

**Bone Morphogenetic Proteins Modulate Mast Cell Responses During  
Inflammation**

A Dissertation Presented for the  
Doctor of Philosophy  
Degree  
The University of Tennessee, Knoxville

Jane Seymour Woodrow  
May 2021

Copyright © 2021 by Jane Seymour Woodrow  
All rights reserved.

## **DEDICATION**

This dissertation is dedicated to my parents who have always supported and encouraged me to do anything I set my mind to. I would not be where I am today without them. I love you.

## **ACKNOWLEDGEMENTS**

I want to acknowledge the help from current and previous lab members who have contributed to the data presented here; Dr. Courtney Thomason, Olivia Good, Henry Hoffman, Dr. Federica Serafini, and Dr. Nicole Corder. Their presence and help in the laboratory were greatly appreciated and made the work more enjoyable. Additionally, I'd like to thank my principal investigator, Dr. Elizabeth Lennon for giving me the opportunity to join her laboratory and help grow the research, both in basic science and clinical research. I want to thank the rest of my committee members, Dr. Barry Rouse (Major Professor), Dr. Melissa Hines, and Dr. Sarah Lebeis for their support and encouragement throughout the years, especially during my move to Philadelphia.

I would also like to express my thanks to the group of people who supported my clinical research while at the University of Tennessee. The large animal internal medicine group, including Dr. Carla Sommardahl, Dr. Melissa Hines, and Dr. Karen McCormick supported my project and helped in recruiting samples. The clinical pathology service was also a very critical component in optimizing and performing the techniques related to the cytology evaluations and I would like to specially thank Dr. Bente Flatland, Aimee Hebrard, and Katie Lee for all their help. Collaborators at NCSU-CVM were also instrumental in sharing samples that were critical for the evaluation, thank you to Dr. Katie Sheats and Dr. Kaori Davis. Dr. Paul Wang and Dr. Yancy Lo at UPenn made additional statistical evaluation possible, thank you. The equine asthma research was

funded by the Morris Animal Foundation (D18EQ-830) and Boehringer Ingelheim Vetmedica, Inc. (Advancement in Equine Research Award 2018).

A large thank you goes to the comparative & experimental medicine program for allowing me to join the program and for their support throughout the years. I would like to specially thank Dr. Stephen Kania, Dr. Michael McEntee, and Kimberly Rutherford for their guidance and support, especially during my move from Knoxville to Philadelphia. Their support has meant a lot and has helped me get to the end of my PhD journey.

Lastly, I'd like to thank my family for their unconditional support. When I was struggling to see the path, they helped me find it again and reach my goals.

## **ABSTRACT**

Chronic inflammatory conditions can lead to detrimental health conditions that become difficult to manage and treat. Ultimately treatment requires modulation of the critical cell types involved in pathology in order to achieve homeostasis. Mast cells, a type of innate immune cell located at areas of high antigen load, are commonly thought of as primarily pro-inflammatory, but are becoming increasingly recognized in other roles, such as wound healing, anti-inflammatory actions, and homeostatic functions. In this study we investigate the role of mast cell subtypes and inflammatory mechanisms via *in vitro* assays using mouse and human mast cells, as well as relate to a clinical disease that involves mast cells, equine asthma.

The first part of the dissertation reviews literature regarding mast cell's presence in two chronic inflammatory conditions, asthma and inflammatory bowel disease (IBD). Additionally, I present a potential protective axis in inflammation, mast cell - bone morphogenetic protein (BMP), that was initially discovered in our laboratory in a mouse model of colitis.

In the second part we evaluated the mast cell – BMP axis in relation to modulating mast cell functions. We observed that mast cells produce BMPs in an inflammatory environment and these BMPs are able to modulate mast cell functions in an anti-inflammatory manner. Additionally, BMP signaling in mast cells has the potential to be both anti- and pro-inflammatory depending on the BMP receptor engaged.

In the third part we investigated the mast cell molecular phenotype found in equine asthma in relation to the inflammatory cytokine/chemokine milieu, in addition to anti-inflammatory and pro-fibrotic factors. We observed that horses with mastocytic asthma have different mast cell phenotypes from healthy or other asthma subtypes. Additionally, there is potential dysregulation of BMPs in severe asthma.

Collectively, these studies identified a potential important role for BMPs and their signaling in mast cell functions and regulating inflammation. Increasing the knowledge of the mast cell – BMP axis may be important in multiple chronic inflammatory conditions, such as asthma and IBD, and be a target for therapy.

## TABLE OF CONTENTS

|                                                                            |     |
|----------------------------------------------------------------------------|-----|
| Chapter 1 Background and Overview .....                                    | 1   |
| Introduction to the mast cell .....                                        | 2   |
| Asthma .....                                                               | 14  |
| Overview .....                                                             | 14  |
| Mast cell and Asthma .....                                                 | 20  |
| Inflammatory Bowel Disease .....                                           | 24  |
| Overview .....                                                             | 24  |
| Mast cell presence in IBD.....                                             | 29  |
| Mouse models of colitis .....                                              | 32  |
| Mast cell's role in mouse models of colitis.....                           | 33  |
| Bone Morphogenetic Proteins .....                                          | 36  |
| Overview .....                                                             | 36  |
| Mast cells and BMP signaling .....                                         | 42  |
| Role in Asthma .....                                                       | 43  |
| Role in IBD .....                                                          | 46  |
| Conclusions .....                                                          | 47  |
| LIST OF REFERENCES .....                                                   | 48  |
| APPENDIX .....                                                             | 80  |
| Chapter 2.....                                                             | 86  |
| BMP – Mast cell axis and its modulation of inflammation .....              | 86  |
| Abstract .....                                                             | 87  |
| Introduction .....                                                         | 88  |
| Materials and Methods .....                                                | 91  |
| Results .....                                                              | 97  |
| Discussion .....                                                           | 103 |
| LIST OF REFERENCES .....                                                   | 109 |
| APPENDIX .....                                                             | 115 |
| Chapter 3 Mast cell phenotype and cytokine profiles in equine asthma ..... | 150 |
| Abstract .....                                                             | 151 |
| Introduction .....                                                         | 152 |
| Material and methods .....                                                 | 155 |
| Results .....                                                              | 161 |
| Discussion .....                                                           | 166 |
| LIST OF REFERENCES .....                                                   | 173 |
| APPENDIX .....                                                             | 178 |
| CHAPTER 4 Conclusions and future directions .....                          | 200 |
| Vita .....                                                                 | 204 |

## LIST OF TABLES

|                                                                                   |     |
|-----------------------------------------------------------------------------------|-----|
| Table 1.1. Examples of molecules stored in mast cell granules.....                | 83  |
| Table 1.2. Tgf $\beta$ superfamily signaling molecules. ....                      | 84  |
| Table 1.3. Chemical BMP receptor inhibitors. ....                                 | 85  |
| Table 3.1. Defined experimental groups.....                                       | 179 |
| Table 3.2. Individual horse's clinical data. ....                                 | 180 |
| Table 3.3. Bronchoalveolar lavage fluid cytology results. ....                    | 182 |
| Table 3.4. Bronchoalveolar lavage fluid characteristics for individual horses.... | 183 |
| Table 3.5. Relative gene expression in BALF cell pellets. ....                    | 185 |
| Table 3.6. Normalized cytokine concentrations in BALF supernatant. ....           | 186 |
| Table 3.7. Individual horse's normalized cytokine concentrations. ....            | 187 |
| Table 3.8. Raw cytokine concentrations for each horse.....                        | 188 |
| Table 3.9. Cytokine percent recovery of spiked samples. ....                      | 189 |
| Table 3.9. Cytokine percent recovery of spiked samples. ....                      | 190 |
| Table 3.10. Taqman Gene Expression Assay IDs. ....                                | 191 |

## LIST OF FIGURES

|                                                                                                                                                                 |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 1.1. Isolation of mast cells using a positive selection kit, with subsequent toluidine blue staining.....                                                | 81  |
| Figure 1.2. Antigen dependent IgE signaling. ....                                                                                                               | 82  |
| Figure 2.1. BMP7 pretreated mast cells have normal degranulation.....                                                                                           | 116 |
| Figure 2.2. BMP7 pretreated mast cells significantly decrease TNF $\alpha$ release upon stimulation.....                                                        | 117 |
| Figure 2.3. Mast cells treated for 72 hours with BMP7 have significantly decreased TNF $\alpha$ release upon stimulation. ....                                  | 118 |
| Figure 2.4. Mast cells treated with BMP7 have stable apoptosis and proliferation status.....                                                                    | 119 |
| Figure 2.5. BMP7 pretreatment modulates mast cell phenotype and response to inflammatory stimulus. ....                                                         | 125 |
| Figure 2.6. Mast cells at baseline express BMP7 and BMP receptors. ....                                                                                         | 127 |
| Figure 2.7. Mast cells exposed to inflammatory stimuli upregulate BMP7 production. ....                                                                         | 129 |
| Figure 2.8. Mast cell degranulation is mildly to unaffected by pretreatment with BMP receptor inhibition.....                                                   | 130 |
| Figure 2.9. Acvr1 blockade results in profoundly decreased TNF secretion upon exposure to inflammatory stimuli.....                                             | 132 |
| Figure 2.10. Acvr1 blockade does not result in significantly increased apoptosis. ....                                                                          | 135 |
| Figure 2.11. Proposed model of pathway activation and BMP7 production upon mast cell stimulation. ....                                                          | 139 |
| Figure 2.12. Mast cells stimulated with inflammatory stimuli have an upregulation in phospho-ERK1/2, with BMMCs additionally having upregulation of p-p38 ..... | 140 |
| Figure 2.13. Pretreatment with BMP7 or LDN-193189 decreases ERK1/2 and p38 phosphorylation in BMMCs, not LAD2 cells. ....                                       | 143 |
| Figure 2.14. LAD2 cells <i>de novo</i> synthesizes more TNF $\alpha$ versus BMMCs have more TNF $\alpha$ stored. ....                                           | 146 |
| Figure 2.15. Proposed model for BMP7 and LDN-193189 reduction in TNF $\alpha$ release upon subsequent stimulation. ....                                         | 148 |
| Figure 3.1. Equine airway mast cells decrease chymase expression in mastocytic asthma. ....                                                                     | 192 |
| Figure 3.2. Protease expression in BALF cell pellets, excluding those horses on corticosteroids. ....                                                           | 193 |
| Figure 3.3. Tgf $\beta$ 1 is elevated in mild/moderate asthma and BMP7 is variably expressed in severe asthma. ....                                             | 194 |
| Figure 3.4. Asthmatic horses have elevated pro-inflammatory cytokines. ....                                                                                     | 195 |
| Figure 3.5. Normalized cytokine concentrations of horses, excluding those on corticosteroids. ....                                                              | 196 |
| Figure 3.6. Dilutional linearity of individual cytokines. ....                                                                                                  | 197 |
| Figure 3.7. Correlation data.....                                                                                                                               | 198 |

|                          |     |
|--------------------------|-----|
| Figure 3.8. PCA/MDS..... | 199 |
|--------------------------|-----|

## **ABBREVIATIONS**

|                      |                                                                                          |
|----------------------|------------------------------------------------------------------------------------------|
| CLP                  | Common lymphoid progenitors                                                              |
| CMP                  | Common myeloid progenitors                                                               |
| PRRs                 | Pattern recognition receptors                                                            |
| PAMPs                | Pathogen-associated molecular patterns                                                   |
| BALF                 | Bronchoalveolar lavage fluid                                                             |
| Th1                  | T helper 1 cells (CD4 <sup>+</sup> T cells)                                              |
| Th2                  | T helper 2 cells (CD4 <sup>+</sup> T cells)                                              |
| Th17                 | T helper 17 cells (CD4 <sup>+</sup> T cells)                                             |
| T <sub>reg</sub>     | CD4 <sup>+</sup> T regulatory cells                                                      |
| MC <sub>T</sub>      | Mast cells that contain only tryptase                                                    |
| MC <sub>TC</sub>     | Mast cells that contain tryptase, chymase, and carboxypeptidase A3                       |
| MC <sub>T/CPA3</sub> | Mast cells that contain high levels of tryptase and carboxypeptidase A3, but low chymase |
| IBD                  | Inflammatory bowel disease                                                               |
| CD                   | Crohn's disease                                                                          |
| UC                   | Ulcerative colitis                                                                       |
| BMPs                 | Bone morphogenetic proteins                                                              |
| EAS                  | Equine asthma syndrome                                                                   |
| mEAS                 | Mild/moderate equine asthma syndrome                                                     |
| sEAS                 | Severe equine asthma syndrome                                                            |
| maEAS                | Mastocytic equine asthma syndrome                                                        |

**CHAPTER 1**  
**BACKGROUND AND OVERVIEW**

## **Introduction to the mast cell**

Mast cells are a type of innate immune cell that is responsible for the body's first line of defense. Their location in the body is ideal for reacting promptly against invaders and tissue damage. Mast cells are found most prominently in locations of high antigen load, for example the skin and mucosal linings such as the airways, intestine, and urogenital tract (1-4). They are also in close proximity to blood and lymphatic vessels in addition to neurons, giving mast cell products the ability to travel to or activate signals at distant sites. Mast cell progenitor cells are derived from the hematopoietic stem cells in the bone marrow, circulate in the blood stream, and then migrate to the tissues where they mature (5). Bone marrow-derived hematopoietic stem cells differentiate into common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). CMPs further differentiate into progenitor cells that result in platelets and red blood cells, as well as a branch that give rise to progenitor cells of granulocyte and monocyte origin. These granulocyte progenitor cells are the branches that give rise to mast cell progenitor cells. Additionally, there are reports to support mast cell progenitor cells can develop from progenitor cell that are bipotent and have the ability to develop into mast cells or basophils (6-8). The source of mast cell progenitors has been and continues to be debated, with some reports indicating mast cell progenitors are more similar to megakaryocyte/erythrocyte progenitor versus the granulocyte/monocyte progenitor (9). Transcription factors that appear to be absolutely required for mast cell development are PU.1, for *in vitro* and *in vivo* differentiation, and Signal Transducer and Activator of Transcription 5 (STAT5), for *in vivo* differentiation (8, 10-12). Recently the carbonic anhydrase 1 gene (Car1) has also been shown to be important in mast cell development

and has been suggested to mark hematopoietic progenitor cell population that can give rise to erythrocytes and mast cells (13, 14). Mast cell progenitor cells can be identified by cell surface receptors which vary as they move from the bone marrow to the peripheral tissues where they fully mature. In the bone marrow, mast cell progenitors are identified as lineage<sup>-</sup> (Lin<sup>-</sup>) c-kit<sup>+</sup> Sca-1<sup>-</sup> Ly6c<sup>-</sup> FcεRIα<sup>-</sup> CD27<sup>-</sup> integrinβ7<sup>+</sup> T1/ST2<sup>+</sup> (15). As they are circulating in the blood mast cell progenitors are identified as Lin<sup>-</sup> c-kit<sup>hi</sup> T1/ST2<sup>+</sup> integrinβ7<sup>hi</sup> CD116/32<sup>hi</sup> (16). Finally, in the peripheral tissues they are identified as CD45<sup>+</sup> Lin<sup>-</sup> CD34<sup>+</sup> integrinβ7<sup>hi</sup> FcεRIα<sup>lo</sup>. As mast cells mature in the tissues, heavily influenced by their environment, their expression of FcεRIα increases and they become increasingly granulated (see Figure 1.1). Homing to tissues relies on integrins and chemokine receptors on the mast cell progenitors. Homing to the intestine relies on integrin α4β7 and chemokine receptor Cxcr2 on the mast cell and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on intestinal epithelium (17, 18). Interestingly, the intestine has higher mast cell progenitor capacity than the bone marrow, which is additionally present in germ-free mice (18, 19). Homing to the lung is reliant on α4β7 and α4β1 on the mast cell and VCAM-1 on lung endothelium. The mast cell progenitor pool in the lung is low, but antigen-challenge will increase recruitment of mast cell progenitor cells (20, 21). Stem cell factor (SCF), the ligand for c-kit receptor, is the most important growth factor for mast cell differentiation and retention in the tissues, which is produced by stromal cells (22). In the tissues mast cells are long lived and have various functions and capabilities. SCF additionally aids in adhesion, migration, IL-6 secretion, and enhances the receptor activation of FcεRI, ST2, and TLR4 (23-28). Downstream SCF signaling augments degranulation and cytokine

production by increasing calcium influx and transcriptional activity (29). Additionally, IL-3 is needed in mouse mast cells for differentiation, survival, and responding *in vivo*, but is not required for human mast cells (30-35).

The role(s) of mast cells in homeostasis and disease has been evaluated mostly in mouse models due to the ability to genetically modify them. Humans that lack mast cells do not exist. A majority of the mast cell knockout mouse models, ex. Kit<sup>W/W-v</sup> and Kit<sup>W-sh/W-sh</sup>, are dependent on Kit (the gene for c-kit) mutations that result in the lack of mast cells. More recent mast cell deficient models do not rely on Kit, but rely on other mast cell-specific molecules, such as the proteases they produce in order to modulate their number and production of certain substances. Depending on the model used there may be conflicting conclusions, therefore mast cell specific inhibitors to investigate mast cell function would be the ideal scenarios, but there are none that are entirely specific to mast cell actions and/or products. Mast cells are traditionally known for their involvement in allergic reactions, with the most severe form being anaphylaxis, although due to their many products and cell-to-cell interactions, mast cells have been identified in a growing number of homeostatic functions and disease pathology.

Paul Ehrlich in the nineteenth century first identified mast cells by their cytoplasmic granules and referred to them as ‘mastung’, German for ‘well-fed’ (36). The cytoplasmic granules contain many different preformed compounds, most notably mast cell-specific proteases, biogenic amines, and tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) (see Table 1.1). Release of the compounds, or degranulation, can be caused by many different external stimuli, such as IgE receptor crosslinking, toxins, complement activation, and neuropeptides (4, 37). Following degranulation the cytoplasmic granules can be re-

granulated, therefore the process does not result in mast cell death (38-41). Upon mast cell activation *de novo* synthesized molecules can be produced, such as leukotrienes, prostaglandins, and various cytokines and chemokines. Mast cell degranulation does not require the entire contents of the granule to be released but can occur through selective exocytosis. Mast cells can also release compounds without degranulation, such as the case with LPS stimulation and specific cytokine release (37). The content of the granules was one of the original way mast cells were distinguished in to two different types, along with their anatomical location. Rodent connective tissue-type mast cells (CTMCs) have a serglycin proteoglycan core protein with attached heparin glycosaminoglycan-chains, where mucosal mast cells (MMC)s have a serglycin proteoglycan core protein with attached chondroitin sulfate chains (42, 43). As the identification of the cytoplasmic content of these mast cells has further progressed, subtyping or phenotyping mast cells have been refined.

Mast cell protease cytoplasmic content has additionally been used to subtype, or phenotype, mast cells into mucosal and connective tissue sub-types in humans because human lung mast cells contained both heparin and chondroitin sulfate (44). The three mast cell proteases used to subtype are tryptase, chymase, and carboxypeptidase A3. Human mucosal mast cells are primarily tryptase containing, where connective tissue mast cells have a mixture of all three proteases; tryptase, chymase, and carboxypeptidase A3 (45). This sub-classification has also been used in other species, such as rodents, and can vary slightly based on homologs to human tryptase and chymase. Mouse mucosal mast cells are noted to primarily contain mast cell protease (mcpt)-1, and -2 (chymases) and mcpt-6 (tryptase); where connective tissue mast cells

contain mast cell protease-4 (chymase), mcpt-6, and -7 (tryptase), and carboxypeptidase A3 (Cpa3) (1, 46, 47). Human tryptase analogs in mice are mcpt-6, and -7; where chymase analogs are mcpt-1, -2, and -4 (48, 49). The contents of the mast cell cytoplasm are heavily influenced by its environment; therefore, the subtype of mast cell can change during disease, even in mature differentiated mast cells, and can have mixed types in a specific tissue (50). The ability to analyze large data sets, including transcriptional profiles has advanced our understanding of cell types. Mouse mast cells, for example, are a transcriptionally distinct cell type and only share one common transcription factor, GATA2, with basophils and also, not unexpectedly, the genes that encode for the  $\alpha$  and  $\beta$  chain of the high affinity IgE receptor (51). Additionally, basophils were found to express the gene for Cpa3; therefore, may not be a protease solely expressed by mast cells. Underappreciated previously is also the mast cell's expression of a broad array of Mrgpr-family members; five in total, with one Mrgprb2 being the homolog to the human Mrgprx2. In the future there is likely to be more refined subtypes of mast cells based on the expression of these receptors, along with protease expression.

The most traditional response described in mast cells is IgE binding via Fc $\epsilon$ RI (the high affinity IgE receptor), with subsequent antigen-IgE crosslinking leading to degranulation. This response is key to type I hypersensitivity response, clinically manifested as anaphylaxis, hives, asthma, and food allergy, for example, and it is well accepted that mast cells are critical to its development (52, 53). Fc $\epsilon$ RI is a high affinity IgE receptor that is present on mast cells and basophils as a tetrameric structure. The structure is composed of one alpha subunit that binds IgE, one beta subunit that spans the membrane, and two gamma subunits (54). The beta and gamma chains contain

immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails. The ITAMS are phosphorylated by cytoplasmic Src family tyrosine kinase (Fyn, Lyn) and Syk (55-58). The kinases themselves are activated by receptor aggregation that occurs upon binding IgE and antigen. IgE-crosslinking via antigen leads ultimately to activation of multiple pathways, for example phosphoinositide 3-kinases (PI3K), phospholipase C/ $\text{Ca}^{2+}$ , and MAPK that can lead to degranulation, cytokine gene transcription, and generation of eicosanoids (see Figure 1.2) (2, 59). Activation of the IgE signaling pathway can be activated to different degrees in the absence of antigen cross-linking (60). Variations of IgE antigen-independent activation are dependent on the type of IgE bound. Highly activating IgE are termed 'highly cytokinergic' and low activating IgE is termed 'poorly cytokinergic' (61). A highly cytokinergic IgE used commonly *in vitro* is IgE-anti-DNP (SPE7 clone) and its high activation is attributed to the ability to make a tri-molecular complex (62). The complex is made of a free IgE cross-linking two-bound IgE. The highly cytokinergic IgE can lead to upregulation of receptor internalization, histamine content, histamine and leukotriene release, IL-6 production, adhesion, migration, DNA synthesis, and survival, similar to IgE antigen-dependent activation (12, 61, 63-81).

In addition to the receptor for IgE, they also have the receptor for IgG. The receptors for IgG, including Fc $\gamma$ RI, Fc $\gamma$ RIIA, and Fc $\gamma$ RIII, are variably expressed on mouse and human mast cells with their activation leading to signaling pathways similar to IgE-antigen activation, such as phosphorylation of Fyn, Lyn, Akt, Erk, p38, and JNK (82-84). The functional role for mast cell IgG receptor activation is not clear and requires further exploration, but some possible roles have been identified. IgG1/Fc $\gamma$ RIII

activation of IL4 primed mouse mast cells can induce the release of TNF $\alpha$ , IL10, and VEGF (85-87). Additionally, IFN $\gamma$  treated human mast cells and Fc $\gamma$ RI aggregation can induce histamine release (88). Besides Fc receptors and the classical IgE-antigen activation, mast cells have several other types of receptors ensuring they are key first-line defenders.

Being an innate immune cell, mast cells can respond rapidly to invaders and cellular damage by use of their vast number of pattern recognition receptors (PRR). PRRs can recognize both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). The most well described PRRs are Toll-like receptors (TLRs), but others include NOD-like receptors, C-type lectin-like receptors, and RIG-I-like receptors. TLRs that have been recognized on mast cells are TLR1-13 (89). TLR2 recognizes the Gram-positive bacterial product peptidoglycan and in mast cells its activation leads to degranulation and the release of products, such as histamine (90, 91). TLR4 is most classically studied in its response to Gram-negative bacterial component lipopolysaccharide (LPS), but it also recognizes other molecules such as Gram-positive teichuronic acid, viral components, and endogenous molecules such as heat shock proteins, to name a few (92-94). Mast cell TLR4 activation typically leads to the secretion of TNF $\alpha$ , IL-1, and IL-6 without degranulation (95, 96). Recognition of PAMPs or DAMPs via mast cell PRRs with subsequent activation enables mast cells to protect the host via direct mechanisms and by indirect mechanisms in which mast cell actions support the immune response and lead to activation of the adaptive immune response. Mechanisms in which mast cells can aid in the recruitment and activation of innate immune cells is via histamine, protease, and TNF $\alpha$  release. In

bacterial peritonitis models, mast cells are necessary for host survival and neutrophil recruitment (97-99). Additionally, release of histamine and proteases aid in general cellular recruitment via vasodilation and vascular leak (100-102).

Depending on the host insult, the adaptive immune response may be required for full protection of the host. The adaptive immune response is characterized by a humoral response, B cell activation and immunoglobulin production; and cellular response, characterized by CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells. Antigen presentation via MHCI on all nucleated cells can activate CD8<sup>+</sup> T cells independent of the aid of other cells. Activation of CD4<sup>+</sup> T cells, also referred to as CD4<sup>+</sup> T helper cells, requires antigen presentation via MHCII by antigen presenting cells (APC). The most classically described APC is the dendritic cell. Mast cells can influence DC maturation, migration, and function (37, 103, 104). Migration of dendritic cells (DCs) to the draining lymph nodes, where naïve T cells home to and initial presentation of antigen occurs, is critical for initiation of the adaptive immune response and mast cells promote the migration in part by their release of TNF $\alpha$  (105-108). Histamine can also influence DC chemotaxis, cytokine secretion, up-regulation of MHCII and co-stimulatory molecules; as well as aid in DC polarization of Th2 immune response (109-112). There is further support for mast cells and their products, such as PGD<sub>2</sub> and PGE<sub>2</sub>, in influencing DCs and a Th2 polarization (113-116). An *in vivo* model took advantage of labeling mast cell granules and investigated the movement of the granules in respect to modulating DCs (117). In this they saw that the granules released by mast cells were taken up by dermal DCs and they were taken to the associated skin-draining lymph node. Additionally, the granules taken up by the DCs were able to promote DC maturation and migration to the lymph node, again partially

attributed to mast cell-derived TNF $\alpha$ . Mast cell-derived products therefore have many roles and ability to modulate other immune cells.

Mast cells can aid in activation of the adaptive immune response via indirect manners, such as previously mentioned support of DC migration, but also via direct mechanisms. Mast cells have the ability to present antigen on their cell surface via MHCI and MHCII in order to activate CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells, respectively (118). Mast cells have been noted to act as APCs in mouse, rat, and human derived mast cells (119-122). It has been further proposed that mast cells are “tissue-localized” APCs that present to effector/memory CD4<sup>+</sup> T cells and can modulate effector functions by secretion of cytokines, such as IL-6 and TNF $\alpha$  (123). In addition to cytokine secretion, mast cells can upregulate co-stimulatory molecules critical for cell-to-cell contact, such as ICOS ligand, PD-L1, PD-L2, OX40L, CD153, Fas, and 4-1BB (124). The combination of TNF $\alpha$  and OX40L is noted to be especially important in CD4<sup>+</sup> T cell activation and OX40L can be upregulated on mast cells via TLR and Fc $\epsilon$ RI engagement. Besides activation and recruitment of CD4<sup>+</sup> T cells, secretion of chemokines, such as CCL5, upon TLR activation can aid in CD8<sup>+</sup> T cell recruitment (125). A model of murine cytomegalovirus infection supports the findings that activated mast cells can recruit CD8<sup>+</sup> T cells via CCL5 release (126). An additional mast cell product, leukotriene B<sub>4</sub>, is also able to recruit CD8<sup>+</sup> T cells in allergy models (127). Chemokine-induced recruitment is not the only mechanism mast cells utilize to modulate CD8<sup>+</sup> T cells. Mast cells are also able to process bacterial antigens from live bacteria and present to CD8<sup>+</sup> T cells via MHC I (128). *In vivo* models of experimental autoimmune encephalitis (EAE)

and Leishmania infection utilizing mast cell deficient mice support mast cell recruitment of T cells and note defective CD4<sup>+</sup> and CD8<sup>+</sup> T cell numbers (118, 129, 130).

In addition to the mast cell's immune responses and aid in initiation of the adaptive immune response, mast cells are also involved in dampening the immune response to return to homeostasis. Mast cells have been shown to be critical for supporting regulatory CD4<sup>+</sup> T cell (T regs) tolerance. Mast cells are protective and support T regs in graft versus host disease and nephrotoxic serum nephritis (131, 132). In both those models it has been suggested that T regs recruit mast cells to their location via the release of IL9 and this recruitment is critical to support T reg numbers. Moreover in a model of papain-induced allergic inflammation mast cells again were shown to support T reg numbers and proliferation via IL33-stimulated mast cell production of IL2 (133). Additional *in vitro* and *in vivo* studies support that in an environment of LPS, mast cells support regulatory T cell numbers and functions via cell-to-cell interactions via MHC II and ICOSL/ICOS mechanisms (134, 135). Besides supporting T regs in an environment of LPS, mast cells appear to aid in regulating cytokine production in response to LPS. In an acute exposure to LPS, mast cells appear to be important in regulating the level of systemic inflammation evidence by aiding in dampening IL6 production, perhaps via production of SOCS-1, an intracellular protein involved in negative feedback loop of IL6 (136). Besides cell-to-cell interactions, the production of anti-inflammatory cytokines is important in regulating immune responses. Two important anti-inflammatory cytokines are Tgf $\beta$  and IL10. Tgf $\beta$  is especially important in T cell differentiation (T regs and Th17), dendritic cell tolerance, and downregulation of mast cell inflammatory products (137-139). IL10 can inhibit the

production of inflammatory cytokines, chemokines, and proliferation of T cells (140-142). Mast cells have been shown to be sources of Tgf $\beta$  and IL10; therefore, the mast cell's ability to downregulate inflammation is not unwarranted (4, 85, 103, 143-145). Mast cell-derived IL10 was shown to reduce pathology of contact hypersensitivity and chronic UVB irradiation models in mice (85, 146). Further, Tgf $\beta$  and IL10 production by mast cells are important in their prevention of graft-host-disease (147, 148). In addition to IL10 and Tgf $\beta$ , IL33-stimulated mast cells are able to help drive differentiation and function of macrophage that are capable of suppressing T cell functions in a model of experimental autoimmune encephalitis (149). The mast cell's ability to do this was associated with its production of IL6 and IL13. This supports that mast cells can have many different roles that can vary depending on the tissue environment to be pro- or anti-inflammatory.

TNF $\alpha$  is a primary cytokine noted in inflammatory conditions and mast cells can be a source of it subsequent to multiple pro-inflammatory stimuli and subsequently activated pathways. As previously mentioned, LPS recognition via TLR4 can result in TNF $\alpha$  release. The production of TNF $\alpha$  in the mast cells is believed to be mainly MyD88 dependent, which is thought to not require receptor internalization in mast cells, although recently receptor internalization has been noted to be required in some instances (150, 151). TLR4 signaling can lead to NF $\kappa$ B and MAPK pathway activation in mast cells. The step in which the signal bifurcates is the activation of the complex made up of Tgfb-activated kinase 1 (TAK1) and TAK1-binding (TAB) proteins. At this bifurcation MAPKs or the inhibitor of nuclear factor- $\kappa$ B-kinase (IKK) complex are activated. IKK's role is crucial in activating NF $\kappa$ B by phosphorylating I $\kappa$ B $\alpha$ , which is

then degraded and allows for phosphorylation of NFκB complex and movement to the nucleus to modulate gene transcription, including TNFα. Activation of MAPK pathways results in AP-1 complex activation, which modulates gene transcription of TNFα. NFκB-dependent TNFα production can also be augmented by PI3K activation in mast cells (152). MAPK activation in mast cells leads to p38 and ERK1/2 activation, which results in TNFα production (153). Further support for both NFκB and p38 activation leading to TNFα production in the mast cell is that both are required to be down-regulated for endotolerance (154). Interestingly TAK1 is a signaling protein involved in both TLR4 and FcεR-mediated mast cell stimulation. The use of a TAK1 inhibitor resulted in reduced cell signaling pathways, IκBα and p38, in both LPS and IgE-antigen stimulation, emphasizing cell signaling pathways are often more complicated with potential cross-talk and sharing of cytoplasmic signaling proteins (154, 155).

Mast cells are in areas of high antigen-load, and near vasculature and nerve endings making them adept at responding to and/or releasing molecules that can go systemically via the help of the vasculature or “talk” with neighboring nerve endings (156, 157). A molecule released by nerve endings that can be sensed by mast cells is substance P (SP) and it is elevated in inflammatory conditions (158, 159). SP can be recognized by mast cells via neurokinin-1 receptor (NK-1R) or Mas-related G-protein coupled receptors (Mrgpr's) Mrgprx2 (Mrgprb2 mouse homolog), and can result in TNFα production, as well as degranulation (160-166). Both of these receptors are G protein-coupled receptors. G protein-coupled receptor activation can lead to NFκB, PI3K, and MAPK activation (167, 168). NK-1 receptor expression on immune cells is broad, including lymphoid and myeloid cells, while Mrgprx(b)2 is only found on mast

cells. Mrgprx2 expression on mast cells is also variable, with human skin mast cells and synovial mast cells having the highest expression and lung mast cells not expressing the receptor (169). NK-1R has the highest affinity for SP, but can bind additional tachykinins such as neurokinin A and neurokinin B (170). Mrgprx2 binds SP, but can additionally bind molecules such as major basic protein, eosinophil peroxidase, and opioids including morphine and hydrocodone (171, 172).

Two locations of high antigen load where mast cells can be found are the lungs and gastrointestinal tract. As described, the role of the mast cell can be multifunctional, therefore increased knowledge of the mast cell's capabilities in these locations is important for understanding disease pathology and treatment options. Two diseases of chronic inflammation in which mast cells are players in the pathology are asthma and inflammatory bowel disease.

## **Asthma**

### ***Overview***

Airway inflammation involves the activation of inflammatory cells and production of inflammatory mediators, which with chronicity can lead to the development of asthma. Asthma is a disease characterized by airway inflammation, enhanced bronchial reactivity, and reversible airflow obstruction. Asthma had been previously thought of as two broad categories, allergic asthma (Th2 high) versus non-allergic asthma (non-Th2), although now this has been accepted to be more complicated. Asthma is a disease that both humans and horses can naturally develop, with some similarities between the two. Recently the human asthma research literature has emphasized further describing asthma by their specific endotypes and phenotypes. Endotype refers to the mechanistic pathway resulting in asthma, and phenotype refers

to the varying clinical presentations that can occur in asthma (173). Asthma is a heterogenous group of affected patients, which hinders targeted therapy, hence why the current emphases of describing endotype and phenotype in human asthma in attempts to better identify effective treatments in individual patients.

The two broad endotypes in human asthma are termed type 2 (T2)-high (allergic asthma) and non-T2 (non-allergic asthma), with each having multiple phenotypes. T2 high endotype and its phenotypes are specified as: atopic, late onset, and aspirin-exacerbated respiratory disease (AERD). Non-T2 endotype and its phenotypes are specified as: Non-atopic, smokers, obesity related, and elderly. The pathophysiology of the T2-high endotype encompasses involvement of alarmins, Th2 cytokines, eosinophils, mast cells, basophils, IgE, and PGD<sub>2</sub>. Asthmatics have reduced barrier integrity with a loss of E-cadherin and claudin-18, which allows for the introduction of allergens and microbes to deeper stromal tissues (174, 175). The airway epithelial cells themselves can recognize intruders and damage via PRRs that results in the production of alarmins. In the case of type 2 immune response, the alarmins most commonly identified are thymic stromal lymphopoietin (TSLP), IL25, and IL33. IL25 and IL33 can activate innate lymphoid cells (ILC)-2, while TSLP can help to prime DCs to further polarize a type 2 immune response. ILC2's activated by alarmins further promote type 2 immune response by producing IL5 and IL13, in addition to that produced by activated Th2 CD4<sup>+</sup> T cells (176, 177). The type 2 cytokines, IL13 and IL4 promote goblet cells and the production of mucus, as well as airway hyperresponsiveness. IL4 is additionally able to induce Th2 cells and B-cell isotype switching to IgE. IL5 promotes the differentiation and maturation of eosinophil progenitors, in addition to supporting mast cells and

basophils (178). In human asthma, eosinophils are the main cell type associated with type 2 asthma. Stimulated eosinophils can release a large array of products, such as IL5 and IL13 that support type 2 immune responses, eotaxins that recruit more eosinophils, cysteinyl leukotrienes, and cytotoxic proteins such as major basic protein and eosinophil peroxidase (179). Eosinophils and their products are involved in airway remodeling and airway smooth muscle contractility (180, 181). Sputum eosinophil evaluation is considered the “gold standard” type 2 biomarker, with a level >3% noted in human eosinophilia asthma. Additionally, in humans, severe asthma is classified by poor to no response to treatment and eosinophilic airway inflammation makes up about 40-60% of these patients (182). Mast cells and basophils express the high affinity IgE receptor, therefore activation via this mechanism can lead to various products, such as histamine, prostaglandins, leukotrienes, type 2 cytokines, and mast cell specific proteases. Mast cell’s role in asthma will be discussed later in greater detail. Serum IgE is measured in human asthmatics as an indicator of atopy, or allergy. In addition to IgE activating mast cells and basophils, airway smooth muscle cells express the high affinity receptor; therefore IgE may be involved in airway remodeling (183). Although classically asthma is thought of as a type 2 immune response, this T2 inflammation is only observed in about half of individuals with asthma and about a third of those with severe asthma (184, 185).

Non-T2, or T2-low asthma, is less understood, but is characterized by neutrophilic (sputum neutrophils >40-60%) or paucigranulocytic (normal sputum neutrophil and eosinophil %) inflammation. The recruitment and maintenance of neutrophilic inflammation is not understood and non-T2 asthma is also noted to be less

responsive to corticosteroids. The immune response appears to be a Th1 and/or Th17 response. A Th1-high signature is the increased production of IFN $\gamma$ , which is seen in about 50% of individuals with severe asthma. IFN $\gamma$  has also been suggested to be involved in increasing airway hyper-responsiveness, which may result in patients being unresponsive to corticosteroids (186, 187). In addition to Th1 inflammation, Th17 inflammation can sometimes be a component of asthma typified by the production of IL17A and IL22. Th17 cytokines can increase smooth muscle cell proliferation and collagen deposition (188). IL17 production is additionally noted to lead to CXCL8 production by airway epithelial cells leading to neutrophil recruitment; therefore this may be a component of neutrophilic inflammation (189). The pathophysiology of these human asthma endotypes and phenotypes, particularly non-T2 asthma is unclear and continues to be studied in order to best treat individual patients.

Equine and human asthma have similarities; including the pathology of both includes reversible airflow obstruction caused by bronchospasms, increased mucus production, airway hyper-responsiveness, and pulmonary remodeling (190, 191). Equine asthma syndrome encompasses two subtypes, severe asthma (or previously known as recurrent airway obstruction), and mild to moderate asthma (or previously known as inflammatory airway disease) (192). The cause of equine asthma is multifactorial as it is in humans, including environmental conditions, feeding substrates, housing, season, preventative medicine practices, distribution of infectious agents, and genetic influences. Severe asthma is typically seen in horses >7-years-old with marked lower airway inflammation and obstruction, frequent coughing, and increased respiratory effort at rest. Mild/moderate asthma affects horses of all ages and clinical signs are

usually not seen at rest. A diagnosis of equine asthma is commonly based on history, clinical signs of lower airway inflammation, and bronchoalveolar lavage fluid (BALF) cytology consisting of >25% neutrophils for severe asthma or >10% neutrophils, >5% mast cells, and/or >5% eosinophils for mild/moderate asthma (192). Cell types found in BALF from healthy horses are <10-5% neutrophils, <1% eosinophils, and <2% metachromatic cells with primarily macrophages and lymphocytes, although there are no discrete reference ranges for macrophages or lymphocytes (192). Severe asthma in horses is thought to be similar to human non-T2 asthma, while mild/moderate asthma is similar to T2 asthma. Further definition of asthma patients, such as phenotype and endotype seen in human asthma is not well understood in equine asthma, although warrants further investigation.

The exact pathophysiology resulting in T2 asthma and non-T2 asthma has not been fully elucidated in horses or humans, but is seen as an allergic reaction versus an environmental, air pollution, ozone or viral exposure, respectively (193). Both human and equine allergic type asthma are thought to involve a Th2 immune response, but this is not always straightforward and both allergic and non-allergic human/equine asthma cases can include a Th1 and/or Th17 immune response (194-196). The cytokine production found in BALF is also thought to reflect the predominant immune response, although a discrete Th1 versus Th2 immune response is not uniformly found in all horses evaluated (197-199). Diagnosis of asthma in humans consists of a history or current respiratory signs and symptoms consistent with asthma, as well as objective observation of airflow obstruction via airway function tests (200). Airway function tests are less available for diagnosing equine asthma due to costs, ease of use of the

equipment, and required knowledge for analysis. Cytological evaluation of the BALF can be used to confirm an increase in inflammatory cells such as eosinophils or neutrophils in human or equine asthma (201). In human medicine though the use of induced sputum has taken the place of BALs and the cellular content is comparable (202, 203). Performing a BAL with cytological evaluation is standard in equine asthma diagnosis, although with the currently defined cellular parameters for mild/moderate and severe asthma, the groups are very heterogenous. Heterogenous group definitions likely affect the ability to properly treat and manage each equine patient. Diagnostics in human asthma also include the use of biomarkers, such as serum IgE (specific or total), eosinophil and neutrophil count, to better categorize the patient's endotype, which may influence treatment.

Treatment, in both humans and horses, includes managing the airway inflammation and increasing bronchodilation to ease breathing. Avoiding the inciting antigen which causes signs of asthma can be done by modulating the environment and is highly relied upon in managing equine asthma, although as one might suspect, can be very hard to manage due to the environment horses live in. In addition to antigen avoidance, medications can be utilized. Physicians have more tools to pull from versus veterinarians in regard to treatment due to costs and availability. Treatment of human asthma includes a stepping up strategy, with assessment of clinical signs, diagnostics, and patient adherence to treatment as guidelines for stepping up treatment (204). Initial treatment includes the use of daily, low dose inhaled corticosteroids to decrease inflammation, with the as-needed use of short-acting beta<sub>2</sub>-agonists for bronchodilation. More severe symptoms may require an increase in corticosteroid dose

and/or switch to oral dosages, and the use of long-acting beta<sub>2</sub>-agonists. Corticosteroid and bronchodilator treatments are used in equine asthma therapy, although the additional use of biologics used in human asthma is not. For example, physicians have the option of adding on anti-IgE, anti-IL5, anti-IL5R $\alpha$ , and/or anti-IL4R $\alpha$ ; but as the targets indicate, are intended for T<sub>2</sub>-asthma (205, 206). Biologics targeted for non-T<sub>2</sub> asthma are not currently available and most likely will not be developed until non-T<sub>2</sub> pathophysiology is further understood. Human and equine asthma similarities may prove beneficial in applying research findings across species and support using the horse as a human model of naturally occurring asthma. The translational research may aid in advancing the diagnosis and treatment of asthma in a quicker timeline.

### ***Mast cell and Asthma***

Mast cells play a role in acute and chronic human asthma, but their role has not been clearly defined (207). Both eosinophilic (T<sub>2</sub>-asthma) and non-eosinophilic (non-T<sub>2</sub> asthma) asthma share a common feature in which airway smooth muscle is infiltrated by activated mast cells (208, 209). There is also evidence of activated mast cells in the airway mucosa releasing mediators such as histamine, TNF $\alpha$ , PGD<sub>2</sub>, IL4, and IL13 that contribute to asthma pathology, such as promoting mucous production, smooth muscle cell proliferation and hypertrophy (210, 211). Even with evidence of mast cells involvement in asthma, they are low to non-detectable percentages, 0.25%-3% of total cells, in individuals with asthma (201, 202, 212, 213). The ease of obtaining human flow cytometry reagents has aided in better identification of mast cells in sputum versus cytological evaluation. A recent study did this technique and reported increased sputum mast cells in humans with asthma that positively correlated with sputum eosinophils

and basophils (214). Sputum eosinophil numbers correlate with peripheral blood counts, but sputum mast cell numbers, most likely due to their long-term tissue residence, are not reflected in periphery counts. Sputum neutrophil counts are also not reflected in peripheral blood counts (215). This emphasizes the need to study mast cells *in situ* or from the primary location of interest. In human medicine, they recognize two mast cell subtypes based on their neutral serine proteases; 1) cells that contain tryptase as well as chymase and carboxypeptidase A<sub>3</sub> (MC<sub>TC</sub>), and 2) cells that contain tryptase only (MC<sub>T</sub>) (216, 217). MC<sub>T</sub> are typically located at mucosal surfaces such as the nasal and bronchial epithelium in rhinitis and asthma patients, and are in the bronchial lamina propria of both healthy and disease-affected humans (217, 218). The MC<sub>TC</sub> group is typically located in the connective tissue of normal human skin and airway smooth muscle bundles in asthma (216, 217, 219). Therefore, in a healthy individual the majority of mast cells in the lung should be MC<sub>T</sub> with less than 20% MC<sub>TC</sub>.

Human asthmatic patients have an increased number of MC<sub>TC</sub>, and those numbers increase with asthma severity (220). Identifying mast cells in BALF or induced sputum is limited by standard immunohistochemistry (IHC) approach because airway luminal mast cells are rarely detectable in induced sputum of humans with asthma (221, 222). Mast cell subtypes therefore have been identified recently using real-time quantitative (q) PCR. Utilizing this technique another mast cell subtype was identified in epithelial brushings and endobronchial biopsies of human asthmatic patients that had high protease levels of tryptase and carboxypeptidase A<sub>3</sub>, but low chymase (MC<sub>T/CPA<sub>3</sub></sub>). This is different from the typical mast cell subtype found in asthmatic patients that contain high concentrations of all 3 proteases, tryptase, chymase and carboxypeptidase

A3 (MC<sub>TC</sub>). The MC<sub>T/CPA3</sub> subtype was found to be increased in patients with Th2-high asthma and it predicted responsiveness to inhaled corticosteroids (223). This new subtype, which is associated with airway eosinophilia and greater corticosteroid responsiveness, was also identified in induced sputum of human asthmatic patients (224, 225). These findings show that mast cell subtype correlates to disease phenotype, response to therapy, and can be identified in induced sputum. It was already known that eosinophils in induced sputum correlated to greater response to corticosteroids, but this was the first link to mast cell subtype (226). Utilizing the new technique of molecular phenotyping of mast cells by gene expression of mast cell proteases, we are able to better characterize and understand the role of the mast cell in asthma.

The equine mast cell has been under-investigated and most of the information is in reference to mastocytosis (increase mast cell population) or mast cell tumors. Mast cell research across species is further defining its role and importance in innate immunity, adaptive immune response, graft versus host disease, helminth infection, inflammatory bowel disease and asthma (131, 132, 227-229). Mast cell subtypes and location are not as well defined in the horse as they are in the human. A study in 2005, investigated lung biopsy samples via IHC of healthy versus severe asthma affected horses. This study identified that in healthy horses the wall of lung bronchi and bronchioles consist mainly of chymase positive mast cells, while around blood vessels there are mainly tryptase positive mast cells (230). No double stained mast cells were seen; meaning the mast cells only had one protease, although carboxypeptidase A3 was not stained for. In comparison to healthy horses, severely asthmatic horses were found to have more chymase positive mast cells in the walls of bronchi. A 2007 study

investigated tryptase expression and production in BALF of healthy versus severely asthmatic horses when naturally challenged and found tryptase production was higher in severely asthmatic horses and it correlated to neutrophil numbers in BALF (231). Further, IHC of lung biopsy samples found a trend for an increase in the tryptase positive mast cells in bronchial and bronchiolar epithelium of severe asthma affected horses versus healthy. Also reported was a significant increase in total airway epithelial mast cells in severely asthmatic horses versus healthy, showing mast cells appear to be recruited to the airways of asthmatic horses and are activated. A downfall of this study was no other mast cell proteases, besides tryptase, were investigated. Identifying mast cells in patients of any species also runs the risks of false negatives when using protein staining of cytoplasmic granules, because as discussed earlier, mast cells can degranulate, therefore resulting in no stain uptake. Unfortunately, these findings do not paint a clear picture as to what mast cell subtypes horses have and how asthma affects the subtypes seen, but there are similarities to the human studies in which mast cell subtypes are influenced by asthma. As noted before mast cells are rarely detectable in human induced sputum but have been identified using molecular phenotyping. Therefore, it is possible to use this technique in routinely acquired BALF for equine asthma diagnosis.

