonal Antibody Purification and Biotinylation

Hybridomas were grown in RPMI-1640 medium containing 2% or less of FBS and grown to a high concentration for high level production of antibody. 500 mL of this medium was resuspended in 50% ammonium sulphate to precipitate the antibody. The antibody was resuspended in 1 X PBS and dialyzed to remove the ammonium sulphate and HPLC was performed on a Mono Q HR column (GE Healthcare). Peak protein fractions were taken and used to stain BLT1 expressing cells to check antibody activity. Those fractions that had the highest amount of activity were taken and biotinylated using the EZ-link sulfo-NHS-LC-biotin kit (Pierce).

Random Chimeragenesis

Random chimeras between human and mouse BLT1 were generated following a protocol used previously to make chimeras of cAMP receptors (75). The plasmid pCDNA3.1+hmB1 was used to produce the hBLT1 and mBLT1 chimeras. The sequence for hemagglutinin-tagged hBLT1 was cloned between the Nhe I and Hind III sites of pCDNA3.1+. The sequence for mBLT1 was then cloned between the EcoR I and Not I sites of the plasmid containing hBLT1. After the chimeragenesis procedure, the chimeric receptor was flanked by Nhe I on the 5' end and Not I on the 3' end. The pcDNA3.1+ vector was linearized by double digestion with EcoR I and Hind III restriction enzymes, which were located between both the genes. After testing by gel electrophoresis whether digestion was complete, the enzymes were inactivated at 65 °C for 30 minutes. The digested DNA was gel-purified. The digested DNA (750 ng) was used to transform *E. coli* strains DH5α and TOP10 (Invitrogen) competent cells by heat shock and plated on

LB plates containing ampicillin. 16 colonies were taken from the DH5α plate and 16 colonies from the TOP10 plate and analyzed by restriction analysis to check for a 1 kb insert. 28 of these clones were determined to contain the correct size insert and were digested separately with 3 restriction endonucleases; Xho I which is found at 243 bases in mBLT1, Kpn I which is found at 517 bases in hBLT1, and EcoN I which is found at 728 bases in hBLT1. This analysis allowed us to break down the clones into 4 quadrants 1-4 where crossover occurred. 9 clones were located in quadrant 1, 6 in quadrant 2, 11 in quadrant 3, and 6 in quadrant 4. The clones were confirmed by sequencing using primers on either side of the multiple cloning site of the vector. 3 to 4 clones were sequenced from each quadrant leading to the identification of 9 unique sites of crossover.

Staining of Human Neutrophils

Human volunteer blood was collected by venapuncture in 60 mL syringes containing 5 mL of 3.8% w/v sodium citrate anticoagulant. An equal volume of 3% Dextran T-500 in PBS was mixed and red blood cells were allowed to sediment for 30 minutes at room temperature. The leukocyte-rich plasma was aspirated above the red cells with a syringe with tubing attached. The plasma was distributed in 15 mL volumes into 50-mL disposable tubes and then under-laid with 10 mL of 1.077 g/mL density Ficoll-Hypaque solution using a 20 mL syringe with tubing on the end. A layer of 1.105 g/mL Ficoll-Hypaque was then placed under the 1.077g/mL Ficoll layer. The tubes were centrifuged for 30 minutes at 1000 g. Each tube contained a red cell pellet and two white interface layers. The lower interface was taken which contained > 98% neutrophils. The neutrophils were pooled into one tube to about 20 mL and 30 mL of PBS was added. The

tubes were mixed and centrifuged at 1200 rpm for 12 minutes. The supernatant was decanted and the tube was blotted on a paper towel leaving the white cell pellet. The pellet was washed twice with PBS and centrifuged at 1000 rpm for 10 minutes. The cells were resuspended in PBS and were counted. 500,000 cells were placed in several FACS tubes and the cells were washed with PBS + 0.1% BSA. The cells were then resuspended in 0.5 mL of PBS + 1% BSA. 10 μL of several culture supernatants from positive hybridomas was added to separate tubes. The remaining procedures were done as above.

Murine Antibody Screening of Primary Cells and Cultured CD8+ T cells

Flow cytometry was performed using four samples from mice; blood, bone marrow, spleen and lymph nodes. Mice were euthanized and the skin on the abdomen and thoracic area were opened up to enable blood collection by cardiac puncture into tubes containing heparin (MP Biomedicals). The entire ventral surface from under the jaw of the mouse down to the base of the hind-limbs had the skin laid open to reveal the lymph nodes. The superficial cervical, axillary, brachial and inguinal lymph nodes and spleen were removed and the leukocytes were isolated. Bone marrow was isolated from the femurs and tibias and leukocytes were isolated. These samples were resuspended in 100 μL of PBS + 1% BSA containing 1 μL of biotinylated 3D7 and a number of conjugated cell surface markers. The tubes were then incubated for 1h at 4˚C and washed with PBS + 0.1% BSA. The cells were resuspended in 200 μL on PBS + 1% BSA containing 1 μl of streptavidin-conjugated APC and incubated for 45m at 4˚C. 2 mL of BD FACS Lysing Solution was added to blood samples and 1 mL added to the remaining samples and incubated at room temperature for 1h. The cells were washed, resuspended

and analyzed by flow cytometry. This same procedure was used to stain CD8+ effector and central memory T cells.

Dexamethasone Up-regulation of BLT1 in CD8+ T cells

Mononuclear cells collected from lymph nodes and spleen of OT-1 TCR transgenic mice were stimulated with 1 μg/mL of SIINFEKL peptide for 90 min. After washing, cells were cultured for 2 days in complete media (RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-ME, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 10 mM HEPES). Cells were then washed and cultured in complete media containing 20 ng/mL of either recombinant murine IL-12 for generation of T_{EFF} cells or IL-15 for T_{CM} cells. To address the effect of DEX on memory CD8+ T cells, dexamethasone 21-phosphate disodium salt (DEX; Sigma-Aldrich) was added to the culture medium during differentiation of these cells. Medium containing cytokine and/or DEX were changed everyday. After 5 days, cultures were shown to contain > 99% αβ/CD8+ T cells. CD4+, CD11c+, NK1.1+ or γδ+ cells were <0.1%. The cells were >90% either CD8+ effector memory or central memory cell phenotype, as shown by the CD62L/CCR7 cell surface marker. T_{EFF} cells derived through this protocol showed phenotypic and functional characteristics of effector memory CD8+ T cells in vivo. Cells were used between days 5 and 8 of culture after addition of either IL-2 or IL-15.

RESULTS

Generation of hBLT1 and mBLT1 Specific Monoclonal Antibodies

300.19, a murine pre-B cell line expressing high levels of human BLT1 was used to immunize BLT1 deficient mice in BALB/c background as described in methods. Two days before sacrifice, a blood sample was taken and the plasma was analyzed for reactivity to hBLT1. This analysis is shown in Figure 4, where the red line represents parental 300.19 cells and the blue line represents 300.19 cells expressing hBLT1. As expected, the plasma from immunized mice showed reactivity against 300.19 cells. However, 300.19 cells expressing hBLT1 showed a considerable increase in mean fluorescence demonstrating specific activity against hBLT1.

Based on this observation, spleens were removed from these mice and fused with SP2/0 myeloma cells to form hybridomas as described in methods. The cells were diluted at a ratio (2.5 X 10^5/mL) so that positive wells should arise from only one hybridoma, thereby making it monoclonal. As the cell growth in a 96-well plate reached approximately 50% confluence the supernatants were taken and analyzed by flow cytometry. A simple screening method using equal amounts of parental 300.19 cells and 300.19 cells expressing HA-tagged hBLT1 was used for rapid detection of positive clones (Figure 5). By this method, we anticipated no reactivity when the supernatants contained no antibody (negative, Figure 5a) whereas a complete shift indicated a 300.19 cell specific antibody (Figure 5c) and half the cells reacting indicated the presence of a potential BLT1 specific antibody (Figure 5b). Results representative of all three scenarios are shown in Figure 5 for hBLT1.

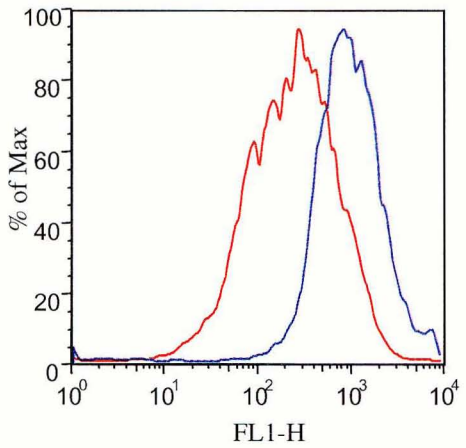


Figure 4: Plasma Screening of hBLT1 Immunized Mice. BLT1 -/- Balb/c mice were immunized with 300.19 cells expressing hBLT1 and boosted twice with the same cells. Plasma was isolated from one of these mice and was used to stain 300.19 parental cells (red line) and 300.19 cells expressing hBLT1 (blue line).

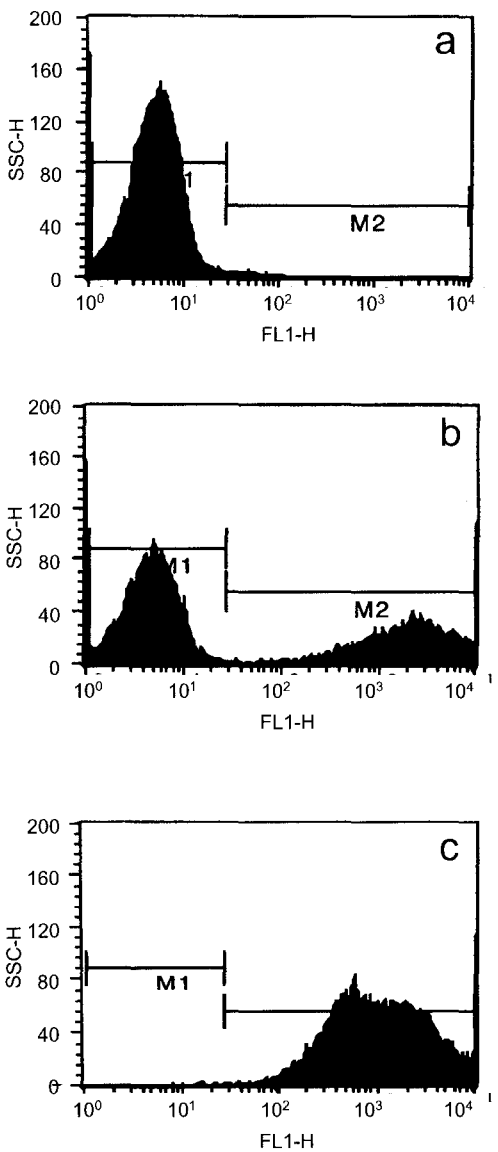


Figure 5: Screening Strategy for Detecting Hybridoma Supernatants with hBLT1-Specific Antibodies. The supernatant of hybridoma culture (10 μL) was added to an equal mixture of 300.19 parental cells and 300.19 cells expressing HA-tagged hBLT1. Goat anti-mouse IgG-FITC was used as secondary antibody. The 300.19 cells were analyzed on the FL1 gate for FITC staining. Three representative results are shown. a) Culture supernant with no detectable antibody b) Culture supernatant containing an antibody that recognized 300.19 cells. c) Parental 300.19 antibody.

Cells from individual wells that showed hBLT1 specific staining (pattern as seen in 5b) were further expanded and antibody specificity of the supernatants to hBLT1 was confirmed by staining parental 300.19 and 300.19 cells expressing hBLT1 separately. Many of these were further analyzed for reactivity against other cell lines expressing human or murine BLT1 as well as human and murine neutrophils. The results of these analyses are shown in Table 1 where the level of binding was rated as – for no binding or + to +++++ for how well they stained cells expressing the indicated forms of BLT1. As seen in Table 1, these monoclonal antibodies stained hBLT1 fused to RFP in 300.19 cells and HA-tagged hBLT1 in rat basophilic leukemia (RBL) cells. There was, however, no staining when the monoclonal antibodies were tested against 300.19 cells expressing HA-tagged mBLT1. In addition, zymosan-elicited murine neutrophils which are known to express BLT1 at high levels also showed no detectable staining.

Since monoclonal antibodies to mBLT1 would be a very useful tool to investigate the role of BLT1 in mice, the same technique as above was used with 300.19 cells expressing mBLT1. The resulting antibodies from this experiment are shown in Table 2. All these antibodies were found to be specific for mBLT1 showing no staining for cells expressing hBLT1.

Clone ID	300.19 hBLT1-RFP	RBL hBLT1	300.19 mBLT1	Murine Neutrophil	Human Neutrophil	300.19 hBLT1-DG
2A8	++++	+++	-	-	++	++++
2A8-SC2	N.D.	+++	N.D.	N.D.	N.D.	++++
5B10	+++	+	N.D.	N.D.	N.D.	+++
6H12	++++	+++	-	-	++	++++
7A5	+	+	+	N.D.	N.D.	++
7G6	+++	++	-	N.D.	N.D.	+++
7G6-SC2	++++	++	-	N.D.	N.D.	+++
8E8	++++	+	-	N.D.	N.D.	+++
8H7	+++	++	-	N.D.	N.D.	+++
9A4	+++	++	-	N.D.	N.D.	++++
9B10	+++	+	-	N.D.	N.D.	+
9E12	+++	++	-	-	N.D.	++++
9G8	++++	++	-	-	N.D.	++++
10C8	+++	++	-	N.D.	N.D.	+++
10C8-SC3	+++++	++++	-	N.D.	N.D.	+++++
11D1	++++	+++	-	N.D.	+	+++++
11G3	+	+	-	N.D.	N.D.	++
12B1	+	+	N.D.	N.D.	N.D.	+++
12F1	++	+	-	-	-	++++
13C3	+++	+	-	N.D.	N.D.	+++
13C3-SC1	++++	+++	-	N.D.	N.D.	+++++
13E2	+++	+	-	-	N.D.	++
13E2-SC8	++++	+++	-	N.D.	N.D.	+++
13F2	++++	+++	-	-	++	+++++
13F8	+	+	-	N.D.	N.D.	++
14A7	++	++	-	N.D.	N.D.	++++
14D9	+++	+	-	-	-	+++++
14E8	+	+	-	N.D.	N.D.	++++
14E8-SC6	+++	++	-	N.D.	N.D.	++++
14F11	+++	+	-	N.D.	-	+++++
15A3	+++	N.D.	-	N.D.	N.D.	N.D.
15E6	++	N.D.	-	N.D.	+	N.D.
16A12	++++	N.D.	-	N.D.	N.D.	N.D.
16D8	++++	N.D.	-	N.D.	N.D.	N.D.
16A10	++	N.D.	-	N.D.	N.D.	N.D.
16D2	++	N.D.	++	N.D.	N.D.	N.D.
17E6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
17G4	N.D.	N.D.	-	N.D.	N.D.	N.D.
16F1	N.D.	N.D.	-	N.D.	N.D.	N.D.
17F9	++++	N.D.	-	N.D.	N.D.	N.D.
6F4	+	N.D.	N.D.	N.D.	N.D.	N.D.
3G9	+	N.D.	-	N.D.	N.D.	N.D.

Table 1: Human BLT1 Monoclonal Antibody Species Specificity. A panel of hBLT1 monoclonal antibodies were used to stain hBLT1 fused to different tags and expressed in various cell types or primary cells. For each cell type, each antibody is ranked as N.D. for not determined, - for negative, + for mean fluorescence (MF) of 20-100, ++ for MF of 101-250, +++ for MF of 251-500, ++++ for MF of 501-1000 and +++++ for MF of 1001 or more.

	300.19 mBLT1	300.19 hBLT1
2E4	+	-
3A5	++	-
3D7	+++++	-
4A12	++	-
5E6	+	-
6B1	+++	-
6E2	+++++	-
6F11	+++++	-

Table 2: Reactivity of Murine BLT1 Monoclonal Antibody. A panel of mBLT1 monoclonal antibodies was used to stain cells expressing mBLT1 or hBLT1 tagged with RFP. For each cell type, the antibody is ranked as N.D. for not determined, - for negative, + for mean fluorescence (MF) of 20-100, ++ for MF of 101-250, +++ for MF of 251-500, ++++ for MF of 501-1000 and +++++ for MF of 1001 or more.

Analysis of parental 300.19 cells (solid line), 300.19 cells expressing hBLT1 (dotted line) and 300.19 cells expressing mBLT1 (dashed line) stained separately with 3 different antibodies is shown Figure 6. These three cell lines were stained with 12CA5, an antibody that recognizes the HA epitope tagged to hBLT1 and mBLT1 (Figure 6a). As seen in Figure 6a, the parental 300.19 cells did not stain with 12CA5, whereas cells expressing the HA-tagged versions of hBLT1 and mBLT1 stained positive for 12CA5. In Figure 6b, 6H12 (a hBLT1 specific mAb) was used to stain the cell lines and only stains 300.19 cells expressing hBLT1. In contrast the 6B1 (a mBLT1 specific mAb) reacted with only 300.19 cells expressing mBLT1. These data are representative of all the mAbs tested against hBLT1 and mBLT1 and demonstrate that the antibodies produced are species specific for the type of BLT1 used to immunize the mice.

Epitope Mapping of hBLT1 Monoclonal Antibodies

We next wanted to determine epitope specificity of anti-BLT1 antibodies. To accomplish this, a series of chimeras between hBLT1 and mBLT1 were made using random chimeragenesis. This method takes advantage of the ability of *E. coli* to carry out homologous recombination between related sequences *in vivo*. The hBLT1 and mBLT1 proteins are highly homologous with ~75% identity. A flow diagram for the strategy is shown in Figure 7 and the methodology described in the Methods section. Utilizing unique restriction enzyme sites in either gene, we were able to divide the resulting chimeras into quadrants where crossover occurred. Based on this information, several chimeras were sequenced to determine the exact region of crossover and representative chimeras are shown in Figure 8, where the hBLT1 region is shown in pink

and the mBLT1 region is shown in blue. The designation below each chimera denotes where the protein sequence ends for hBLT1 and begins for mBLT1. Four chimeras (B1C7, B1C29, B1C22, and B1C8) were transiently transfected into HEK-293 cells and used to test binding of the human mAbs by flow cytometry. The data shows that all chimeras reacted with 12CA5 antibody at similar levels (Figure 9a and 9c) indicating normal surface expression of each chimera. In contrast, only hBLT1, B1C7 and B1C29 reacted with 6H12, the human BLT1 mAb, but not with mBLT1, B1C22 or B1C8 (Figure 9b, 9d and 9e). Since the antibody can only react with extracellular surface domains, these results suggest that the extracellular loop 2 (ECL2) is likely the binding site for the monoclonal antibodies. As this region is replaced with the corresponding region from murine BLT1, loss of binding occurs. Quantification of the data with other hBLT1 mAbs shown in Figure 6e confirms the finding with 6H12 and suggests that all hBLT1 antibodies are likely to react with the similar epitopes located on ECL2. In summary, 12CA5 is shown to stain all six chimeras equally well, as expected, while 6H12, 2A8 and 13F2 only show positive staining for hBLT1, B1C7 and B1C29.

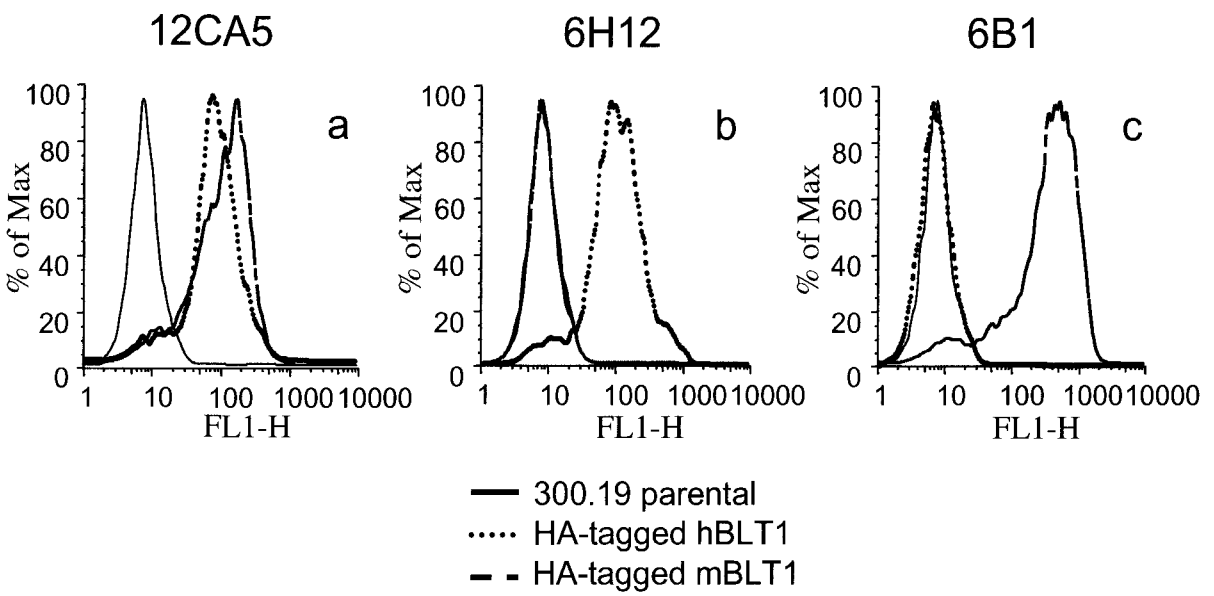


Figure 6: Flow Cytometric Analysis of Species Specific Monoclonal Antibody. Flow cytometry utilizing three cell lines is shown: 300.19 parental cells (solid line), 300.19 cells expressing hBLT1 (dotted line) and 300.19 cells expressing mBLT1 (dashed line). a) Both the cell lines expressing HA-tagged receptor demonstrated positive staining for 12CA5, while 300.19 parental cells are negative. b) HA-tagged hBLT1 only showed positive for 6H12 (human specific BLT1 mAb), where the 300.19 cells expressing mBLT1 as well as parental 300.19 showed negative. c) HA-tagged mBLT1 only showed positive for 6B1 (murine specific BLT1 mAb), where the 300.19 cells expressing hBLT1 as well as parental 300.19 showed negative.

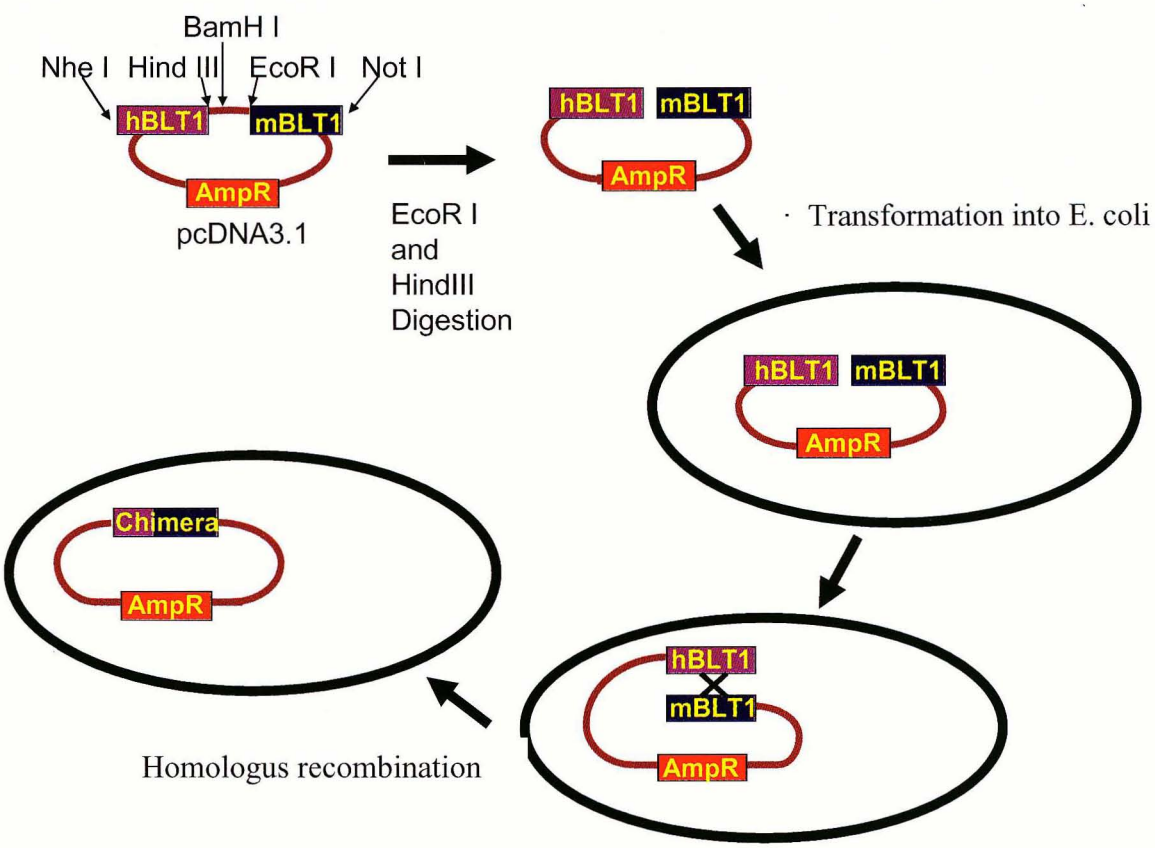


Figure 7. Strategy for Random Chimeragenesis of hBLT1 and mBLT1. hBLT1 was first cloned into the pcDNA3.1 vector at Nhe I and Hind III restriction sites. mBLT1 was next cloned into above construct at EcoR I and Not I sites The pcDNA3.1 vector containing hBLT1 and mBLT1 sequence was linearized by BamH1. The linerized plasmid (750 ng) was transformed into DH5a and TOP 10 E. coli strains. Only plasmids encoding chimeric receptors form colonies.

