LIPID MEDIATORS IN THE DEVELOPMENT AND RESOLUTION OF

EXPERIMENTAL LYME ARTHRITIS

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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EXPERIMENTAL LYME ARTHRITIS

presented by Victoria A. Blaho,

a candidate for the degree of doctor of philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

For Grandma and Grandpa Blaho and Grandma and Grandpa Vlahos,

You made this all possible by taking risks, leaving your homes and families to start over here. Your love and courage to face the unknown has done more for me than you'll ever know. I know you can see what your sacrifices have been for. Thank you so much, and I love you all.

Most importantly, for Mom and Dad,

This work is yours, because I owe everything to you. I hope I've made you both proud. I miss you so much, Daddy, but I know you'll always be with me. I love you both more than words can express, forever.

Vicki

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TABLE I

15ddPGJ$_2$	15-deoxy-Δ (delta)^{12,14}PGJ$_2$ 11-oxo-5,9-*cis*-12,14-*trans*-prostatetraenoic acid
15-PGDH	15-prostaglandin dehydrogenase
5*S*-HPETE	5*S*-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid
8*S*-HETE	8*S*-hydroxy-5*cis*, 9-*trans*, 11-*cis*, 14-*cis*-eicosatetraenoic acid
12-HDH	12-hydroxyeicosanoid dehydrogenase
12*S*-HPETE	12*S*-hydroperoxy-5*cis*, 8-*cis*, 10-*trans*, 14-*cis*-eicosatetraenoic acid
AA	arachidonic acid 20:4, ω-6; 5, 8, 11, 14 eicosatetraenoic acid
αCD40	anti-CD40
ACK	ammonium chloride, potassium buffer
AP-1	activator protein-1
ATP	adenosine triphosphate
Bb	*Borrelia burgdorferi*
BLT$_{1/2}$	leukotriene B$_4$ receptor 1 or 2
BSA	bovine serum albumin
BSK-II	Barbour Stoenner Kelly – II medium
C3H	C3H/HeJ
Ca^{2+}	calcium
CFA	complete Freund's adjuvant
COX	cyclooxygenase
COX-1/2$^{-/-}$	COX-1 or 2 gene knock-out
Coxib	COX-2 inhibitor
cPLA$_2$	cytosolic phospholipase A$_2$

cysLT	cysteinyl (peptidyl) leukotriene(s)
DBA	DBA/2J
diHETE	dihydroxyeicosatetraenoic acid
DMEM-NSP	Dulbucco's Modified Eagle medium- Nutridoma-SP
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EP	E-series prostanoid receptor
ERK	extracellularly-regulated kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FLAP	5-lipoxygenase activating protein
FOXP3	forkhead box P3
FP	F-series prostanoid receptor
HBSS	Hank's balanced salt solution
HRP	horseradish peroxidase
IFN-γ	gamma interferon
Ig	immunoglobulin
IL	interleukin
IL-1β	interleukin-1 beta
IFA	incomplete Freund's adjuvant
KC	CXCL2
kD	kiloDalton

LO	lipoxygenase
LPS	lipopolysaccharide
LT	leukotrienes
LTA$_4$	leukotriene A$_4$ 5S-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LTB$_4$	leukotriene B$_4$ 5S, 12R-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid
LTC$_4$	leukotriene C$_4$ 5S-hydroxy-6R-S-glutathionyl-7,9-trans-11,14-*cis*-eicosatetraenoic acid
LTD$_4$	leukotriene D$_4$ 5S-hydroxy-6R-(S-cysteinylglycinyl)-7,9,11-*trans*-14-*cis*-eicosatetraenoic acid
LTE$_4$	leukotriene E$_4$ 5S-hydroxy, 6R-(S-cysteinyl), 7,9-*trans*-11,14-*cis*-eicosatetraenoic acid
LXA$_4$	lipoxin A$_4$ 5S,6R,15S-trihydroxy-7,9-*trans*-11,13-*cis*,eicosatetraenoic acid
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NF-κB	nuclear factor-kappa B
NSAID	non-steroidal anti-inflammatory drug
Osp	outer surface protein
PBS	phosphate-buffered saline
PE	phycoerythrin
PG	prostaglandins
PGD$_2$	prostaglandin D$_2$ 9S,15S-dihydroxy-11-oxo-5-*cis*, 13-*trans*-prostadienoic acid

PGE$_2$ prostaglandin E$_2$
                      9-oxo-11R,15S-dihydroxy-5-cis, 13-$trans$-prostadienoic acid

PGF$_{2\alpha}$ prostaglandin F$_{2\alpha}$
                      9S,11R,15S-trihydroxy-5-cis, 13-$trans$-prostadienoic acid

PGG$_2$ prostaglandin G$_2$
                      9S,11R,-epidioxy-15S-hydroperoxy-5-cis, 13-$trans$-prostadienoic acid

PGH$_2$ prostaglandin H$_2$
                      9S,11R,-epidioxy-15S-hydroxy-5-cis, 13-$trans$-prostadienoic acid

PGHS   prostaglandin G/H endoperoxide synthase (COX)

PGI$_2$ prostaglandin I$_2$; prostacyclin
                      6,9S-epoxy-11R,15S-dihydroxy-5-cis, 13-$trans$-prostadienoic acid

PGN   peptidoglycan

RANKL   receptor activator of nuclear factor kappa B

RP-HPLC   reverse phase – high pressure liquid chromatography

RT-PCR   reverse transcription-polymerase chain reaction

SRSA   slow-reacting substances of anaphylaxis

STAg   soluble *Toxoplasma* antigen

TBST   Tris-buffered saline with Tween

TGF-β   tumor growth factor-beta

T$_{H1}$/T$_{H2}$ T-helper type-1/T-helper type-2

TLR   Toll-like receptor

TNF-α   tumor necrosis factor-alpha

TP   T-series prostanoid (thromboxane) receptor

TUNEL   (terminal transferase dUTP nick end labeling)

TX   thromboxanes

TXA$_2$ thromboxane A$_2$
                 9S,11S,-epoxy-15S-hydroxy-thromboxa-5-cis, 13-$trans$-dienoic acid

TXB$_2$ thromboxane B$_2$
                 9S,11S,15S-trihydroxy-thromboxa-5-cis, 13-$trans$-dienoic acid

VEGF   vascular endothelial growth factor

Inhibitors

Clx   celecoxib (Celebrex; C$_{17}$H$_{14}$F$_3$N$_3$O$_2$S)
                     4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]
                     benzenesulfonamide

CP-105,696   (+)-1-(3S,4R)-[3-(4-phenyl-benzyl)-4-hydroxy-chroman-7-yl]
                 cyclopentane carboxylic acid

CPX   celecoxib & CP-105,696

Mex   meloxicam (Mobic; C$_{14}$H$_{13}$N$_3$O$_4$S$_2$)
                     4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-
                     benzathiazine-3-carboxamide-1,1-dioxide

Rof   rofecoxib (Vioxx; C$_{17}$H$_{14}$O$_4$S)
                     4-(4-methylsulfonylphenyl)-3-[phenyl-5H-furan-2-one

SC-560   5-(4-chloro-phenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole

SCX   SC-560 & celecoxib

CHAPTER I

INTRODUCTION

A. *The Paradox of Inflammation*

Inflammation is the cardinal phase of the immune response. This essential process of detecting and repairing deviations in homeostasis is incited by injury or invading pathogens (1,2). Thus, initiation of acute inflammation leads to mobilization of an elegantly regulated response that eventually results in clearance of dead and dying cells, repair of damaged tissue matrices, and a resumption of normal tissue and organ functions (3).

Despite the beneficial and indispensable nature of the process, inflammation is largely approached as an obstacle to be overcome or prevent altogether, due to its inevitable involvement in chronic and autoimmune diseases such as atherosclerosis, arthritis, and bronchitis (4). Herein lies the inherent paradox of inflammation: if dysregulated, the very process meant to protect an organism could provoke more damage than the initial injury. It is therefore no surprise that inflammation is a tightly regulated enterprise involving overlapping and often opposing mechanisms. Although the steps necessary for proper evolution of inflammation have long been appreciated, it was believed that once the response had fulfilled its purpose, and the instigating stimulus removed, resolution of inflammation was then a passive process. The recruitment of inflammatory cells and production of pro-inflammatory mediators would cease, and those pro-inflammatory

mediators in the extracellular milieu or in circulation would be catabolized, leading to a steady waning of the inflammatory response. However, research within the past decade has indicated that this belief is a gross oversimplification of a well-orchestrated process of down-regulation, with complex regulatory mechanisms rivaling those governing the development of the response (5,3). More recently, it has been hypothesized that the inhibition of key pro-inflammatory mediators may actually lead to a potentiation of the inflammatory response; a seemingly contradictory outcome.

B. *Eicosanoids and Inflammation*

Eicosanoids are lipid molecules well-known for their roles in induction of the inflammatory response (6). Despite their diminutive size (20 carbon chain compared to a 6 to 70 kD cytokine or chemokine) these molecules are some of the most powerful immune modulators known. Production of eicosanoids begins when an insult or chemical signal leads to elevated intracellular Ca^{2+} levels, resulting in the translocation of phospholipases, particularly cytosolic phospholipase A_2 ($cPLA_2$), to the nuclear membrane (7). Phospholipases act on membrane phospholipids to liberate arachidonic acid (20:4, ω-6; 5, 8, 11, 14 eicosatetraenoic acid) which is then metabolized via three primary enzymatic pathways: cyclooxygenases 1 or 2 (COX-1 or -2), lipoxygenases (5-LO, 15-LO) and cytochrome P_{450} epoxygenase (7,8). Specific synthases coupled to these enzymes further metabolize intermediates to form leukotrienes (LT), prostaglandins (PG), thromboxanes (TX), or lipoxins (LX) in a manner that is dependent upon cell and tissue type, and temporal regulation (9) (Figure 1; 10).
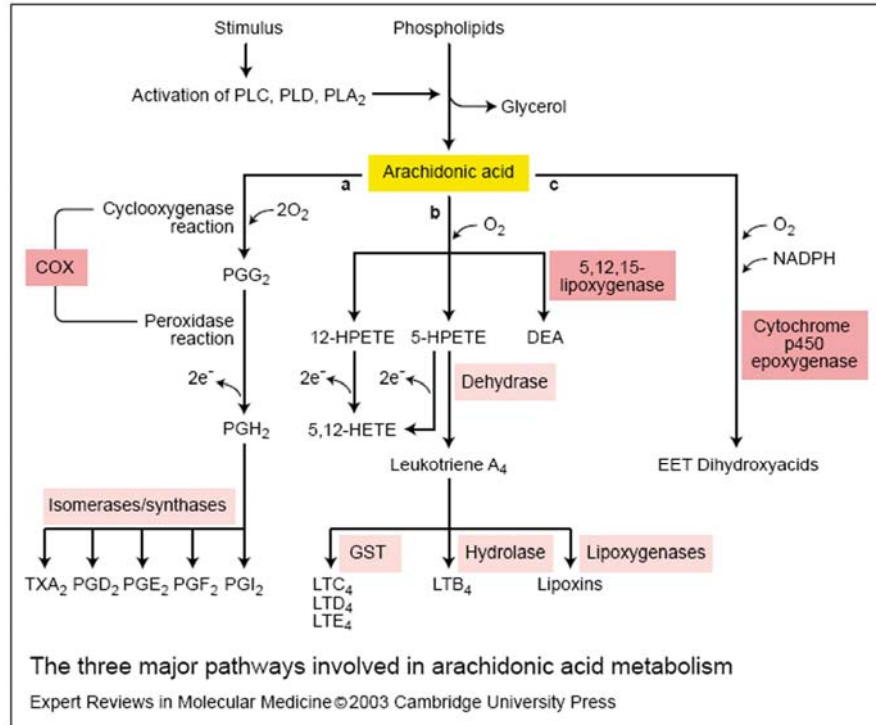
Figure 1. The three major pathways involved in arachidonic acid metabolism. Arachidonic acid is derived directly from linolenic acid or is ingested as a dietary constituent. Arachidonic acid is stored in the cell membrane of virtually all cells and is released in response to stimuli such as histamine and platelet-derived growth factor. Arachidonic acid can be released by three pathways (not shown): (1) conversion of phosphatidyl ethanolamine or phosphatidyl choline to phosphatidic acid in a reaction catalysed by phospholipase D (PLD), followed by formation of diglyceride and monoglyceride and the release of arachidonic acid; (2) degradation of phosphatidylinositol via a sequence of reactions beginning with PLC cleavage of the phosphodiester bond of membrane lipids to yield diacylglycerol, followed by the action of dilglyceride lipase and monoglyceride lipase to release arachidonic acid and glycerol; and (3) direct action of PLA_2 on a phospholipid. (a) The cyclooxygenase (COX) pathway results in the formation of prostaglandin G_2 (PGG_2) from arachidonic acid by a cyclooxygenase reaction. In a subsequent peroxidase reaction, PGG_2 undergoes a two-electron reduction to PGH_2. Both of these reactions are catalysed by COX (prostaglandin synthase H). PGG_2 serves as a substrate for cell-specific isomerases and synthases, producing other eicosanoids such as prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). (b) The lipoxygenase pathway forms hydroperoxyeicosatetraenoic acids (HPETEs) and dihydroxyeicosatetraenoic acid (DEA) by lipoxygenase and subsequently converts these to (1) hydroxyeicosatetraenoic acids (HETEs) by peroxidases, (2) leukotrienes (e.g. LTC_4) by hydrase and glutathione S-transferase (GST), and (3) lipoxins by lipoxygenases. (c) The epoxygenase pathway forms epoxyeicosatrienoic acid (EET) and dihydroxyacids by cytochrome p450 epoxygenase **(fig001dfd)**.

Borrowed from Belton and Fitzgerald. Expert Rev. Mol. Med. 2003.

Once produced, these compounds are quickly released by their producing cells to act in mostly autocrine and paracrine fashions. However, occasionally eicosanoids can be found in the circulation, presumably with endocrine activity (11).

Although often viewed as pro-inflammatory, eicosanoids are critical for maintenance of homeostasis in several organ systems, such as the nervous and circulatory systems (12,13). It is these roles that can make the inhibition of eicosanoids an especially dangerous undertaking. For example, pharmacological inhibition of the COX-2 enzyme was originally proposed to stop the production of prostaglandin E_2, (PGE_2), considered one of the key mediators of the pro-inflammatory response of arthritis (14,15). However, COX-2 is also responsible for production of PGI_2 (prostacyclin), a vasodilator and anti-platelet regulator, and a critical counterbalance to thromboxane A_2 (TXA_2), a vasoconstrictor and platelet activator, produced mainly by COX-1 (16,17). Chronic inhibition of COX-2 can therefore generate unchecked increases in TXA_2 levels in susceptible individuals and lead to unintended cardiovascular side effects (10). The balance in the regulatory functions between different eicosanoids makes the disruption of these pathways all the more complex.

Cyclooxygenases

The best characterized enzymes involved in eicosanoid metabolism are the COX enzymes. Officially named prostaglandin endoperoxide synthases, the COX enzyme is a homodimer possessing two separate active sites (18,19). The COX-1 and -2 isozymes tend to localize to different parts of the cell, with COX-1 mainly found within the

perinuclear envelope and COX-2 in the smooth endoplasmic reticulum (SER), however, they catalyze identical reactions (20,21,22). Both enzymes receive arachidonate from $cPLA_2$ and convert it first to prostaglandin G_2 (PGG_2) via their cyclooxygenase activity, then to PGH_2 by their hydroperoxidase activity. PGH_2 can then be further metabolized by specific synthases into bioactive prostaglandins or thromboxanes (18).

Despite their similarities, the COX isozymes appear to perform dramatically different biological functions. COX-1 is constitutively expressed by most cell types and is rarely up-regulated upon pro-inflammatory stimulation (23,24). Conversely, the COX-2 gene is an early immediate gene, and its protein expression is constitutive in only a few cell types or tissues, such as the kidney, neurons, and gastrointestinal tract (24,25,26,27). In most other cells its expression is stimulation-dependent (28,29). Understanding the regulation of COX-2 production and activity is not a trivial undertaking. Numerous inflammatory stimuli, such as the Toll-like receptor (TLR) ligands lipopolysaccharide (LPS) (30) or peptidoglycan (PGN) (31), the cytokines TNFα and IL-1β (32), or even the cyclooxygenase product PGE_2, can induce up-regulation of COX-2 mRNA and/or protein production (33). Once COX-2 mRNA is synthesized, its stability is one rate-limiting factor in COX-2 protein, and therefore PG, synthesis. PGE_2 itself, p38 mitogen activated protein kinase (MAPK), and extracellularly-regulated kinase (ERK) -1 or -2 appear to mediate increases in COX-2 protein and activity mainly via stabilization of its transcript; however, the exact mechanism by which this stabilization occurs is unknown (34).

The up-regulation and translocation of COX-2 protein to the smooth ER is correlated with increased cytokines, signal transducers, and other molecules involved in the inflammatory response, either dependent or independent of PGE_2 production (34,13,35). These include the cytokines IL-1β, -4, -6, -10, -11, TNFα, and IFNγ (27,14,36,37,38); elevated levels of cytosolic Ca^{2+} (13), activation of AP-1 subunits cFos and cJun (39), and feedback increases in NF-κB, production of vascular endothelial growth factor (VEGF) (40) and matrix metalloproteinases (MMPs) 1, 2, 3, 9, 11, and 13 (41,42,43). Each of these molecules has a respectable role in the regulation of immunity and are also all implicated as participants in the pathogenesis of chronic inflammatory and autoimmune diseases.

The contribution of COX-1 to the inflammatory response is incompletely understood, and appears to be disease-specific, as compared to COX-2, which seems to contribute to most inflammatory responses (44,45). However, since COX-2 protein results from *de novo* synthesis in most cell types, PG production at early time points is thought to be the result of COX-1 activity (46,22). In gastric, vascular, and pulmonary inflammation models, COX-1 regulates a return to homeostasis and the prevention of nonspecific inflammatory responses (47,48,49). Since COX-2-specific inhibitors do not reduce the production of PGs at the site of inflammation to the same extent as nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit both isoforms equally, this implies that there is a role for COX-1 in regulating inflammatory responses (48,50,51).

Attempts have been made to discern a more general contribution of COX-1 to inflammation using the COX-1-specific inhibitor SC-560. Using the reversed passive dermal Arthus reaction, animals were treated with SC-560 or celecoxib (Clx) before dermal injury was induced by intradermal injection of rabbit anti-chicken ovalbumin IgG, followed by immediate intravenous injection of chicken ovalbumin. At the site of injection, TXB levels (a stable metabolite of TXA_2) were increased 10- to 15-fold, and this increase was completely reversed by SC-560 administration. PGE_2 production, which was not increased by the reaction, was also significantly decreased by SC-560 but not Clx treatment. Complicating the issue, the use of a carageenan-induced inflammation model demonstrated that SC-560 and Clx had similar effects on PGE_2 production in the inflamed rat paw, however only the inhibition of COX-2 resulted in decreased edema (24). Since this study was published in *Proceedings of the National Academy of Sciences* by the Pfizer cyclooxygenase group in 1998, very little research has been devoted to the investigation of the role of COX-1 in the inflammatory response. As such, the contribution of COX-1 in regulating the inflammatory response, if any has not been identified to date.

One of the few studies to investigate the contribution of both cyclooxygenase isoforms to host defense compared the contribution of COX-1 and -2 to the immune response against influenza A virus. The authors found that COX-1^{-/-} mice had an enhanced inflammatory response with earlier production of the pro-inflammatory cytokines TNF-α and IL-1β, but lower PGE_2 levels, in the bronchoalveolar lavage fluid; while the COX-2^{-/-} animals exhibited blunted inflammation. The authors concluded that a deficiency in COX-2

appeared to be beneficial, whereas COX-1 deficiency was detrimental during influenza infection (52). This further supports the proposition that COX-1 does play a role in inflammation, which is characterized by regulation and counterbalance of the inflammatory response promoted by COX-2 (53).

Prostanoids: Prostaglandins and Thromboxanes

COX activation and translocation to the nuclear membrane results first in cyclooxygenation of AA to PGG_2, then reduction within the peroxidase catalytic site to PGH_2. The terminal synthases: PGE synthases (of which there are three), and $PGF_{2\alpha}$, PGD, TX, and prostacyclin (PGI) synthases, then isomerize PGH_2 to their specific product. These synthases tend to preferentially associate with one COX isoform more than the other, e.g. TX synthase associates with COX-1, and prostacyclin synthase with COX-2 (9,53,54), although the data regarding COX and synthase co-localization vary depending upon the cell types used and are derived almost exclusively from *in vitro* data.

The products of these synthases, as well as some of their subsequent breakdown products, transduce a broad spectrum of biological signals. PGE_2, perhaps the most thoroughly investigated PG, also demonstrates the widest array of known biological activity. PGE_2 can suppress IL-2 production and induce IL-4 and IL-10 in response to parasite infection (55,56), induce osteolysis via receptor activator of nuclear factor kappa B (RANKL) in synovial fibroblasts (57), induce *FOXP3* gene expression and a regulatory phenotype in $CD4^+CD25^+$ T cells (58), regulate vascular smooth muscle tone (59), alter neuronal excitability and synaptic processing leading to the sensation of pain (60), and induce the

resolution of inflammation via reductions in IL-12 and IFN-γ production and the induction of LXA_4 (33,61). In order to perform these tasks, PGE_2 is the only known lipid with four dedicated receptors, EP1-4 (62,36).

PGD_2 has a similar degree of complexity, mediating both pro-and anti-inflammatory actions, as well as maintaining vascular and gastric homeostasis (63,44,64,65). Currently, it is believed that the other prostanoids appear to perform more specialized functions. PGI_2 is vasodilatory (12) and $PGF_{2\alpha}$ mediates muscle contraction, most importantly during parturition (66). TXA_2 is a counterbalance to PGI_2 in its ability to induce vasoconstriction, however it also induces platelet activation and aggregation (54), potentiates näive T cell function (67), and regulates thymocyte apoptosis (68,69) More recently, it was discovered that these eicosanoids are not cleared purely by cell uptake or non-enzymatic degradation, but there is an enzyme specifically for the deactivation of PGs. 15-prostaglandin dehydrogenase (15-PGDH) is induced by TGF-β and catalyzes the NAD^+-dependent oxidation of PGs to 15-keto metabolites with much lower biological activity (70). Thus, this enzyme promotes the resolution of inflammation by not only altering the PG itself, but it also creates a 15-keto antagonist to the PG receptors that blocks the binding of the unoxidized PG (71,72).

Cyclooxygenase Inhibitor Specificity
Inhibition of COX, and therefore prostanoid production, is not only an important tool for the study of eicosanoid regulation of inflammation, but also for the clinical treatment of the symptoms of inflammation. The ability to differentially inhibit either COX-1 or

COX-2 was viewed as a watershed accomplishment, since scientists at the time believed that COX-2 was responsible only for the induction of the inflammatory response. It was therefore believed that the selective inhibition of COX-2 would result in a beneficial decrease in inflammation, while preserving the homeostatic functions of COX-1 (29,51).

Although we now understand that both isozymes have various homeostatic responsibilities, it was crucial at the time to understand the basis for COX selectivity, whether it was a structural difference between the enzymes themselves, differential cellular localization, or some other factor. Once this knowledge was in hand, the development of COX-2 specific inhibitors progressed rapidly (51,15).

Based on crystal structures of cloned ovine and murine COX-1 and COX-2 protein, respectively, complexed with different known and experimental inhibitors, it was discovered that, despite the highly conserved COX protein structures, COX-2 selectivity was determined primarily via a binding side pocket in the COX-2 structure (19). This was due to a deletion of a methylene group by an isoleucine to valine substitution at position 523, creating a much larger cavity for compounds to bind to the cyclooxygenase active site. Selective inhibitors of COX-2 were then able to bind more tightly to COX-2 than to COX-1. The substitution of different moieties onto the diaryl heterocyclic inhibitors, e. g. celecoxib, allows for enhanced binding in this side pocket via hydrophobic interactions.

5-lipoxygenase

5-lipoxygenase (5-LO) is so named on the basis of its ability to oxygenate the fifth carbon atom of AA, counted from its carboxylic carbon (73). There are two cellular reservoirs for 5-LO, nuclear and cytosolic. Upon activation by Ca^{2+}, ATP, or hydroperoxides, 5-LO translocates from both of these sites to bind the nuclear membrane. Once bound to the membrane, synthesis of eicosanoids requires docking with the transmembrane protein, 5-lipoxygenase activating protein (FLAP), which presumably feeds the AA substrate from $cPLA_2$ to 5-LO (73,74).

Other lipoxygenases have also been described: 8-LO, 12-LO, and 15-LO are named for their ability to affect migration of specific carbon double bonds of arachidonic, linoleic, or linolenic acids (13). This forms specific conjugated dienes, e.g., 8-LO metabolizes AA to 8*S*-hydroxy-5*cis*, 9-*trans*, 11-*cis*, 14-*cis*-eicosatetraenoic acid (8*S*-HETE), and 12-LO metabolizes AA to 12*S*-hydroperoxy-5*cis*, 8-*cis*, 10-*trans*, 14-*cis*-eicosatetraenoic acid (12*S*-HPETE) (75). Although it is known that these LOs can be expressed in leukocytes and platelets, as well as other various tissue cell-types, the biological actions of many of their products, such as different diHPETEs and HETEs, are not well characterized. However, it is known that LXs are produced by 12-LO or 15-LO metabolism of LTA_4 (76,77).

Unlike the COX enzymes, 5-LO is found only in a select group of cells, mainly those of myeloid origin. It is similar to the COX in that it is a bifunctional enzyme, possessing both 5-oxygenase and LTA_4 synthase activity (8,13). Once AA is presented to 5-LO, the

5-lipoxygenase activity results in 5S-hydroperoxyeicosatetraenoic acid (5S-HPETE), itself a biologically active compound (78), which is further metabolized via migration of a double bond and elimination of a hydroxyl moiety from the hydroperoxy group to form LTA$_4$ (79). This highly unstable intermediate is then metabolized to two different classes of leukotrienes: LTB$_4$ via LTA$_4$ hydrolase, or the cysteinyl (peptidyl) LTs, beginning with LTC$_4$, via LTC$_4$ synthase (80,81,82). The cysteinyl-LTs (cysLTs), LTC$_4$, LTD$_4$, and LTE$_4$, are also known as the Slow Reacting Substances of Anaphylaxis, or SRSA (83).

Importantly, the presence of 5-LO in a cell is not necessary for the production of LTs by that cell type. Via the process of transcellular biosynthesis, cells which do not possess 5-LO, such as platelets and endothelial cells, can acquire LTA$_4$ released from neutrophils and produce the cysLTs or LTB$_4$ (84,85). As stated above, 5-LO is expressed mainly by cells of myeloid lineage, such as neutrophils, mast cells, and macrophages, however LTA$_4$ hydrolase is expressed ubiquitously. The existence of transcellular synthesis pathways highlights the importance of well-characterized, physiologically relevant, *in vivo* models for the study of eicosanoid regulation of the inflammatory response.

Leukotrienes: LTB$_4$ and its Receptor BLT$_1$

Neutrophils are the primary cellular source of LTB$_4$, although other cells, such as red blood cells, are capable of LTB$_4$ production. LTB$_4$ synthesis is stimulated by many of the same signals that initiate PG synthesis. IL-1β, TNF-α, and IL-6, as well as PGE$_2$ and LTB$_4$ itself, can induce activation of 5-LO and LTA$_4$ hydrolase (86,87,88). Pathogen-derived molecules such as LPS and STAg (soluble *Toxoplasma* antigen) have also been

shown to up-regulate 5-LO translocation and activity (89,90,91). As an example of the role of 5-LO-derived products in the immune response, genetic deficiency of 5-LO lead to increased infection severity, as judged by footpad swelling during, *Leishmania amazonensis* infection (92).

Once produced, LTB_4 mediates most of its biological effects via two G-protein coupled receptors, BLT_1 and BLT_2 (93). BLT_1, the first LTB_4 receptor described (94), is expressed mainly on leukocytes, and highly expressed on activated neutrophils (95). Engagement of BLT_1 leads to chemotaxis, leukocyte rolling, adhesion to vascular endothelium, and extravasation (96). In neutrophils, ligation of BLT_1 imparts the characteristic of making LTB_4 the most powerful neutrophil chemoattractant known (74,96). Continued stimulation of BLT_1 on neutrophils leads to superoxide anion secretion and degranulation (96,97,95). Further stimulation via BLT_1 prevents neutrophil apoptosis, however studies demonstrating increased neutrophil survival have only been performed *in vitro* (98,99). The other mechanism for LT regulation is via degradation, which is similar to that regulating the prostanoids. An enzyme much like 15-PGDH, 12-hydroxyeicosanoid dehydrogenase (12-HDH) converts LTB_4 to its 12-oxo derivative, which has diminished biological activity but can still bind to BLT_1 (100,101). Thus, this branch of the eicosanoid family shares many of the same types of regulatory mechanisms displayed by the prostanoids.

C. *Role-playing: Regulation of Inflammation*

Certain eicosanoids are believed to always perform pro-inflammatory duties e.g., leukotriene B_4 (LTB_4) recruits and activates neutrophils (74), and cysteinyl leukotrienes (LTC_4, D_4, and E_4) activate mast cells (102). Others perform on a more temporal basis e.g., PGE_2 and PGD_2 are considered pro- or anti-inflammatory based on their temporal production by different cell types in different diseases. The most recently appreciated role of eicosanoids is the ability of some molecules to regulate the resolution of inflammatory responses: lipoxin A_4 (LXA_4), resolvins, and 15-deoxy-$\Delta^{12,14}$ PGJ_2 are solely produced in the body's attempt to down-regulate the inflammatory response (103,104,105). Recent studies suggest that the synthesis of these pro-resolution molecules is dependent upon signals produced by pro-inflammatory molecules generated early during the inflammatory response (33,61). However, models to support this interplay of pro-inflammation regulating pro-resolution are mainly contrived and short term, such as the air pouch model or the carageenan paw edema model (105,106,107). Theses models are not adequate representations of a realistic inflammatory response, such as that which would be seen during a response to infection (the primary purpose of the inflammatory response), and may lead to erroneous conclusions.

Thus, we purposed experimental Lyme arthritis as an ideal model for the study of the pro-inflammation and -resolution mediators of the immune response. Susceptibility to experimental Lyme arthritis was previously thought to be due to differences in the T_H1/T_H2 responses. However, we have shown that arthritis development is dependent solely upon the presence of neutrophils; innate immune cells which are known to be

potent producers and responders to eicosanoids and other lipid mediators of inflammation.

D. Borrelia burgdorferi *and Experimental Lyme Arthritis*

Caused by infection with the spirochete *B. burgdorferi*, Lyme disease is the most common vector-borne disease in the United States, with over 23,000 new cases reported each year according to the Centers for Disease Control (CDC). Lyme disease patients present with diverse symptoms, initially reporting flu-like symptoms and, in 60-80% of reported cases, the notorious "bull's eye" rash erythema migrans (108,109,110). Although the infection generally responds well to antibiotic therapy, if left untreated, infection with *B. burgdorferi* can result in disease states affecting the cardiovascular, nervous, or most commonly, the musculoskeletal system (111,112).

Despite advances in understanding the biology of *B. burgdorferi*, mechanisms underlying the development of pathology remain unclear. Experimental Lyme arthritis recapitulates many aspects of human disease and is generated by infection of arthritis-susceptible mice with *B. burgdorferi* (113,114). While no animal model completely reproduces all aspects of human disease, it is well accepted that the sequelæ observed in the mouse model of Lyme disease are analogous to those which develop in the majority of Lyme disease patients. The arthritis that develops is an inflammatory arthritis dependent upon the presence of the spirochete, responds well to antibiotic therapy, and is not autoimmune in nature (115,116). Susceptible mouse strains (e.g. C3H/HeJ or AKR) develop a severe inflammatory arthritis which peaks about 14-17 days post-infection, then spontaneously

resolves over the next few weeks, whereas resistant strains (e.g. DBA/2J or C57BL/6) develop little or no arthritis (113,117,118). Development of pathology is controlled by murine genetics, with innate immunity determining susceptibility to arthritis development (119,120), and humoral immunity regulating clearance of the spirochete from the joints and presumably disease resolution (121,122,123,123). While the presence of spirochetes in the joint is required for the development of arthritis, the evolution of pathology is not dependent upon the absolute numbers in the joint. Thus, both arthritis-resistant and -susceptible mouse strains can harbor relatively equivalent numbers of *Borrelia* in their joints, and yet retain their phenotypic differences (116,120). This suggests that an overly exuberant or inappropriate host response may be an additional requirement for the development of pathology.

Immunopathogenesis

B. burgdorferi belongs to the group of bacteria known as spirochetes, named for their spiral shape and "corkscrew" pattern of motility. This mode of movement is due to the presence of flagella sandwiched between the cytoplasmic and outer membranes (109). While *B. burgdorferi* does not express LPS, its cell wall consists of the TLR2 agonist peptidoglycan (124) and outer surface proteins (Osp) preferentially expressed during different stages of the spirochete life cycle (125,126). Osps A and B are expressed on spirochetes in the tick midgut, then once inside the mammalian host expression switches to highly-immunogenic OspC (127,128,125,129), although low levels of OspA have been found in inflammatory foci in experimental Lyme disease (130). The Osps are thought to play a role in spirochete dissemination within the mammalian host, however the source of

the tropism of *B. burgdorferi* for the synovium, heart, and nervous system tissues is unknown. *B. burgdorferi* can also bind platelets via $\alpha_{2b}\beta_3$ integrin (131,132), fibronectin (133) and decorin (134) found in synovial tissue and similar tissue matrices, or they can be found associated with collagen fibers (135).

Because all inflammatory arthritides share certain characteristics and the prominent role of T and B cells in the pathogenesis of rheumatoid arthritis, early work focused on the role of these cell types in Lyme arthritis (136,137,138,139). However, numerous studies using mice deficient for T or B cells, or with genetic deletion of various T helper-type 1 (T_H1) or T_H2 cytokines or their receptors, have shown that neither these acquired immune cells, nor the cytokines involved in their regulation, have any appreciable role in the development Lyme arthritis pathology (140,120,141,119,142,143,144). Using mice deficient for the chemokine receptor CXCR2, as well as antibody-mediated cell depletion assays, our lab has demonstrated that neutrophil entry into the joint is absolutely necessary for the development of Lyme arthritis pathology (117,145). Although the murine model of Lyme disease illustrates the pathways mediating the inflammatory response to an infectious organism, recent studies utilizing the K/BxN model of rheumatoid arthritis have also demonstrated the necessity of neutrophils and neutrophil-derived eicosanoids in the development of arthritis pathology (146).