Mast cells are potential therapeutic targets and have been evaluated via different mechanisms. Mast cell stabilizers have been used in racehorses with mild to moderate asthma with improvement in clinical signs and bronchial hyper-responsiveness, although the drug's effects are not only directed to mast cells as the name claims (232). A new therapy that targets mast cells is the tyrosine kinase inhibitor imatinib, which

targets c-kit, a receptor found on the cell surface of mast cells. Most mature immune cells, with the exception of innate lymphoid cells-2 (ILC2), lack c-kit, making it more mast cell specific. This therapy has been effective in the treatment of corticosteroid-refractory severe asthma in human patients and could be a treatment consideration for equine asthma patients in the future (233). However, the role of mast cells and the specific mediators involved in equine asthma needs further characterization. Additionally, improved definitions of equine asthma subtype may prove beneficial in diagnosing, treating, and long-term management.

## **Inflammatory Bowel Disease**

### ***Overview***

Inflammatory Bowel Disease (IBD) is an incurable, chronic inflammatory disorder of the gastrointestinal tract whose etiology continues to be unclear but affects individuals worldwide. Current therapeutic approaches to IBD are focused on systemic immunosuppression, but they are variably effective, do not result in a cure and often cause severe negative side effects, including secondary infections and increased risk of malignancy (234-236). The two main subtypes of IBD are Crohn's Disease (CD) and Ulcerative Colitis (UC). The pathology in CD is typically located to the ileum and the proximal colon, but it can affect any region of the intestine, where UC pathology starts in the rectum and extends proximally. Other differences include CD inflammation is usually transmural and UC is restricted to the mucosa. Each patient requires tailored therapies due to variable remission rates and complications such as neoplasia, strictures, fistulae, and abscesses. Preventing disease, predicting relapse, and preventing complications is limited. Genome wide association studies have identified around 100 genetic risk loci shared between the two main subtypes of IBD, CD and UC (237, 238).

Concordance rates in monozygotic twins were found to be 35% for CD and 16% for UC, indicating a stronger genetic influence in CD, but also indicating there are additional causative factors in the development of disease, such as environmental and microbiome influences (239). Nucleotide-binding oligomerization domain containing 2 (NOD2) was the first susceptibility gene identified in CD. Dendritic cells (DCs) from CD patients with NOD2 susceptibility variants were found to be less able to localize bacteria in to autophagolysosomes and had reduced autophagy, meaning the DCs were less able to rid the body of bacteria and immune inducing antigens (240). Additionally, early-onset IBD is associated with loss-of-function defects in IL10 or its receptor. IL10 is an important cytokine for immune homeostasis and all loss-of-function defects in it or its receptor leads to pathology similar to IBD (241, 242). While some gene mutations and polymorphisms have stronger associations than others with the development of IBD, they cannot explain all cases of IBD or pathology. There are other influences on the genome, such as epigenetics and microRNAs (miRNAs). Deciphering the exact cause of this multifactorial disease is complicated, but it highlights the need for individualized treatment and continued research into the disease pathogenesis.

IBD patients have variable decreased barrier function, increased sensing, and response to antigen that goes unchecked compared to healthy individuals (243). Over exuberant inflammatory response to luminal antigens, such as lipopolysaccharide (LPS), a gram-negative bacterial wall molecule, are part of the initiation and propagation of the disease. Chronic or increased load of antigen leads to increased pro-inflammatory cytokines and chemokine production. Cytokine of most importance and targeted for therapy is TNF $\alpha$ . TNF $\alpha$  is potentially made by various immune cells, such as T

lymphocytes, macrophage/monocytes, mast cells, other granulocytes, as well as fibroblasts and other non-immune cells (244). The production of TNF $\alpha$  can be incited by antigens such as LPS, but also by bacteria, virus, tumor cells, immune complexes, cellular damage due to radiation or even stress (245). TNF $\alpha$  can act through two receptors, TNF receptor-1 found on all nucleated cells, and TNF receptor-2 primarily found on endothelial and hematopoietic cells (246, 247). Activation and release of TNF $\alpha$  results in pro-inflammatory states due to it activating other cell types, resulting in the release of other pro-inflammatory cytokines such as IL6 and IL1 $\beta$ . Further effects of TNF $\alpha$  are expression of adhesion molecules and recruitment of immune cells, proliferation of fibroblasts, activation of coagulation and fibrinolysis, and inhibition of apoptosis (248, 249). *In vitro* work has supported the role of TNF $\alpha$  in recruitment of immune cells, such as neutrophils, increased epithelial permeability, and the formation of granulomas (250-252). Levels of TNF $\alpha$  are additionally noted to be elevated in IBD patients, both in the cells of the intestine and feces, but are less clearly elevated in the serum (253-257). While pro-inflammatory cytokines, such as TNF $\alpha$ , are elevated in IBD patients, but the exact cause or start of the inflammation is not clear.

Genetically susceptible individuals appear to have dysregulated immune responses to intestinal commensal microbiota, which leads to chronic inflammation (258). Studies have reported significantly elevated circulating endotoxin levels in active disease versus inactive disease, as well as versus healthy controls (259, 260). In response to LPS innate immune cells recognize the antigen via pattern recognition receptors, specifically TLR4, and produce molecules in response to and/or present antigen to adaptive immune cells. TLR4 polymorphisms have also been identified as

susceptibility factors of IBD, although their functions are not clear, it further supports a role of LPS-TLR4 signaling in the pathology of IBD (261). In addition to over-exuberant immune responses to microbiota and antigens, IBD patients are noted to have composition changes in their microbiome compared to healthy control microbiome; therefore, manipulation of the microbiome has become a therapeutic target.

Other treatment options include targets of immune suppression which are the most commonly used therapeutics and can have negative side-effects such as increased susceptibility to infections and cancer development, as well as variable remission rates. Corticosteroids are the most common immune suppressant used, first reported to be effective in UC in 1954 (262). Corticosteroids reduce TNF $\alpha$ , IL1 $\beta$ , type I interferon production, and regulate T helper cell differentiation in order to aid in decreasing IBD pathology (263). Other immunosuppressive therapy includes the use of cyclosporine A, methotrexate, and thiopurines. With the development of biologic therapy, anti-TNF $\alpha$  targets have become popular. These anti-TNF $\alpha$  agents have been shown to be beneficial in IBD therapy in several studies and are targeted to TNF $\alpha$  itself, or its receptors (264-268). Anti-TNF $\alpha$  agents were first approved by the FDA for use in CD in 1998 and since then there have been different formulations. Anti-TNF $\alpha$  therapy is thought to help by blocking the TNF $\alpha$  effects and appear to primarily distribute to tissues of high inflammation (246, 269). Examples of anti-TNF $\alpha$  actions are reduced cytokine production, induction of apoptosis in activated T lymphocytes, antibody-dependent cell mediated cytotoxicity, and complement-dependent cytotoxicity (246, 270-272). In general the development of biologics have been beneficial to IBD treatment, but do come with downfalls and negative side-effects (273). A major concern in anti-TNF $\alpha$

treatment is loss of response. Primary non-responders are patients that never respond to anti-TNF $\alpha$  therapy and this happens in up to 30% of patients (274-276). Additionally, secondary non-responders will occur in about 50% of those that initially did respond, requiring dose escalation or switching to a different anti-TNF $\alpha$  therapy. Possible explanations for the non-responders may be due to variations in metabolism and production of antidrug antibodies. This leads to the need for dosage escalation, switch to new anti-TNF $\alpha$  agent, and multidrug therapy, which requires close patient monitoring and treatment tailoring. Due to multifactorial causes of IBD, additional treatment options are always needed. The development of new therapeutics though is reliant on understanding the pathogenesis of the disease, therefore continued investigation of the cells and their role(s) are important.

Chronic inflammation, such as in IBD, is a continuous cycle brought upon by constant activation of T lymphocytes. There are several types of T lymphocytes, including two broad types, CD4<sup>+</sup> helper T cells and CD8<sup>+</sup> cytotoxic T cells. The main pathogenic cell in IBD is currently considered to be a subtype of CD4<sup>+</sup> helper T cell named Th17 (277). This is supported by increased cell numbers and related cytokine, IL17, from inflamed intestinal tissue from IBD patients compared to normal tissue (278, 279). In order to regulate the adaptive immune response and limit T lymphocyte activation, regulatory T cells are imperative. Regulatory T cell numbers have been shown in the periphery of IBD patients to be decreased in active disease versus non-active, but proliferation of T regs within the lamina propria does occur (280, 281). The T regs in the lamina propria also appear to be functional via *in vitro* suppression of effector T cells. Therefore, it has been proposed that the ratio of regulatory T cells to

effector T cells is the important factor and not the total number of T regs for decreased inflammatory functions. A clinical study using antigen-specific regulatory T cell therapy in CD patients with refractory disease has supported the theory that reduced ratio of T regs to effector T cells results in inflammation and increasing the T reg compartment is beneficial (282). Regulatory T cells can help decrease immune activation in all cell types by cell-to-cell interactions and the production of anti-inflammatory cytokines, such as IL10 and Tgf $\beta$ . Regulatory T cell numbers is also supported my mast cells, as previously discussed.

Regulatory T cells secrete anti-inflammatory molecules such as IL-10 and Tgf $\beta$ , which decrease inflammation by decreasing the ability of APCs to stimulate effector T cells, as well as support their own T reg proliferation. LPS and IFN $\gamma$  induce MHCII expression on mast cells, which are then able to present antigen to antigen-specific regulatory T cells with subsequent expansion (135). Mast cells are also able to activate naïve CD4<sup>+</sup> T cells. Mast cells exposed to LPS and IgE for 6 weeks caused TLR4 and NF $\kappa$ B activation that led to the induction of regulatory T cells from a population of naïve CD4<sup>+</sup> T cells. The induction was shown to be dependent on ICOSL/ICOS interaction on the mast cell and T cell, respectively (134). Mast cells and regulatory T cells interact and support each other's anti-inflammatory state, therefore investigating the role of the mast cell in IBD may lead to targets for modulation of regulatory T cell numbers or other anti-inflammatory mechanisms.

### ***Mast cell presence in IBD***

Early investigation of the pathophysiology of the major subtypes of IBD, including the presence of mast cells, involved microscopy-imaging studies. In the

healthy human, mast cells are normally found in the intestines where they are 2-5% of the mononuclear cells in the lamina propria (283). The mucosal mast cells are reported to be primarily of the MC<sub>T</sub> subtype, 98%, and the submucosal mast cells are primarily MC<sub>TC</sub> subtype, 77% (216, 284). Human biopsy samples from CD patients noted mast cell numbers were markedly increased, along with evidence of degranulation as detected using transmission electron microscopy (285). Further investigations have supported an increased number of mast cells in IBD patients versus healthy controls (286-288), although some investigators have found opposing results in other studies that demonstrated no difference or decreased mast cell numbers (289-292). Using protease-labeling techniques, chronic UC patients have increased numbers of tryptase-positive mast cells compared to controls and have increased mast cell counts in active disease versus remission (293, 294). Variation in mast cell numbers across reports is likely in part due to the method of staining. Mast cell degranulation can lead to falsely decreased reported numbers, therefore when staining for mast cells this needs to be taken into consideration.

To further investigate mast cell's role in IBD, evaluation of mast cell-derived products has been evaluated. A key pro-inflammatory cytokine in IBD is TNF $\alpha$  and mast cells are a potential source, although other immune cells are capable of producing it as well. Mast cells in the human intestine express TNF $\alpha$  and increase its production in the presence of IgE-crosslinking, exposure to LPS, and bacteria (295). Further evaluation of TNF $\alpha$ -positive mast cells in CD patients has shown increased mast cell numbers in addition to increased TNF $\alpha$ -positive mast cells during disease, suggesting a role in the disease process (296). In support of the mast cell's involvement in the disease, other

mast cell–derived products, such as histamine is noted to be increased in active CD and UC (297-300). Additionally, urinary excretion of the histamine metabolite N-methylhistamine is increased in IBD patients versus healthy controls (301). Spontaneous secretion of tryptase, a mast cell or basophil-derived product, is also found to be increased in UC (302). Mast cells isolated from ulcerative colitis biopsy samples and further stimulated with IgE actively secrete increased amounts of histamine, leukotrienes, and prostaglandins when isolated from inflamed regions versus un-inflamed regions of the intestine (303). While mast cell number and inflammatory mediators are noted to be increased both *ex vivo* and *in vivo*, mast cells do not solely produce inflammatory mediators, but also have the potential to secrete anti-inflammatory mediators and interact with cells via cell surface proteins to regulate immune responses. A lone paper ventured to hypothesize a suppressive role for the mast cell and noted that mast cell numbers were increased at the border of active UC (304). Unfortunately, this finding was not further clarified, but is suggestive the mast cell may have an accelerating or decelerating role in the inflammatory process.

Investigating the status of the mast cell during IBD treatments suggests that decreased numbers are beneficial, but the treatments in general are downregulating the immune response, therefore they are not specific. Corticosteroids are noted to decrease mast cell numbers in IBD, but there was no correlation between mast cell number and the severity of inflammation noted on histological analysis of biopsy samples (305). Further analysis of the mast cell's role in IBD has transitioned to mouse models to better understand the pathophysiology.

### ***Mouse models of colitis***

Mouse models of colitis can assess acute and chronic stages of inflammation, innate versus adaptive immune responses, and genetic influence depending on the model used. Common models used include chemically induced colitis models or genetically modified mice that are susceptible to colitis (306, 307). Each model has their pros and cons, as well as ability to assess certain physiological mechanisms of the disease. Chemically induced models of colitis using dextran sodium sulfate (DSS) or 2, 4, 6-tri-nitro-benzene-sulfonic acid (TNBS) are common and primarily assess barrier function and acute inflammation. Chemically induced models harness the ability of the chemical administered to damage the intestinal barrier and subsequently lead to intestinal inflammation. Chemically induced models can be modified to partially resemble chronic inflammation by decreasing the chemical dosage and giving multiple rounds, although the mice can still recover. The benefits of the chemically induced models are they have a shorter timeline and therefore can generate data quicker. On the other hand, these models are not spontaneous nor reach a chronic inflammatory stage, which is the mechanism in IBD. Models such as IL10<sup>-/-</sup> and CD4<sup>+</sup> CD45RB<sup>hi</sup> SCID transfer colitis further evaluate the adaptive immune response and specific genes of interest. Cell transfer models use genetically modified mice that lack adaptive immunity that can then receive lymphocytes of interest give the researcher the ability to investigate particular aspects of the lymphocyte subsets. Genetically modified mice allow for deletions, insertion, or up-regulation of genes of interest and genome wide association studies in human IBD patients have identified potential genes of interest that are associated with an increased risk of developing IBD. The ability to genetically

modify mice then gives the researcher the ability to investigate the role of a specific gene in pathology, either in a whole mouse or conditional model. The IL10<sup>-/-</sup> model for example is especially translatable because the colitis pathology spontaneously develops and resembles aspects of actual IBD pathology. Early onset-IBD is also associated with loss of function mutations in the IL10 gene or IL10 receptor gene (308).

### ***Mast cell's role in mouse models of colitis***

A majority of the research evaluating the role of the mast cell in colitis has entailed using chemically induced mouse models of colitis, but has also evolved to use of genetically modified mice (309, 310). TNBS-treated rats are reported to have increased mast cell density and DSS-treated rats are noted to have increased numbers of eosinophils and mast cells versus controls, but a cause and effect was not established (311, 312). Mast cells store and *de novo* synthesize many molecules of various functions. Common molecules evaluated in colitis models have been proteases such as tryptase and chymase, and histamine. Mouse tryptase, mast cell protease (MCP)-6, was found to exacerbate DSS and TNBS-induced colitis, supported by MCP-6 knockout mice having improved colitis (313). Evaluation of mast cell degranulation products in the systemic blood system showed that in rat DSS colitis, rat mast cell protease (RMCP)-I and RMCP-II were elevated, suggesting mast cell proliferation and/or increased degranulation (314). Mast cell proteases cleave proteins into their active form and peritoneal cell cultures from chymase-deficient mice have decreased conversion of proMMP- to MMP-9 compared to wild type (315). Additionally MMP-9 deficient mice had significantly decreased DSS-induced colitis and formation of active MMP-9 has been attributed to chymase activity in colitis models (316, 317).

An increase in mast cell-derived products in the acute time period has been associated with worsened colitis pathology, but increased mast cell numbers and their molecules over a chronic period may be protective. Mast cell numbers and RMCPII have been seen to remain elevated for up to 20 days after DSS treatment (318). This elevation suggests a benefit of mast cell presence in the recovery time. Support of the mast cell's benefit in recovery and healing is suggested to be associated with mast cell breakdown of IL33 in a MCP-4 dependent manner (319). Additional support for the mast cell's role in healing from colitis damage was suggested to be attributed to mast cell dependent IL33/ST2 signaling; therefore, IL33 may be involved in multiple signaling pathways actions of the mast cell (320). Mast cells are additionally linked to maintaining intestinal barrier functions.

The intestinal barrier defends the host against the contents of the intestinal lumen and its permeability can increase with stress and inflammation. Tryptase is involved in barrier integrity by activating protease-activated receptor (PAR) on basolateral and apical membranes of the intestinal epithelial cells resulting in tight junction disruption and increase in permeability (321-325). Mast cells have also been attributed to regulating basal intestinal epithelial migration and barrier functions in a chymase/MCPT4-dependent manner activation of PAR2 and MMP-2 (326, 327). Intestinal barrier permeability is increased during colitis and can be decreased with use of treatments such as dexamethasone or doxantrazole. These treatments decrease the general inflammatory state and cytokines, in addition to down-regulating mast cell protease production; therefore again they are not mast cell specific (328). This highlights the dichotomy of mast cell functions in which an over exuberant or acute

reaction mast cell products can be detrimental, but the same products are necessary for basal functions. Mast cell's role in homeostatic functions in barrier functions is supported in colitis-prone mice that additionally lack mast cells have increased intestinal permeability and reconstitution with mast cells is beneficial (229, 329, 330).

Using the IL10<sup>-/-</sup> mouse as a model of IBD is supported by the finding that anti-TNF $\alpha$  antibody treatment is beneficial, as it is in IBD patients, and mutations in IL10 or its receptor are associated with early-onset IBD (331, 332). Additionally an immunosuppressive agent, everolimus, has a therapeutic effect in this model; likely as a result of decreased CD4<sup>+</sup> T cell infiltrate and IFN $\gamma$  production (333). Evaluating the immunological changes in this mouse model prior to spontaneous development of colitis showed that there is a reduction in the frequency of CD4<sup>+</sup> T regulatory cells and a higher frequency of activated T cells in the colon, as well as increased IL17 and IFN $\gamma$  production in the colon (334). Severity of the colitis has been noted to be dependent on the genetic background of the mouse and housing environment and appears to be most mild in C57Bl/6 mice in specific-pathogen free (SPF) housing (335). The cytokine IL10 is known to decrease production of inflammatory cytokines and inhibit Th1 cell development, therefore its lack would naturally be thought to lead to chronic inflammation (336, 337). IL10<sup>-/-</sup> mice on C57Bl/6 background in SPF housing are noted to have proximal colitis, characterized by mucin depletion, hyper-regenerative or degenerative lesions of the intestinal epithelia, abnormal crypt and villus structures, and lymphoplasmocytic and histiocytic infiltration of the lamina propria (338). Confirming acute colitis mechanisms in a more physiological and pertinent chronic colitis, such as the IL10<sup>-/-</sup>, aids in applying findings to humans with IBD.

Our group, as well as others, has shown that mast cells are protective in a mouse model of spontaneous, chronic colitis using the IL10<sup>-/-</sup> mouse (229, 329, 330). The IL10<sup>-/-</sup> mouse can be bred with a mast cell deficient Kit<sup>W-sh/W-sh</sup> mouse (also referred to as Sash), which results in a mast cell-deficient mouse with spontaneous colitis. Although the mechanism of protection is not clearly defined, further experimentation using reconstitution techniques showed the protective mechanism is independent of IL10. The presence of mast cells may lead to the production of protective molecule(s) and, or cell-to-cell interactions resulting in manipulation of the immune response in an anti-inflammatory manner. Further investigation by our laboratory showed that bone morphogenetic protein (BMP)-2, -4, -6, & -7 were significantly decreased in mast cell deficient colitis mice and BMP-4, -6, & 7 levels were restored to baseline when mast cell reconstitution was performed, suggesting the mast cell as a source or manipulator of BMP production. This finding indicated BMPs as a candidate molecule aiding in the mast cell's protective capability in a model of spontaneous, chronic colitis. This has therefore led to further investigation of this mast cell-BMP axis by our laboratory, such as do mast cells produce BMPs and how do BMPs impact mast cell functions.

## **Bone Morphogenetic Proteins**

### ***Overview***

Bone morphogenetic proteins (BMPs) are a group of 20 different secreted proteins in the Tgfβ superfamily, which also includes Tgfβ and activin, that induce a signal through heterotetrameric receptor complexes (see Table 1.2) (339). BMPs were first identified in its role to induce ectopic bone formation, but are also involved in embryonic development, tissue healing, and regulation of innate and adaptive immune cells (340, 341). BMP-2, -4, -8, and -10 appear to be expressed at the earliest stages of

embryogenesis, as those homozygous knockouts are embryonically lethal (342-345). BMPs are synthesized as large precursors, 400-500 amino acids, which are cleaved by serine endoproteases to their bioactive form of 50-100 amino acids (346-348). BMPs can form homodimers or heterodimers, where heterodimers are noted to activate signaling pathways at a greater degree than their homodimers; for example: BMP2/5, BMP2/6, BMP2/7, and BMP4/7 (347, 349, 350). There are 2 types of BMP receptors based on sequence homology (351). Type 1 receptors include: activin A receptor type I (ACVR1), (also known as activin receptor-like kinase-2 or ALK2); BMP receptor type IA (BMPRIa), (also known as ALK3); ACVRL1, (also known as ALK1); and BMPRIb, (also known as ALK6). Type 2 receptors include: BMPRII, ActRII, and ActRIIb. The receptors are serine/threonine kinase receptors, which are made up of a short extracellular domain, a single trans-membrane domain, and intracellular domain containing the serine/threonine kinase. Both type 1 and type 2 receptors are required for transducing a signal. The type 2 receptor has been shown to phosphorylate the type 1 receptor, which ultimately propagates the signal via the phosphorylation of a group of Smad proteins (referred to as canonical signaling) (352, 353). Smad is a term created by the combination of *C. elegans* *Sma* genes and the *Drosophila* *Mad* genes. Tgf $\beta$  and activin use structurally similar type 1 and type 2 receptor kinases that BMPs utilize. Tgf $\beta$  and activin use type 1 receptors ALK4, ALK5, or ALK7, in combination with type 2 receptors Tgf $\beta$ RII, ActRII, or ActRIIb. Additionally, the Smad proteins they engage are also different. Tgf $\beta$  and activin engage Smad-2/3, while BMPs engage Smad-1,5,8/9 and both require a co-Smad, Smad4.

Specific BMP ligands are noted to engage type 1 versus type 2 receptors first, and then recruit the opposite receptor. For example, BMP-6 and -7 interact with type 2 and then recruit type 1; and BMP-2 and -4 bind to type 1 first and then recruit type 2 (354). Although there has been some information contradicting receptor affinity preference, specifically BMP7 was reported to have equal affinity for type 1 and type 2 receptors (355). The receptor location at the cell surface, its movement, and endocytosis is poorly understood. There is some information to suggest the receptors may be segregated in clathrin coated pits and detergent resistant membranes, which may also lead to Smad-dependent or Smad-independent pathway activation depending on receptor location (347, 356-358). Further work is required to fully understand receptor dynamics and mechanisms.

The ligand-bound receptor complex leads to canonical signaling pathway via phosphorylating Smad-1, -5, & -8/9; this phosphorylated complex associates with Smad-4, which is then translocated to the nucleus to regulate transcription of target genes (359-361). Inhibitor Smad proteins, specifically Smad7 that inhibits both BMP and activin/Tgf $\beta$  signaling, can regulate Smad signaling where Smad6 prefers to block BMP signaling (362). In addition to canonical signaling, Smad-independent, or non-canonical signaling includes activation of several pathways, including PI3K/Akt, ERK, p38, c-Jun, JNK, and NF $\kappa$ B (363, 364). Smad-independent pathway activation is thought to include other signaling proteins such as BRAM1 (Bone morphogenetic protein Receptor Associated Molecule 1), XIAP (X-linked inhibitor of apoptosis protein), TAK1 (TGF-beta activated kinase 1), and TAB1 (TAK1 binding protein) (365-368). Downstream from TAK1 includes activation of p38, JNK, and NF $\kappa$ B; therefore BMP

signaling is more complicated than pure Smad signaling pathways and its subsequent targets (368-370).

Controlling BMP signaling includes inhibitory Smad proteins, as well as BMP ligand and receptor antagonists. There are soluble antagonists that can inhibit ligand/receptor interaction prior to cell signaling activation (371-374). In vertebrates, the structurally distinct classes of inhibitor proteins are twisted gastrulation (TSG), chordin, noggin, and the DAN family. The DAN family includes proteins such as gremlin and sclerostin (375-377). Specific soluble BMP antagonists have also been noted to have a higher affinity for certain ligands, for example noggin and chordin prefer BMP-2, and -4; and sclerostin prefers BMP-6, and -7 (371, 373). Chemical inhibitors have also been discovered and developed as an additional tool to evaluate both Tgf $\beta$  and BMP signaling. Chemical inhibitors targeting the Tgf $\beta$  signaling pathway target receptors ALK4, 5, and 7, while BMP signaling blockade involves inhibition of ALK2, 3, and 6. One of the first chemical inhibitors found to block BMP receptor signaling was dorsomorphin (378). Dorsomorphin and its derivative LDN-193189 have slight differences, including affinities for the receptors and off-target effects (379). In the use of dorsomorphin or LDN, the lowest possible concentration to block BMP signaling is recommended in attempt to decrease off-target effects, such as inhibiting other protein kinases, such as MAPK's; although in light that BMP signaling includes multiple non-canonical pathways, these off-target effects may not be true (380-382). Effects, such as inhibiting VEGF and AMPK are potentially "truer" off-target effects that are more attributed to dorsomorphin versus LDN-193189 (381, 383). There are additional BMP

and Tgf $\beta$  receptor chemical inhibitors that can be utilized in the attempt to understand the cell-signaling pathway (see Table 1.3) (384).

BMP signaling may additionally have cross talk with other signaling pathways, such as the NF- $\kappa$ B signaling. It has been suggested that the p65-subunit of the NF- $\kappa$ B complex may bind to Smad1/4 complex (385). Additionally, BMP signaling and TLR4 activation may be linked via signaling proteins TAK1, a signaling protein previously described in mast cell signaling pathways, and ECSIT (evolutionary conserved signaling intermediate in Toll pathways). TAK1 and ECSIT are key signaling proteins involved in TLR4 signaling and NF- $\kappa$ B activation (386). ECSIT is also important in BMP signaling in mouse embryogenesis, and further TLR signaling in ECSIT knockdowns are noted to have decreased Smad-1,5,8/9 phosphorylation following TLR3 stimulation of connective tissue progenitor cells (387, 388). Further investigation of BMP signaling in immune cells is likely to identify further cross talk or sharing of signaling proteins that will increase our understanding and functions of BMP signaling in immune cells.

The critical importance of BMP signaling in development and basal physiological needs is apparent in their mouse global knock out models. Several of the BMP ligand, receptors, and signaling protein knockouts are embryonically lethal or die shortly after birth. BMP-2 and -4 global knockouts are embryonically lethal, and BMP-1, -7, and -11 global knockouts die shortly after birth (342, 389-391). BMP receptor global knockouts, BMPR1a, Acvr1, and BMPR2 are embryonically lethal, and Acvr2b die shortly after birth (392-395). The cell signaling proteins, Smad-1, -4, -5, and -7, global knockouts are embryonically lethal, while Smad8 is dispensable (396-400).

BMPs and their signaling are becoming increasingly noted to be involved in regulating immune cell populations and actions. BMP ligand and receptor expression is appreciated in the human thymus and BMP4 treatment inhibits T cell differentiation, which supports a role for BMPs in T cell differentiation (401). Cellular proliferation of naïve CD4<sup>+</sup> T cells following activation is inhibited when BMP signaling is blocked, identifying an additional role for BMPs in T cell activation and homeostasis (402, 403). BMP signaling is also indicated to be involved in specific CD4<sup>+</sup> lineages. Using chemical BMP receptor inhibitors, CD4<sup>+</sup> T cell activation under polarizing conditions resulted in significantly decreased Th17 and T reg differentiation, while Th2 polarization remained the same (404). Inhibition of Th17 cells may be linked to signaling via Bmpr1a, where cells that are deficient of this receptor primarily differentiation into IL17-producing CD4<sup>+</sup> T cell (405). BMP ligands and receptors are also indicated in T regulatory cell differentiation. BMP-2 and -4 are noted to have an additive effect with Tgfβ in inducing T regs, which may be linked to Bmpr1a signaling as its inactivation specifically in CD4<sup>+</sup> T cells led to impaired generation of T regs in mice (406, 407). While there is a clear role for BMP signaling in T cell differentiation, additional cell types can also be modulated by BMPs.

BMPs appear to also have a role in driving alternatively activated M2-macrophage phenotype. The M2 phenotype is appears to be specifically associated with BMP7. BMP7 treatment has been seen to polarize M2 macrophage and decrease inflammatory products *in vitro* and *in vivo*, which appears to be attributed to Smad-PI3K-Akt-mTOR pathway and Bmpr1a receptor expression via *in vitro* analysis (408-411). BMP ligands can have different actions; such as BMP6 treated macrophage have

increased inducible nitric oxide synthase and TNF $\alpha$  (412). It is therefore important to investigate several BMP ligand – immune cell interactions because they can vary in their actions.

Dendritic cells (DC) and B cells are also influenced by BMP signaling. Tgf $\beta$  is known to drive Langerhan cell differentiation, but BMP7 is also increased in niches of Langerhan cells and BMP7-deficient mice have reduced Langerhan cell numbers; therefore there may be a role for BMP7 aiding in Tgf $\beta$ -DC effects (413). Human dendritic cells have been reported to produce BMP4 that increases with maturation, and maturation in the face of BMP inhibition resulted in decreased PD-L1 and PD-L2 expression, reducing their ability to modulate other cells (414). Lastly, B cells express BMP receptors and BMP6 treatment induces Smad-1,5,8/9 phosphorylation which has an anti-proliferative effect in naïve and memory B cells (415).

Specific BMP ligand/receptor/signaling in immune cell populations therefore pose a possible interesting area for modulation of immune cell differentiation and function, which could be harnessed for a desired effect. Key questions that remain to be clarified are what BMP signaling pathways, canonical versus non-canonical, are these BMPs effecting in these immune populations and are the downstream effects different based on cell type, BMP ligand, and receptor engaged.

### ***Mast cells and BMP signaling***

Mast cell – BMP interactions are minimally understood. Treatment with BMP4 was found to increase mast cell responsiveness to IgE activation and BMP2 was noted to increase mast cell survival (416). Evaluation of BMP receptors on mouse bone marrow derived mast cells found expression of several type 1 and type 2 receptors (417). Type 1

receptors detected were: Acvr2, Acvr1 (Alk2), Bmpr1a (ALK3), Acvr1b, Tgf $\beta$ r1, and Acvr1c; while Bmpr1b (ALK6) was not detected. Type 2 receptors detected were: Actr2a, Actr2b, Bmpr2, and Tgfbr2. Additionally, overexpression of Acvr1 in mast cells is noted to be pro-inflammatory in models of heterotopic ossification (HO) and fibrodysplasia ossificans progressive (FOP), where specifically mast cell production of TNF $\alpha$ , IL1 $\beta$ , and IL6 are increased (417). Bmpr2 gene expression has been additionally identified as a mast cell signature gene when compared to basophils; therefore potentially suggesting BMP signaling via Bmpr2 is more important in mast cells versus basophils (51). Further investigation of mast cell - BMP signaling is likely to identify additional roles that may be important in chronic inflammatory conditions.

### ***Role in Asthma***

Bone morphogenetic proteins (BMPs) are ligands in the Tgf $\beta$  superfamily and are critically involved in embryogenesis, including lung development. Lung development regulation by BMP4 and BMP's Smad signaling pathway is thought to occur via crosstalk with the Wnt signaling pathway. Genetic deletion of Smad1 leads to disruption of lung branching morphogenesis and eventual neonatal respiratory failure (418). Additionally, Smad8 is required for proper pulmonary vascular remodeling (419). Of the several BMP receptors, specifically deleting Bmpr1a leads to abnormalities of lung branching morphogenesis, airway formation, and neonatal respiratory distress (420, 421). BMP7 also appears to be involved in lung branching morphogenesis and is most highly expressed in the basement membrane (422). This highlights the importance of BMP molecules and their signaling pathway for lung tissue development and homeostasis.

Lung tissue homeostasis is reliant on BMPs and their signaling; therefore, their status in lung inflammatory conditions is of importance for understanding the disease pathology and treatment options. A chronic inflammatory condition of the lung that has similar pathophysiology in humans and horses is asthma. In both species the disease pathology is characterized by reversible airflow obstruction caused by bronchospasms, increased mucus production, airway hyper-responsiveness, and pulmonary remodeling (190, 191). In addition to BMPs being involved in lung morphology and vascular remodeling, BMP4 is able to inhibit fibroblast proliferation and BMP7 is anti-fibrotic (423). BMP7 has been shown to inhibit Tgf $\beta$ 1-induced epithelial-mesenchymal transition, which is associated with decreased expression of collagen type 1 in fibroblasts and lung myofibroblasts (424-426). In support of these individual findings, a study found BMP4 reduced Tgf $\beta$ 1-induced ECM production and BMP7 inhibited Tgf $\beta$ 1 induced myofibroblast-like transformation in normal human lung fibroblasts (427). In models of bleomycin-induced pulmonary fibrosis, Tgf $\beta$ -Smad2/3 signaling is pro-fibrotic, where BMP-Smad1,5,8/9 is anti-fibrotic (428, 429). Remodeling and thickening of the airways are characteristics of asthma and collagen production is one of the components of this thickening, therefore BMP ligands and signaling may be altered in asthma (430).

There has been limited research in the area of asthma and BMP signaling, although significant differences have been found. Bronchial biopsies from asthmatic patients and controls showed that BMP receptor expression is downregulated in asthma and allergen challenge in asthmatics increases BMP7, BMP canonical signaling, and BMP receptor expression (431). Down-regulation during asthma may impede repair and

allergen challenge may lead to increases in attempts to repair and return to homeostasis. In a mouse model of asthma, BMP7 treatment was found to be beneficial, supported by reduced lung inflammation and collagen type 1 production (432). A rhesus macaque model of allergic airway disease noted that *Bmpr1a* expression was not significantly different with the induction of disease, but BMP canonical signaling was significantly decreased with disease (433). Additionally, during recovery both *Bmpr1a* and BMP canonical signaling were significantly elevated, suggesting a role for BMP signaling for maintaining lung homeostasis during asthma. Smad-1,5,8/9 signaling in asthmatic samples supports that in human asthmatics, lung fibroblasts are more responsive to Tgf $\beta$  and hence transitioning to myofibroblasts due to a dysregulation of smad signaling where Smad-2/3 is upregulated and Smad-1,5,8/9 is downregulated; which supports the dichotomy in Smad signaling in pro- anti-fibrotic effects (434).

Additional support for BMP signaling importance in asthma is in the expression level of known BMP inhibitors. For example, a BMP signaling antagonist, Follistatin-like 1 (Fstl1), has been shown to be significantly elevated in asthmatic patients versus healthy control (435). Further, Fstl1 levels are associated with increased smooth muscle mass and basement membrane thickening in asthmatics (436). A mouse model of asthma was used to investigate the possible mechanisms for these findings and deciphered that macrophage/myeloid cells produce Fstl1 which leads to Oncostatin M (OSM) production (437). Administration of Fstl1 to these mice resulted in airway remodeling and increased OSM levels, where anti-OSM antibody administration resulted in decreased airway remodeling, eosinophilic inflammation, and airway hyper-responsiveness. OSM is made primarily by neutrophils in mucosal airway diseases and

is increased in asthma patients with incompletely reversible airflow obstruction (438, 439). The limited described research in asthma supports further investigation of BMP signaling, with the goals being to better understand asthma pathology and how to control it effectively.

### ***Role in IBD***

BMP ligands, receptors, and signaling proteins are expressed in the gastrointestinal tract. In both normal human and mouse colon BMP2, Bmpr1a, Bmpr1b, Bmpr2, and p-Smad1/4 were identified in colonocytes at the epithelial surface (440). In the adult mouse intestine, Bmpr1a and BMP2 were highly expressed in the epithelium of the villus and crypts, while BMP4 was more highly expressed in the stromal and mesenchymal cells around crypts and glands (441-443). Further gene expression profiling in humans suggests BMP-1, -2, -5, and -7, Smad7, and Bmpr2 are highly expressed in the tip regions of the colon, while BMP antagonists chordin-like 1, gremlin 1, and gremlin 2 are highly expressed in the colon crypts (444).

BMP signaling and its role in colitis has been most studied in relation to BMP7, Smad5 and Smad7 expression. BMP dysregulation has been identified in IBD and colitis models. Smad7 is highly expressed in IBD intestinal samples and downregulation of smad7 expression resulted in decreased intestinal inflammation and continues to be evaluated in further human clinical studies for actual clinical use (445-448).

Additionally, Smad5 gene expression was found to be decreased in IBD patients and its deficiency results in the breakdown of the apical junctional complex and worsened experimental colitis; further underscoring the importance of BMP signaling in intestinal homeostasis (449). In rat models of colitis, both TNBS- and DSS-induced, BMP7 has

been described to be anti-inflammatory and reduces colitis pathology (450-452). DSS injury is noted to result in a reduction of BMP ligands and receptors, which is thought to lead to greater inflammation and injury as seen by targeted intestinal genetic deletion of BMP receptors using a villin-Cre; *Bmpr1a*<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup> system which resulted in worsened colitis (453). Furthermore, the use of intestinal organoids and treatment with BMP4 resulted in decreased TNF $\alpha$ -induced CXCL8 (IL8) production. BMP pathway suppression early on during colonic inflammation has also been linked to inflammation-driven development of colon neoplasia in mice, underscoring the importance of this pathway in intestinal health and chronic inflammation (454). BMP signaling modulation as a novel treatment option for IBD is ideal because in addition to modulating inflammation, it can directly affect fibrosis. BMP7 has the ability to decrease intestinal fibrosis primarily by inhibiting Tgf $\beta$ -induced epithelial to mesenchymal transition that results in fibroblast/myofibroblast differentiation, with subsequent production of extracellular matrix proteins resulting in fibrosis (455). Treatment options targeted at blocking epithelial to mesenchymal transition and subsequent fibrosis would be a beneficial tool in the treatment of chronic colitis.

### **Conclusions**

Taken together, mast cell – BMP signaling axis may be a critically important axis in chronic inflammatory conditions. The axis may be an additional mechanism in mast cell roles in chronic inflammation and warrants further investigation. Additionally, defining each of their roles, BMPs and mast cell, in modulating the immune response in chronic inflammatory conditions such as asthma and IBD is intriguing as potential targets for treatment.

## LIST OF REFERENCES

1. Kitamura Y. Heterogeneity of mast cells and phenotypic change between subpopulations. *Annual review of immunology*. 1989;7:59-76.
2. Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. *Nature reviews Immunology*. 2002;2(10):773-86.
3. Galli SJ, Zsebo KM, Geissler EN. The kit ligand, stem cell factor. *Advances in immunology*. 1994;55:1-96.
4. Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiological reviews*. 1997;77(4):1033-79.
5. Hallgren J, Gurish MF. Mast cell progenitor trafficking and maturation. *Advances in experimental medicine and biology*. 2011;716:14-28.
6. Arinobu Y, Iwasaki H, Gurish MF, Mizuno S, Shigematsu H, Ozawa H, et al. Developmental checkpoints of the basophil/mast cell lineages in adult murine hematopoiesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(50):18105-10.
7. Iwasaki H, Mizuno S, Arinobu Y, Ozawa H, Mori Y, Shigematsu H, et al. The order of expression of transcription factors directs hierarchical specification of hematopoietic lineages. *Genes & development*. 2006;20(21):3010-21.
8. Qi X, Hong J, Chaves L, Zhuang Y, Chen Y, Wang D, et al. Antagonistic regulation by the transcription factors C/EBPalpha and MITF specifies basophil and mast cell fates. *Immunity*. 2013;39(1):97-110.
9. Franco CB, Chen CC, Drukker M, Weissman IL, Galli SJ. Distinguishing mast cell and granulocyte differentiation at the single-cell level. *Cell stem cell*. 2010;6(4):361-8.
10. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science (New York, NY)*. 1994;265(5178):1573-7.
11. Walsh JC, DeKoter RP, Lee HJ, Smith ED, Lancki DW, Gurish MF, et al. Cooperative and antagonistic interplay between PU.1 and GATA-2 in the specification of myeloid cell fates. *Immunity*. 2002;17(5):665-76.
12. Shelburne CP, McCoy ME, Piekorz R, Sexl V, Roh KH, Jacobs-Helber SM, et al. Stat5 expression is critical for mast cell development and survival. *Blood*. 2003;102(4):1290-7.
13. Henry EK, Sy CB, Inclan-Rico JM, Espinosa V, Ghanny SS, Dwyer DF, et al. Carbonic anhydrase enzymes regulate mast cell-mediated inflammation. *The Journal of experimental medicine*. 2016;213(9):1663-73.
14. Inclan-Rico JM, Hernandez CM, Henry EK, Federman HG, Sy CB, Ponessa JJ, et al. *Trichinella spiralis*-induced mastocytosis and erythropoiesis are simultaneously supported by a bipotent mast cell/erythrocyte precursor cell. *PLoS pathogens*. 2020;16(5):e1008579.
15. Chen CC, Grimbaldston MA, Tsai M, Weissman IL, Galli SJ. Identification of mast cell progenitors in adult mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(32):11408-13.
16. Dahlin JS, Heyman B, Hallgren J. Committed mast cell progenitors in mouse blood differ in maturity between Th1 and Th2 strains. *Allergy*. 2013;68(10):1333-7.

17. Abonia JP, Austen KF, Rollins BJ, Joshi SK, Flavell RA, Kuziel WA, et al. Constitutive homing of mast cell progenitors to the intestine depends on autologous expression of the chemokine receptor CXCR2. *Blood*. 2005;105(11):4308-13.
18. Gurish MF, Tao H, Abonia JP, Arya A, Friend DS, Parker CM, et al. Intestinal mast cell progenitors require CD49 $\beta$ 7 (alpha4beta7 integrin) for tissue-specific homing. *The Journal of experimental medicine*. 2001;194(9):1243-52.
19. Guy-Grand D, Dy M, Luffau G, Vassalli P. Gut mucosal mast cells. Origin, traffic, and differentiation. *The Journal of experimental medicine*. 1984;160(1):12-28.
20. Abonia JP, Hallgren J, Jones T, Shi T, Xu Y, Koni P, et al. Alpha-4 integrins and VCAM-1, but not MAdCAM-1, are essential for recruitment of mast cell progenitors to the inflamed lung. *Blood*. 2006;108(5):1588-94.
21. Dahlin JS, Ivarsson MA, Heyman B, Hallgren J. IgE immune complexes stimulate an increase in lung mast cell progenitors in a mouse model of allergic airway inflammation. *PloS one*. 2011;6(5):e20261.
22. Gurish MF, Austen KF. Developmental origin and functional specialization of mast cell subsets. *Immunity*. 2012;37(1):25-33.
23. Dastyk J, Metcalfe DD. Stem cell factor induces mast cell adhesion to fibronectin. *Journal of immunology (Baltimore, Md : 1950)*. 1994;152(1):213-9.
24. MacNeil AJ, Junkins RD, Wu Z, Lin TJ. Stem cell factor induces AP-1-dependent mast cell IL-6 production via MAPK kinase 3 activity. *Journal of leukocyte biology*. 2014;95(6):903-15.
25. Meininger CJ, Yano H, Rottapel R, Bernstein A, Zsebo KM, Zetter BR. The c-kit receptor ligand functions as a mast cell chemoattractant. *Blood*. 1992;79(4):958-63.
26. Drube S, Heink S, Walter S, Lohn T, Grusser M, Gerbaulet A, et al. The receptor tyrosine kinase c-Kit controls IL-33 receptor signaling in mast cells. *Blood*. 2010;115(19):3899-906.
27. Iwaki S, Tkaczyk C, Satterthwaite AB, Halcomb K, Beaven MA, Metcalfe DD, et al. Btk plays a crucial role in the amplification of Fc epsilonRI-mediated mast cell activation by kit. *The Journal of biological chemistry*. 2005;280(48):40261-70.
28. Wei JJ, Song CW, Sun LC, Yuan Y, Li D, Yan B, et al. SCF and TLR4 ligand cooperate to augment the tumor-promoting potential of mast cells. *Cancer immunology, immunotherapy : CII*. 2012;61(3):303-12.
29. Hundley TR, Gilfillan AM, Tkaczyk C, Andrade MV, Metcalfe DD, Beaven MA. Kit and FcepsilonRI mediate unique and convergent signals for release of inflammatory mediators from human mast cells. *Blood*. 2004;104(8):2410-7.
30. da Silva EZ, Jamur MC, Oliver C. Mast cell function: a new vision of an old cell. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*. 2014;62(10):698-738.
31. Okayama Y, Kawakami T. Development, migration, and survival of mast cells. *Immunologic research*. 2006;34(2):97-115.
32. Shimizu Y, Matsumoto K, Okayama Y, Sakai K, Maeno T, Suga T, et al. Interleukin-3 does not affect the differentiation of mast cells derived from human bone marrow progenitors. *Immunological investigations*. 2008;37(1):1-17.

33. Iemura A, Tsai M, Ando A, Wershil BK, Galli SJ. The c-kit ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. *The American journal of pathology*. 1994;144(2):321-8.
34. Mekori YA, Oh CK, Metcalfe DD. IL-3-dependent murine mast cells undergo apoptosis on removal of IL-3. Prevention of apoptosis by c-kit ligand. *Journal of immunology (Baltimore, Md : 1950)*. 1993;151(7):3775-84.
35. Lantz CS, Boesiger J, Song CH, Mach N, Kobayashi T, Mulligan RC, et al. Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites. *Nature*. 1998;392(6671):90-3.
36. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. *Nature reviews Immunology*. 2014;14(7):478-94.
37. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. *Nature immunology*. 2005;6(2):135-42.
38. Kobayashi T, Asboe-Hansen G. Degranulation and regranulation of human mast cells. An electron microscopic study of the whealing reaction in urticaria pigmentosa. *Acta dermato-venereologica*. 1969;49(4):369-81.
39. Padawer J. Editorial: The ins and outs of mast cell function. *The American journal of anatomy*. 1974;141(3):299-302.
40. Walker BE. Mast cell turn-over in adult mice. *Nature*. 1961;192:980-1.
41. Xiang Z, Block M, Lofman C, Nilsson G. IgE-mediated mast cell degranulation and recovery monitored by time-lapse photography. *The Journal of allergy and clinical immunology*. 2001;108(1):116-21.
42. Enerbäck L. Mast cells in rat gastrointestinal mucosa. 2. Dye-binding and metachromatic properties. *Acta pathologica et microbiologica Scandinavica*. 1966;66(3):303-12.
43. Rönnberg E, Melo FR, Pejler G. Mast cell proteoglycans. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*. 2012;60(12):950-62.
44. Stevens RL, Fox CC, Lichtenstein LM, Austen KF. Identification of chondroitin sulfate E proteoglycans and heparin proteoglycans in the secretory granules of human lung mast cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1988;85(7):2284-7.
45. Irani AM, Schwartz LB. Human mast cell heterogeneity. *Allergy proceedings : the official journal of regional and state allergy societies*. 1994;15(6):303-8.
46. Welle M. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. *Journal of leukocyte biology*. 1997;61(3):233-45.
47. Xing W, Austen KF, Gurish MF, Jones TG. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(34):14210-5.
48. Hallgren J, Pejler G. Biology of mast cell tryptase. An inflammatory mediator. *The FEBS journal*. 2006;273(9):1871-95.