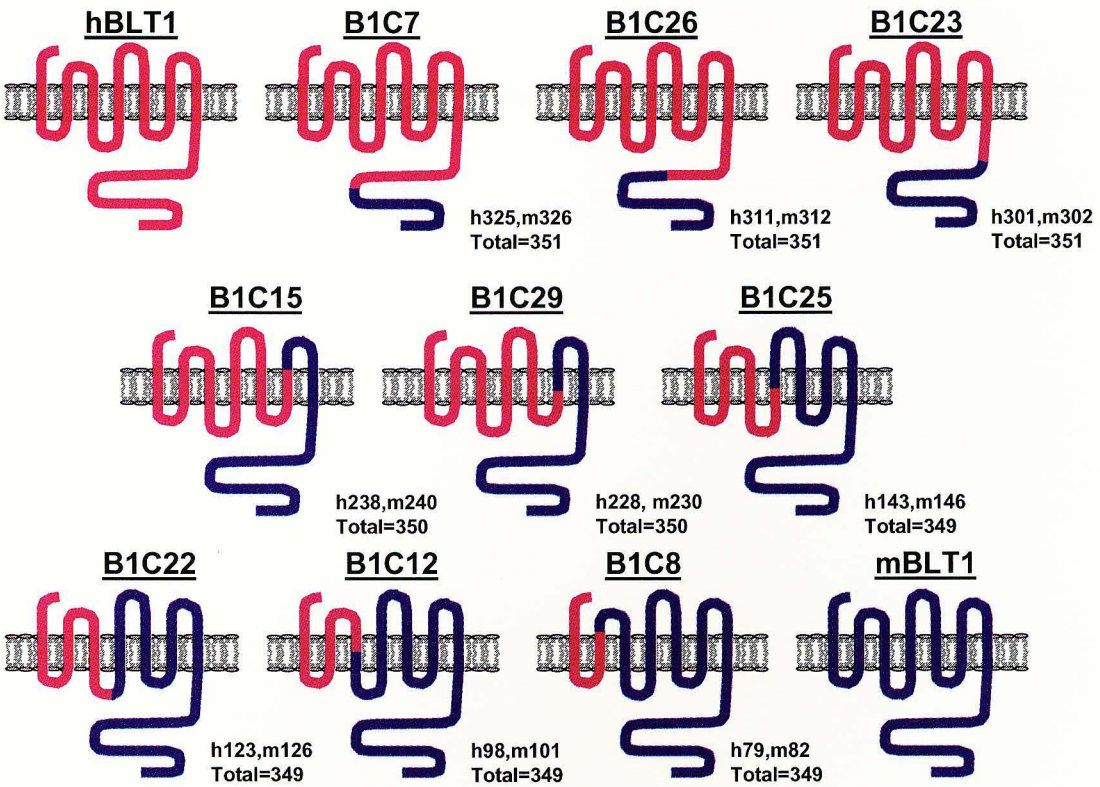


Figure 8: Cartoon Representation of BLT1 Chimeric Receptors. Chimeric receptors resulting from random chimeragenesis were sequenced to determine the exact region of crossover between hBLT1 and mBLT1. Since hBLT1 was on the 5' end of the coding region, all chimeras begin with hBLT1. Wild-type hBLT1 is shown in pink. Wild-type mBLT1 is shown in blue. Chimeras between hBLT1 and mBLT1 represented by the both colors. The total amino acid length of the chimera and at which amino acid residue of hBLT1 ends and mBLT1 begins is noted under each construct.

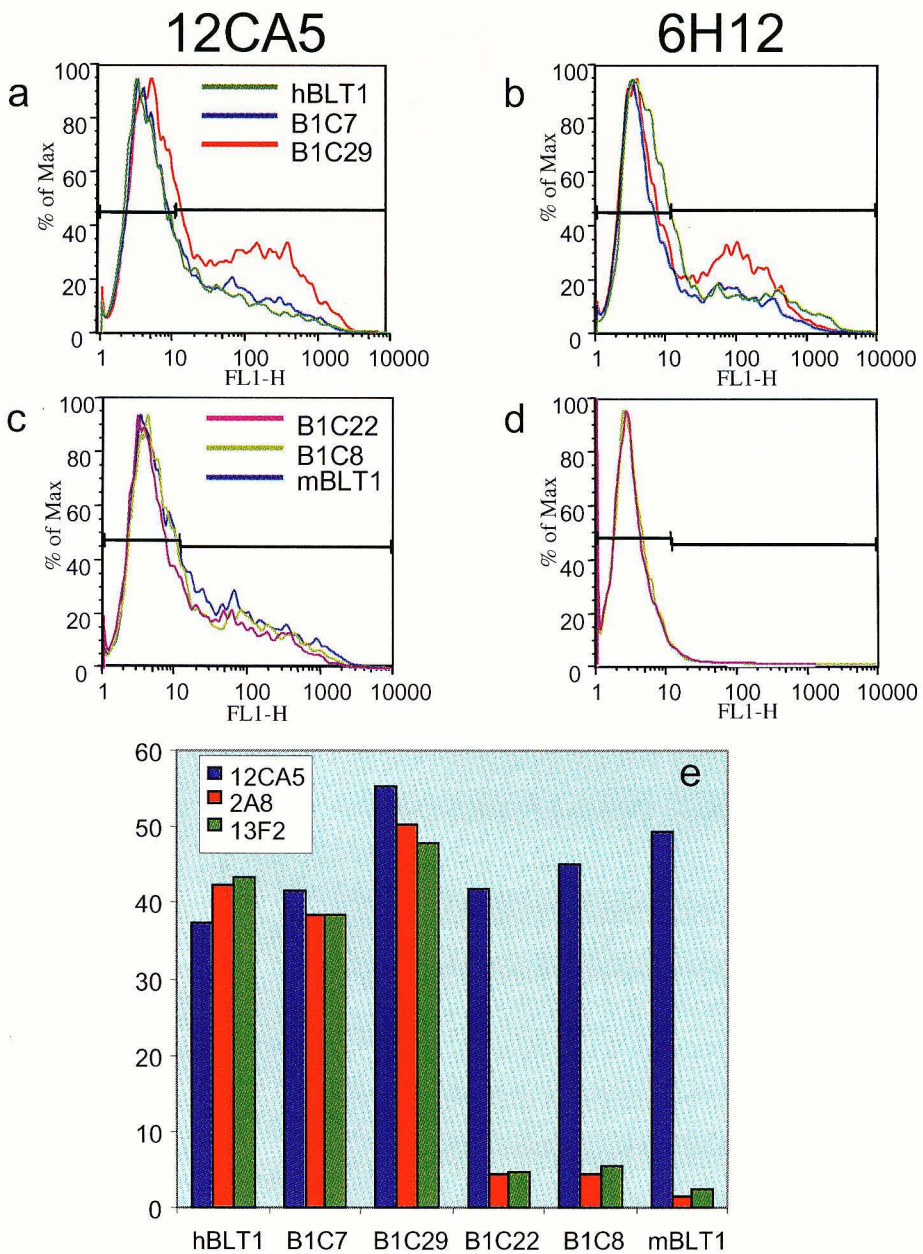


Figure 9: Epitope Mapping for hBLT1 and mBLT1 Monoclonal Antibodies: HEK-293 cells were transiently transfected with HA tagged hBLT1 (green line), B1C7 (blue line), B1C29 (red line), B1C22 (pink line), B1C8 (light green line) or mBLT1 (light blue line). 12CA5 and several hBLT1 monoclonal antibodies were used to stain these transient transfections. 12CA5 is a positive control for each transfection (a and c). Staining with 6H12 is shown in b and d, where only hBLT1, B1C7 and B1C29 demonstrate positive staining, while B1C22, B1C8 and mBLT1 are negative. Quantification of the percentage of positive cells for 12CA5 staining and two different hBLT1 antibodies is shown in f. Once again, 12CA5 is shown to stain all six equally well, while 2A8 and 13F2 only show positive staining for hBLT1, B1C7 and B1C29.

Characterization of hBLT1 Monoclonal Antibodies

To test whether these monoclonal antibodies could bind primary human cells, a blood sample was taken and the neutrophils were isolated and stained with a sampling of the monoclonal antibodies. 300.19 cells expressing hBLT1 were used as controls in the same experiment. All eight antibodies stained 300.19 cells expressing hBLT1 very well, however, only five of the eight stained primary human neutrophils. To test whether this staining difference was due to differential glycosylation status of hBLT1 in murine B cells versus human primary neutrophils cells, a deglycosylation mutant of hBLT1 was generated by standard site directed mutagenesis protocol (Jala et al), in which the two glutamine glycosylation sites were mutated to glutamate (N2Q and N164Q). A stable 300.19 cells expressing N2Q- N164Q-hBLT1 (hBLT1-DG) was generated and tested for antibody specificity. A panel of antibodies were used to stain the 300.19 cells expressing the deglycosylation mutant of hBLT1 and as seen in Table 1 all the antibodies tested stained the deglycosylation mutant at equal or better levels than the wild-type hBLT1 line.

Differential Expression of Murine BLT1 Detected by Monoclonal mBLT1 Antibodies

Using the same hBLT1/mBLT1 chimeras, it was shown that the murine BLT1 monoclonal antibodies were also specific for the ECL2 region of the protein (data not shown). Using these mBLT1 monoclonal antibodies, the expression pattern of murine BLT1 was demonstrated by flow cytometry. BLT1-/- mice served as a negative control to determine the precise expression in primary cells from mice. We also analyzed the

expression of BLT1 in BLT2-/- mice as the BLT1 promoter is located in the BLT2 coding region and the expression pattern of BLT1 may be altered in BLT2-/- mice. Thus, the mAbs are a valuable tool to examine this aspect of BLT1 regulation as this will become important for studies described in Chapter III. We analyzed the expression of mBLT1 in various primary cells isolated from murine blood, bone marrow, lymph nodes and spleen.

a) Neutrophils:

The expression of BLT1 on neutrophils from murine blood, bone marrow, lymph nodes and spleen of wild-type, BLT2-/- and BLT1-/- mice is shown in Figures 10 and 11. The gating strategy by forward and side scatter for neutrophils is shown in Figure 10a for blood, Figure 10c for bone marrow, Figure 11a for spleen, and Figure 11c for lymph nodes. This gated population was further analyzed for expression of GR-1-FITC, a major neutrophil marker (Figures 10b, 10e, 11b, 11e), and GR-1+ cells are analyzed for the expression of BLT1 (Figures 10c, 10f, 11c, 11f). BLT1-/- cells are represented by a red line, BLT2-/- by a blue line and wild-type (BLT1/BLT2+/+) mice by a green line. The highest expression of BLT1 was seen in blood and the bone marrow neutrophils (Figure 10c and 10f). Though few neutrophils are present in the spleen and lymph nodes, they showed positive staining for BLT1 in GR-1+ cells in Figures 11c and 11f suggesting the presence of mBLT1 at low levels compared to blood and bone marrow cells.

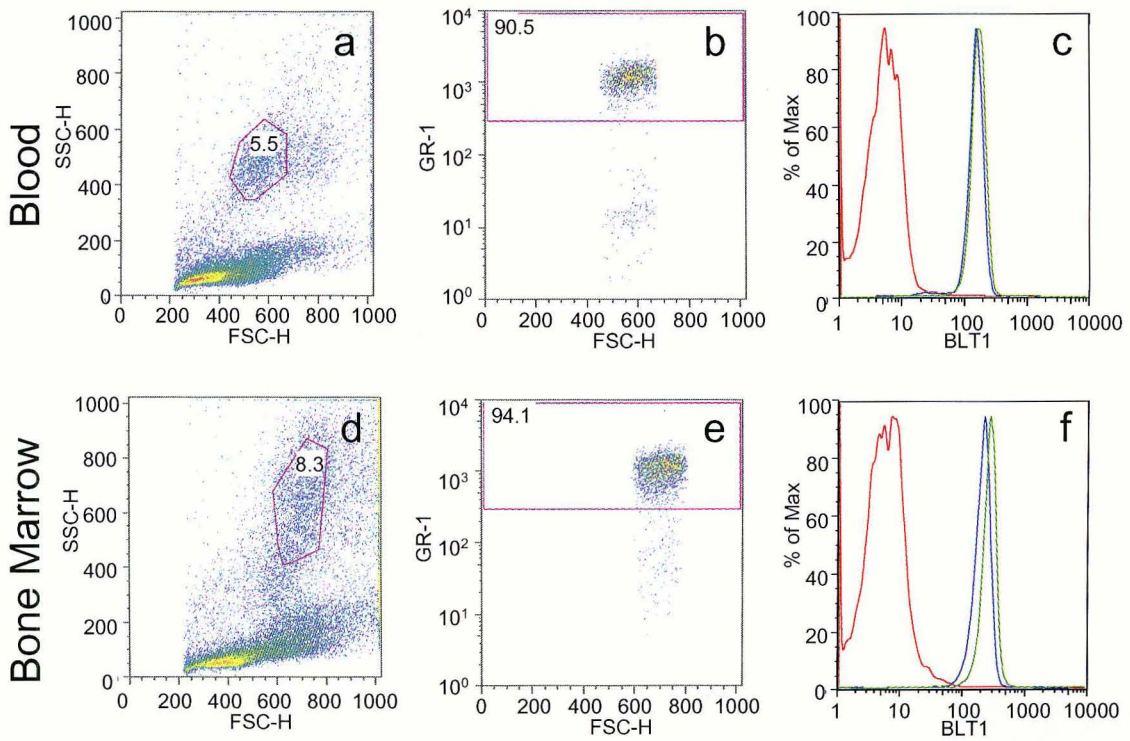


Figure 10: BLT1 Expression on Neutrophils from Murine Blood and Bone Marrow. Whole blood of mice was collected from C57/Bl6 mice by cardiac puncture. Tibias and femurs were removed from mice and flushed with RPMI. The bone marrow was uniformly resuspended, filtered and washed. 1 X 10^6 bone marrow cells were taken for analysis. 50 µL of blood was taken directly for analysis. Blood (a,b and c) and bone marrow (d,e and f) were stained with 3D7 and GR1-FITC. Neutrophils were gated using characteristic forward and side scatter in a and d. Cells which stained positively for the GR1-FITC marker were gated in b and e. GR1-FITC positive neutrophils were analyzed for their expression of BLT1 in c and f. Staining for BLT1 -/- mice (red line), BLT2 -/- mice (blue line) and BLT1/BLT2 +/+ mice (green line) showed BLT1 -/- mice stain negatively with 3D7, while BLT1/BLT2 +/+ and BLT2 -/- both stain well in both bone marrow and blood.

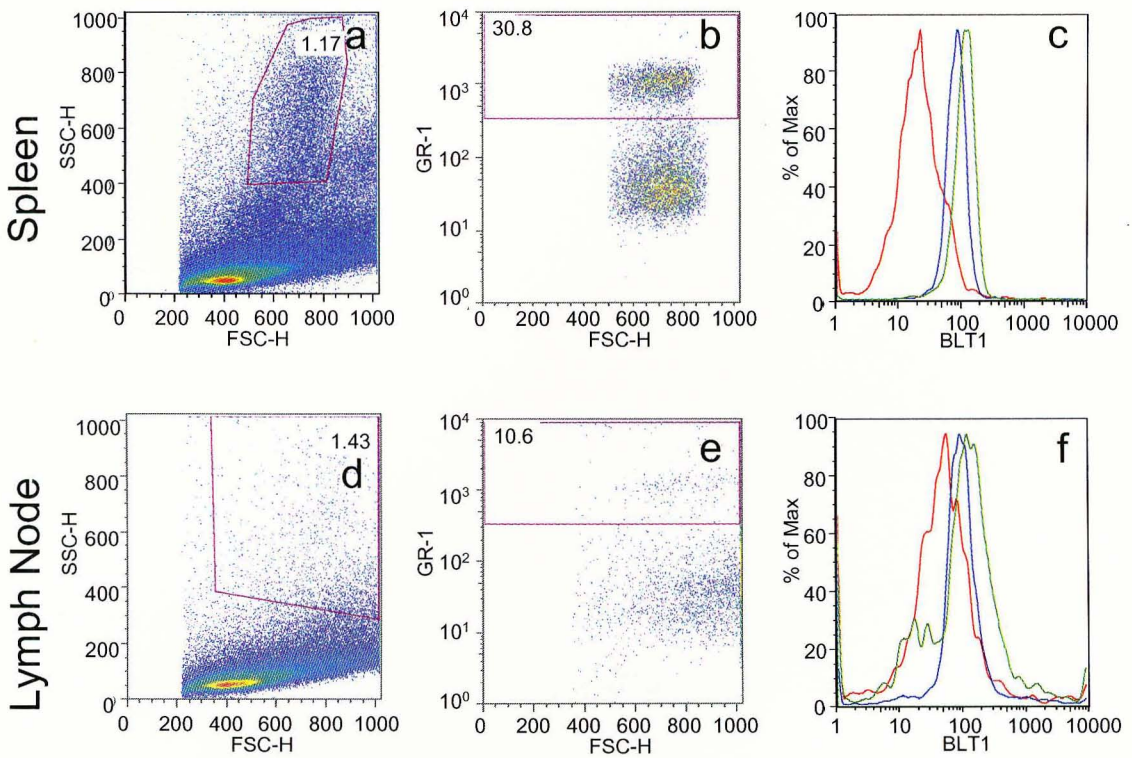


Figure 11: BLT1 Expression on Neutrophils from Spleen and Lymph Nodes. Spleen and lymph nodes were removed from C57/Bl6 mice and single cell suspensions were made by passage through nylon mesh. 1 X 10^6 spleen and lymph node cells were taken for analysis. Spleen cells (a, b and c) and lymph node cells (d, e and f) were stained with 3D7 and GR1-FITC. Neutrophils were gated using characteristic forward and side scatter in a and d. Cells which stained positively for the GR1-FITC marker were gated in b and e. GR1-FITC positive neutrophils were analyzed for their expression of BLT1 in c and f. Staining for BLT1 -/- mice (red line), BLT2 -/- mice (blue line) and BLT1/BLT2 +/+ mice (green line) showed the background staining for BLT1 -/- mice with 3D7 increased, while BLT1/BLT2 +/+ and BLT2 -/- both demonstrated positive staining for BLT1.

b) Eosinophils:

Eosinophils were also found to express BLT1 at high levels in the blood and at lower levels in bone marrow, spleen and lymph nodes. The gating strategy for eosinophils is shown in 12a (blood), 12d (bone marrow cells), 13a (spleen) and 13d (lymph nodes) where all cells were plotted for GR-1-FITC expression on the y-axis and side scatter on the x-axis. Cells which were GR-1-intermediate and showed a high side scatter were gated as eosinophils. The gate was then analyzed in 12b, 12e, 13b and 13e for the expression of Siglec-F-PE, which is a specific marker for murine eosinophils. Siglec-F positive cells were finally analyzed for the expression of BLT1 in 12c, 12f, 13c and 13f. In summary, expression of BLT1 is high in the blood (Figure 12c) and is readily detectible in the bone marrow, spleen and lymph nodes (Figure 12f, 13c and 13f).

c) Macrophages:

Macrophages were analyzed to detect the expression of mBLT1 in the bone marrow, followed by the blood, spleen and lymph nodes of wild type, BLT1-/- and BLT2-/- mice. The gating strategy is shown in 14a (blood), 14d (bone marrow), 15a (spleen) and 15d (lymph node), where all cells were initially plotted by forward and side scatter. The gated cells were then analyzed by fluorescence of CD11b-FITC and CD16-PE (Figure 14b, 14e, 15b and 15f). CD11b is a marker for macrophages and neutrophils which were separated by the forward and side scatter. Those cells which showed high fluorescence for CD11b and CD16 were gated (Figure 14b, 14e, 15b and 15f) and analyzed for the expression of BLT1 (14c, 14f, 15c and 15f). The highest expression of BLT1 in macrophages was seen in the bone marrow (Figure 14f). BLT1 expression was also present in the blood and even though macrophages are a rare population in the spleen

and lymph node, positive staining was seen in those locations as well. It was observed that the expression of mBLT1 decreased in bone marrow of BLT2-/- mice compared to wild-type mice. Whether this difference has any functional impact needs to be studied further. Analysis of macrophages with intermediate levels of CD16 and CD11b staining showed not detectable BLT1 expression (Figure 16).

d) Natural Killer Cells:

The expression of BLT1 was analyzed on natural killer cells (Figure17) from blood, bone marrow, spleen and lymph nodes. Natural killer cells were gated from the side and forward scatter analysis as shown in 17a. The gated cells were then analyzed by NK1.1-PE, a natural killer cell marker, and CD3-FITC (17b) and the NK1.1+ CD3- cells were taken as natural killer cells and were analyzed for their expression of BLT1 in blood (17c), and bone marrow (17d). Natural killer cells that express the T cell marker CD3 are termed natural killer T cells. Using a similar gating strategy as NK cells, natural killer T cells were tested for BLT1 expression. NKT cells were gated by forward and side scatter in 17a with the same gate as NK cells. The gated cells were again analyzed with NK1.1-PE and CD3-FITC, but the cells which stained for both CD3 and NK1.1 were taken as NKT cells. The expression in blood (17e) and bone marrow (17f) is shown and again, no positive staining was seen for BLT1 in the sites tested.

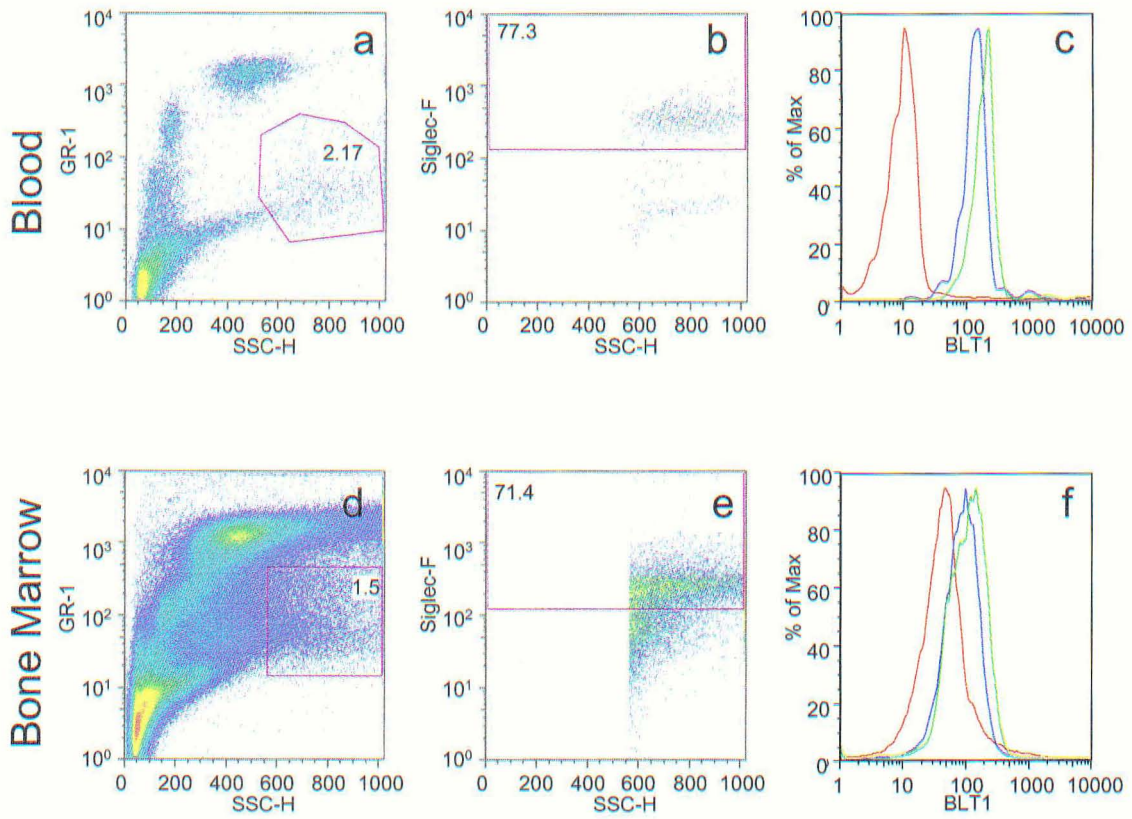


Figure 12: BLT1 Expression on Eosinophils from Blood and Bone Marrow. Whole blood cells and bone marrow cells were isolated from C57/Bl6 mice as described in Figure legend 11. Blood (a,b and c) and bone marrow (d,e and f) were stained with 3D7, GR1-FITC and Siglec-F-PE. Eosinophils were gated as GR1 intermediate and SSC high in a and d. Cells which stained positively for the Siglec-F-PE marker were gated in b and e. Siglec-F positive eosinophils were analyzed for their expression of BLT1 in c and f. Staining for BLT1 -/- mice (red line), BLT2 -/- mice (blue line) and BLT1/BLT2 +/+ mice (green line) demonstrated a large increase in staining of BLT1/BLT2 +/+ and BLT2 -/- mice over BLT1 -/- mice in the blood. The background staining of BLT1 -/- mice increased in the analysis of bone marrow, but positive staining is visible for BLT1/BLT2 +/+ and BLT2 -/- mice.