The availability of mouse strains displaying differential susceptibility to the arthritic inflammatory response, well-characterized physical aspects of disease, and the predictable return to homeostasis under normal disease conditions, suggest that the

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murine model of experimental Lyme arthritis offers an excellent opportunity to investigate not only the mechanisms of Lyme disease pathology, but also the pathways intrinsic to the development and resolution of the inflammatory response.

Lyme Disease and Eicosanoids

Few studies to date have examined the role of eicosanoids in the pathology of Lyme disease. An early clinical study found that synoviocytes from a single patient with chronic Lyme arthritis produced high levels of PGE_2 during *in vitro* culture of synoviocytes; however, this study dates from before the description of *B. burgdorferi* as the causative agent of Lyme disease (147). More recently, murine microglial cells were shown to produce PGE_2 when stimulated with *B. burgdorferi*, suggesting a possible role for pro-inflammatory prostaglandins in neuroborreliosis (148,149). A study which focused on Lyme arthritis found that *Cox-2* gene expression as examined by RT-PCR was increased in the ankles of arthritis-susceptible C3H mice 14 days post-infection with *B. burgdorferi*. Treatment of these mice with the COX-2-specific inhibitor MF-Tricyclic reduced arthritis severity at 14 days post-infection without affecting T or B cell responses (150). Thus, COX-2 appeared to be involved in the development of the inflammatory arthritis seen in experimental Lyme disease.

E. Purpose and Experimental Approach

The focus of my graduate studies was to determine the contribution of members of the eicosanoid cascade, namely the cyclooxygenase enzymes, to the regulation of experimental Lyme arthritis pathology. Using COX-2-specific inhibitors and knock-out

animals, it was determined that COX-2 was not required for the development of Lyme arthritis, but played a prominent role in the resolution of arthritis pathology. Regulation of arthritis resolution by COX-2 was not mediated via alterations in antibody production or an inability to clear *Borrelia* from arthritic joints. Further pharmacological and knockout studies found that COX-1 played a regulatory role during development of arthritis by maintaining a check on the developing inflammatory response. At later time points, COX-1 mediated down-regulation of the inflammatory response. The differential contribution of the COX isozymes to arthritis pathology during infection with *B. burgdorferi* lead to the characterization of a previously unappreciated role for COX-1 in the regulation of B lymphocyte responses and pathogen-specific antibody production using isozymes-specific inhibitors and COX-1 and -2 gene knock-out animals. Finally, the role of LTB_4 in regulation of Lyme arthritis pathology was investigated using a BLT_1-specific antagonist.

CHAPTER II

MATERIALS AND METHODS

A. *Materials*

1. *Animals*

Female C3H/HeJ (C3H) and DBA/2J (DBA) mice, 4-6 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME). COX-2 heterozygous mice (B6;129S7-*Ptgs2^{tm1Jed}*) were purchased as breeders, also from The Jackson Laboratory, and were backcrossed onto the C3H genetic background for ten generations. Heterozygous mice were then intercrossed to produce knockout and wild-type littermates. COX-1 knockout mice (B6;129P2-*Ptgs1^{tm1Unc}*) and wild-type littermate controls were purchased from Taconic Farms (Germantown, NY).

2. *B lymphocyte isolation, stimulation, and culture*

The AutoMacs negative selection B cell isolation kit, AutoMacs Running and Rinsing buffers were from Miltenyi Biotech, Foster City, CA. The rat anti-mouse CD40 and goat F(ab')$_2$ anti-mouse IgM were from Southern Biotech and arachidonate was from Cayman Chemical, Ann Arbor, MI. Dulbecco's Modified Eagle medium (DMEM), Hank's balanced salt solution (HBSS), phosphate buffered saline (PBS), Dulbecco's phosphate buffered saline were all from Invitrogen Life Technologies, Carlsbad, CA. Nutridoma-SP was from Roche Applied Science, Indianapolis, IN.

3. *RNA and DNA isolation and quantitative real-time RT-PCR*

The Bead Beater homogenizer and 2.5 mm zirconia beads were from BioSpec Products, Inc., Bartlesville, OK. 2 mL silicone O-ring tubes were from Sarstedt, Newton, NC. For nucleic acid isolation, TRIzol reagent and DEPC H_2O were from Invitrogen Corp. 100% ethanol was from Aaper Alcohol and Chemical Co., Shelbyville, KY, and chloroform and isopropanol were from Fisher Scientific, Fair Lawn, NJ. The TaqMan Universal PCR Master Mix, EZ RT-PCR kit, the VIC-labeled Nidogen probe, and the ABI Prism 7700 Sequence Detection System were all from Applied Biosystems, Foster City, CA.   All other probes and primers were from Integrated DNA Technologies, Coralville, IA.

4. *SDS-PAGE/Western blot analysis and immunobloting*

The Novex X-Cell gel running and transfer system, gel combs, and Novex cassettes were from Invitrogen. Tris HCl, NaCl, EDTA, Triton X-100, sodium deoxycholate, SDS, glycine, gelatin, Tween-20, and methanol were all from Fisher Scientific. Polyvinyl DF membrane was from Millipore, Bedford, MA.

HRP-conjugated goat anti-mouse Ig was from Southern Biotech. The rabbit anti-mouse COX-1, COX-2, thromboxane receptor (TP), or $PGF_{2\alpha}$ receptor (FP) primary antibodies were all from Cayman Chemical, Ann Arbor, MI.   The rabbit anti-mouse GAPDH antibodies were from Chemicon, Temecula, CA, or Santa Cruz Biotechnology, Inc., Santa Cruz, CA.   The detection systems VectaStain ABC-AmP and Vetor *Nova*Red substrate kits were from Vector Corp, Burlingame, CA.

5. *Flow cytometric detection of cyclooxygenase-2*

Fetal bovine serum was from Invitrogen. The fluorescein (FITC)-conjugated rat anti-mouse CD19 antibody was from eBioscience, San Diego, CA, and the phycoerythrin (PE)-labeled mouse anti-human IgG$_1$ antibody that displays cross-reactivity with mouse COX-2 (151) and the isotype control were from Santa Cruz Biotechnology, Inc. The Fix and Perm kit was from Caltag Laboratories, Burlingame, CA. The FACScan flow cytometer and CellQuest software were both from BD Biosciences.

6. *Bacteria, infections, and vaccination*

B. burgdorferi N40 strain was a kind gift from J. Weis, University of Utah. Barbour, Stoenner, Kelly (BSK) II medium and rabbit serum were purchased from Sigma-Aldrich, St. Louis, MO. The Petroff-Hausser counting chamber was purchased from Hausser Scientific, Horsham, PA. Formalin for spirochete inactivation was from Fisher Scientific, and complete Freund's adjuvant (CFA) was from Gibco. Isofluorane (Novaplus) was from Abbot Laboratories, North Chicago, IL, purchased through the University of Missouri Veterinary Medicine Teaching Hospital pharmacy.

7. *Determination of antibody levels*

Immulon 2B ELISA plates were from Nalgene, Rochester, NY. The rat anti-mouse IgG and goat anti-mouse IgM used for coating plates were from AnaSpec, San Jose, CA, and Southern Biotech, Birmingham, AL, respectively. The ELISA standards, purified mouse total IgG or IgM, IgG$_1$, IgG$_{2a}$, IgG$_{2b}$, and IgG$_3$ were from Equitech Labs, Kerrville, TX or

Bethyl Laboratories, Montgomery, TX, respectively. Alkaline phosphatase-conjugated donkey anti-mouse IgG was from Jackson Immuno Research, West Grove, PA, alkaline phosphatase-conjugated rat anti-mouse IgG_1, G_{2a}, G_{2b}, or G_3 were from BD-PharMingen, San Diego, CA, and alkaline phosphatase-conjugated rat anti-mouse IgM was from Southern Biotech. The Sigma 104 phosphatase substrate tablets were from Sigma-Aldrich and the diethanolamine was from Fisher Scientific.

8. *Measurement of eicosanoids*

Ultra pure water and enzyme immunoassays (EIA) for PGE_2, $PGF_{2\alpha}$, TxB_2 (as a measure of TxA_2), or LTB_4 were from Cayman Chemical, and for 15-deoxy-$\Delta^{12,14}$ PGJ_2 was from Assay Designs, Ann Arbor, MI.

9. *Inhibitors and antagonists*

The COX-2-specific inhibitor celecoxib (Celebrex) was purchased from LKT Laboratories, Inc, St. Paul, MN and incorporated into a normal laboratory diet by Research Diets, New Brunswick, NJ. The COX-1-specific inhibitor SC-560 was from Cayman Chemical. Normal control rodent chow was Purina PicoLab 5053 from Purina Mills, St. Louis, MO. The BLT_1 antagonist CP-105,696 was provided by James Gierse of Pfizer, Inc, Chesterfield, MO.

10. *Apoptosis analysis*

The TUNEL (terminal transferase dUTP nick end labeling) staining kit was from Promega, Madison, WI, and 30% hydrogen peroxide was from Fisher Scientific. The

real-time PCR arrays were from Super Array, Frederick, MD, as was the RT^2 PCR Array

First Strand synthesis kit, and the RT^2 Real-time Sybr Green/Rox PCR master mix. PCR

analysis software was downloaded from the Super Array web site

:http://www.superarray.com/PCRArrayPlate.php.

B. *Methods*

1. *Bacteria and infections*

A virulent, low-passage, clonal isolate of the *B. burgdorferi* N40 strain was used for all

infections. Frozen stocks were placed in 7.5 mL of Barbour, Stoenner, Kelly (BSK) II

medium with 6% rabbit serum and grown to log phase by incubating for 5-6 days at

32˚C. Spirochetes were enumerated using dark field microscopy and a Petroff-Hausser

counting chamber. Spirochete dilutions were made in sterile BSK II medium and mice

were inoculated in both hind footpads with 2.5 x 10^5 *B. burgdorferi* organisms in 50μl of

medium, for a final inoculum of 5 x 10^5, a concentration which reliably produces

arthritis in susceptible animals and is a typical inoculum (117,144,152).

2. *Animals*

COX-2 heterozygous mice (B6;129S7-*Ptgs2^{tm1Jed}*) were purchased as breeders and were

backcrossed onto the C3H/HeJ genetic background for ten generations. Heterozygous

mice were then intercrossed to produce knockout and wild-type littermates. Animals were

housed in a specific pathogen-free facility and given sterile food and water *ad libitum*.

3. *Tissue homogenization for nucleic acid extraction*

All tissues were snap-frozen in liquid nitrogen immediately after collection, then stored at -80ºC until extraction. All tissues were placed in a 2 mL cryotube with a silicone o-ring containing approximately 750 µl of zirconia beads and 1 mL of Trizol or Tri-reagent. Tissues were then minced in Trizol with RNase/DNase-free scissors to decrease the homogenization time needed. Tubes were then placed in the Bead Beater homogenizer and homogenized for 1.5 minutes. After homogenization, the sample was incubated at room temperature for 5 minutes, then transferred to a new 1.5 mL microcentrifuge tube for RNA and DNA extraction.

4. *RNA extraction*

After homogenization and transfer to a new tube, 200 µl chloroform was added to the sample and the tube vortexed for at least 15 seconds. The sample was incubated at room temperature for 15 minutes, and then centrifuged at 12,000 x g for 30 minutes at 4ºC. The interphase (white) and organic (red) phase were set aside for DNA extraction. The aqueous (clear upper) phase was removed to a new 1.5 mL microcentrifuge tube and 10 µg glycogen and 500 µl isopropanol were added. The sample was incubated for 20 minutes at -80ºC to precipitate the RNA, then centrifuged at 12,000 x g for 15 minutes at 4ºC. The supernatant was discarded, 75% ethanol was added, the tube vortexed, and then centrifuged at 12,000 x g for 5 minutes at 4ºC. The supernatant was again discarded, the pellet dried and resuspended in 200 µl DEPC water by freezing at -80ºC, then flash-thawing in a 55ºC water bath. Concentration and purity were determined by measuring the absorbance at 260 nm and 280 nm of a 10x dilution of the sample. Integrity of the

RNA was determined by electrophoresis of 1-10 µL of sample on a 1% agarose gel and UV visualization of the 28S and 18S bands by ethidium bromide staining.

5. *DNA extraction*

To the interphase and organic phase from the RNA Trizol extraction, 500 µl of DNA back-extraction buffer (1 M Tris, pH 8.0; 4 M guanidine thiocyanate; 50 mM sodium citrate) was added and the sample vortexed and incubated at room temperature for 10 minutes. The sample was centrifuged at 12,000 x g for 15 minutes at 4ºC and the aqueous phase transferred to a new 1.5mL microcentrifuge tube. 400 µl of isopropanol was added to the aqueous phase, the sample vortexed, and incubated at -80ºC for 20 minutes before the DNA was pelleted by centrifugation at 12,000 x g for 5 minutes at 4ºC. The pellet was then washed with 75% ethanol, and then resuspended in DEPC H_2O.

6. *Real-time polymerase chain reaction (PCR) for determination of* B. burgdorferi *loads*

Multiplex real-time PCR was performed using the TaqMan Universal PCR Master Mix with the ABI Prism 7700 Sequence Detection System. 2 µl of DNA was added to 23 µl per well of a reaction mixture in a 96-well real-time PCR reaction plate. The reaction mixture consisted of 12.5 µl of 2x master mix, 9.7 µl H_2O, 0.6 µl of 15 µM *Nidogen* 5'/3' working solution (300 nm of each primer), 0.3 µl of 10 pmol/µl *Nidogen* probe (100 nm), 0.6 µl of *Flagellin* 5'/3' working solution (300 nm of each primer), and 0.3 µl of 10 pmol/µl *Flagellin* probe (100 nm) for a final reaction volume of 25 µl.   Final concentrations are stated in parenthesis. The following program was used for real-time PCR: 50ºC for 2 minutes, then 45 cycles of 95ºC for 20 seconds and 60ºC for 1 minute. The mouse *Nidogen* gene is a single copy gene used as an endogenous control as

described previously. Real-time PCR reactions for *Flagellin* were normalized to copies of *Nidogen* DNA within the same sample. Bacterial loads are expressed as copies of *B, burgdorferi Flagellin* per 1000 copies of mouse *Nidogen*.

7. *Real-time reverse-transcription RT-PCR*

Multiplex real-time RT-PCR was performed using the one-step EZ RT-PCR kit. 0.6 µl of RNA was added to 29.4 µl per well of a reaction mixture in a 96 well real-time PCR reaction plate. The reaction master mix consisted of 12.9 µl H_2O, 6 µl 5x TM buffer, 3.6 µl MnOAc (3 mM), 0.9 µl each of dATP, dGTP, dCTP (300 µM each), and dUTP (600 µM), 0.6 µl of 15 µM *Nidogen* 5'/3' working solution (300 nM of each primer), 0.3 µl 10 pmol/µl *Nidogen* probe (100 nM), 0.6 µl of 15 µM *Gene Of Interest (GOI)* 5'/3' working solution (300 nM of each primer, see Appendix for primer and probe sets), 0.3 µl 10 pmol/µl *GOI* probe (100 nM), 1.2 µl r*Tth* polymerase (0.1 U/µl), and 0.3 µl AmpErase UNG (0.01 U/µl) for a final reaction volume of 30.0 µl. Final concentrations are stated in parenthesis. The following program was used for the combined one-step reverse transcription PCR reaction: 50° for 15 minutes, 60° for 30 minutes, and 95° for 10 minutes, then 45 cycles of 20 seconds at 95° and one minute at 60°. The mouse *Nidogen* gene is a single copy gene used as an endogenous control as described previously (117). Real-time RT-PCR reactions were normalized to copies of *Nidogen* mRNA within the same sample using the $^{-\Delta\Delta}C_T$ method. mRNA levels are expressed as fold change in expression level as compared to unstimulated controls or day 0 untreated animals.

8.   *Extraction of tissue eicosanoids by reverse-phase high pressure liquid chromatography (RP-HPLC)*

Tissues were frozen in liquid nitrogen, wrapped in aluminum foil, and replaced in liquid nitrogen. The joint in foil was again removed from the liquid nitrogen and pulverized with a hammer. The resulting powder was placed in 5 mL ice-cold 100% ethanol in a borosilicate glass test tube, vortexed, weighed, and incubated at -20ºC for 72h with periodic vortexing throughout. Samples were centrifuged at 3,500 x g for 30 min at 4ºC, the clear ethanolic supernatant dried under nitrogen, and resuspended in 50mM HEPES with 50% methanol. The LUNA C18-2 column (150mm x 2mm, 5μ) was washed with 100% methanol for 30 minutes before each set of samples to check for proper pressures and flow rates. Standards for LTB_4, LXA_4, PGE_2, and/or 15-deoxy-$\Delta^{12,14}$-PGJ_2 (elution times: LTB_4 = 15.5 min, LXA_4 = 11.5 min, PGE_2 = 12 min, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 = 12.5 min) were applied to the column before every run to verify proper elution times, as judged by monitoring the UV spectra of each standard at 280 nm and 310 nm. 50 μl of sample was applied to the column equilibrated with Solvent A (methanol/acetonitrile/water/acetic acid; 10:15:100:0.2, v/v, pH 6.0). The column was eluted at a flow rate of 1 mL/min with 55% Solvent A and 45% Solvent B (100% methanol) over 5 min. Solvent B was then increased linearly to 75% over 15 min and maintained at this level for an additional 15 min. 2 mL fractions were collected in borosilicate glass test tubes, capped immediately, and stored at -20ºC until they were dried under nitrogen and resuspended in 250 μl enzyme immunoassay (EIA) buffer. Samples resuspended in EIA buffer from the assay kits of different companies can be assayed on all plates, regardless of maker.

9. *B Lymphocyte isolation*

Spleens from each animal were aseptically removed to a 35 mm tissue culture dish containing a 70 μm nylon cell strainer and ~5mL Dulbecco's Modified Eagle Medium supplemented with 1% Nutridoma-SP (DMEM-NSP). Spleens were then pushed through the filter using the plunger of a 3cc syringe to prepare a single cell suspension that was then removed to a 15 mL conical. The dish was washed with fresh DMEM-NSP to collect any remaining cells, which were then removed to the same conical for a total volume of 15mL. Cells were centrifuged at 500 x g for 10 minutes at 4ºC, the supernatants removed, and erythrocytes lysed using 4 mL ammonium chloride, potassium (ACK) buffer. Splenocytes were incubated with ACK lysis buffer at room temperature for 5 minutes, then hand vortexed and incubated for an additional 5 minutes for a total incubation time of 10 minutes. The tubes were each brought to a total volume of 15 mL with Hanks' balanced salt solution (HBSS) to stop cell lysis, and centrifuged at 500 x g for 5 min at 4ºC. Supernatants were discarded, and the cells resuspended in 1 mL DMEM-NSP for counting by trypan blue exclusion. Total splenocyte preparations were labeled using an AutoMacs B cell negative selection isolation kit according to the manufacturer's instructions. Cells were brought to 2 mL in HBSS for AutoMacs separation using the "Depl05" program. Cells were eluted in AutoMacs Running Buffer (PBS, 2 mM EDTA, 0.5% BSA) into a new 15 mL conical and placed on ice until all samples were processed. Each tube was then brought to 15 mL total volume with HBSS and centrifuged at 500 x g for 10 min at 4ºC. Supernatants were discarded, cells resuspended in 1 mL DMEM-NSP, and cell number and viability determined using

trypan blue exclusion prior to culture. B cells were found to be >99% $CD19^+$ and <1% $CD3^+$ or $CD14^+$ as determined by flow cytometry.

10. *Culture conditions*

Preliminary experiments were performed using DMEM supplemented with 10% fetal bovine serum (FBS), however, the serum interfered with antibody and eicosanoid measurements. We then adopted serum-free culture conditions, supplementing media with Nutridoma-SP. Highly purified B lymphocytes were then cultured in DMEM-NSP with or without stimuli for 8, 24, or 36 hours, or 7 days for mRNA, protein, eicosanoid, or immunoglobulin measurement, respectively. B cells were stimulated with rat anti-mouse CD40 (0.5 μg/mL), goat F(ab')$_2$ anti-mouse IgM (10 μg/mL), *B. burgdorferi* spirochetes at a multiplicity of infection (MOI) =1, *B. burgdorferi* total antigen (5μg/mL), arachidonate (10μM), or were untreated. The concentration of *B. burgdorferi* antigen used has been shown to activate B cells and induce their proliferation and differentiation into plasma cells (153,154). For mRNA measurement, B cells were stimulated in triplicate at 5×10^6 cells/ mL in 200μL. Original experiments stimulated cells at 1×10^6 cells/mL, however, the amount of mRNA isolated from B cells is very small, and therefore cell densities were increased to collect enough mRNA for real-time PCR. All other experiments were performed at a cell density of 5×10^6 cells/mL in 200μL for a final concentration of 1×10^6 cells per well. Depending on the number of cells collected, and if both eicosanoid and antibody levels were to be measured, cell stimulations were typically stimulated in triplicate for eicosanoid production and in duplicate for antibody production.

11. *Stimulation with live* B. burgdorferi

For stimulations with live *B. burgdorferi*, the same cultures were used as were used for *in vivo* infections. Spirochetes were washed to remove any residual serum before their addition to B cell cultures. Bacteria were enumerated by darkfield microscopy, then 500 µl of culture were placed in a 2 mL microcentrifuge tube and the total volume was brought to 2 mL with HBSS. The tube was centrifuged at 18,000 x g for 30 minutes at 4ºC. The supernatant was then discarded, and the bacteria were resuspended in a volume of DMEM-NSP that resulted in a final concentration of 1×10^8 spirochetes/mL.

12. *SDS-PAGE/Western blot analysis*

B lymphocytes (1×10^6 cells/mL) were stimulated as described above and total protein was isolated in 40 µl modified radio-immunoprecipitation assay buffer (RIPA; 50 mM Tris HCl pH=7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). 30 µg of protein were fractionated on a 10% SDS-PAGE gel and transferred electrophoretically to polyvinyl DF membrane. Protein from J774.2 cells was loaded at $1/6^{th}$ the concentration of B cell protein. Membranes were incubated with a 1:1000 dilution of rabbit anti-mouse COX-1, COX-2, thromboxane receptor (TP), $PGF_{2\alpha}$ receptor (FP), or GAPDH primary antibody. Secondary antibody and development followed using the VectaStain ABC-AmP kit according to manufacturer's instructions.

13. *Flow cytometric detection of cyclooxygenase-2*

Following stimulation, B lymphocytes were collected and washed with 5% fetal bovine serum in phosphate-buffered saline (FBS-PBS) and stained at a density of 1×10^6 cells/100 μl. Surface staining was performed with fluorescein (FITC)-conjugated rat anti-mouse CD19 antibody in 5% FBS-PBS for 15 minutes at room temperature. Intracellular staining was performed for COX-2 protein using a phycoerythrin (PE)-labeled mouse anti-human IgG$_1$ antibody that displays cross-reactivity with mouse COX-2 (151) or the isotype control and a Fix and Perm kit according to manufacturer's instructions. Flow cytometric data were collected on a FACScan flow cytometer and analyzed using CellQuest software.

14. *Determination of antibody levels*

B. burgdorferi-specific IgM and IgG levels in the sera of infected animals were determined by enzyme-linked immunosorbent assay (ELISA) using alkaline phosphatase-conjugated anti-mouse IgM and IgG as a modification of Bolz, et al (124) and Wooten, et al (155). Immulon 2B ELISA plates were coated with 0.5 μg/mL of *B. burgdorferi* antigen in coating buffer (0.1 M bicarbonate buffer, pH 9.4). Two columns on each plate were reserved for the standard curve. These wells were coated with rat anti-mouse IgG (20 μg/mL) or goat anti-mouse IgM (20 μg/mL) in coating buffer. For total non-specific immunoglobulin from sera or cell culture supernatant, the plate was coated with rat anti-mouse IgG or goat anti-mouse IgM. Plates were coated overnight at 4ºC then washed with wash buffer (0.05% Tween-20 in 1x PBS). Non-specific binding was blocked with 3% BSA in PBS (BSA/PBS) at room temperature for 1.5 h. Standard curves were

created by 1:3 dilution of purified mouse total IgG or IgM, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3. Cell culture supernatants or sera of individual animals were diluted in BSA/PBS and incubated for 2 hours at room temperature. Alkaline phosphatase-conjugated donkey anti-mouse IgG, rat anti-mouse IgG_1, G_{2a}, G_{2b}, or G_3, or rat anti-mouse IgM was applied at a 1:1000 dilution for 45 minutes at room temperature. Plates were washed and read at 409 nm after addition of the phosphatase substrate (Sigma 104 tablets) in 10% diethanolamine buffer, pH 9.2.

15. *Vaccine preparation*

The *B. burgdorferi* vaccine was produced according to Munson, et al (156). Briefly, *B. burgdorferi* organisms were grown to log phase in BSK II media, pelleted by centrifugation (10,000 x g, 15°C, 10 minutes) and washed with PBS (pH 7.4). The washed pellet was resuspended in 1% formalin, incubated at 32°C for 30 minutes, washed, and resuspended in PBS. Formalin inactivation was verified by dark-field microscopy and the formalin-inactivated spirochetes in PBS were mixed with complete Freund's adjuvant (CFA) for a final concentration of 4×10^6 *B. burgdorferi*/mL.

16. *Vaccination and challenge*

Mice were anesthetized with isofluorane and vaccinated subcutaneously in the inguinal region with 0.25 mL (approximately 10^6 *B. burgdorferi*) of the formalin-inactivated vaccine. Sham vaccinated mice were injected with BSK II media in CFA. Twenty-one days after vaccination, mice were infected in the hind footpads with *B. burgdorferi* as described above for normal inoculation.

17. *Immunoblotting to determine antigen specificity of antisera*

Immunoblotting of serum from *B. burgdorferi*-infected animals against *B. burgdorferi* antigen was performed at 1:5000 and 1:10,000 dilutions for unvaccinated and vaccinated animals, respectively. Normal, uninfected mouse serum was used as a control and diluted to 1:5000. Blots were derived from SDS-PAGE of whole-cell lysates of N40 cultures used for infection. Antigen was fractionated on 12% gels and transferred to PVDF membrane. Briefly, after protein transfer, the PVDF membrane was blocked overnight with 5% gelatin in Tris-buffered saline (TBS, pH 7.2), then washed with wash buffer (0.1% gelatin in TBS with 0.05% Tween 20 (TBST)). The membrane was then cut into strips, which were incubated for 2 hours with mouse serum at the indicated dilutions in 1% gelatin in TBST. HRP-conjugated goat anti-mouse Ig was added at a 1:7500 dilution and conjugate visualized using the Vector *Nova*Red substrate kit. The reaction was stopped after ten minutes by rinsing with distilled water.

18. *Measurement of eicosanoids*

B lymphocyte culture supernatants were diluted in enzyme immunoassay (EIA) buffer and analyzed by EIA according to the manufacturer's instructions for the presence of PGE_2, $PGF_{2\alpha}$, TXB_2 (as a measure of TXA_2), or LTB_4 after 36 hours of incubation in the presence of stimulus and/or inhibitor(s). Tissue eicosanoid fractions were diluted in EIA buffer before EIA analysis for PGE_2, LTB_4, and 15-deoxy-$\Delta^{12,14}$ PGJ_2.

19. *Inhibition of cyclooxygenase-1 or -2*

In vitro *inhibition*

Celecoxib and SC-560 were dissolved in 100% ethanol/ 0.01% Tween-20 or 100% ethanol alone, respectively, as stock solutions and stored at -20ºC until dilution to working concentrations (1μM) in cell culture medium. B cells were pre-incubated at 37ºC with inhibitors or vehicle for 30 minutes before the addition of stimuli.

In vivo *inhibition*

Stock solutions of meloxicam, celecoxib, or rofecoxib were dissolved in dimethyl sulfoxide (DMSO) and stored at 4ºC before dilution in 1% methylcellulose for final dosages of 1 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} (meloxicam) or 5 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} (celecoxib and rofecoxib). In later experiments, celecoxib was incorporated into a normal laboratory diet as described by King, et. al. (157), at a concentration of 1,000 ppm, which is equivalent to $\approx$125 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} and results in a plasma concentration of about 2 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} (Charles Loftin, University of Kentucky, personal communication). Animals were fed celecoxib chow beginning day -1 of infection with *B. burgdorferi* and control animals were fed normal rodent chow (Purina PicoLab 5053). For COX-1 inhibition, dilutions were mixed daily in 200μL sterile PBS and animals were treated once daily by oral gavage for a final dosage of 10 mg $\cdot$ kg^{-1} $\cdot$ day^{-1}.

In vivo blockade of BLT$_1$

CP-105,696 is a cyclopropane carboxylic acid compound that selectively blocks binding of leukotriene B$_4$ to the leukotriene B$_4$ receptor BLT$_1$. Stock solutions were made by dissolving 100 mg in 100% ethanol with 0.05% Tween-20 and 1% DMSO. Working

dilutions were made in PBS for either once or twice daily treatments at a concentration of $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$.

20. *TUNEL staining and real-time RT-PCR apoptosis array*

TUNEL staining was used to detect apoptosis in histological sections of tibiotarsal joints. Staining was performed strictly according to manufacturer's instructions. A real-time PCR array was used to measure the expression of the mRNA of a panel of pro- and anti-apoptotic genes. RNA extracted from the ankles of three vehicle-treated (2 µl per sample) or four CP-105,696-treated (1.8 µl per sample) C3H mice was combined to perform reverse transcription (RT) using a First Strand synthesis kit. 182 µl of DEPC H_2O was added to the entire RT reaction, which was then added to 1272 µl 2X SuperArray PCR master mix and 1173 µl H_2O. 25 µl of master mix was then added to each well of the PCR array plate. For the real-time PCR reaction, the program was: 10 minutes at 95ºC, the 50 cycles of 15 seconds at 95ºC, then 1 minute at 60ºC. Once both the control (vehicle-treated) and test (CP-105,696-treated) samples were run, the C_t threshold was set for both plates simultaneously. The reaction data were then analyzed using the Super Array analysis software, available from their web site, as described in *Materials*.

CHAPTER III

CYCLOOXYGENASE-2 REGULATES THE RESOLUTION

PHASE OF ARTHRITIS

A. *Introduction*

Cyclooxygenase-2 (COX-2) is largely viewed as an enzyme whose up-regulation results in the development and propagation of inflammation. Although recent studies also suggest a fundamental role for COX-2 in the down-regulation of the inflammatory response, interpretation of these studies is confounded by the use of models that do not display physiologically relevant, long-term processes of induction and resolution of inflammation. In fact, most of the data concerning the participation of COX-2 in the inflammatory response were garnered from models of autoimmune disease. Although helpful for deciphering the contribution of COX-2 in rheumatoid arthritis, collagen- and adjuvant-induced arthritis models do not provide a faithful representation of the mechanisms that govern a true, acute inflammatory response, such as that which would be induced by infection (36,158). Furthermore, *in vitro* studies demonstrating the mobilization of COX-2 as a pro-resolution mediator in a response must be interpreted with caution, due to the extremely complex circuitry and cellular interactions which govern eicosanoid metabolism (33,159).

Infection with *B. burgdorferi* leads to the development of an inflammatory arthritis, characterized by cellular infiltration into the joints, predominated by neutrophils and macrophages. Both of theses cell types play significant roles in both the induction and resolution of the inflammatory response. They are known to secrete pro-inflammatory mediators and recruit other immune cells (160,161,162), but they can also express pro-apoptotic, -efferocytotic, and –resolution molecules, especially lipid mediators (163,164,165). In murine Lyme arthritis, neutrophils are the cell type responsible for establishment of pathology (117,145). Coincidentally, they are also a well-characterized source of eicosanoids produced by COX-2.

As mentioned earlier, a paper published in 1980 stated that PGE_2 was isolated from synovial fluid of a patient with "Lyme disease", however this was before *B. burgdorferi* was demonstrated to be the etiological agent of Lyme arthritis pathology (147). Two papers have illustrated that *B. burgdorferi* can stimulate PGE_2 production by microglial cells *in vitro* (148,149). Only one paper has addressed the role of COX-2 in experimental Lyme arthritis. Anguita, et al. (150) conducted an abbreviated study of the role of COX-2 during *B. burgdorferi* infection by utilizing the COX-2-preferential inhibitor MF-tricyclic. Previously referred to as "COX-2-specific", MF-tricyclic is now considered "preferential" as compared to drugs with greater selectivity for COX-2, such as celecoxib and rofecoxib. Arthritis-susceptible C3H animals were infected subcutaneously with *B. burgdorferi*, treated daily with MF-tricyclic, and their immune response was assessed at d14 and d60 post-infection. They found that treatment with MF-tricyclic lead to an inhibition of arthritis development, without altering acquired immune system responses.

We wished to expand on these findings for several reasons, primarily because the development of arthritis could have been delayed by the inhibition of COX-2, and therefore might have been overlooked when examining the response only at day 14. Day 60 is a time point by which most animals would have completely resolved their inflammatory response, so no pathology would be expected to be present. Furthermore, the authors of the previous study utilized a low dose subcutaneous inoculation model, and therefore it is uncertain when the spirochetes actually arrived in the joint tissue. This could have created even more difficulty in determining when arthritis development truly began. Also, as stated previously, MF-tricyclic is no longer considered a true COX-2-specific inhibitor, meaning COX-1 could also have been affected. We therefore examined the effect of COX-2-specific inhibition on the development and resolution of arthritis pathology at various time points during *B. burgdorferi* (*Bb)* infection.