49. Hellman L, Thorpe M. Granule proteases of hematopoietic cells, a family of versatile inflammatory mediators - an update on their cleavage specificity, in vivo substrates, and evolution. *Biological chemistry*. 2014;395(1):15-49.
50. Sonoda S, Sonoda T, Nakano T, Kanayama Y, Kanakura Y, Asai H, et al. Development of mucosal mast cells after injection of a single connective tissue-type mast cell in the stomach mucosa of genetically mast cell-deficient W/W<sup>v</sup> mice. *Journal of immunology (Baltimore, Md : 1950)*. 1986;137(4):1319-22.
51. Dwyer DF, Barrett NA, Austen KF. Expression profiling of constitutive mast cells reveals a unique identity within the immune system. *Nature immunology*. 2016;17(7):878-87.
52. Costa JJ, Weller PF, Galli SJ. The cells of the allergic response: mast cells, basophils, and eosinophils. *Jama*. 1997;278(22):1815-22.
53. Bischoff S, Crowe SE. Gastrointestinal food allergy: new insights into pathophysiology and clinical perspectives. *Gastroenterology*. 2005;128(4):1089-113.
54. Blank U, Ra C, Miller L, White K, Metzger H, Kinet JP. Complete structure and expression in transfected cells of high affinity IgE receptor. *Nature*. 1989;337(6203):187-9.
55. Cambier JC. Antigen and Fc receptor signaling. The awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). *Journal of immunology (Baltimore, Md : 1950)*. 1995;155(7):3281-5.
56. Lin S, Cicala C, Scharenberg AM, Kinet JP. The Fc(epsilon)RIbeta subunit functions as an amplifier of Fc(epsilon)RIgamma-mediated cell activation signals. *Cell*. 1996;85(7):985-95.
57. Kinet JP. The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. *Annual review of immunology*. 1999;17:931-72.
58. Donnadieu E, Jouvin MH, Kinet JP. A second amplifier function for the allergy-associated Fc(epsilon)RI-beta subunit. *Immunity*. 2000;12(5):515-23.
59. Turner H, Kinet JP. Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature*. 1999;402(6760 Suppl):B24-30.
60. Kawakami T, Kitauro J. Mast cell survival and activation by IgE in the absence of antigen: a consideration of the biologic mechanisms and relevance. *Journal of immunology (Baltimore, Md : 1950)*. 2005;175(7):4167-73.
61. Kitauro J, Song J, Tsai M, Asai K, Maeda-Yamamoto M, Mocsai A, et al. Evidence that IgE molecules mediate a spectrum of effects on mast cell survival and activation via aggregation of the FcepsilonRI. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100(22):12911-6.
62. Bax HJ, Bowen H, Dodev TS, Sutton BJ, Gould HJ. Mechanism of the antigen-independent cytokinergic SPE-7 IgE activation of human mast cells in vitro. *Scientific reports*. 2015;5:9538.
63. Parravicini V, Gadina M, Kovarova M, Odom S, Gonzalez-Espinosa C, Furumoto Y, et al. Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nature immunology*. 2002;3(8):741-8.
64. Asai K, Kitauro J, Kawakami Y, Yamagata N, Tsai M, Carbone DP, et al. Regulation of mast cell survival by IgE. *Immunity*. 2001;14(6):791-800.

65. Kalesnikoff J, Huber M, Lam V, Damen JE, Zhang J, Siraganian RP, et al. Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity*. 2001;14(6):801-11.
66. Tanaka S, Takasu Y, Mikura S, Satoh N, Ichikawa A. Antigen-independent induction of histamine synthesis by immunoglobulin E in mouse bone marrow-derived mast cells. *The Journal of experimental medicine*. 2002;196(2):229-35.
67. Oka T, Hori M, Tanaka A, Matsuda H, Karaki H, Ozaki H. IgE alone-induced actin assembly modifies calcium signaling and degranulation in RBL-2H3 mast cells. *American journal of physiology Cell physiology*. 2004;286(2):C256-63.
68. Pandey V, Mihara S, Fensome-Green A, Bolsover S, Cockcroft S. Monomeric IgE stimulates NFAT translocation into the nucleus, a rise in cytosol Ca<sup>2+</sup>, degranulation, and membrane ruffling in the cultured rat basophilic leukemia-2H3 mast cell line. *Journal of immunology (Baltimore, Md : 1950)*. 2004;172(7):4048-58.
69. Lam V, Kalesnikoff J, Lee CW, Hernandez-Hansen V, Wilson BS, Oliver JM, et al. IgE alone stimulates mast cell adhesion to fibronectin via pathways similar to those used by IgE + antigen but distinct from those used by Steel factor. *Blood*. 2003;102(4):1405-13.
70. Kitaura J, Kinoshita T, Matsumoto M, Chung S, Kawakami Y, Leitges M, et al. IgE- and IgE+Ag-mediated mast cell migration in an autocrine/paracrine fashion. *Blood*. 2005;105(8):3222-9.
71. Sakurai D, Yamasaki S, Arase K, Park SY, Arase H, Konno A, et al. Fc epsilon RI gamma-ITAM is differentially required for mast cell function in vivo. *Journal of immunology (Baltimore, Md : 1950)*. 2004;172(4):2374-81.
72. Kitaura J, Xiao W, Maeda-Yamamoto M, Kawakami Y, Lowell CA, Kawakami T. Early divergence of Fc epsilon receptor I signals for receptor up-regulation and internalization from degranulation, cytokine production, and survival. *Journal of immunology (Baltimore, Md : 1950)*. 2004;173(7):4317-23.
73. Yamasaki S, Ishikawa E, Kohno M, Saito T. The quantity and duration of FcRgamma signals determine mast cell degranulation and survival. *Blood*. 2004;103(8):3093-101.
74. Kohno M, Yamasaki S, Tybulewicz VL, Saito T. Rapid and large amount of autocrine IL-3 production is responsible for mast cell survival by IgE in the absence of antigen. *Blood*. 2005;105(5):2059-65.
75. Kitaura J, Eto K, Kinoshita T, Kawakami Y, Leitges M, Lowell CA, et al. Regulation of highly cytokinergic IgE-induced mast cell adhesion by Src, Syk, Tec, and protein kinase C family kinases. *Journal of immunology (Baltimore, Md : 1950)*. 2005;174(8):4495-504.
76. Tanaka S, Mikura S, Hashimoto E, Sugimoto Y, Ichikawa A. Ca<sup>2+</sup> influx-mediated histamine synthesis and IL-6 release in mast cells activated by monomeric IgE. *European journal of immunology*. 2005;35(2):460-8.
77. Alfredsson J, Puthalakath H, Martin H, Strasser A, Nilsson G. Proapoptotic Bcl-2 family member Bim is involved in the control of mast cell survival and is induced together with Bcl-XL upon IgE-receptor activation. *Cell death and differentiation*. 2005;12(2):136-44.

78. Yoshikawa H, Nakajima Y, Tasaka K. Glucocorticoid suppresses autocrine survival of mast cells by inhibiting IL-4 production and ICAM-1 expression. *Journal of immunology* (Baltimore, Md : 1950). 1999;162(10):6162-70.
79. Yoshikawa H, Nakajima Y, Tasaka K. Enhanced expression of Fas-associated death domain-like IL-1-converting enzyme (FLICE)-inhibitory protein induces resistance to Fas-mediated apoptosis in activated mast cells. *Journal of immunology* (Baltimore, Md : 1950). 2000;165(11):6262-9.
80. Xiang Z, Ahmed AA, Moller C, Nakayama K, Hatakeyama S, Nilsson G. Essential role of the prosurvival bcl-2 homologue A1 in mast cell survival after allergic activation. *The Journal of experimental medicine*. 2001;194(11):1561-69.
81. Plaut M, Pierce JH, Watson CJ, Hanley-Hyde J, Nordan RP, Paul WE. Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores. *Nature*. 1989;339(6219):64-7.
82. Falanga YT, Chaimowitz NS, Charles N, Finkelman FD, Pullen NA, Barbour S, et al. Lyn but not Fyn kinase controls IgG-mediated systemic anaphylaxis. *Journal of immunology* (Baltimore, Md : 1950). 2012;188(9):4360-8.
83. Pullen NA, Barnstein BO, Falanga YT, Wang Z, Suzuki R, Tamang TD, et al. Novel mechanism for Fc{epsilon}RI-mediated signal transducer and activator of transcription 5 (STAT5) tyrosine phosphorylation and the selective influence of STAT5B over mast cell cytokine production. *The Journal of biological chemistry*. 2012;287(3):2045-54.
84. Pullen NA, Falanga YT, Morales JK, Ryan JJ. The Fyn-STAT5 Pathway: A New Frontier in IgE- and IgG-Mediated Mast Cell Signaling. *Frontiers in immunology*. 2012;3:117.
85. Grimbaldston MA, Nakae S, Kalesnikoff J, Tsai M, Galli SJ. Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. *Nature immunology*. 2007;8:1095.
86. Chong HJ, Andrew Bouton L, Bailey DP, Wright H, Ramirez C, Gharse A, et al. IL-4 selectively enhances Fc gamma RIII expression and signaling on mouse mast cells. *Cellular immunology*. 2003;224(2):65-73.
87. Andreu P, Johansson M, Affara NI, Pucci F, Tan T, Junankar S, et al. FcR gamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer cell*. 2010;17(2):121-34.
88. Okayama Y, Kirshenbaum AS, Metcalfe DD. Expression of a functional high-affinity IgG receptor, Fc gamma RI, on human mast cells: Up-regulation by IFN-gamma. *Journal of immunology* (Baltimore, Md : 1950). 2000;164(8):4332-9.
89. Agier J, Pastwinska J, Brzezinska-Blaszczyk E. An overview of mast cell pattern recognition receptors. *Inflammation research : official journal of the European Histamine Research Society* [et al]. 2018.
90. Varadaradjalou S, Feger F, Thieblemont N, Hamouda NB, Pleau JM, Dy M, et al. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human mast cells. *European journal of immunology*. 2003;33(4):899-906.
91. McCurdy JD, Olynich TJ, Maher LH, Marshall JS. Cutting edge: distinct Toll-like receptor 2 activators selectively induce different classes of mediator production from human mast cells. *Journal of immunology* (Baltimore, Md : 1950). 2003;170(4):1625-9.

92. Yang S, Sugawara S, Monodane T, Nishijima M, Adachi Y, Akashi S, et al. *Micrococcus luteus* teichuronic acids activate human and murine monocytic cells in a CD14- and toll-like receptor 4-dependent manner. *Infection and immunity*. 2001;69(4):2025-30.
93. Haynes LM, Moore DD, Kurt-Jones EA, Finberg RW, Anderson LJ, Tripp RA. Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. *Journal of virology*. 2001;75(22):10730-7.
94. Roelofs MF, Boelens WC, Joosten LA, Abdollahi-Roodsaz S, Geurts J, Wunderink LU, et al. Identification of small heat shock protein B8 (HSP22) as a novel TLR4 ligand and potential involvement in the pathogenesis of rheumatoid arthritis. *Journal of immunology (Baltimore, Md : 1950)*. 2006;176(11):7021-7.
95. Marshall JS. Mast-cell responses to pathogens. *Nature reviews Immunology*. 2004;4(10):787-99.
96. Metz M, Siebenhaar F, Maurer M. Mast cell functions in the innate skin immune system. *Immunobiology*. 2008;213(3-4):251-60.
97. Echtenacher B, Mannel DN, Hultner L. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature*. 1996;381(6577):75-7.
98. Leal-Berumen I, Conlon P, Marshall JS. IL-6 production by rat peritoneal mast cells is not necessarily preceded by histamine release and can be induced by bacterial lipopolysaccharide. *Journal of immunology (Baltimore, Md : 1950)*. 1994;152(11):5468-76.
99. Malaviya R, Ikeda T, Ross E, Abraham SN. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature*. 1996;381(6577):77-80.
100. Huang C, Friend DS, Qiu WT, Wong GW, Morales G, Hunt J, et al. Induction of a selective and persistent extravasation of neutrophils into the peritoneal cavity by tryptase mouse mast cell protease 6. *Journal of immunology (Baltimore, Md : 1950)*. 1998;160(4):1910-9.
101. Schmidlin F, Amadesi S, Dabbagh K, Lewis DE, Knott P, Bunnett NW, et al. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. *Journal of immunology (Baltimore, Md : 1950)*. 2002;169(9):5315-21.
102. Burns AR, Bowden RA, Abe Y, Walker DC, Simon SI, Entman ML, et al. P-selectin mediates neutrophil adhesion to endothelial cell borders. *Journal of leukocyte biology*. 1999;65(3):299-306.
103. Galli SJ, Kalesnikoff J, Grimbaldston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annual review of immunology*. 2005;23:749-86.
104. Galli SJ, Grimbaldston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nature reviews Immunology*. 2008;8(6):478-86.
105. Dudeck A, Dudeck J, Scholten J, Petzold A, Surianarayanan S, Kohler A, et al. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity*. 2011;34(6):973-84.

106. Schubert N, Dudeck J, Liu P, Karutz A, Speier S, Maurer M, et al. Mast cell promotion of T cell-driven antigen-induced arthritis despite being dispensable for antibody-induced arthritis in which T cells are bypassed. *Arthritis & rheumatology* (Hoboken, NJ). 2015;67(4):903-13.
107. Dawicki W, Jawdat DW, Xu N, Marshall JS. Mast cells, histamine, and IL-6 regulate the selective influx of dendritic cell subsets into an inflamed lymph node. *Journal of immunology* (Baltimore, Md : 1950). 2010;184(4):2116-23.
108. Dudeck J, Ghouse SM, Lehmann CH, Hoppe A, Schubert N, Nedospasov SA, et al. Mast-Cell-Derived TNF Amplifies CD8(+) Dendritic Cell Functionality and CD8(+) T Cell Priming. *Cell reports*. 2015;13(2):399-411.
109. Gutzmer R, Diestel C, Mommert S, Kother B, Stark H, Wittmann M, et al. Histamine H4 receptor stimulation suppresses IL-12p70 production and mediates chemotaxis in human monocyte-derived dendritic cells. *Journal of immunology* (Baltimore, Md : 1950). 2005;174(9):5224-32.
110. Mazzoni A, Young HA, Spitzer JH, Visintin A, Segal DM. Histamine regulates cytokine production in maturing dendritic cells, resulting in altered T cell polarization. *The Journal of clinical investigation*. 2001;108(12):1865-73.
111. Caron G, Delneste Y, Roelandts E, Duez C, Bonnefoy JY, Pestel J, et al. Histamine polarizes human dendritic cells into Th2 cell-promoting effector dendritic cells. *Journal of immunology* (Baltimore, Md : 1950). 2001;167(7):3682-6.
112. Mazzoni A, Leifer CA, Mullen GE, Kennedy MN, Klinman DM, Segal DM. Cutting edge: histamine inhibits IFN- $\alpha$  release from plasmacytoid dendritic cells. *Journal of immunology* (Baltimore, Md : 1950). 2003;170(5):2269-73.
113. Kitawaki T, Kadowaki N, Sugimoto N, Kambe N, Hori T, Miyachi Y, et al. IgE-activated mast cells in combination with pro-inflammatory factors induce Th2-promoting dendritic cells. *International immunology*. 2006;18(12):1789-99.
114. Theiner G, Gessner A, Lutz MB. The mast cell mediator PGD<sub>2</sub> suppresses IL-12 release by dendritic cells leading to Th2 polarized immune responses in vivo. *Immunobiology*. 2006;211(6-8):463-72.
115. Kalinski P, Hilkens CM, Snijders A, Snijdwint FG, Kapsenberg ML. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E<sub>2</sub>, promote type 2 cytokine production in maturing human naive T helper cells. *Journal of immunology* (Baltimore, Md : 1950). 1997;159(1):28-35.
116. Mazzoni A, Siraganian RP, Leifer CA, Segal DM. Dendritic cell modulation by mast cells controls the Th1/Th2 balance in responding T cells. *Journal of immunology* (Baltimore, Md : 1950). 2006;177(6):3577-81.
117. Dudeck J, Froebel J, Kotrba J, Lehmann CHK, Dudziak D, Speier S, et al. Engulfment of mast cell secretory granules on skin inflammation boosts dendritic cell migration and priming efficiency. *The Journal of allergy and clinical immunology*. 2019;143(5):1849-64.e4.
118. Stelekati E, Bahri R, D'Orlando O, Orinska Z, Mittrucker HW, Langenhaun R, et al. Mast cell-mediated antigen presentation regulates CD8+ T cell effector functions. *Immunity*. 2009;31(4):665-76.
119. Frandji P, Oskeritzian C, Cacaraci F, Lapeyre J, Peronet R, David B, et al. Antigen-dependent stimulation by bone marrow-derived mast cells of MHC class II-

- restricted T cell hybridoma. *Journal of immunology* (Baltimore, Md : 1950). 1993;151(11):6318-28.
120. Fox CC, Jewell SD, Whitacre CC. Rat peritoneal mast cells present antigen to a PPD-specific T cell line. *Cellular immunology*. 1994;158(1):253-64.
  121. Poncet P, Arock M, David B. MHC class II-dependent activation of CD4+ T cell hybridomas by human mast cells through superantigen presentation. *Journal of leukocyte biology*. 1999;66(1):105-12.
  122. Suurmond J, van Heemst J, van Heiningen J, Dorjee AL, Schilham MW, van der Beek FB, et al. Communication between human mast cells and CD4(+) T cells through antigen-dependent interactions. *European journal of immunology*. 2013;43(7):1758-68.
  123. Gaudenzio N, Laurent C, Valitutti S, Espinosa E. Human mast cells drive memory CD4+ T cells toward an inflammatory IL-22+ phenotype. *The Journal of allergy and clinical immunology*. 2013;131(5):1400-7.e11.
  124. Nakae S, Suto H, Iikura M, Kakurai M, Sedgwick JD, Tsai M, et al. Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF. *Journal of immunology* (Baltimore, Md : 1950). 2006;176(4):2238-48.
  125. Orinska Z, Bulanov E, Budagian V, Metz M, Maurer M, Bulfone-Paus S. TLR3-induced activation of mast cells modulates CD8+ T-cell recruitment. *Blood*. 2005;106(3):978-87.
  126. Ebert S, Becker M, Lemmermann NA, Buttner JK, Michel A, Taube C, et al. Mast cells expedite control of pulmonary murine cytomegalovirus infection by enhancing the recruitment of protective CD8 T cells to the lungs. *PLoS pathogens*. 2014;10(4):e1004100.
  127. Ott VL, Cambier JC, Kappler J, Marrack P, Swanson BJ. Mast cell-dependent migration of effector CD8+ T cells through production of leukotriene B4. *Nature immunology*. 2003;4(10):974-81.
  128. Malaviya R, Twesten NJ, Ross EA, Abraham SN, Pfeifer JD. Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells. *Journal of immunology* (Baltimore, Md : 1950). 1996;156(4):1490-6.
  129. Gregory GD, Robbie-Ryan M, Secor VH, Sabatino JJ, Jr., Brown MA. Mast cells are required for optimal autoreactive T cell responses in a murine model of multiple sclerosis. *European journal of immunology*. 2005;35(12):3478-86.
  130. Maurer M, Lopez Kostka S, Siebenhaar F, Moelle K, Metz M, Knop J, et al. Skin mast cells control T cell-dependent host defense in *Leishmania major* infections. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2006;20(14):2460-7.
  131. Lu LF, Lind EF, Gondek DC, Bennett KA, Gleeson MW, Pino-Lagos K, et al. Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature*. 2006;442(7106):997-1002.
  132. Eller K, Wolf D, Huber JM, Metz M, Mayer G, McKenzie AN, et al. IL-9 production by regulatory T cells recruits mast cells that are essential for regulatory T cell-induced immune suppression. *Journal of immunology* (Baltimore, Md : 1950). 2011;186(1):83-91.

133. Morita H, Arae K, Unno H, Miyauchi K, Toyama S, Nambu A, et al. An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers. *Immunity*. 2015;43(1):175-86.
134. Nie X, Cai G, Zhang W, Wang H, Wu B, Li Q, et al. Lipopolysaccharide mediated mast cells induce IL-10 producing regulatory T cells through the ICOSL/ICOS axis. *Clinical immunology (Orlando, Fla)*. 2012;142(3):269-79.
135. Kambayashi T, Allenspach EJ, Chang JT, Zou T, Shoag JE, Reiner SL, et al. Inducible MHC class II expression by mast cells supports effector and regulatory T cell activation. *Journal of immunology (Baltimore, Md : 1950)*. 2009;182(8):4686-95.
136. Springer JM, Raveendran VV, Zhang M, Funk R, Smith DD, Maz M, et al. Mast Cell Degranulation Decreases Lipopolysaccharide-Induced Aortic Gene Expression and Systemic Levels of Interleukin-6 In Vivo. *Mediators of inflammation*. 2019;2019:3856360.
137. Esebanmen GE, Langridge WHR. The role of TGF-beta signaling in dendritic cell tolerance. *Immunologic research*. 2017;65(5):987-94.
138. Yoshimura A, Muto G. TGF-beta function in immune suppression. *Current topics in microbiology and immunology*. 2011;350:127-47.
139. Gebhardt T, Lorentz A, Detmer F, Trautwein C, Bektas H, Manns MP, et al. Growth, phenotype, and function of human intestinal mast cells are tightly regulated by transforming growth factor beta1. *Gut*. 2005;54(7):928-34.
140. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annual review of immunology*. 2001;19:683-765.
141. Taylor A, Verhagen J, Blaser K, Akdis M, Akdis CA. Mechanisms of immune suppression by interleukin-10 and transforming growth factor-beta: the role of T regulatory cells. *Immunology*. 2006;117(4):433-42.
142. Groux H, Bigler M, de Vries JE, Roncarolo MG. Inhibitory and stimulatory effects of IL-10 on human CD8+ T cells. *Journal of immunology (Baltimore, Md : 1950)*. 1998;160(7):3188-93.
143. Hugle T, Hogan V, White KE, van Laar JM. Mast cells are a source of transforming growth factor beta in systemic sclerosis. *Arthritis and rheumatism*. 2011;63(3):795-9.
144. Qu Z, Liebler JM, Powers MR, Galey T, Ahmadi P, Huang XN, et al. Mast cells are a major source of basic fibroblast growth factor in chronic inflammation and cutaneous hemangioma. *The American journal of pathology*. 1995;147(3):564-73.
145. Woidacki K, Popovic M, Metz M, Schumacher A, Linzke N, Teles A, et al. Mast cells rescue implantation defects caused by c-kit deficiency. *Cell Death & Disease*. 2013;4:e462.
146. Chacon-Salinas R, Limon-Flores AY, Chavez-Blanco AD, Gonzalez-Estrada A, Ullrich SE. Mast cell-derived IL-10 suppresses germinal center formation by affecting T follicular helper cell function. *Journal of immunology (Baltimore, Md : 1950)*. 2011;186(1):25-31.
147. Elieh Ali Komi D, Ribatti D. Mast cell-mediated mechanistic pathways in organ transplantation. *European journal of pharmacology*. 2019;857:172458.
148. de Vries VC, Pino-Lagos K, Elgueta R, Noelle RJ. The enigmatic role of mast cells in dominant tolerance. *Current opinion in organ transplantation*. 2009;14(4):332-7.

149. Finlay CM, Cunningham KT, Doyle B, Mills KHG. IL-33-Stimulated Murine Mast Cells Polarize Alternatively Activated Macrophages, Which Suppress T Cells That Mediate Experimental Autoimmune Encephalomyelitis. *Journal of immunology* (Baltimore, Md : 1950). 2020;205(7):1909-19.
150. Sandig H, Bulfone-Paus S. TLR signaling in mast cells: common and unique features. *Frontiers in immunology*. 2012;3:185.
151. Pérez-Rodríguez MJ, Ibarra-Sánchez A, Román-Figueroa A, Pérez-Severiano F, González-Espinosa C. Mutant Huntingtin affects toll-like receptor 4 intracellular trafficking and cytokine production in mast cells. *Journal of neuroinflammation*. 2020;17(1):95.
152. Hochdorfer T, Kuhny M, Zorn CN, Hendriks RW, Vanhaesebroeck B, Bohnacker T, et al. Activation of the PI3K pathway increases TLR-induced TNF-alpha and IL-6 but reduces IL-1beta production in mast cells. *Cellular signalling*. 2011;23(5):866-75.
153. Hochdorfer T, Tiedje C, Stumpo DJ, Blackshear PJ, Gaestel M, Huber M. LPS-induced production of TNF-alpha and IL-6 in mast cells is dependent on p38 but independent of TTP. *Cellular signalling*. 2013;25(6):1339-47.
154. Poplutz M, Levikova M, Luscher-Firzlauff J, Lesina M, Algul H, Luscher B, et al. Endotoxin tolerance in mast cells, its consequences for IgE-mediated signalling, and the effects of BCL3 deficiency. *Scientific reports*. 2017;7(1):4534.
155. Watson CJF, Maguire ARR, Rouillard MM, Crozier RWE, Yousef M, Bruton KM, et al. TAK1 signaling activity links the mast cell cytokine response and degranulation in allergic inflammation. *Journal of leukocyte biology*. 2020;107(4):649-61.
156. Cheng LE, Hartmann K, Roers A, Krummel MF, Locksley RM. Perivascular mast cells dynamically probe cutaneous blood vessels to capture immunoglobulin E. *Immunity*. 2013;38(1):166-75.
157. Barbara G, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, et al. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology*. 2004;126(3):693-702.
158. Joos GF, Pauwels RA. Pro-inflammatory effects of substance P: new perspectives for the treatment of airway diseases? *Trends in pharmacological sciences*. 2000;21(4):131-3.
159. Sideri A, Bakirtzi K, Shih DQ, Koon HW, Fleshner P, Arsenescu R, et al. Substance P mediates pro-inflammatory cytokine release from mesenteric adipocytes in Inflammatory Bowel Disease patients. *Cellular and molecular gastroenterology and hepatology*. 2015;1(4):420-32.
160. Ansel JC, Brown JR, Payan DG, Brown MA. Substance P selectively activates TNF-alpha gene expression in murine mast cells. *Journal of immunology* (Baltimore, Md : 1950). 1993;150(10):4478-85.
161. Taracanova A, Alevizos M, Karagkouni A, Weng Z, Norwitz E, Conti P, et al. SP and IL-33 together markedly enhance TNF synthesis and secretion from human mast cells mediated by the interaction of their receptors. *Proceedings of the National Academy of Sciences of the United States of America*. 2017;114(20):E4002-e9.
162. Azzolina A, Bongiovanni A, Lampiasi N. Substance P induces TNF-alpha and IL-6 production through NF kappa B in peritoneal mast cells. *Biochimica et biophysica acta*. 2003;1643(1-3):75-83.

163. Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP. Neuropeptides activate human mast cell degranulation and chemokine production. *Immunology*. 2008;123(3):398-410.
164. Ali H. Mas-related G protein coupled receptor-X2: A potential new target for modulating mast cell-mediated allergic and inflammatory diseases. *Journal of immunobiology*. 2016;1(4).
165. van der Kleij HP, Ma D, Redegeld FA, Kraneveld AD, Nijkamp FP, Bienenstock J. Functional expression of neurokinin 1 receptors on mast cells induced by IL-4 and stem cell factor. *Journal of immunology (Baltimore, Md : 1950)*. 2003;171(4):2074-9.
166. Green DP, Limjunyawong N, Gour N, Pundir P, Dong X. A Mast-Cell-Specific Receptor Mediates Neurogenic Inflammation and Pain. *Neuron*. 2019;101(3):412-20.e3.
167. Koh YH, Moolchhala S, Bhatia M. Activation of neurokinin-1 receptors up-regulates substance P and neurokinin-1 receptor expression in murine pancreatic acinar cells. *Journal of cellular and molecular medicine*. 2012;16(7):1582-92.
168. Sun J, Ramnath RD, Zhi L, Tamizhselvi R, Bhatia M. Substance P enhances NF-kappaB transactivation and chemokine response in murine macrophages via ERK1/2 and p38 MAPK signaling pathways. *American journal of physiology Cell physiology*. 2008;294(6):C1586-96.
169. Varricchi G, Pecoraro A, Loffredo S, Poto R, Rivellese F, Genovese A, et al. Heterogeneity of Human Mast Cells With Respect to MRGPRX2 Receptor Expression and Function. *Frontiers in cellular neuroscience*. 2019;13:299.
170. Helke CJ, Krause JE, Mantyh PW, Couture R, Bannon MJ. Diversity in mammalian tachykinin peptidergic neurons: multiple peptides, receptors, and regulatory mechanisms. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1990;4(6):1606-15.
171. Fujisawa D, Kashiwakura J, Kita H, Kikukawa Y, Fujitani Y, Sasaki-Sakamoto T, et al. Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticaria. *The Journal of allergy and clinical immunology*. 2014;134(3):622-33.e9.
172. Lansu K, Karpiak J, Liu J, Huang XP, McCorvy JD, Kroeze WK, et al. In silico design of novel probes for the atypical opioid receptor MRGPRX2. *Nature chemical biology*. 2017;13(5):529-36.
173. Kuruvilla ME, Lee FE, Lee GB. Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease. *Clinical reviews in allergy & immunology*. 2019;56(2):219-33.
174. Heijink IH, Kies PM, Kauffman HF, Postma DS, van Oosterhout AJ, Vellenga E. Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. *Journal of immunology (Baltimore, Md : 1950)*. 2007;178(12):7678-85.
175. Sweerus K, Lachowicz-Scroggins M, Gordon E, LaFemina M, Huang X, Parikh M, et al. Claudin-18 deficiency is associated with airway epithelial barrier dysfunction and asthma. *The Journal of allergy and clinical immunology*. 2017;139(1):72-81.e1.
176. Yang Q, Ge MQ, Kokalari B, Redai IG, Wang X, Kemeny DM, et al. Group 2 innate lymphoid cells mediate ozone-induced airway inflammation and

- hyperresponsiveness in mice. *The Journal of allergy and clinical immunology*. 2016;137(2):571-8.
177. Chen R, Smith SG, Salter B, El-Gammal A, Oliveria JP, Obminski C, et al. Allergen-induced Increases in Sputum Levels of Group 2 Innate Lymphoid Cells in Subjects with Asthma. *American journal of respiratory and critical care medicine*. 2017;196(6):700-12.
  178. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. *The Journal of allergy and clinical immunology*. 2010;125(2 Suppl 2):S73-80.
  179. Persson C. Lysis of primed eosinophils in severe asthma. *The Journal of allergy and clinical immunology*. 2013;132(6):1459-60.
  180. Durrani SR, Viswanathan RK, Busse WW. What effect does asthma treatment have on airway remodeling? Current perspectives. *The Journal of allergy and clinical immunology*. 2011;128(3):439-48; quiz 49-50.
  181. Chung KF. Airway smooth muscle cells: contributing to and regulating airway mucosal inflammation? *The European respiratory journal*. 2000;15(5):961-8.
  182. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clinical pharmacology and therapeutics*. 2001;69(3):89-95.
  183. Samitas K, Delimpoura V, Zervas E, Gaga M. Anti-IgE treatment, airway inflammation and remodelling in severe allergic asthma: current knowledge and future perspectives. *European respiratory review : an official journal of the European Respiratory Society*. 2015;24(138):594-601.
  184. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *American journal of respiratory and critical care medicine*. 2009;180(5):388-95.
  185. Kuo CS, Pavlidis S, Loza M, Baribaud F, Rowe A, Pandis I, et al. A Transcriptome-driven Analysis of Epithelial Brushings and Bronchial Biopsies to Define Asthma Phenotypes in U-BIOPRED. *American journal of respiratory and critical care medicine*. 2017;195(4):443-55.
  186. Raundhal M, Morse C, Khare A, Oriss TB, Milosevic J, Trudeau J, et al. High IFN-gamma and low SLPI mark severe asthma in mice and humans. *The Journal of clinical investigation*. 2015;125(8):3037-50.
  187. Gauthier M, Chakraborty K, Oriss TB, Raundhal M, Das S, Chen J, et al. Severe asthma in humans and mouse model suggests a CXCL10 signature underlies corticosteroid-resistant Th1 bias. *JCI insight*. 2017;2(13).
  188. Al-Ramli W, Prefontaine D, Chouiali F, Martin JG, Olivenstein R, Lemiere C, et al. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *The Journal of allergy and clinical immunology*. 2009;123(5):1185-7.
  189. Linden A. Role of interleukin-17 and the neutrophil in asthma. *International archives of allergy and immunology*. 2001;126(3):179-84.
  190. Leclerc M, Lavoie-Lamoureux A, Lavoie JP. Heaves, an asthma-like disease of horses. *Respirology (Carlton, Vic)*. 2011;16(7):1027-46.
  191. Herszberg B, Ramos-Barbon D, Tamaoka M, Martin JG, Lavoie JP. Heaves, an asthma-like equine disease, involves airway smooth muscle remodeling. *The Journal of allergy and clinical immunology*. 2006;118(2):382-8.

192. Couetil LL, Cardwell JM, Gerber V, Lavoie JP, Leguillette R, Richard EA. Inflammatory Airway Disease of Horses--Revised Consensus Statement. *Journal of veterinary internal medicine*. 2016;30(2):503-15.
193. Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. *Thorax*. 2002;57(7):643-8.
194. Bullone M, Lavoie JP. Asthma "of horses and men"--how can equine heaves help us better understand human asthma immunopathology and its functional consequences? *Molecular immunology*. 2015;66(1):97-105.
195. Linden A, Dahlen B. Interleukin-17 cytokine signalling in patients with asthma. *The European respiratory journal*. 2014;44(5):1319-31.
196. Giguere S, Viel L, Lee E, MacKay RJ, Hernandez J, Franchini M. Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. *Veterinary immunology and immunopathology*. 2002;85(3-4):147-58.
197. Richard EA, Depecker M, Defontis M, Leleu C, Fortier G, Pitel PH, et al. Cytokine concentrations in bronchoalveolar lavage fluid from horses with neutrophilic inflammatory airway disease. *Journal of veterinary internal medicine*. 2014;28(6):1838-44.
198. Beekman L, Tohver T, Leguillette R. Comparison of cytokine mRNA expression in the bronchoalveolar lavage fluid of horses with inflammatory airway disease and bronchoalveolar lavage mastocytosis or neutrophilia using REST software analysis. *Journal of veterinary internal medicine*. 2012;26(1):153-61.
199. Hue E, Orard M, Toquet MP, Depecker M, Couroucé A, Pronost S, et al. Asymmetrical Pulmonary Cytokine Profiles Are Linked to Bronchoalveolar Lavage Fluid Cytology of Horses With Mild Airway Neutrophilia. *Frontiers in veterinary science*. 2020;7:226.
200. Kaplan AG, Balter MS, Bell AD, Kim H, McIvor RA. Diagnosis of asthma in adults. *CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne*. 2009;181(10):E210-20.
201. Kelly C, Ward C, Stenton CS, Bird G, Hendrick DJ, Walters EH. Number and activity of inflammatory cells in bronchoalveolar lavage fluid in asthma and their relation to airway responsiveness. *Thorax*. 1988;43(9):684-92.
202. Pin I, Gibson PG, Kolendowicz R, Girgis-Gabardo A, Denburg JA, Hargreave FE, et al. Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax*. 1992;47(1):25-9.
203. Grootendorst DC, Sont JK, Willems LN, Kluin-Nelemans JC, Van Krieken JH, Veselic-Charvat M, et al. Comparison of inflammatory cell counts in asthma: induced sputum vs bronchoalveolar lavage and bronchial biopsies. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 1997;27(7):769-79.
204. Castillo JR, Peters SP, Busse WW. Asthma Exacerbations: Pathogenesis, Prevention, and Treatment. *The journal of allergy and clinical immunology In practice*. 2017;5(4):918-27.
205. McGregor MC, Krings JG, Nair P, Castro M. Role of Biologics in Asthma. *American journal of respiratory and critical care medicine*. 2019;199(4):433-45.

206. Yilmaz I. Biologics for oral corticosteroid-dependent asthma. *Allergy and asthma proceedings*. 2020;41(3):151-7.
207. Bradding P. Asthma: eosinophil disease, mast cell disease, or both? *Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology*. 2008;4(2):84-90.
208. Berry M, Morgan A, Shaw DE, Parker D, Green R, Brightling C, et al. Pathological features and inhaled corticosteroid response of eosinophilic and non-eosinophilic asthma. *Thorax*. 2007;62(12):1043-9.
209. Begueret H, Berger P, Vernejoux JM, Dubuisson L, Marthan R, Tunon-de-Lara JM. Inflammation of bronchial smooth muscle in allergic asthma. *Thorax*. 2007;62(1):8-15.
210. Broide DH, Gleich GJ, Cuomo AJ, Coburn DA, Federman EC, Schwartz LB, et al. Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. *The Journal of allergy and clinical immunology*. 1991;88(4):637-48.
211. Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. *The Journal of allergy and clinical immunology*. 2006;117(6):1277-84.
212. Tomioka M, Ida S, Shindoh Y, Ishihara T, Takishima T. Mast cells in bronchoalveolar lumen of patients with bronchial asthma. *The American review of respiratory disease*. 1984;129(6):1000-5.
213. Flint KC, Leung KB, Hudspith BN, Brostoff J, Pearce FL, Johnson NM. Bronchoalveolar mast cells in extrinsic asthma: a mechanism for the initiation of antigen specific bronchoconstriction. *British medical journal (Clinical research ed)*. 1985;291(6500):923-6.
214. Fricker M, Qin L, Niessen N, Baines KJ, McDonald VM, Scott HA, et al. Relationship of sputum mast cells with clinical and inflammatory characteristics of asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2020.
215. Schleich FN, Manise M, Sele J, Henket M, Seidel L, Louis R. Distribution of sputum cellular phenotype in a large asthma cohort: predicting factors for eosinophilic vs neutrophilic inflammation. *BMC pulmonary medicine*. 2013;13:11.
216. Irani AA, Schechter NM, Craig SS, DeBlois G, Schwartz LB. Two types of human mast cells that have distinct neutral protease compositions. *Proceedings of the National Academy of Sciences of the United States of America*. 1986;83(12):4464-8.
217. Schwartz LB. Analysis of MC(T) and MC(TC) mast cells in tissue. *Methods in molecular biology (Clifton, NJ)*. 2006;315:53-62.
218. Carter RJ, Bradding P. The role of mast cells in the structural alterations of the airways as a potential mechanism in the pathogenesis of severe asthma. *Current pharmaceutical design*. 2011;17(7):685-98.
219. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *The New England journal of medicine*. 2002;346(22):1699-705.
220. Balzar S, Fajt ML, Comhair SA, Erzurum SC, Bleecker E, Busse WW, et al. Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma

- Research Program. American journal of respiratory and critical care medicine. 2011;183(3):299-309.
221. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. *Respirology* (Carlton, Vic). 2006;11(1):54-61.
  222. Cai Y, Carty K, Henry RL, Gibson PG. Persistence of sputum eosinophilia in children with controlled asthma when compared with healthy children. *The European respiratory journal*. 1998;11(4):848-53.
  223. Dougherty RH, Sidhu SS, Raman K, Solon M, Solberg OD, Caughey GH, et al. Accumulation of intraepithelial mast cells with a unique protease phenotype in T(H)2-high asthma. *The Journal of allergy and clinical immunology*. 2010;125(5):1046-53.e8.
  224. Wang G, Baines KJ, Fu JJ, Wood LG, Simpson JL, McDonald VM, et al. Sputum mast cell subtypes relate to eosinophilia and corticosteroid response in asthma. *The European respiratory journal*. 2016;47(4):1123-33.
  225. Berthon BS, Gibson PG, Wood LG, MacDonald-Wicks LK, Baines KJ. A sputum gene expression signature predicts oral corticosteroid response in asthma. *The European respiratory journal*. 2017;49(6).
  226. Brown HM. Treatment of chronic asthma with prednisolone; significance of eosinophils in the sputum. *Lancet* (London, England). 1958;2(7059):1245-7.
  227. Zhang W, Wu K, He W, Gao Y, Huang W, Lin X, et al. Transforming growth factor beta 1 plays an important role in inducing CD4(+)CD25(+)forhead box P3(+) regulatory T cells by mast cells. *Clinical and experimental immunology*. 2010;161(3):490-6.
  228. Leveson-Gower DB, Sega EI, Kalesnikoff J, Florek M, Pan Y, Pierini A, et al. Mast cells suppress murine GVHD in a mechanism independent of CD4+CD25+ regulatory T cells. *Blood*. 2013;122(22):3659-65.
  229. Chichlowski M, Westwood GS, Abraham SN, Hale LP. Role of mast cells in inflammatory bowel disease and inflammation-associated colorectal neoplasia in IL-10-deficient mice. *PloS one*. 2010;5(8):e12220.
  230. van der Haegen A, Kunzle F, Gerber V, Welle M, Robinson NE, Marti E. Mast cells and IgE-bearing cells in lungs of RAO-affected horses. *Veterinary immunology and immunopathology*. 2005;108(3-4):325-34.
  231. Dacre KJ, McGorum BC, Marlin DJ, Bartner LR, Brown JK, Shaw DJ, et al. Organic dust exposure increases mast cell tryptase in bronchoalveolar lavage fluid and airway epithelium of heaves horses. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2007;37(12):1809-18.
  232. Hare JE, Viel L, O'Byrne PM, Conlon PD. Effect of sodium cromoglycate on light racehorses with elevated metachromatic cell numbers on bronchoalveolar lavage and reduced exercise tolerance. *Journal of veterinary pharmacology and therapeutics*. 1994;17(3):237-44.
  233. Cahill KN, Katz HR, Cui J, Lai J, Kazani S, Crosby-Thompson A, et al. KIT Inhibition by Imatinib in Patients with Severe Refractory Asthma. *The New England journal of medicine*. 2017;376(20):1911-20.
  234. Colia R, Corrado A, Cantatore FP. Rheumatologic and extraintestinal manifestations of inflammatory bowel diseases. *Annals of medicine*. 2016:1-9.

235. Scheinfeld N. A comprehensive review and evaluation of the side effects of the tumor necrosis factor alpha blockers etanercept, infliximab and adalimumab. *The Journal of dermatological treatment*. 2004;15(5):280-94.
236. Waljee AK, Wiitala WL, Govani S, Stidham R, Saini S, Hou J, et al. Corticosteroid Use and Complications in a US Inflammatory Bowel Disease Cohort. *PloS one*. 2016;11(6):e0158017.
237. Franke A, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nature genetics*. 2010;42(12):1118-25.
238. Anderson CA, Boucher G, Lees CW, Franke A, D'Amato M, Taylor KD, et al. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nature genetics*. 2011;43(3):246-52.
239. Spehlmann ME, Begun AZ, Burghardt J, Lepage P, Raedler A, Schreiber S. Epidemiology of inflammatory bowel disease in a German twin cohort: results of a nationwide study. *Inflammatory bowel diseases*. 2008;14(7):968-76.
240. Cooney R, Baker J, Brain O, Danis B, Pichulik T, Allan P, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nature medicine*. 2010;16(1):90-7.
241. Glocker EO, Kotlarz D, Boztug K, Gertz EM, Schaffer AA, Noyan F, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *The New England journal of medicine*. 2009;361(21):2033-45.
242. Shouval DS, Ouahed J, Biswas A, Goettel JA, Horwitz BH, Klein C, et al. Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. *Advances in immunology*. 2014;122:177-210.
243. Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature*. 2011;474(7351):307-17.
244. Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacology & therapeutics*. 2008;117(2):244-79.
245. Van Deventer SJ. Tumour necrosis factor and Crohn's disease. *Gut*. 1997;40(4):443-8.
246. Mitoma H, Horiuchi T, Tsukamoto H, Ueda N. Molecular mechanisms of action of anti-TNF-alpha agents - Comparison among therapeutic TNF-alpha antagonists. *Cytokine*. 2018;101:56-63.
247. Vandenabeele P, Declercq W, Beyaert R, Fiers W. Two tumour necrosis factor receptors: structure and function. *Trends in cell biology*. 1995;5(10):392-9.
248. Begue B, Wajant H, Bambou JC, Dubuquoy L, Siegmund D, Beaulieu JF, et al. Implication of TNF-related apoptosis-inducing ligand in inflammatory intestinal epithelial lesions. *Gastroenterology*. 2006;130(7):1962-74.
249. Sanchez-Munoz F, Dominguez-Lopez A, Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. *World journal of gastroenterology*. 2008;14(27):4280-8.
250. Mullin JM, Snock KV. Effect of tumor necrosis factor on epithelial tight junctions and transepithelial permeability. *Cancer research*. 1990;50(7):2172-6.

251. Rampart M, De Smet W, Fiers W, Herman AG. Inflammatory properties of recombinant tumor necrosis factor in rabbit skin in vivo. *The Journal of experimental medicine*. 1989;169(6):2227-32.
252. Amiri P, Locksley RM, Parslow TG, Sadick M, Rector E, Ritter D, et al. Tumour necrosis factor alpha restores granulomas and induces parasite egg-laying in schistosome-infected SCID mice. *Nature*. 1992;356(6370):604-7.
253. Murch SH, Braegger CP, Walker-Smith JA, MacDonald TT. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. *Gut*. 1993;34(12):1705-9.
254. Yapali S, over Hamzaoglu H. Anti-TNF treatment in inflammatory bowel disease. *Annals of gastroenterology*. 2007.
255. Avdagic N, Babic N, Seremet M, Delic-Sarac M, Drace Z, Denjalic A, et al. Tumor necrosis factor-alpha serum level in assessment of disease activity in inflammatory bowel diseases. *Medicinski glasnik : official publication of the Medical Association of Zenica-Doboj Canton, Bosnia and Herzegovina*. 2013;10(2):211-6.
256. Braegger CP, Nicholls S, Murch SH, Stephens S, MacDonald TT. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet (London, England)*. 1992;339(8785):89-91.
257. Murch SH, Lamkin VA, Savage MO, Walker-Smith JA, MacDonald TT. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. *Gut*. 1991;32(8):913-7.
258. Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature*. 2011;474(7351):298-306.
259. Wellmann W, Fink PC, Benner F, Schmidt FW. Endotoxaemia in active Crohn's disease. Treatment with whole gut irrigation and 5-aminosalicylic acid. *Gut*. 1986;27(7):814-20.
260. Pastor Rojo O, Lopez San Roman A, Albeniz Arbizu E, de la Hera Martinez A, Ripoll Sevillano E, Albillos Martinez A. Serum lipopolysaccharide-binding protein in endotoxemic patients with inflammatory bowel disease. *Inflammatory bowel diseases*. 2007;13(3):269-77.
261. Cheng Y, Zhu Y, Huang X, Zhang W, Han Z, Liu S. Association between TLR2 and TLR4 Gene Polymorphisms and the Susceptibility to Inflammatory Bowel Disease: A Meta-Analysis. *PloS one*. 2015;10(5):e0126803.
262. Truelove SC, Witts LJ. Cortisone in ulcerative colitis; preliminary report on a therapeutic trial. *British medical journal*. 1954;2(4884):375-8.
263. Flammer JR, Rogatsky I. Minireview: Glucocorticoids in autoimmunity: unexpected targets and mechanisms. *Molecular endocrinology (Baltimore, Md)*. 2011;25(7):1075-86.
264. Derkx B, Taminiau J, Radema S, Stronkhorst A, Wortel C, Tytgat G, et al. Tumour-necrosis-factor antibody treatment in Crohn's disease. *Lancet (London, England)*. 1993;342(8864):173-4.
265. van Dullemen HM, van Deventer SJ, Hommes DW, Bijl HA, Jansen J, Tytgat GN, et al. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). *Gastroenterology*. 1995;109(1):129-35.

266. Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* (London, England). 2002;359(9317):1541-9.
267. Sands BE, Anderson FH, Bernstein CN, Chey WY, Feagan BG, Fedorak RN, et al. Infliximab maintenance therapy for fistulizing Crohn's disease. *The New England journal of medicine*. 2004;350(9):876-85.
268. Cholakapane A, Hazlewood GS, Kaplan GG, Peyrin-Biroulet L, Ananthakrishnan AN. Systematic review with meta-analysis: comparative efficacy of biologics for induction and maintenance of mucosal healing in Crohn's disease and ulcerative colitis controlled trials. *Alimentary pharmacology & therapeutics*. 2017;45(10):1291-302.
269. Berns M, Hommes DW. Anti-TNF-alpha therapies for the treatment of Crohn's disease: the past, present and future. *Expert opinion on investigational drugs*. 2016;25(2):129-43.
270. Eissner G, Kirchner S, Lindner H, Kolch W, Janosch P, Grell M, et al. Reverse signaling through transmembrane TNF confers resistance to lipopolysaccharide in human monocytes and macrophages. *Journal of immunology* (Baltimore, Md : 1950). 2000;164(12):6193-8.
271. Eissner G, Kolch W, Scheurich P. Ligands working as receptors: reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system. *Cytokine & growth factor reviews*. 2004;15(5):353-66.
272. ten Hove T, van Montfrans C, Peppelenbosch MP, van Deventer SJ. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. *Gut*. 2002;50(2):206-11.
273. Mao EJ, Hazlewood GS, Kaplan GG, Peyrin-Biroulet L, Ananthakrishnan AN. Systematic review with meta-analysis: comparative efficacy of immunosuppressants and biologics for reducing hospitalisation and surgery in Crohn's disease and ulcerative colitis. *Alimentary pharmacology & therapeutics*. 2017;45(1):3-13.
274. Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Colombel JF, Panaccione R, et al. Adalimumab induction therapy for Crohn disease previously treated with infliximab: a randomized trial. *Annals of internal medicine*. 2007;146(12):829-38.
275. Yarur AJ, Rubin DT. Therapeutic Drug Monitoring of Anti-tumor Necrosis Factor Agents in Patients with Inflammatory Bowel Diseases. *Inflammatory bowel diseases*. 2015;21(7):1709-18.
276. Sandborn WJ, Abreu MT, D'Haens G, Colombel JF, Vermeire S, Mitchev K, et al. Certolizumab pegol in patients with moderate to severe Crohn's disease and secondary failure to infliximab. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. 2010;8(8):688-95.e2.
277. Lee SH, Kwon JE, Cho ML. Immunological pathogenesis of inflammatory bowel disease. *Intestinal research*. 2018;16(1):26-42.
278. Zenewicz LA, Antov A, Flavell RA. CD4 T-cell differentiation and inflammatory bowel disease. *Trends in molecular medicine*. 2009;15(5):199-207.
279. Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, et al. Increased expression of interleukin 17 in inflammatory bowel disease. *Gut*. 2003;52(1):65-70.

280. Eastaff-Leung N, Mabarrack N, Barbour A, Cummins A, Barry S. Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease. *Journal of clinical immunology*. 2010;30(1):80-9.
281. Maul J, Loddenkemper C, Mundt P, Berg E, Giese T, Stallmach A, et al. Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. *Gastroenterology*. 2005;128(7):1868-78.
282. Desreumaux P, Foussat A, Allez M, Beaugerie L, Hebuterne X, Bouhnik Y, et al. Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease. *Gastroenterology*. 2012;143(5):1207-17.e2.
283. Jakate S, Demeo M, John R, Tobin M, Keshavarzian A. Mastocytic enterocolitis: increased mucosal mast cells in chronic intractable diarrhea. *Archives of pathology & laboratory medicine*. 2006;130(3):362-7.
284. Irani AM, Bradford TR, Kepley CL, Schechter NM, Schwartz LB. Detection of MCT and MCTC types of human mast cells by immunohistochemistry using new monoclonal anti-tryptase and anti-chymase antibodies. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*. 1989;37(10):1509-15.
285. Dvorak AM, Monahan RA, Osage JE, Dickersin GR. Crohn's disease: transmission electron microscopic studies. II. Immunologic inflammatory response. Alterations of mast cells, basophils, eosinophils, and the microvasculature. *Human pathology*. 1980;11(6):606-19.
286. Tunc B, Filik L, Altintas E, Turhan N, Ulker A, Dagli U. Mucosal mast cells in irritable bowel syndrome and inflammatory bowel disease. *Acta medica (Hradec Kralove)*. 2005;48(3-4):163-4.
287. Gelbmann CM, Mestermann S, Gross V, Kollinger M, Scholmerich J, Falk W. Strictures in Crohn's disease are characterised by an accumulation of mast cells colocalised with laminin but not with fibronectin or vitronectin. *Gut*. 1999;45(2):210-7.
288. Nolte H, Spjeldnaes N, Kruse A, Windelborg B. Histamine release from gut mast cells from patients with inflammatory bowel diseases. *Gut*. 1990;31(7):791-4.
289. Lloyd G, Green FH, Fox H, Mani V, Turnberg LA. Mast cells and immunoglobulin E in inflammatory bowel disease. *Gut*. 1975;16(11):861-5.
290. Sarin SK, Malhotra V, Sen Gupta S, Karol A, Gaur SK, Anand BS. Significance of eosinophil and mast cell counts in rectal mucosa in ulcerative colitis. A prospective controlled study. *Digestive diseases and sciences*. 1987;32(4):363-7.
291. Bischoff SC, Wedemeyer J, Herrmann A, Meier PN, Trautwein C, Cetin Y, et al. Quantitative assessment of intestinal eosinophils and mast cells in inflammatory bowel disease. *Histopathology*. 1996;28(1):1-13.
292. Farhadi A, Keshavarzian A, Fields JZ, Jakate S, Shaikh M, Banan A. Reduced immunostaining for c-kit receptors in mucosal mast cells in inflammatory bowel disease. *Journal of gastroenterology and hepatology*. 2007;22(12):2338-43.
293. Stoyanova, II, Gulubova MV. Mast cells and inflammatory mediators in chronic ulcerative colitis. *Acta histochemica*. 2002;104(2):185-92.
294. Kurosawa M, Nagai H. Accumulation of Mast Cells in the Lesions and Effects of Antiallergic Drugs on the Patients with Inflammatory Bowel Disease. *Ulcers*. 2013;2013:7.

295. Bischoff SC, Lorentz A, Schwengberg S, Weier G, Raab R, Manns MP. Mast cells are an important cellular source of tumour necrosis factor alpha in human intestinal tissue. *Gut*. 1999;44(5):643-52.
296. Lilja I, Gustafson-Svard C, Franzen L, Sjobahl R. Tumor necrosis factor-alpha in ileal mast cells in patients with Crohn's disease. *Digestion*. 2000;61(1):68-76.
297. Knutson L, Ahrenstedt O, Odland B, Hallgren R. The jejunal secretion of histamine is increased in active Crohn's disease. *Gastroenterology*. 1990;98(4):849-54.
298. Raithel M, Matek M, Baenkler HW, Jorde W, Hahn EG. Mucosal histamine content and histamine secretion in Crohn's disease, ulcerative colitis and allergic enteropathy. *International archives of allergy and immunology*. 1995;108(2):127-33.
299. Raithel M, Schneider HT, Hahn EG. Effect of substance P on histamine secretion from gut mucosa in inflammatory bowel disease. *Scandinavian journal of gastroenterology*. 1999;34(5):496-503.
300. Fox CC, Lichtenstein LM, Roche JK. Intestinal mast cell responses in idiopathic inflammatory bowel disease. Histamine release from human intestinal mast cells in response to gut epithelial proteins. *Digestive diseases and sciences*. 1993;38(6):1105-12.
301. Winterkamp S, Weidenhiller M, Otte P, Stolper J, Schwab D, Hahn EG, et al. Urinary excretion of N-methylhistamine as a marker of disease activity in inflammatory bowel disease. *The American journal of gastroenterology*. 2002;97(12):3071-7.
302. Raithel M, Winterkamp S, Pacurar A, Ulrich P, Hochberger J, Hahn EG. Release of mast cell tryptase from human colorectal mucosa in inflammatory bowel disease. *Scandinavian journal of gastroenterology*. 2001;36(2):174-9.
303. Fox CC, Lazenby AJ, Moore WC, Yardley JH, Bayless TM, Lichtenstein LM. Enhancement of human intestinal mast cell mediator release in active ulcerative colitis. *Gastroenterology*. 1990;99(1):119-24.
304. King T, Biddle W, Bhatia P, Moore J, Miner PB, Jr. Colonic mucosal mast cell distribution at line of demarcation of active ulcerative colitis. *Digestive diseases and sciences*. 1992;37(4):490-5.
305. Goldsmith P, McGarity B, Walls AF, Church MK, Millward-Sadler GH, Robertson DA. Corticosteroid treatment reduces mast cell numbers in inflammatory bowel disease. *Digestive diseases and sciences*. 1990;35(11):1409-13.
306. Kiesler P, Fuss IJ, Strober W. Experimental Models of Inflammatory Bowel Diseases. *Cellular and molecular gastroenterology and hepatology*. 2015;1(2):154-70.
307. Oh SY, Cho KA, Kang JL, Kim KH, Woo SY. Comparison of experimental mouse models of inflammatory bowel disease. *International journal of molecular medicine*. 2014;33(2):333-40.
308. Zhu L, Shi T, Zhong C, Wang Y, Chang M, Liu X. IL-10 and IL-10 Receptor Mutations in Very Early Onset Inflammatory Bowel Disease. *Gastroenterology research*. 2017;10(2):65-9.
309. Mizoguchi A. Animal models of inflammatory bowel disease. *Progress in molecular biology and translational science*. 2012;105:263-320.
310. Mizoguchi A, Takeuchi T, Himuro H, Okada T, Mizoguchi E. Genetically engineered mouse models for studying inflammatory bowel disease. *The Journal of pathology*. 2016;238(2):205-19.

311. Tobin JM, Delbridge LM, Di Nicolantonio R, Bhathal P. Development of colorectal sensitization is associated with increased eosinophils and mast cells in dextran sulfate sodium-treated rats. *Digestive diseases and sciences*. 2004;49(7-8):1302-10.
312. Magro F, Fraga S, Azevedo I, Soares-da-Silva P. Intestinal 5-hydroxytryptamine and mast cell infiltration in rat experimental colitis. *Digestive diseases and sciences*. 2006;51(3):495-501.
313. Hamilton MJ, Sinnamon MJ, Lyng GD, Glickman JN, Wang X, Xing W, et al. Essential role for mast cell tryptase in acute experimental colitis. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(1):290-5.
314. Iba Y, Sugimoto Y, Kamei C. Participation of mast cells in colitis inflammation induced by dextran sulfate sodium. *Methods and findings in experimental and clinical pharmacology*. 2002;24(1):15-8.
315. Tchougounova E, Lundequist A, Fajardo I, Winberg JO, Abrink M, Pejler G. A key role for mast cell chymase in the activation of pro-matrix metalloprotease-9 and pro-matrix metalloprotease-2. *The Journal of biological chemistry*. 2005;280(10):9291-6.
316. Ishida K, Takai S, Murano M, Nishikawa T, Inoue T, Murano N, et al. Role of chymase-dependent matrix metalloproteinase-9 activation in mice with dextran sodium sulfate-induced colitis. *The Journal of pharmacology and experimental therapeutics*. 2008;324(2):422-6.
317. Santana A, Medina C, Paz-Cabrera MC, Diaz-Gonzalez F, Farre E, Salas A, et al. Attenuation of dextran sodium sulphate induced colitis in matrix metalloproteinase-9 deficient mice. *World journal of gastroenterology*. 2006;12(40):6464-72.
318. Iba Y, Sugimoto Y, Kamei C, Masukawa T. Possible role of mucosal mast cells in the recovery process of colitis induced by dextran sulfate sodium in rats. *International immunopharmacology*. 2003;3(4):485-91.
319. Rigoni A, Bongiovanni L, Burocchi A, Sangaletti S, Danelli L, Guarnotta C, et al. Mast Cells Infiltrating Inflamed or Transformed Gut Alternatively Sustain Mucosal Healing or Tumor Growth. *Cancer research*. 2015;75(18):3760-70.
320. He Z, Song J, Hua J, Yang M, Ma Y, Yu T, et al. Mast cells are essential intermediaries in regulating IL-33/ST2 signaling for an immune network favorable to mucosal healing in experimentally inflamed colons. *Cell death & disease*. 2018;9(12):1173.
321. Compton SJ, Renaux B, Wijesuriya SJ, Hollenberg MD. Glycosylation and the activation of proteinase-activated receptor 2 (PAR(2)) by human mast cell tryptase. *British journal of pharmacology*. 2001;134(4):705-18.
322. Bueno L, Fioramonti J. Protease-activated receptor 2 and gut permeability: a review. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society*. 2008;20(6):580-7.
323. Jacob C, Yang PC, Darmoul D, Amadesi S, Saito T, Cottrell GS, et al. Mast cell tryptase controls paracellular permeability of the intestine. Role of protease-activated receptor 2 and beta-arrestins. *The Journal of biological chemistry*. 2005;280(36):31936-48.

324. Li S, Guan J, Ge M, Huang P, Lin Y, Gan X. Intestinal mucosal injury induced by tryptase-activated protease-activated receptor 2 requires beta-arrestin-2 in vitro. *Molecular medicine reports*. 2015;12(5):7181-7.
325. Enjoji S, Ohama T, Sato K. Regulation of epithelial cell tight junctions by protease-activated receptor 2. *The Journal of veterinary medical science*. 2014;76(9):1225-9.
326. Groschwitz KR, Ahrens R, Osterfeld H, Gurish MF, Han X, Abrink M, et al. Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(52):22381-6.
327. Groschwitz KR, Wu D, Osterfeld H, Ahrens R, Hogan SP. Chymase-mediated intestinal epithelial permeability is regulated by a protease-activating receptor/matrix metalloproteinase-2-dependent mechanism. *American journal of physiology Gastrointestinal and liver physiology*. 2013;304(5):G479-89.
328. Stein J, Ries J, Barrett KE. Disruption of intestinal barrier function associated with experimental colitis: possible role of mast cells. *The American journal of physiology*. 1998;274(1):G203-9.
329. Zhang H, Xue Y, Wang H, Huang Y, Du M, Yang Q, et al. Mast cell deficiency exacerbates inflammatory bowel symptoms in interleukin-10-deficient mice. *World journal of gastroenterology*. 2014;20(27):9106-15.
330. Lennon EM, Borst LB, Edwards LL, Moeser AJ. Mast Cells Exert Anti-Inflammatory Effects in an IL10(-/-) Model of Spontaneous Colitis. *Mediators of inflammation*. 2018;2018:7817360.
331. Gratz R, Becker S, Sokolowski N, Schumann M, Bass D, Malnick SD. Murine monoclonal anti-tNF antibody administration has a beneficial effect on inflammatory bowel disease that develops in IL-10 knockout mice. *Digestive diseases and sciences*. 2002;47(8):1723-7.
332. Scheinin T, Butler DM, Salway F, Scallion B, Feldmann M. Validation of the interleukin-10 knockout mouse model of colitis: antitumour necrosis factor-antibodies suppress the progression of colitis. *Clinical and experimental immunology*. 2003;133(1):38-43.
333. Matsuda C, Ito T, Song J, Mizushima T, Tamagawa H, Kai Y, et al. Therapeutic effect of a new immunosuppressive agent, everolimus, on interleukin-10 gene-deficient mice with colitis. *Clinical and experimental immunology*. 2007;148(2):348-59.
334. Gomes-Santos AC, Moreira TG, Castro-Junior AB, Horta BC, Lemos L, Cruz DN, et al. New insights into the immunological changes in IL-10-deficient mice during the course of spontaneous inflammation in the gut mucosa. *Clinical & developmental immunology*. 2012;2012:560817.
335. Mahler M, Leiter EH. Genetic and environmental context determines the course of colitis developing in IL-10-deficient mice. *Inflammatory bowel diseases*. 2002;8(5):347-55.
336. Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A. IL-10 inhibits cytokine production by activated macrophages. *Journal of immunology (Baltimore, Md : 1950)*. 1991;147(11):3815-22.

337. Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *Journal of immunology (Baltimore, Md : 1950)*. 1991;146(10):3444-51.
338. Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993;75(2):263-74.
339. Kingsley DM. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes & development*. 1994;8(2):133-46.
340. Urist MR. Bone: formation by autoinduction. *Science (New York, NY)*. 1965;150(3698):893-9.
341. Chen D, Zhao M, Harris SE, Mi Z. Signal transduction and biological functions of bone morphogenetic proteins. *Frontiers in bioscience : a journal and virtual library*. 2004;9:349-58.
342. Zhang H, Bradley A. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. *Development (Cambridge, England)*. 1996;122(10):2977-86.
343. Winnier G, Blessing M, Labosky PA, Hogan BL. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes & development*. 1995;9(17):2105-16.
344. Zhao GQ, Hogan BL. Evidence that mouse Bmp8a (Op2) and Bmp8b are duplicated genes that play a role in spermatogenesis and placental development. *Mechanisms of development*. 1996;57(2):159-68.
345. Chen H, Shi S, Acosta L, Li W, Lu J, Bao S, et al. BMP10 is essential for maintaining cardiac growth during murine cardiogenesis. *Development (Cambridge, England)*. 2004;131(9):2219-31.
346. Xiao YT, Xiang LX, Shao JZ. Bone morphogenetic protein. *Biochemical and biophysical research communications*. 2007;362(3):550-3.
347. Sieber C, Kopf J, Hiepen C, Knaus P. Recent advances in BMP receptor signaling. *Cytokine & growth factor reviews*. 2009;20(5-6):343-55.
348. Nelsen SM, Christian JL. Site-specific cleavage of BMP4 by furin, PC6, and PC7. *The Journal of biological chemistry*. 2009;284(40):27157-66.
349. Little SC, Mullins MC. Bone morphogenetic protein heterodimers assemble heteromeric type I receptor complexes to pattern the dorsoventral axis. *Nature cell biology*. 2009;11(5):637-43.
350. Israel DI, Nove J, Kerns KM, Kaufman RJ, Rosen V, Cox KA, et al. Heterodimeric bone morphogenetic proteins show enhanced activity in vitro and in vivo. *Growth factors (Chur, Switzerland)*. 1996;13(3-4):291-300.
351. Fujii M, Takeda K, Imamura T, Aoki H, Sampath TK, Enomoto S, et al. Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. *Molecular biology of the cell*. 1999;10(11):3801-13.
352. Massague J, Seoane J, Wotton D. Smad transcription factors. *Genes & development*. 2005;19(23):2783-810.
353. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J. Mechanism of activation of the TGF-beta receptor. *Nature*. 1994;370(6488):341-7.

354. de Caestecker M. The transforming growth factor-beta superfamily of receptors. *Cytokine & growth factor reviews*. 2004;15(1):1-11.
355. Heinecke K, Seher A, Schmitz W, Mueller TD, Sebald W, Nickel J. Receptor oligomerization and beyond: a case study in bone morphogenetic proteins. *BMC biology*. 2009;7:59.
356. Nohe A, Keating E, Underhill TM, Knaus P, Petersen NO. Dynamics and interaction of caveolin-1 isoforms with BMP-receptors. *Journal of cell science*. 2005;118(Pt 3):643-50.
357. Hartung A, Bitton-Worms K, Rechtman MM, Wenzel V, Boergermann JH, Hassel S, et al. Different routes of bone morphogenic protein (BMP) receptor endocytosis influence BMP signaling. *Molecular and cellular biology*. 2006;26(20):7791-805.
358. Bragdon B, Thinakaran S, Bonor J, Underhill TM, Petersen NO, Nohe A. FRET reveals novel protein-receptor interaction of bone morphogenetic proteins receptors and adaptor protein 2 at the cell surface. *Biophysical journal*. 2009;97(5):1428-35.
359. Miyazono K, Maeda S, Imamura T. BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine & growth factor reviews*. 2005;16(3):251-63.
360. Zwijsen A, Verschueren K, Huylebroeck D. New intracellular components of bone morphogenetic protein/Smad signaling cascades. *FEBS letters*. 2003;546(1):133-9.
361. Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. Two major Smad pathways in TGF-beta superfamily signalling. *Genes to cells : devoted to molecular & cellular mechanisms*. 2002;7(12):1191-204.
362. Hanyu A, Ishidou Y, Ebisawa T, Shimanuki T, Imamura T, Miyazono K. The N domain of Smad7 is essential for specific inhibition of transforming growth factor-beta signaling. *The Journal of cell biology*. 2001;155(6):1017-27.
363. Guo X, Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell research*. 2009;19(1):71-88.
364. Strong AL, Spreadborough PJ, Pagani CA, Haskins RM, Dey D, Grimm PD, et al. Small molecule inhibition of non-canonical (TAK1-mediated) BMP signaling results in reduced chondrogenic ossification and heterotopic ossification in a rat model of blast-associated combat-related lower limb trauma. *Bone*. 2020;139:115517.
365. Shim JH, Greenblatt MB, Xie M, Schneider MD, Zou W, Zhai B, et al. TAK1 is an essential regulator of BMP signalling in cartilage. *The EMBO journal*. 2009;28(14):2028-41.
366. Yamaguchi K, Nagai S, Ninomiya-Tsuji J, Nishita M, Tamai K, Irie K, et al. XIAP, a cellular member of the inhibitor of apoptosis protein family, links the receptors to TAB1-TAK1 in the BMP signaling pathway. *The EMBO journal*. 1999;18(1):179-87.
367. Kurozumi K, Nishita M, Yamaguchi K, Fujita T, Ueno N, Shibuya H. BRAM1, a BMP receptor-associated molecule involved in BMP signalling. *Genes to cells : devoted to molecular & cellular mechanisms*. 1998;3(4):257-64.
368. Lu M, Lin SC, Huang Y, Kang YJ, Rich R, Lo YC, et al. XIAP induces NF-kappaB activation via the BIR1/TAB1 interaction and BIR1 dimerization. *Molecular cell*. 2007;26(5):689-702.
369. Nohe A, Keating E, Knaus P, Petersen NO. Signal transduction of bone morphogenetic protein receptors. *Cellular signalling*. 2004;16(3):291-9.

370. Guicheux J, Lemonnier J, Ghayor C, Suzuki A, Palmer G, Caverzasio J. Activation of p38 mitogen-activated protein kinase and c-Jun-NH<sub>2</sub>-terminal kinase by BMP-2 and their implication in the stimulation of osteoblastic cell differentiation. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2003;18(11):2060-8.
371. Lein P, Drahushuk KM, Higgins D. Effects of bone morphogenetic proteins on neural tissues. In: Vukicevic S, Sampath KT, editors. *Bone Morphogenetic Proteins: From Laboratory to Clinical Practice*. Basel: Birkhäuser Basel; 2002. p. 289-319.
372. Piccolo S, Sasai Y, Lu B, De Robertis EM. Dorsoventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell*. 1996;86(4):589-98.
373. Yanagita M. BMP antagonists: their roles in development and involvement in pathophysiology. *Cytokine & growth factor reviews*. 2005;16(3):309-17.
374. Zimmerman LB, De Jesus-Escobar JM, Harland RM. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell*. 1996;86(4):599-606.
375. Bouwmeester T, Kim S, Sasai Y, Lu B, De Robertis EM. Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature*. 1996;382(6592):595-601.
376. Hsu DR, Economides AN, Wang X, Eimon PM, Harland RM. The *Xenopus* dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Molecular cell*. 1998;1(5):673-83.
377. Stanley E, Biben C, Kotecha S, Fabri L, Tajbakhsh S, Wang CC, et al. DAN is a secreted glycoprotein related to *Xenopus* cerberus. *Mechanisms of development*. 1998;77(2):173-84.
378. Yu PB, Hong CC, Sachidanandan C, Babbitt JL, Deng DY, Hoyng SA, et al. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nature chemical biology*. 2008;4(1):33-41.
379. Vogt J, Traynor R, Sapkota GP. The specificities of small molecule inhibitors of the TGF $\beta$  and BMP pathways. *Cellular signalling*. 2011;23(11):1831-42.
380. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, et al. The selectivity of protein kinase inhibitors: a further update. *The Biochemical journal*. 2007;408(3):297-315.
381. Cannon JE, Upton PD, Smith JC, Morrell NW. Intersegmental vessel formation in zebrafish: requirement for VEGF but not BMP signalling revealed by selective and non-selective BMP antagonists. *British journal of pharmacology*. 2010;161(1):140-9.
382. Boergermann JH, Kopf J, Yu PB, Knaus P. Dorsomorphin and LDN-193189 inhibit BMP-mediated Smad, p38 and Akt signalling in C2C12 cells. *The international journal of biochemistry & cell biology*. 2010;42(11):1802-7.
383. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of clinical investigation*. 2001;108(8):1167-74.
384. Dinter T, Bocobo GA, Yu PB. Pharmacologic Strategies for Assaying BMP Signaling Function. *Methods in molecular biology (Clifton, NJ)*. 2019;1891:221-33.

385. Urata M, Kokabu S, Matsubara T, Sugiyama G, Nakatomi C, Takeuchi H, et al. A peptide that blocks the interaction of NF- $\kappa$ B p65 subunit with Smad4 enhances BMP2-induced osteogenesis. *Journal of cellular physiology*. 2018;233(9):7356-66.
386. Wi SM, Moon G, Kim J, Kim ST, Shim JH, Chun E, et al. TAK1-ECSIT-TRAF6 complex plays a key role in the TLR4 signal to activate NF- $\kappa$ B. *The Journal of biological chemistry*. 2014;289(51):35205-14.
387. Xiao C, Shim JH, Klüppel M, Zhang SS, Dong C, Flavell RA, et al. Ecsit is required for Bmp signaling and mesoderm formation during mouse embryogenesis. *Genes & development*. 2003;17(23):2933-49.
388. Wang H, Behrens EM, Pignolo RJ, Kaplan FS. ECSIT links TLR and BMP signaling in FOP connective tissue progenitor cells. *Bone*. 2018;109:201-9.
389. Suzuki N, Labosky PA, Furuta Y, Hargett L, Dunn R, Fogo AB, et al. Failure of ventral body wall closure in mouse embryos lacking a procollagen C-proteinase encoded by Bmp1, a mammalian gene related to Drosophila tolloid. *Development (Cambridge, England)*. 1996;122(11):3587-95.
390. Dudley AT, Lyons KM, Robertson EJ. A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes & development*. 1995;9(22):2795-807.
391. McPherron AC, Lawler AM, Lee SJ. Regulation of anterior/posterior patterning of the axial skeleton by growth/differentiation factor 11. *Nature genetics*. 1999;22(3):260-4.
392. Mishina Y, Suzuki A, Ueno N, Behringer RR. Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes & development*. 1995;9(24):3027-37.
393. Komatsu Y, Scott G, Nagy A, Kaartinen V, Mishina Y. BMP type I receptor ALK2 is essential for proper patterning at late gastrulation during mouse embryogenesis. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2007;236(2):512-7.
394. Beppu H, Kawabata M, Hamamoto T, Chytil A, Minowa O, Noda T, et al. BMP type II receptor is required for gastrulation and early development of mouse embryos. *Developmental biology*. 2000;221(1):249-58.
395. Oh SP, Li E. The signaling pathway mediated by the type IIB activin receptor controls axial patterning and lateral asymmetry in the mouse. *Genes & development*. 1997;11(14):1812-26.
396. Lechleider RJ, Ryan JL, Garrett L, Eng C, Deng C, Wynshaw-Boris A, et al. Targeted mutagenesis of Smad1 reveals an essential role in chorioallantoic fusion. *Developmental biology*. 2001;240(1):157-67.
397. Chang H, Huylebroeck D, Verschueren K, Guo Q, Matzuk MM, Zwijsen A. Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development (Cambridge, England)*. 1999;126(8):1631-42.
398. Arnold SJ, Maretto S, Islam A, Bikoff EK, Robertson EJ. Dose-dependent Smad1, Smad5 and Smad8 signaling in the early mouse embryo. *Developmental biology*. 2006;296(1):104-18.

399. Sirard C, de la Pompa JL, Elia A, Itie A, Mirtsos C, Cheung A, et al. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes & development*. 1998;12(1):107-19.
400. Chen Q, Chen H, Zheng D, Kuang C, Fang H, Zou B, et al. Smad7 is required for the development and function of the heart. *The Journal of biological chemistry*. 2009;284(1):292-300.
401. Cejalvo T, Sacedón R, Hernández-López C, Diez B, Gutierrez-Frías C, Valencia J, et al. Bone morphogenetic protein-2/4 signalling pathway components are expressed in the human thymus and inhibit early T-cell development. *Immunology*. 2007;121(1):94-104.
402. Martinez VG, Sacedon R, Hidalgo L, Valencia J, Fernandez-Sevilla LM, Hernandez-Lopez C, et al. The BMP Pathway Participates in Human Naive CD4+ T Cell Activation and Homeostasis. *PloS one*. 2015;10(6):e0131453.
403. Varas A, Martínez VG, Hernández-López C, Hidalgo L, Entrena A, Valencia J, et al. Role of BMP signalling in peripheral CD4+ T cell proliferation. *Inmunología*. 2009;28(3):125-30.
404. Yoshioka Y, Ono M, Osaki M, Konishi I, Sakaguchi S. Differential effects of inhibition of bone morphogenetic protein (BMP) signalling on T-cell activation and differentiation. *European journal of immunology*. 2012;42(3):749-59.
405. Browning LM, Pietrzak M, Kuczma M, Simms CP, Kurczewska A, Refugia JM, et al. TGF- $\beta$ -mediated enhancement of T(H)17 cell generation is inhibited by bone morphogenetic protein receptor 1 $\alpha$  signaling. *Science signaling*. 2018;11(545).
406. Lu L, Ma J, Wang X, Wang J, Zhang F, Yu J, et al. Synergistic effect of TGF-beta superfamily members on the induction of Foxp3+ Treg. *European journal of immunology*. 2010;40(1):142-52.
407. Kuczma M, Kurczewska A, Kraj P. Modulation of bone morphogenetic protein signaling in T-cells for cancer immunotherapy. *Journal of immunotoxicology*. 2014;11(4):319-27.
408. Rocher C, Singla R, Singal PK, Parthasarathy S, Singla DK. Bone morphogenetic protein 7 polarizes THP-1 cells into M2 macrophages. *Canadian journal of physiology and pharmacology*. 2012;90(7):947-51.
409. Singla DK, Singla R, Wang J. BMP-7 Treatment Increases M2 Macrophage Differentiation and Reduces Inflammation and Plaque Formation in Apo E-/- Mice. *PloS one*. 2016;11(1):e0147897.
410. Rocher C, Singla DK. SMAD-PI3K-Akt-mTOR pathway mediates BMP-7 polarization of monocytes into M2 macrophages. *PloS one*. 2013;8(12):e84009.
411. Ihle CL, Straign DM, Provera MD, Novitskiy SV, Owens P. Loss of Myeloid BMPR1a Alters Differentiation and Reduces Mouse Prostate Cancer Growth. *Frontiers in oncology*. 2020;10:357.
412. Hong JH, Lee GT, Lee JH, Kwon SJ, Park SH, Kim SJ, et al. Effect of bone morphogenetic protein-6 on macrophages. *Immunology*. 2009;128(1 Suppl):e442-50.
413. Yasmin N, Bauer T, Modak M, Wagner K, Schuster C, Köffel R, et al. Identification of bone morphogenetic protein 7 (BMP7) as an instructive factor for human epidermal Langerhans cell differentiation. *The Journal of experimental medicine*. 2013;210(12):2597-610.

414. Martínez VG, Hidalgo L, Valencia J, Hernández-López C, Entrena A, del Amo BG, et al. Autocrine activation of canonical BMP signaling regulates PD-L1 and PD-L2 expression in human dendritic cells. *European journal of immunology*. 2014;44(4):1031-8.
415. Kersten C, Sivertsen EA, Hystad ME, Forfang L, Smeland EB, Myklebust JH. BMP-6 inhibits growth of mature human B cells; induction of Smad phosphorylation and upregulation of Id1. *BMC immunology*. 2005;6:9.
416. Motakis E, Guhl S, Ishizu Y, Itoh M, Kawaji H, de Hoon M, et al. Redefinition of the human mast cell transcriptome by deep-CAGE sequencing. *Blood*. 2014;123(17):e58-67.
417. Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, et al. Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an Acvr1(R206H) Mouse Model of Fibrodysplasia Ossificans Progressiva. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2018;33(2):269-82.
418. Xu B, Chen C, Chen H, Zheng SG, Bringas P, Jr., Xu M, et al. Smad1 and its target gene Wif1 coordinate BMP and Wnt signaling activities to regulate fetal lung development. *Development (Cambridge, England)*. 2011;138(5):925-35.
419. Huang Z, Wang D, Ihida-Stansbury K, Jones PL, Martin JF. Defective pulmonary vascular remodeling in Smad8 mutant mice. *Human molecular genetics*. 2009;18(15):2791-801.
420. Eblaghie MC, Reedy M, Oliver T, Mishina Y, Hogan BL. Evidence that autocrine signaling through Bmpr1a regulates the proliferation, survival and morphogenetic behavior of distal lung epithelial cells. *Developmental biology*. 2006;291(1):67-82.
421. Sun J, Chen H, Chen C, Whitsett JA, Mishina Y, Bringas P, Jr., et al. Prenatal lung epithelial cell-specific abrogation of Alk3-bone morphogenetic protein signaling causes neonatal respiratory distress by disrupting distal airway formation. *The American journal of pathology*. 2008;172(3):571-82.
422. Vukicevic S, Latin V, Chen P, Batorsky R, Reddi AH, Sampath TK. Localization of osteogenic protein-1 (bone morphogenetic protein-7) during human embryonic development: high affinity binding to basement membranes. *Biochemical and biophysical research communications*. 1994;198(2):693-700.
423. Jeffery TK, Upton PD, Trembath RC, Morrell NW. BMP4 inhibits proliferation and promotes myocyte differentiation of lung fibroblasts via Smad1 and JNK pathways. *American journal of physiology Lung cellular and molecular physiology*. 2005;288(2):L370-8.
424. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, et al. BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nature medicine*. 2003;9(7):964-8.
425. Zeisberg M, Bottiglio C, Kumar N, Maeshima Y, Strutz F, Muller GA, et al. Bone morphogenetic protein-7 inhibits progression of chronic renal fibrosis associated with two genetic mouse models. *American journal of physiology Renal physiology*. 2003;285(6):F1060-7.
426. Izumi N, Mizuguchi S, Inagaki Y, Saika S, Kawada N, Nakajima Y, et al. BMP-7 opposes TGF-beta1-mediated collagen induction in mouse pulmonary myofibroblasts

- through Id2. American journal of physiology Lung cellular and molecular physiology. 2006;290(1):L120-6.
427. Pegorier S, Campbell GA, Kay AB, Lloyd CM. Bone morphogenetic protein (BMP)-4 and BMP-7 regulate differentially transforming growth factor (TGF)-beta1 in normal human lung fibroblasts (NHLF). Respiratory research. 2010;11:85.
428. Liu YL, Chen BY, Nie J, Zhao GH, Zhuo JY, Yuan J, et al. Polydatin prevents bleomycin-induced pulmonary fibrosis by inhibiting the TGF- $\beta$ /Smad/ERK signaling pathway. Experimental and therapeutic medicine. 2020;20(5):62.
429. Jiang Q, Liu C, Liu S, Lu W, Li Y, Luo X, et al. Dysregulation of BMP9/BMP2/SMAD Signaling Pathway Contributes to the Bleomycin-induced Pulmonary Fibrosis and Pulmonary Hypertension in a Rat Model. British journal of pharmacology. 2020.
430. Nomura A, Uchida Y, Sakamoto T, Ishii Y, Masuyama K, Morishima Y, et al. Increases in collagen type I synthesis in asthma: the role of eosinophils and transforming growth factor-beta. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology. 2002;32(6):860-5.
431. Kariyawasam HH, Xanthou G, Barkans J, Aizen M, Kay AB, Robinson DS. Basal expression of bone morphogenetic protein receptor is reduced in mild asthma. American journal of respiratory and critical care medicine. 2008;177(10):1074-81.
432. Stumm CL, Halcsik E, Landgraf RG, Camara NO, Sogayar MC, Jancar S. Lung remodeling in a mouse model of asthma involves a balance between TGF-beta1 and BMP-7. PloS one. 2014;9(4):e95959.
433. Lynn TM, Molloy EL, Masterson JC, Glynn SF, Costello RW, Avdalovic MV, et al. SMAD Signaling in the Airways of Healthy Rhesus Macaques versus Rhesus Macaques with Asthma Highlights a Relationship Between Inflammation and Bone Morphogenetic Proteins. American journal of respiratory cell and molecular biology. 2016;54(4):562-73.
434. Wnuk D, Paw M, Ryzek K, Bochenek G, Śladek K, Madeja Z, et al. Enhanced asthma-related fibroblast to myofibroblast transition is the result of profibrotic TGF- $\beta$ /Smad2/3 pathway intensification and antifibrotic TGF- $\beta$ /Smad1/5/(8)9 pathway impairment. Scientific reports. 2020;10(1):16492.
435. Miller M, Esnault S, Kurten RC, Kelly EA, Beppu A, Das S, et al. Segmental allergen challenge increases levels of airway follistatin-like 1 in patients with asthma. The Journal of allergy and clinical immunology. 2016;138(2):596-9.e4.
436. Liu Y, Liu T, Wu J, Li T, Jiao X, Zhang H, et al. The Correlation between FSTL1 Expression and Airway Remodeling in Asthmatics. Mediators of inflammation. 2017;2017:7918472.
437. Miller M, Beppu A, Rosenthal P, Pham A, Das S, Karta M, et al. Fstl1 Promotes Asthmatic Airway Remodeling by Inducing Oncostatin M. Journal of immunology (Baltimore, Md : 1950). 2015;195(8):3546-56.
438. Pothoven KL, Norton JE, Suh LA, Carter RG, Harris KE, Biyasheva A, et al. Neutrophils are a major source of the epithelial barrier disrupting cytokine oncostatin M in patients with mucosal airways disease. The Journal of allergy and clinical immunology. 2017;139(6):1966-78.e9.

439. Simpson JL, Baines KJ, Boyle MJ, Scott RJ, Gibson PG. Oncostatin M (OSM) is increased in asthma with incompletely reversible airflow obstruction. *Experimental lung research*. 2009;35(9):781-94.
440. Hardwick JC, Van Den Brink GR, Bleuming SA, Ballester I, Van Den Brande JM, Keller JJ, et al. Bone morphogenetic protein 2 is expressed by, and acts upon, mature epithelial cells in the colon. *Gastroenterology*. 2004;126(1):111-21.
441. Batts LE, Polk DB, Dubois RN, Kulessa H. Bmp signaling is required for intestinal growth and morphogenesis. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2006;235(6):1563-70.
442. Haramis AP, Begthel H, van den Born M, van Es J, Jonkhoeer S, Offerhaus GJ, et al. De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science (New York, NY)*. 2004;303(5664):1684-6.
443. He XC, Zhang J, Tong WG, Tawfik O, Ross J, Scoville DH, et al. BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nature genetics*. 2004;36(10):1117-21.
444. Kosinski C, Li VS, Chan AS, Zhang J, Ho C, Tsui WY, et al. Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104(39):15418-23.
445. Boirivant M, Pallone F, Di Giacinto C, Fina D, Monteleone I, Marinaro M, et al. Inhibition of Smad7 with a specific antisense oligonucleotide facilitates TGF-beta1-mediated suppression of colitis. *Gastroenterology*. 2006;131(6):1786-98.
446. Monteleone G, Neurath MF, Ardizzone S, Di Sabatino A, Fantini MC, Castiglione F, et al. Mongersen, an oral SMAD7 antisense oligonucleotide, and Crohn's disease. *The New England journal of medicine*. 2015;372(12):1104-13.
447. Feagan BG, Sands BE, Rossiter G, Li X, Usiskin K, Zhan X, et al. Effects of Mongersen (GED-0301) on Endoscopic and Clinical Outcomes in Patients With Active Crohn's Disease. *Gastroenterology*. 2018;154(1):61-4.e6.
448. Monteleone G, Kumberova A, Croft NM, McKenzie C, Steer HW, MacDonald TT. Blocking Smad7 restores TGF-beta1 signaling in chronic inflammatory bowel disease. *The Journal of clinical investigation*. 2001;108(4):601-9.
449. Allaire JM, Darsigny M, Marcoux SS, Roy SA, Schmouth JF, Umans L, et al. Loss of Smad5 leads to the disassembly of the apical junctional complex and increased susceptibility to experimental colitis. *American journal of physiology Gastrointestinal and liver physiology*. 2011;300(4):G586-97.
450. Maric I, Poljak L, Zoricic S, Bobinac D, Bosukonda D, Sampath KT, et al. Bone morphogenetic protein-7 reduces the severity of colon tissue damage and accelerates the healing of inflammatory bowel disease in rats. *Journal of cellular physiology*. 2003;196(2):258-64.
451. Maric I, Kucic N, Turk Wensveen T, Smoljan I, Grahovac B, Zoricic Cvek S, et al. BMP signaling in rats with TNBS-induced colitis following BMP7 therapy. *American journal of physiology Gastrointestinal and liver physiology*. 2012;302(10):G1151-62.
452. Hao Z, Yang X, Lv Y, Li S, Purbey BK, Su H. Intracolonic administered adeno-associated virus-bone morphogenetic protein-7 ameliorates dextran sulphate sodium-induced acute colitis in rats. *The journal of gene medicine*. 2012;14(7):482-90.

453. Ji T, Takabayashi H, Mao M, Han X, Xue X, Brazil JC, et al. Regulation and function of bone morphogenetic protein signaling in colonic injury and inflammation. *American journal of physiology Gastrointestinal and liver physiology*. 2017;312(1):G24-g33.
454. Karagiannis GS, Afaloniati H, Karamanavi E, Poutahidis T, Angelopoulou K. BMP pathway suppression is an early event in inflammation-driven colon neoplasmatogenesis of uPA-deficient mice. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2016;37(2):2243-55.
455. Flier SN, Tanjore H, Kokkotou EG, Sugimoto H, Zeisberg M, Kalluri R. Identification of epithelial to mesenchymal transition as a novel source of fibroblasts in intestinal fibrosis. *The Journal of biological chemistry*. 2010;285(26):20202-12.

## **APPENDIX**

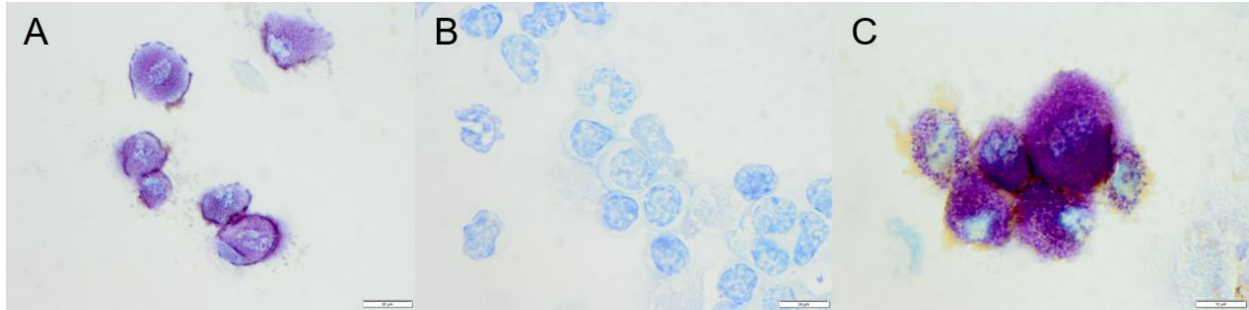


**Figure 1.1. Isolation of mast cells using a positive selection kit, with subsequent toluidine blue staining.** A) Positive fraction stained with toluidine blue to stain mast cells granules (500x total magnification). B) Negative fraction stained with toluidine blue and not granulated cells are visible (500x total magnification). C) Cluster of positive fraction cells stained with toluidine blue that clearly show cytoplasmic granules consistent with mast cell (1000x total magnification). Sample used for selection was mouse peritoneal fluid and positively selected for using Miltenyi Biotec CD117 selection kit. All images obtained with the Olympus BX41.

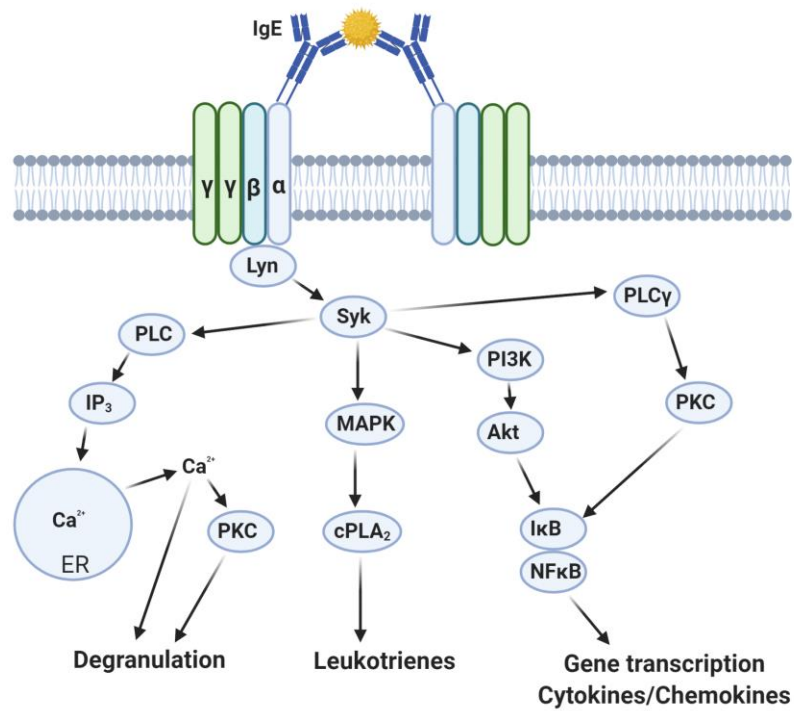


**Figure 1.2. Antigen dependent IgE signaling.** A simplified version of the cell signaling pathway occurring in mast cells upon antigen binding and IgE-cross linking. Activation results in degranulation of cytoplasmic granules and their contents, leukotriene production, and cytokine/chemokine production. Created with BioRender.com

**Table 1.1. Examples of molecules stored in mast cell granules.**

| <b>Molecule Type</b> | <b>Molecule Name</b>                                       |
|----------------------|------------------------------------------------------------|
| Proteases            | Mast cell specific: tryptase, chymase, carboxypeptidase A3 |
| Cytokines            | TNF, IL4                                                   |
| Chemokines           | CXCL8                                                      |
| Lysosomal enzymes    | B-hexosaminidase                                           |
| Bioactive monoamines | Histamine, serotonin                                       |
| Proteoglycans        | Chondroitin sulphates, heparin                             |
| Peptides             | Substance P                                                |

Adapted from: Wernersson, S. and G. Pejler, *Mast cell secretory granules: armed for battle*. Nat Rev Immunol, 2014. 14(7): p. 478-94.

**Table 1.2. Tgf $\beta$  superfamily signaling molecules.**

| <b>Ligand</b> | <b>Type I Receptor</b>                         | <b>Type II Receptor</b>     | <b>Receptor regulated-Smad (R-Smad)</b> | <b>Co-Smad</b> | <b>Inhibitory Smad (I-Smad)</b> |
|---------------|------------------------------------------------|-----------------------------|-----------------------------------------|----------------|---------------------------------|
| BMP2/4        | ALK2 (Acvr1)<br>ALK3 (Bmpr1a)<br>ALK6 (Bmpr1b) | BMPRII                      | Smad1<br>Smad5<br>Smad8                 | Smad4          | Smad7                           |
| BMP7          | ALK2 (Acvr1)<br>ALK3 (Bmpr1a)<br>ALK6 (Bmpr1b) | BMPRII<br>ActRII<br>ActRIIB | Smad1<br>Smad5<br>Smad8                 | Smad4          | Smad7                           |
| Tgf $\beta$ s | ALK5 (T $\beta$ RI)                            | T $\beta$ RII               | Smad2<br>Smad3                          | Smad4          | Smad6<br>Smad7                  |
| Activins      | ALK4 (Acvr1b)                                  | ActRII<br>ActRIIB           | Smad2<br>Smad3                          | Smad4          | Smad6<br>Smad7                  |

Adapted from: Massague, J., J. Seoane, and D. Wotton, *Smad transcription factors*.