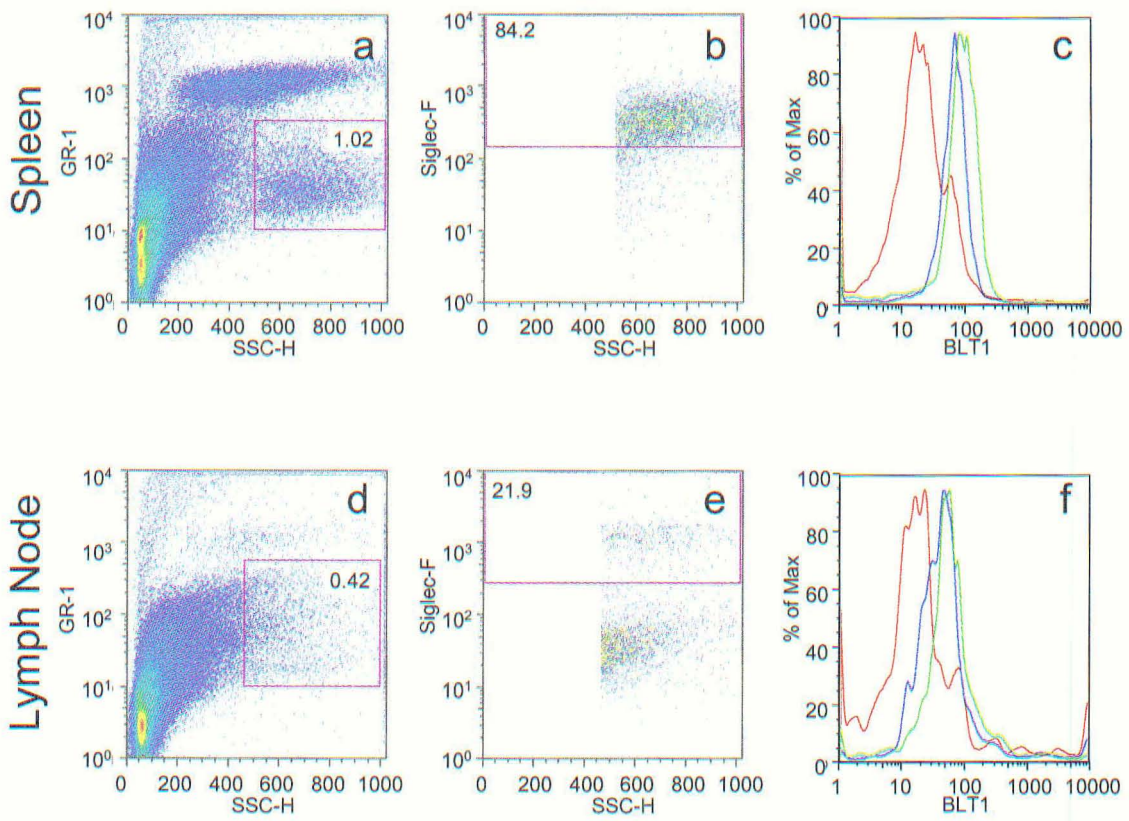


Figure 13: BLT1 Expression on Eosinophils from Spleen and Lymph Nodes. Spleen and lymph nodes were isolated as described in Figure 12 legend. Spleen cells (a,b and c) and lymph node cells (d,e and f) were stained with 3D7, GR1-FITC and Siglec-F-PE. Eosinophils were gated as GR1 intermediate and side scatter high in a and d. Cells which stained positively for the Siglec-F-PE marker were gated in b and e. Siglec-F positive eosinophils were analyzed for their expression of BLT1 in c and f. Staining for BLT1 -/- mice (red line), BLT2 -/- mice (blue line) and BLT1/BLT2 +/+ mice (green line) showed background staining for BLT1 -/- mice with 3D7 increased, while BLT1/BLT2 +/+ and BLT2 -/- both demonstrated positive staining for BLT1.

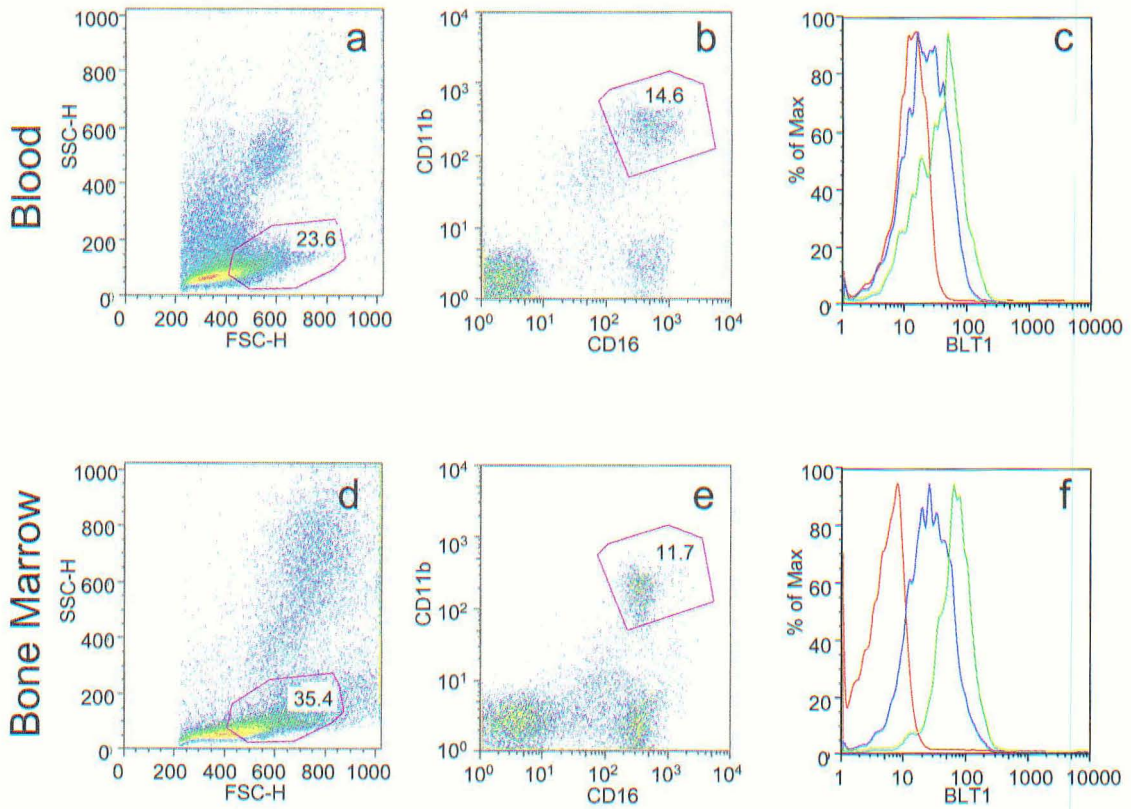


Figure 14: BLT1 Expression on CD11b High and CD16 High Macrophages from Blood and Bone Marrow. Blood (a,b and c) and bone marrow (d,e and f) were stained with 3D7, CD11b-FITC and CD16-PE. Macrophages were gated by characteristic forward and side scatter (a and d). Cells which stained CD11b high and CD16 high were gated (b and e). CD11b high and CD16 high macrophages were analyzed for their expression of BLT1 (c and f). Staining for BLT1 -/- mice (red line), BLT2 -/- mice (blue line) and BLT1/BLT2 +/+ mice (green line) demonstrated a noticeable increase in staining of BLT1/BLT2 +/+ and BLT2 -/- mice over BLT1 -/- mice in the blood. A definite increase in BLT1 staining was seen in bone marrow for BLT1/BLT2 +/+ mice and a moderate increase in BLT2 -/- mice.

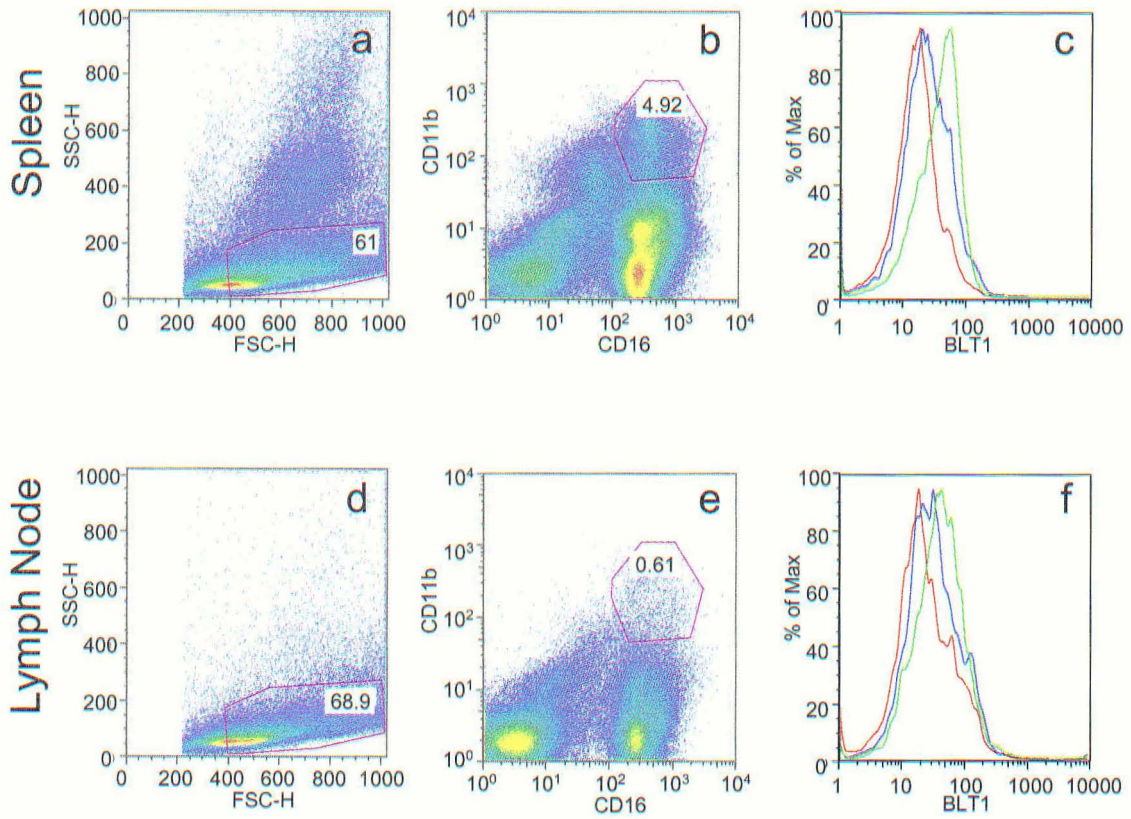


Figure 15: BLT1 Expression on CD11b High and CD16 High Macrophages from Spleen and Lymph Nodes. Spleen cells (a,b and c) and lymph node cells (d,e and f) were stained with 3D7, CD11b-FITC and CD16-PE. Macrophages were gated by characteristic forward and side scatter in a and d. Cells which stained CD11b high and CD16 high were gated in b and e. CD11b high and CD16 high macrophages were analyzed for their expression of BLT1 in c and f. Staining for BLT1 -/- mice (red line), BLT2 -/- mice (blue line) and BLT1/BLT2 +/+ mice (green line) showed high background staining for BLT1 -/- mice and an increase in staining is seen in macrophages from BLT1/BLT2 +/+ and BLT2 -/- mice.

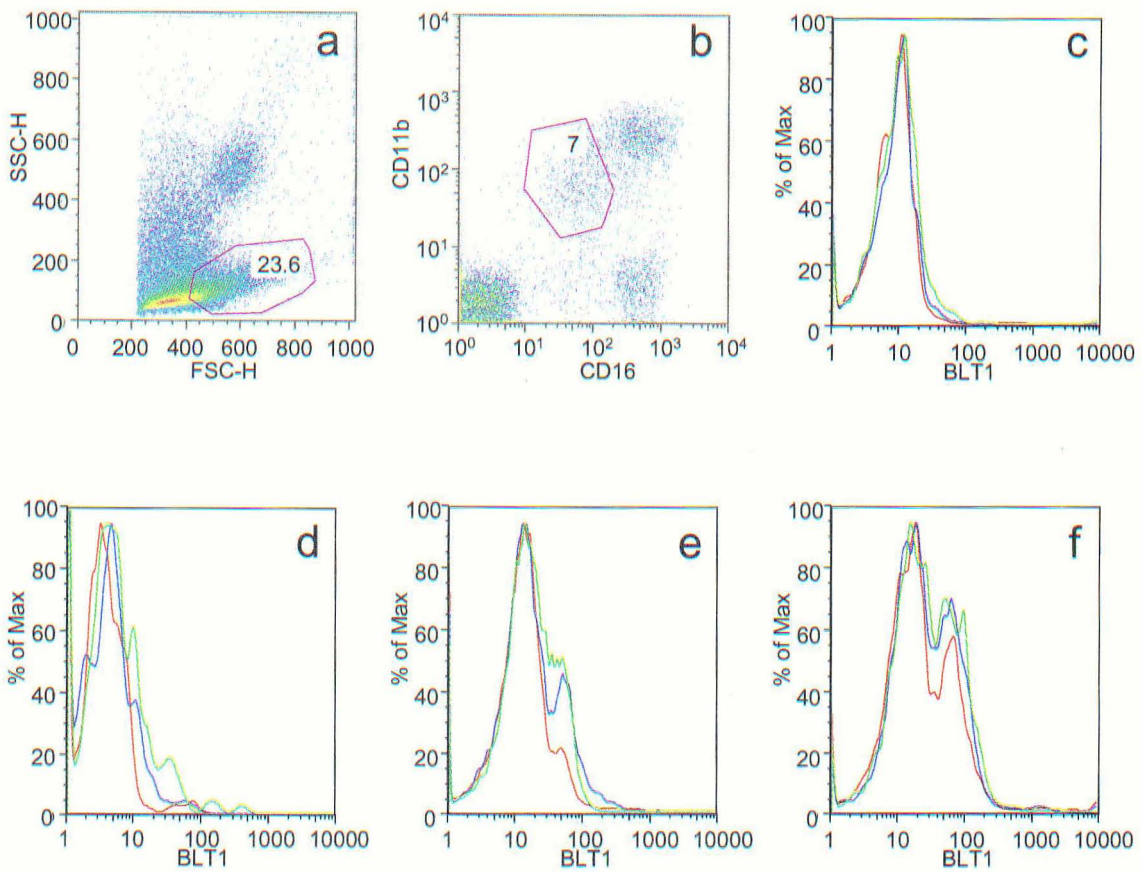


Figure 16: Lack of Detectable BLT1 Staining on CD11b Intermediate and CD16 Intermediate Macrophages. Blood (a,b and c), bone marrow (d), spleen cells (e) and lymph node cells (f) were stained with 3D7, CD11b-FITC and CD16-PE. Macrophages were gated by characteristic forward and side scatter in a. Cells that were CD11b intermediate and CD16 intermediate were gated in b. CD11b intermediate and CD16 intermediate macrophages were analyzed for their expression of BLT1 in c,d,e and f. Staining for BLT1 -/- mice (red line), BLT2 -/- mice (blue line) and BLT1/BLT2 +/+ mice (green line) demonstrated no detectable expression of BLT1 on CD11b intermediate and CD16 intermediate macrophages in any sample tested.

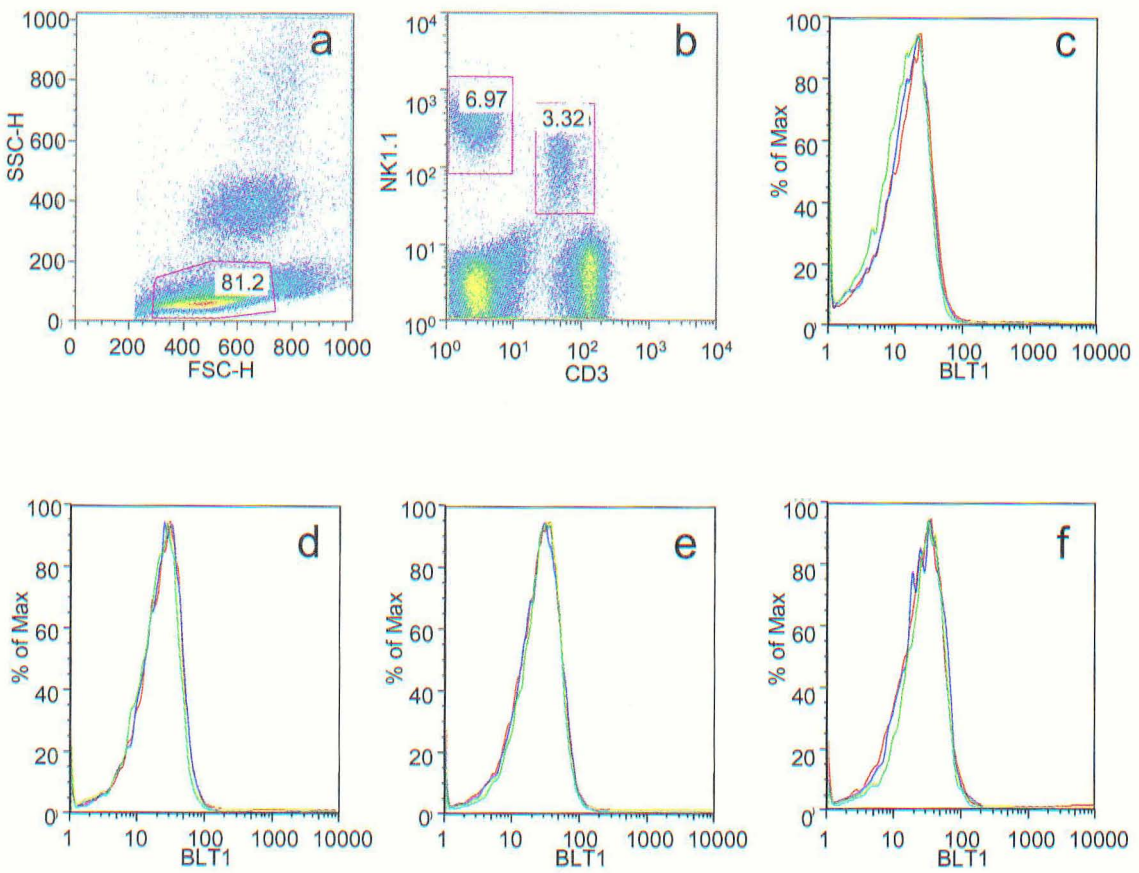


Figure 17: Lack of Detectable BLT1 Staining on Natural Killer or Natural Killer T Cells. Blood (a,b,c and e) and bone marrow (d and f) were stained with 3D7, CD3-FITC and NK1.1-PE. Natural killer and natural killer T cells were gated by characteristic forward and side scatter in a. Cells which stained NK1.1 positive and CD3 negative and cells which stained NK1.2 positive and CD3 positive were gated in b. NK1.1 positive natural killer cells were analyzed for their expression of BLT1 in blood (c) and bone marrow (d). NK1.1 and CD3 positive natural killer t cells were analyzed for their expression of BLT1 in blood (e) and bone marrow (f). Staining for BLT1 -/- mice (red line), BLT2 -/- mice (blue line) and BLT1/BLT2 +/+ mice (green line) demonstrated no detectable expression of BLT1 on natural killer cells or natural killer T cells in blood or bone marrow.

e) B cells:

BLT1 had not been identified on B cells previously and our tests showed no expression on B cells at any site tested. The gating strategy is shown in Figure 18a from all cells being shown by forward and side scatter. The gated cells were analyzed for their expression of B220, a B cell marker (Figure 18b). B220 positive cells were analyzed for their expression of BLT1 in blood (18c), bone marrow (18d), spleen (18e) and lymph nodes (18f). There was no detectable expression of mBLT1 observed in B cells from any locale.

f) T cells:

Expression of BLT1 has been shown in populations of activated T cells. Analysis of T cells in various sites in the mouse, however, demonstrated no detectable expression of BLT1 on T cells at any site tested. The same forward and side scatter gate was used in (Figure 19a) as for the B cells, but the gated cells were tested for their expression of CD3-FITC, a T cell marker, (Figure 19b). CD3 positive cells were again analyzed for their expression of BLT1 in blood (Figure 19c), bone marrow (Figure 19d), spleen (Figure 19e) and lymph node (Figure 19f).

Recent studies show that activated effector T cells, but not naïve T cells express BLT1 at detectible levels (16). It is apparent that the T cells analyzed in our unstimulated mice are not effector cells. To test for BLT1 expression on activated T cells, we received CD8+ effector and central memory T cell cultures from the Gelfand laboratory. These cells were stained with the naked 3D7-mBLT1 antibody along with CD8-PE or CD62L-PE. CD8+ effector T cells should stain highly with CD62L, while central memory cells stain CD62L low. In Figure 20, staining with 3D7-mBLT1 and CD8 shows a definite

increase in staining for effector T cells (31% in a) when compared to central memory T cells (0.3 %). A similar result is seen upon staining with 3D7-mBLT1 and CD62L, where effector T cells are CD62L high and demonstrate 28% positive staining for BLT1, while central memory T cells are CD62L low and shows only a background level of 0.4% positive staining. In studies carried out in Dr. Gelfand's laboratory using the biotinylated 3D7-mBLT1 antibody, dexamethasone was shown to up-regulate the expression of BLT1 in CD8+ effector and but not in CD8+ central memory T cells (Figure 21). Furthermore, the increase in expression was correlated with increased functional activity as measured by calcium mobilization and ERK-activation (data not shown).

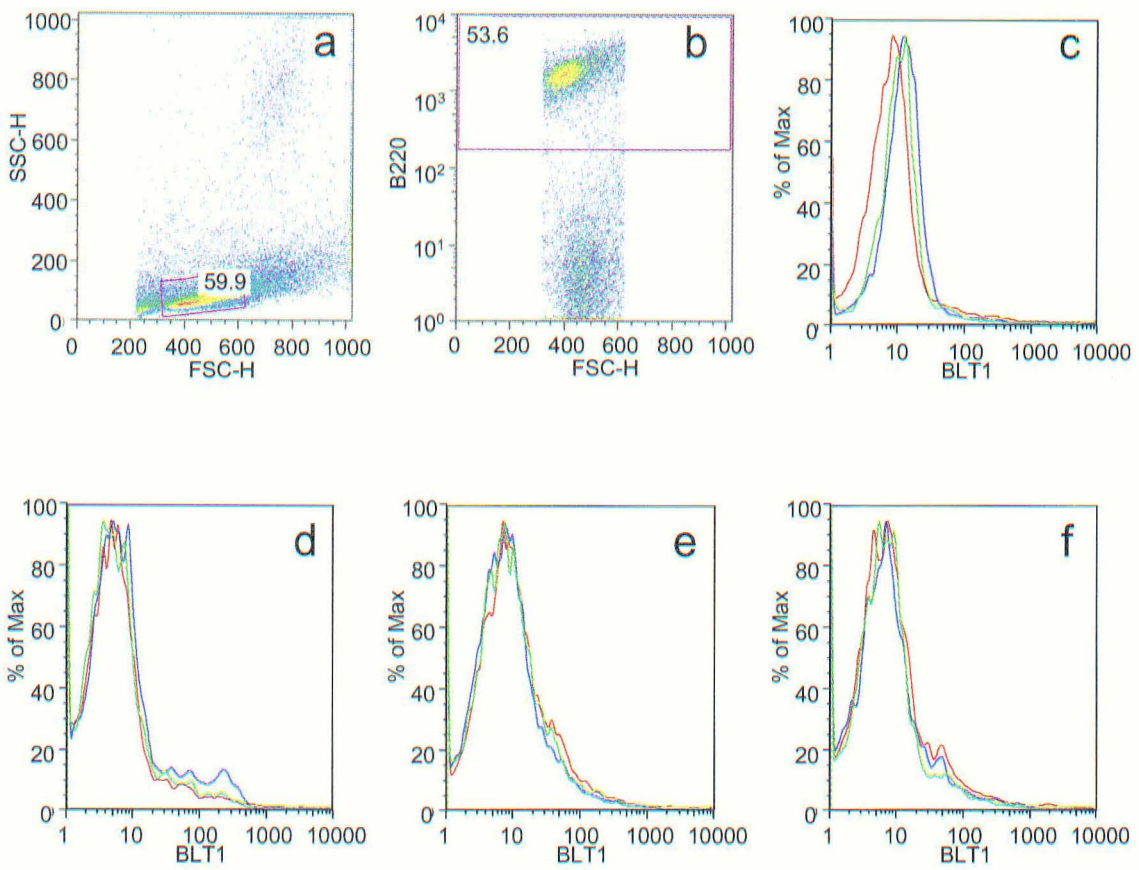


Figure 18: Lack of Detectable BLT1 Staining on B cells. Blood (a,b and c), bone marrow (d), spleen cells (e) and lymph node cells (f) were stained with 3D7 and B220-PE. B cells were gated by characteristic forward and side scatter (a). Cells which stained B220 positive were gated in (b). B220 positive B cells were analyzed for their expression of BLT1 (c,d,e and f). Staining for BLT1 -/- mice (red line), BLT2 -/- mice (blue line) and BLT1/BLT2 +/+ mice (green line) demonstrated no detectable expression of BLT1 on B cells in any sample tested.