B. *Results*

1. *COX-2 inhibition results in prolonged Lyme arthritis pathology*

To elucidate the mechanism of COX-2 regulation of the inflammatory response during *B. burgdorferi* infection, arthritis-resistant DBA and -susceptible C3H mice were treated with a COX-2 inhibitor or vehicle alone for one day, and were then inoculated in both hind footpads with *Bb*. The development of arthritis was followed by measuring ankle diameters at the indicated time points. Ankle swelling is usually indicative of the underlying inflammatory response in experimental Lyme arthritis, however some exceptions have been noted. Resistant DBA mice demonstrated no differences in ankle diameter between inhibitor- and vehicle-treated groups (Figure 2); however, susceptible C3H mice demonstrated differences in ankle swelling dependent on COX-2 selectivity of the inhibitor used. Meloxicam, a COX-2 preferential inhibitor, did not affect the development of ankle swelling, however, at later time points, ankle diameters did not decrease as compared to vehicle-treated animals (Figure 3). Specific inhibition of COX-2 by the more selective compounds celecoxib (Clx) and rofecoxib (Rof) often resulted in no difference in ankle swelling or decreased ankle swelling (Figure 4 a and b). Although increases in ankle diameter are usually indicative of the underlying pathology, COX-2 products are well-known vasodilators, and thus decrease tissue edema. It was therefore possible that the decreased ankle diameter induced by the most specific Coxib, rofecoxib (13), was due only to decreased edema, and therefore not an accurate representation of the actual arthritis severity, *per se*.
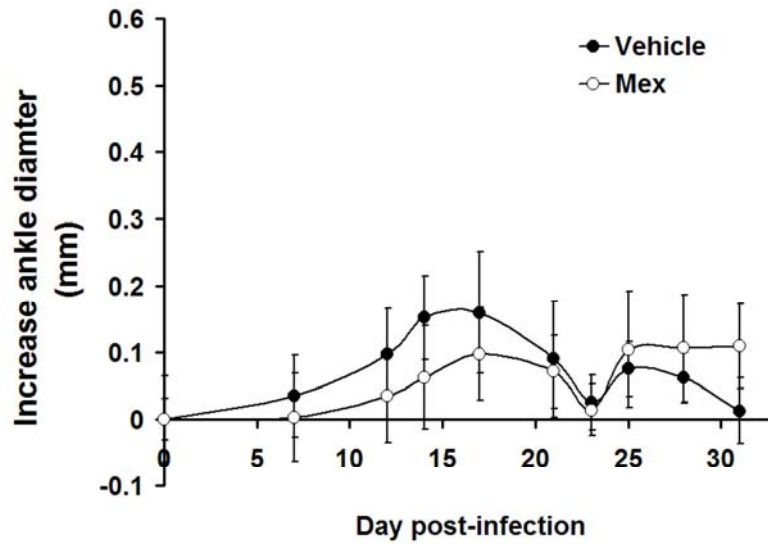
Figure 2. Effect of meloxicam treatment on ankle diamter increase in *B. burgdorferi*-infected DBA/2J mice. Mice were treated daily with either vehicle (closed circles) or 10 mg/kg meloxicam p.o.(Mex; open circles). Values are $\pm$ s.d. $n = 6$ mice per group
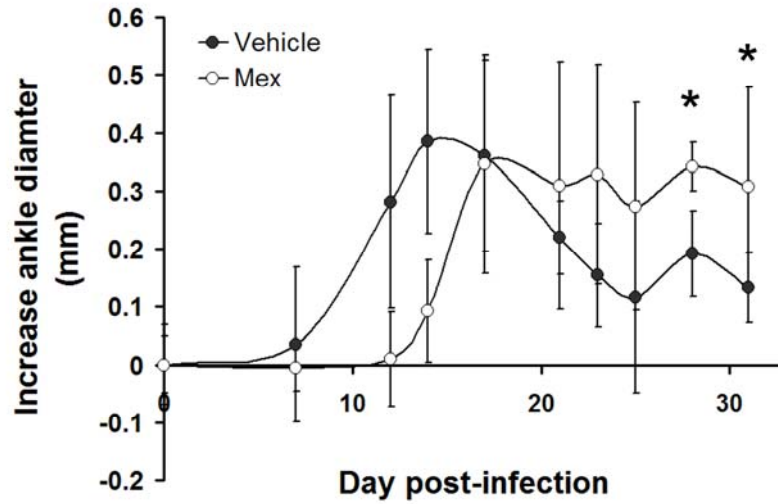
Figure 3. Effect of meloxicam treatment on ankle diameter increase in *B. burgdorferi*-infected C3H/HeJ mice. Ankle diamters were significantly increased at days 28 and 32 post-infection in meloxicam treated mice (open circles) as compared to vehicle-treated mice (closed circles). Delays in the development of ankle swelling at days 10-14 were not consistently demonstrated (data not shown). Meloxicam treatment: 10 mg/kg p.o. Values are $\pm$ s.d. $p \leq 0.05$. $n = 6$.
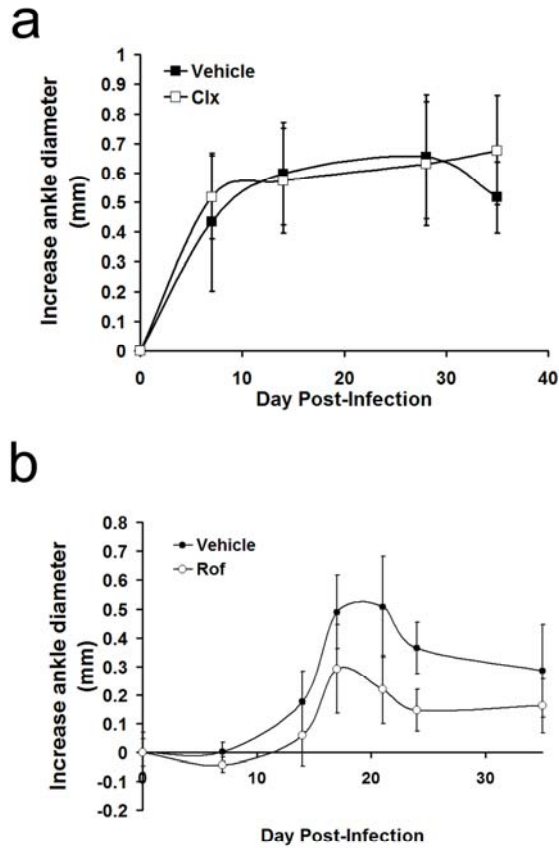
Figure 4. Effect of different COX-2-specific inhibitors on ankle diamter increase induced by *B. burgdorferi* infection. C3H mice were treated with celecoxib (a, Clx) or rofecoxib (b, Rof) or vehicle. Ankle diameters of COX-2 inhibitor-treated mice (open symbols) are compared to their vehicle-treated controls (closed symbols). Clx and Rof treatment: 5 mg/kg/day p.o. Values are $\pm$ s.d. *n* = 6-8 mice per group.

Severity of arthritis pathology was therefore determined by histological scoring at days 7, 14, 28, or 35 post-infection. Our laboratory has previously shown that neutrophil infiltration into the infected joints was necessary for the development of experimental Lyme arthritis (117,145). We were therefore surprised to see similar arthritis pathology, indicated primarily by high levels of neutrophil, and to a lesser extent, macrophage infiltration into the joints, between vehicle- and COX-2 inhibitor (Coxib) -treated mice (Figure 5 a and b). Occasionally, arthritis development was delayed in inhibitor-treated animals, as they demonstrated limited pathology or ankle swelling at day 14 post-infection; however, by day 17 these mice had developed arthritis indistinguishable from their vehicle-treated counterparts (data not shown). COX-2 inhibition did not lead to the development of arthritis in the resistant DBA mice (Figure 6 a and b), indicating that COX-2 products do not appear to play a role in determining genetic susceptibility or resistance to the development of Lyme arthritis pathology.

Intriguingly, while vehicle-treated animals proceeded to resolve their arthritis, Coxib-treated animals still displayed a severe inflammatory response in their joints at d35 post-infection, as demonstrated by histology in Figure 7 histology d35. Whereas the joints of control animals have few inflammatory cells visible (Figure 7 a and c), the joints of Coxib-treated animals still contain a high concentration of infiltrating neutrophils, indicative of an ongoing inflammatory response (Figure 7 b and d). Thus, not only did COX-2 inhibition fail to prevent the development of arthritis pathology, but once the inflammatory response was ongoing, inhibition of COX-2 prevented the initiation of disease resolution.
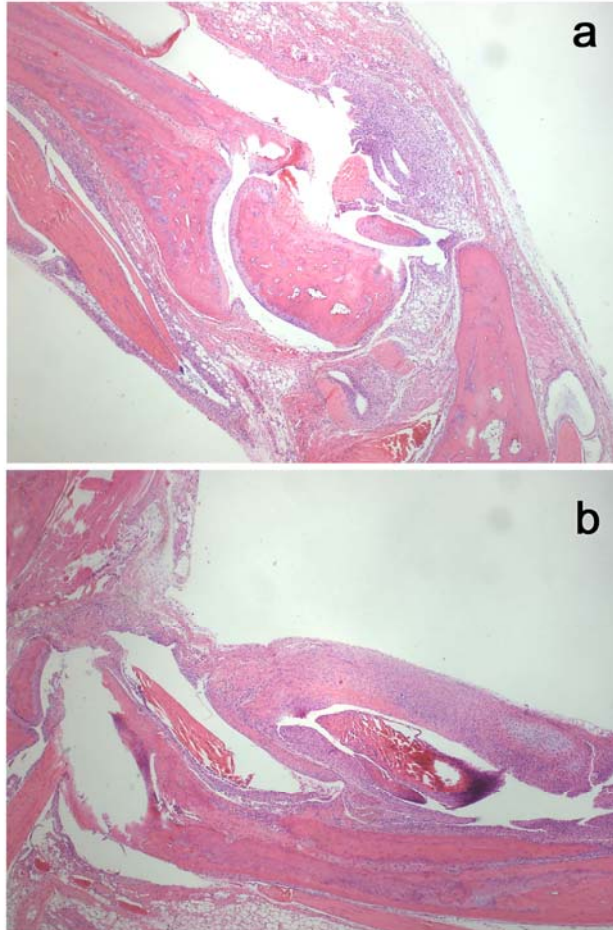
Figure 5. Hemotoylin and eosin (H & E) staining of tibiotarsal joints from vehicle- (a) and meloxicam- (b) treated mice at d21 post-infection. Meloxicam treatment: 10 mg/kg/day p.o. Magnification = 10x
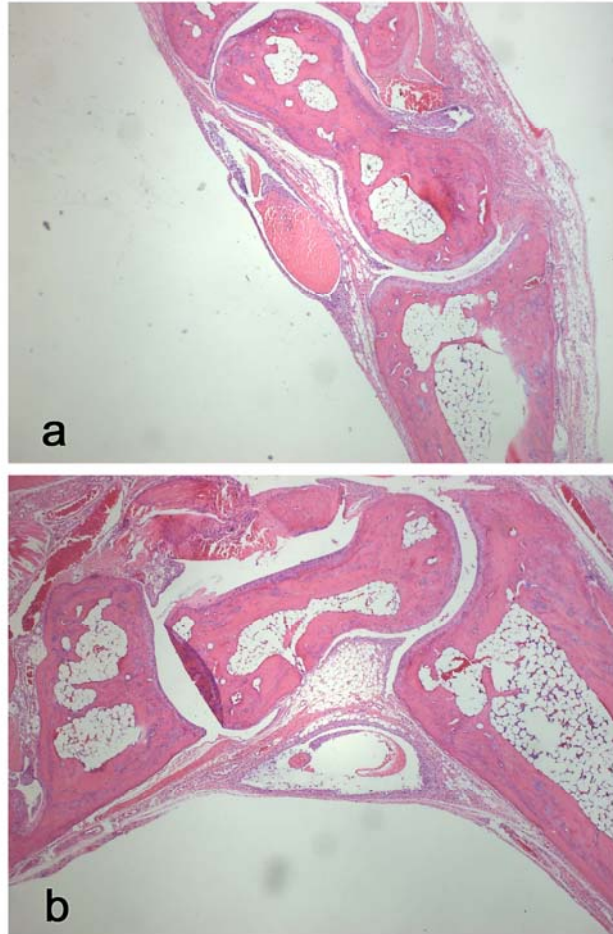
Figure 6. H & E stain of DBA tibiotarsal joints at d21 post-infection. Mice were treated with vehicle (a) or meloxicam (b). Meloxicam treatment: 10 mg/kg/day p.o. Magnification = 10x
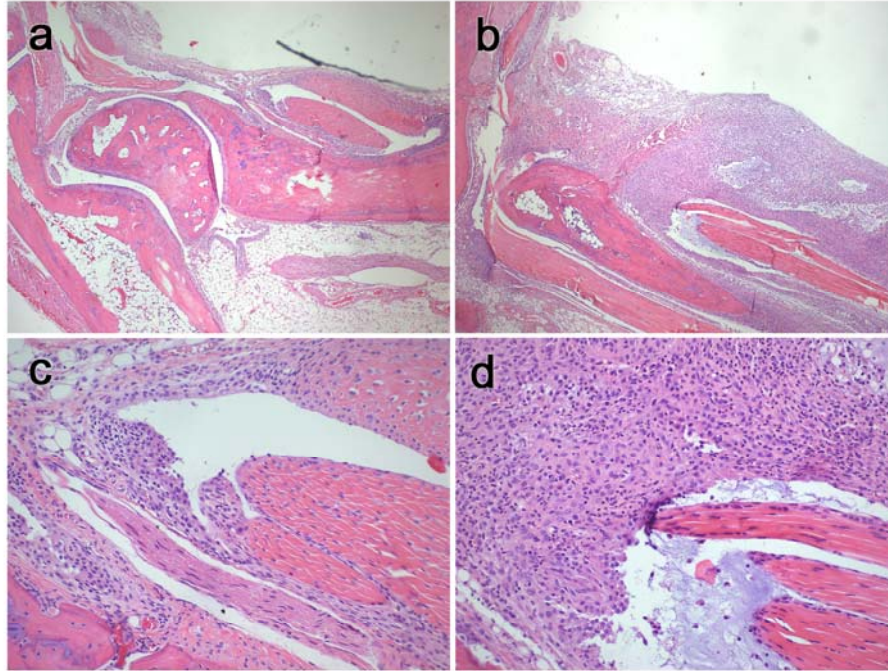
Figure 7. H & E staining of tibiotarsal joints of C3H mice at d35 post-infection. Mice were treated with vehicle (a & c) or rofecoxib (b & d). a & b: 10x magification; c & d: 40x magnification of the joints pictured in a & d. Rofecoxib treatment: 5 mg/kg/day p.o.

Since COX-2 inhibition appeared to affect arthritis resolution and not the development of arthritis pathology, we treated C3H and DBA mice with Mex beginning one day prior to *B. burgdorferi* infection, and once then daily thereafter until d14 post-infection. Administration of the COX-2 inhibitor was then ceased and the infection was allowed to continue until d35 post-infection, at which time arthritis severity was assessed by histology. The time course of COX-2 inhibition did not lead to arthritis development in the DBA mice (Figure 8 a). As shown in Figure 8 b, administration of the COX-2 inhibitor through d14 of infection was sufficient for inhibition of arthritis resolution in C3H animals. Thus, it appears that a pro-resolution signal is produced early during the normal course of *B. burgdorferi* infection, prior to day 14. This supports the premise that the inflammatory response is a dynamic process, and that COX-2 is responsible for the production or induction of a molecule that initiates the resolution of inflammation.

2. *Inability to resolve Lyme arthritis in COX-2 inhibitor-treated mice is not due to altered humoral immunity or increased spirochetemia*

These data were in apparent opposition to the previous study which found that inhibition of COX-2 prevented development of arthritis. However, as our use of different Coxibs implies, examination of COX-2 inhibitor-treated mice at d14 post-infection alone may have allowed a slight delay in arthritis development in these animals to be mistaken for a complete inhibition of pathology. Alternatively, the use of an intradermal inoculation model may have led to a slight delay in arthritis development not seen in the footpad inoculation model. In our study, arthritis pathology developed in both vehicle-treated and
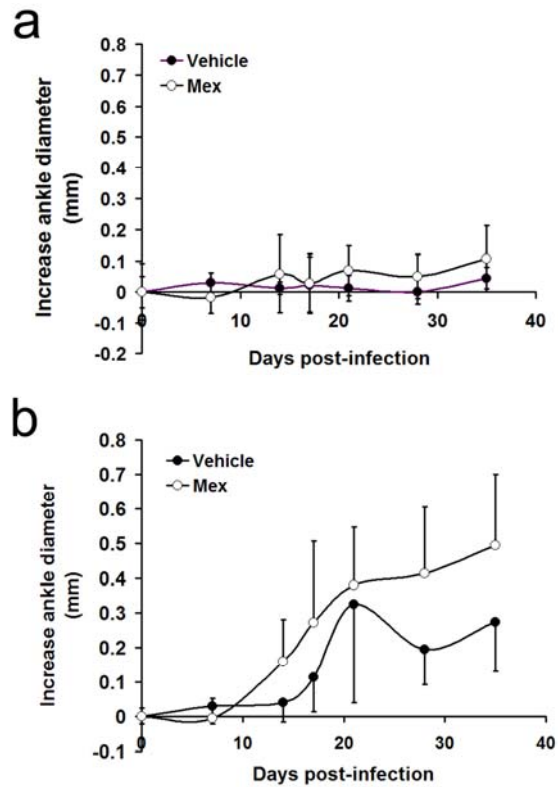
Figure 8. Early administration of a COX-2 inhibitor is sufficient to prevent the induction of resolution. DBA (a) and C3H (b) mice were treated daily with vehicle (closed circles) or meloxicam (Mex; open circles) beginning the day before infection until d14 post-infection. C3H mice (b) still failed to resolve their ankle swelling, despite withdrawl of inhibitor. Withdrawl of treatment had no affect on DBA development of ankle swelling. Meloxicam treatment: 10 mg/kg/day. Error bars represent $\pm$ s.d. n = 5 mice per time point. These data are representative of two.

Clx-treated C3H mice, however there appeared to be a delay or inhibition of the natural resolution of the inflammatory response seen in vehicle-treated mice when the mice were treated with the COX-2 inhibitor (see Figure 7). Spirochætal clearance from infected tissues is thought to be mediated by *Bb*-specific antibodies, leading to the down-regulation of the inflammatory response and the return to homeostasis. Thus it was possible that the production of *Bb*-specific antibodies might be altered in COX-2 inhibitor-treated animals, leading to a deficit in spirochete clearance and a delay in arthritis resolution. As shown in Figure 9, *Borrelia*-specific antibody production was not significantly altered by COX-2 inhibition at either of the time points tested in C3H (Figure 9 b) or DBA (Figure 9 a) mice, indicating that the failure of arthritis resolution in the COX-2 inhibitor-treated mice was not due to an alteration in antibody responses. We also measured the levels of *Bb* in the joints by real-time PCR at various time points post-infection to determine if COX-2 inhibition resulted in increased bacterial burden, resulting in the sustained inflammatory response observed histologically. Figure 10 demonstrates that no differences in spirochete numbers were found at any of the time points examined in the joints of either DBA (Figure 10 a) or C3H (Figure 10 b) mice treated with Clx. Thus, although COX-2 inhibition resulted in the inability of C3H mice to resolve their arthritis, their ability to produce *Borrelia*-specific antibody responses and clear spirochetes from infected tissues was not impaired. This suggests that the clearance of spirochetes alone from the tissues is not sufficient to trigger arthritis resolution. Another factor, or factors, under the influence of COX-2 or its products must also control the initiation of arthritis resolution.
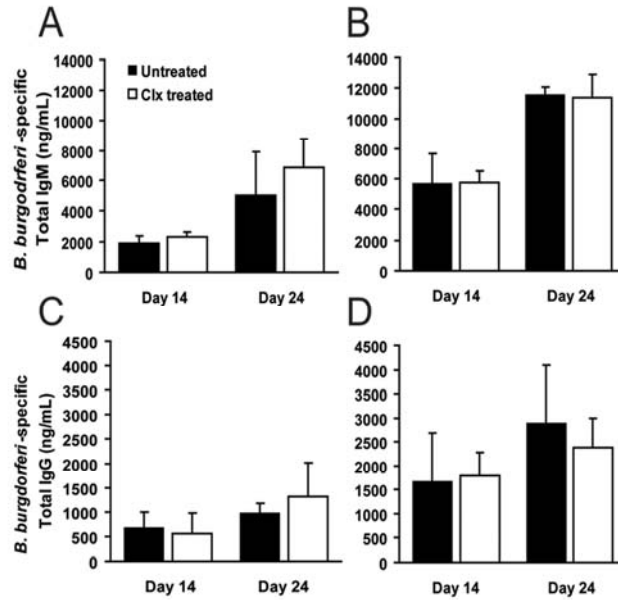
Figure 9. Celecoxib treatment does not inhibit antibody development. Antibody responses of DBA (A & C) and C3H (B & D) mice are illustrated. Mice were treated with vehicle (filled bars) or celecoxib (Clx; open bars) and *B. burgdorferi*-specific antibody levels measured at d14 and d24 post-infection. Error bars represent $\pm$ s.d.
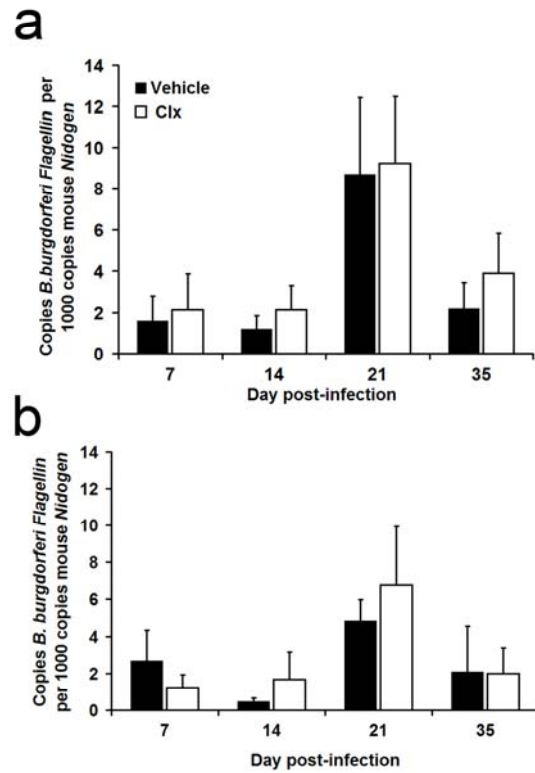
Figure 10. *B. burgdorferi* loads in the joints are not altered by COX-2 inhibition. Spirochete burdens were measured by real-time PCR in the joints of DBA (a) and C3H (b) mice at d7, 14, 21, and 35 post-infection treated with vehicle (closed bars) or celecoxib (Clx; open bars). Error bars represent ± s.d. *n* = 5 mice per group per time point.

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3. *COX-1, COX-2, 5-LO, FLAP, and cPLA$_2$ mRNA expression in response to COX-2 inhibition*

COX-2 mRNA expression is up-regulated in response to pro-inflammatory stimuli, and its continued expression *in vitro* is mediated by stabilization of transcript. Products of COX-2 feed back to partially regulate this stabilization, resulting in increased transcript and COX-2 products (34). Previous *in vitro* studies have also shown that the expression of COX-2 appears to be bimodal, with initial increases in its expression correlating with the beginning of the inflammatory response, and its later expression initiating the resolution of the inflammatory response (166). It was therefore of interest to determine the kinetic expression of COX-2 mRNA in the joints of arthritis-resistant DBA and -susceptible C3H mice in response to *Bb* infection. The expression of COX-1 and COX-2 mRNA were analyzed at time points between d3 and d35 post-infection and compared to d0 expression levels (Figure 11). As stated earlier, COX-1 is constitutively expressed in most tissues and is not thought to play a significant role in inflammatory responses.

As expected, our results show that COX-1 mRNA expression was similar between C3H and DBA vehicle-treated mice, and not significantly up-regulated in response to *Bb* infection (Figure 11 a). COX-2 transcript was also expressed at a low basal level in the joints, but this expression was significantly increased in infected tissues from both C3H and DBA mice (Figure 11 b). Although slightly different kinetics were displayed between resistant and susceptible mouse strains, vehicle-treated C3H and DBA joints expressed COX-2 mRNA in the bimodal pattern predicted by the *in vitro* studies. COX-1
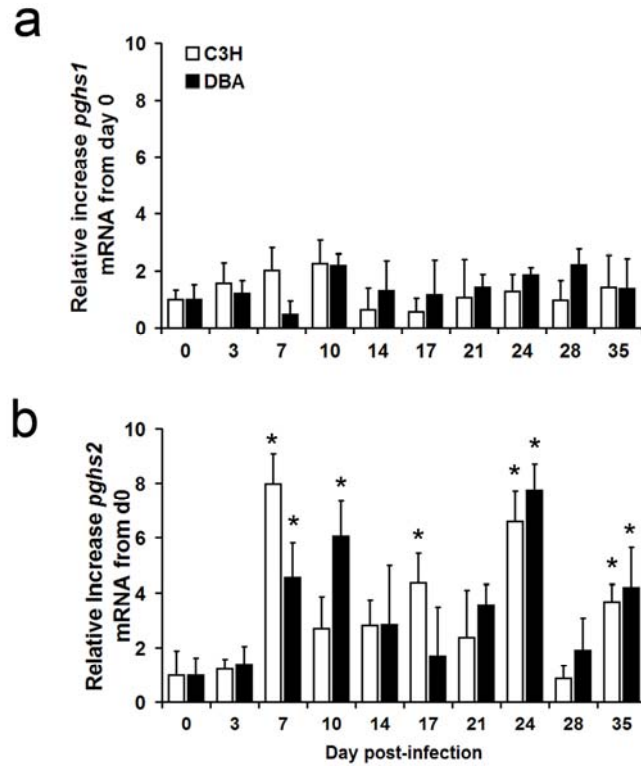
Figure 11. COX-1 and COX-2 mRNA expression in the joints of *B. burgdorferi* infected mice. C3H (open bars) and DBA (closed bars) mice did not alter their expression of COX-1 mRNA (a) in response to infection. COX-2 expression (b) was significantly increased at several time points during infection in both mouse strains. Error bars represent ± s.d.. *$p < 0.001$ versus day 0. $n = 5$ mice per time point. These data are representative of three experiments.

mRNA expression was unaltered in either mouse strain by COX-2 inhibition (Figure 12 a and b). Whereas DBA expression of COX-2 appeared to be unaffected by COX-2 inhibition (Figure 13 a), COX-2 mRNA expression in the joints of Clx-treated C3H animals was significantly decreased at d7 and d24 post-infection, with sustained increases from d10 to d14 post-infection, as compared to the joints of control animals (Figure 13 b). This alteration in COX-2 expression in the susceptible, but not the resistant mouse strain, indicates that COX-2 inhibition may disrupt the normal kinetics of regulatory lipid production controlling the inflammatory response, thus leading to the lack of arthritis resolution observed in *Bb*-infected Coxib-treated C3H mice.

We also assayed the expression of 5-LO, FLAP, and cPLA$_2$ mRNA. As described earlier, 5-LO metabolizes AA to LTs with the help of FLAP, and cPLA$_2$ is responsible for beginning the arachidonate cascade by releasing AA from membrane phospholipids for utilization by the COX and LO enzymes. Production of the mRNA for these proteins has been shown to be up-regulated by inflammatory stimuli (167,162). Thus, we wished to ascertain whether their expression might be altered in the joints of celecoxib-treated mice in comparison to those of vehicle-treated mice. Expression of 5-LO, FLAP, and cPLA$_2$ mRNAs were measured by real-time RT-PCR at the same time points during *B. burgdorferi* infection as COX-1 and COX-2 mRNA.

C3H vehicle-treated mice up-regulate expression of cPLA$_2$ at day 7, 3 days earlier than DBA animals (Figure 14). Clx did not alter expression of cPLA$_2$ in the joints of DBA mice (Figure 14 a), however the joints of C3H animals treated with Clx demonstrated
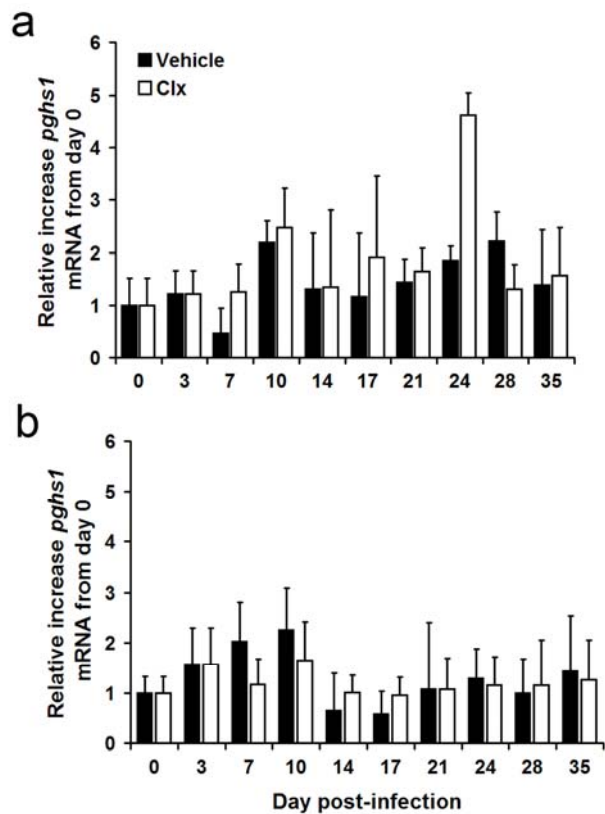
Figure 12. COX-1 mRNA production was unaltered by COX-2 inhibition. COX-1 mRNA was measured from the joints of DBA (a) and C3H (b) mice treated with vehicle (closed bars) or celecoxib (Clx; open bars). Error bars represent $\pm$ s.d. n = 5 mice per time point. These data are representative of three experiments.
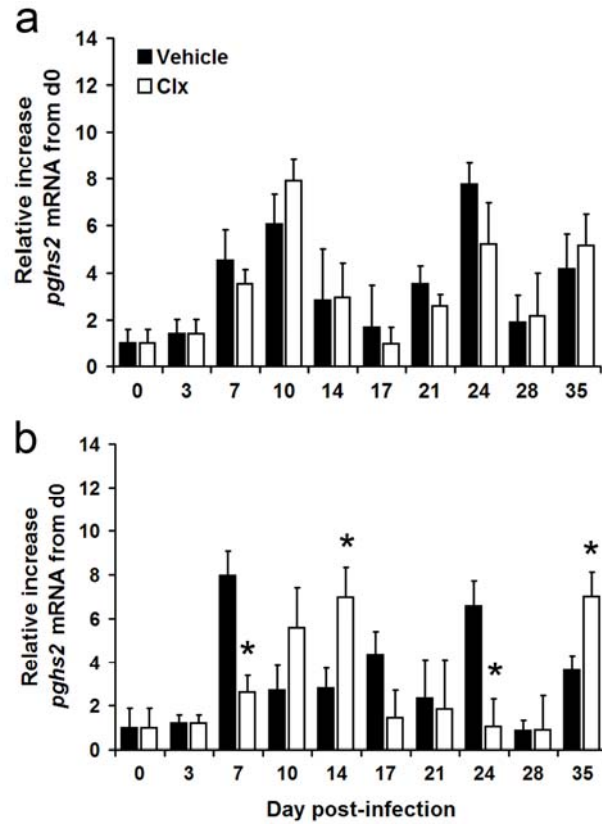
Figure 13. COX-2 inhibition altered production of COX-2 mRNA by C3H animals. COX-2 mRNA was measured in the joints of DBA (a) and C3H (b) mice treated with vehicle (closed bars) or celecoxib (Clx; open bars). COX-2 inhibition did not alter COX-2 mRNA production in DBA joints, but affected COX-2 mRNA production in C3H mice at several time points. Error bars represent $\pm$ s.d. * $p < 0.001$ versus vehicle-treated controls. $n = 5$ mice per time point.
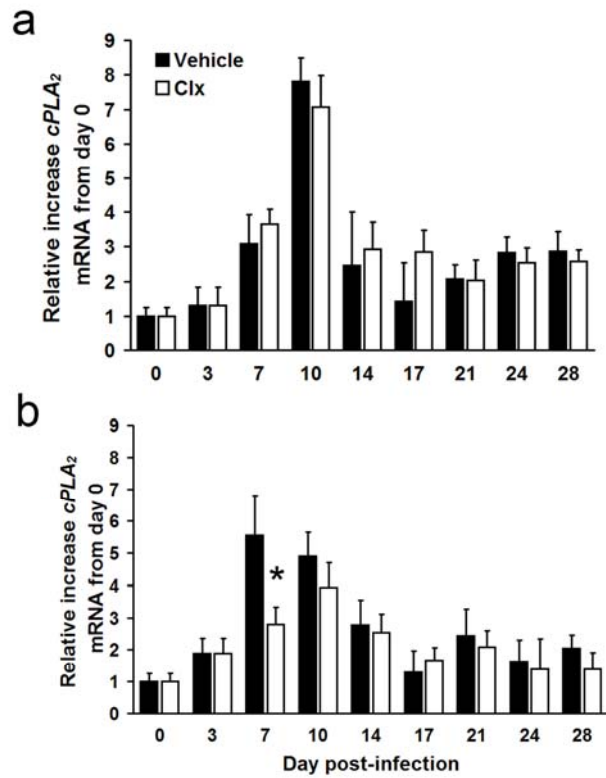
Figure 14. cPLA$_2$ mRNA production is up-regulated by *B. burgdorferi* infection and affeted by COX-2 inhibition in C3H mice. cPLA$_2$ mRNA was measured in the joints of *B. burgdorferi* infected DBA (a) and C3H (b) mice treated with vehicle (closed bars) or celecoxib (Clx; open bars). cPLA$_2$ was up-regulated in both DBA and C3H mice. COX-2 inhibition affected cPLA$_2$ expression in C3H mice at d7 post-infection. Error bars represent $\pm$ s.d. *$p < 0.001$ versus untreated controls. $n = 5$ mice per time point.