Genes Dev, 2005. 19(23): p. 2783-810

**Table 1.3. Chemical BMP receptor inhibitors.**

| <b>Compound</b> | <b>Affinity for these receptors</b>                   |
|-----------------|-------------------------------------------------------|
| Dorsomorphin    | ALK2, ALK3, ALK6                                      |
| LDN-193189      | ALK2, ALK3, ALK5, ALK6, T $\beta$ RII                 |
| LDN-212854      | ALK2, ALK3, ALK6                                      |
| LDN-214117      | ALK2                                                  |
| Ko2288a         | ALK2, ALK3, ALK6                                      |
| DMH1            | ALK2, ALK3                                            |
| DMH2            | ALK2, ALK3, ALK5, ALK6, BMP $\alpha$ 2, T $\beta$ RII |
| SB-431542       | ALK4, ALK5, ALK7                                      |

Adapted from: Dinter, T., G.A. Bocobo, and P.B. Yu, *Pharmacologic Strategies for*

*Assaying BMP Signaling Function*. Methods Mol Biol, 2019. 1891: p. 221-233.

## **CHAPTER 2**

### **BMP – MAST CELL AXIS AND ITS MODULATION OF INFLAMMATION**

## **Abstract**

Mast cells are a type of innate immune cell with its role primarily constrained to the tissues. Mast cells are in high concentration at locations of high antigen exposure where they are able to recognize intruders and respond quickly to help coordinate the immune response. Mast cells are not only inflammatory drivers, as commonly thought of with antigen-IgE mediated responses; they are additionally able to help maintain barrier homeostasis and are beneficial in models of lung and intestinal inflammation. Transforming growth factor (Tgf)- $\beta$  and bone morphogenetic proteins (BMPs) are in the same superfamily, with each previously found to have the ability to modulate the immune response in anti-inflammatory manners. The interaction between mast cells and BMPs, which there a several different BMP ligands, is not clearly defined, although mast cells are noted to express high amounts of a specific BMP receptor, Bmpr2, and increased activity of another BMP receptor, Acvr1, results in increased pro-inflammatory cytokine production. Due to BMP ligands having the ability to modulate immune responses in an anti-inflammatory manner, and the mast cell – BMP axis is poorly defined; we chose to investigate this further. We hypothesized that during inflammation BMPs modulate mast cell responses in an anti-inflammatory manner. Results showed that preincubation of mouse and human mast cells with BMP7 resulted in decreased TNF $\alpha$  secretion without affecting degranulation. Chemical inhibition of Acvr1 resulted in further significant TNF $\alpha$  secretion, which in mouse mast cells appears to be related to decreased p-p38 and pERK1/2 but is not evident in human mast cells. These results show both an anti- and pro-inflammatory role of the BMP – mast cell axis with BMP7 treatment leading to a decrease in TNF $\alpha$ , while blocking Acvr1 signaling

leads to further decreased TNF $\alpha$  release. Although further work is needed to clarify the mechanism by which TNF release is decreased in human mast cell, we believe that Acvr1 is critical in mast cells for appropriate TNF production and release. Based on preliminary results, investigating the steps involved in subsequent release of vesicles containing TNF $\alpha$  may be of interest. Ultimately, increased understanding of the BMP ligand – Mast cell axis could be an area with novel therapeutic options in diseases of inflammation.

## **Introduction**

Mast cells are located in the lung, intestine, skin, and urogenital tract where antigen exposure is concentrated; therefore as a member of the innate defense system they are in a location of optimal antigen recognition and response (1-4). Mast cells play a critical role in early responses to pathogens, which are recognized via complement receptors, mannosylated receptor protein, PAMP receptors (such as TLR4), and they contain the NLRP3 inflammasome (5-11). Mast cells also have the high affinity IgE receptor (Fc $\epsilon$ RI) and are classically known to be activated in antigen dependent IgE manners that lead to degranulation. Activated mast cells are able to release preformed and *de novo* synthesized molecules, in addition to interacting with other cells with cell surface receptors (12-15). Pro-inflammatory preformed mast cell-derived products include histamine, TNF $\alpha$ , and tryptase; while *de novo* synthesized molecules include additional TNF $\alpha$ , arachidonic acid-derived metabolites, interleukins, and chemokines. Classical pro-inflammatory actions of mast cells, such as IgE-mediated activation and LPS-TLR4 activation are widely studied, but anti-inflammatory or protective roles of the mast cell is less well understood.

The beneficial role of mast cells has been more recently identified in models of lung and intestinal inflammation. In a model of papain-induced lung inflammation, mast cells were found to decrease lung inflammation, likely associated with supporting T regulatory cell proliferation (16). Additionally, in a house dust mite model of lung inflammation mast cell derived protease, MCP-4, was found to be beneficial in decreasing inflammatory infiltrate (17). Mucosal healing of the intestinal tract was also identified as a role of mast cells in a model of colitis, attributed to IL33/ST2 signaling and production of IL22 and IL13 (18). Furthermore, our group, as well as others, has shown that mast cells are protective in a mouse model of spontaneous, chronic colitis using the IL10<sup>-/-</sup> mouse (19-21). Although the mechanism of protection is not clearly defined, further experimentation by our lab using reconstitution techniques showed the protective mechanism is independent of IL10 and may be linked to the direct or indirect production of bone morphogenetic protein (BMP)-2, -4, -6, & -7. Bone morphogenetic proteins are a group of 20 different proteins in the Tgf $\beta$  superfamily that induce a signal through heterotetrameric receptor complexes (22). The BMP-mast cell axis is not well described, but BMPs have the ability to support anti-inflammatory and anti-fibrotic mechanisms; therefore, the BMP-mast cell axis may be particularly important and possible novel treatment in chronic inflammatory conditions of the lung or intestine.

BMP ligands have been shown both *in vitro* and *in vivo* to be anti-inflammatory. Foxp3 T regulatory cell differentiation and proliferation is primarily Tgf $\beta$ 1-driven, but BMP2 and BMP4 have been shown to be synergistic in T regulatory cell induction (23). In addition to regulating cell induction, BMPs are able to regulate cytokine production. Blockade of BMP receptors with chemical inhibitors *in vitro* resulted in decreased pro-

inflammatory cytokines in response to stimuli; furthermore, BMP7 treatment in a model of atherosclerosis aids in M2 macrophage differentiation and decreased pro-inflammatory cytokine production such as MCP-1 and TNF $\alpha$  (24, 25). BMP7 is additionally beneficial in models of colitis where treatment with BMP7 reduced disease activity, pathology, and myeloperoxidase activity (26). In addition to BMP7 decreasing inflammation, it is also anti-fibrotic and blocks Tgf $\beta$ -induced epithelial to mesenchymal transition that is especially beneficial in diseases of chronic inflammation (27, 28). Interestingly, specific BMPs can have opposite effects. BMP6 was found *in vitro* to decrease macrophage proliferation, but induced pro-inflammatory molecules, such as inducible nitric oxide synthase and TNF $\alpha$  (29). Initial evaluation of the mast cell – BMP interaction has been noted to be more pro-inflammatory as BMP4 is noted to aid in mast cell survival and recovery to second IgE-antigen stimulation and overexpression of a specific BMP receptor, Acvr1, in mast cells results in increased production of pro-inflammatory cytokines (30, 31). The BMP - mast cell axis, specifically in relation to BMP7 is potentially very interesting due to BMP7's anti-inflammatory and anti-fibrotic functions, and because BMP2, a receptor for BMP7, is a gene expression profiling marker that separates basophils and other immune cells from mast cells (32).

BMP receptor-ligand complex signals via phosphorylating Smad-1, -5, & -8/9; this phosphorylated BR-Smad complex associates with Smad-4 that is then translocated to the nucleus to regulate transcription of target genes such as a group of proteins referred to as inhibitors of DNA binding and cell differentiation (Id) (33-35). The canonical BMP receptor signaling pathway is Smad-dependent, but non-canonical, Smad-independent signaling also occurs which involves TAK1, MAPKs, and NF $\kappa$ B (36).

When BMP ligand is bound, BMP type 2 receptor phosphorylates the type 1 receptor, which ultimately propagates the signal in a canonical or non-canonical pathway. BMP receptor chemical inhibitors are utilized to evaluate BMP signaling, which work by binding to BMP receptors with variable affinity and block the receptor's serine-threonine kinase ability. The first BMP receptor chemical inhibitor identified was dorsomorphin. Dorsomorphin and its derivative, LDN-193189, have the highest affinity for Acvr1 compared to other chemical inhibitors.

We hypothesized that during inflammation BMPs modulate mast cell responses in an anti-inflammatory manner and that BMP receptors differentially modulate mast cell inflammatory responses. The objectives were to define the role of BMPs in regulating mast cell functions by evaluating classical mast cell degranulation and the release of TNF $\alpha$ , a key cytokine in driving inflammation.

## **Materials and Methods**

**Reagents.** Lipopolysaccharide (LPS, L3024), anti-DNP IgE (D8406), substance P (SP, S6883), A23187 (C7522), DNP-HSA (A6661), dorsomorphin (P5499), SB-431542 (S4317), DMH1 (D8946), and DMH2 (SML1535) were obtained from Sigma-Aldrich (St. Louis, MO). LDN-193189 (6053) was obtained from Tocris Bioscience (Bristol, UK). p-nitrophenyl-N-acetyl-beta-D-glucosaminide (NAG, 229411000) was obtained from Acros Organics (Fair Lawn, NJ). Mouse IL3 (403-ML), mSCF (455-MC), mBMP7 (5666-BP), mTgfb $\beta$ 1 (7666-MB), human SCF (255-SC), and hBMP7 (354-BP) were obtained from R&D Systems (Minneapolis, MN).

**Antibodies.** Anti- $\beta$ -actin (4970), anti-phospho-NF- $\kappa$ B p65 (Ser536, 3033), anti-NF- $\kappa$ B p65 (8242), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Ty204, 9101), anti-

p44/42 MAPK (Erk1/2, 4695), anti-phospho-p38 MAPK (Thr180/Tyr182, 4511), anti-p38 MAPK (8690), anti-phospho-TAK1 (Ser412, 9339), anti-rabbit IgG HRP-linked (7074), and anti-mouse IgG HRP-linked (7076) were purchased from Cell Signaling Technology (Danvers, MA). Anti-calnexin (MA3-027) was purchased from Thermo Fisher Scientific (Waltham, MA). Anti-BMP7 (MAB4350) was purchased from Millipore Sigma. Anti-BMP7 (ab130206) was purchased from Abcam (Cambridge, UK).

**Mice.** C57Bl/6J mice were used in this study. Mice were housed in specific-pathogen free barrier facility. Mice were euthanized via CO<sub>2</sub> inhalation. All procedures were approved by the University of Tennessee and University of Pennsylvania Institutional Animal Care and Use Committee.

**Bone marrow derived mast cells (BMMCs).** Female wild type mice, C57Bl/6 (Jackson mice), age 6-8 weeks old were used to collect femurs for bone marrow isolation. Cells were cultured in RPMI 1640 with L-glutamine, supplemented with 10% fetal bovine serum, penicillin/streptomycin, HEPES, sodium pyruvate, essential amino acids, plus IL3 and SCF at 10 ng/ml (cytokines from R&D, FBS from Gibco (part of Thermo Fisher), and all other reagents from Corning (Corning, NY)). Mast cells were cultured in 175 cm<sup>2</sup> tissue culture flasks, incubated in a humidified incubator at 37°C with 5% CO<sub>2</sub>. For the first 1-2 weeks the medium was changed three times per week, and thereafter 2 times per week. Mast cell differentiation was not assessed until at least 4 weeks of culture was met. Purity of mast cells was determined by flow cytometry using anti-CD117 and anti-FcεRIα cell surface markers (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were used when ≥90% double labeled.

**Cell lines.** RBL-2H3 cells, a rat mast cell line, were cultured in EMEM complete media supplemented with 10% FBS (GE Life Sciences, Marlborough, MA) and penicillin/streptomycin, with passaging performed every 3 to 4 days. LAD2 cells, a human mast cell line, were obtained from the NIH and cultured in Stem Pro complete media (Thermofisher), supplemented with L-Glutamine, penicillin/streptomycin, and 100 ng/ml SCF. Medium was changed and cells passaged via hemidepletion once a week. Both cell types were cultured in 75 cm<sup>2</sup> tissue culture flasks, incubated in a humidified incubator at 37°C with 5% CO<sub>2</sub>.

**Beta-hexosaminidase assay.** To evaluate degranulation, RBL-2H3 cells (500,000 cells/ml) and LAD2 cells (500,000 cells/ml) were seeded in to 96 well plates. RBL-2H3 cells were sensitized overnight with 0.5 ug/ml anti-DNP-IgE. RBL-2H3 cells were induced to degranulate with the addition of 100 ng/ml DNP-BSA for 2 hours. LAD2 cells were induced to degranulate with the addition of 1 uM SP for 2 hours. Modulation of degranulation by certain molecules was assessed by preincubation for 24 hours with BMP7 or BMP receptor chemical inhibitors at specified dosages. Both cell types were also exposed to a calcium ionophore, 1 uM A23187, as a positive control. Degranulation was assessed by first collecting the supernatant, then lysing the cells to collect the protein and then incubating both with p-nitrophenyl-N-acetyl-beta-D-glucosaminide (NAG) for 1 hr at 37°C. The reaction was stopped with the addition of carbonate buffer and then the absorbance was read at 405 nm using a microplate reader. Percent degranulation was calculated by adding the absorbance readings for the supernatant and cell lysate, then dividing the absorbance of the supernatant by the total absorbance and multiply by 100 to give a percent.

***TNF $\alpha$  detection.*** Cells were pretreated with BMP7 or BMP receptor inhibitors at indicated dosages and times. BMMCs ( $1 \times 10^6$  cell/ml) were co-stimulated with IgE at 0.5  $\mu$ g/ml when exposed to LPS stimulation in order to optimize TNF $\alpha$  production. LAD2 cells (500,000 cell/ml) were stimulated with SP to induce TNF $\alpha$  secretion. A subset of treated cells was treated with A23187 (calcium ionophore) for about 2 hours prior to supernatant collection in order to force mast cell degranulation. Supernatant was collected for TNF $\alpha$  detection by ELISA (Invitrogen, Carlsbad, CA).

***BMP7 detection.*** BMMCs were stimulated for 24hrs with pro-inflammatory stimuli. A subset of treated cells was treated with A23187 (calcium ionophore) about 2 hours prior to supernatant collection in order to force mast cell degranulation. BMP7 was then detected via ELISA (Thermo Fisher).

***RNA sequencing and analysis.*** BMMCs were stimulated, as indicated, at  $1 \times 10^6$  and then cell pellets were collected, washed with PBS, and final cell pellets made at  $2 \times 10^6$  cells. Pellets were immediately stored at  $-80^\circ\text{C}$  and then sent to a commercial company for RNA extraction and RNA sequencing. Samples were analyzed with an Illumina HiSeq machine. Raw sequence files (fastq) for 27 samples were mapped to the genome with using salmon against the mouse transcripts described in genecode. Transcript counts were summarized to the gene level using tximport and normalized and tested for differential expression using DESeq2.

***Real-time polymerase chain reaction (RT-qPCR).*** RNA was extracted from BMMC pellets per manufacturer's instructions (Qiagen). Quantification and purity of RNA extracted was assessed via NanoDrop 2000c Spectrophotometer (Thermo Fisher). Reverse transcription and cDNA synthesis was performed on 200 ng of RNA

using Maxima First Strand cDNA synthesis kit (Thermo Fisher) and stored at -80°C. Taqman Gene Expression Assays were used for all target genes;  $\beta$ -actin (Mm02619580\_G1), BMP1a (Mm00477650\_m1), BMP2 (Mm00432134\_m1), Acvr1 (Mm01331069\_m1), and BMP1b (Mm03023971\_m1) (Applied Biosystems, Foster City, CA). Real time PCR was performed on 10 ng cDNA, with Taqman Fast Advanced Master Mix (Applied Biosystems). QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems) was used for thermal cycling at recommended settings. Results were normalized to  $\beta$ -actin and relative quantification was performed using  $2^{\Delta Ct}$  method.

***Apoptosis Analysis.*** Apoptosis was evaluated using a commercially available Annexin V apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ). BMMCs (1 x 10<sup>6</sup> cell/ml) and LAD2 cells (500,000 cell/ml) were treated with BMP7 or BMP receptor inhibitors for 24 hours at indicated dosages. Hydrogen peroxide was used as a positive control and for FMO controls. Staining was evaluated via flow cytometry and analyzed with FlowJo software (BD Bioscience).

***MTS cell proliferation analysis.*** Cellular proliferation was evaluated in cells treated with LPS/IgE, SP, BMP7, or BMP receptor inhibitors after 24-hour exposure. LAD2 cells (500,000 cell/ml) and BMMCs (1 x 10<sup>6</sup> cell/ml) were seeded into a 96 well plate and MTS/PMS was added per manufacturer's instructions (Promega, Madison, WI) and absorbance was read after 2 hours at 490 nm using a microplate reader.

***Evaluation of BMP ligands and receptors via Western blot.*** Naïve cell pellets were resuspended in 50  $\mu$ L of RIPA buffer supplemented with protease and phosphatase inhibitors. Lysate protein concentration was assessed with a commercially available kit per manufacturer's instructions (Thermo Fisher, Cat. No. A53226). Twenty

to thirty ug of protein sample, plus laemmli sample buffer was separated via SDS-PAGE and then transferred to a nitrocellulose membrane. The membranes were blocked with 5% BSA in TBS-T buffer for phosphorylated protein detection or 5% milk in TBS-T buffer for one hour at room temperature. Primary antibodies were incubated overnight at 4°C in 5% BSA in TBS-T buffer (1:1000 dilution, except for  $\beta$ -actin at 1:2000). The following morning the primary antibody was removed, and the membrane was washed three times with TBS-T. Respective secondary antibody (1:2000) was incubated at room temperature for 1 hr in 5% milk TBS-T buffer. The membrane was then washed three times and the protein bands were detected with chemiluminescent HRP substrate (Thermo Fisher). Densitometry was analyzed using Li-Cor Odyssey imager and Image studio software (Lincoln, NE).

***Evaluation of cell signaling pathways in association with TNF $\alpha$  production via Western blot.*** BMMCs were stimulated at a concentration of  $1 \times 10^6$  cells/ml and LAD2 cells were stimulated at a concentration of 500,000 cells/ml. Cells were stimulated with LPS/IgE or SP, respectively, at indicated times and dosages. Some experiments included pretreatment with BMP7 or BMP receptor inhibitors for indicated times and dosages in order to evaluate modulation of LPS/IgE and SP signaling resulting in TNF $\alpha$  release. Cell pellets were collected at twice the concentration of stimulation and were washed with PBS supplemented with protease and phosphatase inhibitors. Two million BMMCs or 1 million LAD2 cell pellets were resuspended in 50  $\mu$ L of RIPA buffer supplemented with protease and phosphatase inhibitors. Protein quantification, SDS-PAGE, and protein detection was performed in same manner as stated above.

**Statistical Analysis.** GraphPad Prism 8 (San Diego, CA, USA) was used for statistical analysis. Normality of data was assessed using Shapiro-Wilk test and data was analyzed using one-way analysis of variance with Tukey's multiple comparisons in normally distributed data, and by Kruskal-Wallis with Dunn's multiple comparisons in non-normally distributed data. Statistical significance was set at  $p < 0.05$ .

## **Results**

### ***Pre-treatment of mast cells with BMP7 results in decreased TNF $\alpha$ release, while not affecting degranulation.***

The potential immuno-modulatory effects of BMP7 treated mast cells is unknown; therefore, we first set out to determine if mast cells pretreated with BMP7 would affect the mast cell's ability to degranulate and/or release TNF $\alpha$ . Mast cells pretreated with BMP7 at indicated dosages for 24 hours prior to stimulation with IgE/DNP or SP resulted in no to very mild decrease in degranulation in both RBL-2H3 and LAD2 cells (Figure 2.1). Interestingly, cells pretreated with BMP7 at varying dosages for 24hrs with subsequent LPS or SP stimulation, resulted in significantly decreased TNF $\alpha$  release in both BMMCs and LAD2 cells (Figure 2.2). Additionally, BMP7 pretreatment resulted in a decrease in TNF $\alpha$  production in a dose-dependent manner. Prolonged exposure to BMP7, 72-hour pretreatment, also resulted in significantly decreased TNF $\alpha$  production (Figure 2.3).

Since BMP7 pretreatment did not result in a decrease of degranulation, but did decrease TNF $\alpha$  release, BMP7 effects appear to be more targeted and not due to cell death. Apoptosis and proliferation assays were performed and confirmed these thoughts. Apoptosis is not induced by BMP7 pretreatment in either BMMCs or LAD2

cells, as seen in Figure 2.4. Additionally, proliferation status appears unchanged with BMP7 treatment versus untreated. These results support that BMP7 treatment is not resulting in decreased TNF $\alpha$  release due to increased cell death. Taken together, the data support that BMP7 treatment is anti-inflammatory and affects the mast cell's ability to produce and/or release TNF $\alpha$ . The resultant decrease in TNF $\alpha$  may be attributed to change in mast cell phenotype, or effect on cell signaling pathways that result in TNF $\alpha$  production and release.

***BMP7 treatment appears to drive mast cell phenotype.***

Mast cells are generally accepted to have two main subtypes, or phenotypes, based on the mast cell proteases they contain. Human mucosal mast cells are primarily tryptase containing, where connective tissue mast cells have a mixture of all three proteases; tryptase, chymase, and carboxypeptidase A3 (37). This sub-classification can vary slightly between species but is relatively similar. Mouse mucosal mast cells are noted to primarily contain mast cell protease (mcpt)-1, and -2 (chymases) and mcpt-6 (tryptase); where connective tissue mast cells contain mast cell protease-4 (chymase), mcpt-6, and -7 (tryptase), and carboxypeptidase A3 (1, 38, 39). Human tryptase analogs in mice are mcpt-6, and -7; where chymase analogs are mcpt-1, -2, and -4 (40, 41). Due to these two possible phenotypes, we wanted to evaluate *in vitro* if BMP7 treated BMDCs drove a specific phenotype. As seen in Figure 2.5, BMP7 treatment prior to LPS stimulation results in significantly increased tryptase, mcpt1, and mcpt2; consistent with mucosal mast cells. These results support that specific mast cell phenotypes may be more anti-inflammatory, which is important to know for driving mast cell responses and could be used as a treatment target.

### ***Inflammatory stimuli upregulate BMP7 production.***

Mast cells pretreated with BMP7 have a specific effect, decreasing pro-inflammatory TNF $\alpha$  release, but our next question was if mast cells produce BMP7 themselves when in an inflammatory milieu, which may be a component of their beneficial roles. At baseline both BMMCs and LAD2 cells express BMP7 and BMMCs express BMP receptors (Figure 2.6). At baseline BMMCs do not express BMP1b and most highly express BMP2. Additionally, stimulation with pro-inflammatory cytokines caused an upregulation in BMP7 production in BMMCs (Figure 2.7). These results suggest that in an environment of inflammatory milieu, mast cells could be a source of BMP7, which then has the ability to activate BMP signaling in it or other surrounding cells. Since BMPs can bind to several different receptors and form heterotetrameric receptor complexes, this led us to investigate what happens to mast cell degranulation and TNF $\alpha$  secretion when BMP receptors are inhibited by use of chemical inhibitors. BMP7 is thought to interact with a BMP type 2 receptor and then recruit a type 1, but there has also been a report to support equal receptor affinity (42). Additionally, type 1 receptor affinity for BMP7 is said to be Acvr1, but can also engage Bmpr1a (43). BMP7 engagement of receptors is likely to be influenced by what receptors each cell type has; therefore, requires investigation for each cell type of interest.

### ***Acvr1 blockade results in decreased TNF $\alpha$ release.***

BMP ligands engage both a BMP type 1 and type 2 receptor for signaling activation. BMP type 2 receptor has been shown to phosphorylate the type 1 receptor, which then ultimately propagates the signal via canonical signaling, via Smad-1,5,8/9, or non-canonical signaling via TAK1, MAPKs, and NF $\kappa$ B. BMP7 is reported to use type 2

receptors: Bmpr2, Actr2, or Actr2b; and type 1 receptors: Acvr1 or Bmpr1b (44). Mast cells highly express Bmpr2 and do not express Bmpr1b; therefore, BMP7 likely engages Bmpr2 and Acvr1 in an ideal scenario. Chemical inhibition of BMP receptors can be done using several different molecules, which have varying affinity for specific BMP receptors, but all work by inhibiting the receptors kinase ability. We found that mast cells pretreated with BMP receptor inhibitors did not have significantly decreased degranulation, as would be expected based on BMP7 not affecting degranulation (Figure 2.8). Interestingly, as seen in Figure 2.9, the use of LDN-193189 or dorsomorphin alone, without BMP7, resulted in significantly decreased TNF $\alpha$  secretion upon stimulation in LAD2 cells versus other chemical inhibitors. This significant decrease in TNF $\alpha$  release was also seen in BMDCs pretreated with dorsomorphin or LDN-193189. LDN-193189, dorsomorphin, DMH1, and DMH2 target BMP type 1 receptors, while SB-431542 targets Tgf $\beta$  type 1 receptors. SB-431542 had a mild decrease in TNF $\alpha$  release, although not as pronounced as dorsomorphin or LDN-193189; therefore, the effect appears strongest with inhibition of BMP receptors. The BMP receptor chemical inhibitors have varying receptor affinities with highest affinities for LDN-193189 and dorsomorphin noted to be Acvr1 and Bmpr1b amongst the other inhibitors used (45). Taking into account receptor affinities, TNF $\alpha$  results, and that mast cells do not express Bmpr1b; it is likely the dramatic effect of LDN/dorsomorphin is due to their high affinity for Acvr1. The effect is not likely due to cell viability as apoptosis status is unchanged, and while proliferation states are mildly, variably affected, degranulation is normal at those dosages (Figure 2.10). Therefore, it is more likely that LDN/dorsomorphin effects are linked to changes in cell signaling pathways, which result in TNF $\alpha$  production and secretion, such as

MAPKs and NFkB. These pathways are also able to be activated by BMP ligands through non-canonical signaling; therefore it may be that Acvr1 activation is more closely linked to non-canonical signaling resulting in TNF $\alpha$  production, where canonical signaling via Smad-1,5,8/9 results in decreased TNF $\alpha$  production and release (Figure 2.11).

***Phospho-ERK1/2 is upregulated upon stimulation in BMMCs and LAD2 cells.***

In order to further evaluate how LDN-193189 and dorsomorphin are resulting in pronounced decrease in TNF $\alpha$  we first chose to evaluate some of the pathways associated with TNF $\alpha$  production, such as NFkB p65 subunit, p38, ERK1/2, and TAK1 to see what pathways are upregulated in our cells. Results showed that upon stimulation, in both BMMCs and LAD2 cells, ERK1/2 is upregulated, while BMMCs additionally had upregulation in p38 (Figure 2.12). Both p65 and TAK1 were unchanged at the time points evaluated.

***BMP7 and LDN-193189 decrease baseline p-p38 and induction of pERK1/2 in stimulated BMMCs.***

We further evaluated activation of these pathways with 72hr pretreatment of BMP7 or LDN-193189 to see if TNF $\alpha$  release was due to down regulation of these pathways. BMMCs had significant downregulation in both baseline of p-p38 and induction of pERK1/2 15 minutes post-stimulation, which was not seen in LAD2 cells (Figure 2.13). These results support that in BMMCs, BMP7 and LDN-193189 may be decreasing TNF $\alpha$  release via downregulating pathways, MAPKs, that can result in its production. The effect of BMP7 and LDN-193189 on LAD2 cells remains unknown. We chose to focus on a few key pathways, but other pathways not investigated may be

affected in LAD2 cells, or BMMCs, and warrant further investigation, such as PI3K, PKC, and Lyn (46-49). Another explanation for decreased TNF $\alpha$  detection in the supernatant is that the cells are unable to release TNF $\alpha$  from the storage vesicle, whether it be pre-formed or newly synthesized.

***LAD2 cells appear to primarily de novo synthesizes TNF $\alpha$ , while BMMCs have more stored TNF $\alpha$ .***

Mast cells can store preformed molecules in large cytoplasmic vesicles (granules); which are then released via exocytosis (degranulation). Mast cells can also newly synthesize molecules upon stimulation and form new secretory vesicles via the Golgi apparatus. Secretion of the *de novo* synthesized molecules entails vesicular trafficking and recycling, which can involve different signals and vesicular trafficking proteins versus degranulation. Acvr1 inhibition may result in the blockage of a signal that results in release of TNF $\alpha$  from its cytoplasmic vesicle. TNF $\alpha$  in mast cells can be pre-stored and *de novo* synthesized upon stimulation and therefore can be packaged and re-packaged into various types of vesicles. In our hands it appears the human mast cell line, LAD2, *de novo* synthesizes TNF $\alpha$  upon stimulation, while mouse BMMCs have a lot of TNF $\alpha$  pre-stored as shown by BMMC negative controls subject to forced degranulation have more TNF $\alpha$  versus LAD2 cells (Figure 2.14). This increased pre-stored TNF $\alpha$  may be associated with higher baseline p-p38 seen in our BMMCs versus little to no detection in LAD2 cells. We would expect if LDN-193189 treatment was resulting in a build-up of TNF $\alpha$  that was unable to be released and subsequently detected in the supernatant, the addition of A23187, a calcium ionophore, may result in increased release and detection of TNF $\alpha$ . Mast cell degranulation is largely reliant on

calcium; therefore A23187 is used as a positive control for mast cell degranulation as it causes an influx of calcium. A23187 treatment resulted in increased TNF $\alpha$  secretion in BMMCs versus its same treatment without A23187, and LDN-193189 pretreatment resulted in significantly lower TNF $\alpha$  suggesting BMMCs when stimulated increase TNF $\alpha$  production, but primarily release the TNF $\alpha$  that was prestored. This suggests that LDN-193189 treatment in BMMCs may affect both prestored and *de novo* synthesized TNF $\alpha$ . Similar results were not seen in LAD2 cells. LAD2 cells, upon A23187 treatment, resulted in similar TNF $\alpha$  release versus its same treatment without A23187 treatment, suggesting that LDN-193189 treatment has more of an effect on *de novo* synthesized TNF $\alpha$  or that TNF $\alpha$  in LAD2 cells are in different secretory vesicles not sensitive to A23187 treatment.

## Discussion

Our findings support a dual role of BMP signaling in the ability to modulate mast cell release of TNF $\alpha$ . The treatment of mast cells with BMP7 or LDN-193189 (Acvr1 inhibitor) prior to stimulation with pro-inflammatory molecules resulted in decreased TNF $\alpha$  release while not affecting mast cell degranulation. Pathways associated with TNF $\alpha$  production upon LPS or SP stimulation, such as NF $\kappa$ B, TAK1, and MAPKs (including p38 and ERK1/2) were evaluated as the possible mechanism for the decreased TNF $\alpha$  release. Of the pathways we investigated, in both BMMCs and LAD2s, it appears there are different effects of BMP7 and LDN-193189 in each cell type. BMP7 and LDN-193189 pretreatment was noted to decrease baseline p-p38 and post-stimulation p-ERK1/2 in BMMCs; which may be the mechanism(s) by which less TNF $\alpha$  release was detected in these cells. LAD2 cells did not have any modulation of the

pathways investigated with BMP7 or LDN-193189 pretreatment. Other pathways in LAD2 cells may be affected by BMP7 or LDN-193189 pretreatment that were not investigated here, or physical release of TNF $\alpha$  from storage vesicles may be affected and warrant further investigation.

In order to modulate the BMP pathway, we utilized BMP receptor chemical inhibitors that work by binding to and blocking the receptor's serine/threonine kinase ability. Unexpectedly, treatment with BMP receptor chemical inhibitors alone resulted in decreased TNF $\alpha$  release without affecting degranulation. Of note, specific inhibitors appeared to inhibit the TNF $\alpha$  release better than others and of those that were most affective, LDN-193189 and dorsomorphin, these have the highest affinity for Acvr1. Mast cells that were genetically engineered to have a gain-of-function mutation in Acvr1, that is ligand-independent and ligand-responsive have been noted to have an increase in pro-inflammatory cytokine production upon stimulation with SP and BMP4; therefore blocking Acvr1 may not be surprising that it results in a decrease in TNF $\alpha$  release (31). What was slightly surprising was there was not an exogenous source of BMPs applied in our scenario, only LPS or SP for BMMC or human LAD2 cell stimulation, respectively. This suggests possible low-level constitutive release of BMPs and/or Acvr1-mediated BMP pathway activation, or crosstalk between the BMP pathway and LPS/SP signaling pathways.

Our results also suggest the synthesis and storage of TNF $\alpha$  in human LAD2 cells versus mouse BMDCs is slightly different based on forced degranulation utilizing A23187. LAD2 cells appear to make their TNF $\alpha$  *de novo* upon stimulation with SP, while BMDCs have more stored, with the addition of *de novo* synthesis upon stimulation with

LPS. BMMCs pretreated with LDN-193189, with no other stimulation, had less TNF $\alpha$  stored in their granules versus negative control which may be attributed to the decreased p-p38 at baseline seen on western blot. Pretreatment with LDN-193189, with no other stimulation, in LAD2 cells did not result in decreased TNF $\alpha$  versus negative control likely due to the fact that they primarily *de novo* synthesizes TNF $\alpha$ . The mechanism by which the cells store their TNF $\alpha$  is important in further deciphering the mechanism of action of both BMP7 and LDN-193189. Release of specific molecules from their associated vesicle is suggested to involve different machinery in mast cells, such as VAMP-8 involved in the release of  $\beta$ -hexosaminidase and cathepsin D; and VAMP-3 involved with release of TNF $\alpha$  and histamine (50, 51). Further evaluation of TNF $\alpha$  release in association with BMP7 and LDN-193189 may identify an additional mechanism of BMP related modulation of immune cells. Pathways modulated by BMP signaling may aid in the activation and mobilization of SNARE and VAMP proteins needed for vesicle movement to the plasma membrane. Both ERK1/2 and NF $\kappa$ B signaling has been previously indicated to be important for activation of machinery needed for vesicle movement and physical release of TNF $\alpha$  in mast cells (52, 53).

BMPs are known to induce the production of a group of proteins referred to as inhibitors of DNA binding and cell differentiation (Id) which are able to modulate the transcription of genes important in cell proliferation and differentiation. BMPs have been noted to induce these in several different cell types such as smooth muscle cells, stem cells, and B cells (54-58). Importantly these Id proteins also have roles in immune cell functions and differentiation, such as promoting T regulatory cells, controlling CD8<sup>+</sup> and CD4<sup>+</sup> T cell subsets and effector functions, and B cell lymphopoiesis (59-65). It is

unknown if these proteins are induced in mast cells upon BMP ligand treatment or what their roles could be in mast cell functions. Induction of these proteins may be related to TNF $\alpha$  production and/or release and warrant further investigation.

Confirmation of what receptors BMP7 is binding to on mast cells would aid in clarification of the mast cell – BMP axis. Specific BMP ligand - receptor engagement may result in different signaling, with specific ones resulting in canonical versus non-canonical signaling, or a mix thereof. Mast cells in inflammatory milieu upregulate BMP7, which may be released and lead to autocrine/paracrine BMP signaling that results in modulation in mast cell functions. When a specific BMP receptor is blocked, such as with Acvr1, BMP7 may be engaging Bmpr2 and Bmpr1a instead, which avoids activation of non-canonical BMP signaling through Acvr1 and only results in canonical signaling, which is thought to be anti-inflammatory. Ultimately, it may be the specific BMP pathway activated, canonical versus non-canonical, that has the ability to modulate downstream inflammatory actions, such as the production and/or release of TNF $\alpha$ .

Signaling pathways in mast cells that result in TNF $\alpha$  production have been studied more thoroughly in mouse BMMCs versus human LAD2 cells. Pathways upregulated upon stimulation with SP in LAD2 cells has been previously investigated, but in conjunction with IL33 stimulation and only select components of the NF $\kappa$ B pathway were evaluated (66). LAD2 stimulation with SP and/or IL33 resulted in changes in the NF $\kappa$ B pathway, specifically phosphorylation of I $\kappa$ B- $\alpha$ , seen only with IL33 alone. Additionally, TNF $\alpha$  release post-SP stimulation was 50% decreased when NK-1R was blocked, suggesting SP is recognized by other receptors. NK-1R binding of

SP is noted to lead to NFκB, MAPKs, and PI3K activation, and more recently SP has been noted to bind to Mrgprx2 (mouse homolog, Mrgprb2) and not solely NK-1R (67-70). Activation of the Mrgprx2 receptor can lead to calcium mobilization important for degranulation, but also to PI3K activation and subsequent MAPK activation; therefore other pathways besides NFκB may be activated that subsequently lead to TNFα production in LAD2 cells (71). Our results support activation of ERK1/2 signaling pathway in SP stimulated LAD2 cells. Stimulation of BMMCs with LPS has been extensively investigated and several pathways have been indicated in TNFα production. Initially TLR4 signaling in mast cells was thought to only be MyD88-dependent, which did not require receptor complex internalization, but receptor complex internalization may be required for activation of specific pathways. BMMC stimulation with LPS can lead to NFκB; MAPKs, such as p38 and ERK1/2; and PI3K activation (46, 52, 72-74). Additionally, intracellular trafficking of the TLR4 complex is important, perhaps more so for ERK1/2 activation (52). Our results in BMMCs support activation of ERK1/2 and p38 in BMMCs upon stimulation with LPS. Further investigation of additional pathways, such as PI3K is required, especially in LAD2 cells, as our data here has not identified pathways modulated by BMP7 or LDN-193189.

Ultimately the mast cell – BMP axis needs to be furthered evaluated *in vivo*. Global knockouts of components of the BMP family are typically embryonically lethal, although targeted deletion of BMP ligands and/or receptors is possible and is currently being pursued in our laboratory. Mast cell's high expression of BMP2 and the fact that it is a gene expression marker that separates it from basophils is a particularly interesting area of further investigation. Defining the requirement for BMP2 in mast

cell functions will aid in clarifying it as a target of modulation in diseases where the mast cell is involved, such as anaphylaxis, allergies, colitis, etc.

A limitation of this study is the inability to easily compare LPS stimulation in both BMMCs and LAD2 cells. LAD2 cells are minimally, to not responsive to LPS (75). We chose to focus on LPS with the BMMCs because of the desire to relate to what would be encountered in the colon during colitis, although SP is also upregulated during inflammation. Future experiments may include other human derived mast cells, such as embryonic cord blood, and/or the use of SP in BMMCs.

In conclusion our results support a dual role of the BMP – mast cell axis in the regulation of inflammation (Figure 2.15). BMP7 treatment and Acvr1 blockade both resulted in decreased TNF $\alpha$  release upon stimulation with pro-inflammatory molecules. In BMMCs, it is suggested here that BMP7 and Acvr1 blockade with LDN-193189 results in the reduction of signaling via the p38 and ERK1/2 pathway. Further investigation is required to determine the mechanism of action in LAD2 cells. Modulation of cell signaling pathways may be associated with both a decrease in TNF $\alpha$  production and release of TNF $\alpha$  from its storage vesicle. Ultimately these results further help to define potential anti-inflammatory mechanisms of the BMP – mast cell axis, which may help to explain mast cell's beneficial roles in chronic inflammation and identify novel therapeutic targets.

## LIST OF REFERENCES

1. Kitamura Y. Heterogeneity of mast cells and phenotypic change between subpopulations. *Annual review of immunology*. 1989;7:59-76.
2. Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol*. 2002;2(10):773-86.
3. Galli SJ, Zsebo KM, Geissler EN. The kit ligand, stem cell factor. *Advances in immunology*. 1994;55:1-96.
4. Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiological reviews*. 1997;77(4):1033-79.
5. Vines CM, Prossnitz ER. Mechanisms of G protein-coupled receptor-mediated degranulation. *FEMS microbiology letters*. 2004;236(1):1-6.
6. Gommerman JL, Oh DY, Zhou X, Tedder TF, Maurer M, Galli SJ, et al. A role for CD21/CD35 and CD19 in responses to acute septic peritonitis: a potential mechanism for mast cell activation. *Journal of immunology (Baltimore, Md : 1950)*. 2000;165(12):6915-21.
7. Malaviya R, Ross EA, MacGregor JI, Ikeda T, Little JR, Jakschik BA, et al. Mast cell phagocytosis of FimH-expressing enterobacteria. *Journal of immunology (Baltimore, Md : 1950)*. 1994;152(4):1907-14.
8. Supajatura V, Ushio H, Nakao A, Akira S, Okumura K, Ra C, et al. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *The Journal of clinical investigation*. 2002;109(10):1351-9.
9. Orinska Z, Bulanov E, Budagian V, Metz M, Maurer M, Bulfone-Paus S. TLR3-induced activation of mast cells modulates CD8+ T-cell recruitment. *Blood*. 2005;106(3):978-87.
10. Heib V, Becker M, Warger T, Rechtsteiner G, Tertilt C, Klein M, et al. Mast cells are crucial for early inflammation, migration of Langerhans cells, and CTL responses following topical application of TLR7 ligand in mice. *Blood*. 2007;110(3):946-53.
11. Nakamura Y, Kambe N, Saito M, Nishikomori R, Kim YG, Murakami M, et al. Mast cells mediate neutrophil recruitment and vascular leakage through the NLRP3 inflammasome in histamine-independent urticaria. *The Journal of experimental medicine*. 2009;206(5):1037-46.
12. Frandji P, Oskeritzian C, Cacaraci F, Lapeyre J, Peronet R, David B, et al. Antigen-dependent stimulation by bone marrow-derived mast cells of MHC class II-restricted T cell hybridoma. *Journal of immunology (Baltimore, Md : 1950)*. 1993;151(11):6318-28.
13. Fox CC, Jewell SD, Whitacre CC. Rat peritoneal mast cells present antigen to a PPD-specific T cell line. *Cellular immunology*. 1994;158(1):253-64.
14. Poncet P, Arock M, David B. MHC class II-dependent activation of CD4+ T cell hybridomas by human mast cells through superantigen presentation. *Journal of leukocyte biology*. 1999;66(1):105-12.
15. Stelekati E, Bahri R, D'Orlando O, Orinska Z, Mittrucker HW, Langenhaun R, et al. Mast cell-mediated antigen presentation regulates CD8+ T cell effector functions. *Immunity*. 2009;31(4):665-76.

16. Morita H, Arae K, Unno H, Miyauchi K, Toyama S, Nambu A, et al. An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers. *Immunity*. 2015;43(1):175-86.
17. Waern I, Lundequist A, Pejler G, Wernersson S. Mast cell chymase modulates IL-33 levels and controls allergic sensitization in dust-mite induced airway inflammation. *Mucosal immunology*. 2013;6(5):911-20.
18. He Z, Song J, Hua J, Yang M, Ma Y, Yu T, et al. Mast cells are essential intermediaries in regulating IL-33/ST2 signaling for an immune network favorable to mucosal healing in experimentally inflamed colons. *Cell death & disease*. 2018;9(12):1173.
19. Lennon EM, Borst LB, Edwards LL, Moeser AJ. Mast Cells Exert Anti-Inflammatory Effects in an IL10(-/-) Model of Spontaneous Colitis. *Mediators of inflammation*. 2018;2018:7817360.
20. Chichlowski M, Westwood GS, Abraham SN, Hale LP. Role of mast cells in inflammatory bowel disease and inflammation-associated colorectal neoplasia in IL-10-deficient mice. *PloS one*. 2010;5(8):e12220.
21. Zhang H, Xue Y, Wang H, Huang Y, Du M, Yang Q, et al. Mast cell deficiency exacerbates inflammatory bowel symptoms in interleukin-10-deficient mice. *World journal of gastroenterology*. 2014;20(27):9106-15.
22. Kingsley DM. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes & development*. 1994;8(2):133-46.
23. Lu L, Ma J, Wang X, Wang J, Zhang F, Yu J, et al. Synergistic effect of TGF-beta superfamily members on the induction of Foxp3+ Treg. *European journal of immunology*. 2010;40(1):142-52.
24. Varas A, Valencia J, Lavocat F, Martínez VG, Thiam NN, Hidalgo L, et al. Blockade of bone morphogenetic protein signaling potentiates the pro-inflammatory phenotype induced by interleukin-17 and tumor necrosis factor- $\alpha$  combination in rheumatoid synoviocytes. *Arthritis research & therapy*. 2015;17(1):192.
25. Singla DK, Singla R, Wang J. BMP-7 Treatment Increases M2 Macrophage Differentiation and Reduces Inflammation and Plaque Formation in Apo E<sup>-/-</sup> Mice. *PloS one*. 2016;11(1):e0147897.
26. Hao Z, Yang X, Lv Y, Li S, Purbey BK, Su H. Intracolonicly administered adeno-associated virus-bone morphogenetic protein-7 ameliorates dextran sulphate sodium-induced acute colitis in rats. *The journal of gene medicine*. 2012;14(7):482-90.
27. Stumm CL, Halcsik E, Landgraf RG, Camara NO, Sogayar MC, Jancar S. Lung remodeling in a mouse model of asthma involves a balance between TGF-beta1 and BMP-7. *PloS one*. 2014;9(4):e95959.
28. Duangkumpha K, Techasen A, Loilome W, Namwat N, Thanan R, Khuntikeo N, et al. BMP-7 blocks the effects of TGF- $\beta$ -induced EMT in cholangiocarcinoma. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2014;35(10):9667-76.
29. Hong JH, Lee GT, Lee JH, Kwon SJ, Park SH, Kim SJ, et al. Effect of bone morphogenetic protein-6 on macrophages. *Immunology*. 2009;128(1 Suppl):e442-50.

30. Motakis E, Guhl S, Ishizu Y, Itoh M, Kawaji H, de Hoon M, et al. Redefinition of the human mast cell transcriptome by deep-CAGE sequencing. *Blood*. 2014;123(17):e58-67.
31. Convente MR, Chakkalakal SA, Yang E, Caron RJ, Zhang D, Kambayashi T, et al. Depletion of Mast Cells and Macrophages Impairs Heterotopic Ossification in an Acvr1(R206H) Mouse Model of Fibrodysplasia Ossificans Progressiva. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2018;33(2):269-82.
32. Dwyer DF, Barrett NA, Austen KF. Expression profiling of constitutive mast cells reveals a unique identity within the immune system. *Nature immunology*. 2016;17(7):878-87.
33. Miyazono K, Maeda S, Imamura T. BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine & growth factor reviews*. 2005;16(3):251-63.
34. Zwijsen A, Verschuere K, Huylebroeck D. New intracellular components of bone morphogenetic protein/Smad signaling cascades. *FEBS letters*. 2003;546(1):133-9.
35. Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. Two major Smad pathways in TGF-beta superfamily signalling. *Genes to cells : devoted to molecular & cellular mechanisms*. 2002;7(12):1191-204.
36. Guo X, Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell research*. 2009;19(1):71-88.
37. Irani AM, Schwartz LB. Human mast cell heterogeneity. *Allergy proceedings : the official journal of regional and state allergy societies*. 1994;15(6):303-8.
38. Welle M. Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. *Journal of leukocyte biology*. 1997;61(3):233-45.
39. Xing W, Austen KF, Gurish MF, Jones TG. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(34):14210-5.
40. Hallgren J, Pejler G. Biology of mast cell tryptase. An inflammatory mediator. *The FEBS journal*. 2006;273(9):1871-95.
41. Hellman L, Thorpe M. Granule proteases of hematopoietic cells, a family of versatile inflammatory mediators - an update on their cleavage specificity, in vivo substrates, and evolution. *Biological chemistry*. 2014;395(1):15-49.
42. de Caestecker M. The transforming growth factor-beta superfamily of receptors. *Cytokine & growth factor reviews*. 2004;15(1):1-11.
43. Heinecke K, Seher A, Schmitz W, Mueller TD, Sebald W, Nickel J. Receptor oligomerization and beyond: a case study in bone morphogenetic proteins. *BMC biology*. 2009;7:59.
44. Macías-Silva M, Hoodless PA, Tang SJ, Buchwald M, Wrana JL. Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *The Journal of biological chemistry*. 1998;273(40):25628-36.