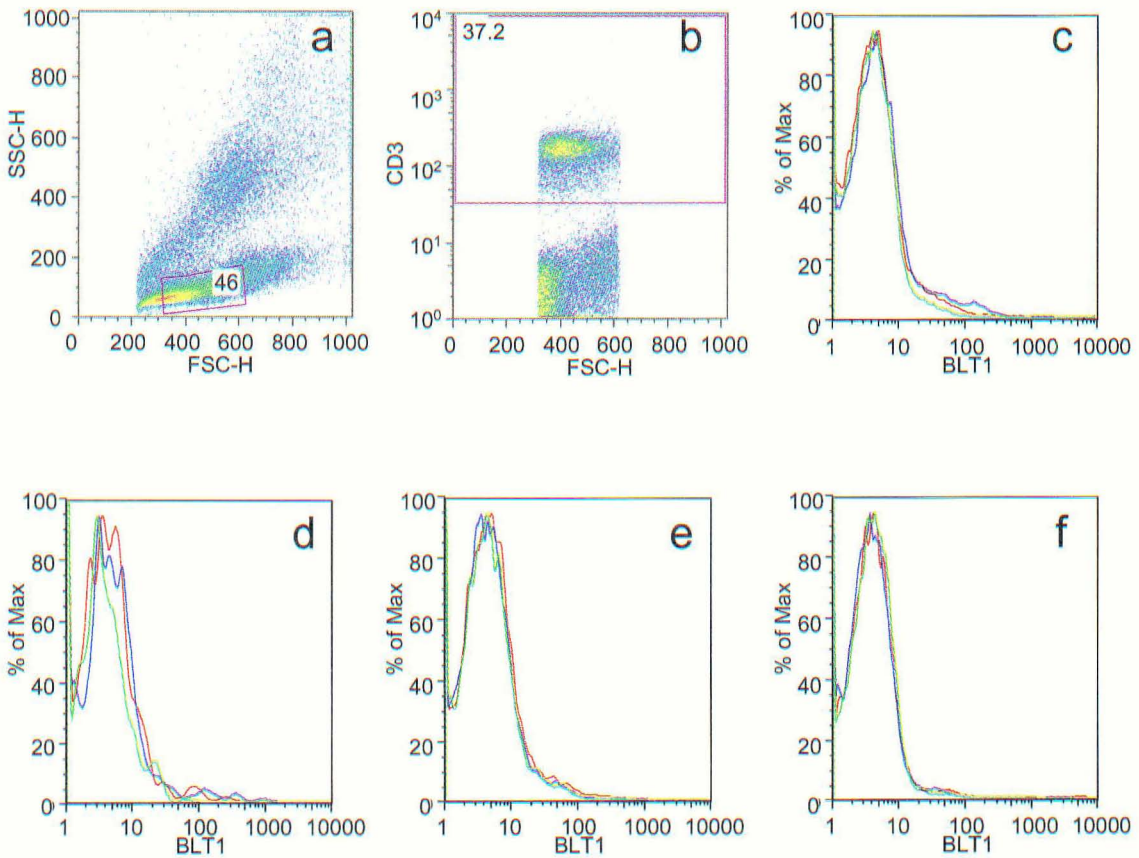


Figure 19: Lack of Detectable BLT1 Staining on Resting T cells. Blood (a,b and c), bone marrow (d), spleen cells (e) and lymph node cells (f) were stained with 3D7 and B220-PE. T cells were gated by characteristic forward and side scatter in a. Cells which stained CD3 positive were gated in b. CD3 positive T cells were analyzed for their expression of BLT1 in c,d,e and f. Staining for BLT1 -/- mice (red line), BLT2 -/- mice (blue line) and BLT1/BLT2 +/+ mice (green line) demonstrated no detectable expression of BLT1 on T cells in any sample tested.

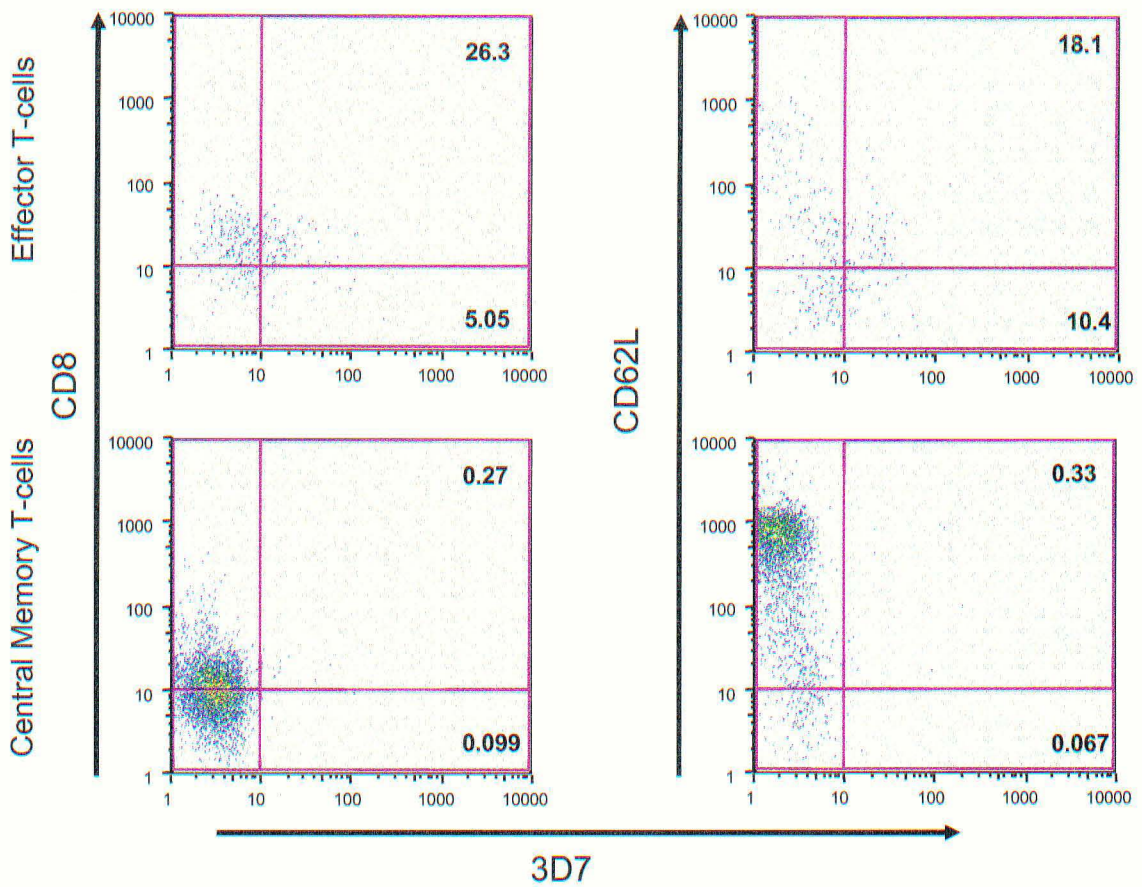


Figure 20: BLT1 is Expressed on CD8+ Effector, but not on Central Memory T cells. CD8+ T cells cultures were prepared and shipped to our laboratory. CD8+ effector and central memory T cells were stained with unconjugated 3D7 and either CD8-PE or CD-62L-PE. Both populations are CD8+, but effector T cells are CD62L low and central memory T cells are CD62L high. CD8 or CD62L staining are shown on the y-axis and 3D7 staining is shown on the x-axis. CD8+ effector T cells demonstrate positive staining for BLT1, but not CD8+ central memory T cells.

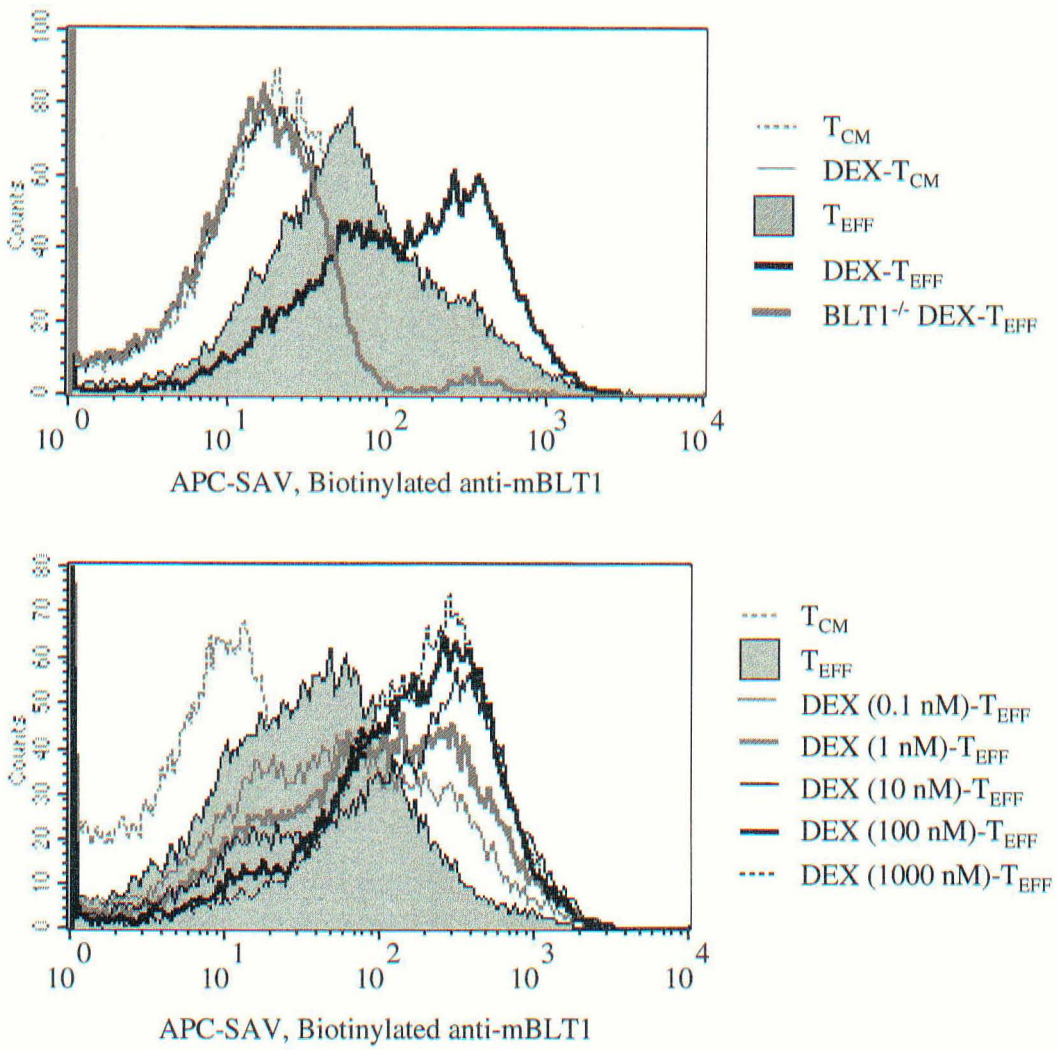


Figure 21: Dexamethasone Up-regulates BLT1 on T Effector but not T Central Memory Cells. (a) surface expression of BLT1 on CD8+ central memory T cells (T_{CM}), DEX-treated T_{CM}, CD8+ effector T cells (T_{EFF}) and BLT1-deficient DEX-treated T_{EFF} cells. DEX (100nM) was added in culture media during differentiation of T_{EFF}, T_{CM} or BLT1-deficient T_{EFF} cells. Cells after 7 days of culture were stained with biotinylated 3D7, followed by APC-conjugated streptavidin. Staining of these cells with isotype control showed similar staining as T_{CM} cells. (b) Surface expression of BLT1 on T_{EFF} cells treated with different doses of DEX (0-1000 nM). The data shown are representative of three independent experiments.

DISCUSSION

This section describes the generation and characterization of monoclonal antibodies against both human and murine BLT1. Membrane proteins in general and GPCRs in particular are known to be difficult immunogens for making monoclonal antibodies. Use of knock out mice for generating high affinity antibodies to membrane proteins such as L-selectin was highly successful (74). The general presumption being that in knock-out animals the protein becomes non-self and therefore, immunogenic. The immunization scheme of using 300.19 cells to express the protein of interest and the use of BLT1-/- mice worked well to produce three sets of antibodies: those to HA-tag, mBLT1 and hBLT1. Though we intended to produce antibodies that were cross-reactive between human and murine BLT1, the resulting antibodies were found to be specific for the immunized form of BLT1. Thus, despite the two proteins being highly homologous, we generated antibodies specific to the unique regions of the receptors. Interestingly, while the antibodies did not cross-react, both the human and murine BLT1 antibodies were specific for the ECL2 of the respective form of BLT1. This result may be placed in the context that ECL2 is the largest of the extra cellular loops and therefore likely to have contributed most epitopes. The fact that nearly 30 different antibodies for human BLT1 as well as the 8 antibodies for murine BLT1 were all reactive against the ECL2 region suggests that this region is probably immuno-dominant. In this context, it is interesting to note that the most diverse region between the human and the murine BLT1 is ECL2. While the mBLT1 and hBLT1 display ~79% homology the ECL2 region as shown below is only 41.7% identical with only 10 identical residues out of 24.

```
hBLT1-AYRTVVPWKTNMSLCFPRYPSEGH
      .:::::  :.    .: : ::   :
mBLT1-VYRTVK-WNNRTLICAPNYPNKEH
```

This result raises the question whether all of these antibodies may be against a single epitope and originated from the same clone. However, it was found that antibodies of the classes IgG1, IgG2a and IgG2b subtype were found in the analysis of representative antibodies (data not shown). This demonstrates that these antibodies originated from separate and distinct plasma cells. However, we could not reach firm conclusions about the specific epitopes on murine and human BLT1 recognized by our panel of monoclonal antibodies. Further studies with additional chimeras in ECL2 region and peptides corresponding to this region of mBLT1 and hBLT1 will delineate the binding sites for all of the antibodies described here.

As all the hBLT1antibodies were identified by screening with HA-tagged hBLT1 expressing 300.19 cells they were further tested and were reactive against RFP-fused hBLT1. Moreover, they also recognized hBLT1 expressed in a rat basophilic leukemia (RBL) cell line suggesting that there are no cell type-dependent differences in epitope recognition for exogenously expressed BLT1. To examine the expression of BLT1 in primary cells we stained human neutrophils and observed positive staining with most antibodies. However, as described in Table 1 not all hBLT1 antibodies were reactive against human neutrophils. Since both primary and transfected cells were stained on the same day it is not clear why some of these antibodies did not react with human neutrophils. Since we used murine cells expressing the human BLT1 for immunization, it was possible species specific differences in glycosylation status might have contributed

to differences in the reactivity of human neutrophils and transfected cells. However, when mutants of BLT1 that are defective in glycosylation were tested, all antibodies reacted equally well indicating no effect of glycosylation status in BLT1 epitope recognition. Another possible explanation for the observed result is potential polymorphisms in BLT1 gene; however this remains to be explored further.

A monoclonal antibody for hBLT1 has been generated and used to show the expression of BLT1 in monocytes and granulocytes from human peripheral blood by flow cytometry (72). Minor subpopulations of CD19 and CD22 positive B cells and CD8 positive cells showed some BLT1 staining. In their studies, binding of antibodies to BLT1 was not blocked by adding synthetic peptides representing the extracellular loops of BLT1 suggesting that the epitopes may have been conformation-dependant. In contrast, our use of chimeric receptors allowed mapping of the antibody binding site to the ECL2. Since the antibodies described by Sabirsh et al also displayed species specific reactivity, they might be binding to the similar sites in ECL2. Both antibodies were found to block the function of the receptors as determined by increase in EC50 for calcium, chemotaxis and MAP kinase activation (73). In preliminary experiments with partially purified 6H12 antibody, we observed ~50% inhibition of dose dependent calcium mobilization. Recent studies from our laboratory showed that ECL2 is a critical region for LTB_4 binding in BLT1 (12). Thus, the current demonstration that ECL2 is also the binding site for BLT1 antibodies offers an explanation for their functional blocking capacity. Their potential use in the clinic, however, requires further *in vitro* and *in vivo* characterization.

Because our intent was to generate antibodies against both murine and human BLT1, we initiated experiments to produce murine BLT1 antibodies. The anti mBLT1 antibodies would be extremely useful in characterizing different knock-out and transgenic animals developed in our laboratory as well as to address specific expression patterns and regulation of BLT1 in murine primary cells. As described in Results, we were quite successful and generated eight independent monoclonal antibodies for mBLT1. LTB_4 responsive receptors have been demonstrated by function in multiple cell types of mice and these antibodies have allowed us to begin probing these cells for expression. In humans and in mice, neutrophils have been shown to be a key BLT1 expressing cell. The results presented here confirm that neutrophils are indeed the highest BLT1 expressing primary immune cells. Moreover, as might be expected the BLT1-/- mice did not show any detectable expression of BLT1. But more importantly, the new mBLT1 antibodies have allowed analysis of BLT1 expression in BLT2-/- mice. Since both BLT1 and BLT2 are closely linked in the genome and separated only by ~4.0 kb of intergenic region, the influence of disrupting one gene on the expression of the other could be quite significant. In this context, the data presented here shows that both in neutrophils and in eosinophils the BLT1 expression is similar to WT mice in BLT2-/- mice. This observation is particularly relevant to the studies reported in Chapter III.

Analysis of BLT1 expression in cells isolated from different tissues revealed interesting patterns. Neutrophils and eosinophils showed high levels of expression when analyzed from different tissues such as bone marrow, blood, lymph nodes and spleen. In contrast, the expression of BLT1 in macrophages showed significant variation based on the tissue from which they were isolated. This may be related to maturation status of

macrophages. Macrophages with high CD11b and CD16 expression showed significant BLT1 expression, whereas macrophages with low CD11b and CD16 showed complete lack of BLT1 expression. In addition, macrophages from bone marrow displayed higher levels of BLT1 expression relative to blood monocytes. Previous studies from our and other laboratories have shown functional expression of BLT1 in murine and human monocyte/macrophages. However, the current results suggest a potential for tissue and maturation stage-dependent regulation of BLT1 in macrophages. Since macrophages express high levels of BLT1 in the bone marrow, low levels of LTB_4 produced from bone marrow-derived leukocytes might act as a retention signal for these cells. We propose under conditions of inflammation, the balance shifts to increased tissue levels of LTB_4 and as a consequence higher systemic levels of LTB_4. Indeed, increased tissue and systemic levels of LTB_4 were reported in diverse inflammatory diseases including atherosclerosis and arthritis. Increased levels of LTB_4 have been observed in the blood and synovial fluids of patients with rheumatoid arthritis (76, 77). This could then serve as mechanism for rapid mobilization of inflammatory cells to the site of inflammation. The decrease in BLT1 levels in peripheral tissues could be related to the receptor activation and down regulation due to increased LTB_4 levels.

Although we did not detect any BLT1 staining in our analysis of T or B lymphocytes in peripheral blood or lymph nodes, several laboratories have demonstrated functional expression on effector T cells. In previous studies expression of BLT1 was observed on activated T cells and the T cells we studied were resting and unactivated which explains their lack of BLT1 expression. When effector and central memory CD8+ T cells were tested for expression of BLT1, effector cells showed positive staining, while

central memory cells were completely negative. This is in line with the previous functional data reported by Ott et al. (78) and later by Miyahara et al. (50) who showed that BLT1 expression on CD8+ T cells is critical in murine models of airway hyper-responsiveness. More recently, using the biotinylated-3D7 antibody described in this thesis, Ohno in Dr. Gelfand's laboratory identified that dexamethasone up regulates BLT1 expression in effector memory CD8+ T cells (unpublished data). A certain percentage of asthma patients treated with corticosteroids are insensitive to the drugs and in some cases might exasperate the disease. The dramatic increase in the expression and function of BLT1 by dexamethasone offers a potential explanation for steroid resistance in asthma.

A large number of diseases have been associated with LTB_4 and many would likely be benefited by an antibody which blocks BLT1 function. That is an ultimate goal of this research. What both the hBLT1 and mBLT1 antibodies allow for at this point is the characterization of BLT1 in disease scenarios and in pre-clinical mouse models of disease. For example, the mBLT1 antibodies will allow us to determine which cells differentially express BLT1 during the development of different diseases given the ease of being able to follow disease progression in mouse models. The current studies confirmed that neutrophils, eosinophils and macrophages express BLT1. Beyond that, differential regulation of BLT1 in macrophages was identified. In addition, steroid induced up-regulation of BLT1 levels in T cells was demonstrated. Thus, the studies presented are just a beginning of what could become an important area of study. This understanding might allow us to proceed towards treating human disease.

CHAPTER III

NON-REDUNDANT AND CRITICAL ROLES FOR LEUKOTRIENE B_4 RECEPTORS BLT1 AND BLT2 IN MOUSE MODELS OF INFLAMMATORY ARTHRITIS

INTRODUCTION

Inflammation

The role of inflammation in disease processes of humans has become an important topic recently even prompting a cover story on TIME magazine. Many diseases which had been simply associated with genetics have recently been intimately associated with inflammation. Atherosclerosis is one such case where it had been thought that the disease was caused by faulty regulation of lipid metabolism, but is now known to be largely due to chronic inflammation. Inflammation was historically described by the Latin words calor, dolor, rubor and tumor which mean heat, pain, redness and swelling. Inflammation brings about these results by the action of cytokines, chemokines and other mediators with their effects on leukocytes and the vasculature (79). These mediators cause vasodialation, which causes the redness, heat and swelling and brings in inflammatory cells, which by their actions cause the pain. In the initial stages

of inflammation, neutrophils are the main cell type present followed by macrophages. These two types of immune cells form the first line of defense in infection. Either through additional cytokine and chemokine production, or through antigen presentation by dendritic cells, the adaptive immune system becomes involved at later stages and enables the immune system to be more focused in its attack. These cells can once again cause the recruitment of neutrophils and macrophages and in the case of acute infection cause the clearance of the infectious agent and resolve the inflammation. In chronic infections, the inflammation is ongoing and is not cleared. Chronic infections have been linked to a number of auto-immune diseases (80-84). There are several mechanisms by which this is thought to occur including molecular mimicry by the infectious agent (82). A brief overview of what auto-immune disease is and what connections have been made follows.

Auto-immune diseases are conditions where an individual's immune system causes damage to the individual chronically. Examples of auto-immune disease are multiple sclerosis, systemic lupus erythematosus, myasthenia gravis and rheumatoid arthritis. In multiple sclerosis, the body attacks the myelin sheath around nerves which disrupts normal nerve function (85). In lupus, auto-antibodies are made against common molecules such as double stranded DNA, nucleus or phospholipids and leads to a multitude of problems in the joints, skin, kidneys, heart, lungs, blood or brain (86). Many viruses have been associated with multiple sclerosis including Epstein-Barr virus (EBV) (81, 83). EBV is also thought to lead to the development of systemic lupus erythematosus due to EBNA-1 of the virus having cross-reactivity with certain lupus auto-antigens (80, 82).

Rheumatoid Arthritis

Rheumatoid arthritis is the emphasis in this thesis. Rheumatoid arthritis (RA) affects 2.1 million Americans, which is 1% of the population. In RA, multiple joints are usually inflamed in a symmetrical pattern and usually involve the small joints of the hands or feet (87). Rarely, rheumatoid arthritis can even affect the joint that is responsible for the tightening of the vocal cords to change the tone of the voice, the cricoarytenoid joint (88). Though RA is a disease of mostly the joints, it does affect other parts of the body. RA can cause Sjogren's syndrome, which is characterized by inflammation that affects the glands of the mouth and eyes making them particularly dry (89). Pleuritis, or inflammation of the lung, also occurs in RA patients, which causes tightness of the chest and trouble breathing (90). Rheumatoid nodules occur under the skin around the elbows and fingers (89). Other secondary effects of RA are pericarditis, anemia, decrease in white blood cells due to enlarged spleen, and vasculitis. The main presentation of RA is in the joints, however.