significantly lower levels of cPLA$_2$ mRNA at d7 compared to their vehicle-treated counterparts (Figure 14 b). When the expression of 5-LO and FLAP were compared within the strains, although the absolute numbers were different, the two patterns were similar for FLAP and 5-LO produced by DBA mice (Figure 15 a) and FLAP and 5-LO produced by C3H mice (Figure 15 b), with one primary peak of up-regulation around 10 post-infection. As FLAP regulates 5-LO activity, we were pleased to see the expected expression pattern. FLAP mRNA was increased in vehicle-treated mice of both resistant and susceptible mouse strains and was not altered in either strain by COX-2 inhibition (Figure 16 a and b). Curiously, DBA animals did demonstrate significantly altered production of 5-LO mRNA during treatment with Clx (Figure 17 a), while C3H animals did not (Figure 17 b). Since this is the only "inflammatory" mRNA measured (between 5-LO, FLAP, COX-2, and cPLA$_2$) that was altered by COX-2 inhibition in DBA animals, and conversely, the only mRNA not different between vehicle and Clx-treated C3H animals, it is possible that 5-LO products could play a disease modulating role. Production of 5-LO mRNA could have been altered in Coxib-treated DBA mice in order to maintain the "proper" inflammatory response demonstrated by the vehicle-treated DBA mice, thus COX-2 inhibition did not effect Lyme arthritis pathology in this resistant mouse strain. Likewise, C3H mice have altered production of both COX-2 and cPLA$_2$. In this case, 5-LO might not be receiving the "modulation" signals that are present in DBA mice and mediate the maintenance of homeostasis. Thus, 5-LO production of modulating compounds was not adjusted to compensate for altered production of lipid mediators.
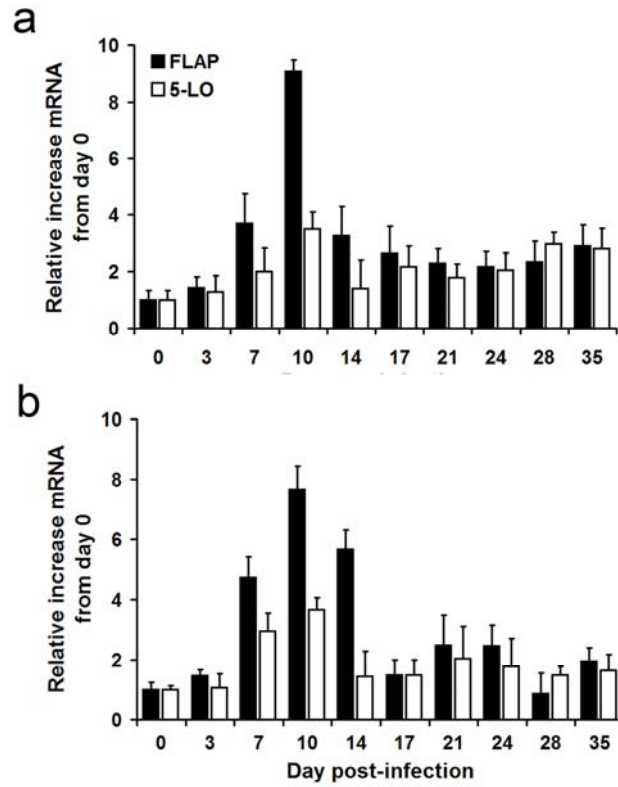
Figure 15. Expression of FLAP versus 5-LO mRNA in the joints of DBA (a) and C3H (b) *B. burgdorferi* infected mice. FLAP production (closed bars) is shown in the context of 5-LO (open bars) mRNA production from the same joints. Both DBA and C3H mice demonstrate a primary peak of production at d10 post-infection. Error bars represent $\pm$ s.d. n = 5 mice per time point. Data are representative of two experiments.
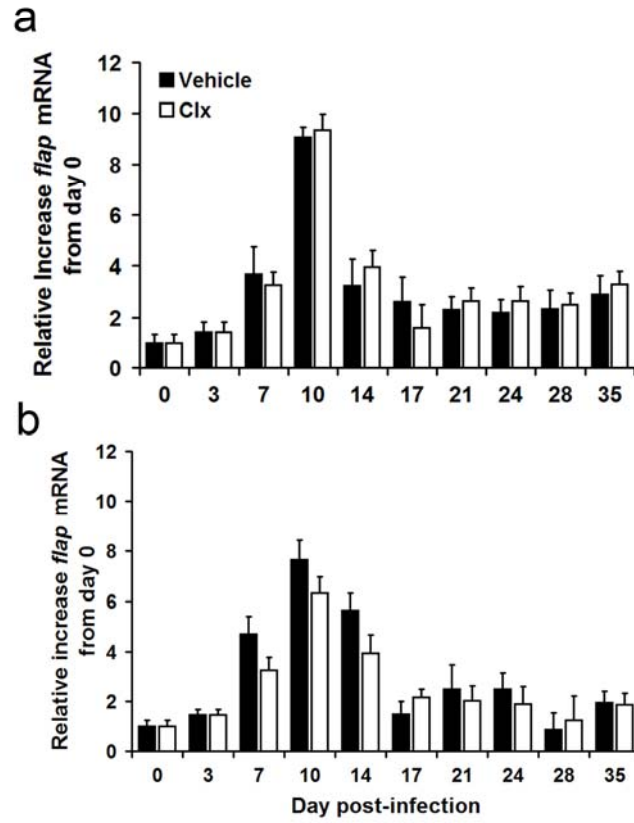
Figure 16. FLAP expression induced by *B. burgdorferi* infection is not altered by celecoxib (Clx) treatment. DBA (a) and C3H (b) mice were treated with vehicle (closed bars) or Clx (open bars) and FLAP mRNA expression was measured. Error bars represent $\pm$ s.d. n = 5 mice per time point. Data are representative of two experiments.
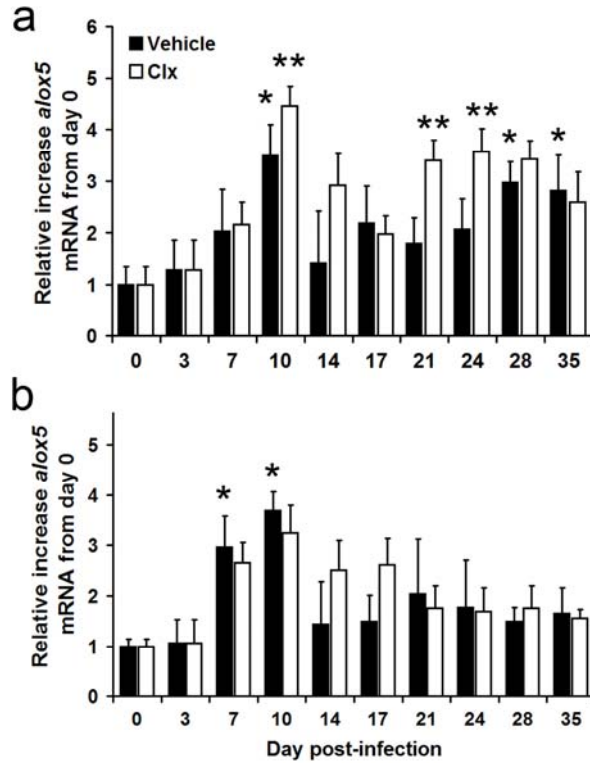
Figure 17. 5-LO (*alox5*) mRNA production was altered in the joints of DBA (a) but not C3H (b) mice upon inhibition of COX-2. Animals were treated with vehicle (closed bars) or celecoxib (Clx; open bars). * & **$p < 0.05$. *versus d0. **versus vehicle-treated control. $n = 5$ mice per time point.

4. *COX-2-deficient mice develop non-resolving Lyme arthritis similar to Coxib-treated animals*

There was the possibility that COX-2 inhibition was incomplete in our system, thus allowing for the development of a full, albeit altered, inflammatory response. To further investigate the role of COX-2 in development and resolution of the inflammatory response to *Bb* infection, we obtained mice genetically deficient in COX-2 expression (B6.129*Ptgs2*^{tm1Jed}). These mice were backcrossed in our laboratory for 10 generations onto both the C3H and DBA backgrounds. COX-2$^{-/-}$ animals and their wild-type (WT) littermates were infected with *Bb,* and ankle swelling and arthritis severity scores were compiled, as described earlier. Increases in ankle diameter were inhibited in a gene dilution manner, as wild-type mice displayed the normal pattern of ankle swelling, COX-2$^{-/-}$ littermates had reduced ankle swelling curves, and heterozygous littermates displayed an intermediate phenotype (Figure 18). C3H COX-2$^{-/-}$ displayed an initial inflammatory response similar to their WT littermates, however, they also demonstrated the inability to resolve their arthritis as seen in the C3H COX-2 inhibitor-treated animals, with arthritis pathology still evident 60 days post-infection (Figure 19 a and b). DBA mice deficient in COX-2 were refractory to the development of Lyme arthritis pathology, similar to their wild-type Coxib-treated counterparts (Figure 20 a and b). Also similar to inhibitor-treated C3H and DBA mice, neither *Bb*-specific antibody levels (Figure 21) nor bacterial burdens (Figure 22) were altered in COX-2$^{-/-}$ animals compared to WT littermate controls, further implying a pivotal role for COX-2 in innate immune system down-modulation of the inflammatory response.

a

b
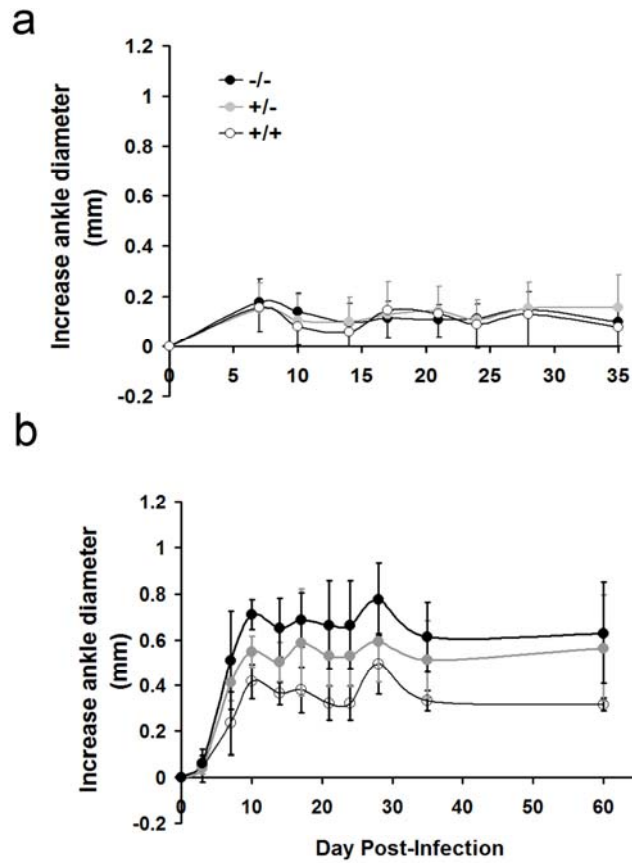
Figure 18. Effect of COX-deficiency on ankle diameter increase. The COX-2 gene knockout was backcrossed onto both the DBA and C3H backgrounds. DBA (a) and C3H (b) COX-2$^{-/-}$ (closed symbols), COX-2$^{+/-}$ (grey symbols), and COX-2$^{+/+}$ (open symbols) littermates were infected with *B. burgdorferi* and increases in ankle diameter were measured at various time points until d35 (DBA) or d60 (C3H) post-infection. *n* = 6 COX-2$^{+/+}$, *n* = 10 COX-2$^{+/-}$, *n* = 11 COX-2$^{-/-}$ mice per group. This experiment is representative of four seperate experiments.
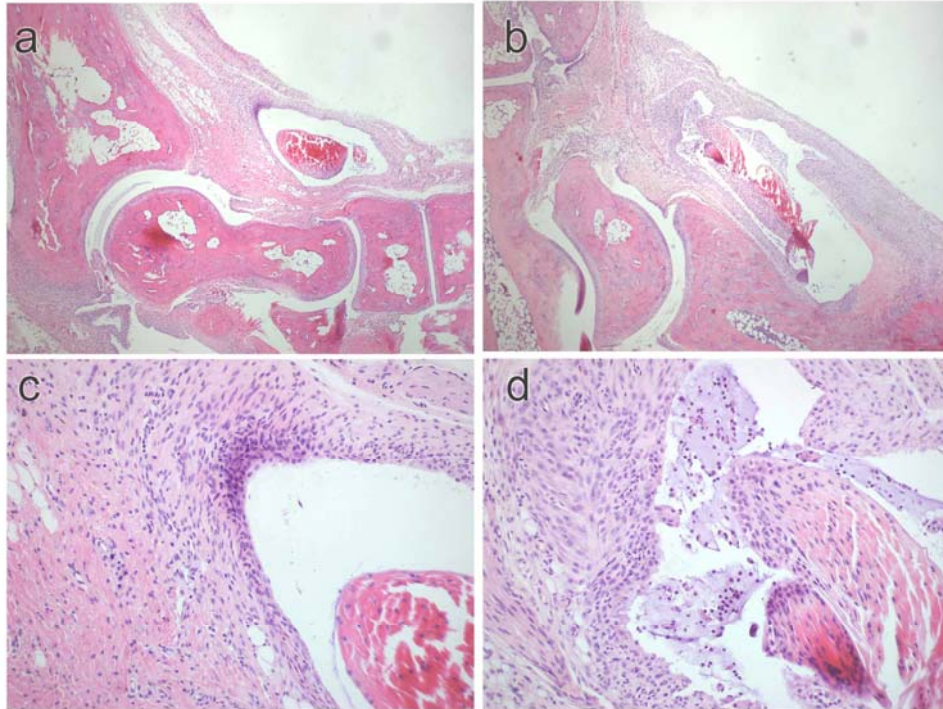
Figure 19. H & E stain of tibiotarsal joints from C3H COX-2$^{+/+}$ (a & c) and COX-2$^{-/-}$ (b & d) at d60 post-infection. a & b: 10x magnification. c & d: 40x magnification of the joints pictured in a & b.
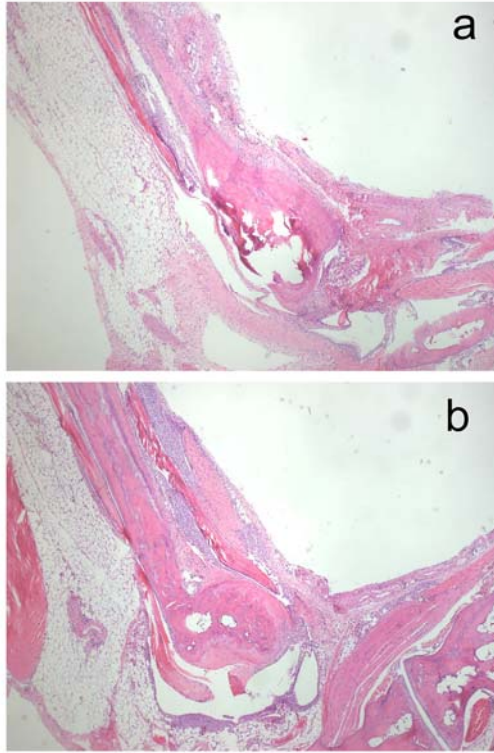
Figure 20. H & E staining of tibiotarsal joints from COX-2$^{+/+}$ (a) and COX-2$^{-/-}$ (b) DBA mice at d35 post-infection. Magnification: 10x.
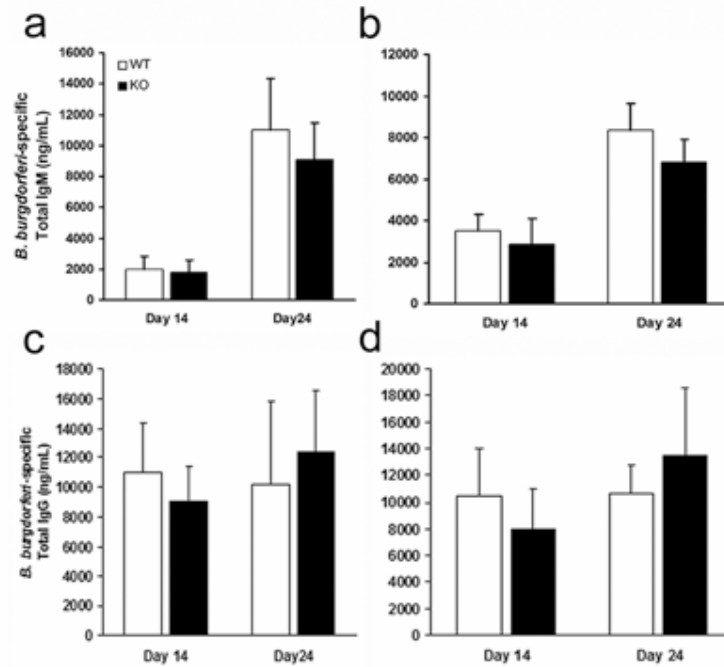
Figure 21. Comparison of antibody production by COX-2$^{-/-}$ and COX-2$^{+/+}$ DBA (a & c) and C3H (b & d) mice. IgM (a & b) and IgG (c & d) levels were measured by ELISA at d14 and d24 post-infection in COX-2 wild-type (WT; open bars) and knockout (KO; closed bars). Error bars represent $\pm$ s.d. *n* = 8-11 mice per group. This is representative of three experiments.
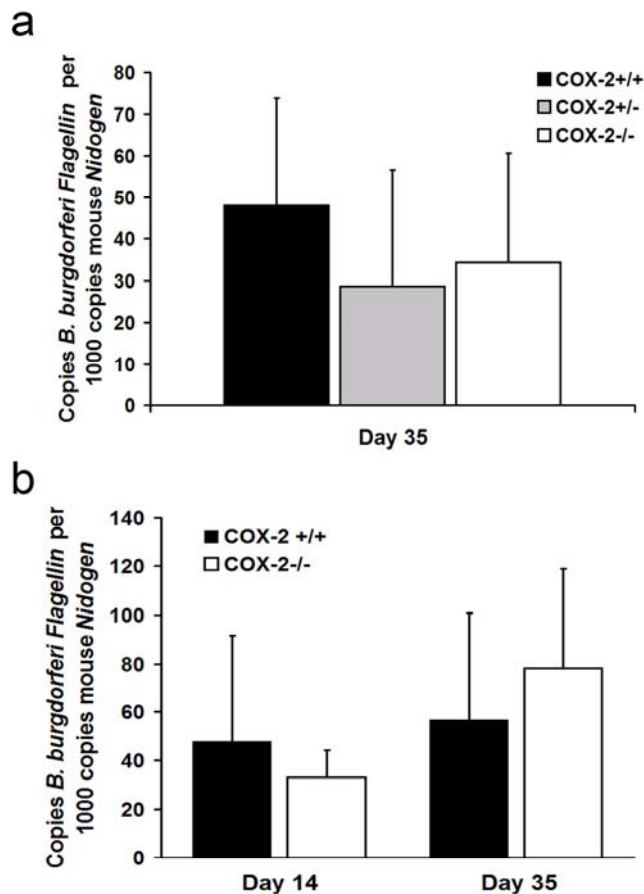
Figure 22. Bacterial loads in COX-2⁻/⁻ DBA (a) and C3H (b) mice. *B. burgdorferi* levels in the joint were quantitated by real-time PCR in COX-2⁺/⁺ (closed bars), COX-2⁺/⁻ (grey bar), and COX-2⁻/⁻ (open bars) mice. Since no pathology was observed, bacterial loads were exmined at only one time point in DBA animals. Error bars represent $\pm$ s.d. n = 5-7 mice per group. This experiment is representative of five different experiments.

5. B. burgdorferi *infection induces production of both pro- and anti-inflammatory lipid molecules, which are altered by COX-2 inhibition*

Incubation of murine microglia with *B. burgdorferi in vitro* has been shown to induce production of PGE_2; however it is unknown what lipid mediators may be produced *in vivo* during *B. burgdorferi* infection, particularly with regard to the arthritic inflammatory response. We therefore extracted and isolated eicosanoids from knee joints of vehicle-, as well as Clx-treated animals, to determine if certain lipids were produced in response to *B. burgdorferi* infection, and whether that production was altered by COX-2 inhibition. Figure 23 shows that the COX products PGE_2 and 15-deoxy-$\Delta^{12, 14}$ PGJ_2 ($15ddPGJ_2$), as well as the 5-LO product LTB_4 were all up-regulated in the joints of *B. burgdorferi*-infected animals. Of interest is the production of pro-resolution cyclopentenone $15ddPGJ_2$ at early time points of infection, indicating a regulatory role by this COX-2 product early in the inflammatory response.

COX-2 inhibition had both expected and unexpected effects on production of these lipids. Figure 23 a and b show the anticipated decrease in PG production as a result of COX-2 inhibition. Curiously, however, the 5-LO product LTB_4 was dramatically increased as a result of COX-2 inhibition in these joints (Figure 23 c). This increased LTB_4 may be the result of two different mechanisms. The first possibility is a "LT shunting" mechanism, which operates much like a water faucet and a sink. COX-2 inhibition blocks the "drain" of the "sink", which closes the primary exit for AA metabolism. However, the AA faucet continues to flow, leading to increasing levels of AA. 5-LO then functions as an overflow valve. AA is still freed from the membrane, and 5-LO is available to
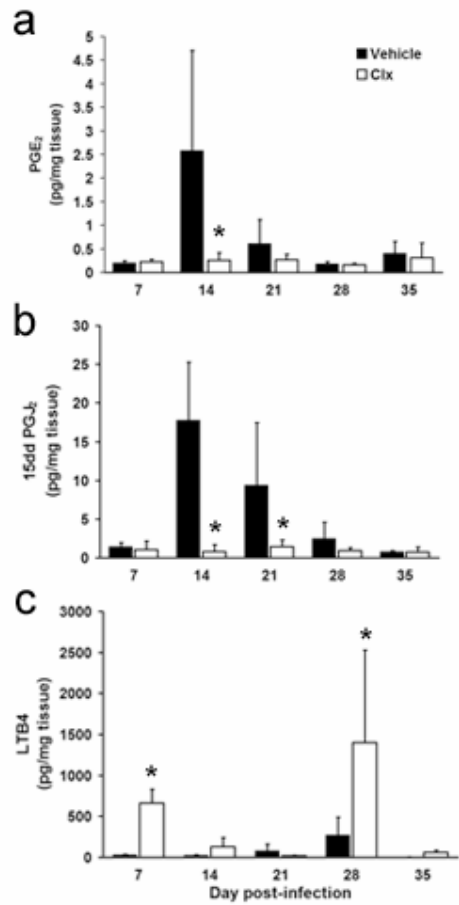
Figure 23. Production of eicosanoids in ankle joints of C3H mice infected with *B. burgdorferi*. Levels of PGE_2 (a), 15-deoxy-$\Delta^{12,14}$ PGJ_2 (15ddPGJ$_2$; b), and LTB_4 (c), were meausred from the joints of vehicle (normal chow)- (closed bars) and celecoxib chow- (Clx, open bars) treated C3H animals. Error bars represent $\pm$ s.d. *$p < 0.05$, $n = 5$ animals per group. This experiment is representative of two.

metabolize it. Thus, COX-2 inhibition might result in this "shunt" from the COX-2 "drain" to the 5-LO "overflow valve", leading to increased LTB_4 production. Another potential mechanism could be that inhibition of COX-2 induced the production of other pro-inflammatory stimuli or triggers a feedback mechanism, which further up-regulates LTB_4 production. The exact mechanism for the increased production of LTB_4 seen in the COX-2 inhibitor-treated animals is still a matter for debate and further exploration.

6. *Blockade of the BLT_1 does not restore resolution to COX-2 inhibitor-treated animals*

The data supporting a potential *in vivo* pro-resolution role for $15ddPGJ_2$ are unclear; however the pro-inflammatory nature of LTB_4 is indisputable. We therefore determined if the increased production of LTB_4 during COX-2 inhibition was the driving force behind the inability of these Coxib-treated mice to resolve their Lyme arthritis. A blockade of receptor signaling was chosen over the enzymatic inhibition of 5-LO or LTA_4 hydrolase for several reasons. The first was to prevent any further disruptions in substrate availability, i.e., inhibition of the LTA_4 hydrolase could possibly lead to increased substrate for the cysteinyl LT (cysLT) LTC_4 synthase, or 5-LO inhibition could lead to increased AA availability for COX-1. Also, inhibition of 5-LO would not only disrupt LTB_4 production, but also cysLT production and potentially alter pro-resolution lipoxin production, complicating our results. Therefore, antagonism of the LTB_4 receptor (BLT_1) was the most logical experimental approach.

CP-105,696 is a cyclopropane carboxylic acid that demonstrates potent antagonism of BLT_1. Although there is a BLT_2, relatively little is known about its function, and it does

not appear to play an appreciable role in neutrophil responses to LTB_4. To examine the effect of BLT_1 antagonism during COX-2 inhibition, C3H mice were infected with *B. burgdorferi*, and divided in to three treatment groups: vehicle, Clx, and Clx plus CP-105,696 (CPX), with the initiation of treatment on the day before infection. Changes in ankle diameter over the time course of infection are illustrated in Figure 24. Blocking BLT_1 in addition to COX-2 inhibition did not alter ankle swelling until d28 post-infection, at which time it actually increased. It did not, however, appear to affect arthritis severity, as shown by histology in Figure 25. There are several possible explanations for this outcome: 1.) if there is a feedback mechanism involved, blocking BLT_1 may induce even greater production of LTB_4 and thus overcome the blockade; 2.) either separately or in conjunction with the first possibility, BLT_2 may actually be involved in this arthritic response, and therefore BLT_1 antagonism would be ineffective at blocking LTB_4 signaling; 3.) although it is produced upon infection and COX-2 inhibition increases that production, it is possible that LTB_4 is only a generic by-product of the inflammatory response and not the primary mechanism whereby the lack of arthritis resolution is mediated. Due to the complicated nature of eicosanoid regulation, all of these explanations are within the realm of possibility; likewise, there may be some other previously unappreciated regulatory pathway involved. Further experiments must be conducted to fully decode the mechanisms responsible.

C. *Conclusions*

In the present study, we demonstrated a lack of arthritis resolution induced by specific inhibition of COX-2 during infection with the etiologic agent of Lyme disease, the
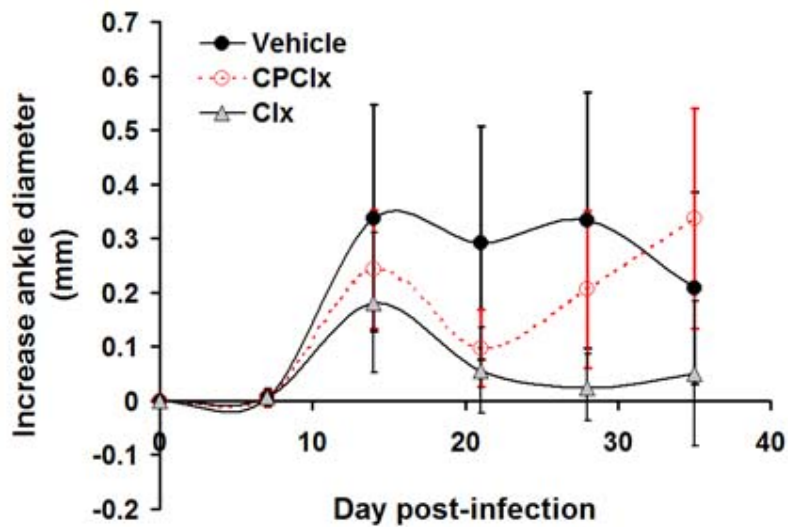
Figure 24. Affect of BLT$_1$ antagonism in conjunction with COX-2 inhibition on development of ankle swelling. Ankle diamters were measured in C3H mice infected with *B. burgdorferi* and treated with either vehicle (closed circle), celecoxib chow (Clx; grey triangle), or both Clx and CP-105,696 (CPClx; open red circle). Error bars represent $\pm$ s.d. n = 5 mice per treatment group.
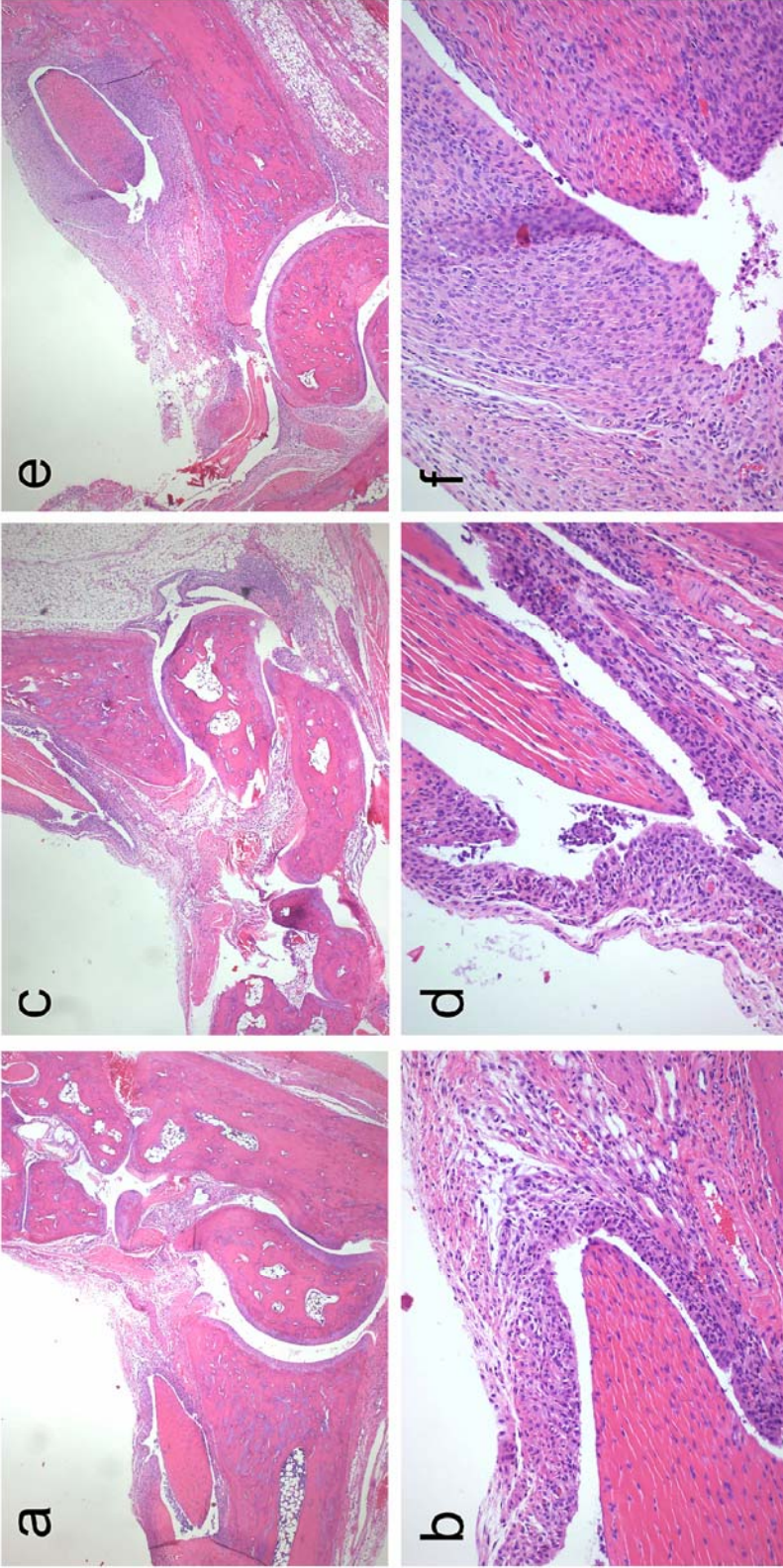
Figure 25. H & E staining of tibiotarsal joints from vehicle- (a & b), celecoxib chow- (Clx; c & d), and celecoxib + CP-105,696-treated (CPX; e & f) at d35 post-infection. a, c, & e: 10x magnification. b, d, & f: 40x magnification of the joints pictured in a, c, & e.

spirochete *Borrelia burgdorferi*. While no differences in the development of pathology were evident in Lyme arthritis-resistant DBA/2J (DBA) mice, in arthritis-susceptible C3H/HeJ mice, arthritis development and severity were not altered, however the inflammation induced by *B. burgdorferi* infection was not resolved in the presence of COX-2 inhibitors. Withdrawal of COX-2 inhibition at d14 did not lead to arthritis resolution, implying an early "on" switch for induction of pro-resolution mechanisms. This inability to resolve inflammation was not due to an increase in bacterial loads or altered antibody responses, indicating that humoral immunity was not affected by COX-2 inhibition. Surprisingly, normal up-regulation of COX-2 mRNA was altered by COX-2 inhibition in C3H mice, most notably at d7, indicating an effect on the feed-back mechanisms involved in regulation of COX-2. Similarly, $cPLA_2$ mRNA expression was also altered at d7 in C3H animals treated with Clx. FLAP mRNA expression was increased in the joints of both DBA and C3H animals, but was not altered in either strain by Clx treatment. The expression pattern of 5-LO mRNA by vehicle-treated animals was similar to that of FLAP mRNA. However, whereas $cPLA_2$ and COX-2 mRNAs were altered by Clx-treatment in C3H animals but not in DBA animals, the inverse was true of 5-LO expression; joints from DBA animals treated with Clx demonstrated altered 5-LO mRNA expression, whereas those from C3H Clx-treated animals did not. Furthermore, deletion of COX-2 on the susceptible genetic background resulted in an inability to resolve arthritis similar to that seen in inhibitor-treated wild-type mice. COX-2$^{-/-}$ DBA animals also responded similarly to their inhibitor-treated counterparts, in that there was no difference in arthritis pathology between knock-out and wild-type animals.

Although production of the anti-inflammatory cyclopentenone prostaglandin (PG) 15-deoxy-$\Delta^{12,14}$ PGJ$_2$, and the highly pro-inflammatory leukotriene B$_4$ (LTB$_4$) were induced in arthritic joints at similar time points during the normal course of infection, we found that COX-2 inhibition not only decreased production of 15-deoxy-$\Delta^{12,14}$ PGJ$_2$, but lead to elevated LTB$_4$ levels. Despite the dramatic increase in LTB$_4$ produced in the context of COX-2 inhibition at several time points, blockade of the LTB$_4$ receptor BLT$_1$ did not induce arthritis resolution in Coxib-treated animals. We propose that, upon infection with *B. burgdorferi*, production of both pro- and anti-inflammatory lipid mediators is initiated, and that inhibition of COX-2 leads to dysregulation of pro-resolution pathways, ultimately resulting in an inability to resolve Lyme arthritis pathology. Furthermore, withdrawal of pro-inflammatory signaling, i.e., LTB$_4$ via BLT$_1$, is not sufficient for the induction of resolution; pro-resolution signal is also crucial for down-regulation of the inflammatory response. This suggests a common mechanism whereby pro-inflammatory and pro-resolution signals work in concert to initiate down-modulation of an infection-induced inflammatory response.