45. Augeri DJ, Langenfeld E, Castle M, Gilleran JA, Langenfeld J. Inhibition of BMP and of TGF $\beta$  receptors downregulates expression of XIAP and TAK1 leading to lung cancer cell death. *Molecular cancer*. 2016;15:27.
46. Hochdorfer T, Kuhny M, Zorn CN, Hendriks RW, Vanhaesebroeck B, Bohnacker T, et al. Activation of the PI3K pathway increases TLR-induced TNF-alpha and IL-6 but reduces IL-1beta production in mast cells. *Cellular signalling*. 2011;23(5):866-75.
47. Avila M, Martinez-Juarez A, Ibarra-Sanchez A, Gonzalez-Espinosa C. Lyn kinase controls TLR4-dependent IKK and MAPK activation modulating the activity of TRAF-6/TAK-1 protein complex in mast cells. *Innate immunity*. 2012;18(4):648-60.
48. Abdel-Raheem IT, Hide I, Yanase Y, Shigemoto-Mogami Y, Sakai N, Shirai Y, et al. Protein kinase C-alpha mediates TNF release process in RBL-2H3 mast cells. *British journal of pharmacology*. 2005;145(4):415-23.
49. Li G, Lucas JJ, Gelfand EW. Protein kinase C alpha, betaI, and betaII isozymes regulate cytokine production in mast cells through MEKK2/ERK5-dependent and -independent pathways. *Cellular immunology*. 2005;238(1):10-8.
50. Tiwari N, Wang CC, Brochetta C, Ke G, Vita F, Qi Z, et al. VAMP-8 segregates mast cell-preformed mediator exocytosis from cytokine trafficking pathways. *Blood*. 2008;111(7):3665-74.
51. Puri N, Roche PA. Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(7):2580-5.
52. Pérez-Rodríguez MJ, Ibarra-Sánchez A, Román-Figueroa A, Pérez-Severiano F, González-Espinosa C. Mutant Huntingtin affects toll-like receptor 4 intracellular trafficking and cytokine production in mast cells. *Journal of neuroinflammation*. 2020;17(1):95.
53. Madera-Salcedo IK, Cruz SL, Gonzalez-Espinosa C. Morphine prevents lipopolysaccharide-induced TNF secretion in mast cells blocking I $\kappa$ B kinase activation and SNAP-23 phosphorylation: correlation with the formation of a  $\beta$ -arrestin/TRAF6 complex. *Journal of immunology (Baltimore, Md : 1950)*. 2013;191(6):3400-9.
54. Yang J, Li X, Li Y, Southwood M, Ye L, Long L, et al. Id proteins are critical downstream effectors of BMP signaling in human pulmonary arterial smooth muscle cells. *American journal of physiology Lung cellular and molecular physiology*. 2013;305(4):L312-21.
55. Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell*. 2003;115(3):281-92.
56. Kersten C, Sivertsen EA, Hystad ME, Forfang L, Smeland EB, Myklebust JH. BMP-6 inhibits growth of mature human B cells; induction of Smad phosphorylation and upregulation of Id1. *BMC immunology*. 2005;6:9.
57. Kowanetz M, Valcourt U, Bergström R, Heldin CH, Moustakas A. Id2 and Id3 define the potency of cell proliferation and differentiation responses to transforming growth factor beta and bone morphogenetic protein. *Molecular and cellular biology*. 2004;24(10):4241-54.

58. Mody AA, Wordinger RJ, Clark AF. Role of ID Proteins in BMP4 Inhibition of Profibrotic Effects of TGF- $\beta$ 2 in Human TM Cells. *Investigative ophthalmology & visual science*. 2017;58(2):849-59.
59. Liu C, Wang HC, Yu S, Jin R, Tang H, Liu YF, et al. Id1 expression promotes T regulatory cell differentiation by facilitating TCR costimulation. *Journal of immunology* (Baltimore, Md : 1950). 2014;193(2):663-72.
60. Rauch KS, Hils M, Lupar E, Minguet S, Sigvardsson M, Rottenberg ME, et al. Id3 Maintains Foxp3 Expression in Regulatory T Cells by Controlling a Transcriptional Network of E47, Spi-B, and SOCS3. *Cell reports*. 2016;17(11):2827-36.
61. Yang CY, Best JA, Knell J, Yang E, Sheridan AD, Jesionek AK, et al. The transcriptional regulators Id2 and Id3 control the formation of distinct memory CD8+ T cell subsets. *Nature immunology*. 2011;12(12):1221-9.
62. Knell J, Best JA, Lind NA, Yang E, D'Cruz LM, Goldrath AW. Id2 influences differentiation of killer cell lectin-like receptor G1(hi) short-lived CD8+ effector T cells. *Journal of immunology* (Baltimore, Md : 1950). 2013;190(4):1501-9.
63. Lin YY, Jones-Mason ME, Inoue M, Lasorella A, Iavarone A, Li QJ, et al. Transcriptional regulator Id2 is required for the CD4 T cell immune response in the development of experimental autoimmune encephalomyelitis. *Journal of immunology* (Baltimore, Md : 1950). 2012;189(3):1400-5.
64. Nakatsukasa H, Zhang D, Maruyama T, Chen H, Cui K, Ishikawa M, et al. The DNA-binding inhibitor Id3 regulates IL-9 production in CD4(+) T cells. *Nature immunology*. 2015;16(10):1077-84.
65. Cochrane SW, Zhao Y, Perry SS, Urbaniak T, Sun XH. Id1 has a physiological role in regulating early B lymphopoiesis. *Cellular & molecular immunology*. 2011;8(1):41-9.
66. Taracanova A, Alevizos M, Karagkouni A, Weng Z, Norwitz E, Conti P, et al. SP and IL-33 together markedly enhance TNF synthesis and secretion from human mast cells mediated by the interaction of their receptors. *Proceedings of the National Academy of Sciences of the United States of America*. 2017;114(20):E4002-e9.
67. Garcia-Recio S, Gascón P. Biological and Pharmacological Aspects of the NK1-Receptor. *BioMed research international*. 2015;2015:495704.
68. Guhl S, Lee HH, Babina M, Henz BM, Zuberbier T. Evidence for a restricted rather than generalized stimulatory response of skin-derived human mast cells to substance P. *Journal of neuroimmunology*. 2005;163(1-2):92-101.
69. Azimi E, Reddy VB, Shade KC, Anthony RM, Talbot S, Pereira PJS, et al. Dual action of neurokinin-1 antagonists on Mas-related GPCRs. *JCI insight*. 2016;1(16):e89362.
70. Fujisawa D, Kashiwakura J, Kita H, Kikukawa Y, Fujitani Y, Sasaki-Sakamoto T, et al. Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticaria. *The Journal of allergy and clinical immunology*. 2014;134(3):622-33.e9.
71. Subramanian H, Gupta K, Ali H. Roles of Mas-related G protein-coupled receptor X2 on mast cell-mediated host defense, pseudoallergic drug reactions, and chronic inflammatory diseases. *The Journal of allergy and clinical immunology*. 2016;138(3):700-10.

72. Sandig H, Bulfone-Paus S. TLR signaling in mast cells: common and unique features. *Frontiers in immunology*. 2012;3:185.
73. Hochdorfer T, Tiedje C, Stumpo DJ, Blackshear PJ, Gaestel M, Huber M. LPS-induced production of TNF-alpha and IL-6 in mast cells is dependent on p38 but independent of TTP. *Cellular signalling*. 2013;25(6):1339-47.
74. Poplutz M, Levikova M, Luscher-Firzlaff J, Lesina M, Algul H, Luscher B, et al. Endotoxin tolerance in mast cells, its consequences for IgE-mediated signalling, and the effects of BCL3 deficiency. *Scientific reports*. 2017;7(1):4534.
75. Kubo Y, Fukuishi N, Yoshioka M, Kawasoe Y, Iriguchi S, Imajo N, et al. Bacterial components regulate the expression of Toll-like receptor 4 on human mast cells. *Inflammation research : official journal of the European Histamine Research Society [et al]*. 2007;56(2):70-5.

## **APPENDIX**

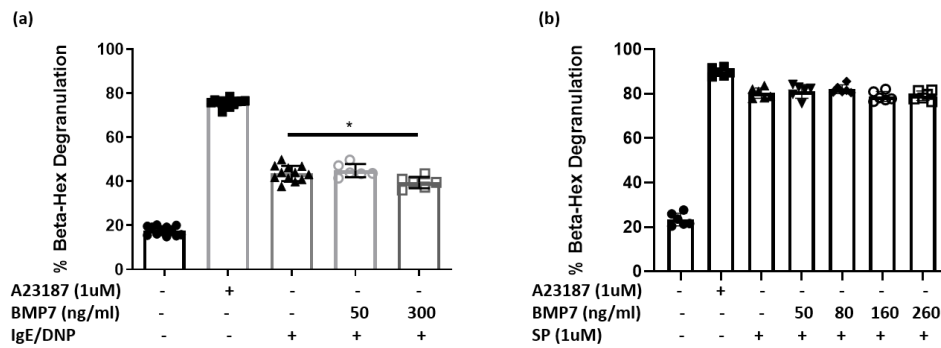


**Figure 2.1. BMP7 pretreated mast cells have normal degranulation.** Two mast cell lines, (a) RBL-2H3 (rat) and (b) LAD2 (human) are minimally to not affected in the ability to degranulate determined by  $\beta$ -hexosaminidase release with pretreatment of BMP7. RBL-2H3 and LAD2 cells were pretreated with BMP7 at indicated dosages for 24 hours and subsequently stimulated for 2 hours with A23187 (positive control), IgE/DNP, or SP and degranulation evaluated. Cells stimulated with IgE/DNP were pretreated with IgE at 0.5 ug/ml 24hrs prior to exposure to DNP at 100ng/ml. Data is displayed as mean with standard deviation. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < .001$ ; \*\*\*\*  $p < 0.0001$ .

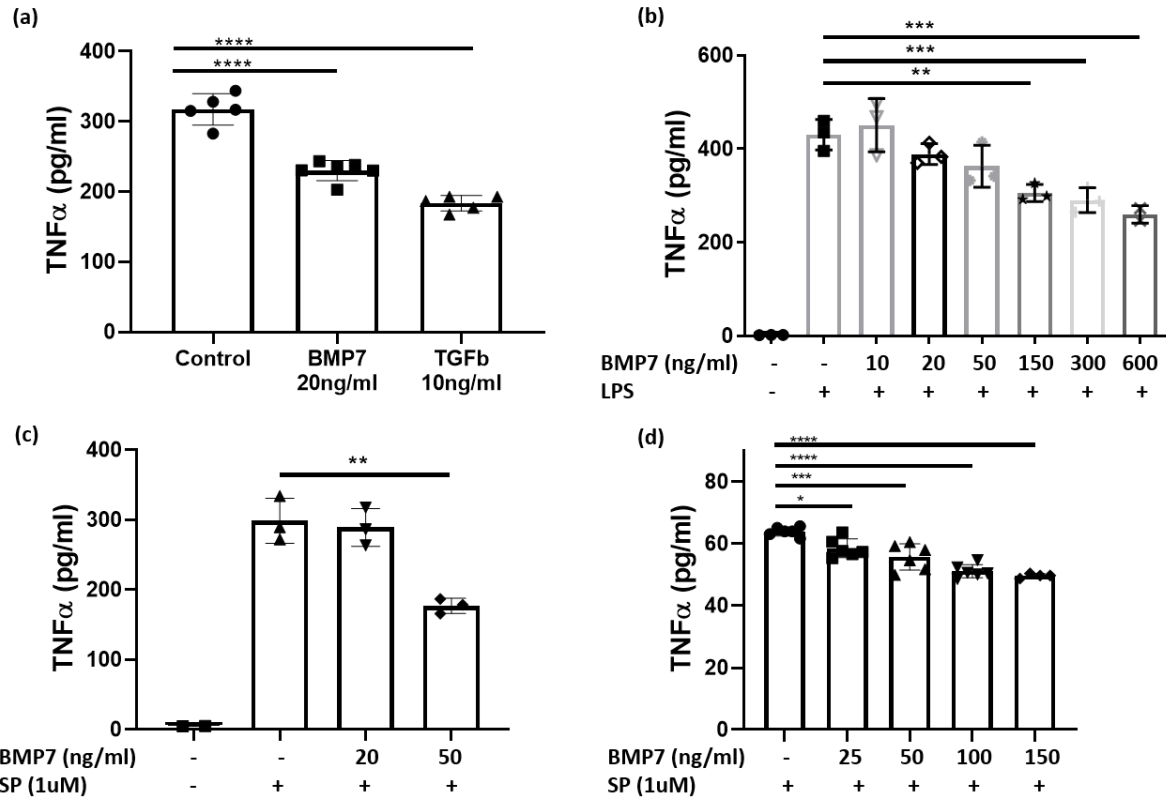


**Figure 2.2. BMP7 pretreated mast cells significantly decrease TNFα release upon stimulation.** Two mast cell lines, (a) & (b) BMMCs (mouse) and (c) & (d) LAD2 (human), have significantly decreased TNFα secretion upon stimulation when pretreated for 24 hours with BMP7. Cells were pretreated with BMP7 at  $1 \times 10^6$  cell/ml for BMMCs and 500,000 cell/ml for LAD2 for 24 hours and subsequently stimulated for 16 to 24 hours with LPS (0.5 ug/ml) or SP (1 uM), respectively. Tgfβ1 pretreatment was used as a positive control in BMMCs. Data is displayed as mean with standard deviation. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

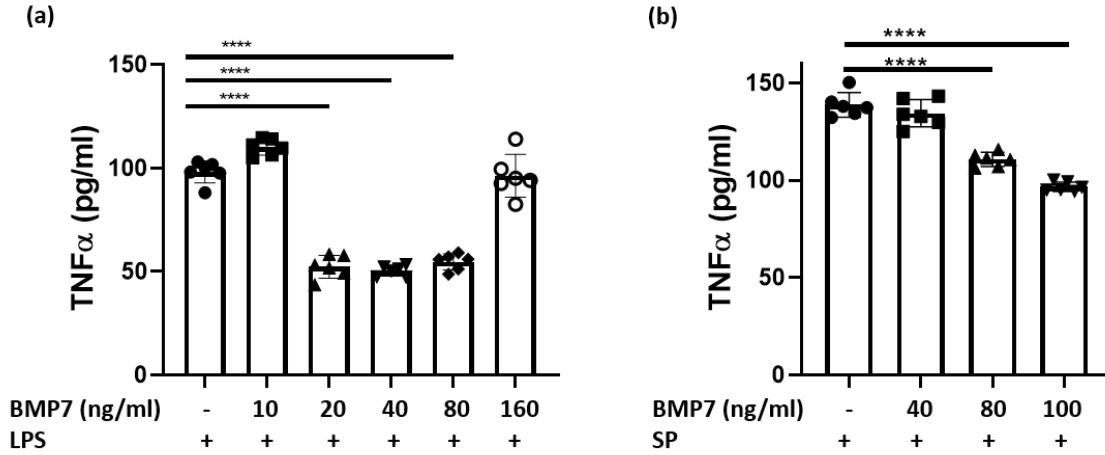


**Figure 2.3. Mast cells treated for 72 hours with BMP7 have significantly decreased TNFα release upon stimulation.** Two mast cell lines, (a) BMMCs (mouse) and (b) LAD2 (human), have significantly decreased TNFα secretion upon stimulation when pretreated for 72 hours with BMP7. Cells were pretreated with BMP7 at  $1 \times 10^6$  cell/ml for BMMCs and 500,000 cell/ml for LAD2 for 72 hours and subsequently stimulated for 16 to 24 hours with LPS (0.5 ug/ml) or SP (1 uM), respectively. Data is displayed as mean with standard deviation. \*,  $p<0.05$ ; \*\*,  $p<0.01$ ; \*\*\*,  $p<0.001$ ; \*\*\*\*  $p<0.0001$ .

(a)

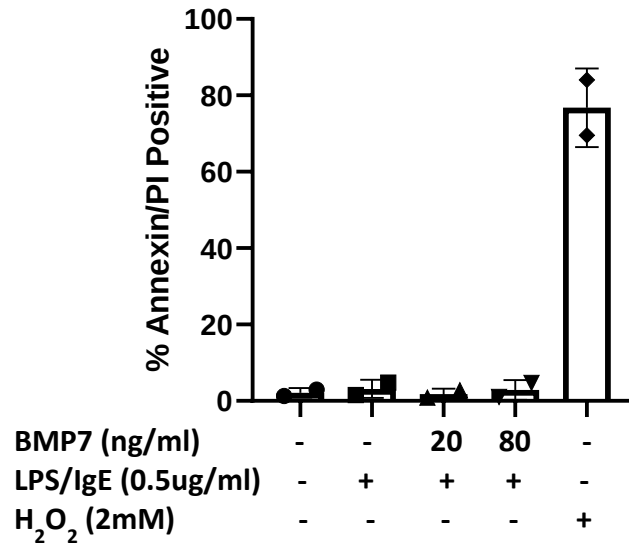
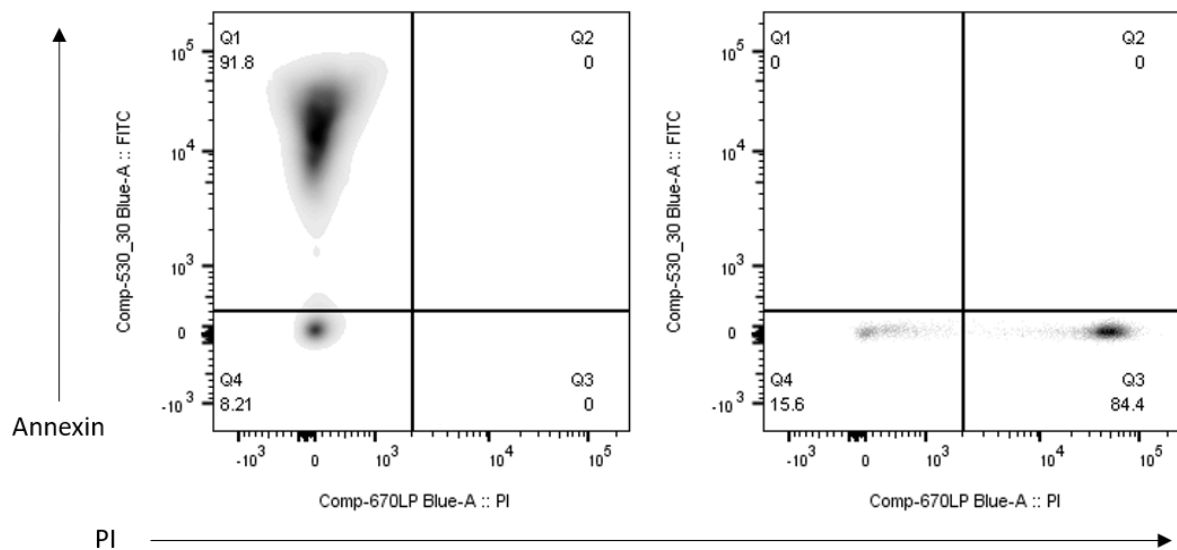


**Figure 2.4. Mast cells treated with BMP7 have stable apoptosis and proliferation status.** (a) & (b) Mouse BMMCs pretreated for 24hrs with BMP7, with secondary LPS/IgE stimulation do not have an increase in apoptotic cells. (c) BMMCs stimulated for 24hrs with BMP7 do not have a significant change in cellular proliferation as compared to the treatment with LPS/IgE. (d) & (e) Human mast cells, LAD2, pretreated for 24hrs with BMP7, with secondary SP stimulation do not have an increase in apoptotic cells. (f) LAD2 cells stimulated for 24hrs with BMP7 have no change in cellular proliferation. Hydrogen peroxide was applied to the cells for about 2-4 hours at concentrations indicated as a positive control for apoptosis analysis. Data is displayed as mean with standard deviation. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .

**(b)**  
**FMO controls:**



**Experiment examples:**

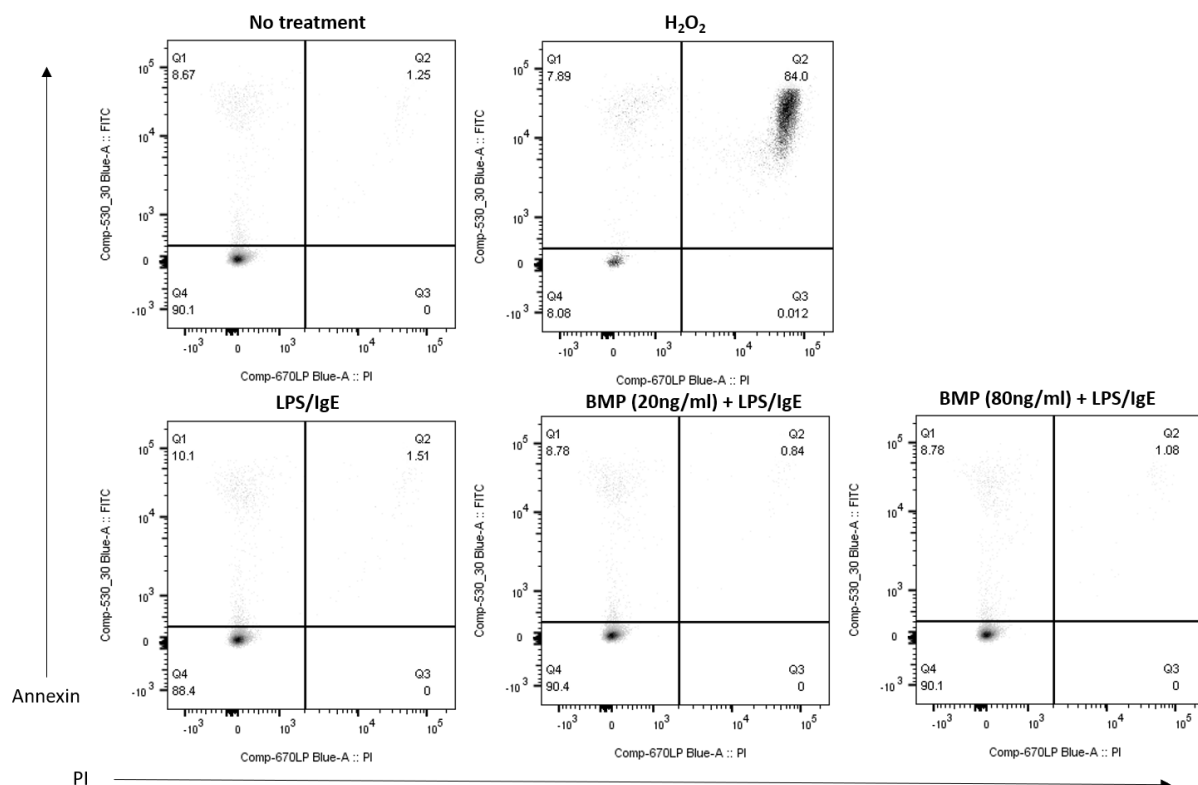
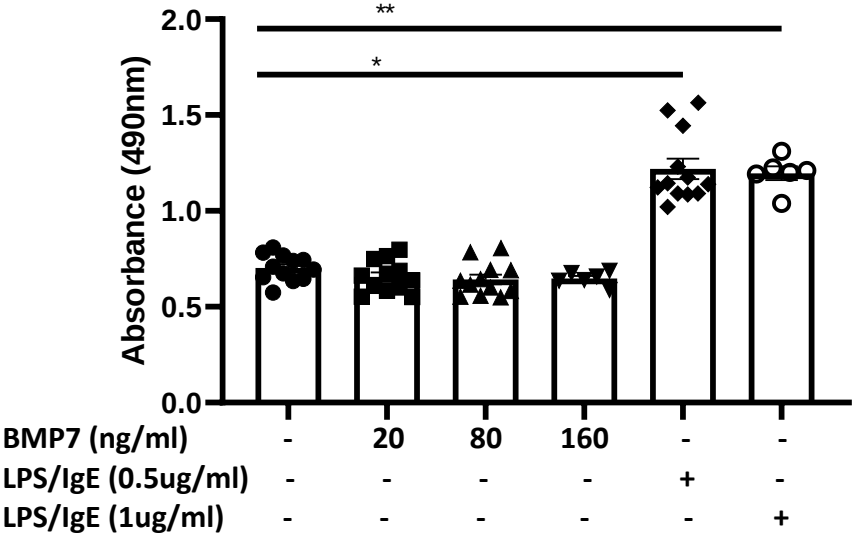


**Figure 2.4 continued**

(c)



(d)

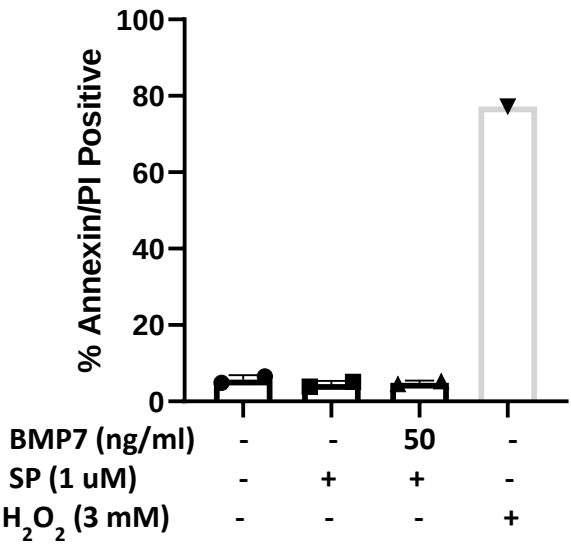


Figure 2.4 continued

**(e)**  
**FMO controls:**

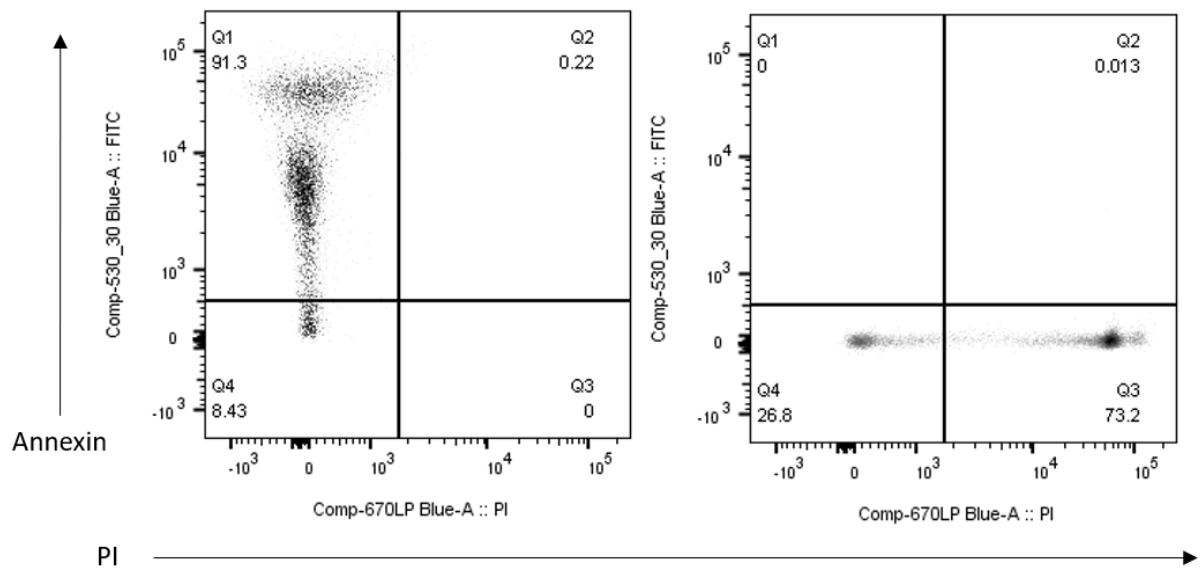


**Figure 2.4 continued**

Experiment examples:

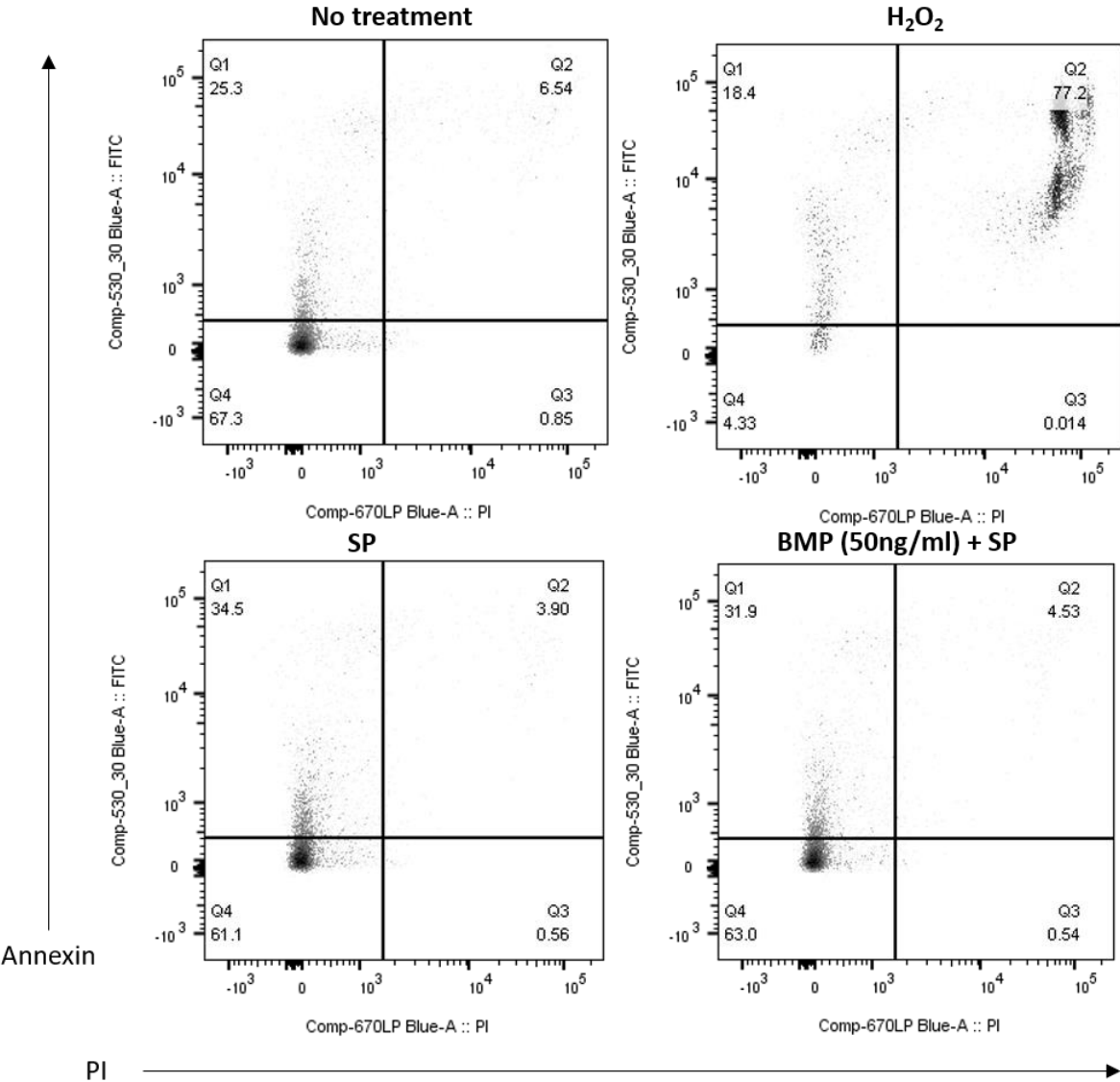


Figure 2.4 continued

(f)

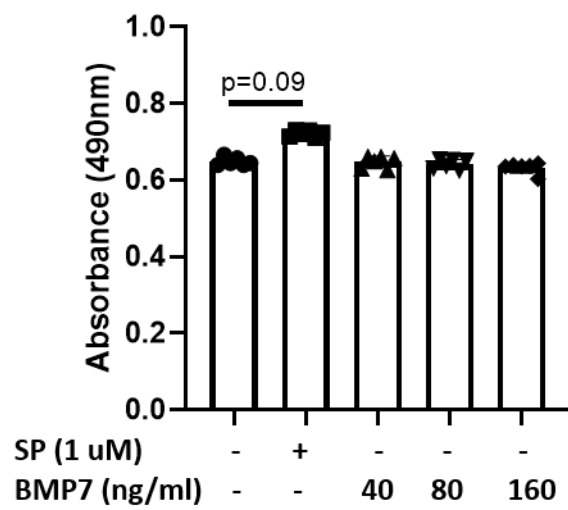


Figure 2.4 continued

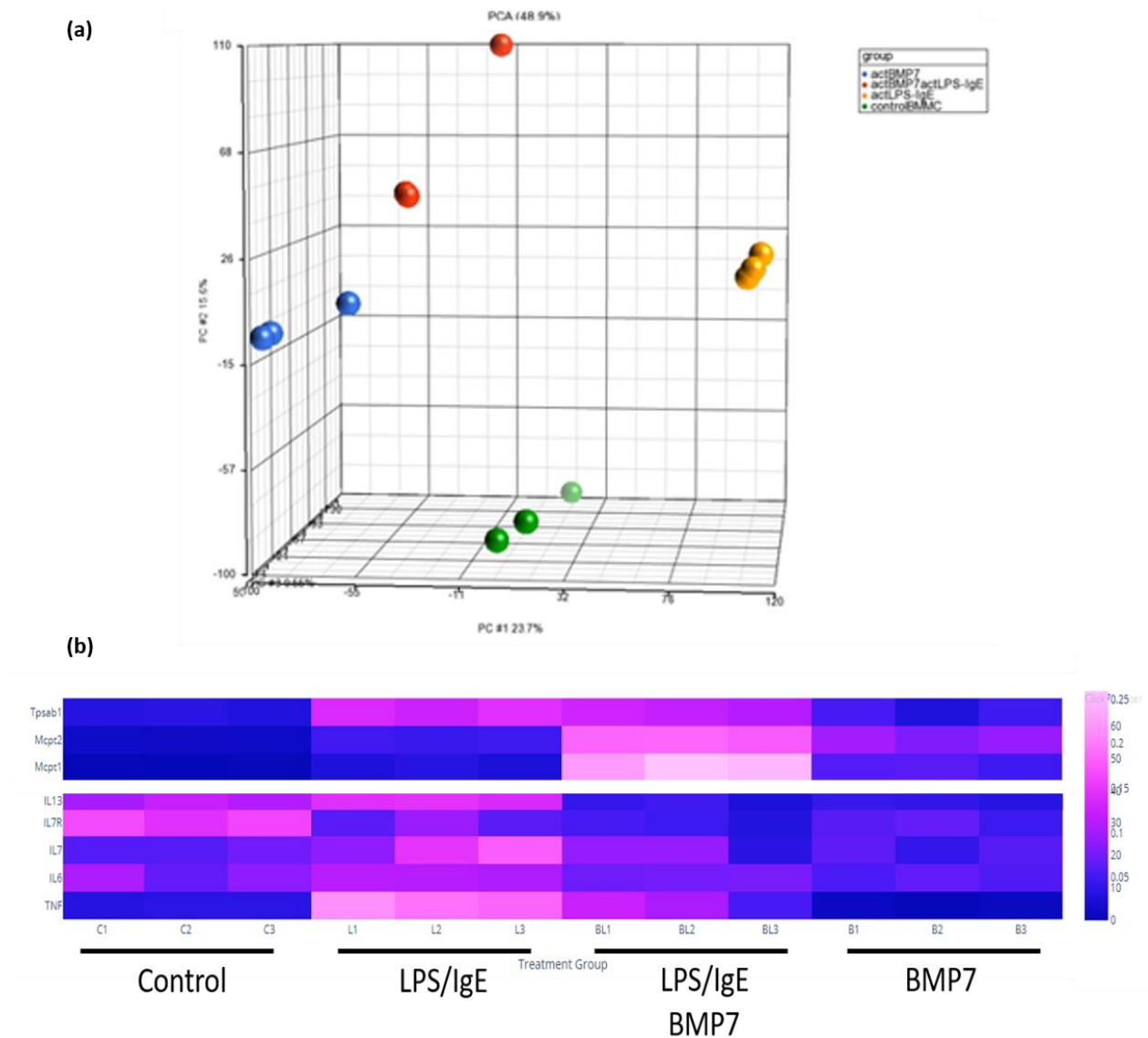
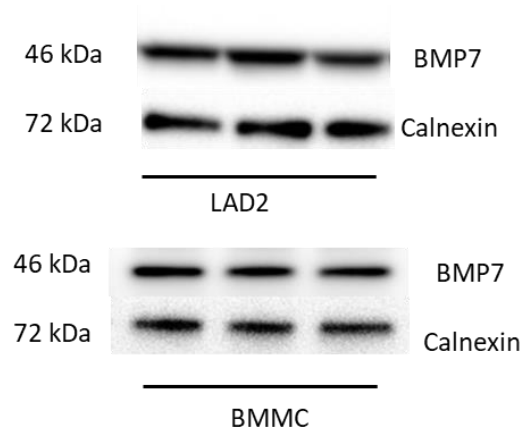


**Figure 2.5. BMP7 pretreatment modulates mast cell phenotype and response to inflammatory stimulus.** BMMCs that were pretreated with BMP7 prior to inflammatory stimulus have decreased inflammatory cytokine production but increased protease expression. BMMCs were pretreated with vehicle or BMP7 at 20 ng/ml for 24hrs, and then selective pretreated cells were subject to LPS/IgE (0.5 ug/ml) for 24hrs. Cells were then harvested for RNA sequencing. (a) Depicts PCA of 4 different

treatments and (b) is a heat map of significantly differentially expressed genes. Blue dots = BMP7, Red dots = BMP7 + LPS/IgE, Yellow dots = LPS/IgE, Green dots = Negative control.

(a)



(b)

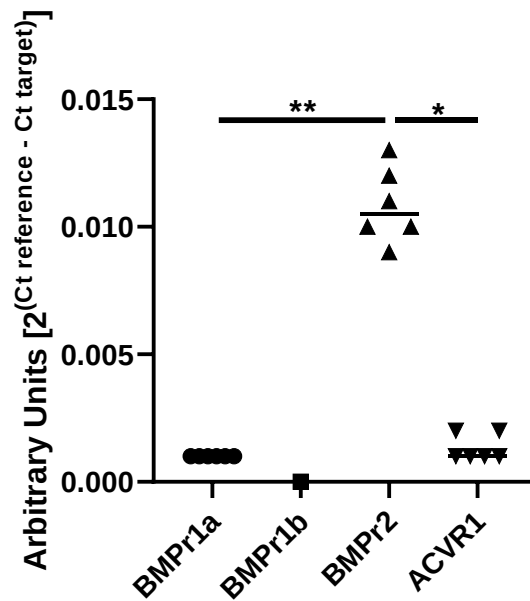


**Figure 2.6. Mast cells at baseline express BMP7 and BMP receptors. (a)**

BMP7 is expressed in both LAD2 cells and BMMCs. (b) BMP receptor expression of BMPr1a, BMPr2, and ACVR1 at baseline is detected at the RNA level in BMMCs, with BMPr2 expressed at the highest level and BMPr1b was not detected. (c) BMMCs, LAD2, and RBL-2H3 cells express BMPr2 at the protein level. Data is displayed as mean with standard deviation. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

**(c)**

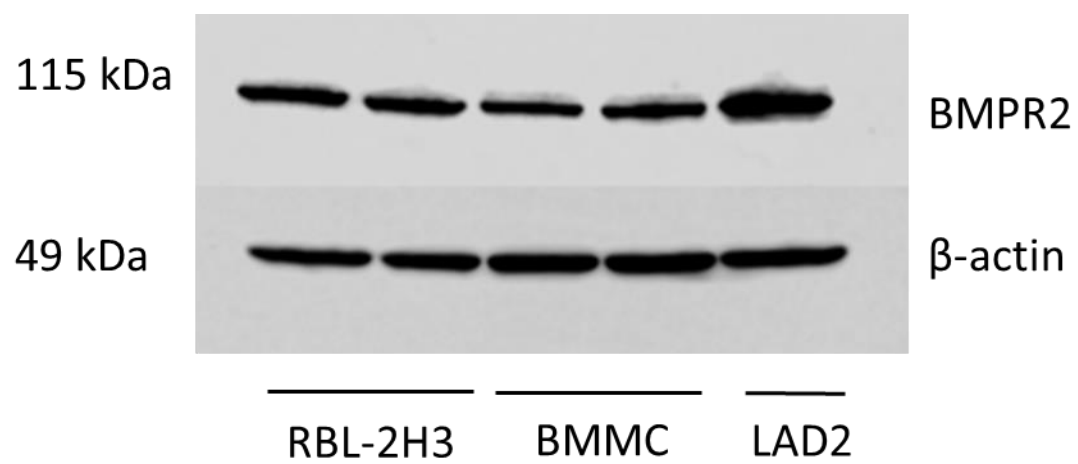


**Figure 2.6 continued**

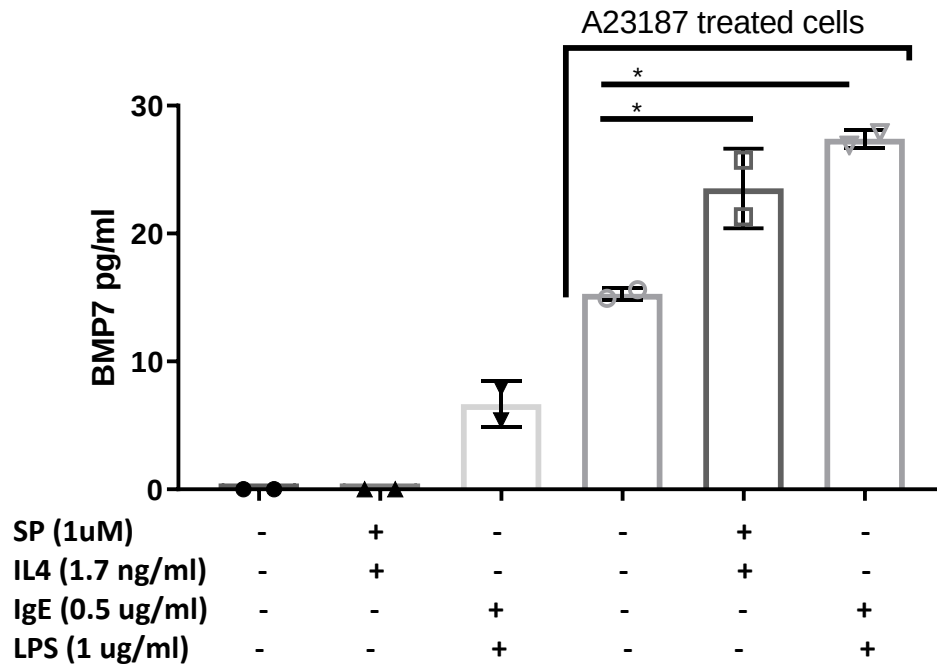


**Figure 2.7. Mast cells exposed to inflammatory stimuli upregulate BMP7 production.** BMP7 is upregulated in BMNCs when exposed to inflammatory stimuli for 24hrs. A23187 (1 uM), a calcium ionophore, induces mast cell degranulation and was used to increase detection of BMP7. Data is displayed as mean with standard deviation.

\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

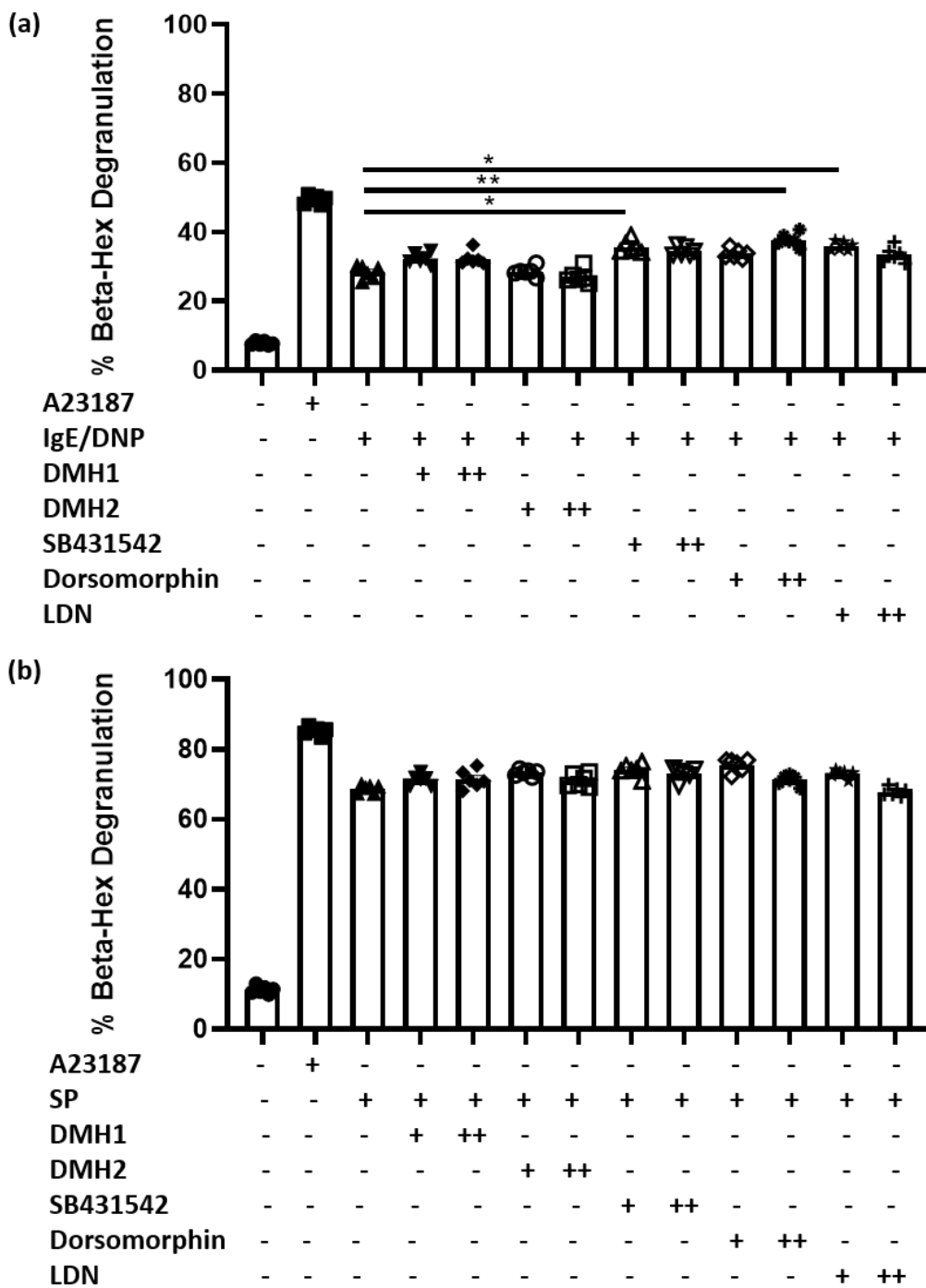


**Figure 2.8. Mast cell degranulation is mildly to unaffected by pretreatment with BMP receptor inhibition.** (a) RBL-2H3 and (b) LAD2 cells are able to

degranulate normally in the face of BMP receptor inhibition, with minimal changes. Cells were pretreated with BMP receptor inhibitors at 500,000 cell/ml for 24 hours and subsequently stimulated for 2 hours with A23187 (1  $\mu$ M, positive control), IgE/DNP, or SP (1  $\mu$ M) and degranulation evaluated by detection of  $\beta$ -hexosaminidase. Cells stimulated with IgE/DNP were pretreated with IgE at 0.5  $\mu$ g/ml 24hrs prior to exposure to DNP at 100 ng/ml. BMP receptor inhibitors were applied at two different dosages, +, 0.25  $\mu$ M and ++, 1  $\mu$ M. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .

(a)

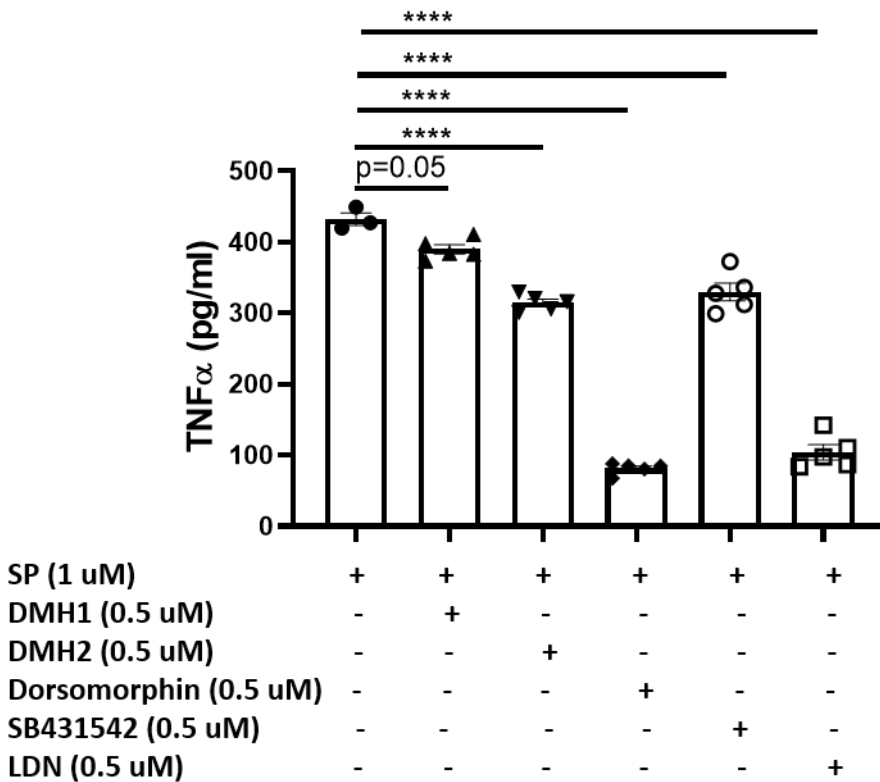
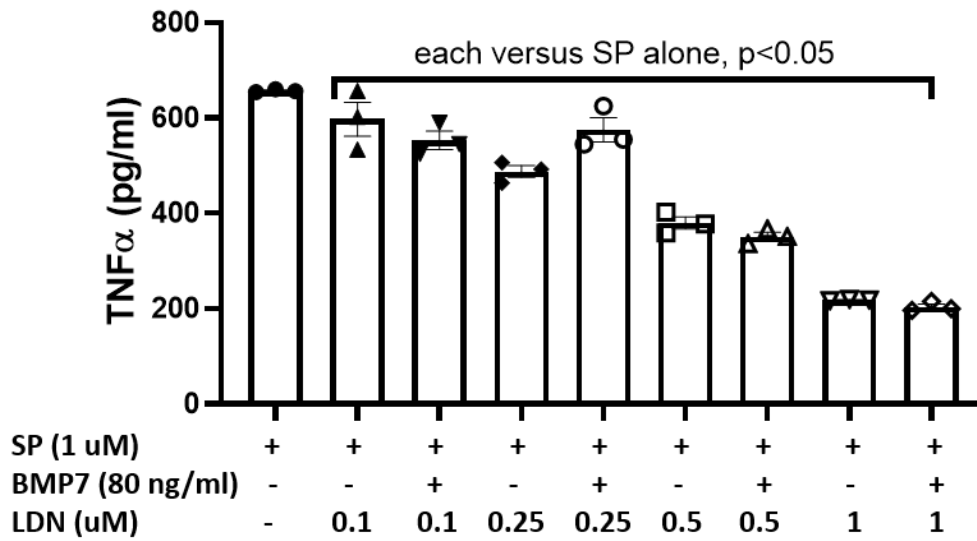


**Figure 2.9. Acvr1 blockade results in profoundly decreased TNF secretion upon exposure to inflammatory stimuli.** LAD2 (a, b & c) and BMMCs (d & e) have significantly decreased TNF $\alpha$  secretion upon stimulation when pretreated for 24 hours with BMP receptor inhibitors with high affinity for Acvr1 (LDN-193189 and dorsomorphin). Cells were pretreated with BMP receptor inhibitors at  $1 \times 10^6$  cell/ml for BMMCs and 500,000 cell/ml for LAD2 for 24 hours and subsequently stimulated for 16 to 24 hours with LPS (0.5 ug/ml) or SP (1 uM), respectively. Data is displayed as mean with standard deviation. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < .001$ ; \*\*\*\*  $p < 0.0001$ .

(b)



(c)

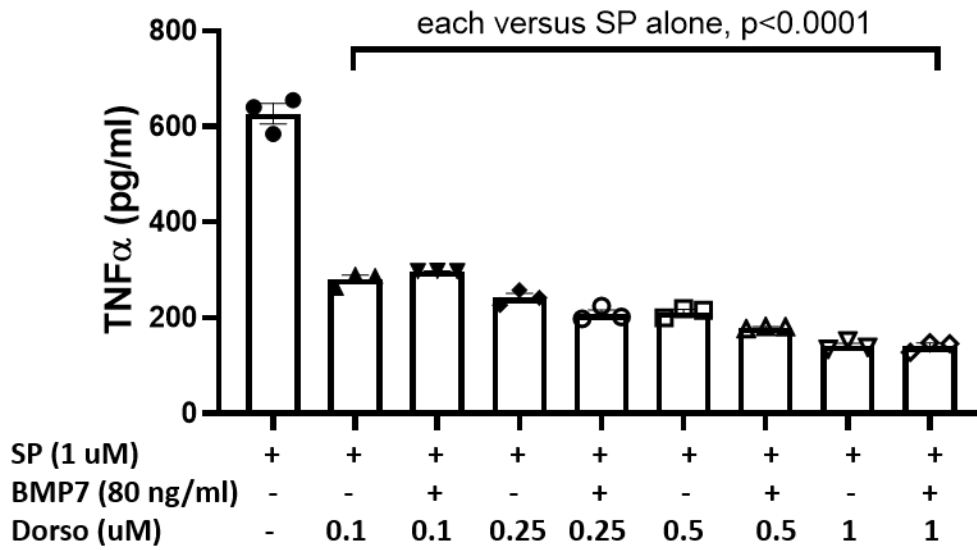
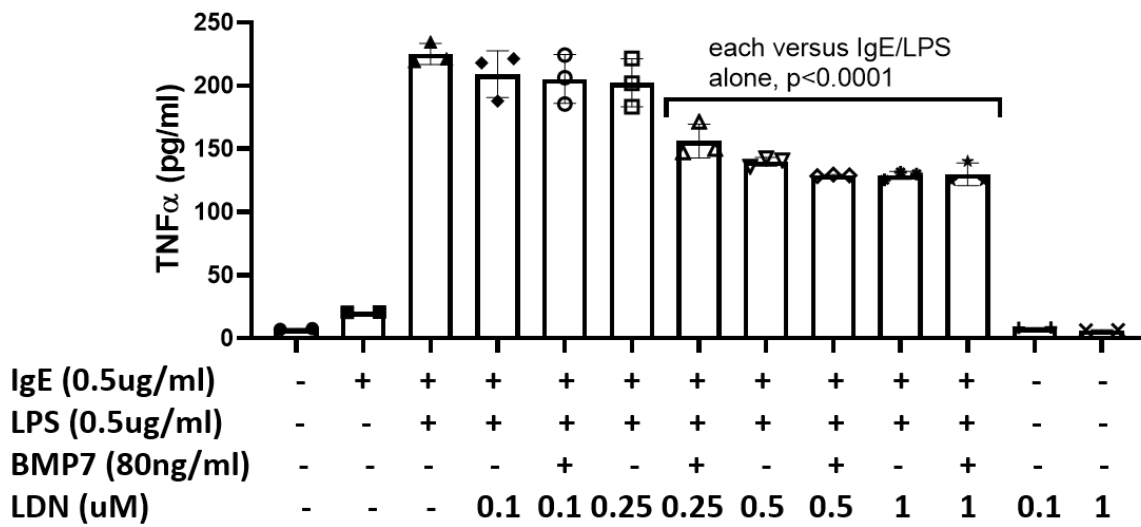


Figure 2.9 continued

(d)



(e)

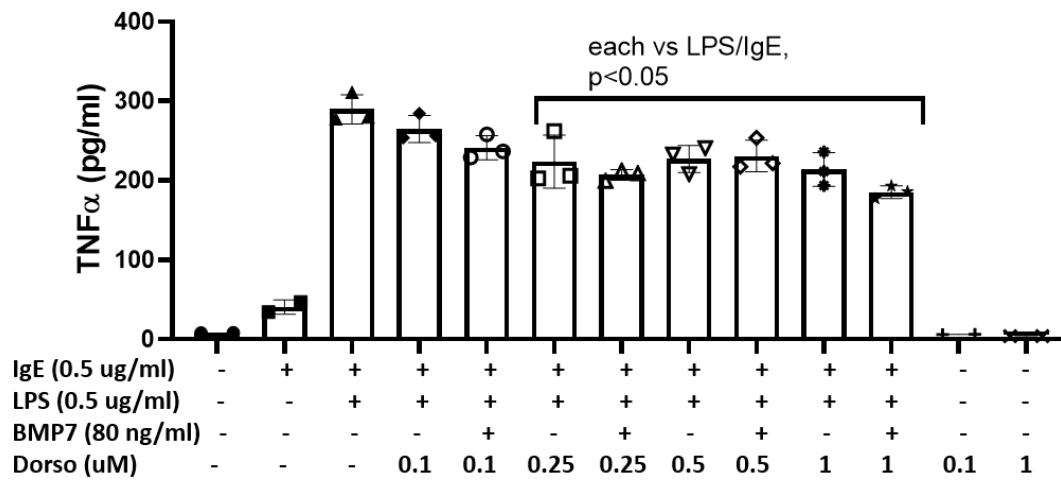


Figure 2.9 continued

(a)

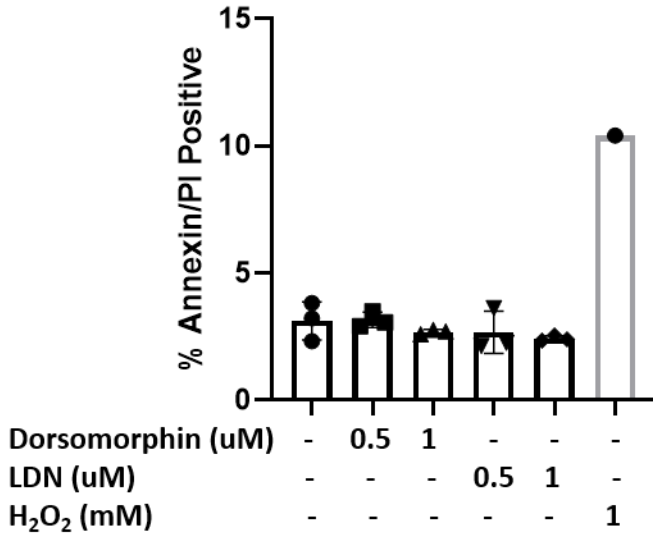
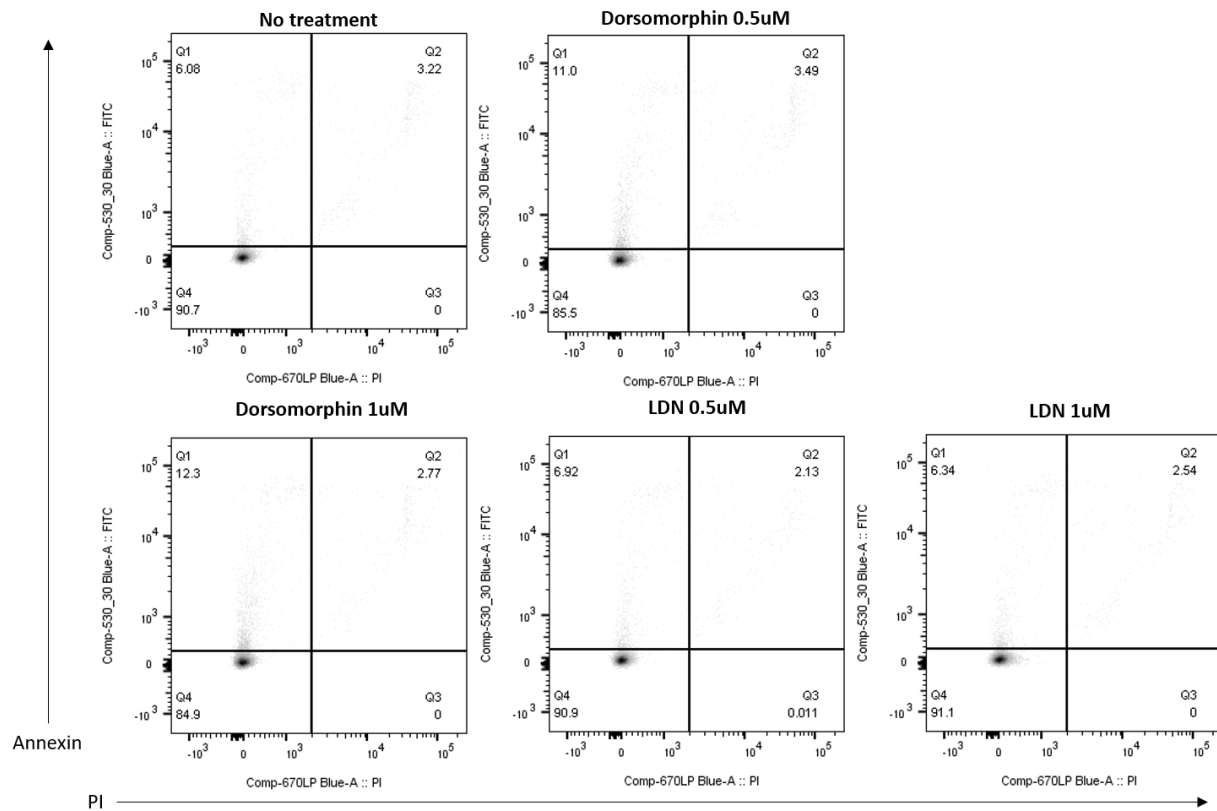


**Figure 2.10. Acvr1 blockade does not result in significantly increased apoptosis.** Mouse BMMCs (a) & (b) and LAD2 cells (d) & (e) pretreated for 24hrs with dorsomorphin or LDN have stable apoptosis status. LAD2 cells had significantly increased annexin V/PI positive cells with 1 uM dorsomorphin treatment, although this is likely not physiologically relevant when compared to the positive control apoptosis inducer and the previous finding that degranulation is normal at this dose. Hydrogen peroxide was applied to the cells for about 2-4 hours at concentrations indicated as a positive control and FMOs were done in same way as previously shown. Cellular proliferation was assessed in (c) BMMCs and (f) & (g) LAD2s after 24hr treatment with BMP receptor inhibitors and was mildly, variably affected, although degranulation is noted to be normal at these dosages. Data is displayed as mean with standard deviation.

\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

(b)



(c)

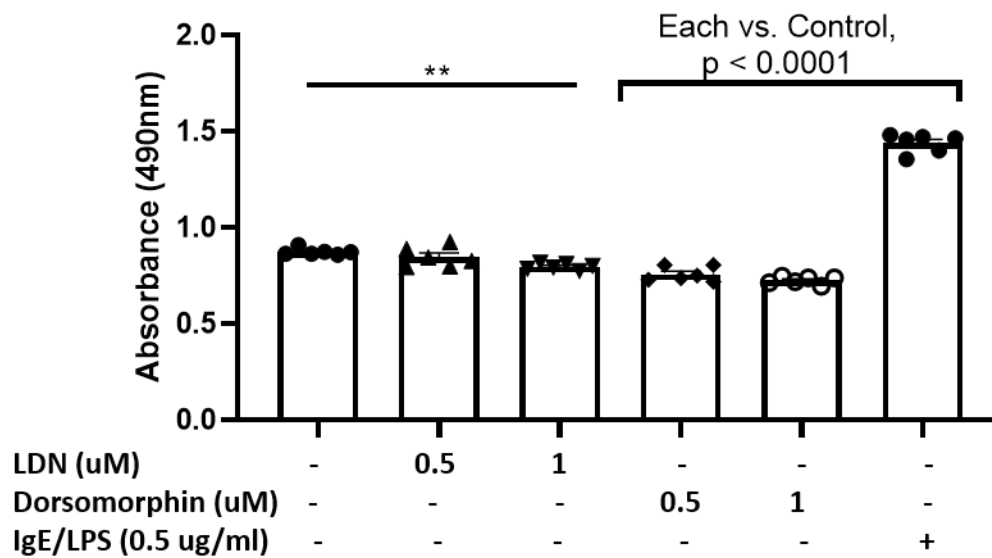
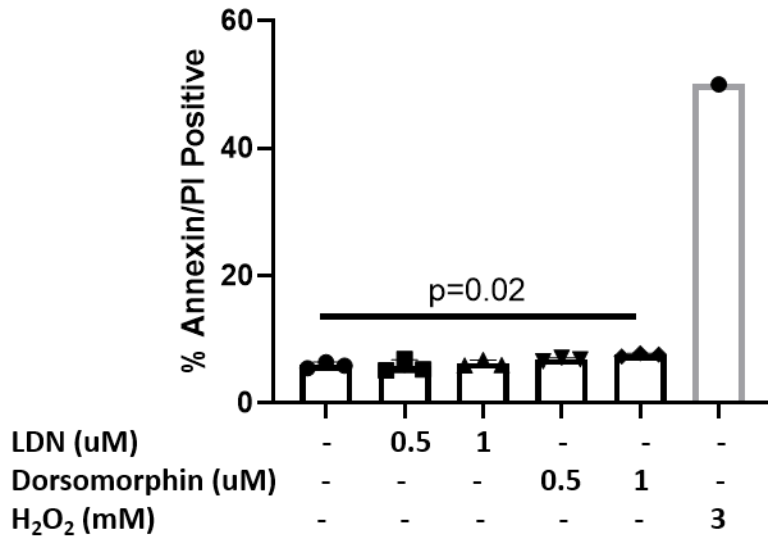


Figure 2.10 continued

(d)



(e)

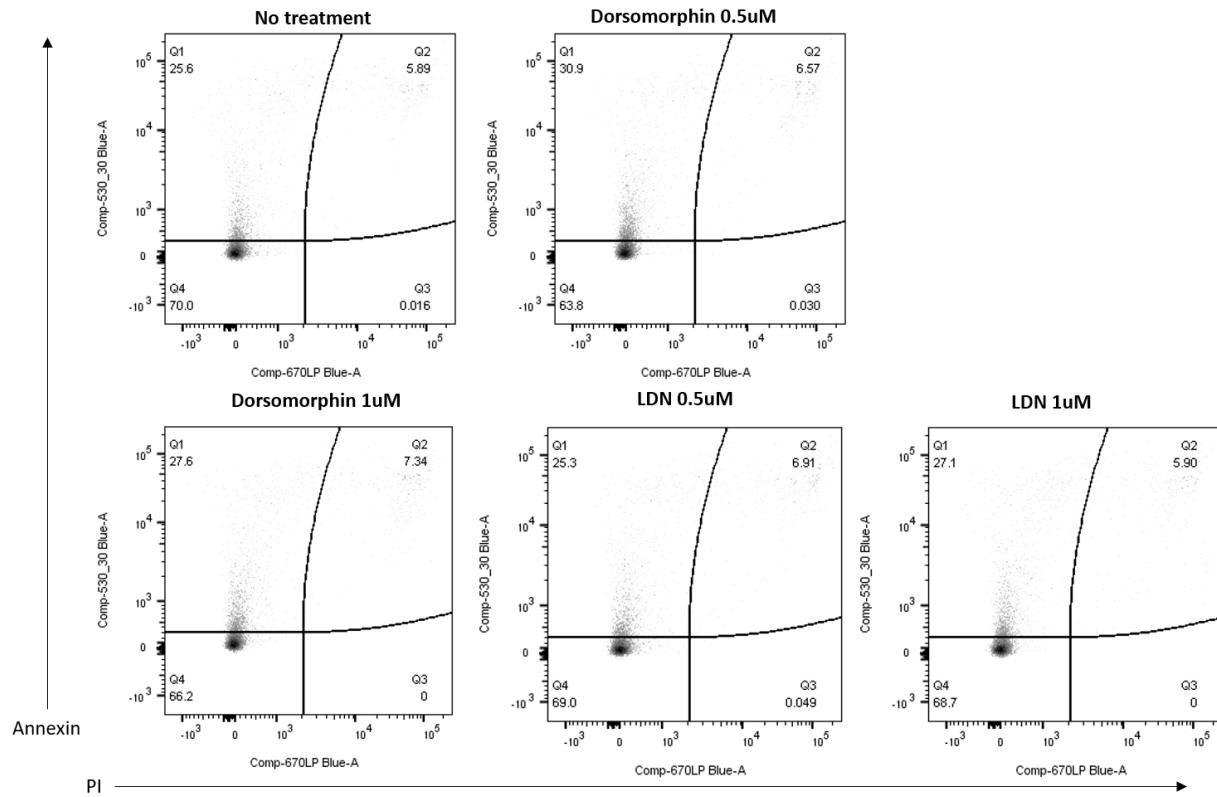
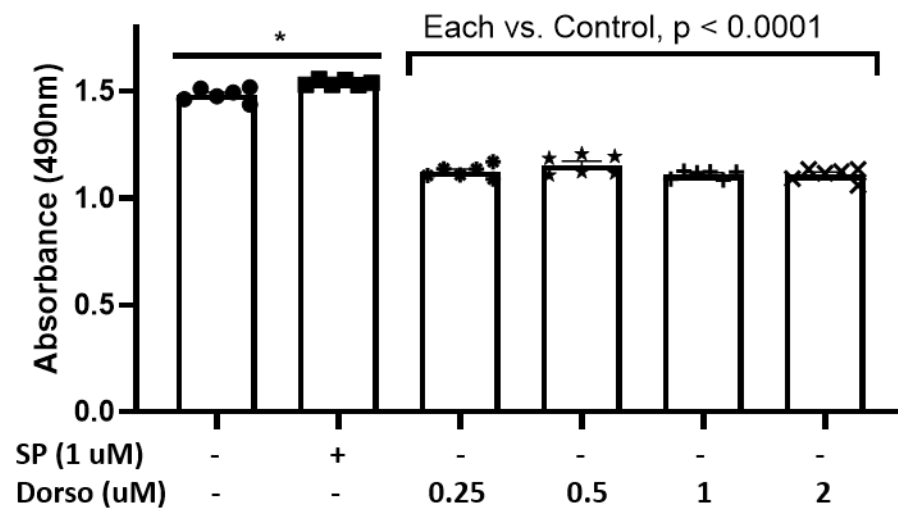


Figure 2.10 continued

(f)



(g)

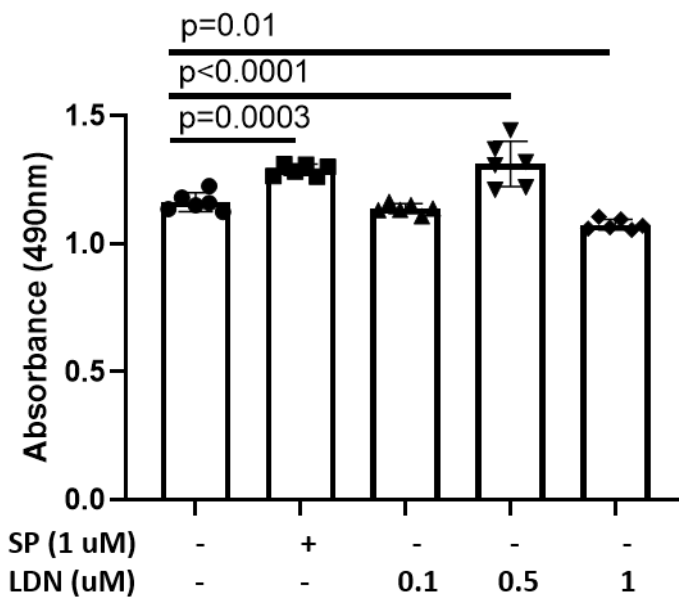


Figure 2.10 continued

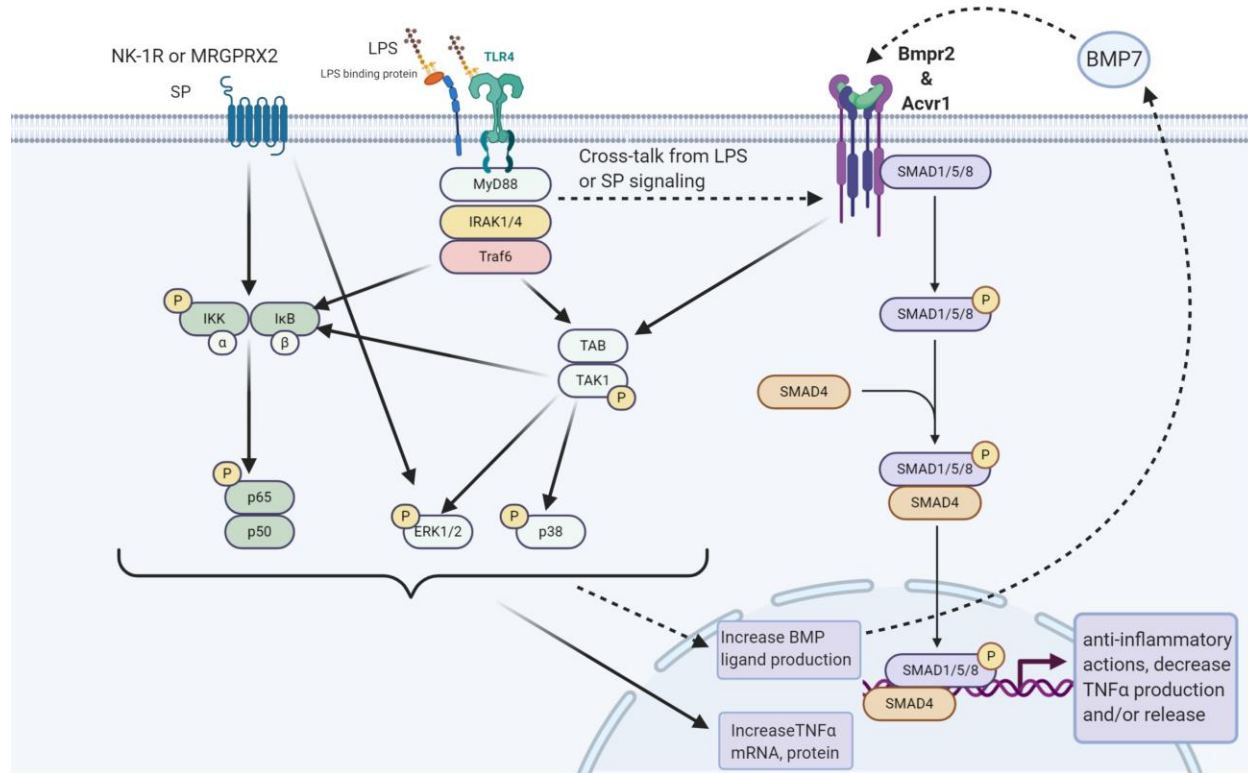


**Figure 2.11. Proposed model of pathway activation and BMP7 production upon mast cell stimulation.** Created with BioRender.com.

**(a)**

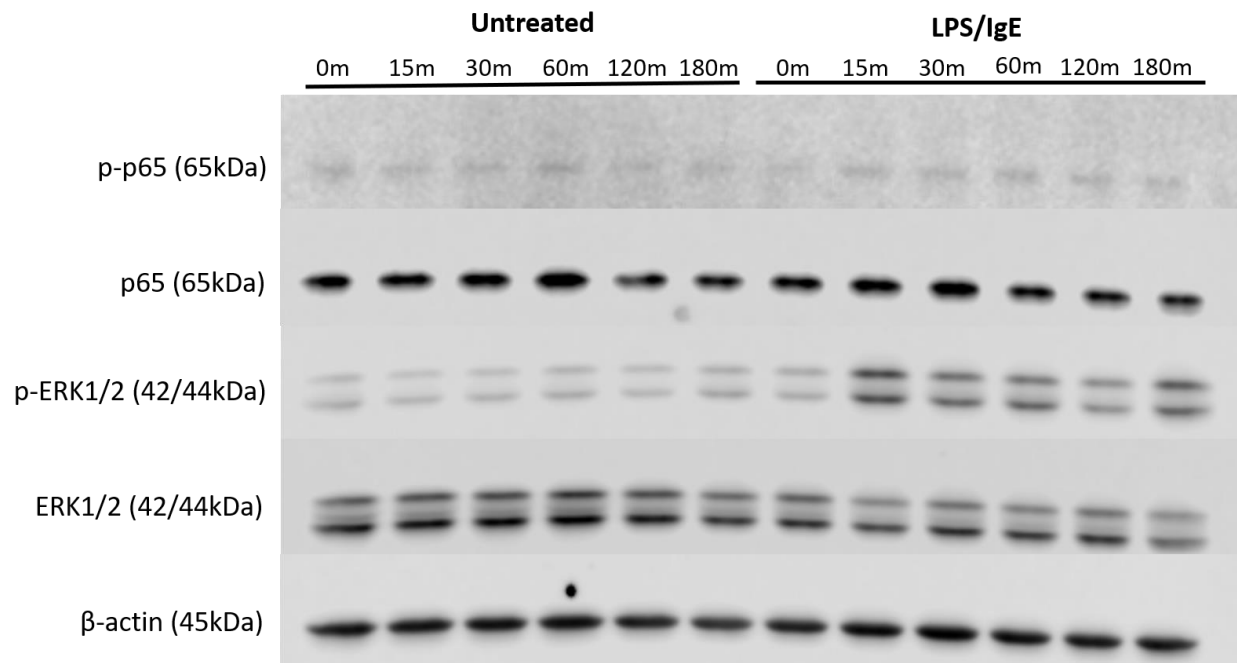
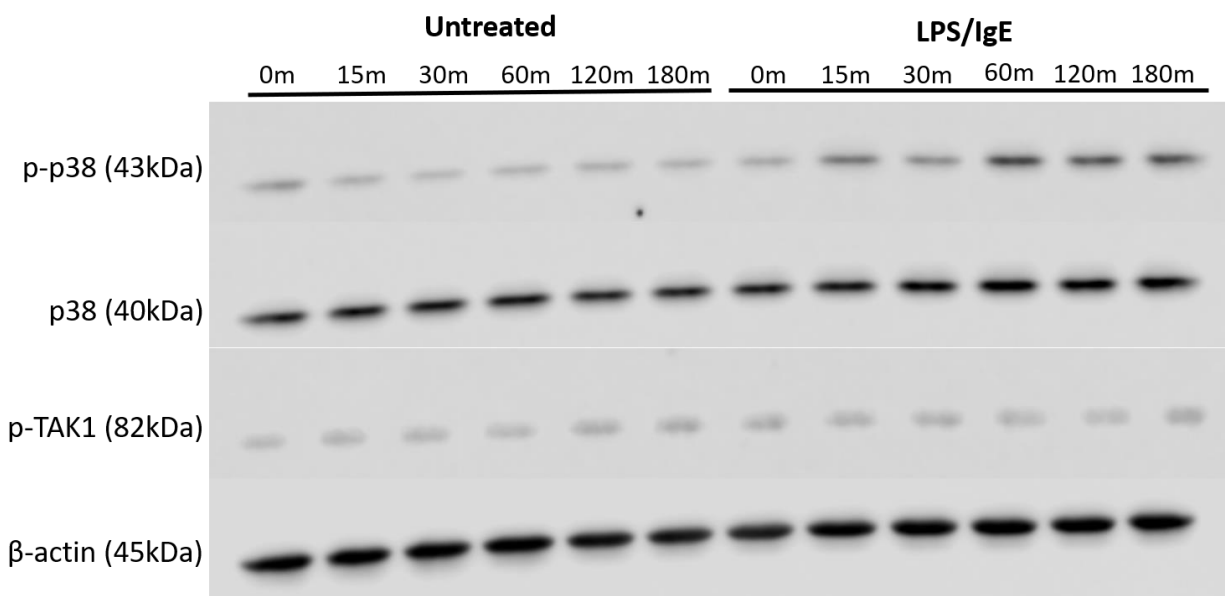


**Figure 2.12. Mast cells stimulated with inflammatory stimuli have an upregulation in phospho-ERK1/2, with BMMCs additionally having upregulation of p-p38.** BMMC (a) & (b) and LAD2 cells (c) & (d) show upregulation of pERK1/2 upon stimulation with LPS/IgE or SP for 24 or 16hr, respectively. BMMCs additionally appear to have upregulation in p-p38 upon stimulation.

**(b)**



**(c)**

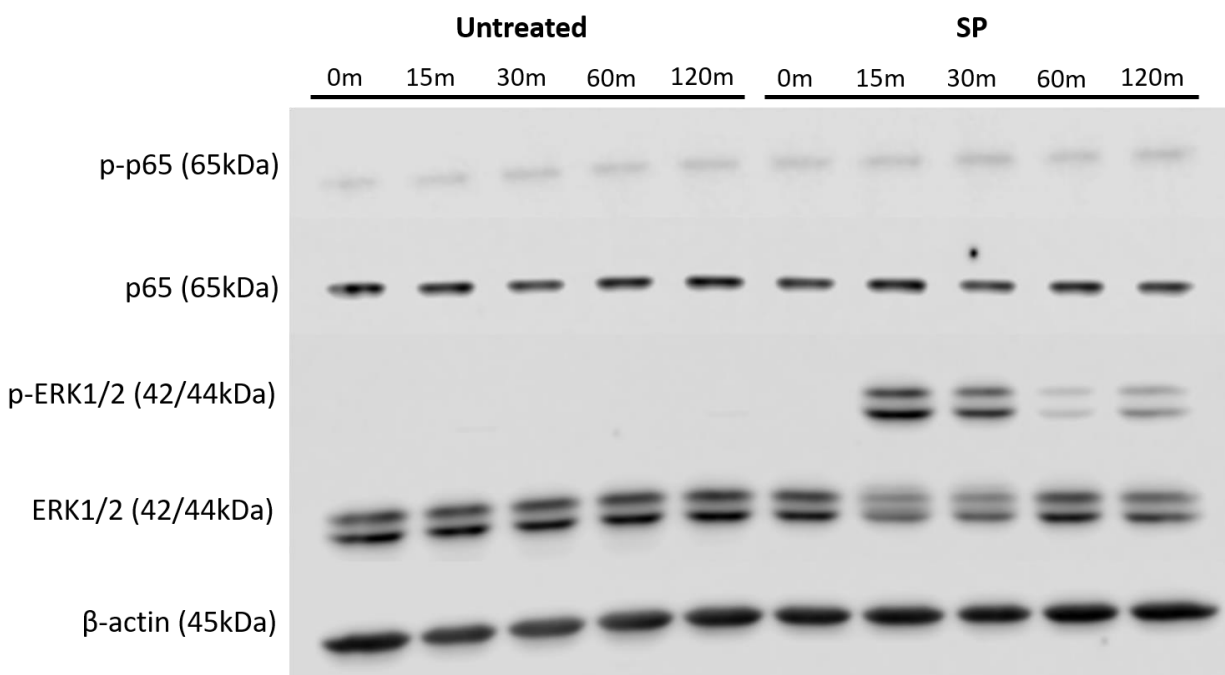


**Figure 2.12 continued**

**(d)**

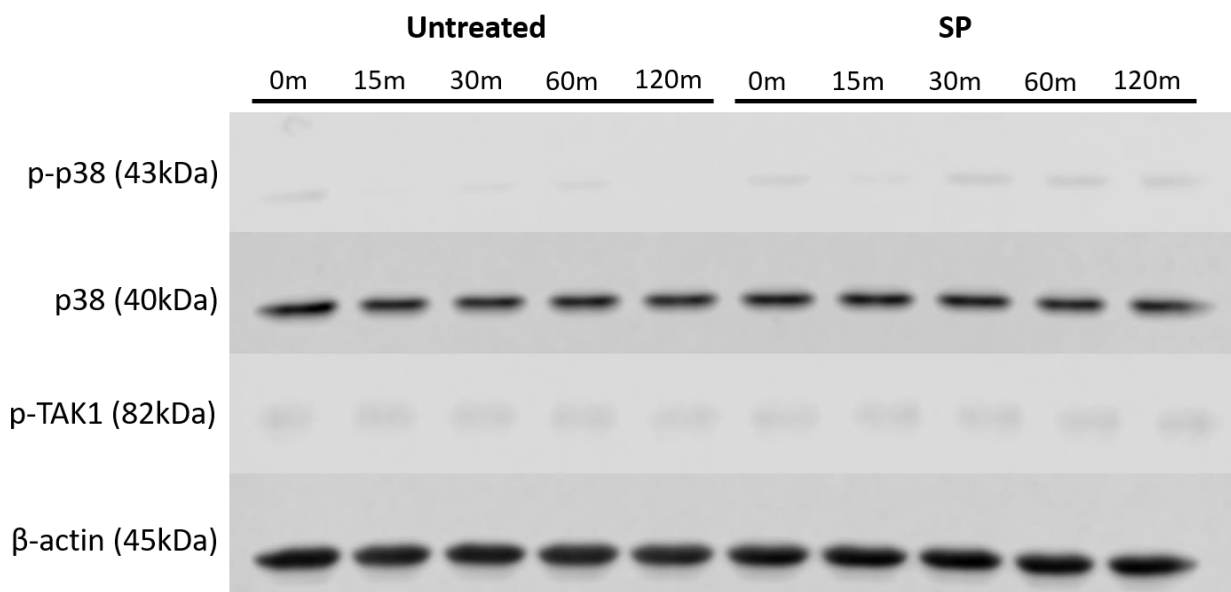


**Figure 2.12 continued**

(a)

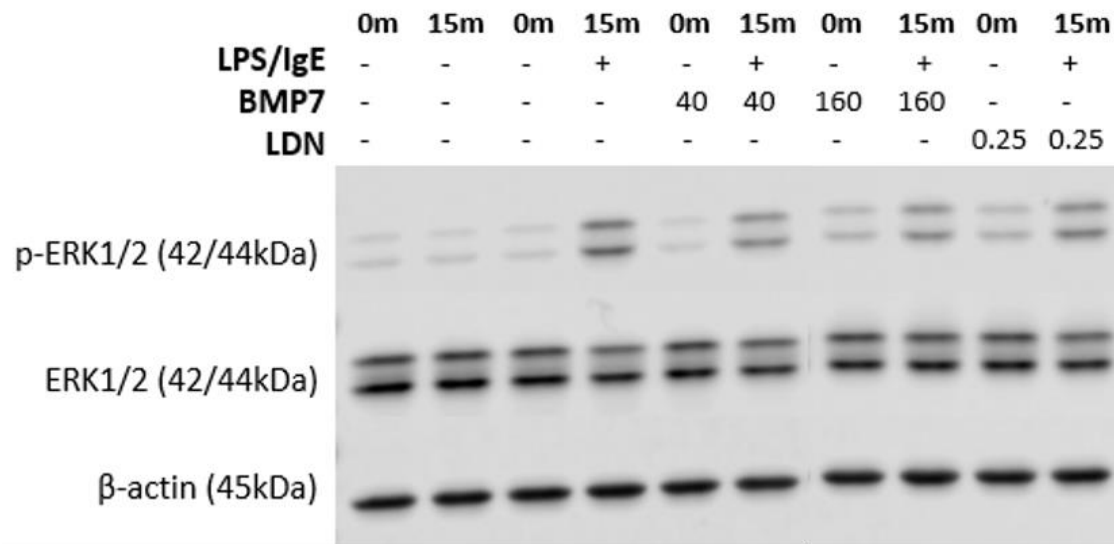
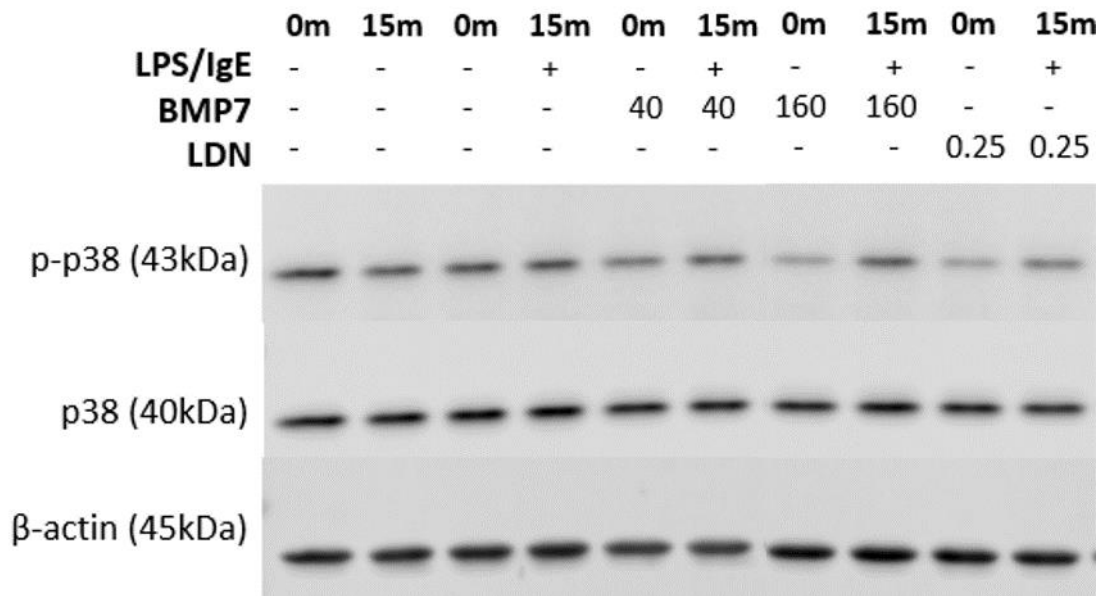


**Figure 2.13. Pretreatment with BMP7 or LDN-193189 decreases ERK1/2 and p38 phosphorylation in BMMCs, not LAD2 cells.** BMMCs (a) & (b) and LAD2 cells (e) were pretreated with BMP7 (BMMCs: 40 or 160 ng/ml; LAD2 40 or 80 ng/ml) or LDN-191389 (BMMCs: 0.25 uM; LAD2: 0.25 or 0.5 uM) for 72hrs and then subsequently stimulated with LPS/IgE or SP, respectively. Cell pellets were collected at 0 min and 15 min post LPS/IgE or SP stimulation. Pathways previously identified as induced upon stimulation, ERK1/2 and p38, were evaluated. Pretreated BMMCs with BMP7 and LDN-193189 had (c) significantly decreased pERK1/2 at 15 minutes post stimulation and interestingly had (d) significantly decreased p-p38 at baseline. LAD2 cells did not have any significant decrease in ERK1/2 induction with BMP7 or LDN-191389 pretreatment.

(b)



(c)

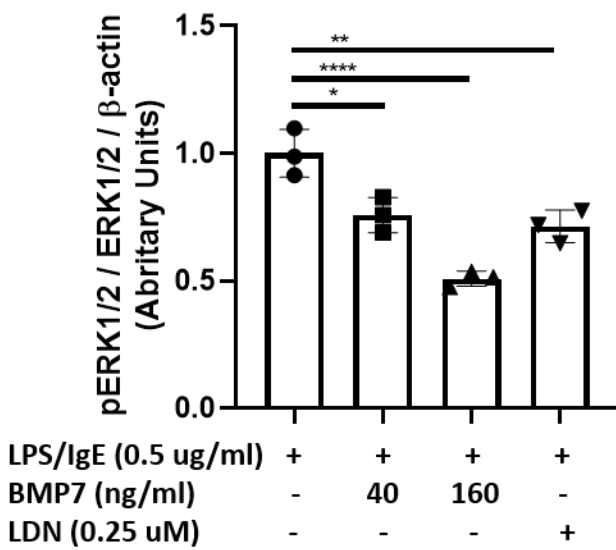
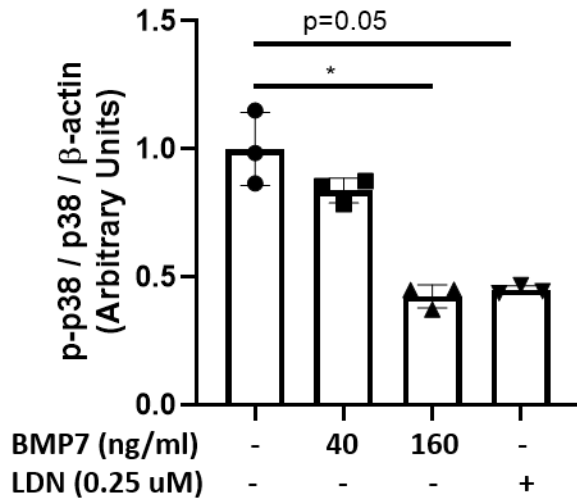


Figure 2.13 continued

(d)



(e)

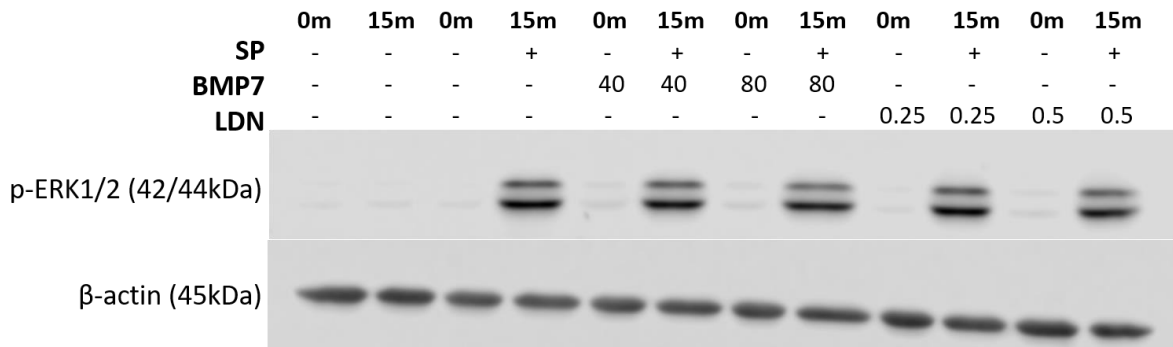
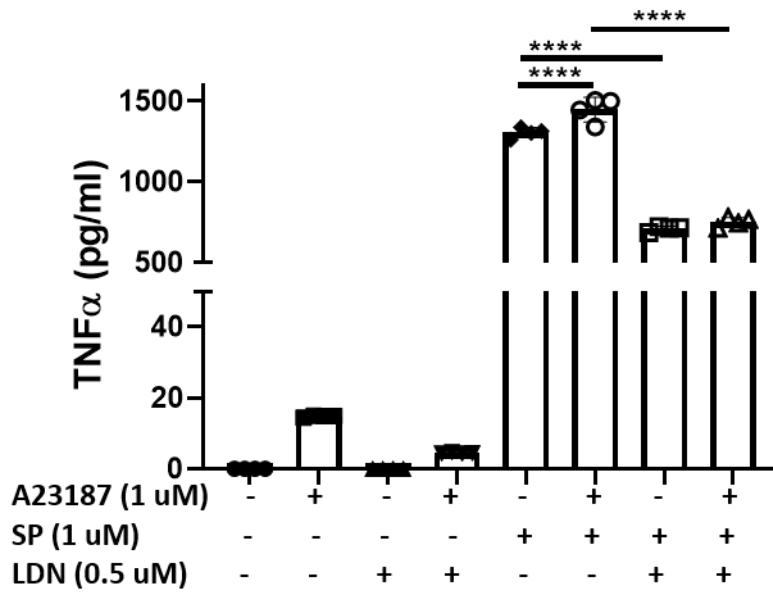


Figure 2.13 continued

(a)



(b)

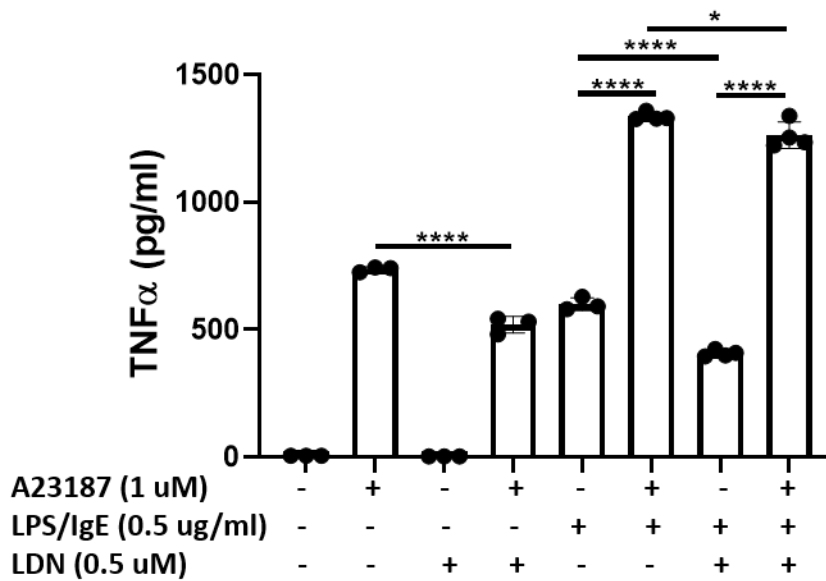


**Figure 2.14. LAD2 cells *de novo* synthesizes more TNF $\alpha$  versus BMMCs have more TNF $\alpha$  stored.** LAD2 cells (a) and BMMCs (b) were pretreated with LDN-193189 for 24hrs and subsequently stimulated with SP or LPS/IgE for 16 to 24hrs, respectively.