RA demonstrates alternating periods of flares and remission. Flares can present as fatigue, lack of appetite, low grade fever, stiffness, and muscle and joint aches or can cause red, swollen, painful and tender joints due to excessive synovial fluid production and, of course, intense inflammation. Remissions can occur naturally or through interventions. They can last a few weeks or for years. During remissions, individuals with RA can live a normal and healthy life. What controls this cycle of flare and remission is unknown, but drugs have been developed that allow remission periods to be more frequent and to last for a greater length of time.

Rheumatoid arthritis (RA) has had links to several infectious agents over the years including *Chlamydia pneumoniae* (91), periodontal flora (84) and EBV (92). Indeed, one of the models that will be discussed below absolutely requires the presence of a bacterial stimulus (mycobacteria) for the model to work properly. Absence or a lower concentration of the mycobacteria causes the immunization protocol to be ineffective (93). However, a definite connection between an infectious agent and RA has not been proven.

Activation of synovial fibroblasts has been linked to infectious agents through Toll Like Receptors (TLRs). The activated synovial fibroblasts produce pro-inflammatory cytokines, chemokines and matrix degrading enzymes (94). TLRs have been recently demonstrated to be involved in the initial stage of synovial activation (95). Microbial products or even RNA from necrotic cells within the synovium (96) cause activation of the synovial fibroblasts and subsequent release of pro-inflammatory cytokines and chemokines (97). Another possible initiation step involves the formation of immune complexes resulting from auto-antibody production. Immune complexes have been demonstrated to induce the production of leukotrienes by mast cells (98). This production was absolutely necessary in an immune complex-induced peritonitis model in mice. Recently, a pathway of joint inflammation involving the production of LTB_4 by mast cells was demonstrated. The mast cell generated LTB4 activates macrophages leading to the production of TNFα (99). TNFα induces further production of LTB_4 by mast cells, which generates an amplification loop for the production of LTB_4 and persistent inflammation in the synovium.

Many mechanisms are potentially involved in the resolution of inflammation. One of these are the switch from 5-LO and COX enzymes to 15-LO in leukocytes. Where leukotrienes and prostaglandins are pro-inflammatory, the 15-LO products called lipoxins are anti-inflammatory. In murine air pouch model was used to demonstrate the temporal manner in which this transition occurs. Injection of sterile air on days 3 and 6 led to a significant production of prostaglandin E_2 (PGE_2), little LTB_4 production and few PMNs. When TNFα was administered, a large increase in LTB_4 was demonstrated that reached a maximum at 1 hour and preceded a large influx of PMNs that reached a maximum at 2.5 hours. The maximum amount of PGE_2 was seen at this time point as well. A large increase in lipoxin A_4 was seen by 4 hours and remained elevated while the number of PMNs and level of PGE_2 was reduced (100). Lipoxins have been demonstrated to retard the entry of new neutrophils to the site (101), reduce vascular permeability (102) and stimulate macrophages to phagocytose apoptotic neutrophils (103).

Another set of molecules that down-regulate inflammation are the resolvins. Resolvins are derivatives of eicosapentaenoic acid have also been demonstrated in the air pouch model to inhibit leukocyte trafficking to the air pouch (104). These molecules act directly on leukocytes and have even been demonstrated recently to bind to BLT1 specifically to down-regulate its pro-inflammatory effects (105). As stated earlier, down-stream effects of LTB_4-BLT1 interaction are at least in part mediated through NFκB. It was demonstrated that resolvin E1 dampens NFκB signaling upon interaction with its receptors, ChemR23 (104), which again illustrates the importance of BLT1 in inflammation.

Treatment of RA

There is a long list of treatments for RA. General anti-inflammatory drugs such as aspirin are used quite often, but more specific therapies have emerged such as anti-TNFα treatment that has greatly advanced the treatment of RA. Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are what are commonly referred to as the first line drugs (106). Some examples of NSAIDs are acetylsalicylate (aspirin), naproxen, ibuprofen, and etodolac. NSAIDs are drugs that are used to reduce tissue inflammation, pain and swelling. Aspirin has been used for centuries for headache and other aches and pains. In higher doses than for headache, it has been shown to be effective in treating RA (107). In these high doses, NSAIDs have side-effects which include stomach upset, abdominal pain, ulcers, and even gastrointestinal bleeding. Other potential side effects may include damage to the liver and kidneys, ringing in the ears (tinnitus), fluid retention and high blood pressure. To minimize the gastro-intestinal side effects, NSAIDs should be taken with food and typically antacids and proton-pump inhibitors are used. Aspirin is a COX-1 inhibitor. COX-1 and COX-2 both are enzymes that produce pro-inflammatory prostaglandins from arachidonic acid. In an attempt to block prostaglandin synthesis without the gastro-intestinal side-effects, COX-2 inhibitors were developed. These compounds will be discussed further below.

Corticosteroids are the other first line treatment which can be taken orally or directly injected into the joint (108, 109). Examples of corticosteroids are prednisone and methylprednisolone. Corticosteroids are more effective in reducing inflammation than NSAIDs and are better at increasing joint mobility. They are typically used for short

periods during flares or when NSAIDs are not effective. Corticosteroids are used for short periods due to side effects which include diabetes, weight gain, facial puffiness, thinning of the skin and bone, easy bruising, cataracts, risk of infection, muscle wasting, and destruction of large joints, such as the hips.

First line drugs are effective in blocking inflammation and pain, but are not effective in stopping cartilage and bone destruction (110). For this, more specific drugs are necessary which are termed disease-modifying anti-rheumatic drugs (DMARDs). The first line drugs work quickly, but DMARDs can take months or even years to work effectively. Due to the slow method of action and the fact that the DMARDs must be tailored to specific patients, early treatment is critical in RA. Some examples of DMARDs are hydroxychloroquine, sulfasalazine, gold salts, D-penicillamine and methotrexate. Sulfasalazine and methotrexate are commonly used because they have few side effects. Methotrexate is the treatment of choice of the second line DMARDs (111). Methotrexate is an immunosuppressive drug. Most immunosuppressive drugs are only used in patients with severe disease or complications of RA. Methotrexate is used often due to the ability to adjust dosing for the patient's specific needs.

Another type of second line drugs are biologic modifiers. One of the therapies that is working well and gaining acceptance in the clinic is use of anti-TNF drugs such as etanercept, infliximab, and adalimumab. These modifiers bind to TNF and block it from interacting with its receptor and, thus, blocking the robust inflammation to which TNF leads. TNF blockers are generally taken in combination with methotrexate which increases the efficacy of both treatments (112). Blocking of IL-1R with anakinra has

shown promise in the clinic (113). It too is commonly used in combination with methotrexate, but has not been as effective as the other biologic modifiers. Rituxan is an antibody which binds to B cells and eliminates them. It has been effective in treating RA in patients who do not respond to the anti-TNF therapies (114). Another treatment for those who do not respond to anti-TNF therapies is abatacept, which blocks the activation of T cells (115).

Treatments such as use of a Prosorba column are being studied currently (116). The Prosorba column basically uses Protein A to bind antibody. The patient's blood is pumped out into an apheresis machine to separate the plasma from the blood cells and the plasma is passed over a Protein A column to remove the auto-antibodies present and then pumped back into the patient. This procedure is not currently in use, but it is being evaluated for effectiveness in the clinic.

Failure of COX-2 Inhibitors in Arthritis Therapy

One of the most effective RA treatments in recent years was the use of the COX-2 inhibitors. These include Celebex (Pfizer), Vioxx (Merck) and Bextra (Eli-Lilly). Again, COX-2 inhibitors were investigated and used because COX-1 inhibitors had gastro-intestinal side effects. COX-2 inhibitors were found to have a decreased incidence of these side effects; therefore, these compounds were given to patients for treatment of RA. These patients were found to have an increased risk of myocardial infarction (117) and the drugs were taken off the market quickly. RA has been shown to be linked to coronary heart disease and cardiovascular mortality rate previously (118-122). It has been recognized for a half century that patients with RA die prematurely (123). The

proportion of these deaths attributable to cardiovascular causes range from 39% to 50% (124-126). One possibility is that RA leads to increases in pro-inflammatory molecules in the periphery such as TNFα, IL-1β and IL-6 (127). Another possibility for the increase is the treatment of RA itself. Corticosteroids have been shown to increase the risk of atherosclerosis-associated cardiovascular events by five-fold in those taking high doses of corticosteroids (128). A number of researchers are looking into this important linkage between RA and heart disease. Another interesting connection to RA is a new insight that, while cancer incidence is identical between normal individuals and RA patients, cancer mortality is significantly higher in RA patients (129). Once again, the mechanisms are not clear, but likely include altered states of the immune system, prior treatments for RA and mental health.

Mechanisms of RA

Over and above the association of RA and heart disease already made, patients treated with COX-2 inhibitors had an increase in cardiovascular events compared to patients who did not take COX-2 inhibitors. It is obviously a complex situation involving many parameters that must be studied further, but one simple explanation is that blocking of COX-2 leads to increased production of leukotrienes. The COX enzymes compete with 5-LO for arachidonic acid. Blocking COX-2 could lead to increased levels of arachidonic acid substrate for 5-LO to produce LTA_4 and thereby, LTB_4. It has been shown in animal models that mice deficient in the leukotriene pathway or treated with pathway inhibitors are protected from developing atherosclerosis (43, 130). Specifically,

part of my work has demonstrated that loss of BLT1 leads to a significant reduction in plaque size in the ApoE deficient mouse model of atherosclerosis (35).

In both RA and atherosclerosis, inflammation is the culprit of the disease process. In RA, inflammation leads to joint destruction which is characterized by inflammatory influx into the joint synovium and into synovial tissue, synovial hyperplasia, and abnormal cellular and humoral immune responses. Neutrophils, mast cells, T cells, B cells, and macrophages all play an important role in disease progression and can be seen in the joints of RA patients. The presence of all of these cells and the destructive results listed are all due to the inflammatory process. As stated above, these cells are brought to the site by cytokines, chemokines and other inflammatory mediators such as fMLP and complement fragments. The complex interaction of these molecules and the cells that produce them must be understood for treatment of the disease to progress.

Cytokines are small proteins that are released from cells, usually upon activation, that modulate cells for particular tasks by binding specific receptors. Cytokines can work in an autocrine manner where they act directly on the cell that produced them, a paracrine manner where they act on cells that are close in proximity or in an endocrine manner where they act on cells distant to the source of the production. They are grouped by structure into the hematopoietins, interferons, immunoglobulin superfamily, TNF family and others. Cytokines are categorized as inflammatory or anti-inflammatory, but this is an over-simplification. Since many cytokines are released in most situations, the effect they have on a cell depends on the other factors present in the system. Many cytokines have been implicated in RA including IL-2, IL-6, IL-12 and IL-18 as positive regulators

and IL-4 and IL-10 as negative regulators of disease (131-133). Most importantly, TNFα and IL-1β have been shown to be intimately involved in RA (134) and blocking these cytokines has been particularly effective in the clinic in treating RA (135, 136).

Chemokines are a sub-group of cytokines and include small proteins, anaphylatoxins, bacterial proteins (fMLP), and lipids. Of the small protein chemokines, there are many groups such as the ELR+ CXC, ELR- CXC, CC and CX3C chemokines. These are grouped based on characteristic cysteines that these proteins contain which in turn define the receptors that they bind. MCP-1 (137), CCL5 (138, 139), CXCL8 (140), CXCL12 (141) and many other chemokines have been shown to be important for recruitment of cells to the synovium in RA. Quite potent lipid chemokines also exist which include the prostaglandins, platelet-activating factor, and leukotrienes. These lipid mediators are quickly produced upon cell stimulation because no transcription or translation is needed to produce them. The enzymes needed to produce them are always present and the substrate needed is found in the cell membrane.

Mouse Models of Arthritis

Models of arthritis have allowed for the elucidation of which cytokines, chemokines and other molecules are important in RA. Commonly used models include streptococcal cell wall-induced arthritis, collagen-induced arthritis and the K/BxN model. The streptococcal cell wall-induced arthritis model is typically done in rats and involves the i.p. injection of cell wall components of Group A streptococci. This model is preferential for females and leads to recurrent cycles of inflammation in the joints (142, 143). The pathology is similar to RA in the joints. Two very important models of RA in

mice are the collagen-induced arthritis and the K/BxN models of arthritis in mice. Collagen-induced arthritis (CIA) has been the most widely used model of arthritis initiated by intradermal immunization with type II collagen (144). The immunization protocol varies due to a difference in susceptibility of the strains of mice, which is thought to be due to MHC class linking. DBA/1 ($H\text{-}2^q$) and B10-RIII ($H\text{-}2^r$) mice develop disease following immunization with collagen in Complete Freund's Adjuvant (CFA) for the first injection and using incomplete Freund's adjuvant (IFA) for the booster two to three weeks later. CIA in mice has been shown to have several features in common with human RA (145). In particular, the cytokine requirement and the effects of cytokines on the development and progression of the disease appear similar to human RA (146). In this regard, TNF-α and IL-1β appear critical in the development and progression of RA (134). IL-2, IL-6, IL-12, and IL-18 act as positive modulators and IL-4 and IL-10 are negative modulators of the disease activity (131-133).

Two K/BxN mouse models of RA have attracted significant attention in recent years (147, 148). The K/BxN model based on a T cell receptor (KRN) transgenic mouse which produces a T cell repertoire that recognizes and makes auto antibodies to the ubiquitous glycolytic enzyme, glucose-6-phosphate isomerase (GPI) and develops an aggressive form of arthritis (149). In this case disease may also be transferred with serum or purified antibodies. Disease development in this model is T and B cell independent while neutrophils and mast cells are required for joint inflammation (150, 151). Within CIA models and the K/BxN model, a prominent role for LTB_4 in arthritis has been demonstrated.

Role of Leukotriene B_4 Receptors in Arthritis

A role for LTB_4 in rheumatoid arthritis was suggested by several observations over the past two decades. Neutrophils from RA patients undergoing methotrexate therapy displayed both acute and chronic suppression of LTB_4 synthesis ex vivo (152). This means that part of the efficacy of methotrexate therapy is likely due to this reduction in LTB_4 production in patients. In a recent study, a significant increase in the mRNA levels of BLT1 and BLT2 was seen in the joint tissues and cells from RA patients relative to OA patients suggesting a role for these receptors in RA (153).

Targeted deletions in, or use of antagonists in, the leukotriene biosynthesis pathway as well as deletions of the LTB_4 receptors themselves have allowed for a fuller understanding of the role LTB_4 has in arthritis (Table 3). Much of the early data were provided by antagonists. In 1995, it was seen that the LTB_4 receptor inhibitor CP-105,696 was able to greatly reduce disease severity in an IL-1α accelerated CIA model (154). Two more LTB_4 receptor antagonists were used in the IL-1α accelerated CIA model with similar results (155). In an LPS accelerated CIA model, the LTA_4H inhibitor SA6541 was used and reduced the severity of disease in that study (156). Data utilizing mice deficient in the leukotriene biosynthesis pathway in models of arthritis quickly followed. FLAP knock-out mice were put through the IL-1α accelerated CIA model and found to have a 73% reduction in disease severity, which was associated with a 23% decrease in disease incidence. FLAP heterozygous mice were shown to have a 37% decrease in disease severity and a similar decrease in disease incidence as the FLAP knock-out mice, suggesting that FLAP is a potential drug target (157). Though this

pathway looked like a good target for arthritis treatment, attempts to use the antagonists in clinical trials of arthritis often failed to improve RA.

Mouse Strain	Model of Arthritis	Experimental Results	References
DBA/1LacJ	IL-1 accelerated CIA	Complete protection against disease development by administration of BLT1 antagonist CP-105,696	(154)
DBA/1	IL-1 Accelerated CIA	A 38% reduction in disease severity by administration of BLT1 antagonist LY293111Na or CGS25019C	(155)
DBA/1	LPS-accelerated CIA	Reduced severity of disease by administration of LTA_4H inhibitor SA6541	(156)
DBA/1	IL-18 accelerated CIA	5-LO inhibitor MK-886 reduced the severity of disease	(99)
FLAP-/- DBA/1	IL-1 Accelerated CIA	Reduced incidence and severity of disease in FLAP-/- mice relative to WT mice	(157)
C57Bl/6	K/BxN	Complete protection by preventive administration and reduced severity by therapeutic treatment with 5-LO inhibitor L-739,010 and with BLT1 antagonist CP-105,696	(158)
BLT1-/- and BLT1/BLT2-/- C57Bl/6	CIA	Complete protection from disease development in BLT1-/- and BLT1/BLT2-/- mice. Similar levels of auto antibody (anti-C II) production in receptor deficient animals	(21)
5-LO-/- and LTA4H-/- C57Bl/6	K/BxN	Complete protection from disease development in 5-LO-/- and LTA4H-/- mice. Adoptive transfer of WT neutrophils is sufficient for disease development in 5LO-/- mice	(151)
BLT1-/- C57BL/6	K/BxN	Complete protection from disease development in BLT1-/- mice. Adoptive transfer of WT neutrophils is sufficient for disease development in BLT1-/- mice	(158)

Recent data has renewed interest in the LTB_4 pathway as a target in arthritis. Chen et al. showed that neither 5-LO nor LTA_4 hydrolase knock-out mice develop disease in the K/BxN model, while LTC_4 synthase knock-out mice develop full disease. Prophylactic treatment with a 5-LO antagonist also completely blocked disease from occurring in wild-type mice. They went on to show in mast cell and neutrophil transfer experiments that neutrophils are the primary source of LTB_4 in K/BxN arthritis (151). Using the BLT1 knock-out animals, Luster's group showed a critical role for BLT1 in

K/BxN arthritis (158). BLT1-/- mice do not show any signs of arthritis. Adoptive transfer of neutrophils from wild-type mice restored disease in these mice but most of the recruited neutrophils in the synovium were BLT1 negative suggesting that BLT1 is required only for initiation of joint inflammation but not maintenance. CP-105,696 was also shown to block disease occurrence in this model (158). Our studies with BLT1 and BLT1/BLT2 deficient mice in a CIA model also suggested a critical role for BLT1 in arthritis. Both these mice showed similar levels of anti-collagen antibody levels indicating normal immune response to collagen but complete absence of joint inflammation (21).

These data demonstrate a need for the development of strategies to block the actions of LTB_4 in RA. In fact, a recent clinical trial was performed with BIIL-284 which is claimed to be an antagonist of both BLT1 and BLT2. It was found in this study that the antagonist did improve the disease in patients given the antagonist, but the results were not significant compared to the placebo group (159). They concluded that LTB_4 was not critical in the disease because it was only partially effective. A major problem with this study is the fact that the specificity of this antagonist has not been fully characterized. Only preliminary experiments have been done. Another problem is that the dosage that was used in the clinical trial was 10X less than those necessary for efficacy in mouse models. Specific inhibitors for BLT2 and dual inhibitors for BLT1 and BLT2 are needed, especially since, as will be described below, BLT2 was identified as a critical mediator of arthritis in the present study.

Our laboratory is currently involved in studies to produce these new antagonists. Using molecular modeling, we have been able to demonstrate the amino acids necessary

for the binding of LTB_4 to BLT1 and the residues which are required for activation of the receptor (12). Mutation of the residues predicted to be involved in binding resulted in loss of binding affinity demonstrating that predictions made from the modeling were likely to be correct. This useful model of BLT1 has allowed us to screen compound libraries for novel antagonists of BLT1, which are yet to be tested (Basu et al. unpublished). Modeling is currently underway for BLT2 and we will be able to screen for novel antagonists targeting both BLT1 and BLT2. In screening both receptors, we hope to discover dual inhibitors which would potentially be useful in treating human disease.

Since the loss of BLT1 was enough for mice to be protected from arthritis development, it was no surprise that BLT1/BLT2-/- mice did not develop disease (21). This result suggests that BLT2 might not be important in RA. However, as shown below, loss of BLT2 also protects mice from developing arthritis. Using the K/BxN model, we showed that BLT2-/- mice developed very minimal signs of disease and using bone marrow transfer experiments, the requirement for BLT2 expression by bone marrow derived cells was shown. Moreover, using assays for BLT1 expression and function, it was determined that BLT1 is expressed and functional in BLT2-/- mice. Hence, both BLT1 and BLT2 may be required for the occurrence of RA.

MATERIALS AND METHODS

Mice

BLT1-/- mice on the Balb/c background were characterized and described in (59). BLT1-/- mice on the C57Bl/6 were characterized in (21). BLT1-/- were backcrossed 9 generations onto the DBA/1 background and are described for the first time within this thesis. BLT1/BLT2-/- mice on the C57Bl/6 background were characterized and described in (21). BLT2 -/- were generated at the Duke University Transgenic Mouse Core Facility and were subsequently backcrossed onto the C57Bl/6 background for 7 generations. BLT2-/- mice are described for the first time in this thesis. All studies and procedures were approved by the Animal Care and Use Committee of University of Louisville Research Resources Center.

RNA isolation and Reverse Transcription

Total RNA was isolated from bone marrow cells (BMC) using Trizol Reagent and followed by mini RNeasy kit from Qiagen. Total RNA was treated with Turbo DNAase from Ambion to remove the traces of genomic DNA contamination from RNA samples. cDNA was synthesized from 250 ng of total RNA with random hexamer primers using Taqman Reverse Transcription reagents (Applied Biosystems).

PCR for mBLT1 and mBLT2

mBLT1, mBLT2 and β-2 microglobulin (control gene) genes were PCR amplified by HotStart Taq Polymerase (USB) using above synthesized cDNA (2 μL per reaction) as template with following primers in 20 μL total reaction volume. The PCR was also

performed on the cDNA reaction mix that did not contain reverse transcriptase as a negative control (data not shown).

mBLT1: Forward primer: 5' ATGGCTGCAAACACTACATCTCCT 3'

mBLT1: Reverse Primer : 5'-CACTGGCATACATGCTTATTCCAC-3'

mBLT2: Forward primer: 5' ATGTCTGTCTGCTACCGTCC 3'

mBLT2: Reverse Primer: 5' AGGTGCAGCA CAAGTGTGGC 3'

mβ-2 microglobulin FP: 5' CATACGCCTG CAGAGTTAAG CA 3'

mβ-2 microglobulin RP: 5' GATCACATGT CTCGATCCCA GTAG 3'

The PCR conditions used to amplify these products were as follows. The cDNA (2 μl) and forward and reverse primers (7.5 pmoles) were added to PCR tubes containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 U of Hot start Taq DNA polymerase along with the PCR buffer provided with enzyme 1X concentration. The PCR conditions were: template denaturation at 95 °C for 4 min followed by 45 cycles at 95 °C for 30 sec (denaturation), 56 °C (annealing) and 72 °C for 30 sec (extension). The reaction was continued for 5 min to complete the extension. The expected sizes of the PCR products for mBLT1, mBLT2, β-2 microglobulin were 319, 191, 73 bp respectively.

Calcium Flux

Calcium mobilization was monitored in Indo-I-loaded cells stimulated with various concentrations of LTB_4. Each experiment represents an analysis of 3 x 10^6 cells. Neutrophils were elicited with zymosan for 4 hours and macrophages were elicited for 48 hours and were flushed from the peritoneum. They were then spun down, resuspended at

2 X 10^6/mL in PBS + 1% BSA and loaded with 1.2 µl of pluronic acid (200 mg/ml in Me_2SO) and 1.5 µl of Indo-1 (1 mM solution) and incubated for 30 min at 37 °C. After incubation, the cells were washed with PBS + 0.1% BSA. The cells (2 x 10^6 cells/ml) were resuspended in Hank's balanced salt solution containing 1 mM $CaCl_2$. The response to various concentrations of ligand was recorded using a spectrofluorometer (F2500, Hitachi, San Jose, CA).

CIA on the DBA/1 Background

Complete freund's adjuvant (CFA) was prepared by mixing 100 mg of heat-killed *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories) in 20 ml of IFA (Sigma-Aldrich). An emulsion was formed by dissolving 2.0 mg/ml chick CII (CII; Sigma-Aldrich) overnight at 4°C in 10 mM acetic acid and combining it with an equal volume of CFA. CII solution and the emulsion with CFA were always freshly prepared. Mice were anaesthetized with ketamine HCl/xylazine HCl (Sigma-Aldrich) and were injected i.d. at the base of the tail with a total of 100 µl of emulsion containing 100 µg of CII and 250 µg of *M. tuberculosis*. The same injection was repeated at day 21 in IFA.