CHAPTER IV

LTB$_4$ PRODUCTION DURING DEVELOPMENT OF ARTHRITIS IS

NECESSARY FOR CONTROL AND RESOLUTION OF LYME

ARTHRITIS

A. *Introduction*

Eicosanoids are lipid mediators that have complex regulatory mechanisms and perform a

wide range of functions in the immune system (13). Many eicosanoids have seemingly

contradictory effects, for example, PGE$_2$ production is correlated with severe arthritis, but

it is also a well-characterized immunosuppressant (168). LTB$_4$, however, does not have

this double personality - its known functions are pro-inflammatory, regulating immune

cells, mainly neutrophil migration and activation (88). Earlier we showed that LTB$_4$ was

produced during *B. burgdorferi* infection, however it is unknown if it is responsible for

development of Lyme arthritis pathology, or if it is merely present as a generic marker of

inflammation.

There are several potential pathways for examining the contribution of LTB$_4$ to Lyme

arthritis pathology, indeed to any inflammatory response. Many researchers have utilized

5-LO inhibitors or knock-out mice (169,170,90). However, we now understand that 5-

LO activity is essential for the production of pro-resolution lipids, primarily the lipoxins,

and thus its inhibition or deficit is often undesirable (171,85). Inhibition of LTA$_4$

hydrolase or use of LTA_4 hydrolase-deficient animals is another option, however this could potentially lead to an excess of substrate. Much like the concept of leukotriene shunting, where inhibition of COX-2 leads to excess substrate (arachidonate) available for the 5-LO enzymes, inhibition of LTA_4 hydrolase could allow for LTA_4 to be utilized by LTC_4 synthase, which produces pro-inflammatory cysteinyl LTs. Due to the complex nature of eicosanoid metabolism, the method least likely to interfere with other pathways is receptor antagonism.

LTB_4 has two receptors: BLT_1 is expressed mainly on immune cells, and the lower affinity BLT_2 which is more ubiquitously expressed (172,93). BLT_1 is considered to be the receptor involved in inflammation (173,174). More specifically, it has been demonstrated to mediate LTB_4 signaling in other animals models of arthritis (175,176). We therefore acquired the highly-specific BLT_1 antagonist CP-105,696 (172), and examined the effect of BLT_1 antagonism during *B. burgdorferi* infection.

B. *Antagonism of BLT_1 results in more severe and extended arthritis pathology*

To determine the role of LTB_4 in the development of experimental Lyme arthritis, resistant and susceptible mice were treated with either vehicle or 10mg/kg/day of CP-105,696, beginning the day before infection with *B. burgdorferi*. At this dosage, the development and progression of murine collagen-induced arthritis was inhibited (174). Ankle diameters were measured at d7, 10, 14, 21, and 35 post-infection and compared to d0 measurements. Ankle swelling was increased beginning at d21 post-infection when BLT_1 was blocked in C3H mice (Figure 26). This increase in ankle diameter was correlated with development of arthritis pathology. At early time points, arthritis pathology in CP-105,696-treated joints was more severe than that seen in the joints of vehicle-treated C3H animals (Figure 27), and at later time points this arthritis did not appear to resolve (Figure 28). No difference in arthritis development as compared with control mice was seen in DBA mice treated with the BLT_1 antagonist (data not shown). Hence, a moderating role is suggested for LTB_4 in the development of Lyme arthritis.

C. *CP-105,696 results in increased IgG at d35 but does not affect bacterial loads*

To determine the mechanism underlying this increase in arthritis pathology, *B. burgdorferi*-specific immunoglobulin levels were measured from sera collected from both DBA and C3H mice at d7, 10, 14, 21, and 35 post-infection. Figure 29 a & b shows that IgM levels were not altered by inhibition of LTB_4 signaling, nor is IgG production changed at any time point in DBA mice, or during early time points in C3H animals (Figure 29 c & d). At d35, however, C3H mice treated with CP-105,696 demonstrated significantly higher levels of IgG than vehicle-treated animals.
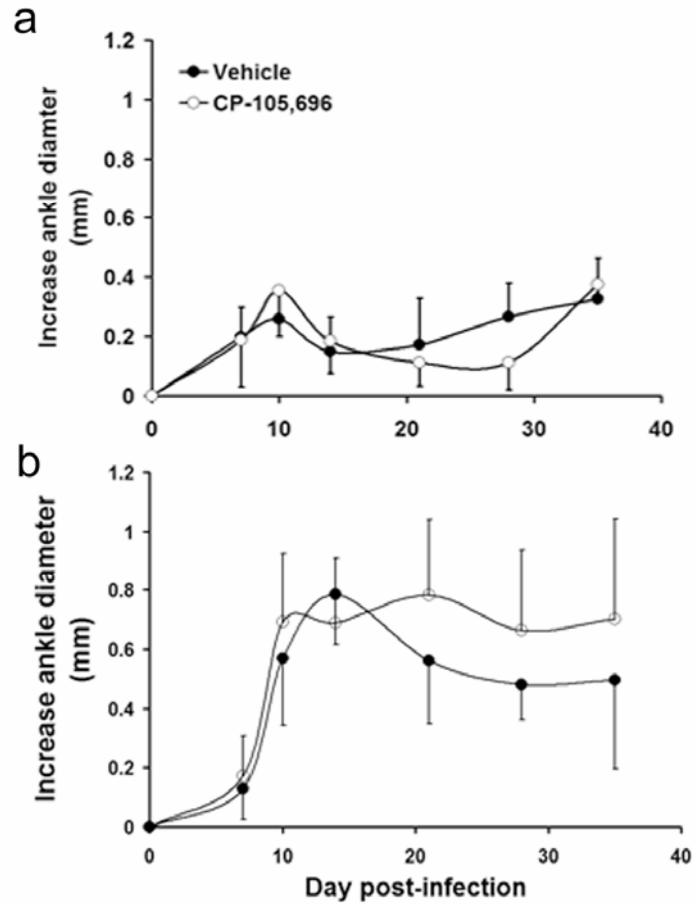
Figure 26. Ankle swelling during BLT$_1$ antagonism. DBA (a) and C3H (b) mice were treated daily with vehicle (closed circles) or CP-105,696 (open circles) and ankle diameters mesaured at the indicated time points. Error bars represent $\pm$ s.d. $n =$ 5 mice per time point. Data are representative of two experiments.
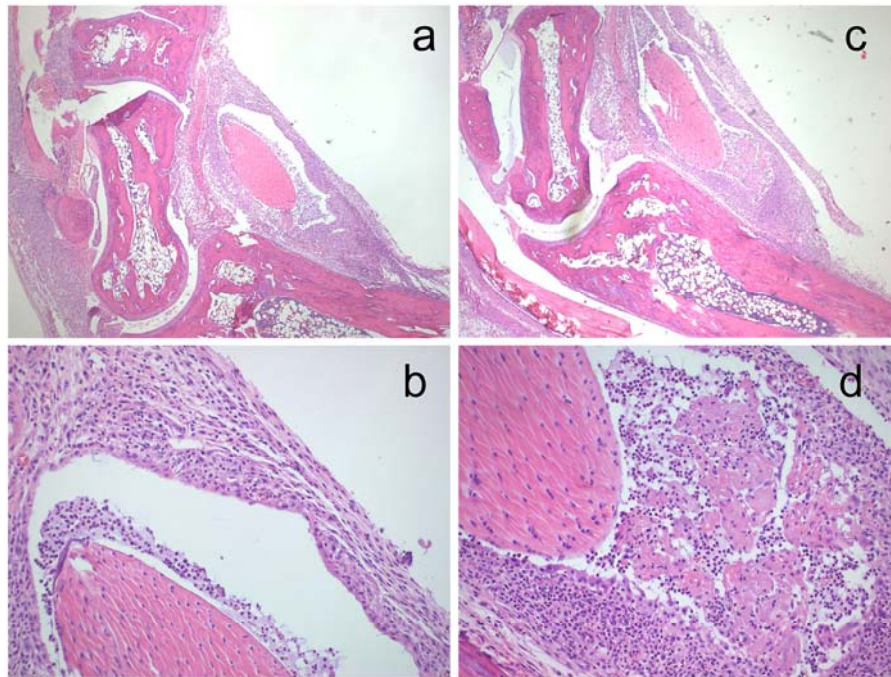
Figure 27. H & E staining of tibiotarsal joints from vehicle- (a & b) or CP-105,696-treated (c & d) C3H mice at d14 post-infection. a & c: 10x magnification; b & d: 40x magnification of the joints pictured in (a) & (c).
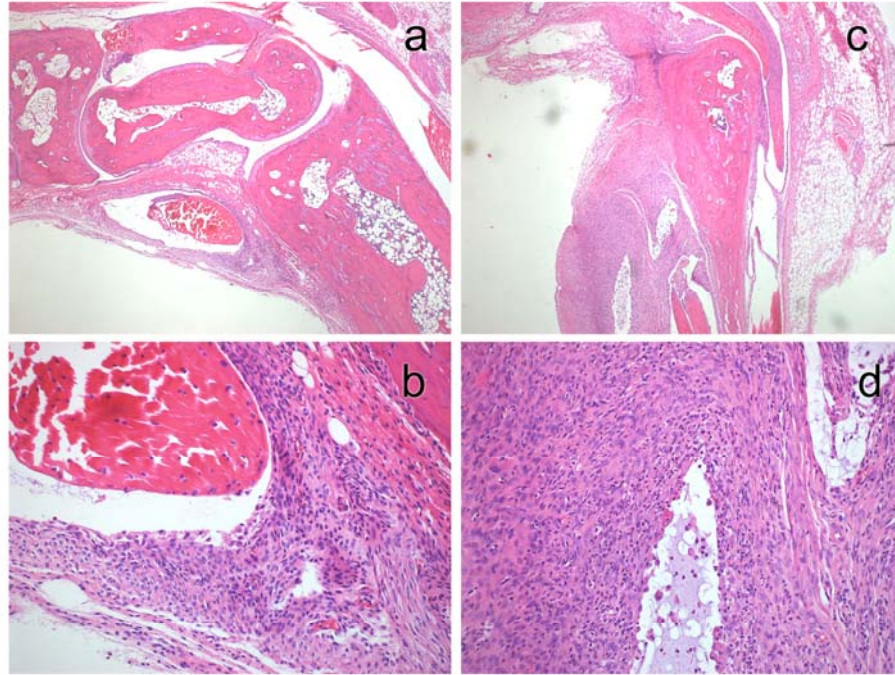
Figure 28. Day 35 H & E staining of tibiotarsal joints from C3H mice treated with vehicle (a & b) or CP-105,696 (c & d). a & c: 10x magnification. b & d: 40x magnification of the joints pictured in (a) & (c).
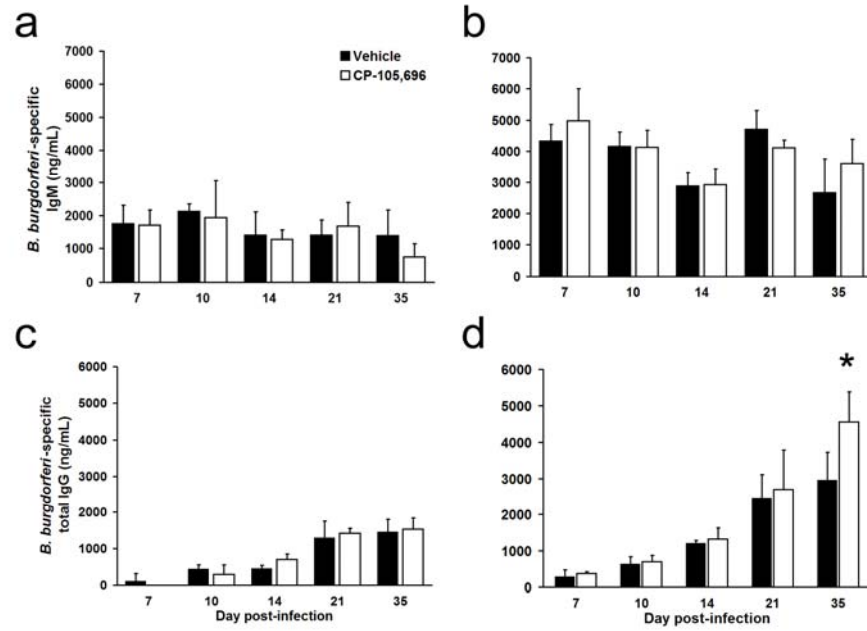
Figure 29. *B. burgdorferi*-specific IgM (a & c) and total IgG (b & d) production at d7, 10, 14, 21, and 35 post-infection. Vehicle- (closed bars) and CP-105,696- (open bars) treated DBA (a & c) and C3H (b & d) mice. Error bars represent ± s.d. *$p < 0.05$ versus vehicle control. $n = 5$ mice per time point. These data are representative of two experiments.

We hypothesized that this increase in antibodies would lead to increased bacterial clearance, since immunoglobulins are thought to be essential for clearance of *B. burgdorferi* from infected tissues (123). Figure 30 demonstrates that this was not the case, since bacterial loads did not differ between the joints of antagonist- or vehicle-treated animals. Therefore, while antibody levels were altered at a late time point, this change did not affect clearance of bacteria from the joints, which probably occurs mainly at earlier time points during the course of infection. Thus, the increased arthritis severity in the CP-105,696-treated mice cannot be attributed to changes in bacterial burdens in the joints of susceptible animals.

D. *COX-2 mRNA is decreased early in infection in the joints of C3H BLT$_1$ antagonist-treated animals.*

Since *B. burgdorferi* loads were not responsible for increased arthritis severity, we next investigated whether the production of key enzymes involved in eicosanoid metabolism was altered by antagonism of BLT$_1$. COX-2 mRNA is frequently up-regulated in inflamed tissues, and production of 5-LO mRNA may be increased in some inflammatory states (167,177). mRNA for the COX-2 and 5-LO enzymes was measured from knee joints of vehicle- and CP-105,696-treated DBA and C3H animals at d7, 10, 14, 21, and 35.

The joints of DBA mice did not demonstrate a difference in expression of either COX-2 or 5-LO mRNA (data not shown). We had anticipated a change in 5-LO mRNA in the joints of C3H mice, since LTB$_4$ is a product of 5-LO metabolism, however this was not
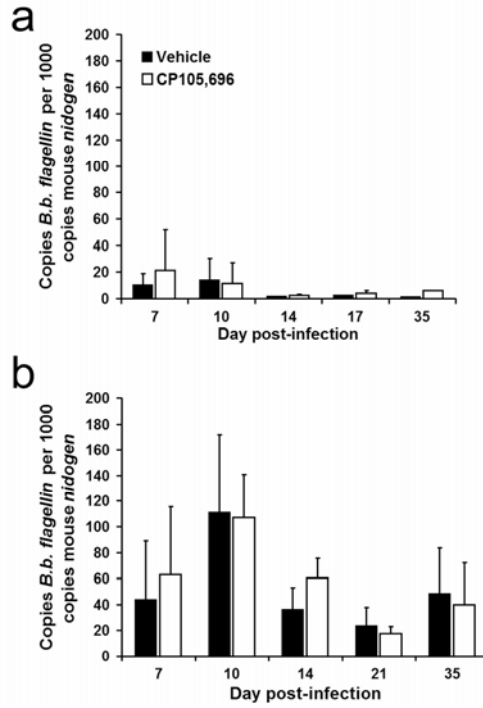
Figure 30. *B. burgdorferi* loads in the tibiotarsal joints of DBA (a) and C3H (b) mice at d7, 10, 14, 21, or 35 post-infection. Mice were treated with vehicle (closed bars) or CP-105,696 (open bars). Error bars represent $\pm$ s.d. n = 5 mice per time point. Data are representative of two experiments.

the case. 5-LO mRNA levels were not different between vehicle- and CP-105,696-treated C3H mice (Figure 31 a). Surprisingly, COX-2 mRNA was significantly decreased in antagonist-treated animals as compared to their control counterparts at d7 post-infection (Figure 31 b). While this might appear to be of little consequence, as shown previously in Chapter III, during the normal course of infection the first phase of the bimodal expression of COX-2 mRNA occurs at d7 post-infection. When this increase does not occur on d7, as was seen with COX-2 inhibition, the normal course of inflammation and resolution does not occur. Therefore, this change in COX-2 mRNA induced by CP-105,696 treatment may contribute to the increased arthritis severity and duration of inflammation seen in these animals.

E. *Blockade of BLT_1 results in decreased TUNEL-staining in inflammatory foci of the joints of* B. burgdorferi-*infected mice*

Another possibility was that LTB_4 was acting primarily via a mechanism that has receives little attention; *in vitro* studies have demonstrated that extended periods of LTB_4 signaling affect the induction of apoptosis by various cell types (95,88). Apoptosis and subsequent regulated phagocytosis of apoptotic cells, termed efferyotosis, is a critical aspect of the inflammatory response. Perturbation of this aspect of inflammation resolution could result in an altered immune response to the *Borrelia* present in the joints, leading to increased arthritis pathology (92). We therefore examined the extent of apoptosis induction in joints by performing a terminal transferase dUTP nick end labeling (TUNEL) assay on histological sections. TUNEL staining exploits a characteristic
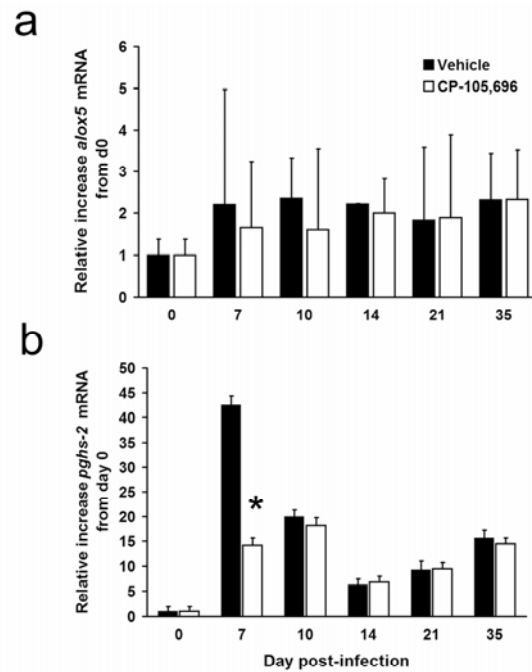
Figure 31. 5-LO (*alox5*; a) and COX-2 (*pghs2;* b) mRNA levels in the tibiotarsal joints of C3H mice treated with vehicle (closed bars) or CP-105,696 (open bars). Error bars represent $\pm$ s.d. *$p < 0.001$ versus vehicle treated controls. $n = 5$ animals per time point. This experiment is representative of two.

common to apoptotic cells, the organized degradation of DNA. By labeling the ends of DNA fragments, TUNEL staining imparts a dark color to cells undergoing apoptosis.

Histological sections of ankle joints collected from both vehicle- and CP-105,696-treated on d35 post-infection were stained by TUNEL and analyzed by light microscopy. Although the joints of vehicle-treated animals demonstrated a large number apoptotic cells in the tendon sheaths and other parts of the joint, the joints of CP-105,696-treated animals did not display the same high level of staining. Figure 32 a and b demonstrates the level of TUNEL staining in a representative joint from a vehicle-treated animal, whereas a representative joint from a CP-105,696 animal has far fewer TUNEL-positive staining cells, especially in the context of the high level of hypertrophy still present in these joints at d35 post-infection (Figure 32 c and d). Therefore, it appears that decreased induction of apoptosis could have been partially responsible for the continuation of severe arthritis pathology seen in the inflammatory foci of BLT_1 antagonist-treated animals infected with *B. burgdorferi*.

F. *Decreased TUNEL staining is accompanied by altered mRNA expression of key pro- and anti-apoptotic gene.*

In an effort to quantify the degree of apoptosis induction, or lack thereof, in the joints of CP-105,696-treated C3H animals, a real-time RT-PCR array was performed. This array assayed the mRNA expression of a panel of 84 pro- and anti-apoptotic genes from the joints of *B. burgdorferi*-infected, control or CP-105,696-treated animals (Appendix II). Since d35 was the time point at which histology demonstrated the dramatic differences in
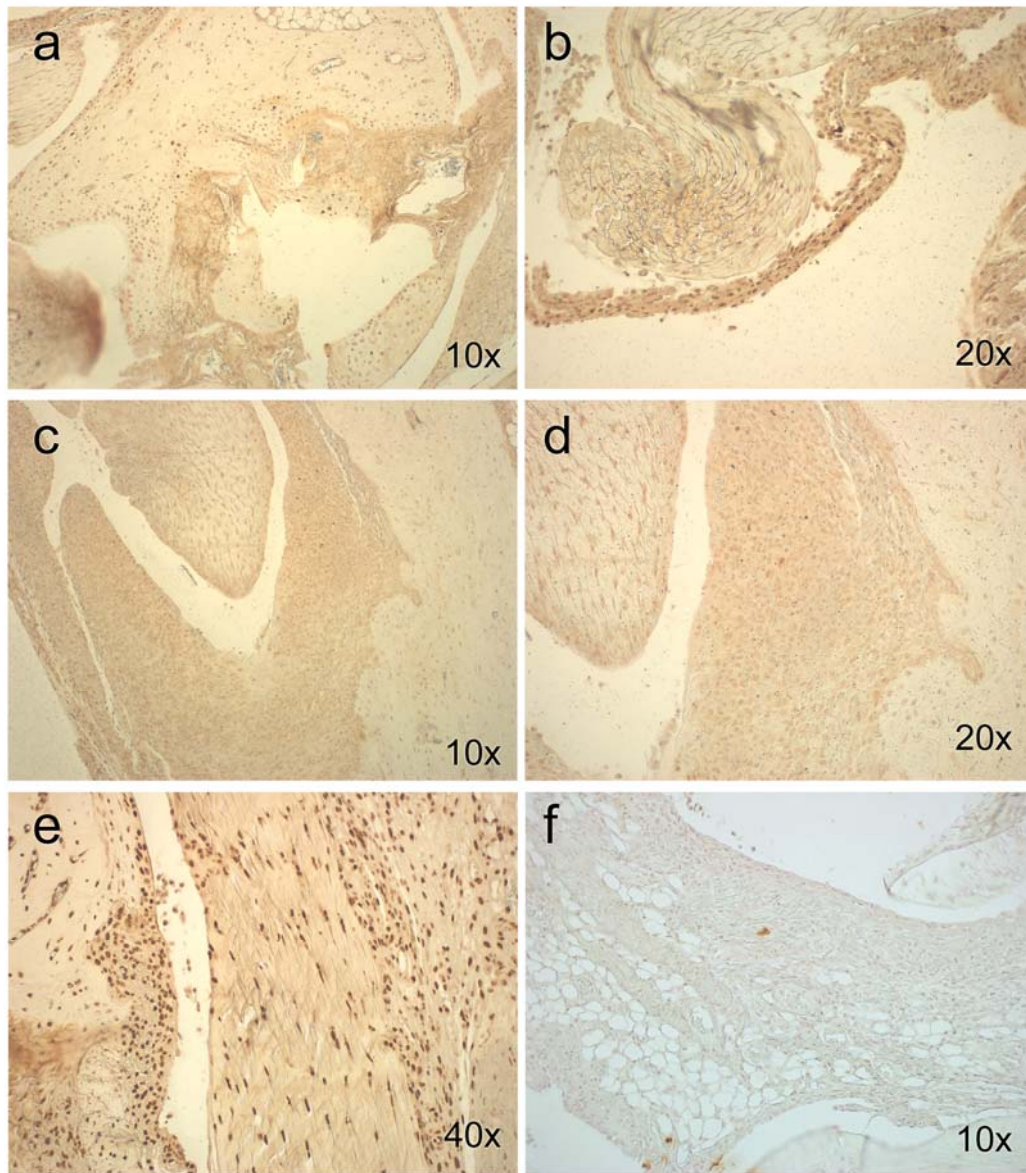
Figure 32. Detection of apoptosis by TUNEL staining in representative tibiotarsal joints of C3H mice at d35 post-infection. Mice were treated with vehicle (a, b, e, & f) or CP-1025,696 (c & d). e, TUNEL positive control; f, TUNEL negative control. Magnification is indicated in the lower right-hand corner of each panel.

TUNEL staining between vehicle- and CP-105,696-treated animals, mRNA from d35 ankle joints was also used in these arrays. Overall, 21 genes were up-regulated and 30 down-regulated in CP-105,696-treated compared to vehicle treated joints (Figure 33). Up-regulation was considered a greater than two-fold change in expression from the vehicle-treated control (Figure 33). Although several pro-survival genes were up-regulated in CP-105,696-treated animals (*Akt* (+6.18), *Bag/RAP46* (+3.31), *Nf-κB* (+62.12)), the most significant differences were found in the decreased pro-apoptotic factors. Eight pro-apoptotic genes were down-regulated by 15-fold or greater: *Bcl2l10* (-23.85), *Casp14* (-28.76), *FasL* (-16.56), *Lhx4* (-36.05), *Mcl1* (-30.51), *Cd70* (-15.19), and *Trp73* (-38.56). Most significantly, sphingosine kinase 2 (*Sphk2*), which is involved in induction of apoptosis (178), as well as regulation of the inflammatory response (179), was calculated as down-regulated by 3782.17-fold. This means that this gene was almost below the limit of detection of all other genes examined, once the C_t threshold was set. It is certain that cDNA was added to the well, because cDNA is added to the master mix, and this is then added to all wells equally.

Further examination of gene expression using more specific TaqMan primers and probes, as well as sphingosine kinase-2-specific *in vivo* experiments, will be necessary to determine the true contribution of this gene to the lack of apoptosis induction seen in the CP-105,696-treated animals. However, it is of interest that the one gene that was most significantly down-regulated in CP-105,696-treated animals is regarded as primarily involved in lipid mediator regulation.
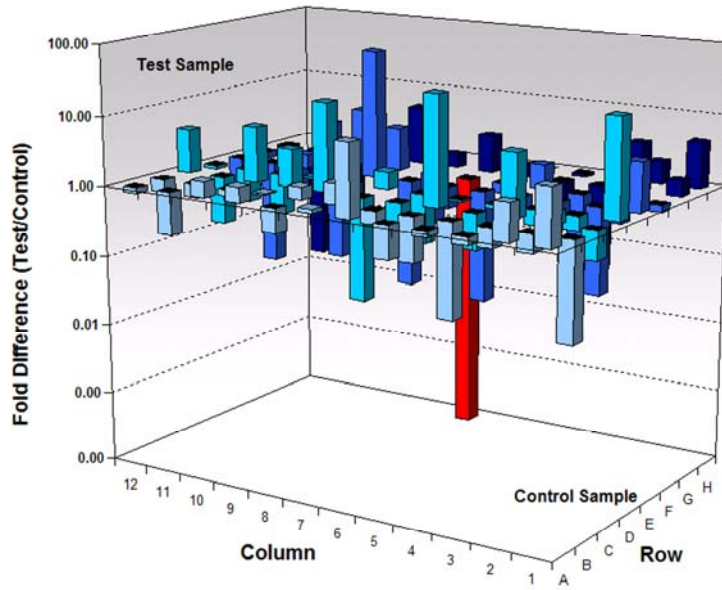
Figure 33. 3-dimensional plot of the 84 genes assayed by real-time RT-PCR. Row and column indicate the position of the well assayed. Fold difference is calculated by the ΔC_t method. Sphingosine kinase-2 (*Sphk-2*) is represnted by the red column.

G. *Conclusions*

There has previously been little evidence to assign any anti-inflammatory or pro-resolution role to LTB_4 during an inflammatory response. LTB_4 has many known pro-inflammatory properties, such as chemoattraction of immune cells, up-regulation of B cell activation, activation of neutrophils, and induction of other pro-inflammatory mediators by endothelial cells (180,181). To our surprise, blockade of LTB_4 signaling in *B. burgdorferi*-infected C3H arthritis-susceptible mice did not prevent or ameliorate arthritis, but actually resulted in an increased arthritis severity and duration during infection. Also, contrary to the predicted results, *B. burgdorferi*-specific IgG levels are higher in CP-105,696-treated mice at d35 post-infection. Despite this difference in antibody levels, bacterial loads did not differ significantly between treated and control animals at this time point.

Although seemingly contradictory, the blockade of LTB_4 signaling could reasonably lead to more severe arthritis pathology. Typically thought of as a neutrophil chemoattractant, LTB_4 also has direct effects on neutrophil activation as well as migration (173,95). Furthermore, continued LTB_4 signaling leads to altered induction of apoptosis in activated neutrophils (95,99). We have previously described the importance of the chemokine KC in the recruitment of neutrophils to *B. burgdorferi*-infected joints (117). It is therefore not surprising that neutrophil chemotaxis occurred in the absence of LTB_4 signaling. The mechanisms underlying the regulation of neutrophil activity are far less clear. Could LTB_4 signaling be required, not for neutrophil activation, but inactivation via apoptosis during Lyme arthritis? Staining specific for apoptotic cells revealed

decreased numbers of cells undergoing apoptosis in the inflammatory lesions of CP-105,696-treated animals (Figure 32), indicating that LTB_4 signaling was required for the induction of apoptosis during the resolution of the inflammatory response. More precise staining of cell surface markers in combination with TUNEL staining, observed by confocal microscopy, will be performed in the future to determine exactly which cell type is undergoing apoptosis in the normal context of arthritis resolution.

Further studies are necessary to rule out compensation by BLT_2 and induction of pro-inflammatory mediators by CP-105,696 treatment during *B. burgdorferi* infection. One might speculate that if CP-105,696 treatment and BLT_1 blockade lead to increases in pro-inflammatory mediators, COX-2 production would also be increased. The decrease in COX-2 mRNA seen at the critical d7 time point indicated that, under normal infection conditions, COX-2 up-regulation is a necessary component of inflammation regulation. Furthermore, these data imply a previously unappreciated anti-inflammatory role for LTB_4, whereby production of this pro-inflammatory lipid mediator leads to a *less* robust inflammatory response. There are also important implications for understanding the complex mechanisms involved in avoiding an unbridled inflammatory response and the appreciation of heretofore undiscovered roles for inflammatory modulators such as LTB_4.

CHAPTER V

CYCLOOXYGENASE-1 PREDOMINATES OVER

CYCLOOXYGENASE-2 IN REGULATION OF THE HUMORAL

RESPONSE TO *B. BURGDORFERI*

A. *Introduction*

Prostaglandins (PG) are major arachidonate metabolites synthesized by prostaglandin G/H synthase, widely known as cyclooxygenase (COX), and play critical roles in the regulation of the immune response. Most cells express a constitutive cyclooxygenase, COX-1, whose known activities include regulation of platelet function and maintenance of gastric mucosa (44,182,183). A second cyclooxygenase isoform, COX-2, is normally undetectable in most healthy tissues (29). Induction of COX-2 occurs in response to a number of inflammatory stimuli, such as lipopolysaccharide (LPS) (184), pro-inflammatory cytokines (38,25), and its own product, PGE_2 (34). Though produced in nano- to micromolar amounts and possessing a short half-life, prostaglandins are hormones crucial for the maintenance of homeostasis, as well as regulation of the immune response (185,186,33). For example, PGE_2 plays a role in the induction of T cell tolerance (187), impairs the T cell response to pathogens such as HIV (188), and modulates the production of T-helper type-1 and -2 cytokines (189). Thromboxane A_2, primarily a COX-1 product, negatively regulates DC-dependent T cell proliferation (190)

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and induces apoptosis of $CD4^+CD8^+$ thymocytes in a concentration-dependent manner (68).

PGE_2 also appears to exert a regulatory effect on the activation and proliferation of B lymphocytes, although its role *in vivo* is not as well understood. Signaling via the prostaglandin E_2 receptors EP_2 and EP_4 following activation by LPS and IL-4 can enhance mitogen- and cytokine-driven isotype switching from IgM to IgE, concurrently inhibiting indicators of B cell activation such as the expression of class II major histocompatability complex (MHC II) and cell enlargement (191,192,193). More recent data from *in vitro* studies indicate that human B lymphocytes are not exclusively responders to PGE_2, but can also be induced to express COX-2 mRNA and protein, and are thus capable of PGE_2 production (194). COX-2-dependent PGE_2 production may provide a critical parameter for modulating antibody production in response to pro-stimulatory signals, as some cell types appear to regulate COX-2 mRNA and protein via a positive feed-back loop in response to PGE_2 (34). Thus, it has been hypothesized that the wide use of nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-2 specific inhibitors could potentially thwart host efforts to produce humoral responses to pathogens or the induction of an effective vaccine response.

While the *in vitro* evidence is intriguing, *in vivo* evidence to support the premise that regulation of antibody production could be impaired via the inhibition of COX-2, and thereby PGE_2 production, is limited. Using models of autoimmune disease, it was shown that COX inhibition may affect total immunoglobulin production (195) or alter isotype

switching (193). Additionally, a recent study demonstrated that mice deficient in COX-2 do not switch from IgM to IgG production after vaccination with Human Papillomavirus-type 16 virus-like particles (HPV 16 VLPs) (196). However, these models do not mimic infectious disease. Antibody production directed toward pathogens constitutes a major defense mechanism of the host immune response, and the possible role of COX in the regulation of humoral responses to pathogens is still unknown.

To address this issue we utilized a murine model of Lyme disease, caused by infection with the spirochete *Borrelia burgdorferi*, to investigate the role of COX in the antibody response to a model infectious agent. *B. burgdorferi* infection of both humans and animals leads to multi-systemic disease, with the greatest pathology affecting the heart and joints (197). When untreated near the time of infection, 60% of individuals may develop a severe arthritis characterized by inflammatory cell infiltrate, synovial hypertrophy, and tendonitis (197). The intense pain and swelling associated with Lyme arthritis in humans is commonly treated with NSAIDs or COX-2 inhibitors (116). The predictability of experimental disease pathology combined with well-developed vaccine models (152), as well as the use of COX inhibitors in the treatment of human Lyme arthritis, make experimental Lyme disease an excellent tool with which to study the effects of COX inhibition on humoral immunity.