K/BxN Arthritis

Serum from K/BxN arthritic mice was kindly donated by David Lee (Harvard University). Sera were pooled to assure uniformity in the injections. 100 µL of serum was injected i.p. on days 0 and 2 and mice were evaluated for clinical symptoms.

Paw and Ankle Measurement and Clinical Scoring

Paw and ankle measurements were taken using a pocket thickness gauge (Mitutoyo). All mice were examined every two to three days following appearance of disease. Arthritis of each individual limb was graded using the following scoring system: 0, normal; 1, apparent swelling and redness limited to one digit; 2, swelling in more than one digit; 3, severe redness and swelling of the entire paw; and 4, maximally inflamed limb with involvement of paw and ankle joint. The maximum score per mouse was 16. Mice were scored as arthritic if the clinical score was at least 2.

Bone Marrow Transfer

Recipient mice were irradiated with 900 rads to ablate all peripheral and bone marrow cells. The following day, donor mice were sacrificed and the femurs and tibias were removed and placed in RPMI medium. The bone marrow was flushed from the bones with RPMI, washed, and resuspended in PBS. The bone marrow from one donor mouse was used to reconstitute two recipient mice. Bone marrow was injected i.v. into recipient mice and the mice were housed in sterile caging for at least four weeks. Mice were allowed 8 weeks to engraft before they were taken for an experiment.

Histopathology

Upon termination of the experiment, mice were sacrificed and ankle joints were removed from both hind legs. Ankles were placed in buffered 10% formalin for 16 hours followed by transfer into 70% ethanol until samples were taken for sectioning. Ankles were decalcified and paraffin embedded. Joint sections (5mm) were stained with H&E

and examined for histological changes such as inflammation and bone destruction. Representative pictures were taken using a Nikon Eclipse E400 microscope.

RESULTS

Characterization of BLT1-/-, BLT2-/- and BLT1/BLT2-/- Mice

We have previously generated BLT1-/- and BLT1/BLT2-/- mice. In the current studies we have generated BLT2-/- mice. The genomic locus spanning the BLT1 and BLT2 genes and the strategy for making individual and double KO mice is shown in Figure 22. Three primer PCR reactions were developed for determining the genotypes at the BLT1 and BLT2 loci. Routinely, mice were genotyped from DNA isolated from tail snips using previously described procedures (21, 59). Figure 23a shows the genotyping of all three BLT deficient mice used in the current studies. The same genomic DNA was used in both PCR protocols to demonstrate clean recognition of the genotype of knock-out mice. BLT1/BLT2+/+ PCR demonstrates only a wild-type band for both BLT1 and BLT2. BLT1-/- PCR demonstrates a wild-type band for BLT2 and a KO band for BLT1. BLT2-/- PCR demonstrates a wild-type band for BLT1 and a KO band for BLT2. BLT1/BLT2-/- PCR demonstrates a KO band for both BLT1 and BLT2. BLT1/BLT2 +/- PCR demonstrates both the wild-type and KO bands for both BLT1 and BLT2. BLT1+/- PCR demonstrates a wild-type band for BLT2 and both a wild-type and KO band of BLT1. BLT2 +/- PCR demonstrates a wild-type band for BLT1 and both a wild-type and KO band of BLT2.

Analysis of BLT1 and BLT2 Expression in Murine Bone Marrow

In Figure 23b, RT-PCR analysis was done using RNA isolated from the bone marrow of BLT1/BLT2+/+, BLT1-/-, BLT2-/- and BLT1/BLT2-/- mice. BLT1/BLT2+/+ bone marrow demonstrates expression of both BLT1 and BLT2. BLT1-/- bone marrow

shows no signal for BLT1, but demonstrates BLT2 signal. BLT2-/- bone marrow shows no signal for BLT2, but demonstrates BLT2 signal. BLT1/BLT2-/- are negative for both BLT1 and BLT2 signal.

BLT1 is Functional in BLT2-/- Mice

Since the experiments described in Chapter II showed some difference in BLT1 surface expression in BLT2-/- mice compared to wild-type mice, we determined the functional expression of BLT1 in BLT2-/- mice. For this, zymosan-elicited neutrophils were tested for intracellular calcium release in response to LTB_4. The expression of BLT1 on the elicited neutrophils is shown in Figure 24. Any difference in BLT1 expression between BLT1/BLT2+/+ and BLT2-/- mice is not statistically significant. The calcium flux response to LTB_4 of wild-type neutrophils is shown in 25a. In 25b, the calcium flux response of neutrophils from BLT2-/- mice to LTB_4 is shown and is virtually identical to the wild-type response if not slightly better. In 25c and 25d, the calcium flux response of neutrophils from BLT1-/- and BLT1/BLT2-/- mice, respectively, is shown demonstrating no response at all.

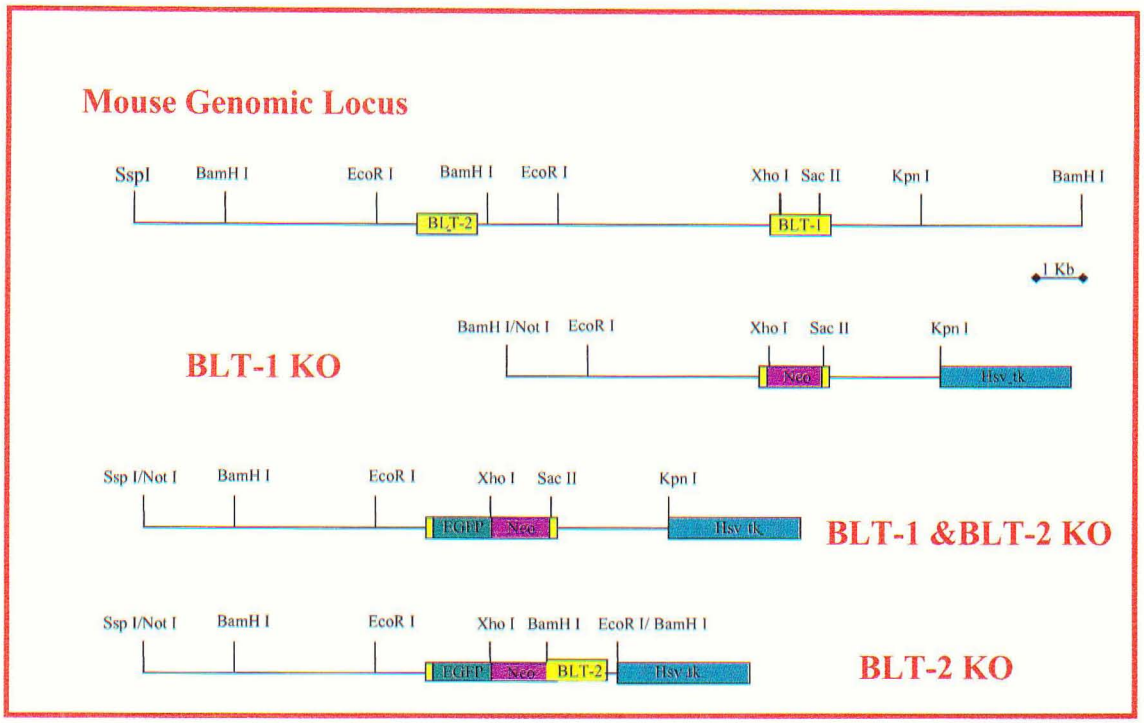


Figure 22. Targeted Disruption of Leukotriene B_4 Receptors. The strategy utilized to produce knock-out animals for the LTB_4 receptors is shown. A diagram of the locus on BLT1 and BLT2 is shown at the top to demonstrate the regions deleted in each construct. The targeting construct for BLT2 is shown on the bottom. The construct contains the same long arm fragment as the BLT1/BLT2 knock-out construct with a short N-terminal stretch of BLT2 followed by an EGFP and a neomycin cassette. Following neomycin, the remainder of the BLT2 sequence was kept due to the fact that the coding sequence of BLT2 contains the promoter of BLT1, though no transcription should occur since the neomycin cassette contains a stop sequence.

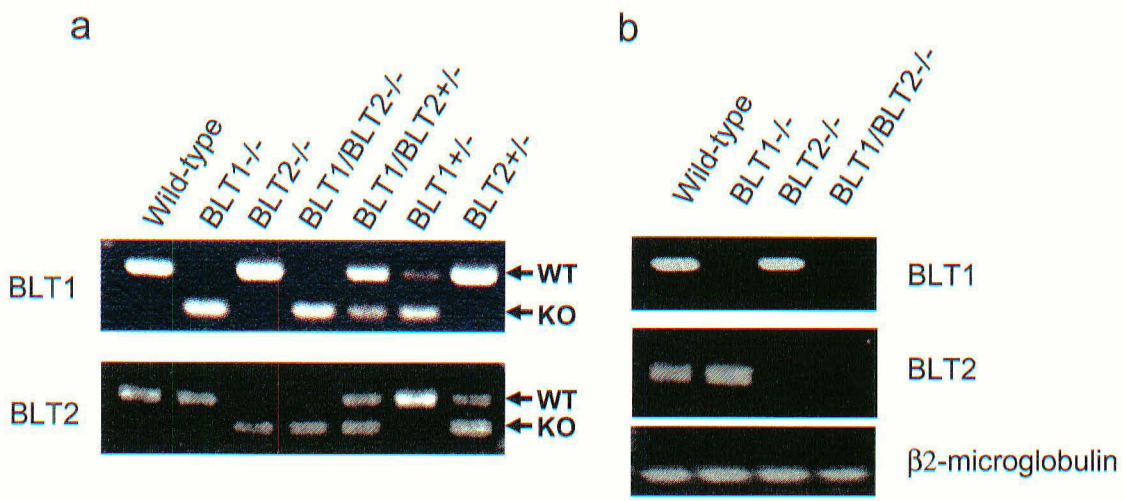


Figure 23. Targeted Deletions of LTB_4 Receptors. ***(a) Genotyping:*** Genomic DNA was isolated from mouse tails and PCR was performed for both BLT1 and BLT2 utilizing separate three primer reactions as described in methods ***(b) RT PCR:*** Total RNA was isolated from Bone marrow cells (BMC) and cDNA was synthesized from 250 ng of total RNA with random hexamer primers. mBLT1, mBLT2 and β-2 microglobulin (control gene) genes were PCR amplified and the results shown. The products for mBLT1, mBLT2, β-2 microglobulin were 319, 191, 73 bp respectively.

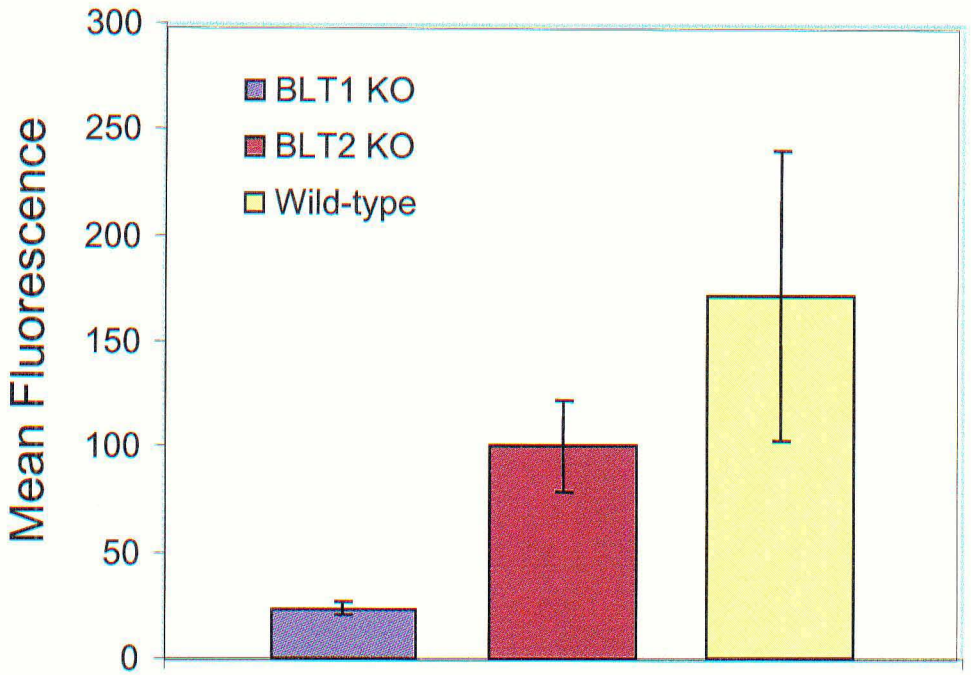


Figure 24: BLT1 Expression on Zymosan-elicited Neutrophils. Mice were injected with 1 mL of 1mg/mL zymosan-a i.p. Following four hours incubation, the peritoneum was washed with RPMI-1640 containing 2% FBS and 0.2 mM EDTA. A sample was taken and incubated with biotinylated-3D7 mouse monoclonal antibody and followed by streptavidin-APC. The cells were analyzed on BD FACSCalibur with FL4 channel. The mean fluorescence values of BLT1 KO (blue bar), BLT2 KO (red bar) and wild-type (beige bar) neutrophils are shown (n=3).

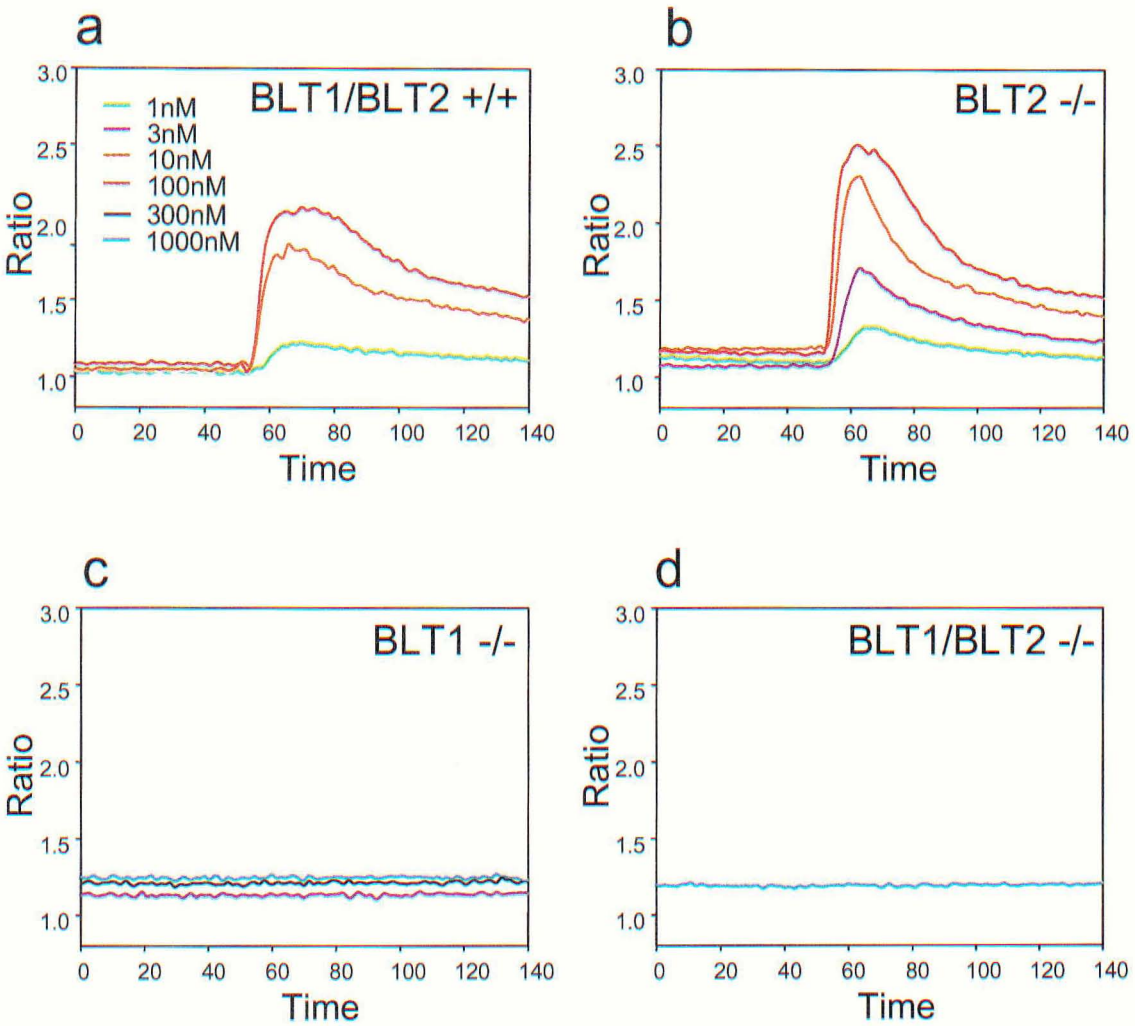


Figure 25: Calcium Flux of Zymosan-elicited Neutrophils. Mice were injected with 1 mL of 1mg/mL zymosan-a i.p. Following four hours incubation, the peritoneum was washed with RPMI-1640 containing 2% FBS and 0.2 mM EDTA. The cells were washed with 1 X PBS + 1% BSA and processed for calcium flux. Concentrations of LTB_4 used were 1 nM (green), 3 nM (purple), 10 nM (orange), 100 nM (brown), 300 nM (black) and 1000 nM (light blue). a) BLT1/BLT2 +/+ (wild type) b) BLT2-/- c) BLT1-/1 d) BLT1/BLT2-/- (double knock out)

BLT1-/- Mice are Protected from CIA on the DBA/1 Background

Our laboratory has shown that BLT1 is critical in the development of CIA on the C57Bl/6 background in mice (21). To test whether BLT1 was important in the more commonly used model of CIA on the DBA/1 background, we bred our BLT1-/- mice onto the DBA/1 background 9 generations. The mice paws were measured on day 0 of the experiment for a baseline thickness and were measured on day 21 for a baseline thickness of the ankle joint. Following the booster injection of collagen on day 21, the mice were analyzed at least every 2-3 days for 3 parameters; clinical score, paw thickness and ankle thickness. Heterozygous mice for BLT1 were bred to give BLT1+/+, +/- and -/- mice as littermates and were immunized for the development of CIA using protocols described in Methods. Figures 26 and 27 are representative of two independent experiments. In both experiments, BLT1-/- mice were nearly completely protected from clinical disease development, whereas BLT1+/+ mice demonstrated severe arthritis reaching a 100% incidence by day 58 of the experiment (26a). BLT1+/+ mice started to show an increase in ankle thickness (27b), paw thickness (27a) and clinical score (26b) starting at day 24 and all these parameters continued to increase until the termination of the experiment at day 70. BLT1-/- mice were protected from development of any clinical signs of disease until day 49 when only 1 mouse showed clinical signs of disease (26a). There is no drastic increase seen at any point in paw thickness (27a) or ankle thickness (27b) in BLT1-/- mice. BLT1+/- mice showed a definite delay in developing disease as compared to BLT1+/+ mice. BLT1+/- mice did not show any signs of disease until day 34 and then the clinical score increased until the end of the experiment (26b). No drastic increases in paw thickness (27a) or ankle thickness (27b) were seen until day 63 where

both began to increase. Whereas +/+ mice reached 100% incidence on day 59, the +/- mice only reached 100% incidence on the final day of the experiment (26a). On day 70, the difference between the clinical score of BLT1+/+ and BLT1+/- was significant ($p < 0.05$). Clinical scores were analyzed by non-parametric t-test in GraphPad Prism version 2.01.

Protection of Both BLT1-/- and BLT2-/- Mice from K/BxN Arthritis

Arthritis was induced in BLT1/BLT2+/+, BLT1-/- and BLT2-/- mice on the C57Bl/6 background with 100 μL of K/BxN arthritogenic serum injected i.p. on day 0 and day 2 and were followed every 1-2 days for signs of arthritis. This experiment is representative of 3 experiments with BLT1/BLT2+/+ and BLT2-/- mice and 2 experiments with BLT1-/- mice. The results of this experiment are shown in Figures 28 and 29. The clinical score is shown in 28b and demonstrates that BLT1/BLT2+/+ mice get a very severe disease. BLT1-/- mice had a slight clinical score from day 6 to 10, but what little inflammation occurred resolved. BLT2-/- mice showed a similar fluctuation in clinical score between day 6 to 10, but a minimal amount of inflammation remained which is shown in 28b. In 28a, the incidence of arthritis is shown. BLT1/BLT2+/+ mice reach 100% incidence by day 8, BLT1-/- mice have no incidence of disease and BLT2-/- mice have a 20% incidence of disease starting at day 13. This incidence was due to one BLT2-/- mouse getting arthritis in one paw as compared to BLT1/BLT2+/+ mice where all five mice get disease in at least two paws. In 29a, it is shown that BLT1/BLT2+/+ mice began to have an increase in paw thickness at day 1 and the thickness continued to increase until it reached a maximum at day 11. Neither the BLT1-/- nor the BLT2-/-

mice showed any significant increase in paw thickness at any point in the experiment. The ankle thickness shown in 29b had similar kinetics to the paw thickness with the BLT1/BLT2+/+ mice beginning to increase at day 1 and reaching a maximum ankle thickness at day 9, while neither the BLT1-/- mice nor BLT2-/- mice showed any considerable increase. Representative histology is shown in Figure 30. A large influx of inflammatory cells was seen in the bottom left of the BLT1/BLT2+/+ joint section at 40X magnification. When the section was viewed at 400X magnification, individual inflammatory cells could be seen. Joint sections from BLT1-/- and BLT2-/- mice were almost completely devoid of any signs of inflammation.

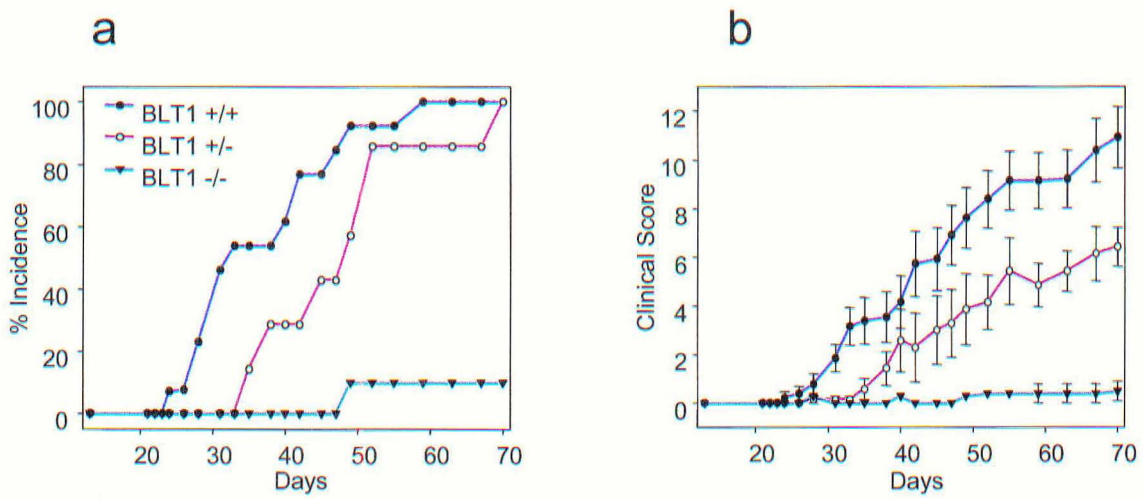


Figure 26: Collagen Induced Arthritis (CIA) on DBA/1 Background: BLT1 +/+ (blue), BLT1 +/- (pink) and BLT1 -/- (light blue) mice on DBA/1 back ground were used in the CIA mouse model. These mice were observed every 2-3 days for visual signs of arthritis. (a) Mice were considered arthritic when they demonstrated a stable clinical score of at least 2 (b) Clinical score was determined as described in Methods BLT1 +/+ (n=13), BLT1 +/- (n=7), BLT1 -/- (n=10)

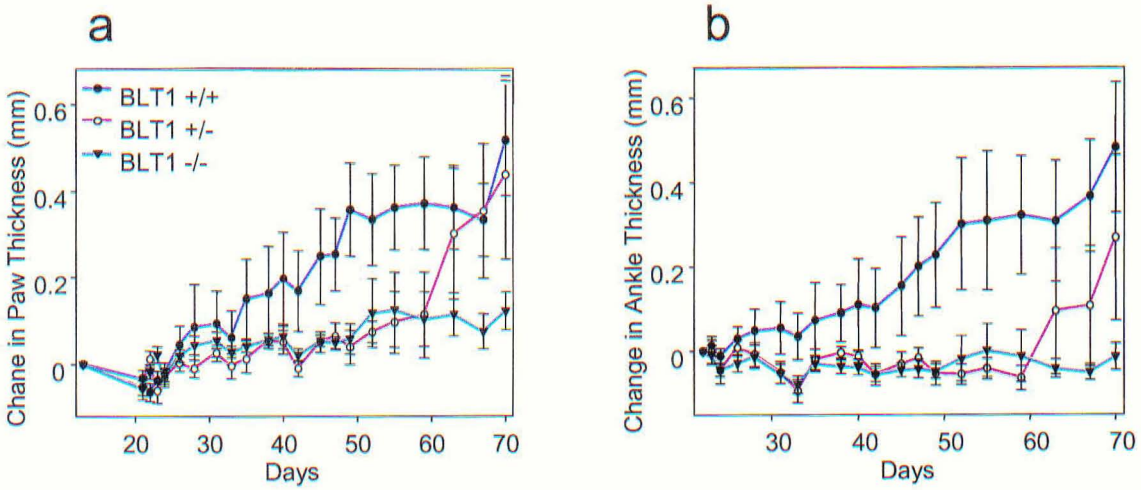


Figure 27: Change in Paw and Ankle Thickness in DBA/1 CIA. BLT1 +/+ (blue), BLT1 +/- (pink) and BLT1 -/- (light blue) mice on DBA/1 back ground were used in CIA model. These mice were followed every 2-3 days for increases in paw (a) and ankle (b) thickness using a dial pocket thickness gauge.

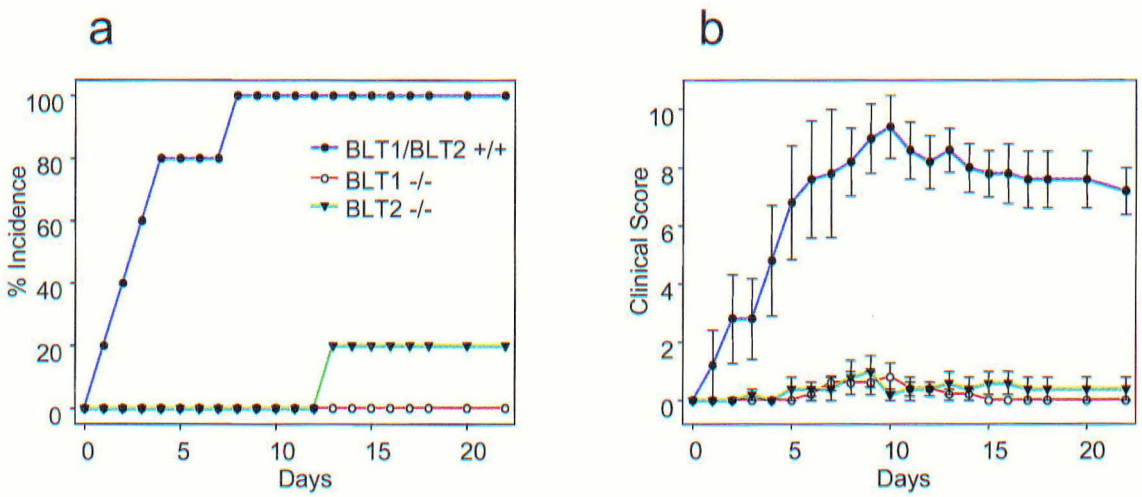


Figure 28: K/BxN Serum Induced Arthritis Model. K/BxN serum (100 µl) was injected i.p on days 0 and 2 in BLT1/BLT2 +/+ (blue), BLT1 -/- (red) and BLT2 -/- (green) mice. These mice were observed every 1-2 days for visual signs of arthritis. (a) Mice were considered arthritic when they demonstrated a stable clinical score of at least 2 (b) Clinical score was determined as described in Methods BLT1 +/+ (n=13), BLT1 +/- (n=7), BLT1 -/- (n=10)

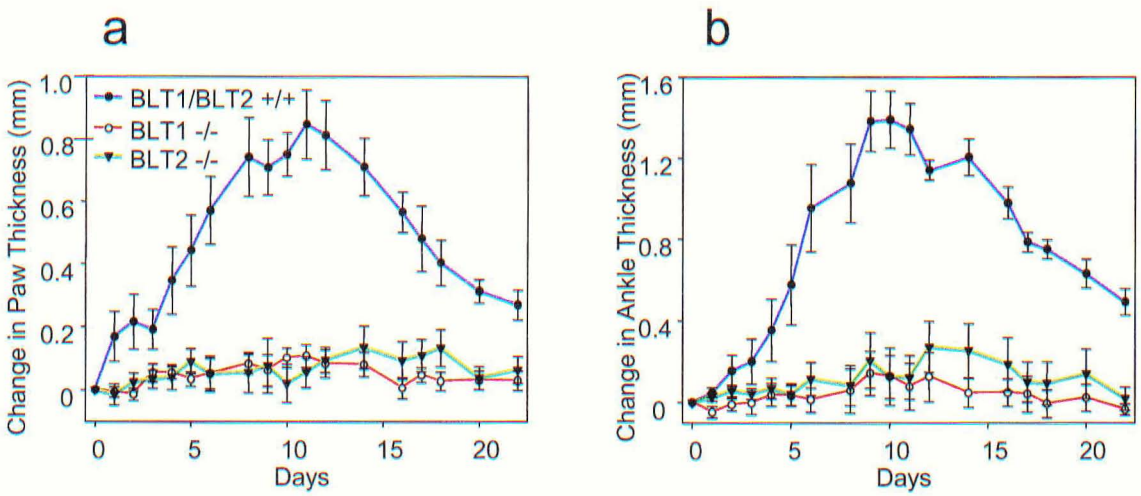


Figure 29: Change in Paw and Ankle Thickness of BLT1/BLT2 +/+, BLT1 -/-, and BLT2 -/- Mice in K/BxN Arthritis. BLT1/BLT2 +/+ (blue), BLT1 -/- (red) and BLT1 -/- (green) mice were followed every 1-2 days for increases in paw (a) and ankle (b) thickness using a dial pocket thickness gauge.

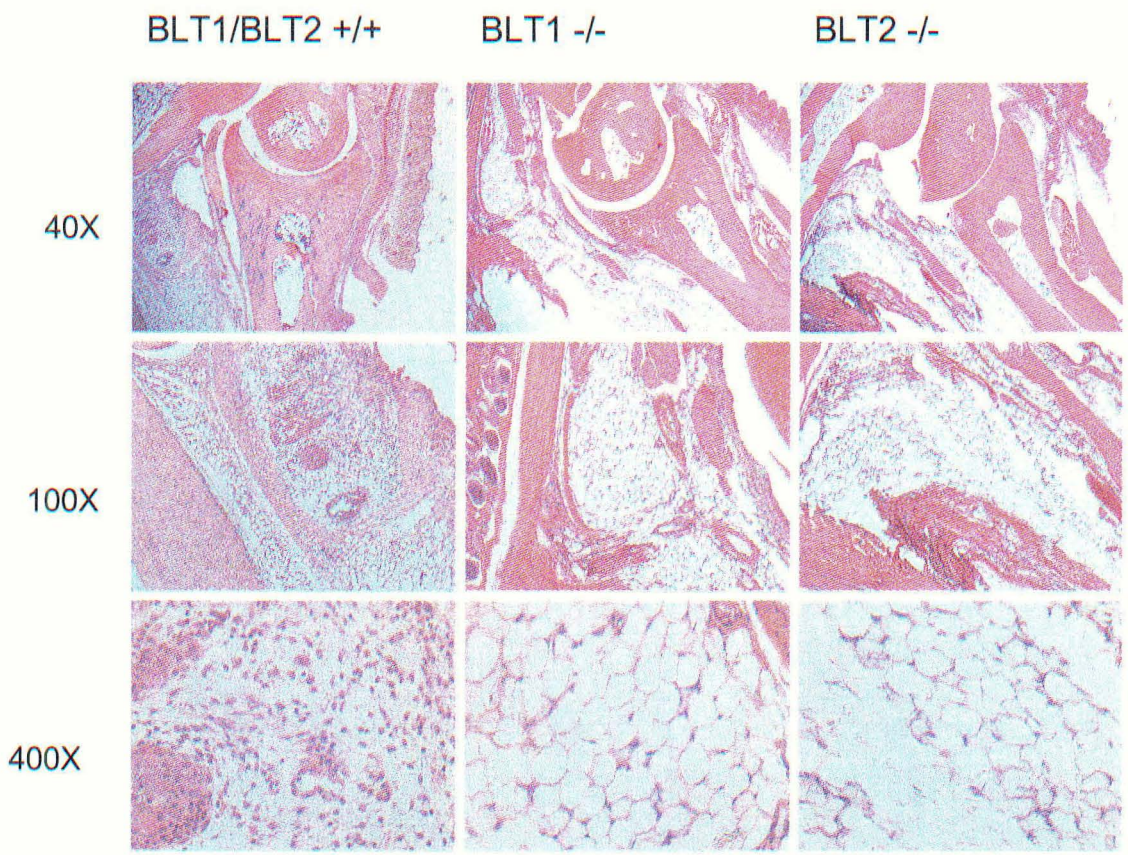


Figure 30: Histology of K/BxN Ankle Joints. The ankle joints of BLT1/BL2 +/+, BLT1 -/- and BLT2 -/- mice from mice subjected to induction of K/BxN arthritis were sectioned and stained with H and E. The images were obtained using Nikon Eclipse E400 microscope. Images at 40X magnification demonstrate infiltration of inflammatory cells in BLT1/BLT2 +/+ mice, but not in BLT1 -/- and BLT2 -/- mice. Inflammatory cells become more apparent at 100X magnification. At 400X magnification, multiple individual inflammatory cells are seen in the BLT1/BLT2 +/+, while no inflammatory cells are present in the BLT1 -/- and BLT2 -/- pictures.

BLT2 is Required on Bone Marrow-Derived Cells for the Development of Arthritis

To determine whether the presence of BLT2 is required on bone marrow-derived cells or other lineages, adoptive transfer experiments were undertaken. Recipient mice were irradiated with 900 rads to completely eliminate all bone marrow and peripheral blood leukocytes. The following day, bone marrow was isolated from donor animals and injected i.v. into the recipient mice. Mice which are congenic for the SJL subtype of CD45 antigen (CD45.1 or Ly5.1) were used in these experiments to distinguish reconstituted leukocytes from any remaining recipient leukocytes (CD45.2 or Ly5.2). Commercially available antibodies to these subtypes were used. The mice were tested for their CD45 expression to determine how well the transplantation worked. The CD45 flow cytometry analysis is shown in Figure 31 for BLT2+/+ mice receiving BLT2+/+ bone marrow (31a), BLT2-/- mice receiving BLT2+/+ bone marrow (31b) and BLT2+/+ mice receiving BLT2-/- bone marrow (31c) and demonstrate ~ 95-98% donor neutrophils in the peripheral blood. Recipient mice were allowed to engraft for at least 8 weeks and were then started on the K/BxN serum transfer arthritis protocol. In Figures 32 and 33, the results of this experiment are shown. These data are representative of three independent experiments. The clinical scoring of these mice is shown in 32b and shows BLT2+/+ mice receiving BLT2+/+ bone marrow (+/+D/+/+R (blue line)) and BLT2-/- mice receiving BLT2+/+ bone marrow (+/+D/-/-R mice (purple line)) have an almost identical course of disease reaching a maximum on day 9 while BLT2+/+ mice receiving BLT2-/- bone marrow (-/-D/+/+R mice (orange line)) show very minimal signs of disease. The incidence of disease is shown in 32a where it is seen that both +/+D/+/+R and +/+D/-/-R mice reach 100% incidence on day 7 and -/-D/+/+R mice reach 20% on

the last day of the experiment again due to one mouse getting disease in one paw. In +/+D/+/+R mice, an increase in paw thickness is seen by day 2 of the experiment and reaches a maximum around day 8. +/+D/-/-R mice show the same kinetics as the BLT2+/+ recipients demonstrating an increase in paw thickness by day 2 and reaching a maximum at day 7. -/-D/+/+R mice were completely protected from signs of increased paw thickness more than some transient increases. A similar trend as 33a is seen in 33b where both +/+D/+/+R and +/+D/-/-R show an increase in ankle thickness on day 2 and reach a maximum around day 9 and -/-D/+/+R showing no drastic increase in ankle thickness. Analysis of H & E staining of joint sections from these mice is shown in Figure 34. BLT2+/+ mice reconstituted with BLT2+/+ bone marrow and BLT2-/- mice reconstituted with BLT2+/+ bone marrow both show a drastic influx of inflammatory cells at 40X magnification. At 400X magnification, inflammatory cells are easily seen in these mice. BLT2+/+ mice receiving BLT2-/- bone marrow show no signs of inflammation at 40X magnification. The same region was taken at 400X magnification as the mice reconstituted with BLT2+/+ showed no inflammatory cells.

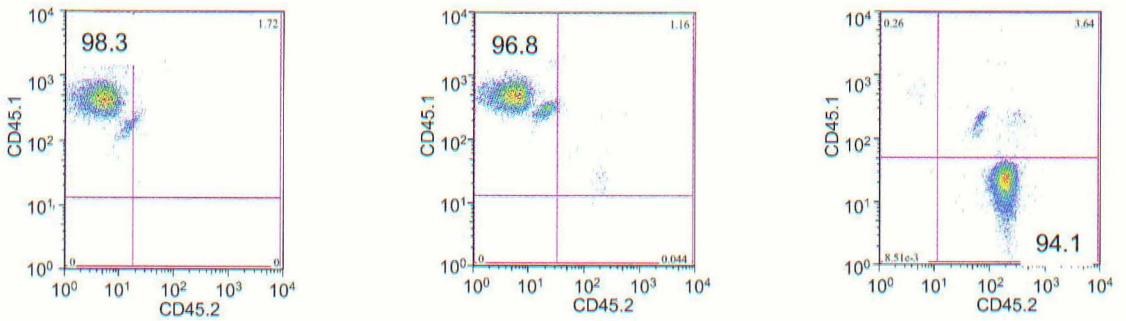


Figure 31: CD45 Expression Pattern of BLT2 +/+ and BLT2 -/- Reciprocal Bone Marrow Transplants. Whole blood from mice was stained with CD45.1-PE and CD45.2-FITC. CD45.2+ BLT2 +/+ and BLT2 -/- mice were reconstituted with CD45.1+ BLT2 +/+ bone marrow from C57Bl/6 SJL congenic mice. CD45.1+ BLT2 +/+ mice were reconstituted with CD45.2+ BLT2 -/- bone marrow. Both BLT2 +/+ and BLT2 -/- recipients of BLT2 +/+ bone marrow demonstrated > 96% of peripheral neutrophils were of donor origin. BLT2 +/+ recipients of BLT2 -/- bone marrow demonstrated > 94% of peripheral neutrophils were of donor origin.

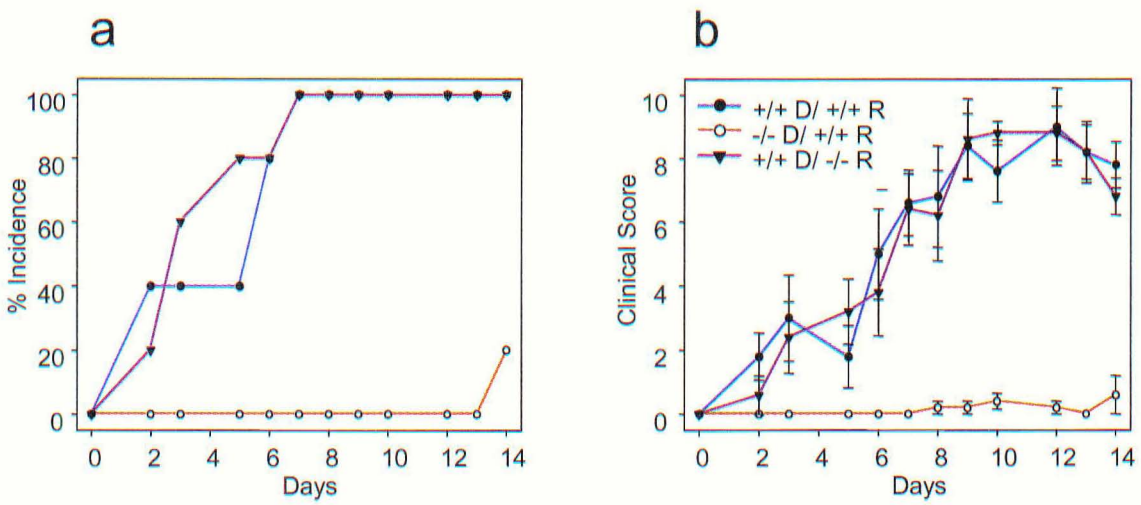


Figure 32: Reciprocal Bone Marrow Transplants Between BLT2 +/+ and BLT2 -/- Mice in K/BxN Arthritis. BLT2 +/+ recipient mice receiving BLT2 +/+ bone marrow (+/+ D/ +/+ R), BLT2 +/+ recipient mice receiving BLT2 -/- bone marrow (-/- D/ +/+ R) and BLT2 -/- recipient mice receiving BLT2 +/+ bone marrow (+/+ D/ -/- R) were observed every 1-2 days for visual signs of arthritis. ***(a) The incidence of clinical disease*** is shown demonstrating +/+ D/ +/+ R (blue), +/+ D/ -/- R (purple) and -/- D/ +/+ R (orange) Mice were considered to have disease when they reached a clinical score of 2. ***(b) Clinical score*** was determined as described in Methods.

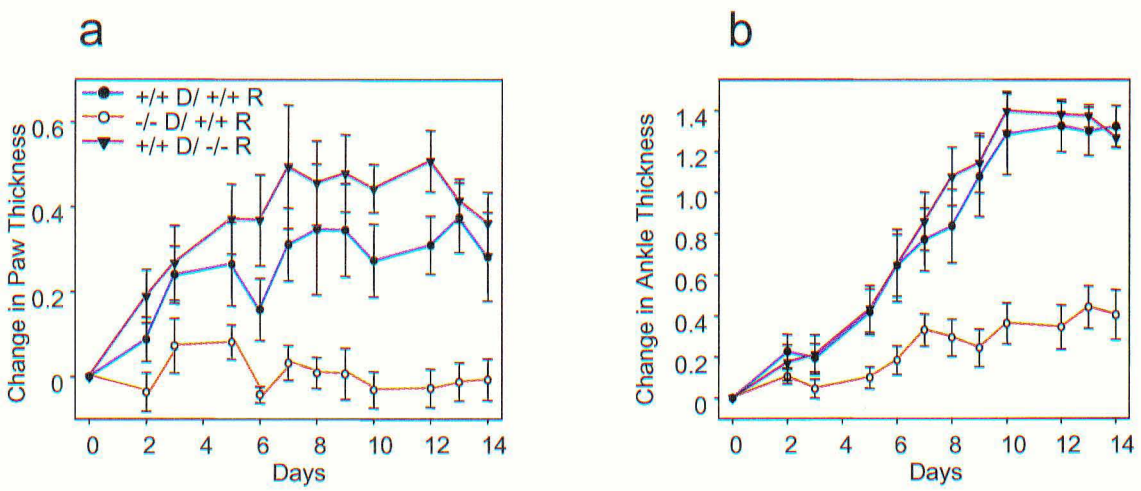


Figure 33: Change in Paw and Ankle Thickness of Reciprocal Bone Marrow Transplant Mice in K/BxN Arthritis. +/+ D/ +/+ R (blue), -/- D/ +/+ R (orange) and +/+ D/ -/- R (purple) mice were followed every 1-2 days for increases in ***paw (a)*** and ***ankle (b)*** thickness using a dial pocket thickness gauge.

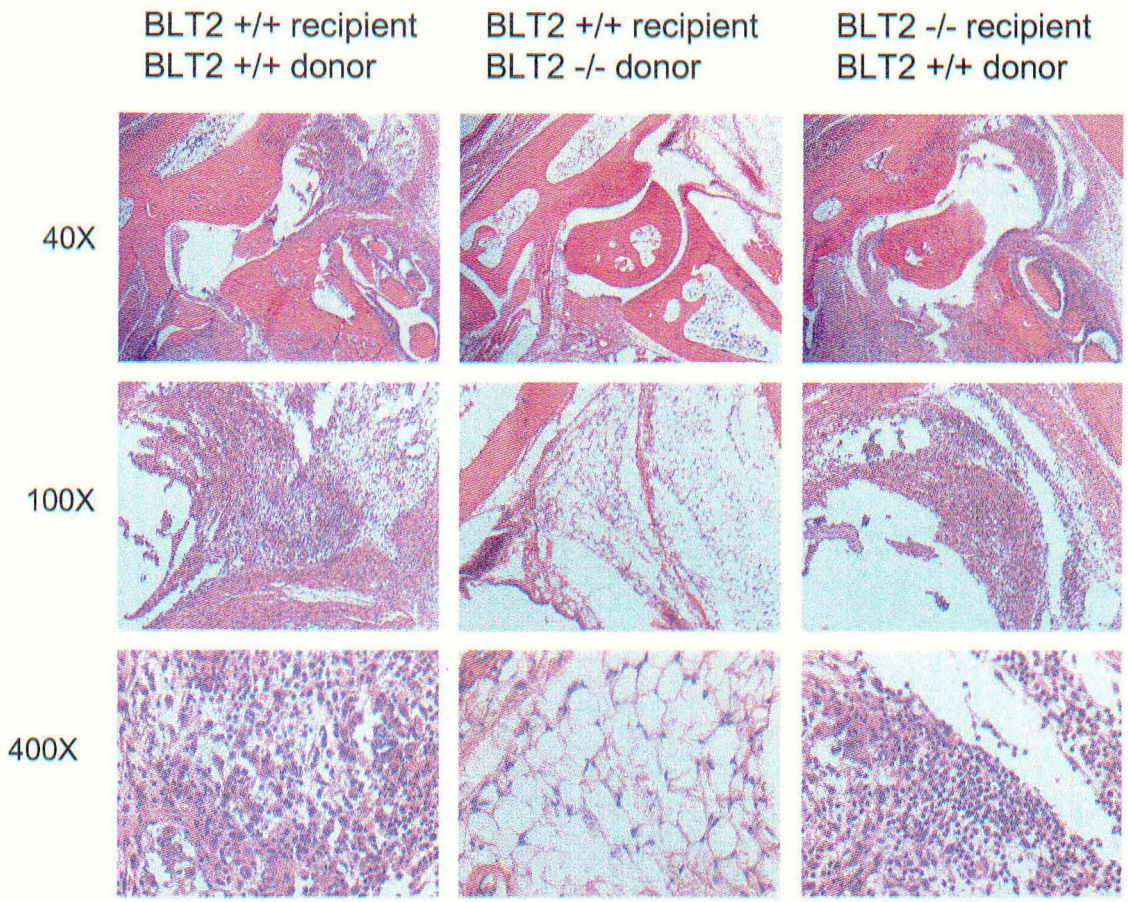


Figure 34: Histology of Ankle Joints for Reciprocal Bone Marrow Transplant Mice in K/BxN Arthritis Experiments. The ankle joints of were sectioned and stained with H and E. The images were obtained using Nikon Eclipse E400 microscope. Pictures at 40X magnification demonstrate infiltration of inflammatory cells in BLT2 +/+ mice reconstituted with BLT2 +/+ bone marrow and BLT2 -/- mice reconstituted with BLT2 +/+ bone marrow, but not in BLT2 +/+ mice reconstituted with BLT2 -/- bone marrow. Inflammatory cells become more apparent at 100X magnification. At 400X magnification, multiple individual inflammatory cells are seen in the BLT2 +/+ reconstituted with BLT2 +/+ bone marrow and BLT2 -/- reconstituted with BLT2 +/+ bone marrow pictures, while no inflammatory cells are present in the BLT2 +/+ reconstituted with BLT2 -/- bone marrow.