In this report, we demonstrate that normal murine B lymphocytes constitutively express COX-1 mRNA and protein and up-regulate the production of COX-2 mRNA and protein upon activation by *B. burgdorferi* antigen, a potent B cell mitogen (154). In response to

live *B. burgdorferi*, B cells produced $PGF_{2\alpha}$, its receptor FP, and TXB_2 (a stable hydrolysis product of TXA_2), and low levels of PGE_2. *In vivo*, COX-2-deficient mice susceptible to the development of experimental Lyme arthritis and carditis (C3H/HeJ) (144,145) infected with *B. burgdorferi* demonstrated IgM and IgG levels comparable to their wild-type littermates. In a model of *B. burgdorferi* vaccination, mice treated with the COX-2 inhibitor celecoxib before and after vaccination were capable of developing full antibody responses upon challenge. We then investigated the contribution of COX-1 *in vitro* in response to *B. burgdorferi*. Surprisingly, inhibition of COX-1 by SC-560 not only significantly reduced the levels of $PGF_{2\alpha}$, TXB_2, and PGE_2 produced, but also lead to greater decreases in IgM and IgG levels, as compared to COX-2 inhibition. COX-2-null cells generated higher basal levels of Ig and eicosanoids than wild-type controls and did not alter their production of these molecules in response to stimulation, however, these cells did respond to COX-1 and COX-2 inhibition by reducing their Ab and eicosanoid production, indicating a role for COX-1 and -2-independent mechanisms of action by these inhibitors *in vitro*. *In vivo,* some Ab responses were significantly reduced by COX-1 inhibition, whereas inhibition of COX-2 did not lead to reduced *B. burgdorferi*-specific IgM and IgG. Unlike *in vitro* inhibition, simultaneous inhibition of both COX isoforms lead to the most significant decreases in Ab production *in vivo*. Most significantly, COX-1-deficient animals produced lower levels of *B. burgdorferi* IgM and IgG, and their sera displayed decreased *B. burgdorferi* Ag recognition compared to their wild-type counterparts. These findings demonstrate that the humoral response to an infectious agent does not rely on COX-2, as previous studies implied, but that COX-1 is the dominant isotype in regulation of the humoral response to an infectious agent.

Furthermore, although treatment of patients with COX-2-specific inhibitors may not impede their humoral responses to either infectious agents or vaccines, care must be taken with the use of traditional nonsteroidal anti-inflammatory drugs (NSAIDs) or COX-1-specific inhibitors.

B. *Results*

1. *Expression of COX-1 and up-regulation of COX-2 mRNA and protein during B lymphocyte stimulation*

To determine if murine B cells express COX-1 or COX-2 in response to bacterial stimulation, splenic B cells were isolated from C3H mice and exposed to various stimuli. Purified B cells (1 x 10^6 cells/mL) were incubated in the presence of anti-CD40, anti-IgM, or *B. burgdorferi* antigen (BbAg) for 8 hours *in vitro*. Total RNA was extracted and analyzed by real-time RT-PCR, and the products visualized by gel electrophoresis (Figure 34). A previous study examining human B cells has shown that COX-2 mRNA was up-regulated after 8 hours of stimulation (194). As this was the first report of COX-2 mRNA production by non-transformed B cells, human or mouse, we choose to examine murine B cell COX-2 mRNA expression at this time point. Figure 34 a and b show constitutive COX-1 mRNA expression which was unaffected by any of the tested stimuli. Unstimulated B cells (Figure 34 a and b, Lane 1) and B cells stimulated with anti-IgM (Figure 34 a and b, Lane 3) expressed no detectable COX-2 mRNA after 8 hours of stimulation. However, stimulation with anti-CD40 up-regulated B cell COX-2 mRNA (Figure 34 a and b, Lane 2), as did incubation with BbAg, consistent with its role as a B cell mitogen (Figure 34 a and b, Lane 4) 198. These increases were significant, as 8 hours of incubation with BbAg induced a greater than 200-fold increase in COX-2 mRNA whereas unstimulated cells expressed no detectable COX-2 mRNA as measured by quantitative real-time PCR (Figure 34 b).
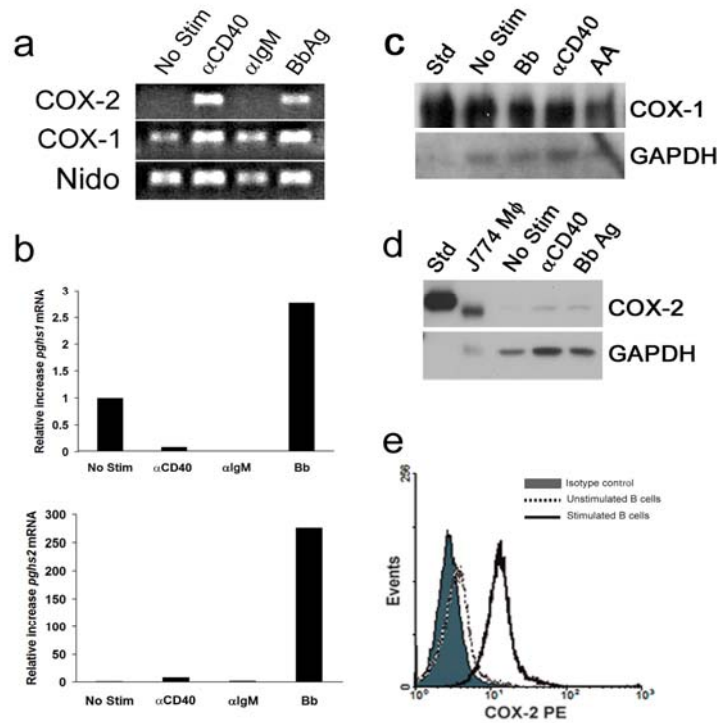
Figure 34. Murine B cells express COX-1 and -2 mRNA and protein, as determined by real-time PCR (b). The products were visualized on an agarose gel by ethidium bromide (a). B cells constitutively express COX-1 protein (c) and up-regulate COX-2 mRNA as determined by Western blot (d) and flow cytometry (e).

COX-2 transcription and translation are positively and negatively regulated by numerous signals (35,39), and although traditionally considered constitutive, post-transcriptional regulation of COX-1 protein expression has been shown in some disease conditions (199,200). We were therefore curious as to how the expression of COX protein would compare to mRNA expression. As a positive control for COX-2 expression, protein was isolated from 7.5 x 10^5 LPS-stimulated J774.2 murine macrophages, a cell line known to constitutively express COX-1 and abundantly express COX-2 protein upon activation with LPS (201). B cells were stimulated with arachidonic acid (AA) as a positive control for COX-1 stimulation (22). Western blot analyses (Figure 34 c and d) are consistent with our RNA results, illustrating the unaltered expression of COX-1 and the up-regulation of COX-2 protein in BbAg-stimulated B cells. Of note is the difference in expression of COX-2 protein by B cells relative to the J774 macrophage cell line. Although B cells can clearly up-regulate COX-2 protein after maximal stimulation, it is only a fraction of the level produced by activated macrophages, in which COX-2 products play a prominent role in activation and modulation of effector function (6,30,182). Neither did B cells express COX-2 to the same extent as the constitutive levels of the COX-1 protein.

Western blot analysis demonstrated that BbAg effectively up-regulated COX-2 protein in B cells, albeit at low levels. To verify the ability of activated murine B lymphocytes to produce COX-2 protein, BbAg was used to stimulate freshly isolated B cells from C3H mice to measure COX-2 protein expression by flow cytometry. After purification, cells were incubated with vehicle or BbAg for 24 hours, then stained intracellularly for COX-2

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protein. Histogram analyses indicate that >95% of CD19$^+$ cells contained detectable COX-2 protein after stimulation (Figure 35 e), results which corroborate our Western blot data.

2. B. burgdorferi-stimulated B lymphocytes produce TXB$_2$ and PGF$_{2\alpha}$ and up-regulate expression of the PGF$_{2\alpha}$ receptor FP

Human B cells have been shown to express PGE$_2$ and PGF$_{2\alpha}$ (194) and have the capacity to produce LTB$_4$ (202). We chose to assay the ability of murine B lymphocytes to produce these eicosanoids, as well as TXB$_2$, in response to 36 hours of incubation with *B. burgdorferi*. In preliminary studies, we found that, although total *B. burgdorferi* antigen was sufficient to up-regulate production of COX-2 mRNA and protein, incubation of B cells with BbAg alone did not stimulate eicosanoid production (data not shown). B cells were therefore stimulated with live *B. burgdorferi* at an MOI=1, anti-CD40, or arachidonate, since exogenous arachidonic acid is a non-specific activator of the COX pathways. LTB$_4$ was not produced under any of the incubation conditions (data not shown). Unstimulated B cells in the presence of vehicle produced low levels of PGF$_{2\alpha}$ and TXB$_2$ (Figure 35 a). Anti-CD40 stimulation increased only PGF$_{2\alpha}$ production. As expected, exogenous AA stimulated the most significant production of PGE$_2$ and PGF$_{2\alpha}$. We also found that AA stimulated these highly purified B cells to produce TXB$_2$. Incubation with live *B. burgdorferi* also stimulated high levels of PGF$_{2\alpha}$ and TXB$_2$. Unexpectedly, B cells were induced to produce only low levels PGE$_2$.
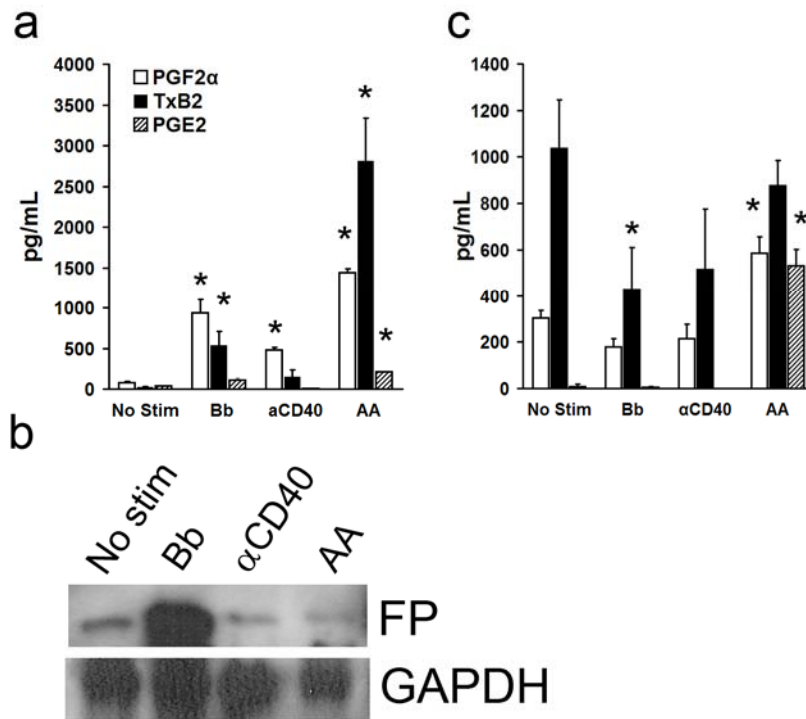
Figure 35. Eicosanoid production by wild-type (a) and COX-2$^{-/-}$ (c) B cells. B cells from 4 mice were stimulated in the presence of live *B. burgdorferi* (Bb), αCD40, or AA for 36h. PGF$_{2\alpha}$, TXB$_2$, and PGE$_2$ were measured from culture supernatnats. Expression of the FP receptor was also induced by incubation with Bb (b), as compared to the other stimuli. Error bars represent $\pm$ s.d. *$p < 0.005$. $n = 4$ mice.

A previous study demonstrated B lymphocyte expression of the PGE_2 receptors EP2 and EP4 (191), however expression of the $PGF_{2\alpha}$ receptor FP or the thromboxane receptor TP by B cells has never been examined. B cells stimulated for 36h were assayed for possible expression of FP and TP after stimulation with live *B. burgdorferi*, αCD-40, or AA. Incubation of B cells with any of the stimuli did not induce protein expression of TP (data not shown), implying that B cell production of TX targets other cells. A basal level of FP protein was detected, and highly up-regulated by incubation with *B. burgdorferi,* but not αCD40 or AA (Figure 35 b). This implies a possible role for autocrine production of $PGF_{2\alpha}$ by B lymphocytes *in vitro*.

We studied the effect of COX-2 deficiency on the ability of murine B cells to produce this same panel of eicosanoids. Therefore, B cells derived from C3H COX-$2^{-/-}$ animals were stimulated in the same fashion as previously described for wild-type cells. Supernatants were then analyzed for secreted TXB_2, PGE_2, and $PGF_{2\alpha}$. Curiously, COX-$2^{-/-}$ B lymphocytes expressed higher basal levels of $PGF_{2\alpha}$ and TXB_2, but not PGE_2 (Figure 35 c), implying COX-2-mediated production of this eicosanoid by B cells. COX-$2^{-/-}$ B lymphocytes were unresponsive to stimulation with *B. burgdorferi* and αCD40 as illustrated by their unaltered eicosanoid concentration. AA, however, induced small increases in $PGF_{2\alpha}$ and PGE_2, but not TXB_2 (Figure 35 c). Also of note is the fact that the traditional B cell stimuli tested induced only low levels of PGE_2 production. Even incubation with arachidonate, a COX substrate and non-specific activator, induced minimal PGE_2 production by murine B lymphocytes (Figure 35 a and c).

3. *Total, non-specific IgG and* Borrelia-*specific IgM and IgG levels in COX-2-deficient mice*

A previous study found that basal IgM and IgG levels in serum from B6.129 COX-2$^{-/-}$ were significantly decreased compared to levels in wild-type mice (194). However, it is unknown if these differences at homeostasis will translate to impaired antigen-specific responses. To determine how COX-2 deficiency might affect humoral responses to an infectious agent, we infected C3H/HeJ COX-2$^{-/-}$ mice with *B. burgdorferi*. The primary method for clearance of *B. burgdorferi* from the host is the production of "traditional" antibodies, as well as "nontraditional" complement-independent *Borreliacidal* antibodies (123). Therefore, sera were collected at days 14 and 24 post-infection and assayed for total non-specific IgG, *B. burgdorferi*-specific IgM and total IgG levels, as well as the IgG subtypes G$_1$, G$_{2a}$, G$_{2b}$, and G$_3$. These time points correlate to the peak of disease and the resolution phase, respectively. Day 0 sera were also collected and analyzed for total IgM and IgG.

According to previous research, a lack of COX-2 and therefore PGE$_2$ production could lead to two potential outcomes: 1.) an increase in IgM and coordinate decrease in total IgG, as PGE$_2$ induces class switching *in vitro* from IgM to other isotypes (203,193) 2.) or as more recently postulated, a decrease in total immunoglobulin production triggered by the interruption of a putative positive feed-back loop occurring in some cells expressing the EP4 prostaglandin receptor, which is also expressed on murine B lymphocytes (191).

Surprisingly, unlike the previous report using B6.129 COX-2$^{-/-}$ mice, COX-2$^{-/-}$ mice on the C3H/HeJ background produced constitutive IgM and IgG levels comparable to their wild type littermates (Figure 36 a and b). Furthermore, *B. burgdorferi*-specific IgM, total IgG, and IgG$_{2a}$, G$_{2b}$, and G$_3$ responses at days 14 and 24 post-infection were also equivalent to their littermate controls (Figure 36 c-f). Thus, our results demonstrate that a pathogen-specific immunoglobulin response indistinguishable from that of wild-type mice can be mounted despite a complete lack of COX-2 activity.

*4. Anti-*Borrelia *antibody responses after vaccination in celecoxib-treated animals*

A recent report examined the potential for COX-2 deficiency to alter the development of the humoral response to vaccination (196). B cells from B6.129 COX-2$^{-/-}$ mice injected with human papilloma virus-type 16 virus-like particles (HPV 16 VLPs) did not undergo class switching, but instead produced significantly higher titers of anti-HPV 16 VLP IgM than their wild-type counterparts. Although vaccinated mice could not be challenged with HPV infection, *in vitro* virus-specific neutralizing antibody titers from vaccinated COX-2$^{-/-}$ mice were lower than those of wild-type mice.

Our studies using COX-2-deficient mice clearly demonstrate that COX-2 activity in B lymphocytes is not necessary for the successful production of antibodies specific to the pathogen *B. burgdorferi*. To better define the role of COX-2 and the potential effects of its inhibition on the ability of vaccination to induce protective antibodies to an infectious agent, we utilized a well-defined model of *B. burgdorferi* immunization (152). *B. burgdorferi* utilizes the white footed mouse as a natural reservoir (204), therefore
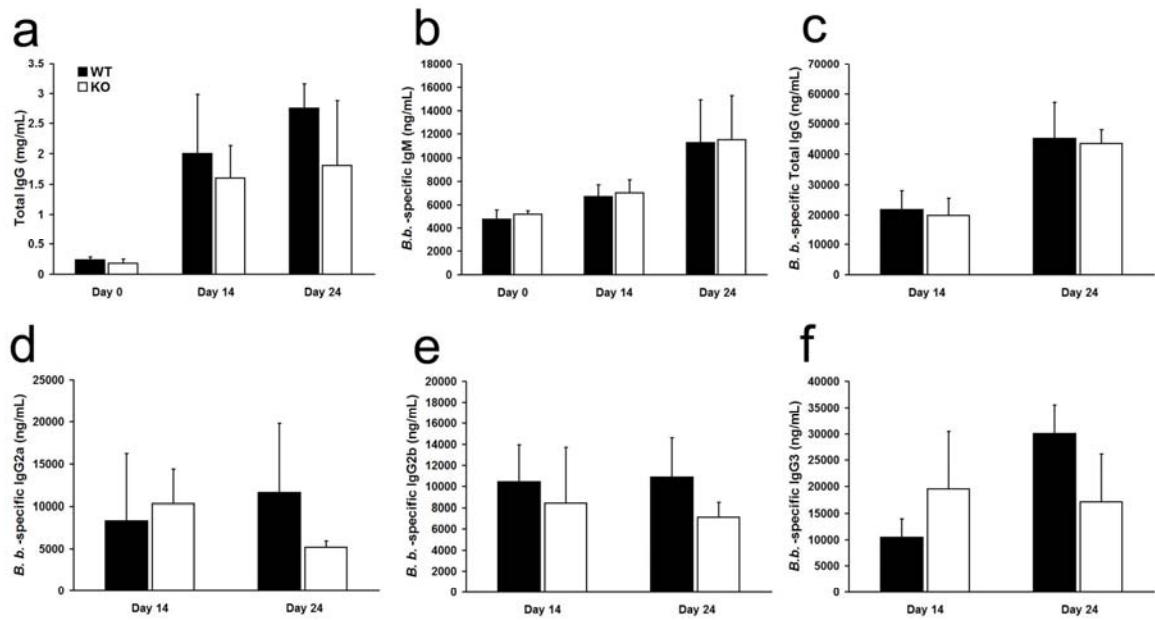
Figure 36. Antibody levels from COX-2$^{-/-}$ (KO; open bars) are not significantly different from those of wild-type (WT; closed bars) mice. Basal total, non-sepcific IgG (a) and *B. burgdorferi*-specific IgM (b) were measured. Additionally, total non-specific IgG, and *B. burgdorferi-sepcific* IgM (b), IgG (c) IgG$_{2a}$ (d), IgG$_{2b}$ (e), and IgG$_3$ (f) were measured at d14 and d24 post-infection. Values are $\pm$ s.d. *n* = 8 - 10 mice per group.

experimental vaccination and challenge in mice provide an excellent opportunity to examine a more physiological model of vaccine-induced humoral immunity than many other experimental models of vaccination. Vaccination against *B. burgdorferi* has been widely studied and has lead to vaccines for both human and animal use (205,206).

To determine the potential role of COX-2 in the development of vaccine-induced immunity, vehicle- or celecoxib-treated Lyme disease-susceptible C3H mice were vaccinated with a whole-organism formalin-inactivated *B. burgdorferi* vaccine and challenged 21 days later by footpad inoculation with *B. burgdorferi*. Fourteen days after challenge, total anti-*Borrelia* IgM and IgG levels were measured by ELISA (Figure 37 a and b). Spirochete-specific antibody concentrations were comparable between vaccinated mice which had received normal chow and animals that had received celecoxib chow.

It was possible that the pattern of antigens recognized, as opposed to the actual antibody concentration, could be altered by COX-2 inhibition in normal or vaccinated mice, since many microbial products stimulate the immune response to *B. burgdorferi*. To address this possibility, Western blot analysis was performed to determine if COX-2 inhibition altered the complexity of antigens recognized. Sera from untreated and celecoxib-treated mice were used in immunoblotting against total *B. burgdorferi* antigen. Key antigens known to induce immunity against the Lyme spirochete (207) were recognized by sera from both untreated (Figure 37 c, lanes 1-3) and celecoxib-treated (Figure 37 c, lanes 4-6) animals: flagellin and outer surface proteins (Osp) A, B, and C.
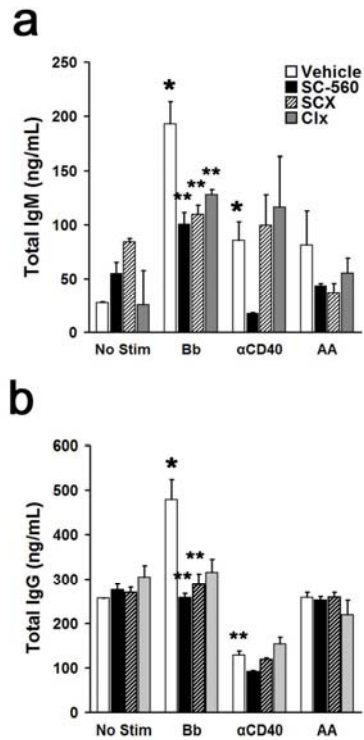
Figure 37. COX-1 inhibition most significantly affects production of IgM and IgG induced by incubation with live *B. burgdorferi*. Supernatants of B cells pre-incubated with vehicle, SC-560, SC-560 and celecoxib (SCX), or celecoxib (Clx) were measured for IgM (a) and IgG (b) by ELISA after 7 d of incubation with respective stimuli. Values are ± s.d. of four mice. *$P \leq 0.045$ versus unstimulated controls. **$P \leq 0.027$ versus vehicle-treated controls. Data are representative of three experiments.

Furthermore, sera from vaccinated mice treated with celecoxib (Figure 37 d, lanes 3-5) recognized the same antigens as untreated animals (Figure 37 d, lanes 1 and 2). These are the same antigens recognized by unvaccinated mice, however, they elicited a much stronger antibody response, despite the increased dilution of the immune sera. These findings further demonstrate the ability of the host to mount a humoral response to *B. burgdorferi* upon primary infection or after vaccination and challenge, in spite of a lack of COX-2 activity.

5. *Inhibition of COX-1 dramatically reduces eicosanoid and immunoglobulin production by B lymphocytes*

COX-2 has been portrayed as the dominant COX isoform in mediating the immune response, primarily via its modulation of inflammation (19). Previous studies predicted that COX-2 should be regulating the humoral response, and we were therefore surprised to discover that COX-2 deficiency did not alter antibody responses to *B. burgdorferi,* after either primary infection or upon challenge after vaccination. Furthermore, the eicosanoid profile generated *in vitro* implicated COX-1, not COX-2, involvement in B lymphocyte responses to live *B. burgdorferi*. Although differences were found between vehicle-treated human B cells and those treated with the nonspecific COX inhibitor indomethacin, no previous studies have examined the effect of highly specific COX-1 inhibition or antibody responses. We therefore choose to examine the response of purified murine B cells to various stimuli in the absence or presence of the COX-1-specific inhibitor SC-560, the COX-2-specific inhibitor celecoxib, or both. B

lymphocytes were subsequently incubated with *B. burgdorferi* at a multiplicity of infection (MOI) =1, anti-CD40, arachidonic acid (AA), or no stimulus.

Unstimulated B cells pre-incubated with SC-560 separately, exhibited decreased basal expression of $PGF_{2\alpha}$ and PGE_2 (Table I) to undetectable levels. COX inhibition had no effect on basal TX production (Table I). Inhibition of COX-1, not COX-2, caused the most significant decreases. Whereas COX-2 inhibition significantly decreased the levels of $PGF_{2\alpha}$ and PGE_2 induced by incubation with *B. burgdorferi* (Table I), TXB_2 was not significantly inhibited (Table I). This is in agreement with the fact that COX-1 is usually responsible for TXB_2 production (208). Furthermore, levels of PGE_2, $PGF_{2\alpha}$, and TXB_2 induced by *B. burgdorferi* stimulation were reduced to undetectable levels in the presence of SC-560.

As COX-1 inhibition alone appeared to have the greatest affect on *in vitro* eicosanoid production by murine B cells, we determined whether this translated to an alteration in *vitro* Ab production. After 7 days of stimulation *in vitro*, supernatants were measured for Ab secretion in response to the three stimuli tested for induction of eicosanoid production, or were incubated without stimulus. Anti-CD40 stimulation induced a small increase in total IgM, but not IgG. This increase was negated by inhibition of COX-1 alone (Figure 38 a and b). As expected, stimulation of B lymphocytes with live *B. burgdorferi* induced significant increases in production of both IgM and IgG (Figure 38 a and b). *Borrelia*-induced antibody production was decreased most dramatically by inhibition of COX-1, followed by dual inhibition, with the least effect induced by

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Table 1. COX inhibition of eicosanoid production by B lymphocytes

Inhibitor	Stimulus	$PGF_{2\alpha}$ (pg/mL)	TXB_2 (pg/mL)	PGE_2 (pg/mL)
vehicle	No stim	77.67 ± 14.68	26.36 ± 6.49	37.46 ± 10.38
	Bb	938.21 ± 166.33	538.92 ± 172.63	113.40 ± 18.87
	αCD40	481.91 ± 26.1	151.03 ± 83.99	7.51 ± 2.84
	AA	1439.60 ± 49.64	2810.15 ± 526.6	213.81 ± 6.63
SC-560 (1 µM)	No stim	0.00 ± 0.00[b]	69.12 ± 38.95	0.00 ± 0.00[c]
	Bb	2.36 ± 4.09[a]	53.60 ± 22.69[a]	1.31 ± 1.31[b]
	αCD40	0.00 ± 0.00[a]	62.21 ± 25.71	0.00 ± 0.00
	AA	400.11 ± 89.92[a]	107.50 ± 33.40[c]	151.85 ± 10.04[c]
SCX (1 µM each)	No stim	130.68 ± 74.73	86.05 ± 39.56	0.00 ± 0.00[c]
	Bb	74.25 ± 66.71[a]	87.18 ± 43.74[a]	0.00 ± 0.00[b]
	αCD40	42.59 ± 73.77[a]	78.25 ± 43.29	0.00 ± 0.00
	AA	424.19 ± 86.23[a]	114.26 ± 38.90[c]	141.74 ± 15.62[b]
Clx (1 µM)	No stim	183.64 ± 55.64[c]	199.14 ± 95.21[c]	8.98 ± 6.05
	Bb	261.13 ± 90.73[a]	254.70 ± 139.22[c]	4.58 ± 3.67[c]
	αCD40	148.70 ± 106.11[a]	181.81 ± 94.57	6.88 ± 4.29
	AA	980.55 ± 274.36[a]	1275.90 ± 182.74	204.60 ± 17.66

B lymphocytes were pre-incubated with vehicle or the indicated inhibitor before stimulation. Eicosanoids were measured from cell supernatants after 36 h in the presence or absence of stimulus. [a]$P \leq 0.001$; [b]$P < 0.01$; [c]$P < 0.05$; versus vehicle-treated controls. Values are mean ± s.d. $n = 4$ mice. These data are representative of four performed. SCX, SC-560 plus celecoxib; Clx, celecoxib; AA, arachidonic acid.
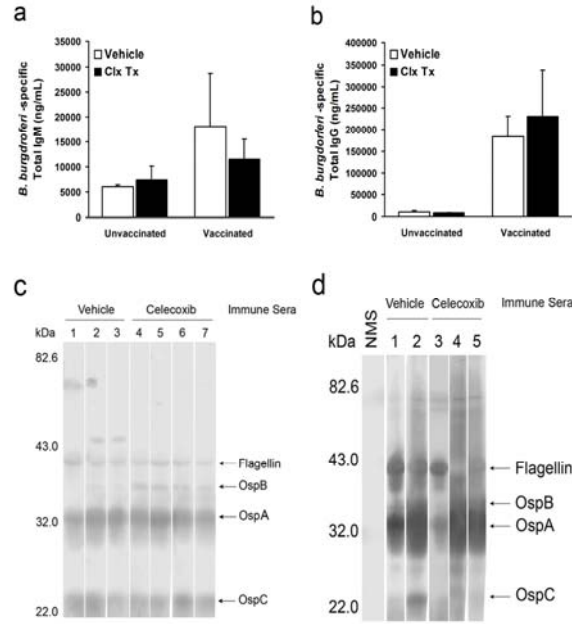
Figure 38. Celecoxib-treatment (closed bars) does not alter the production of vaccine-induced antibodies (a & b) or recognition of *B burgdorferi*-specific antigens (c & d) as compared to vehicle-treated mice (open bars). After challenge, IgM (a) and IgG (b) production by *Borrelia* vaccinated mice was measured at d24 post-infection. Sera were then used in immunoblotting (c & d) against total Bb Ag to determine if antigen recognition was altered by Clx-treatment during primary infection (c) or during vaccination and challenge (d). Values are ± s.d.

incubation with celecoxib. Celecoxib significantly inhibited IgM production; however the decrease in IgG levels was not significant (p=0.48), although there was a decrease. Arachidonate, the major substrate for cyclooxygenase, did not itself induce production of either IgM or IgG. These data directly contradict an earlier study which found that inhibition of COX-2 lead to an increase in IgM due to an inability to class switch, and consequently a decrease in IgG production (196). In the current *in vitro* studies, the production of pathogen-specific antibodies is affected by inhibition of COX-1 activity more so than inhibition of COX-2.

6. B lymphocytes from COX-2$^{-/-}$ mice respond to both COX-1 and COX-2 inhibitors by reducing eicosanoid and antibody production

Since eicosanoid production was altered in the B cells of COX-2$^{-/-}$, we next determined if treatment with COX-1- or COX-2-specific inhibitors would affect eicosanoid production or immunoglobulin production by these cells. The effect of COX-2 inhibition was tested in COX-2$^{-/-}$ because COX-2-independent effects have been described for celecoxib, the inhibitor utilized in these studies (209). COX-2$^{-/-}$ B cells were then treated in a similar fashion to wild-type cells. PGF$_{2\alpha}$, TXB$_2$, and PGE$_2$, as well as total IgM and IgG were then measured from the supernatants.

As with wild-type cells, COX-2$^{-/-}$ B lymphocyte production of PGF$_{2\alpha}$ and TXB$_2$ were reduced to almost undetectable levels by COX-1 inhibition (Figure 39 a and b). Surprisingly, treatment with celecoxib also lead to significant decreases in production of these eicosanoids and PGE$_2$ induced by AA stimulation (Figure 39 a-c), indicating a
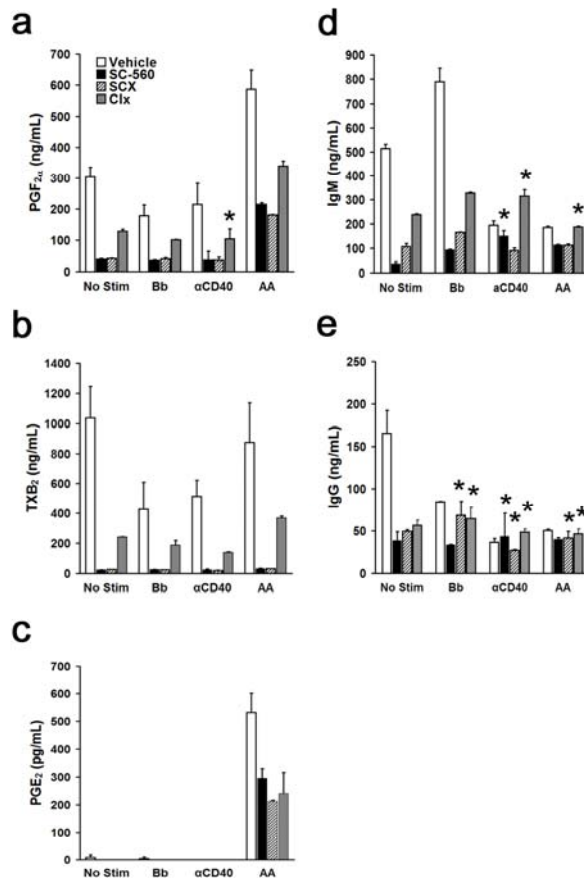
Figure 39. COX-2$^{-/-}$ B cell *in vitro* production of PGF$_{2\alpha}$ (a), TXB$_2$ (b), and PGE$_2$ (c) as well as IgM (d) and IgG (e). B cells were stimulated with live *B. burgdorferi* (Bb), αCD40, or AA for 36h (a-c) or 7 days (d & e) in the presence of vehicle (open bar), SC-560 (closed bar), SC-560 + Clx (SCX; hatched bar), or Clx (grey bar). Error bars represent $\pm$ s.d. ***p > 0.05 (not significant) versus vehicle treated controls.** *n* = 4 mice. Data are representative of two experiments.

possible role for COX-2 independent mechanisms for regulation of eicosanoid production by B cells.

Measurement of Ig production in COX-2$^{-/-}$ demonstrated that basal IgM was increased compared to wild-type cells, however incubation with *B. burgdorferi* stimulated a further increase in IgM production (Figure 39 d). Although basal IgG production was unchanged, incubation with *B. burgdorferi* did not stimulate increased IgG production (Figure 39 e). All stimuli tested actually decreased IgG production (Figure 39 e). COX-1 inhibition caused the most significant decrease in IgM and IgG production during *B. burgdorferi* incubation. As with eicosanoid production, COX-2 inhibition affected both basal IgM and IgG, as well as IgM production in the presence of *B. burgdorferi* (Figure 40 d and e). However, since *B. burgdorferi* stimulation did not result in IgG production in COX-2$^{-/-}$ B cells, COX-2 inhibition did not affect its production (Figure 39 e). Therefore, if appears that *in vitro*, COX-2 deficiency can alter eicosanoid and Ig production by B cells, although our *in vivo* studies demonstrated the ability of COX-2$^{-/-}$ mice to develop full antibody responses to *B. burgdorferi* infection.

7. *Specific inhibition demonstrates the* in vivo *significance of COX-1 to* B. burgdorferi-*specific antibody responses.*

In order to determine the relative contribution of the two COX isoforms *in vivo*, we next examined the effect of COX-1- and COX-2-specific inhibition on the ability of C3H mice to mount a humoral response to *B. burgdorferi* infection. Antibody responses during

administration of SC-560, Clx, or both, were compared to vehicle-treated controls at days 14 and 24 post-infection. Surprisingly, *in vivo*, dual inhibition of COX-1 and -2 had the most dramatic affect on antibody production, significantly reducing both *B. burgdorferi*-specific IgM (Figure 40 a) and IgG (Figure 40 c) at d14, as well as reducing the IgG2$_{2b}$ isotype at this time point (Figure 40 e). SC-560 did significantly decrease total *B. burgdorferi*-specific IgG, as well as IgG$_3$, at d24 post-infection (Figure 40 c and f). Most importantly, in accordance with our *in vitro* data, as well as our studies utilizing COX-2$^{-/-}$ animals, inhibition of COX-2 had the least effect on immunoglobulin production and did not result in significant decreases in any of the antibodies assayed, non- or *B. burgdorferi*-specific. Although *B. burgdorferi*-specific antibody production was decreased, non-specific total IgG was not significantly decreased by any of the treatments (Figure 40 b). These data further demonstrate the importance of the COX-1 isozyme to antibody production.