BLT1 and BLT2 are Non-Redundantly Required for Arthritis Development

Kim et al. previously demonstrated that BLT1-/- mice do not develop arthritis in the K/BxN model, but develop disease if they are given BLT1+/+ neutrophils (158). However, greater than 90% of the neutrophils in the synovium at the end of the experiment were all BLT1-/- as determined by CD45 phenotyping. We believe BLT1-/- neutrophils are likely recruited to the synovium via expression of BLT2. Given that we have mice deficient in BLT1 and BLT2, we have an excellent system to test this hypothesis. If the preliminary stages of arthritis require BLT1 expressing neutrophils and the latter stages BLT2 expressing neutrophils, then reconstituting BLT1/BLT2-/- mice with equal numbers of BLT1-/- bone marrow cells (which should have functional BLT2) and BLT2-/- bone marrow cells (which were shown above to express functional BLT1) should lead to development of arthritis in these mice. If this hypothesis is wrong (if simultaneous or sequential expression of BLT1 and BLT2 is required), then no disease will occur. The CD45 flow cytometry analysis for BLT1/BLT2-/- mice given BLT1/BLT2+/+ bone marrow (35a) and BLT1/BLT2+/+ mice receiving a mixture of BLT1-/- and BLT2-/- bone marrow (35b) and analysis of BLT1/BLT2+/+ mice given a mixture of BLT1-/- and BLT2-/- bone marrow with 3D7 and GR-1-PE (35c) is shown in Figure 35. All the mice used in this experiment displayed ~ 95-98% donor neutrophils in the peripheral blood with examples shown in 35a and 35b. Analysis of BLT1/BLT2+/+ which were reconstituted with a mixture of BLT1-/- and BLT2-/- bone marrow, showed that ~ 50% of the peripheral neutrophils arose from BLT2-/- bone marrow while the other 50% was from BLT1-/- bone marrow in 35c. Results shown in Figures 36 and 37 are those generated from BLT1/BLT2+/+ or BLT1/BLT2-/- mice reconstituted with a

mixture of 50% BLT1-/- and 50% BLT2-/- bone marrow or with BLT1/BLT2+/+ bone marrow. Controls are shown in Figures 36 and 37 where wild-type mice were reconstituted with BLT1-/- or BLT2-/- bone marrow. Both BLT1/BLT2+/+ (blue line) and BLT1/BLT2-/- mice (red line) reconstituted with BLT1/BLT2+/+ bone marrow reached 100% incidence, though the BLT1/BLT2-/- recipients were delayed in reaching 100% by four days compared to the BLT1/BLT2+/+ recipients. This showed in the clinical score in Figure 36b also, where BLT1/BLT2+/+ recipients reached a higher maximum clinical score and reached it four days sooner than the BLT1/BLT2-/- recipient mice. Though reconstitution with BLT2-/- bone marrow (orange line) or BLT1-/- bone marrow (light blue line) led to no disease, there was a definite increase in clinical score in both BLT1/BLT2+/+ (pink line) and BLT1/BLT2-/- mice (green line) reconstituted with 50% BLT1-/- and 50% BLT2-/- bone marrow peaking at day 9 for the BLT1/BLT2+/+ recipients and day 10 for the BLT1/BLT2-/- recipients. The fact that BLT1/BLT2-/- recipients showed an increase in clinical score compared to BLT1/BLT2+/+ recipients is due in part because BLT1/BLT2-/- recipients had a 60% incidence of disease, while BLT1/BLT2+/+ recipients had a 40% incidence of disease, as shown in Figure 36a. In Figure 37a, it is shown once again that BLT1/BLT2+/+ mice reconstituted with BLT1/BLT2+/+ bone marrow developed arthritis quickly with an increase in paw thickness seen on day 2 and reaching a maximum at day 4. BLT1/BLT2-/- mice reconstituted with BLT1/BLT2+/+ bone marrow showed a similar maximum paw thickness, though the kinetics were delayed such that these mice did not reach a maximum until day 9. BLT1/BLT2+/+ or BLT1/BLT2-/- mice reconstituted with a mixture of BLT1-/- and BLT2-/- bone marrow did not show any drastic increase in paw

thickness at any point. In Figure 37b, BLT1/BLT2+/+ mice reconstituted with BLT1/BLT2+/+ bone marrow started to increase in ankle thickness by day 2 of the experiment and reached a maximum thickness at day 9. Similar to the results seen with the paw thickness measurement, BLT1/BLT2-/- reconstituted with BLT1/BLT2+/+ bone marrow reached the same maximum level of ankle thickness, though the kinetics were slightly delayed. A much more noticeable increase was seen in both BLT1/BLT2+/+ and BLT1/BLT2-/- mice reconstituted with 50% BLT1-/- and 50% BLT2-/- bone marrow.

In Figures 36 and 37, wild-type mice reconstituted with BLT1-/- or BLT2-/- bone marrow are shown. In Figure 36a, no disease incidence is shown in wild-type mice reconstituted with either knockout bone marrow. In Figure 36b, a transient increase in BLT1-/- reconstituted mice is seen that quickly wanes, while no increase is seen in BLT2-/- reconstituted mice. No significant increases in paw or ankle thickness were seen in either set of mice in Figures 37a or b.

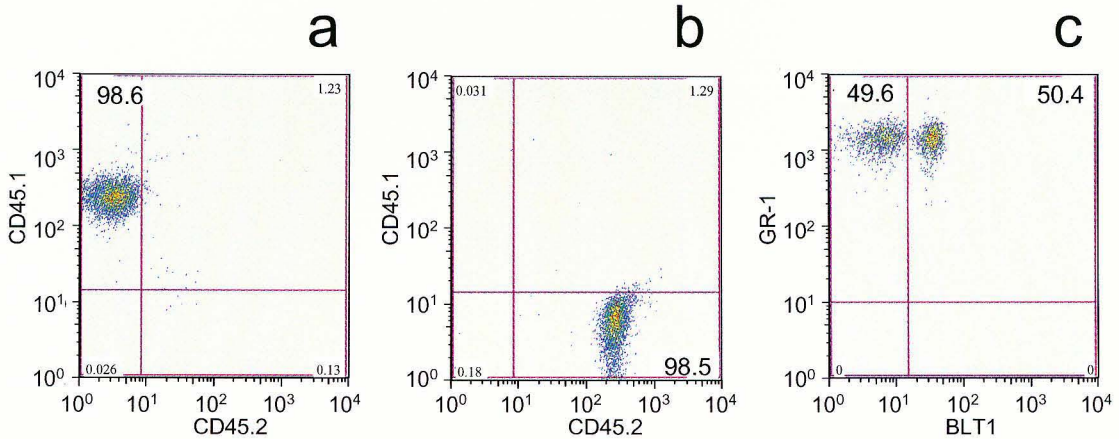


Figure 35: Surface Receptor Characterization of Bone Marrow Transplant Recipient Mice. Whole blood was stained with CD45.1-PE, and CD45.2-FITC. CD45.2+ BLT1/BLT2 -/- mice were reconstituted with CD45.1+ BLT1/BLT2 +/+ bone marrow. CD45.1+ BLT1/BLT2 +/+ mice were reconstituted with a 50% mixture of CD45.2+ BLT2 -/- bone marrow and 50% of CD45.2+ BLT1 -/- bone marrow. a) CD45.2+ BLT1/BLT2 -/- mice reconstituted with CD45.1+ BLT1/BLT2 +/+ bone marrow displayed > 98% of peripheral neutrophils were of donor origin . b) CD45.1+ BLT1/BLT2 +/+ mice reconstituted with a 50% mixture of CD45.2+ BLT2 -/- bone marrow and 50% of CD45.2+ BLT1 -/- bone marrow displayed > 98% peripheral neutrophils were of donor origin. c) Staining of the bone marrow cells with 3D7 (mBLT1 specific monoclonal antibody) and GR1 antibody.

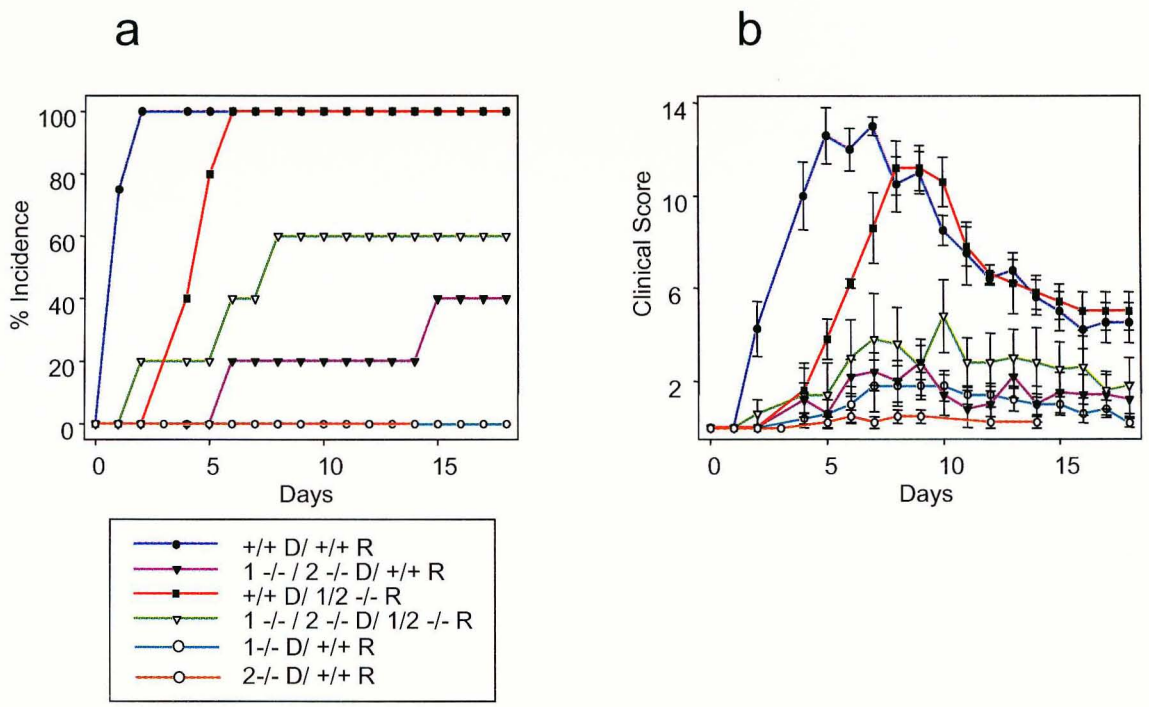


Figure 36: Mixed BLT1 -/- / BLT2 -/- Bone Marrow Transplants in K/BxN Arthritis. BLT1/BLT2 +/+ recipient mice reconstituted with BLT1/BLT2 +/+ bone marrow (+/+ D/ +/+ R), BLT1/BLT2 +/+ recipient mice reconstituted with 50% BLT1 -/- and 50% BLT2 -/- bone marrow (1 -/- / 2 -/- D/ +/+ R), BLT1/BLT2 -/- recipient mice reconstituted with BLT1/BLT2 +/+ bone marrow (+/+ D/ 1/2 -/- R) and BLT1/BLT2 -/- recipient mice reconstituted with 50% BLT1 -/- and 50% BLT2 -/- bone marrow bone marrow (1 -/- / 2 -/- D/ 1/2 -/- R were observed every 1-2 days for visual signs of arthritis. ***(a) The incidence of clinical disease*** is shown demonstrating +/+ D/ +/+ R (blue), +/+ D/ 1/2 -/- R (red), 1 -/- / 2 -/- D/ +/+ R (pink) and 1 -/- / 2 -/- D/ 1/2 -/- R (green) mice. . Mice were considered to have disease when they reached a clinical score of 2. ***(b) Clinical score*** was determined as described in Methods

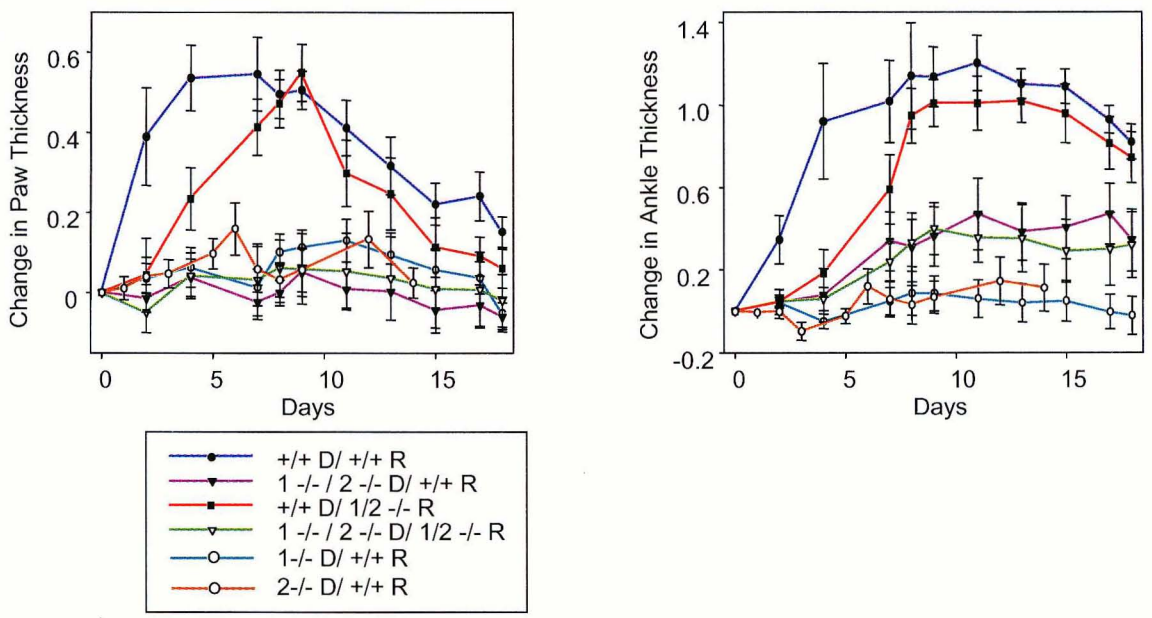


Figure 37: Change in Paw and Ankle Thickness of Mixed BLT1 -/- and BLT2 -/- Bone Marrow Transplant Mice in K/BxN Arthritis. +/+ D/ +/+ R (blue), 1 -/- / 2 -/- D/ +/+ R (pink), +/+ D/ 1/2 -/- R (red) and 1 -/- / 2 -/- D/ 1/2 -/- R (green) mice were followed every 1-2 days for increases in paw (a) and ankle (b) thickness using a dial pocket thickness gauge.

DISCUSSION

Previous data from our laboratory showed that mice deficient in BLT1 or BLT1/BLT2 are completely protected from CIA on the C57Bl/6 background. BLT1/BLT2 +/- mice displayed a delay in the onset and a lower incidence of disease, but those mice that got disease had close to the same level of disease as wild-type mice. The data presented in this thesis confirm all the previous findings of an essential role for BLT1 in murine arthritis. More importantly, they provide the first direct evidence for a biological activity associated with BLT2 because targeted disruption of BLT2 led to a complete protection against the development of serum transfer inflammatory arthritis.

BLT1 is Critical for Arthritis Development

A large body of data accumulated over the last decade has implicated LTB_4 in arthritis. Despite persistent efforts from the pharmaceutical industry, no clear therapeutics targeting this pathway are available for arthritis. The current experiments showed complete protection of BLT1-/- mice against development of arthritis the DBA/1 background for CIA. The BLT1 +/- mice are delayed in incidence of disease compared to wild-type littermates. They do reach 100% incidence by the end of the experiment, but even at that time-point, the clinical score was significantly reduced compared to the wild-type mice ($p< 0.05$). Measurement of the thickness of both the paw and ankle demonstrated a direct measure of the inflammation seen in these mice and once again, a delay in thickness is seen in the BLT1 +/- mice in both the paw and ankle. In all these parameters, BLT1-/- mice were greatly protected showing very minimal signs of disease

in one mouse only. These data confirm that loss of BLT1 protects mice from collagen induced arthritis regardless of the background of the mice. It is also in agreement with the BLT1 antagonist study presented by Griffiths et al. in which they showed the antagonist CP-105,696 was almost completely effective in blocking clinical signs of disease (154). When they gave the mice an injection of IL-1 to accelerate the disease, they saw a significant increase in disease severity in the mice treated with the lower concentration of antagonist and a small increase in the animals treated with the higher concentration of antagonist (157). Subsequent publications all used accelerated arthritis models and only saw partial protection from arthritis. When two other antagonists, LY293111Na and CGS25019C, were used in an accelerated model of CIA, the decrease was not as significant as the previous study (only 38% reduced) (155). It is entirely possible that the antagonists used in Kuwabara et al. were not as efficacious as CP-105,696. However, the fact that CP-105,696 was almost completely protective in those experiments until IL-1 was given to boost the mice demonstrates one simple point. BLT1 antagonism works well to block disease in non-accelerated CIA and IL-1 acceleration results in an inflammatory process that is not completely blocked by BLT1 antagonism. A straight-forward reason could be increased production of LTB_4 in the synovium beyond the point where CP-105,696 or the other antagonists are effective. In fact, when Kuwabara et al. tested the levels of LTB_4 in the paws of these mice with and without the IL-1 acceleration, they saw a significant increase in LTB_4 levels in the IL-1 accelerated mice over saline injected CIA mice at 6 hours (155). When they checked the LTB_4 levels at 24 hours, IL-1 accelerated mice had 3X the levels of LTB_4 in the paw than saline injected CIA mice. It is logical to suggest that if the levels of LTB_4 in a mouse triple,

that the same amount of BLT1 antagonist would not be as effective. Another possible explanation for reduced effectiveness of BLT1 antagonists in IL-1 accelerated CIA models is that at high concentrations of LTB_4, maintenance of arthritis in these mice could be switched to BLT2 (see below). In this case, while a strong BLT1 inhibitor would work at initiation steps, once disease is initiated it might be more dependent on BLT2. In the same study, they also found increased levels of prostaglandin E_2 (PGE_2) in IL-1 accelerated paws over saline controls. PGE_2 is an inflammatory molecule and could explain this increase in clinical disease also. In an IL-18 accelerated CIA protocol, a 5-LO inhibitor was shown effective in lowering the severity of disease, while it did not lower disease incidence by any significant level (99). Again in these experiments, if 5-LO is activated by accelerated arthritis, then the inhibitor of 5-LO would not be nearly as effective in blocking activity. When both the BLT1 antagonists and 5-LO antagonists were used in the K/BxN model which is not an accelerated disease, they were capable of almost entirely eliminating signs of disease. Thus, previous findings of effective inhibition of murine arthritis by BLT1 antagonists but clinical failure of the same inhibitors as arthritis treatment will have to be reexamined in relapsing and remitting type models of arthritis, similar to the EIA models. Unfortunately no such models are currently available and further experimentation might have to rely on repeated immunization or aggressive disease induction by cytokines like IL-1 or use of repeated induction of disease with the serum transfer.

BLT2 is Critical for Serum Transfer Inflammatory Arthritis

The K/BxN model of arthritis has proven to be useful in determining which cells are important in the effector phase of arthritis, by bypassing the steps of antigen processing and display of auto-reactive peptides on dendritic cells and T and B cell involvement. Having seen the result in CIA that BLT1 -/- mice and BLT1/BLT2 -/- both were protected completely from disease development, we were not certain BLT2 played much of a role in arthritis. When we used BLT2 -/- mice in the K/BxN arthritis model, however, we found that these mice were almost completely protected from disease development. Very minimal signs of disease were noted with only one mouse showing disease in one leg. This finding was also visualized in sectioning of the ankle joint, where a large influx of inflammatory cells and bone destruction was seen in BLT2 +/+ sections, while little to no inflammation and bone destruction was seen in BLT2 -/- sections. Our results also confirm the data of Kim et al. who saw a protection conferred by the loss of BLT1 (158). We used our BLT1 -/- mice in this experiment and also did not see any signs of disease in these mice either by macroscopic measures or in the analysis of ankle sections. Thus, there is an apparent non-redundant requirement for both BLT1 and BLT2 in serum transfer-induced arthritis.

Since the expression of BLT2 in mice is not clearly established, it was difficult to infer the cell type specificity of BLT2 expression required for induction of arthritis. To more fully understand this requirement, we performed reciprocal bone marrow transfer experiments between BLT2 +/+ and BLT2 -/- mice with a BLT2 +/+ mouse receiving BLT2 +/+ bone marrow serving as a positive control. Using this strategy, if the expression of BLT2 is critical on bone marrow-derived cells, then recipient mice which

receive BLT2 +/+ bone marrow will get disease whether they express BLT2 or not, thus making BLT2 -/- mice capable of developing arthritis and BLT2 +/+ mice unable to get arthritis. If the expression of BLT2 is required on non-bone marrow derived cells, then expression of BLT2 on the donor bone marrow would have no effect and BLT2 +/+ mice would get arthritis receiving BLT2 +/+ or BLT2 -/- bone marrow. To determine the extent of bone marrow engraftment, we utilized C57Bl/6.SJL BLT2 +/+ mice which carry the SJL CD45.1 marker, while our C57Bl/6 BLT2-/- mice have the CD45.2 marker. Utilizing this strategy, we found that only about 2-5% of circulating neutrophils were derived from recipient bone marrow, which means 95-98% were produced from the donor bone marrow. This experiment demonstrated an absolute requirement for BLT2 on bone marrow-derived cells in arthritis induction. BLT2 +/+ and BLT2 -/- mice receiving BLT2 +/+ bone marrow both developed arthritis at similar levels. In fact, the BLT2 -/- recipients consistently demonstrated slightly higher disease parameters than the BLT2 +/+ recipients receiving BLT2+/+ marrow (positive control). BLT2 +/+ mice receiving BLT2 -/- bone marrow were protected from development of arthritis indicating absolutely no role for BLT2 on non-bone marrow-derived cells in arthritis development. Histopathological examination revealed massive inflammatory cell influx in the BLT2-/- mice receiving BLT2+/+ marrow. Since both of these mice express similar levels of BLT1, it is clear that expression of BLT1 alone in BLT2-/- mice is not sufficient for arthritis induction.

Induction of Arthritis in BLT1/BLT2-/- mice with bone marrow reconstitution from both BLT1 -/- and BLT2 -/- deficient mice

We and others have shown that BLT1-/- mice do not develop arthritis in the K/BxN model. The data from Kim et al suggest that this is at least in part due to the fact that BLT1 must be present on neutrophils at early time-points, but is not necessary at later time points (158). One could hypothesize that BLT1 is absolutely necessary on neutrophils to initiate disease pathology. Data from Chen et al. suggested that neutrophils are the main source of LTB_4 production in the synovium once they arrive (151). This potentially leads to an accumulation of LTB_4 to a level at which effectual chemotaxis of neutrophils is not possible through BLT1. In this case, BLT2 could be the chemotactic receptor to sense these higher concentrations of LTB_4 at the site such that BLT1 is no longer necessary. Indeed, we showed above that BLT2-/- mice are protected from development of arthritis. BLT1/BLT2 -/- mice should not develop K/BxN arthritis given the results seen with the single knockout mice and the fact that BLT1/BLT2 -/- mice did not develop CIA (21). The question we sought to answer was if we reconstitute a BLT1/BLT2 -/- mouse with both BLT1 -/- BLT2 +/+ bone marrow and BLT1 +/+ BLT2 -/- bone marrow, could the presence of BLT1 in a portion of neutrophils and BLT2 on the remainder of the neutrophils lead to disease development? In other words, does expression of BLT1 and BLT2 on separate cells induce arthritis in a mouse that lacks both receptors?

To answer this question, we lethally irradiated both BLT1/BLT2 +/+ and BLT1/BLT2 -/- mice and reconstituted them with either BLT1/BLT2 +/+ bone marrow or an equal mixture of BLT1 -/- and BLT2 -/- bone marrow. After checking a blood sample

for CD45 subtype expression, the mice with the best engraftment were taken through the K/BxN model of arthritis. BLT1/BLT2 +/+ mice reconstituted with BLT1/BLT2 +/+ bone marrow demonstrated a rapid progression of disease as seen in the paw and ankle thickness and clinical score. BLT1/BLT2 -/- mice reconstituted with BLT1/BLT2 +/+ bone marrow reached similar levels of disease as BLT1/BLT2 +/+ recipients, but the kinetics of the disease process were slowed by a few days. We then demonstrated above that BLT2 +/+ mice reconstituted with BLT2 -/- bone marrow did not develop arthritis. A control group of BLT1/BLT2 +/+ mice was reconstituted with BLT1 -/- bone marrow and these mice also did not develop any major signs of arthritis. Reconstituting BLT1/BLT2 +/+ and BLT1/BLT2 -/- mice with a mixture of both knock-out mouse bone marrow, however, led to an increase in signs of arthritis over reconstitution with either single knock-out bone marrow. Both BLT1/BLT2 +/+ and BLT1/BLT2 -/- recipients showed an increase in ankle thickness, clinical score and incidence over time with BLT1/BLT2 -/- recipients showing more of an increase in clinical score and incidence than that of BLT1/BLT2 +/+ recipients. Though the recipient mice did not get a wild-type level of arthritis when reconstituted with a mixture of BLT1 -/- and BLT2 -/- bone marrow, there is an undeniable increase in signs of arthritis. One possible explanation for this result is that BLT1 and BLT2 have to be found on the same cell for full disease progression. Kim et al. demonstrated a definite increase in disease parameters when BLT1 -/- mice were reconstituted with BLT1 +/+ neutrophils, but the level of the increase in ankle thickness and clinical score was about half of the level seen in BLT1 +/+ mice (158). This is similar to the level of disease that was seen in our BLT1 -/- and BLT2 -/- bone marrow mixture experiment. Since the coding regions of BLT1 and BLT2 are so close on the

chromosome and the promoter for BLT1 is found in the coding region of BLT2, it is possible that the two receptors could regulate one another and this is the reason BLT1 and BLT2 expression on separate cells is not enough to develop full disease, but still produce enough inflammation for partial disease progression.

In summary, the results presented here suggest a novel and unexpected role for the low affinity LTB_4 receptor, BLT2, in arthritis. This result could explain, at least in part, the failure of many clinical trials using specific antagonists for the high affinity BLT1 receptor. Targeting both LTB_4 receptors with highly specific dual inhibitors might offer an attractive treatment option for human RA.

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CURRICULUM VITAE

NON-REDUNDANT AND CRITICAL ROLES FOR LEUKOTRIENE B_4 RECEPTORS, BLT1 AND BLT2, IN MOUSE MODELS OF INFLAMMATORY ARTHRITIS

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