8. *COX-1 deficiency results in decreased* B. burgdorferi-*specific antibodies and* Borrelia *antigen recognition.*

Thus far, results from both our *in vitro* and our *in vivo* studies implied a pivotal role for COX-1 in the generation of humoral immunity to *B. burgdorferi*. To confirm this, we utilized mice deficient in COX-1 and examined their ability to develop a *B. burgdorferi*-specific humoral response. Basal antibody levels of B6.129 COX-1$^{-/-}$ mice were assayed, as were antibody levels at d14 and d24 post-infection.
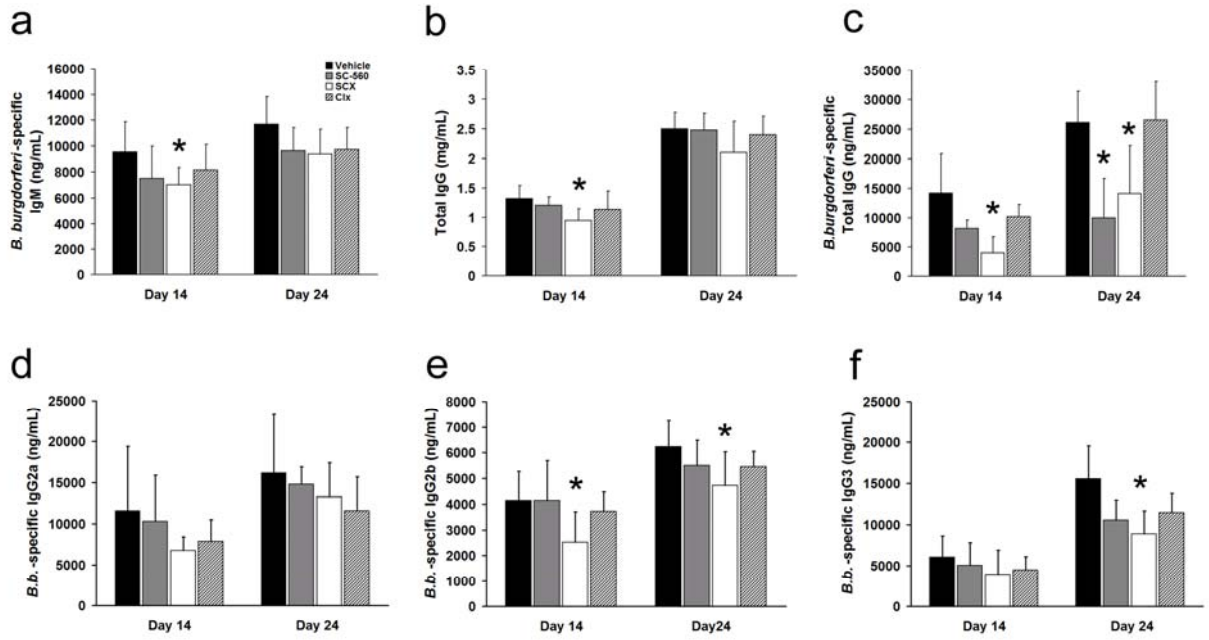
Figure 40. Antibody production is altered in dual inhibitor- and COX-1-specific inhibitor-treated mice. C3H mice were treated with vehicle (closed bars), SC-560 (dark grey bars), SC-560 + Clx (SCX; open bars), or Clx (light grey bars) antibody levels were measured at d14 and d24 post-infection. Total, non-specific IgG was measured (b) as was *B. burgdorferi*-specific IgM (a), total IgG (c), and the IgG isotypes IgG2a (d), IgG2b (e), and IgG3 (f). Error bars represent $\pm$ s.d. *$p < 0.05$. n = 6-10 mice per group. Data are representative of at least two experiments.

COX-1$^{-/-}$ mice demonstrated significantly higher basal levels of IgM, as well as significantly higher IgM at both d14 and d24 post-infection (Figure 41 a). Although non-specific total IgG was not different between knock-out and wild-type animals (Figure 41 b), *B. burgdorferi*-specific IgG was significantly lower at d24 post-infection (Figure 41 c). While the IgG subtypes IgG$_1$ (Figure 41 d) and IgG$_{2b}$ (Figure 41 f) demonstrated a trend toward decreased lower antibody levels, concentrations of IgG$_{2a}$ (Figure 41 e) were significantly decreased. IgG$_3$ was produced at the lowest levels, and was also not affected by COX-1 deficiency (Figure 41 g).

We then determined if antigen recognition was altered in COX-1$^{-/-}$ mice, as compared to their wild-type controls. Sera collected at d24 post-infection were used in immunoblotting against total *B. burgdorferi* antigen (Figure 41 h). Importantly, we found that sera from COX-1$^{-/-}$ animals (Figure 41 h, Lanes 4-7) demonstrated dramatically decreased reactivity with the four primary *Borrelia* antigens, as compared to sera from wild-type control mice (Figure 41 h, Lanes 1-3). Thus, we have demonstrated that a deficiency in COX-1 results in increased IgM and decreased IgG, as a result of decreased isotype switching. Furthermore, the antibodies that are produced by COX-1$^{-/-}$ animals recognize fewer *B. burgdorferi* antigens than sera from wild-type mice. These data clearly demonstrate a previously unappreciated role for COX-1 in the development of the humoral response to bacterial pathogens.
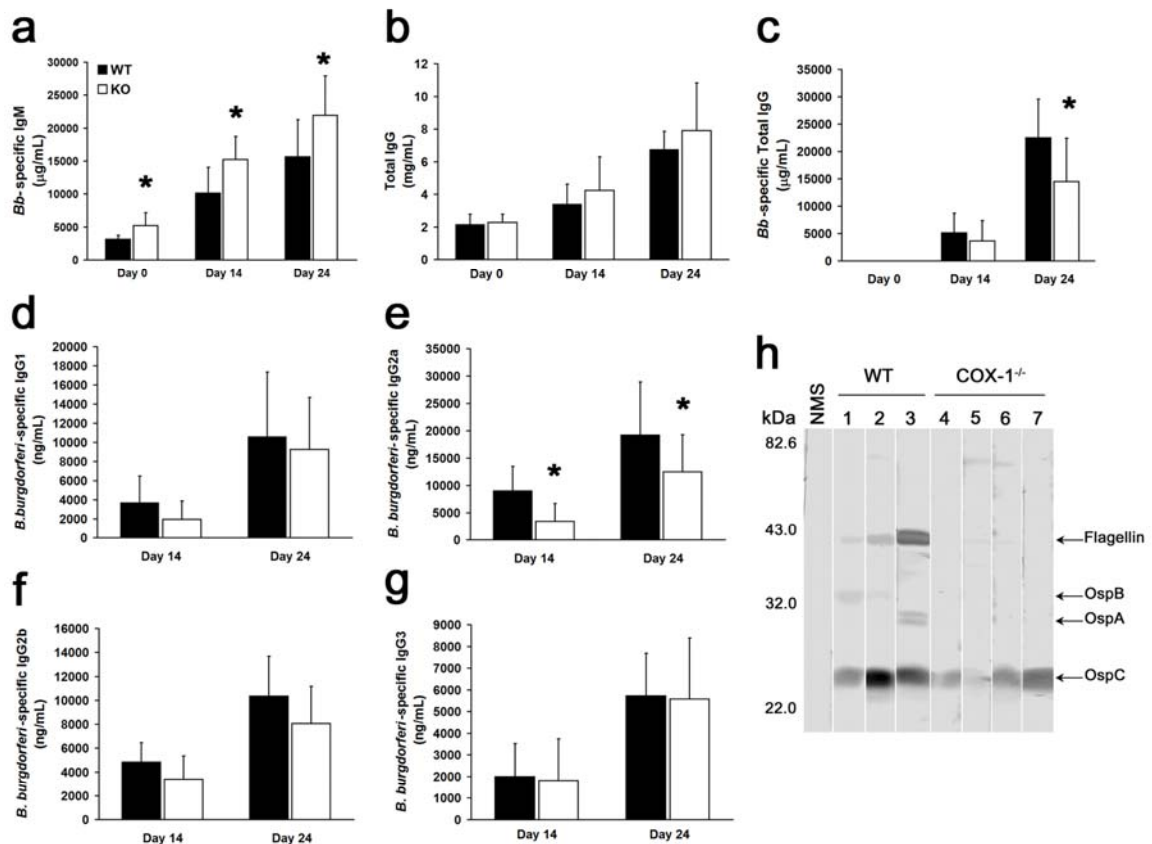
Figure 41. COX-1 deficiency significantly alters antibody responses and antigen recognition. COX-1$^{-/-}$ mice (open bars) and wild-type controls (closed bars) were infected with *B. burgdorferi* and their levels of *B. burgdoreri*-specific IgM (a), toal IgG (c), IgG$_1$ (d), IgG$_{2a}$ (e), IgG$_{2b}$ (f) and IgG$_3$ (g), as well as total non-specific IgG (b) were measured at d14 and d24 post-infection. Basal levels of IgM (a) and total non-specific IgG (b) were also measured. *$P < 0.05$. Error bars represent $\pm$ s.d. $n = 10$ mice per genotype.

C. *Conclusions*

Only recently has the necessity of the COX isozymes in the development of acquired immunity been appreciated, however, no studies have examined the effects of inhibition of both COX isoforms in response to infection. We therefore determined if there was a role for COX-1 or -2 production by murine B lymphocytes. Our data show that primary murine B cells constitutively express COX-1 mRNA and protein, and up-regulate COX-2 mRNA in response to traditional B cell stimuli, similar to the results obtained with human cells (194). Since expression of COX-2 protein is regulated both transcriptionally and translationally (20), we assessed the expression of COX-2 protein in activated B cells by Western blot and flow cytometry, and found that upon stimulation, protein expression of COX-2 was up-regulated, albeit at low levels.

An earlier study showed that day 14 C3H B or T cell responses to intradermal *B. burgdorferi* infection were not altered by treatment with the COX-2 inhibitor MF-Tricyclic (150). We wished to expand on these findings for several purposes: primarily, multiple studies have shown that the site of inoculation with *B. burgdorferi* can modulate disease (210,211); we utilized a footpad inoculation model. Also, only sera from day 14 post-infection were analyzed, whereas it was possible that differences in antibody production could become evident later in the time course, when resolution should be occurring. A deficiency in MyD88 signaling, an up-stream molecule in the COX-2 signaling pathway, alters antibody responses after this time point (124). Finally, differences in COX-2 selectivity and kinetics of the different Coxibs (212) could alter how inhibition affects B cell responses. In our experiments, we utilized celecoxib, a

COX-2 inhibitor that is typically prescribed to patients suffering from Lyme arthritis (116).

Infection of arthritis-susceptible animals treated with celecoxib lead to the development of *B. burgdorferi*-specific antibody levels comparable to untreated controls. Induction of antibody responses is also an integral aspect of vaccine immunology, and using a model of *B. burgdorferi* vaccination, we found that vaccinated mice treated with celecoxib produced full anti-*B. burgdorferi* antibody responses, similar to untreated controls. Analysis of sera from vaccinated, celecoxib-treated and untreated animals demonstrated that sera from vaccinated, COX-2 inhibitor-treated mice retained the ability to recognize the same four key immunogenic proteins: flagellin, OspA, OspB, and OspC, as untreated controls.

We then examined a potential role in B cell responses for COX-1, as compared to COX-2, *in vitro*. We demonstrated that murine B cells possessed the capacity to produce $PGF_{2\alpha}$ and low levels of PGE_2 in response to live *B. burgdorferi*, and also produced high levels of TXB_2, a primarily COX-1-derived eicosanoid. B cells also demonstrated basal FP protein expression, and increased their FP expression in response to *B. burgdorferi*-stimulation, and therefore could respond to $PGF_{2\alpha}$ in an autocrine fashion. We were surprised to see that the most significant decreases in eicosanoid and Ig production, by both wild-type and COX-2$^{-/-}$ cells, were in response to COX-1, and not COX-2, inhibition. Also of note: while AA predictably induced increases in eicosanoid production, this stimulus was not enough to alter Ig production. Only the most

physiological stimulus, live *B. burgdorferi*, was able to stimulate Ig production, although AA induced the highest eicosanoid production. Thus, induction of eicosanoids alone is not sufficient for Ig production. Additionally, the ability of COX-2$^{-/-}$ B cells to down-regulate Ig production in response to both COX-1 and -2 inhibition implies that COX-independent mechanisms might be activated.

In vivo, examination of the effects of COX inhibition clearly demonstrated that COX-1, not COX-2, regulated the humoral response to *B. burgdorferi*. Since *in vivo*, dual inhibition had the greatest effect on Ig production, this implies that compensation may be a factor when inhibition of only one COX isotype is employed. This also indicates that the use of non-specific COX inhibitors (NSAIDs) may have a greater effect clinically with regards to antibody production. The use of COX-1$^{-/-}$ mice clearly illustrates the pivotal role of the COX-1 isotype in development of pathogen-specific antibody responses. Although these mice developed higher levels of IgM compared to their wild-type controls, they did not produce the same levels of *B. burgdorferi*-specific IgG. Furthermore, the antibodies that were produced did not recognize *B. burgdorferi* antigens to the same extent as those from wild-type animals.

In summary, the findings reported herein, with the use of the *B. burgdorferi* infection model demonstrates that in a physiologically relevant system, COX-1 predominates in regulation of the humoral response to bacterial pathogens. Thus, humoral immune responses to infectious agents or vaccine preparations are not likely to be significantly

altered in patients undergoing COX-2-inhibitor therapy, whereas COX-1-specific

inhibition or the use of NSAIDs must be utilized with caution.

CHAPTER VI

CYCLOOXYGENASE-1 MODULATES

THE SEVERITY OF ARTHRITIS

A.   *Introduction*

Cyclooxygenase-1 (COX-1) is traditionally regarded as a housekeeping enzyme, since it appears to be constitutively expressed in most tissues (182). Two of the most widely appreciated roles for COX-1 are gastric cytoprotection and regulation of platelet activation and aggregation, and both activities are tied to alterations in vascular permeability and production of vasoactive mediators (182,183,213). Despite the indispensable nature of COX-1 in regulation of the vasaculature, its role in inflammation is viewed as secondary to the COX-2 isozyme which, unlike its constitutively expressed sister, is an early immediate gene normally up-regulated by pro-inflammatory stimuli (20,18). Earlier research hinted at the role of COX-1 in inflammatory states, however most inflammation research has ignored COX-1 in favor of COX-2 (19,23). As we had seen changes in resolution due to COX-2 deficiencies, we wished to determine what role COX-1 might play in the development or resolution of the inflammatory response to *B. burgdorferi* infection. Unfortunately, the COX-1 gene knockout is not available on a Lyme disease susceptible background and backcrossing to the C3H and full B6 backgrounds is underway in our laboratory. These mice are available, however, on the mixed B6.129 background, which is considered resistant to development of Lyme

arthritis (114). It was still of interest to determine if any differences in arthritis pathology would be evident in the context of COX-1 deficiency, even on this mixed genetic background. COX-1$^{-/-}$ mice (B6;129P2-*Ptgs1^{tm1Unc}*) and their wild-type controls were infected with *B. burgdorferi*, and ankle diameter and antibody responses measured at various time points. Mice were sacrificed at d24 post-infection and arthritis severity and bacterial loads were determined at this time point.

B. *Results*

1. *COX-1 deficiency results in increased joint swelling*

COX-1$^{-/-}$ mice and wild-type controls were inoculated with *B. burgdorferi*, and ankle diameter measurements were taken at days 7, 10, 14, 17, 21, and 24 post-infection and compared to pre-infection measurements. Figure 42 illustrates that, while neither group displayed increased ankle diameters comparable to those seen in susceptible mouse strains, the animals deficient in COX-1 demonstrated greater ankle swelling compared to their wild-type counterparts. Joint swelling in both groups appeared to peak at about day 17 post-infection. We hypothesize that this increase in susceptibility to ankle swelling is due in part to the contribution of COX-1 to thromboxane (TX) production, which is a known vasoconstrictor. Under inflammatory conditions, the levels of vasoconstrictive and vasodilatory mediators varies to regulate cellular influx and control migration of pathogens (6,214). It is possible that under normal conditions, COX-1-mediated TX production is responsible for the counter-regulation of dilatory compounds, such as COX-2-produced prostacyclin (PGI$_2$), and that the loss of COX-1 resulted in a loss of this counterbalance, leading to increased in vasodilation and therefore joint swelling.

2. *COX-1 regulates antibody responses to* B. burgdorferi

Immunoglobulin production in response to *B. burgdorferi* was assessed in COX-1$^{-/-}$ as compared to wild-type animals. Sera were collected at d14 and d24 post-infection, and total *B. burgdorferi*-specific IgM and IgG were measured. We found that both *B. burgdorferi*-specific IgM and IgG were significantly decreased in COX-1$^{-/-}$ mice at both

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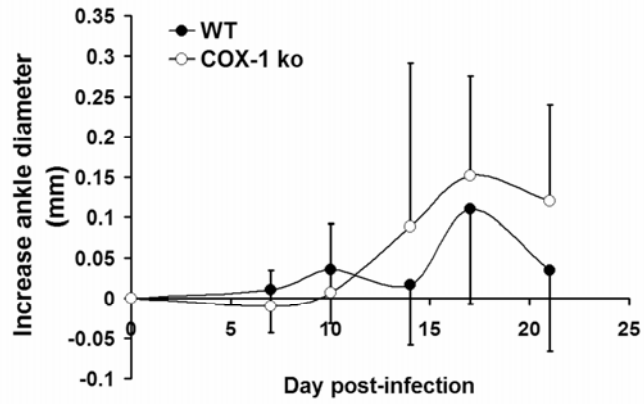
Figure 42. COX-1$^{-/-}$ mice demonstrate a trend in increased ankle swelling. Increases in ankle diamters were measured for both wild-type (WT; closed circle) and COX-1$^{-/-}$ (open circle) mice at d7, 14, 21, and 24 post-infection. Error bars represnt $\pm$ s.d. n = 10 mice.

time points as compared to wild-type controls. Antibody responses of these mice are examined in greater detail in Chapter V.

3. *COX-1 deficiency results in increased arthritis severity and altered cellular infiltrate*

To determine the effect of COX-1 deficiency on arthritis pathology, histological sections of ankle joints at d24 were scored for arthritis severity. Figure 43 a and b illustrates a typical wild-type joint at d24 post-infection, whereas a joint from a COX-1$^{-/-}$ mouse is shown in Figure 43 c-e. The differences in cellular morphology can be seen upon higher magnification, as the COX-1$^{-/-}$ mice have cells that display nuclear morphology typical of neutrophils, however they appear to still contain granules (Figure 43 d & e), unlike the wild-type neutrophils, which do not (Figure 43 b). It is unknown what the significance of these morphological differences is with regards to the development of arthritis pathology.

4. B. burgdorferi *loads are lower in the joints of COX-1$^{-/-}$ mice*

A lack of COX-1 leads to increased arthritis severity and altered cellular recruitment, but could this be the result of increased bacterial loads as a result of decreased total *Borrelia*-specific antibody production? To determine *B. burgdorferi* loads, *B. burgdorferi* DNA was quantitated by real-time PCR in ankle joints contralateral to those used for histology. We found that mice deficient for COX-1 harbored lower levels of *B. burgdorferi* in their joints as compared to their wild-type counterparts, as shown in Figure 44. This decrease in bacterial burden in the joints of the COX-1$^{-/-}$ mice is the opposite of what would be predicated in light of the current dogma in the Lyme disease field, which holds that anti-*Borrelia* antibodies are crucial for clearance of *B. burgdorferi* from the host. As shown
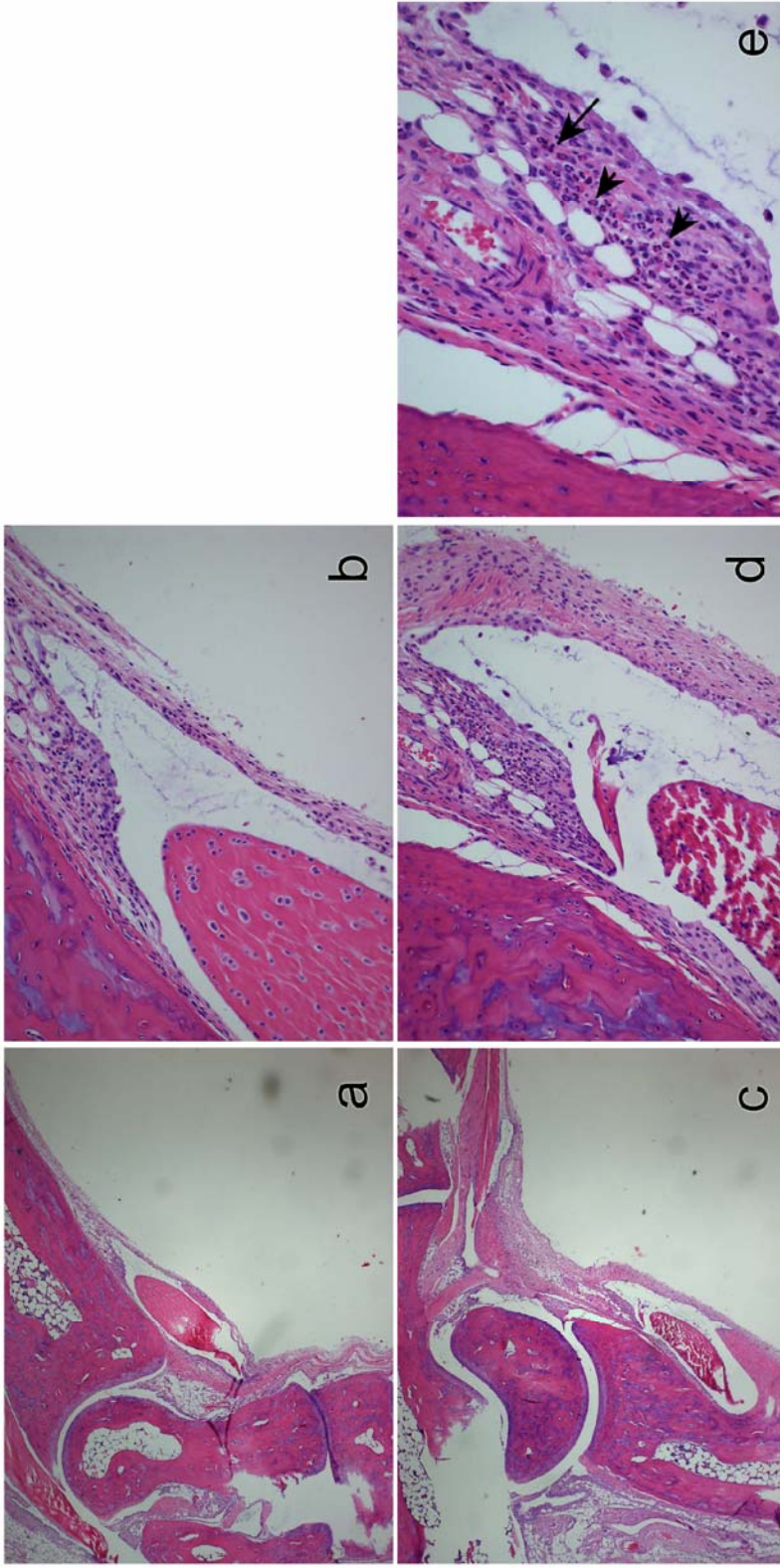
Figure 43. H & E stain of tibiotarsal joints from wild-type (a & b) and COX-1$^{-/-}$ mice at d24 post-infection. a & c: 5x magnification; b & d: 20x magnification; e: 40x magnification. Arrowheads denote cells with representative of the unusual neutrophil nuclear morphology and granular staining. Arrows indicate typical neutrophil morphology.
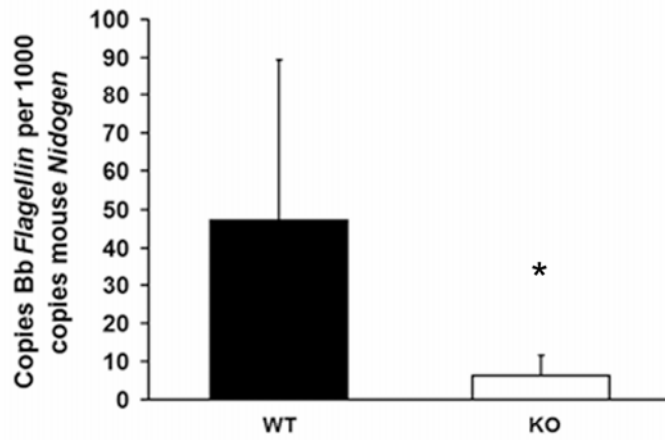
Figure 44. *Borrelia* loads in the joints of wild-type (closed bar) and ko (open bar) C3H mice at d24 post-infection, as measured by real-time PCR. Error bars represent ± s.d. * $p < 0.05$. $n = 10$ mice per group.

in more detail in Chapter V, mice deficient in COX-1 have decreased *Borrelia*-specific antibody responses. Others have shown that antibody production appears to correlate with spirochete levels present in the joints, e.g., IL-10^{-/-} mice demonstrate an inverse correlation between decreased *B. burgdorferi*-specific antibody responses and increased *Borrelia* loads (215). However, this correlation does not appear to hold true in the case of COX-1 deficiency. This is in the context of a resistant mouse strain, and it will be interesting to see if bacterial loads are similarly lowered in the joints of COX-1^{-/-} Lyme arthritis-susceptible animals.

5. *Conclusions*

Data regarding COX-1 involvement in the inflammatory response have been compiled, for the most part, with the view that COX-1 was a control, used as a comparator for COX-2 regulation. However, most of these studies were conducted in the context of autoimmune disease models (216,28,217). The few studies that have investigated the role of COX-1 in the inflammatory response to infection, such as influenza A (52), have found that COX-1 deficiency actually lead to increased disease severity.

In our studies utilizing COX-1^{-/-} mice, on a resistant background, we also found that COX-1 may play a modulatory or protective role in regulation of the inflammatory response to *B. burgdorferi* infection. There was an obvious trend toward increased ankle swelling in the COX-1^{-/-} animals, and antibody levels in response to *B. burgdorferi* were also altered. Histologically, severity was increased in COX-1-deficient mice and there was a dramatic change in the cellular morphology of the inflammatory infiltrate of the

joints. Bacterial loads were drastically decreased in these animals, in direct opposition to the decreased antibody levels. While COX-1 appears to regulate the humoral response to *B. burgdorferi,* it may also regulate immune cell infiltration into the joints, thus increasing bacterial clearance, but also leading to increased arthritis. Further studies utilizing COX-1-deficient C3H/HeJ mice will help to elucidate the impact of COX-1 in the context of arthritis susceptibility.

CHAPTER VII

DISCUSSION

The hallmarks of inflammation are easily listed by most people: redness, swelling, pain, fever, and loss of function (3). Stating what actually regulates the development of these symptoms is not as simple a task. Although scientists have long known that eicosanoids are integral components of any inflammatory response; how they affect their regulatory actions is incompletely understood, and the focus of intense research. My graduate studies began with the goal of understanding the contribution of COX-2 to experimental Lyme pathology. We have come to find that, instead of merely investigating the role of eicosanoids in Lyme disease, we are now using experimental Lyme arthritis as a model system to study the regulation of inflammation by eicosanoids.

We first conducted a detailed analysis of the contribution of COX-2 to the development of experimental Lyme arthritis pathology by utilizing various inhibitors of COX-2. The inhibitors used varied in their COX-2-specificity, i.e., meloxicam is considered to be a COX-2-preferential inhibitor, celecoxib is COX-2 specific, and rofecoxib is even more specific (13), yet their administration during *B. burgdorferi* infection resulted in similar effects on underlying arthritis pathology.

Anguita, et. al. (150), published a brief study utilizing treatment with the COX-2-preferential inhibitor MF-tricyclic during *B. burgdorferi* infection. At the time this study was published, I had just begun experiments examining the possible contribution of COX-2 to Lyme arthritis; however, we were already observing results that differed from those described by Anguita, et. al., and therefore continued with more in-depth studies of COX-2. Now, with a greater understanding of the complexities of COX and eicosanoid regulation, it is not surprising that our experiments would produce data that differed from those previously published (150). There are actually several reasons for these differences. For instance, the published study utilized a different inoculation model, examined only one early (d14) and one late (d60) time point, and this was done using only the arthritis-susceptible C3H mouse strain.

Our initial studies were conducted with meloxicam, and in later experiments, we used primarily celecoxib. The rationale for this change is three-fold: we wanted to be sure the effects we observed were due to COX-2 inhibition, and thus switched to a specific COX-2 inhibitor; rofecoxib was removed from the market by Merck, thus prompting another change, and celecoxib could be obtained for free from pharmacy drug samples. Despite the differences in COX-2-specificity, the effects on underlying Lyme arthritis pathology were similar: inhibition of COX-2 lead to an inability to resolve Lyme arthritis (Figures 4 and 7).

When we first observed this effect on arthritis, there were few reports in the literature to explain this phenomenon. Most COX-2 studies focused on a pro-inflammatory role in

autoimmune diseases, especially models of rheumatoid arthritis (218,177,216). Some studies described anti-inflammatory roles for COX-2 products (64,219); however, these were different from the results we had recorded. Our use of COX-2 inhibitors did not prevent the development of arthritis (Figure 5), thus, COX-2 products did not appear to play a role in modulating the initiation of the inflammatory response, and would not be considered anti-inflammatory. Rather, COX-2 inhibition affected the induction of resolution of Lyme arthritis. Indeed, we found that cessation of COX-2 inhibition at the peak of inflammation did not allow for induction of the proper pro-resolution mechanisms (Figure 6). Therefore, in Lyme arthritis, COX-2 products do not appear to be anti-inflammatory (i.e., they do not prevent arthritis), but are pro-resolution, necessary for down-regulating the inflammatory response.

This view was supported by our data, which demonstrated that concurrent inhibition of COX-2 and LTB_4 signaling did not allow for the beginning of resolution that the pharmacological blockade of COX-2 alone inhibits. Although our original hypothesis asserted that the lack of resolution due to COX-2 inhibition was the result of the increased production of LTB_4 observed in Clx-treated C3H mice (Figure 23), our data clearly illustrate that blocking the signaling of this inflammatory mediator is not sufficient for resolution (Figure 24). This is an important finding with regards to the treatment of inflammation. Thus far, pharmaceutical companies have focused on inhibiting inflammation (220,15), a misguided endeavor, considering most patients will initiate treatment well after the inflammatory process has commenced. Inhibition of selective pro-inflammatory mediators is not sufficient for treatment of inflammatory

disease, e.g., even when you disconnect a plug from a socket, you don't use your finger in its place, because electricity is still flowing! Similarly, disconnecting one desk lamp will not bring darkness to the entire Empire State building - one must throw the circuit breaker to make the flow stop completely. Thus, to stop the inflammatory response, the pro-resolution breaker switch must be thrown - pulling one pro-inflammatory plug will not be enough. For this reason, further studies must be conducted using pro-resolution molecules in addition to COX-2 inhibition, to verify this mechanism in Lyme arthritis.

That the blockade of the LTB_4 receptor BLT_1 did not induce arthritis resolution during COX-2 inhibition, despite it's production during infection, and increased production during COX-2 inhibition, lead us to examine the role of LTB_4 itself in the pathology of experimental Lyme arthritis. C3H mice were treated with the BLT_1 receptor antagonist CP-105,696 during *B. burgdorferi* infection to further elucidate the possible regulation of pathology development due to LTB_4.

The importance of neutrophils to the development of Lyme arthritis (117,145), as well as the contribution of eicosanoids to neutrophil activity (173,221), implied that LTB_4 could contribute to Lyme arthritis development. We were therefore surprised to find that BLT_1 antagonism did not ameliorate arthritis. Furthermore, CP-105,696 treatment actually resulted in a trend toward increased ankle swelling and arthritis severity (Figures 26-28). While our COX-1 studies (Chapter VI) illustrated that an increased inflammatory response could lead to decreased *B. burgdorferi* loads, spirochete burdens were not affected by the increased inflammation in the CP-105,696 (Figure 45). Even more

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surprising was the fact that, while levels of *B. burgdorferi* in the joints were not altered, anti-*Borrelia* IgG levels were significantly increased at d35 post-infection, further calling into question the role of antibodies in control of *B. burgdorferi* in infected tissues.

LTB_4 has been implicated in regulation of cell survival and induction of apoptosis (222,223). We therefore attempted to measure apoptosis induction both qualitatively by TUNEL assay and quantitatively by real-time RT-PCR array. TUNEL staining illustrated decreased apoptosis in the joints of CP-105,696-treated mice compared to their vehicle-treated controls at d35 post-infection, implying decreased apoptosis induced by BLT_1 blockade (Figure 32). Analysis of mRNA from joints of antagonist-treated animals demonstrated changes in key pro- and anti-apoptotic genes. Most notably, expression of the pro-apoptotic gene sphingosine kinase-2 was almost completely ablated in treated mice (Figures 33 and 34). The down-regulation of this gene was of great interest to us, since its primary function is in regulation of lipid mediator production during the inflammatory response (224,208,225). As stated previously, more in-depth studies must be undertaken to determine which cell types BLT_1 antagonism was affecting in arthritic joints.

Just as COX-2 appeared to mediate pro-resolution of experimental Lyme arthritis, studies using COX-1$^{-/-}$ mice implied an anti-inflammatory role for the COX-1 isotype. Periodically, studies demonstrated a protective role for COX-1 during inflammatory responses (44,52). Unfortunately, since research revealed that anti-inflammatory effects of NSAIDs were due primarily to an inducible COX isozyme (COX-2) (226,227), COX-1

has generally been ignored when examining the inflammatory response. Research regarding this isozyme tends to focus on its homeostatic influences on the vasculature (228,10). However, the studies that have examined COX-1 in the context of an acute inflammatory response (i.e., not a chronic or autoimmune disease model) have put forth an image of a "new and improved" COX-1 that is required as a regulator of the inflammatory response. Those studies portray this isozymes as "the good girl", the one that keeps her "partying" pro-inflammatory sister isozyme COX-2 in check. For instance, when COX-2 induces pro-inflammatory mediators in the lungs of mice infected with influenza A virus, COX-1 "pushes back", providing an anti-inflammatory counterbalance to the pro-inflammatory immune response. COX-1 deficient animals demonstrated more severe illness at earlier time points than wild-type mice infected with influenza A (52). In experimental Lyme arthritis, since COX-2 appears to mediate a pro-resolution response, it is unlikely that COX-1 and -2 are antagonistic. This does not rule out COX-1 as a regulatory molecule, since it appears to antagonize the general inflammatory response, just not specifically through antagonism of COX-2.

The influence of COX-1 was therefore examined in the context of COX-1 deficiency on a Lyme arthritis-resistant background. As mentioned previously in Chapters V and VI, these mice are currently not available on an arthritis-susceptible background. This was somewhat propitious, with regard to examining the protective effects of COX-1. If COX-1 is indeed protective, then there is a greater likelihood that increased arthritis severity would be more readily apparent if, under normal conditions arthritis does not develop.

This was confirmed by our Lyme arthritis studies using COX-1$^{-/-}$ mice. These mice developed greater ankle swelling and exhibited more severe arthritis pathology (Figures 43 and 44), and a correlative decrease in *B. burgdorferi* loads in arthritic joints (Figure 45). Curiously, these mice also had decreased antibody class switching and their sera demonstrated a decreased ability to recognize certain *B. burgdorferi* antigens (Chapter V).

The results of these experiments challenge the current dogma in both the eicosanoid and Lyme disease fields. Despite the attention devoted to COX-2 modulation of the inflammatory response, our data clearly demonstrate that COX-1 can also play a pivotal, possibly antagonistic role in inflammation. Thus, this poses a serious question about the use of COX-1 inhibitors and NSAIDs, such as keterolac, ibuprofen, and naproxen (13): how safe are these "newly re-discovered" pain relievers/anti-inflammatory agents? They have seen increased use recently because of the cardiovascular issues related to COX-2-specific inhibitors (13,229), but what of these previously unappreciated side-effects on the immune response to pathogens? Further *in vivo* studies are needed to investigate these issues.

The examination of COX-1 in the immune response to *B. burgdorferi* also brings into question the closely-held belief in the Lyme field that anti-*Borrelia* antibodies are the crucial determinant in clearance of *B. burgdorferi* from infected tissues (123). In the context of COX-1 deficiency, this was clearly not the case. Despite significantly decreased *B. burgdorferi*-specific antibody responses and the almost ablated ability of

serum antibodies to recognize *B. burgdorferi* antigens (Figure 42); bacterial loads were not increased, but were significantly decreased at d24 post-infection (Figure 45).

Antibody participation in *B. burgdorferi* clearance is supported primarily by passive immunization studies (230,231) and the use of severe-combined immunodeficient (SCID) (141). Thus, we propose that hyper-induction of an inflammatory response to *B. burgdorferi*, such as that demonstrated by IL-10$^{-/-}$ mice (232), leads to increased arthritis severity via greater stimulation of innate immune responses, thus allowing for clearance of *Borrelia* in the absence of specific IgG responses. More studies are necessary to decipher the mechanisms that underlie the increased induction of arthritis and its effect on bacterial clearance.

The importance of antibodies during *B. burgdorferi* infection in humans (233) and normal animals is not under debate and has well-characterized (233,123,234). Only recently has the necessity of COX-2 in the development of acquired immunity been appreciated, although *in vivo* studies have mostly concentrated on T cells (235,236). *In vitro* data imply a role for B cell-derived COX-2 during developmental stages such as expression of MHC II and isotype switching (192, 193). Curiously, few *in vivo* studies have investigated a potential role for COX-2 in the regulation of B cell development and activation. It has been shown that basal levels of total IgM and IgG were lower in C57B/6 COX-2$^{-/-}$ mice (194), and these mice also have decreased isotype switching from IgM to IgG in response to vaccination with HPV-VLPs (196). However, no studies have examined the effects of inhibition of both COX isoforms in response to infection.

Therefore, it was uncertain if the decreased antibody levels described by Ryan, et. al., (194) were functionally relevant.

We wished to determine if there was a role for COX-1 or -2 production by primary murine B lymphocytes, and therefore investigated whether these cells could express either isozyme in response to well-known B cell activation signals: anti-CD40 and *B. burgdorferi* total antigen—bacterial antigens with B cell mitogenic properties as well as the capacity to induce the production of antigen-specific antibodies (123). Our data show that primary murine B cells constitutively express COX-1 mRNA and protein, and up-regulate COX-2 mRNA in response to traditional B cell stimuli, similar to the results obtained with human cells (194). Since expression of COX-2 protein is regulated both transcriptionally and translationally (39, therefore we assessed the expression of COX-2 protein in activated B cells by western blot and flow cytometry. Of interest is the difference between the high level of RNA production and the lower levels of COX-2 protein by both mouse strains in our studies. Because eicosanoid metabolism is tightly regulated, it is possible that in an artificial, *in vitro* environment, initial stimulation leads to mRNA production, but the additional environmental signals normally produced by interactions with surrounding cells and tissues are not present in a purified culture, and therefore the RNA levels do not translate to high protein levels. This assessment is supported by the flow cytometry data, which indicate that the majority of stimulated B cells are producing COX-2 protein, but at low levels.

An earlier report examining the *in vitro* effects of COX-2 inhibitors on isolated human B cells postulated that the decrease in B cell activation displayed after inhibitor treatment *in vitro* would translate *in vivo* to a decreased capacity of the host to respond to microbial challenge. The importance of the antibody response to clearance of the *B. burgdorferi* organism makes the experimental Lyme disease model an excellent tool for the investigation of the contribution of COX-2 to humoral immunity. *B. burgdorferi* infection induces the production of T cell-dependent and –independent antigen-specific IgM and IgG antibodies in mice (231,121,142). Several reports have examined the production of antibodies in response to *B. burgdorferi* in the context of toll-like receptor 2 (TLR2) or MyD88 deficiency. Their data demonstrate the complexities of humoral immunity regulation. $TLR2^{-/-}$ mice did not have altered antibody responses (237). $MyD88^{-/-}$ animals had altered *B. burgdorferi*-specific antibody titers, however total IgM and IgG levels were unaltered from wild-type littermates (124). Those studies highlighted the importance of the interplay between cells of the acquired immune system and those of the innate system in compensating for the deficiency of a key signaling protein.

Induction of antibody responses is an integral aspect of vaccine immunology. The possibility that COX-2 inhibition alters antibody responses illustrates a serious issue in this regard, as COX-2 inhibitors are widely prescribed for the treatment of pain and common inflammatory conditions. Using a model of *B. burgdorferi* vaccination, we found that vaccinated mice treated with celecoxib produced full anti-*B. burgdorferi*

antibody responses, similar to untreated controls and retained the ability to recognize the same four key immunogenic proteins as untreated controls.

COX-2 did not appear to play an appreciable role in *B. burgdorferi*-specific antibody responses in COX-2$^{-/-}$ animals, or those treated with a COX-2-specific inhibitor which were vaccinated and challenged with *B. burgdorferi*. We then examined the potential role for COX-1 in B cell responses, as compared to COX-2, *in vitro*.

We demonstrated that murine B cells possessed the capacity to produce PGF$_{2\alpha}$ and low levels of PGE$_2$ in response to live *B. burgdorferi*, and also produced TXB$_2$, a primarily COX-1-derived eicosanoid. B cells also demonstrated basal FP protein expression, and increased their FP expression in response to *B. burgdorferi*-stimulation, and therefore could respond to PGF$_{2\alpha}$ in an autocrine fashion. Since the previous study demonstrated significant responses to COX-2 inhibition, we were surprised to see that the most significant decreases in eicosanoid and Ig production, by both wild-type and COX-2$^{-/-}$ cells, were in response to COX-1, and not COX-2, inhibition. One of the possible explanations for the different findings might be the different concentrations of COX-2 inhibitors used. When preliminary studies were conducted in our laboratory, we found dramatically increased apoptosis of B cells cultured in the presence of celecoxib concentrations above 10μM. This increased apoptosis is a well-known side effect, as celecoxib is used for this purpose in cancer studies, and concentrations above 20μM are considered "high" (238). The COX-2 specific inhibitor utilized by Ryan, et. al, SC-58125, has been shown to decrease cell proliferation and colony size *in vitro* by almost

50% at a concentration of 10µM, and by over 90% at 50µM concentration (239). As their inhibitor studies used concentrations of 40µM (194) or a range of concentrations beginning at 10µM (196), it is not surprising that they found such dramatic decreases in the numbers of cells producing antibodies. We attempted to prevent the possibility of growth arrest or apoptosis induction by incubating B lymphocytes with the minimal concentration required for inhibition. To examine this possibility, cells were incubated with increasing concentrations of inhibitors for at least 36h, since COX-2 inhibitor assay kits only expose cells to inhibitors for a total of 10 minutes; not long enough to test for induction of apoptosis. Thus, both celecoxib and SC-560 were used at a concentration of 1µM in our studies.

Also of note is that while AA predictably induced increased eicosanoid production, this stimulus was not enough to alter Ig production. Only the most physiological stimulus, live *B. burgdorferi*, was able to stimulate Ig production, although AA induced the highest eicosanoid production. Thus, induction of eicosanoids alone is not sufficient for Ig production. Additionally, the ability of COX-2$^{-/-}$ B cells to down-regulate Ig production in response to both COX-1 and -2 inhibition implies that COX-independent mechanisms might be activated. These *in vitro* data also demonstrate the importance of caution in evaluation of data garnered from *in vitro* experiments with regards to eicosanoid regulation.

In vivo, examination of the effects of COX inhibition clearly demonstrated that COX-1, not COX-2, regulated the humoral response to *B. burgdorferi*. Since *in vivo*, dual

inhibition had the greatest effect on Ig production, this implies that compensation may be a factor when inhibition of only one COX isotype is employed. This also indicates that the use of non-specific COX inhibitors (NSAIDs) may have a greater effect clinically with regards to antibody production. The use of COX-1$^{-/-}$ clearly illustrates the pivotal role of the COX-1 isotype in development of pathogen-specific antibody responses. Although these mice developed higher levels of IgM compared to their wild-type controls, they did not produce the same levels of *B. burgdorferi*-specific IgG. Furthermore, the antibodies that were produced did not recognize *B. burgdorferi* antigens to the same extent as those from wild-type animals.

Previous studies have shown a decrease in antibody production after COX-2 inhibition. However, those studies were performed to examine the effect of COX-2 inhibition in models of autoimmune disease, such as arthritis, where COX-2 is known to play a role in the propagation of pathology (14,240). Inhibition of COX-2 in these models led to decreased antibody production. Despite these data and in light of our findings, there is no evidence that this was due to a direct effect on B cell COX-2. Rather, we hypothesize that it may be due to decreased COX-2 activity in tissue and innate immune cells, which can affect B lymphocyte activity via eicosanoid and cytokine production (191,241).

The disparities between our results and the previous *in vitro* findings of a significant role for COX-2 may be due to the vital interactions between B cells and their environment that cannot be adequately simulated *in vitro*, such as their proximity to tissue fibroblasts and macrophages. This is true of other immune cells as well: type of stimuli, presence of

certain co-stimulators, and soluble signals, can alter eicosanoid regulation and response in T lymphocytes (13). Studies have also implied the necessity of PGE_2 exposure prior to antigen stimulation in order for B cells to be affected by COX-2 inhibition, as activated B lymphocytes do not alter their activation state upon exposure to PGE_2 (193). This indicates that *in vivo,* B lymphocytes contact *B. burgdorferi* antigen before encountering cogent concentrations of PGE_2.

In summary, our findings clearly demonstrate a crucial *in vivo* role for COX-1, beyond that of COX-2, in the development and maintenance of the humoral response to *B. burgdorferi*. The differences between the effects of COX inhibition *in vitro* and *in vivo* also illustrate the importance of the microenvironment upon the proper development of pathogen-specific immunoglobulins, and the potential effects of COX inhibition. The use of the *B. burgdorferi* infection model demonstrates that in a physiologically relevant system, COX-1 predominates in regulation of the humoral response to bacterial pathogens. Thus, humoral immune responses to infectious agents or vaccine preparations are not likely to be significantly altered in patients undergoing COX-2-inhibitor therapy. However, this new role for COX-1 in humoral immunity warrants a re-evaluation of attitudes toward "safe" non-specific and COX-1-specific inhibitors.

The work contained in this thesis represents substantial progress in our understanding of both the mechanisms underlying both experimental Lyme arthritis pathology, as well as the contribution of eicosanoids to the regulation of the inflammatory response. Previously unappreciated roles for COX-1 and -2 in the modulation of inflammation have

been described. Using a physiologically relevant *in vivo* model of *B. burgdorferi* infection, COX-1 and COX-2 were shown to affect regulatory actions on both the induction and resolution of the inflammatory response to *B. burgdorferi*. It was further demonstrated that COX-1 is the predominant COX isozyme in the regulation of B lymphocyte responses to *B. burgdorferi*, both *in vitro* and *in vivo*. Studies demonstrating the necessity of COX-1 activity for proper antibody production *in vivo* are the first to ascribe a role to COX-1 in not only B cell activity but the humoral immune response. Furthermore, these studies are the first to describe a pro-resolution role for LTB_4 in the regulation of inflammation. Studies are planned to investigate the molecular mechanisms whereby these molecules affect their crucial immune modulatory functions.

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APPENDIX I

REAL-TIME PCR PRIMER AND PROBE SETS

	sequence 5' to 3'	exon	Tm	%GC	G/C	length
COX-2 *Ptgs2*						
5'	TTC CTC CCG TAG CAG ATG AC	e 4	54	55	3\8	20
3'	TCC ACT CCA TGG CCC AGT	e 5	53	61	4\7	18
probe	AGA TCA TAA GCG AGG ACC TGG GTT CAC C	e 5	63	54	8\7	28
COX-1 *Ptgs1*						
5'	ACA AGA GTA CAG CTA CGA GCA GTT	e 9	56	46	6\5	24
3'	ATC TCT CGG GAC TCC TTG ATG ACA	e 10	57	50	5\7	24
probe	TGA CTA TCA TGT TCT GCA TGT GGC TGT GG	e 10	62	48	9\5	29
cPLA$_2$ *Pla2g4a*						
5'	TCA CAC TGA TGG ATG CCA ACT A	e 5	53	45	4\6	22
3'	CTG ATC ACA CAG TGC CAT G	e 6	54	52	4\6	19
probe	TGG AAG TTT GTT CAT GCC CAG ACC TAC GGT T	e 7	63	48	8\7	31
5-LO *Lox5*						
5'	AAC CTG TTC ATC AAC CGC TTC ATG	e 5	56	46	3\8	24
3'	ACT GGT AGC CAA ACA TGA GGT CTT	e 5-6	56	46	5\6	24
probe	AAC ACT ATA TCT GAG CGA GTC AAG AAC CAC TGG CA	e 6	64	46	8\8	35
FLAP *Alox5ap*						
5'	ATC AAG AGG CTG TGG GCA ACG TT	e 1	57	52	8\4	23
3'	AGT CCA GAG TAC CAC AAG GAA AGT	e 2-3	56	46	6\5	24
probe	TGC CAA CCA GAA CTG CGT AGA TGC GTA CC	e 3	64	55	7\9	29

RAW DATA OF APOPTOSIS PCR ARRAY

Symbol	Well	AVG ΔC$_t$ (Ct(GOI) - Ave Ct (HKG))		2^-ΔC$_t$		Fold Difference	Fold Up- or Down- Regulation
		Test Sample	Control Sample	Test Sample	Control Sample	Test Sample /Control Sample	Test Sample /Control Sample
Akt1	A01	4.33	6.96	5.0E-02	8.0E-03	6.18	6.18
Apaf1	A02	8.04	9.77	3.8E-03	1.1E-03	3.31	3.31
Api5	A03	6.30	5.92	1.3E-02	1.7E-02	0.77	-1.30
Atf5	A04	5.97	5.71	1.6E-02	1.9E-02	0.83	-1.20
Bad	A05	8.76	7.19	2.3E-03	6.9E-03	0.34	-2.97
Bag1	A06	4.35	7.74	4.9E-02	4.7E-03	10.48	10.48
Bag3	A07	4.67	4.89	3.9E-02	3.4E-02	1.17	1.17
Bak1	A08	15.33	14.21	2.4E-05	5.3E-05	0.46	-2.17
Bax	A09	5.13	5.88	2.9E-02	1.7E-02	1.68	1.68
Bcl10	A10	8.65	9.38	2.5E-03	1.5E-03	1.66	1.66
Bcl2	A11	8.42	6.41	2.9E-03	1.2E-02	0.25	-4.03
Bcl2l1	A12	4.64	4.36	4.0E-02	4.9E-02	0.83	-1.21
Bcl2l10	B01	12.74	8.17	1.5E-04	3.5E-03	0.04	-23.85
Bcl2l2	B02	8.24	7.36	3.3E-03	6.1E-03	0.54	-1.84
Bid	B03	8.07	7.13	3.7E-03	7.1E-03	0.52	-1.91
Birc1a	B04	10.74	6.34	5.9E-04	1.2E-02	0.05	-21.14
Birc1b	B05	8.10	5.97	3.6E-03	1.6E-02	0.23	-4.39
Birc2	B06	6.69	6.07	9.7E-03	1.5E-02	0.65	-1.53
Birc3	B07	8.10	9.07	3.6E-03	1.9E-03	1.96	1.96
Birc4	B08	6.11	6.71	1.4E-02	9.6E-03	1.51	1.51
Birc5	B09	8.13	8.18	3.6E-03	3.4E-03	1.04	1.04
Bnip2	B10	4.60	4.30	4.1E-02	5.1E-02	0.82	-1.23
Bnip3	B11	4.80	4.21	3.6E-02	5.4E-02	0.66	-1.51
Bnip3l	B12	4.07	3.46	6.0E-02	9.1E-02	0.66	-1.52
Bok	C01	9.00	7.66	1.9E-03	4.9E-03	0.39	-2.53
Card10	C02	7.30	7.87	6.3E-03	4.3E-03	1.48	1.48
Nod1	C03	7.68	10.61	4.9E-03	6.4E-04	7.59	7.59
Card6	C04	8.39	6.72	3.0E-03	9.5E-03	0.31	-3.18
Casp1	C05	8.25	13.38	3.3E-03	9.4E-05	34.99	34.99
Casp12	C06	11.24	9.93	4.1E-04	1.0E-03	0.40	-2.48
Casp14	C07	17.94	13.09	4.0E-06	1.1E-04	0.03	-28.76
Casp2	C08	8.45	12.61	2.9E-03	1.6E-04	17.94	17.94
Casp3	C09	7.03	8.79	7.6E-03	2.3E-03	3.40	3.40
Casp4	C10	10.08	12.71	9.2E-04	1.5E-04	6.19	6.19
Casp6	C11	8.01	5.83	3.9E-03	1.8E-02	0.22	-4.52

Casp7	C12	8.96	11.05	2.0E-03	4.7E-04	**4.27**	**4.27**
Casp8	D01	7.13	11.76	7.2E-03	2.9E-04	**24.85**	**24.85**
Casp9	D02	13.61	12.92	8.0E-05	1.3E-04	0.62	-1.61
Cflar	D03	6.20	5.51	1.4E-02	2.2E-02	0.62	-1.61
Cidea	D04	13.12	12.92	1.1E-04	1.3E-04	0.87	-1.15
Cideb	D05	12.81	12.72	1.4E-04	1.5E-04	0.94	-1.06
Cradd	D06	9.58	7.27	1.3E-03	6.5E-03	**0.20**	**-4.98**
Dad1	D07	4.22	5.03	5.4E-02	3.1E-02	1.76	1.76
Dapk1	D08	7.97	7.00	4.0E-03	7.8E-03	0.51	-1.95
Dffa	D09	6.39	6.48	1.2E-02	1.1E-02	1.07	1.07
Dffb	D10	9.34	7.48	1.5E-03	5.6E-03	**0.28**	**-3.62**
Tsc22d3	D11	2.45	0.98	1.8E-01	5.1E-01	0.36	**-2.77**
Fadd	D12	9.02	8.86	1.9E-03	2.2E-03	0.89	-1.12
Fas	E01	8.74	10.99	2.3E-03	4.9E-04	**4.78**	**4.78**
Fasl	E02	10.75	6.70	5.8E-04	9.6E-03	**0.06**	**-16.56**
Hells	E03	7.45	6.46	5.7E-03	1.1E-02	0.50	-1.99
Il10	E04	11.41	10.88	3.7E-04	5.3E-04	0.69	-1.44
Lhx4	E05	12.32	7.15	2.0E-04	7.1E-03	**0.03**	**-36.05**
Ltbr	E06	7.73	6.73	4.7E-03	9.4E-03	0.50	**-2.01**
Mcl1	E07	12.38	7.45	1.9E-04	5.7E-03	**0.03**	**-30.51**
Nfkb1	E08	7.90	13.86	4.2E-03	6.7E-05	**62.12**	**62.12**
Nme5	E09	10.48	6.37	7.0E-04	1.2E-02	**0.06**	**-17.27**
Nol3	E10	6.09	6.72	1.5E-02	9.5E-03	1.55	1.55
Pak7	E11	10.84	5.99	5.5E-04	1.6E-02	**0.03**	**-28.82**
Pim2	E12	9.14	7.86	1.8E-03	4.3E-03	0.41	**-2.43**
Polb	F01	5.10	4.73	2.9E-02	3.8E-02	0.78	-1.29
Prdx2	F02	9.46	8.34	1.4E-03	3.1E-03	0.46	**-2.17**
Pycard	F03	9.28	6.70	1.6E-03	9.6E-03	**0.17**	**-5.99**
Ripk1	F04	7.34	8.46	6.2E-03	2.8E-03	**2.16**	**2.16**
Rnf7	F05	5.50	5.04	2.2E-02	3.0E-02	0.73	-1.37
Sphk2	F06	17.94	6.05	4.0E-06	1.5E-02	**0.00**	**-3782.17**
Tnf	F07	10.07	7.74	9.3E-04	4.7E-03	**0.20**	**-5.03**
Tnfrsf10b	F08	10.21	12.24	8.5E-04	2.1E-04	**4.09**	**4.09**
Tnfrsf11b	F09	8.64	11.35	2.5E-03	3.8E-04	**6.56**	**6.56**
Tnfrsf1a	F10	5.55	7.44	2.1E-02	5.8E-03	**3.72**	**3.72**
Cd40	F11	9.10	7.48	1.8E-03	5.6E-03	**0.33**	**-3.07**
Tnfsf10	F12	7.35	7.03	6.1E-03	7.7E-03	0.80	-1.26
Tnfsf12	G01	6.77	7.47	9.2E-03	5.6E-03	1.63	1.63
Cd40lg	G02	15.45	17.69	2.2E-05	4.7E-06	**4.72**	**4.72**
Cd70	G03	17.39	13.46	5.8E-06	8.8E-05	**0.07**	**-15.19**
Traf1	G04	9.27	8.08	1.6E-03	3.7E-03	0.44	**-2.27**
Traf2	G05	7.03	7.53	7.7E-03	5.4E-03	1.42	1.42
Traf3	G06	8.01	9.79	3.9E-03	1.1E-03	**3.42**	**3.42**
Trp53	G07	5.88	6.50	1.7E-02	1.1E-02	1.54	1.54
Trp53bp2	G08	8.57	11.31	2.6E-03	4.0E-04	**6.64**	**6.64**
Trp53inp1	G09	5.88	5.20	1.7E-02	2.7E-02	0.62	-1.60

Trp63	G10	9.51	6.73	1.4E-03	9.4E-03	**0.15**	**-6.87**
Trp73	G11	12.54	7.27	1.7E-04	6.5E-03	**0.03**	**-38.56**
Zc3hc1	G12	7.55	6.79	5.3E-03	9.0E-03	0.59	-1.69
Gusb	H01	6.70	8.85	9.6E-03	2.2E-03	**4.43**	**4.43**
Hprt1	H02	4.70	5.73	3.9E-02	1.9E-02	**2.04**	**2.04**
Hsp90ab1	H03	0.75	0.38	6.0E-01	7.7E-01	0.77	-1.29
Gapdh	H04	-1.79	-1.93	3.5E+00	3.8E+00	0.91	-1.10

APPENDIX III

SUPERARRAY REAL-TIME RT-PCR ARRAY GENE TABLE

Position	UniGene	APHS-033 RefSeq	Symbol	Description	Gene Name
A01	Mm.6645	NM_009652	Akt1	Thymoma viral proto-oncogene 1	Akt/PKB
A02	Mm.220289	NM_009684	Apaf1	Apoptotic peptidase activating factor 1	6230400I06Rik/Apaf-1
A03	Mm.181824	NM_007466	Api5	Apoptosis inhibitor 5	AAC-11/AI196452
A04	Mm.389890	NM_030693	Atf5	Activating transcription factor 5	AFTA/Atf7
A05	Mm.4387	NM_007522	Bad	Bcl-associated death promoter	AI325008/Bbc2
A06	Mm.688	NM_009736	Bag1	Bcl2-associated athanogene 1	Rap46
A07	Mm.84073	NM_013863	Bag3	Bcl2-associated athanogene 3	AA407278/Bis
A08	Mm.2443	NM_007523	Bak1	BCL2-antagonist/killer 1	Bak/N-BAK1
A09	Mm.19904	NM_007527	Bax	Bcl2-associated X protein	BAX
A10	Mm.239141	NM_009740	Bcl10	B-cell leukemia/lymphoma 10	AI132454/BCL-10
A11	Mm.257460	NM_009741	Bcl2	B-cell leukemia/lymphoma 2	AW986256/Bcl-2
A12	Mm.238213	NM_009743	Bcl2l1	Bcl2-like 1	Bcl(X)L/Bcl-XL
B01	Mm.25988	NM_013479	Bcl2l10	Bcl2-like 10	AA420380/AU023065
B02	Mm.6967	NM_007537	Bcl2l2	Bcl2-like 2	AW048834/Bcl-w
B03	Mm.235081	NM_007544	Bid	BH3 interacting domain death agonist	2700049M22Rik/AI875481
B04	Mm.6898	NM_008670	Birc1a	Baculoviral IAP repeat-containing 1a	AV364616/D13Lsd1
B05	Mm.89961	NM_010872	Birc1b	Baculoviral IAP repeat-containing 1b	Naip-rs6/Naip2
B06	Mm.335659	NM_007465	Birc2	Baculoviral IAP repeat-containing 2	AW146227/Api1
B07	Mm.2026	NM_007464	Birc3	Baculoviral IAP repeat-containing 3	AW107670/Api1
B08	Mm.259879	NM_009688	Birc4	Baculoviral IAP repeat-containing 4	Aipa/Api3
B09	Mm.8552	NM_009689	Birc5	Baculoviral IAP repeat-containing 5	AAC-11/Api4
B10	Mm.159777	NM_016787	Bnip2	BCL2/adenovirus E1B interacting protein 1, NIP2	5730523P12Rik/BNIP2beta
B11	Mm.378890	NM_009760	Bnip3	BCL2/adenovirus E1B interacting protein 1, NIP3	Nip3
B12	Mm.29820	NM_009761	Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	C86132/D14Ertd719e
C01	Mm.3295	NM_016778	Bok	Bcl-2-related ovarian killer protein	AI847676/AU021146
C02	Mm.17629	NM_130859	Card10	Caspase recruitment	AI449026/Bimp1

				domain family, member 10	
C03	Mm.28498	NM_172729	Nod1	Nucleotide-binding oligomerization domain containing 1	C230079P11/Card4
C04	Mm.31252	XM_139295	Card6	Caspase recruitment domain family, member 6	D730008L15
C05	Mm.1051	NM_009807	Casp1	Caspase 1	ICE/Il1bc
C06	Mm.42163	NM_009808	Casp12	Caspase 12	CASP12
C07	Mm.20940	NM_009809	Casp14	Caspase 14	MICE/mini-ICE
C08	Mm.3921	NM_007610	Casp2	Caspase 2	Caspase-2/ICH-1
C09	Mm.34405	NM_009810	Casp3	Caspase 3	A830040C14Rik/Apopain
C10	Mm.1569	NM_007609	Casp4	Caspase 4, apoptosis-related cysteine peptidase	Casp11/Caspase-11
C11	Mm.281379	NM_009811	Casp6	Caspase 6	Mch2/mCASP-6
C12	Mm.35687	NM_007611	Casp7	Caspase 7	AI314680/CMH-1
D01	Mm.336851	NM_009812	Casp8	Caspase 8	Caspase-8/FLICE
D02	Mm.88829	NM_015733	Casp9	Caspase 9	AI115399/AW493809
D03	Mm.11778	NM_009805	Cflar	CASP8 and FADD-like apoptosis regulator	2310024N18Rik/A430105C05Rik
D04	Mm.449	NM_007702	Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	AW212747
D05	Mm.435554	NM_009894	Cideb	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B	1110030C18Rik/AI790179
D06	Mm.218009	NM_009950	Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	RAIDD
D07	Mm.319038	NM_010015	Dad1	Defender against cell death 1	AI323713
D08	Mm.24103	NM_029653	Dapk1	Death associated protein kinase 1	2310039H24Rik/2810425C21Rik
D09	Mm.41433	NM_010044	Dffa	DNA fragmentation factor, alpha subunit	A330085O09Rik/DFF35
D10	Mm.388918	NM_007859	Dffb	DNA fragmentation factor, beta subunit	40kDa/5730477D02Rik
D11	Mm.22216	NM_010286	Tsc22d3	TSC22 domain family 3	DIP/Dsip1
D12	Mm.5126	NM_010175	Fadd	Fas (TNFRSF6)-associated via death domain	Mort1/FADD
E01	Mm.1626	NM_007987	Fas	Fas (TNF receptor superfamily member)	AI196731/APO-1
E02	Mm.3355	NM_010177	Fasl	Fas ligand (TNF superfamily, member 6)	APT1LG1/CD178
E03	Mm.57223	NM_008234	Hells	Helicase, lymphoid specific	AI323785/E130115I21Rik
E04	Mm.874	NM_010548	Il10	Interleukin 10	CSIF/Il-10
E05	Mm.103624	NM_010712	Lhx4	LIM homeobox protein 4	Gsh-4/Gsh4
E06	Mm.3122	NM_010736	Ltbr	Lymphotoxin B receptor	AI256028/CD18

E07	Mm.1639	NM_008562	Mcl1	Myeloid cell leukemia sequence 1	AW556805/Mcl-1
E08	Mm.256765	NM_008689	Nfkb1	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	NF-KB1/NF-kappaB
E09	Mm.275430	NM_080637	Nme5	Expressed in non-metastatic cells 5	1700019D05Rik
E10	Mm.204876	NM_030152	Nol3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	ARC/B430311C09Rik
E11	Mm.131572	NM_172858	Pak7	P21 (CDKN1A)-activated kinase 7	2900083L08Rik/6430627N20
E12	Mm.347478	NM_145737	Pim2	Proviral integration site 2	DXCch3/Pim-2
F01	Mm.123211	NM_011130	Polb	Polymerase (DNA directed), beta	A430088C08Rik
F02	Mm.347009	NM_011563	Prdx2	Peroxiredoxin 2	AL022839/Band-8
F03	Mm.24163	NM_023258	Pycard	PYD and CARD domain containing	9130417A21Rik/Asc
F04	Mm.374799	NM_009068	Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	D330015H01Rik/RIP
F05	Mm.28235	XM_905787	Rnf7	Ring finger protein 7	Rbx2/SAG
F06	Mm.24222	NM_020011	Sphk2	Sphingosine kinase 2	C76851
F07	Mm.1293	NM_013693	Tnf	Tumor necrosis factor	DIF/TNF-alpha
F08	Mm.193430	NM_020275	Tnfrsf10b	Tumor necrosis factor receptor superfamily, member 10b	DR5/KILLER
F09	Mm.15383	NM_008764	Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	OCIF/Opg
F10	Mm.1258	NM_011609	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	CD120a/FPF
F11	Mm.271833	NM_011611	Cd40	CD40 antigen	AI326936/Bp50
F12	Mm.1062	NM_009425	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	A330042I21Rik/AI448571
G01	Mm.344820	NM_011614	Tnfsf12	Tumor necrosis factor (ligand) superfamily, member 12	APO3L/DR3L
G02	Mm.4861	NM_011616	Cd40lg	CD40 ligand	CD154/Cd40l
G03	Mm.42228	NM_011617	Cd70	CD70 antigen	CD27LG/Cd27l
G04	Mm.239514	NM_009421	Traf1	Tnf receptor-associated factor 1	4732496E14Rik
G05	Mm.3399	NM_009422	Traf2	Tnf receptor-associated factor 2	AI325259
G06	Mm.27431	NM_011632	Traf3	Tnf receptor-associated factor 3	AI528849/CAP-1
G07	Mm.222	NM_011640	Trp53	Transformation related protein 53	bbl/bfy
G08	Mm.287450	NM_173378	Trp53bp2	Transformation related protein 53 binding protein 2	53BP2/AI746547

G09	Mm.393018	NM_021897	Trp53inp1	Transformation related protein 53 inducible nuclear protein 1	2700057G22Rik/SIP
G10	Mm.20894	NM_011641	Trp63	Transformation related protein 63	AI462811/Ket
G11	Mm.78015	NM_011642	Trp73	Transformation related protein 73	p73
G12	Mm.29780	NM_172735	Zc3hc1	Zinc finger, C3HC type 1	1110054L24Rik/AU01 8540
H01	Mm.3317	NM_010368	Gusb	Glucuronidase, beta	AI747421/Gur
H02	Mm.299381	NM_013556	Hprt1	Hypoxanthine guanine phosphoribosyl transferase 1	C81579/HPGRT
H03	Mm.2180	NM_008302	Hsp90ab1	Heat shock protein 90kDa alpha (cytosolic), class B member 1	90kDa/AL022974
H04	Mm.343110	NM_008084	Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Gapd
H05	Mm.328431	NM_007393	Actb	Actin, beta, cytoplasmic	Actx/E430023M04Rik