Two hours prior to collection, A23187 was applied to force degranulation. TNF $\alpha$  in the supernatant was subsequently detected with ELISA. Data is displayed as mean with standard deviation. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < .001$ ; \*\*\*\*  $p < 0.0001$ .

(a)

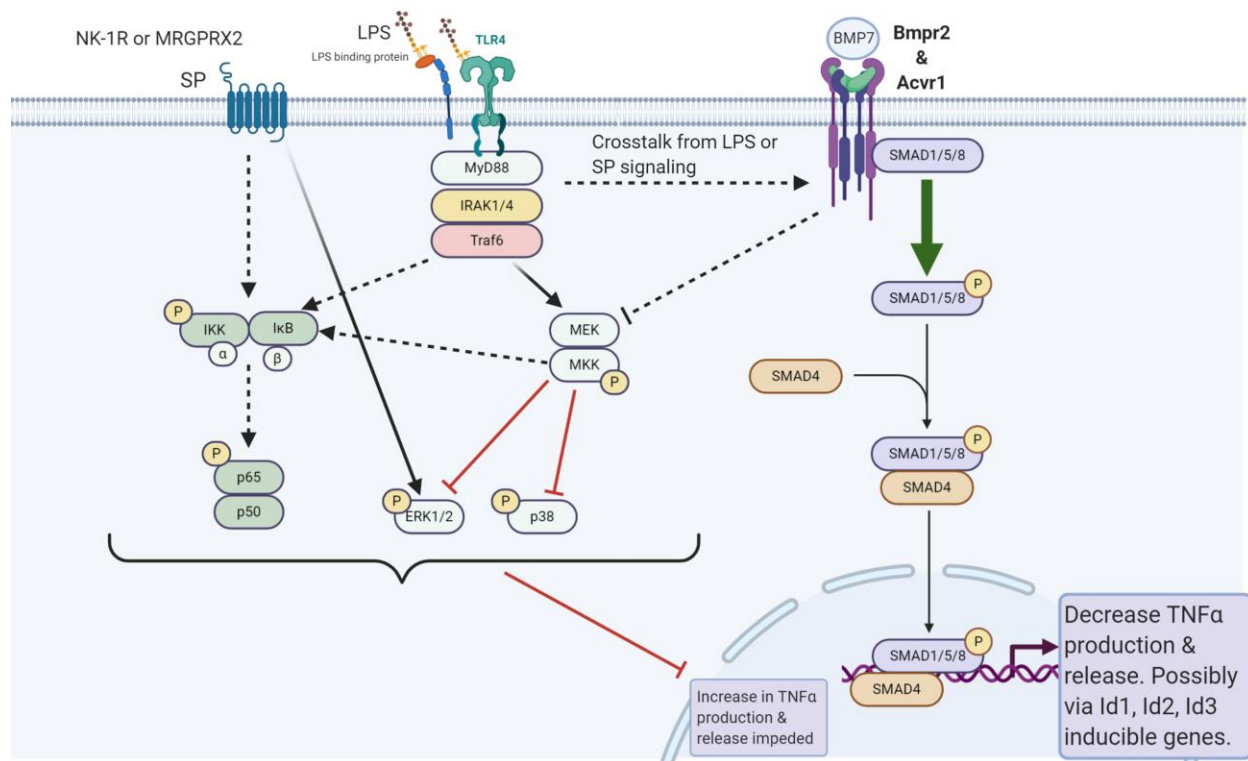


**Figure 2.15. Proposed model for BMP7 and LDN-193189 reduction in TNFα release upon subsequent stimulation.** (a) BMP7 pretreatment has been shown to decrease TNFα release in both BMMCs and LAD2 cells. We have shown that BMP7 pretreatment in BMMCs is able to downregulate baseline p-p38 and induced pERK1/2 upon stimulation with LPS, which likely plays a role in decreasing the TNFα released by these cells. (b) Additionally, LDN-193189 pretreatment in BMMCs showed similar effects. BMP7 treatment is likely driving a canonical signaling pathway, Smad-dependent, which results in anti-inflammatory actions, such as downregulating p-p38 and pERK1/2. Downregulation of these pathways may be due to inducing the production of Id-proteins but is unknown at this time. LDN-193189 inhibits Acvr1 signaling that is likely more closely linked to non-canonical signaling and therefore its blockade leads to decreased activation of those non-canonical signaling pathways, p-p38 and pERK1/2. Acvr1 signaling may help amplify LPS and SP signaling pathways independent of binding BMP ligand, or there may be low grade constitutive signaling occurring in a ligand-dependent or independent manner. Created with BioRender.com.

(b)

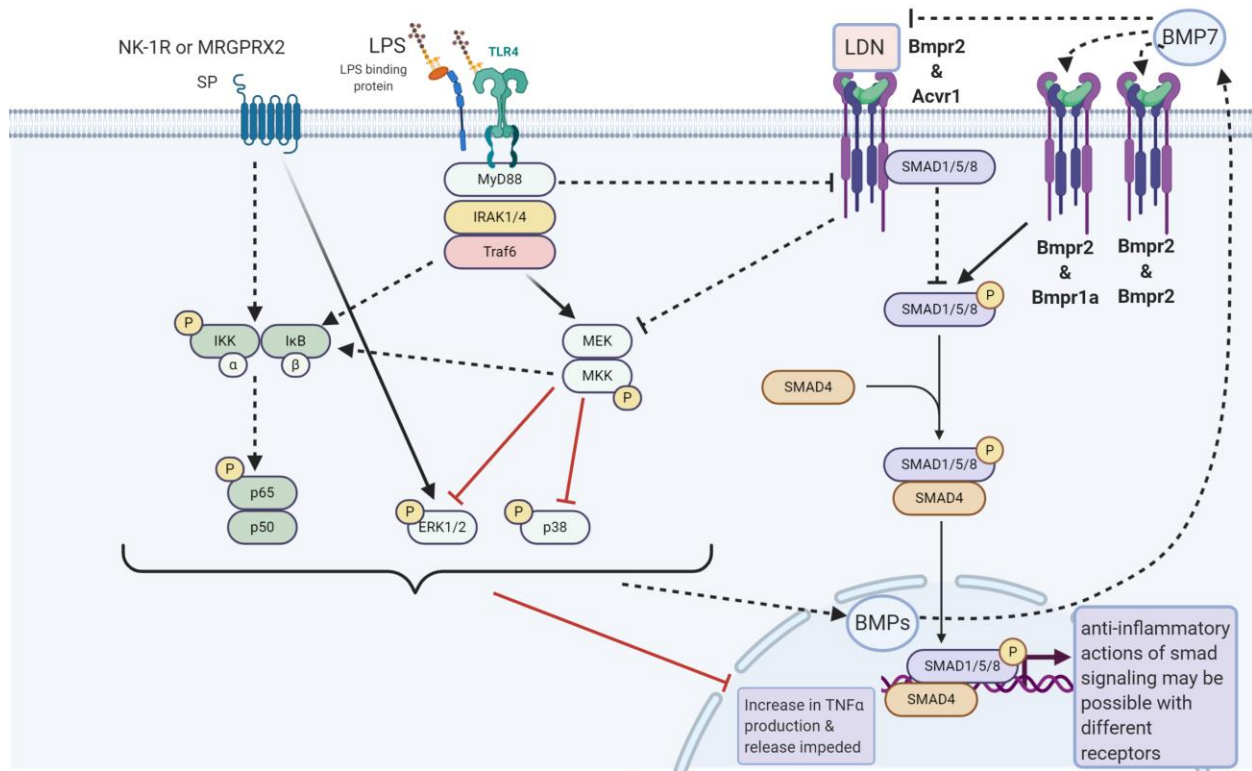


Figure 2.15 continued

## **CHAPTER 3**

### **MAST CELL PHENOTYPE AND CYTOKINE PROFILES IN EQUINE ASTHMA**

## **Abstract**

Equine asthma syndrome is a naturally occurring lung disease characterized by chronic, partially reversible airway obstruction, pulmonary remodeling and lower airway inflammation. The syndrome is classically defined in to two major groups, mild to moderate asthma (mEAS), and severe asthma (sEAS), but each remains a heterogeneous group with further subtyping potentially required. Further subtyping may be supported by significant differences in the cellular and cytokine profiles present in the BALF of asthmatic horses. The cytokine profiles that distinguish asthma groups in horses have not been systematically classified, and mast cell phenotypes, which, in human asthma, correlate with asthma type, lung function, and response to therapy, have not been well described in horses. The purpose of this study was to: (1) compare mast cell protease mRNA expression between healthy and asthmatic horses, (2) analyze the cytokine profile present in BALF of currently defined equine asthma groups, and (3) use these data to evaluate potential biomarkers of defined asthma groups. Mast cell protease gene expression and select cytokine gene expression of the BALF cellular component, and multiplex bead immunoassay for cytokine concentrations in the BALF supernatant were performed. Samples for gene expression profiling were enrolled from two institutions in order to include a mast cell predominant mEAS group. Chymase mRNA expression, a mast cell-specific protease, was significantly decreased in horses with mastocytic asthma, supporting the need for further subtyping in the mEAS group. Horses with severe asthma had elevated TNF $\alpha$ , CXCL8, and IFN $\gamma$  in the BALF supernatant. Multidimensional analysis demonstrated healthy and mEAS horses have the most overlap, with sEAS having the most separation from the others influenced

primarily by neutrophil and lymphocyte concentrations. These results will help further define EAS immunopathology, which could improve understanding and definitions of asthma groups; while also potentially identifies novel therapeutic strategies.

## **Introduction**

Equine asthma is a chronic inflammatory lung disease characterized by enhanced bronchial reactivity, and chronic, partially reversible airflow obstruction, pulmonary remodeling, and lower airway inflammation (1). The most common environmental triggers for asthma development in susceptible horses include pollens, dust, mold, and bacterial components. Equine asthma prevalence in the Northern hemisphere is about 14% and the disease can be frustrating to manage, while also being career or life ending for the horse (2, 3). Recently it has also been shown that prevalence can change with season (4). The diagnosis is based on history, physical examination, bronchoalveolar lavage fluid (BALF) cytology, and lung function tests. Lung function tests are performed less frequently due to cost, required skill for equipment use and data analysis, and availability of the apparatus. History and physical exam findings supporting asthma include clinical signs of lower airway inflammation, such as cough, increased respiratory rate and effort, poor performance/exercise intolerance, and serous, mucoid, or mucopurulent nasal discharge at rest and/or at exercise. A 2016 consensus statement has defined two categories of equine asthma syndrome (EAS), currently designated as mild to moderate asthma (mEAS) and severe asthma (sEAS) (1). Horses with sEAS, auscultation of the lungs at rest or during rebreathing exam can reveal wheezes, crackles, cough, and/or tracheal rattle. Abnormalities upon auscultation may not be apparent, but recovery from a rebreathing exam may be prolonged in mEAS or sEAS

horses. In contrast to horses with sEAS, horses with mEAS do not have clinical signs at rest (1). BALF cytological findings supporting asthma include an increased percentage of neutrophils (most pronounced in severe asthma), mast cells, eosinophils, or some combination of increase in these cells.

In both horses and humans, asthma immune responses have been broadly divided into allergic asthma, with a Th2-high immune response; and non-allergic asthma, with a non-Th2 immune response. However, the two are not always clearly defined as such and can include horses from both mEAS and sEAS group (5). A Th1 immune response has been shown to be associated with a generalized increase in BALF cell numbers in lower airway inflammation, although cytokine profiles, either at the protein or mRNA level, may support more of a Th1, Th2, Th17, or mixed immune response, depending on the study (6-18). Regulation of inflammation is in part attributed to transforming growth factor (Tgf)- $\beta$ 1 production, but Tgf $\beta$ 1 also has the ability to lead to collagen deposition and airway remodeling (19, 20). The extent of airway remodeling in a mouse model of asthma has been linked to the balance of Tgf $\beta$ 1 and bone morphogenetic protein (BMP)-7 due to BMP7's ability to block Tgf $\beta$ 1 pro-fibrotic effects and its anti-inflammatory effects seen in both humans and mice (21-23). One report in horses noted no significant differences in Tgf $\beta$ 1, but has not been followed up, nor has BMP7 been investigated (24). Due to inconsistencies in reported cytokine profiles and focusing on a single category of EAS, this has limited our ability to fully describe the pathogenesis of EAS and its categories, which ultimately limits our diagnostic, treatment, and management abilities.

Human asthma additionally describes asthma type by the predominant inflammatory cells present in the airways, such as eosinophilic, neutrophilic, or paucigranulocytic (4, 25, 26). Recently, the presence of mast cells in human asthmatics' BALF and induced sputum samples has been shown to correlate with asthma type and response to therapy (27-30). Mast cells are classically identified by their expression of CD117 (c-kit), the receptor for stem cell factor (SCF); and FcεRI, the high affinity receptor for IgE. In humans, two main phenotypes of mast cells are recognized based on the proteases they contain; tryptase-only containing mast cells (MC<sub>T</sub>), and tryptase, chymase, and carboxypeptidase A<sub>3</sub> containing mast cells (MC<sub>TC</sub>) (31, 32). Healthy human lungs predominantly contain mast cells of the mucosal-type (MC<sub>T</sub>), and asthma is associated with an increase in MC<sub>T</sub> more so in mild asthma and increasing chymase expression in severe asthma (27, 33). Cytological identification of mast cells in induced human sputum is difficult due to low cell prevalence and mast cells can release their cytoplasmic protease content; therefore, protease mRNA expression is used to avoid these confounders. Using this technique, a third mast cell type with increased tryptase and carboxypeptidase A<sub>3</sub>, but decreased chymase expression was identified in human asthmatics that correlated with eosinophilic asthma and increased responsiveness to corticosteroids (28, 29). Mast cell lung phenotypes identified in humans have not been thoroughly evaluated in horses, but warrant further investigation (34, 35).

We hypothesized that healthy and asthmatic horses would have significantly different BALF mast cell protease mRNA expression and cytokine/chemokine levels that would correlate with BALF cytology results and health status. The objectives of this study were to: (1) compare mast cell protease mRNA expression between healthy and

asthmatic horses, (2) analyze the cytokine profile present in BALF of currently defined equine asthma groups, and (3) use these data to evaluate potential biomarkers of defined asthma groups. The results showed significant differences in mast cell protease expression and cytokine levels across asthma groups. The results potentially support further subdivision of the mild to moderate group to include a mastocytic category and identified cytokine targets of interest for treatment of asthma.

## **Material and methods**

### **Main study population – (UT-CVM)**

Horses were enrolled from the institution's equine hospital caseload and research herd from February 2018 to April 2019. Procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee (protocol 2533). Horses from the clinical caseload were enrolled on a volunteer basis with written, informed owner consent, and completed standardized questionnaire. The institution's research herd guidelines for care and use are in accordance with USDA guidelines.

Table 1 contains the diagnostic criteria for each experimental group, in part defined using consensus guidelines (1). Attending large animal internal medicine clinicians at the time of client-owned horse enrollment evaluated the horses. The institution's research herd was evaluated by the same two investigators for all horses (JW and CS). Exclusion criteria included horses less than one year of age, history of systemic or respiratory disease (besides suspected asthma) in the past three months, and corticosteroid (systemic or intranasal) administration begun or significant dosage changes within two weeks of enrollment. All horses underwent evaluation via history and physical exam, including a rebreathing exam, CBC/fibrinogen measurement, and

BALF cytology. Horses were excluded from analysis if they did not meet final inclusion criteria.

### **Sub-population – (NCSU-CVM)**

As data collection progressed and BALF results of the UT-CVM horses were evaluated, it was observed that only 2 mEAS horses enrolled had  $\geq 1\%$  mast cells. Therefore, additional samples were obtained from NCSU-CVM collaborators (KD & KS), who had previously identified horses with mastocytic asthma (maEAS), as defined by the collaborator (Table 1). These additional samples were used for investigation of gene expression only because using a standardized cDNA input for all targets of interest could normalize this analysis. The NCSU-CVM samples are identified as a sub-population.

### **Sample collection**

#### **Main Population (UT-CVM)**

Blood was collected from the jugular vein into K<sub>2</sub>EDTA tubes (Becton, Dickson, and Co., Franklin Lakes, NJ) prior to sedation and was submitted to the institution's clinical pathology laboratory for CBC determination using an Advia 2120i hematology instrument (Siemens Healthcare Diagnostics, Inc. m Tarrytown, NY) and heat-precipitated fibrinogen measurement using an analogue Goldberg TS meter clinical refractometer (Reichert Technologies, Buffalo, NY) by a licensed medical technologist.

Bronchoalveolar lavage procedure was performed on standing, sedated horses using a blind technique with a BAL catheter (Mila International, Florence, KY). Sedation for each horse was determined by the attending clinician and consisted of xylazine (0.2 – 0.5 mg/kg) or detomidine (5 - 10  $\mu$ g/kg), combined with butorphanol (10 – 20  $\mu$ g/kg) IV. The BAL catheter was passed nasotracheally until wedged. Once wedged, the cuff

was inflated and 200 mL of sterile saline was infused and re-aspirated by 60 mL syringe. The first 10 mL of aspirated sample was discarded as dead space, with subsequent BALF collected and pooled for analysis.

### **BALF Cell Count and Cytologic Analysis (UT-CVM)**

BALF clinicopathologic analysis consisted of a TNCC and cytologic examination of cytocentrifuged BALF. To optimize preservation of cell morphology, BALF was placed in K<sub>2</sub>EDTA tubes for TNCC and cytologic evaluation.

TNCC was performed using a scil Vet ABC hematology analyzer (scil animal care, Gurnee, IL) by a trained laboratory assistant or medical technologist. The WBC reported by the instrument was reported as TNCC. To prepare BALF for TNCC, 200 µL of well-mixed BALF was placed into a previously prepared hyaluronidase-containing cryo tubes, in order to break down any mucus that could clog the instrument (36, 37). All TNCC measurements were done in duplicate to mitigate measurement imprecision, and average of the duplicate TNCC was reported.

For cytologic evaluation, 100 µL of well-mixed BALF was placed in a cytocentrifuge (Aerospray 7120 Slide Stainer and Cytocentrifuge, Wescor Incorporated, Logan UT). Cytocentrifuged specimens were air-dried and stained with aqueous Wright's stain. A 300-cell differential cell count, or all cells when <300 cells on a slide, was performed by a blinded clinical pathologist; respiratory epithelial cells were excluded from these differential counts.

### **BALF Preparation for RNA and Cytokine Analysis (UT-CVM)**

Pooled BALF placed in non-K<sub>2</sub>EDTA tubes was refrigerated at 4°C until further processing, within two hours of collection. To prepare specimens for cytokine and RNA

analysis, BALF was strained through sterile 4x4 gauze, followed by a 70 µm nylon cell strainer to remove mucus, and then centrifuged. Aliquots of supernatant were made and stored at -80°C until further analysis. Cell pellets were washed (1x PBS) two additional times and were stored dry at -80°C until further processing.

### **Sub-Population (NCSU-CVM)**

BALF was collected in a similar manner, with the exception of a larger infusion volume. Following sedations with detomidine (0.005 – 0.01 mg/kg IV) and butorphanol (0.02 – 0.04 mg/kg IV), a cuffed catheter (Bivona, outside diameter 11 mm, 244 cm length, Smith Medical) was passed nasotracheally until wedged. The cuff was then inflated and 300 mL of warmed sterile saline solution was infused and re-aspirated by 60 mL syringe, 150 mLs at a time. The aspirated fluid was pooled, placed on ice and processed within 30 minutes of collection. Of the recovered fluid, 10 mL was submitted to NCSU's Clinical Pathology Service for a total nucleated cell count and 300-cell differential cell count performed by blinded clinical pathologists. Slides were prepared within 2 hours of sample collection, and both direct smear and cytocentrifuge slides were examined. The remaining fluid was filtered to remove mucous and centrifuged to collect the cell pellet. Aliquots were stored at -80°C until further analysis.

### **RNA extraction and gene expression (UT and NCSU-CVM)**

The corresponding institution extracted RNA. For the main population, RNA was extracted from BALF cell pellets using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) per manufacturer instructions. For the sub-population, RNA was isolated using a RNeasy Mini Kit with the addition of RNase-free DNase kit (Qiagen) per manufacturer's

instructions. Samples from the sub-population were then sent to the main institution for analysis. RNA samples were stored at -80°C for up to 18 months.

For both populations, quantification and purity of RNA extracted was assessed via NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity was analyzed on a subset of samples using 2100 Bioanalyzer and the RNA 6000 Nano kit (Agilent, Santa Clara, CA). Reverse transcription and cDNA synthesis was performed on 200 ng of RNA using Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific) and stored at -80°C. Taqman Gene Expression Assays were used for all target genes;  $\beta$ -actin (*actb*), CD117 (*ckit*), tryptase (*tpsb2*), chymase (*cmat1*), carboxypeptidase A3 (*cpa3*), Tgf $\beta$ 1 (*tgfb1*), BMP7 (*bmp7*), BMP4 (*bmp4*), ACVR1 (*acvr1*), and BMP2 (*bmp2*) (Applied Biosystems, Foster City, CA) (Table 3.10 for primer information). Real time PCR was performed on 10 ng cDNA, with Taqman Fast Advanced Master Mix (Applied Biosystems). QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems) was used for thermal cycling at recommended settings. Results were normalized to  $\beta$ -actin and relative quantification was performed using  $2^{-\Delta\Delta Ct}$  method. Plate results were used when  $\beta$ -actin standard deviations were  $\leq 0.7$ . Average standard deviation of all plates run was 0.55.

### **Multiplex Bead Immunoassay Analysis (UT-CVM)**

BALF supernatant was thawed at room temperature and centrifuged to remove any precipitates formed during thawing. Cytokines/chemokines included in the assay were: interleukin (IL)-2, IL-4, IL-5, IL-17A, CXC motif chemokine ligand (CXCL)-8, interferon gamma (IFN $\gamma$ ), and tumor necrosis factor alpha (TNF $\alpha$ ). Cytokine concentrations were quantified using an equine-specific Milliplex® Map Magnetic Bead

Panel (EMD Millipore, St Louis, MO, USA) according to the manufacturer's instructions using a Luminex® 200 instrument and Luminex xPONENT® software run in duplicate (Luminex, Austin, TX, USA). Dilutional linearity and percent recovered were determined using low cytokine containing BALF spiked with kit cytokine standard.

Data analysis was done using Milliplex Analyst v5.1 software (EMD Millipore). A bead count of at least 50 beads per well was used for inclusion in analysis and individual samples with a coefficient of variation (CV) > 15% were not included. The Analyst® software assigned the lowest detectable concentration for the individual analytes in which the cytokine/chemokine concentrations fell below the lower limit of detection (LLOD) of the assay. Best-fit standard curve for each analyte was used to calculate analyte concentrations. Analyte concentrations for each horse was normalized to BALF volume recovered.

### **Statistical analysis**

Normality of data was assessed using Shapiro-Wilk test. Descriptive data are expressed as median with interquartile range for both normal and non-normally distributed data for consistency. Descriptive data, protease expression, and cytokine concentrations were analyzed using one-way analysis of variance with Tukey's multiple comparisons in normally distributed data, and by Kruskal-Wallis with Dunn's multiple comparisons in non-normally distributed data. Pairwise correlation of variables including age, sex, cell concentrations, gene expression fold changes, and normalized cytokine concentrations was performed, with additional multidimensional scaling (MDS) analysis in order to visualize on a two-dimensional figure the clustering of samples based on age, sex, cell concentrations, gene expression fold changes, and

normalized cytokine concentrations. Bray-Curtis distance function was applied to calculate the dissimilarity matrix of dataset, which ignored missing gene expression values in 4 samples. To evaluate which variable contribute the most to sample clustering, an additional principal components analysis (PCA, which is a specific solution of MDS based on Euclidean distance of complete data) on the samples with no missing values was performed, followed by quantification of the contributions of each variable to the first two principal components. Significance was set at  $p \leq 0.05$ .

## **Results**

### **Main study population – University of Tennessee College of Veterinary Medicine (UT-CVM)**

A total of 54 horses were sampled for evaluation. Twenty-four horses were excluded because they did not meet our inclusion criteria (Table 3.1). Final groups were comprised of 11 healthy and 19 asthmatic horses (5 mild/moderate and 14 severe) (Table 3.2). Median age was not significantly different between groups. Various breeds were represented in each group.

### **Sub-population – North Carolina State University College of Veterinary Medicine (NCSU-CVM)**

Samples from 8 horses with mastocytic asthma (maEAS) sampled at NCSU were included to fully evaluate mast cell protease expression due to the limited detection of mast cells in the UT-CVM asthma cohort (Table 3.2). Although the ages of some of the horses were unknown, the median age of the NCSU-CVM group was not significantly different from the UT-CVM groups.

### **BALF Cell Count and Cytologic Analysis (UT & NCSU-CVM)**

Results of BALF cell count and cytological analysis are described in Table 3.3 and 3.4. The median total nucleated cell count (TNCC) of each group was not statistically different, although 5 outliers in the sEAS group had TNCC > 1,000 cell/ $\mu$ L; therefore, was close to significantly different versus healthy,  $p = 0.08$ . As expected, based on standard diagnostic criteria, horses with severe asthma had significantly higher percentages of neutrophils compared to healthy horses and those with maEAS ( $p < 0.0001$  &  $p = 0.0001$ , respectively). Horses with sEAS had lower percentages of lymphocytes in their BALF when compared to healthy, mEAS, or those with maEAS ( $p = 0.01$ ,  $p = 0.0001$ , &  $p = 0.0003$ , respectively). The composition of leukocytes in healthy horses was predominated by macrophages compared to sEAS ( $p < 0.0001$ ). Three horses with mEAS had eosinophils detected in their BALF. No eosinophils were detected in BALF from any healthy horses or horses with sEAS. Mast cells were detected in two mEAS horses and one healthy horse. Mast cells were only consistently detected in maEAS horses.

### **Mast cell protease gene expression in BALF (UT & NCSU-CVM)**

In order to determine whether BALF mast cells have altered protease expression, tryptase, chymase, and/or carboxypeptidase A3 in equine asthma, gene expression analysis was performed on BALF cell pellets (Table 3.5, Fig 3.1). Samples from 4 sEAS were excluded due to poor quality and/or concentrated RNA. Tryptase expression trended higher in horses with maEAS compared to sEAS ( $p = 0.1$ ) but was not statistically different between any groups. Chymase was significantly lower in horses with maEAS (fold change: 0.18 [0.08 – 0.54]) versus control (1.88 [0.33 – 2.46];  $p =$

0.02), or sEAS horses (0.92 [0.65 – 1.97];  $p = 0.04$ ). Protease expression was not significantly changed by removing horses that had clinical signs but use of corticosteroids (Fig 3.2). Taken together, these results suggest that healthy horses and asthmatic horses without mast cells identified on cytology have airway mast cells that express both tryptase and chymase, whereas maEAS horses have mast cells that primarily express tryptase. As expected, CD117(c-kit), expression was significantly increased in horses with maEAS (fold change: 2.32, [1.31 – 3.85]) compared to sEAS (0.38 [0.27 – 1.50];  $p = 0.01$ ). Additionally, carboxypeptidase A3 was not amplified in any sample, with either primer set identified, but whether this was due to a technical issue or lack of carboxypeptidase A3 gene expression in airway mast cells has not been determined.

#### **Analysis of BMP ligands, receptors, and Tgf $\beta$ 1 expression (UT & NCSU-CVM)**

Potential anti-inflammatory and airway remodeling cytokines, BMP7 and Tgf $\beta$ 1, were evaluated in BALF isolated cells at the gene expression level due to availability of reagents (Table 3.5, Fig 3.3). Additionally, BMP4 and two BMP receptors, Acvr1 and Bmpr2, were evaluated alongside to get a broader evaluation of BMP ligand and receptor expression. BMP7 expression was not significantly different across the groups, although a subset of horses diagnosed with sEAS had markedly elevated BMP7 expression and trended higher versus maEAS ( $p = 0.08$ ). Tgf $\beta$ 1 was significantly elevated in mEAS (fold change: 1.43, [1.2 – 1.83]  $p=0.04$ ) versus healthy (fold change: 1.06, [0.81 – 1.22]). Tgf $\beta$ 1 to BMP7 ratios did not explain the subgrouping of sEAS seen in BMP7 expression results, although 3 horses in the sEAS group (1 in low BMP7, 2 in

high BMP7) were unable to have Tgf $\beta$ 1 analyzed due to lack of remaining RNA for analysis. Additional BMP ligand and receptor evaluation was not statistically different across groups but were able to be detected. Due to the inability to perform lung function tests on any of the horses, it is unknown if these horses with high BMP7 or Tgf $\beta$ 1 could represent ones with worse or better lung function. Lung biopsy samples were also not obtained which could have given more information in regard to level of fibrosis and BMP7, Tgf $\beta$ 1 expression levels seen in asthma groups.

### **Multiplex Bead Immunoassay Analysis (IL-2, IL-4, IL-5, IL-17A, CXCL8, IFN $\gamma$ , TNF $\alpha$ ) (UT-CVM)**

Evaluation of BALF supernatant cytokine concentrations was only performed on the UT-CVM study population for consistency of the BALF procedure (Table 3.6, 3.7 & 3.8). Multiplexing allowed for conservation of sample and reducing technical errors and time when measuring multiple analytes. Significant differences between groups were found for normalized concentrations for TNF $\alpha$ , CXCL8 (formerly known as IL-8), and IFN $\gamma$ . Analysis showed that horses with severe asthma had significantly higher TNF $\alpha$  ( $p = 0.0005$ ), CXCL8 ( $p < 0.0001$ ), and IFN $\gamma$  ( $p = 0.006$ ) compared to healthy horses (Fig 3.4). There were no statistical differences in cytokine concentrations between horses with mEAS or sEAS, but comparison is limited due to small sample size. These results support horses with severe asthma, type 1 and neutrophil recruiting immune responses is predominant. No significant differences between groups were found for the remaining analytes, although some analytes were poorly to not detected. Results were not significantly changed by removing horses that had a history of corticosteroid use with continued clinical signs (Fig 3.5).

The equine multiplex bead immunoassay has been used to analyze other more common samples, such as serum, but to the authors' knowledge has not been used to evaluate BALF; therefore, further validation of the assay was performed. A spike and recovery assay using the kit standard and low cytokine concentration BALF was performed, which found the percent recovered was lowest for IL-17A and high at low concentrations for IL-4 and TNF $\alpha$  (Table 3.9). Additionally, dilutional linearity was achieved for all analytes (Fig 3.6).

### **Analysis of Similarity Between Samples (UT-CVM)**

Pairwise correlation of data was done on the UT-CVM cohort (Fig 3.7). Analysis showed a positive, very strongly correlated expression of CD117 and tryptase ( $r = 0.94$ ), suggesting mast cells primarily express tryptase. Pairwise correlation analysis of the consistently detected cytokines (CXCL8, IFN $\gamma$ , and TNF $\alpha$ ) demonstrated a positive correlation between neutrophil concentration and CXCL8 and IFN $\gamma$  concentrations (Pearson's  $r = 0.38$  and  $0.93$ , respectively), suggesting a stronger association between IFN $\gamma$  production and increasing neutrophil concentration. Neutrophil concentration was nearly significantly positively correlated with TNF $\alpha$ , with a  $p$  value of  $0.06$  and  $r = 0.36$ . CXCL8 and TNF $\alpha$  were positively, very strongly correlated with lymphocyte and macrophage concentration, suggesting that CXCL8 and TNF $\alpha$  production are closely related to increasing lymphocyte and macrophage numbers. The data does not allow interpretation of what cells are making the cytokines, but further evaluation of the strong correlations warrants further investigation in regard to production and function. The significant correlations shown of the other cytokines, IL5, IL2, and IL4 are hard to interpret due to low to minimal detection. Additionally, significant correlations with

mast cell concentration are difficult to interpret due to only 2 horses with mild/moderate asthma and one healthy horse having detectable mast cells in the UT-CVM cohort.

Multidimensional scaling (MDS) analysis showed separation between samples from horses with sEAS, mEAS, and healthy horses (Fig 3.8). The healthy samples and mEAS samples did not form distinct clusters, but a gradual trend from healthy to mEAS to sEAS was observed on both axes of the two-dimensional projection. Further principal component analysis on samples with complete data showed that the first two principal components (PCs) explained the majority of the variance, with the first PC explaining 99.97%, and the second PC explaining 94.38% of the variance. The absolute value of the loadings of the first two PCs quantified the contribution of each variable to the observed clustering pattern. By ranking the variables on the maximum absolute loadings from PC1 and PC2, neutrophil concentration and lymphocyte concentration were the main drivers of the observed clusters (Fig 5B). The increase in neutrophils and decrease in lymphocytes observed in sEAS, therefore, is an important association involved in the severity of asthma pathology.

## **Discussion**

This study, to our knowledge, is the first to examine BALF supernatant cytokine/chemokine concentrations and mast cell protease expression in BALF isolated cells in horses with asthma. These results potentially help to clarify pathophysiology of asthma, while also supporting further subtyping of the two primary categories of asthma. Including a mastocytic asthma group in the sample analysis identified the dysregulation of the mast cell protease chymase, potentially supporting the further

subtyping of the mild to moderate asthma group to include a mastocytic group. Loss of chymase may contribute to disease progression since chymase has anti-inflammatory activities in allergic asthma models via modulating IL-33 concentrations, and may be protective of lung function (38, 39). We additionally demonstrated that mast cells in equine BALF express both tryptase and chymase mRNA but fail to express carboxypeptidase A3. Previous work evaluating mast cell types in horses have investigated lung tissue samples, but not BALF, and reports have been conflicting. A report comparing types in sEAS versus control after moldy hay challenge found no MC<sub>TC</sub>, although severely asthmatic horses had significantly more MC<sub>C</sub> in the wall of the bronchi (34). In conflict to this report, another study found increased tryptase expression in lung tissue from asthmatic horses versus control (35). BALF IgE-bearing cells have been evaluated in asthmatic horses and were not different from control horses, although this is not mast cell-specific or evaluates protease expression (40). The current study suggests protease dysregulation, and changes in mast cell type, occur in horses with mastocytic asthma.

Further investigation of mast cell phenotypes in horses is supported by human studies in which mast cells and their type is associated with asthma pathology. Increased numbers of MC<sub>T</sub> in smooth muscle of subjects with asthma have been associated with airway hyper-reactivity (27, 41). Additionally, an increase in MC<sub>TC</sub> type has been suggested to be protective of lung function in severe, steroid-dependent human asthmatics and corticosteroids are noted to decrease MC<sub>T</sub> numbers (27, 39). Molecular analysis of lung samples revealed that tryptase and carboxypeptidase A3 were among the most differentially expressed genes in asthmatics versus control, with Th2-high

asthma associated with expression of tryptase and carboxypeptidase A3, but not chymase (42, 43). Taken together this suggests the importance of continued investigation of mast cell phenotype in relation to asthma type, pathogenesis, and response to therapy in equine asthma.

Utilizing a multiplex bead immunoassay allowed simultaneous detection of a panel of cytokines/chemokines in BALF supernatant. In this study, cytokine/chemokine concentrations in BALF of the main study population (UT-CVM), revealed elevated TNF $\alpha$  and CXCL8 in sEAS horses; therefore, these are potential targets for treatment in asthma. Elevated TNF $\alpha$  and CXCL8 parallels findings in human asthma, in which asthmatics had increased TNF $\alpha$  and CXCL8 versus control, and neutrophilic asthma was associated with further increased CXCL8 (44, 45). A caveat to this immunoassay was a few analytes were poorly detected; therefore, lower sensitivity assays would be beneficial in order to most clearly define the type of immune response at the protein level. We utilized an unbiased approach and multidimensional scaling to integrate clinical data, cytokine/chemokine concentrations, and mast cell protease expression, in order to investigate potential biomarkers of equine asthma. This analysis indicated that neutrophil and lymphocyte concentrations are key drivers of equine asthma differences, in particular, severe equine asthma in which neutrophils increase and lymphocytes decrease. This suggests, that in sEAS, both neutrophils and lymphocytes should be targets of treatment, both their numbers and role in inflammation. Corticosteroids can reduce inflammation by general mechanisms such as inhibiting gene expression of inflammatory cytokines, but targeted cellular therapy against neutrophil/lymphocyte

ratios may be more effective, such as reducing CXCL8, which is key to recruiting and activating neutrophils.

In addition to airway hyper-responsiveness, airway remodeling is another key feature of asthma. Tgf $\beta$ 1 and BMP7 expression in asthma models and human asthma highlights their balance as an important factor in asthma airway remodeling (21-23). This study is the first to evaluate BMP7 expression in the airway of the horse. Our results demonstrated that a subset of horses with sEAS had elevated BMP7 expression compared to all other groups. The potential role of BMP7 in human asthma has been suggested to be anti-inflammatory and anti-fibrotic, with BMP7's ability to block detrimental Tgf $\beta$ 1 effects, such as fibroblast activation with subsequent airway remodeling, production of extracellular matrix, and collagen deposition (22, 23). The sEAS BMP7-high group may have an over exuberant BMP7 response in attempt to counteract airway remodeling and worsening lung function. Further studies to define the horses with BMP7-high and BMP-7 low sEAS may elucidate further subtyping of sEAS and further define the pathophysiology. Further investigating Tgf $\beta$ 1/BMP7 dynamics in the horse may be especially interesting in regard to a translational human asthma model and fibrosis investigation because the horse is noted to develop similar histologic lesions of airway remodeling, including peribronchiolar fibrosis (46).

Primary limitations of the study are small sample size, which limits extrapolation of results to other areas of the country and our ability to identify significant differences. Additionally, experimental group classification relied on history, clinical signs, physical exam, and BALF cytology. Further objective evaluation, such as lung function testing, would be optimal for final diagnosis. Innate variations of a blind BALF collection, such

as the location sampled or volume of return, even when performed in the same manner, could have influenced results. Evaluating protein concentrations in BALF supernatant does not have a universally accepted way to normalize the cytokine/chemokine concentrations across patients. Normalization of analyte concentration to fluid volume recovered during the BAL procedure has been recommended, but is not consistently employed in the literature (47). Our results were normalized to BALF volume returned. Additional manipulation of the supernatant, such as concentrating techniques, may result in protein loss and inconsistent concentrating due to technical error; therefore, if possible, not manipulating the samples is best (48, 49).

The inclusion of a mastocytic asthma group and their results may have been influenced by the BAL technique being performed in a slightly different manner than the main population. Due to this difference, only gene expression was evaluated due to the ability to normalize RNA sample input. The mastocytic asthma group showed significant differences in CD117/c-kit and chymase expression. Tryptase and chymase are primarily made by mast cells, and although mast cell protease knockout models have suggested a mild decrease in basophil number, suggesting low protease expression; basophils are not noted as of yet to be in equine BALF (50-53). In our study, CD117/c-kit expression increased in maEAS horses and highly correlated with tryptase expression levels, therefore the tryptase gene expression is likely to be of mast cell origin, since basophils do not retain CD117 expression once differentiated (54, 55). CD117 can also be expressed by other immune cell types, specifically type 2 innate lymphoid cells, however these cells have not yet been described in the horse (56). Increased CD117 expression has also been linked to increased mast cell responses to SCF; therefore, our results may also support

that mast cells in the mastocytic asthma group are more reactive; although further evaluation of SCF concentrations and *ex vivo* responses is needed (57, 58). It is unclear whether the negative CPA3 amplification in all horses in this study is due to a technical problem with the assay or whether horse BALF mast cells truly do not express CPA3. Ideally mast cell protease expression would not be evaluated in a heterogeneous cell population but would be mast cell specific via mast cell isolation and/or flow cytometry techniques in order to obtain cell specific cell numbers. Due to low mast cell numbers in BALF, even in mastocytic asthma, further optimization is required for mast cell isolation and/or flow cytometry and will be further evaluated.

We propose that further evaluation of protease and cytokine/chemokine profiles in a larger cohort will identify biomarkers that support defining equine asthma subtypes based on BALF cellular infiltrate, including eosinophilic mild/moderate asthma, mastocytic mild/moderate asthma, neutrophilic mild/moderate asthma, and neutrophilic severe asthma. These groups likely have differences that will require specific environmental management and treatments for optimal management of the patient. Lung function tests would be additional useful information for support of diagnosis, treatment recommendations, and evaluating treatment effects. A staging scale for severe asthma could be used in addition to, or substitution of, lung function tests, but a staging scale for mEAS does not exist at this time (59).

In conclusion, this study demonstrates that, similar to human patients with asthma, horses with sEAS have increases in CXCL8, TNF $\alpha$ , and IFN $\gamma$ . In addition, as seen in humans with Th2-high asthma, maEAS is associated with an increase in tryptase

and decrease in chymase. Therefore, horses may serve as a naturally occurring model of human asthma and potential model for chymase dysregulation.

## LIST OF REFERENCES

1. Couetil LL, Cardwell JM, Gerber V, Lavoie JP, Leguillette R, Richard EA. Inflammatory Airway Disease of Horses--Revised Consensus Statement. *Journal of veterinary internal medicine*. 2016;30(2):503-15.
2. Pirie RS. Recurrent airway obstruction: a review. *Equine veterinary journal*. 2014;46(3):276-88.
3. Hotchkiss JW, Reid SW, Christley RM. A survey of horse owners in Great Britain regarding horses in their care. Part 2: Risk factors for recurrent airway obstruction. *Equine veterinary journal*. 2007;39(4):301-8.
4. Sheats MK, Davis KU, Poole JA. Comparative Review of Asthma in Farmers and Horses. *Current allergy and asthma reports*. 2019;19(11):50.
5. Bond S, Leguillette R, Richard EA, Couetil L, Lavoie JP, Martin JG, et al. Equine asthma: Integrative biologic relevance of a recently proposed nomenclature. *Journal of veterinary internal medicine*. 2018;32(6):2088-98.
6. Hughes KJ, Nicolson L, Da Costa N, Franklin SH, Allen KJ, Dunham SP. Evaluation of cytokine mRNA expression in bronchoalveolar lavage cells from horses with inflammatory airway disease. *Veterinary immunology and immunopathology*. 2011;140(1-2):82-9.
7. Lavoie JP, Cesarini C, Lavoie-Lamoureux A, Moran K, Lutz S, Picandet V, et al. Bronchoalveolar lavage fluid cytology and cytokine messenger ribonucleic Acid expression of racehorses with exercise intolerance and lower airway inflammation. *Journal of veterinary internal medicine*. 2011;25(2):322-9.
8. Beekman L, Tohver T, Leguillette R. Comparison of cytokine mRNA expression in the bronchoalveolar lavage fluid of horses with inflammatory airway disease and bronchoalveolar lavage mastocytosis or neutrophilia using REST software analysis. *Journal of veterinary internal medicine*. 2012;26(1):153-61.
9. Richard EA, Depecker M, Defontis M, Leleu C, Fortier G, Pitel PH, et al. Cytokine concentrations in bronchoalveolar lavage fluid from horses with neutrophilic inflammatory airway disease. *Journal of veterinary internal medicine*. 2014;28(6):1838-44.
10. Montgomery JB, Husulak ML, Kosolofski H, Dos Santos S, Burgess H, Meachem MD. Tumor necrosis factor-alpha protein concentrations in bronchoalveolar lavage fluid from healthy horses and horses with severe equine asthma. *Veterinary immunology and immunopathology*. 2018;202:70-3.
11. Giguere S, Viel L, Lee E, MacKay RJ, Hernandez J, Franchini M. Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. *Veterinary immunology and immunopathology*. 2002;85(3-4):147-58.
12. Lavoie JP, Maghni K, Desnoyers M, Taha R, Martin JG, Hamid QA. Neutrophilic airway inflammation in horses with heaves is characterized by a Th2-type cytokine profile. *American journal of respiratory and critical care medicine*. 2001;164(8 Pt 1):1410-3.
13. Debrue M, Hamilton E, Joubert P, Lajoie-Kadoch S, Lavoie JP. Chronic exacerbation of equine heaves is associated with an increased expression of interleukin-

- 17 mRNA in bronchoalveolar lavage cells. *Veterinary immunology and immunopathology*. 2005;105(1-2):25-31.
14. Padoan E, Ferraresso S, Pegolo S, Castagnaro M, Barnini C, Bargelloni L. Real time RT-PCR analysis of inflammatory mediator expression in recurrent airway obstruction-affected horses. *Veterinary immunology and immunopathology*. 2013;156(3-4):190-9.
15. Tessier L, Cote O, Clark ME, Viel L, Diaz-Mendez A, Anders S, et al. Impaired response of the bronchial epithelium to inflammation characterizes severe equine asthma. *BMC genomics*. 2017;18(1):708.
16. Bond SL, Hundt J, Leguillette R. Effect of injected dexamethasone on relative cytokine mRNA expression in bronchoalveolar lavage fluid in horses with mild asthma. *BMC veterinary research*. 2019;15(1):397.
17. Hansen S, Otten ND, Birch K, Skovgaard K, Hopster-Iversen C, Fjeldborg J. Bronchoalveolar lavage fluid cytokine, cytology and IgE allergen in horses with equine asthma. *Veterinary immunology and immunopathology*. 2019;220:109976.
18. Hue E, Orard M, Toquet MP, Depecker M, Couroucé A, Pronost S, et al. Asymmetrical Pulmonary Cytokine Profiles Are Linked to Bronchoalveolar Lavage Fluid Cytology of Horses With Mild Airway Neutrophilia. *Frontiers in veterinary science*. 2020;7:226.
19. Yoshimura A, Wakabayashi Y, Mori T. Cellular and molecular basis for the regulation of inflammation by TGF-beta. *Journal of biochemistry*. 2010;147(6):781-92.
20. Walton KL, Johnson KE, Harrison CA. Targeting TGF- $\beta$  Mediated SMAD Signaling for the Prevention of Fibrosis. *Frontiers in pharmacology*. 2017;8:461.
21. Stumm CL, Halcsik E, Landgraf RG, Camara NO, Sogayar MC, Jancar S. Lung remodeling in a mouse model of asthma involves a balance between TGF-beta1 and BMP-7. *PloS one*. 2014;9(4):e95959.
22. Pegorier S, Campbell GA, Kay AB, Lloyd CM. Bone morphogenetic protein (BMP)-4 and BMP-7 regulate differentially transforming growth factor (TGF)-beta1 in normal human lung fibroblasts (NHLF). *Respiratory research*. 2010;11:85.
23. Kariyawasam HH, Xanthou G, Barkans J, Aizen M, Kay AB, Robinson DS. Basal expression of bone morphogenetic protein receptor is reduced in mild asthma. *American journal of respiratory and critical care medicine*. 2008;177(10):1074-81.
24. Desjardins I, Theoret C, Joubert P, Wagner B, Lavoie JP. Comparison of TGF-beta 1 concentrations in bronchoalveolar fluid of horses affected with heaves and of normal controls. *Veterinary immunology and immunopathology*. 2004;101(3-4):133-41.
25. Bullone M, Lavoie JP. Asthma "of horses and men"--how can equine heaves help us better understand human asthma immunopathology and its functional consequences? *Molecular immunology*. 2015;66(1):97-105.
26. Leclerc M, Lavoie-Lamoureux A, Lavoie JP. Heaves, an asthma-like disease of horses. *Respirology (Carlton, Vic)*. 2011;16(7):1027-46.
27. Balzar S, Fajt ML, Comhair SA, Erzurum SC, Bleecker E, Busse WW, et al. Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma Research Program. *American journal of respiratory and critical care medicine*. 2011;183(3):299-309.

28. Wang G, Baines KJ, Fu JJ, Wood LG, Simpson JL, McDonald VM, et al. Sputum mast cell subtypes relate to eosinophilia and corticosteroid response in asthma. *The European respiratory journal*. 2016;47(4):1123-33.
29. Berthon BS, Gibson PG, Wood LG, MacDonald-Wicks LK, Baines KJ. A sputum gene expression signature predicts oral corticosteroid response in asthma. *The European respiratory journal*. 2017;49(6).
30. Fricker M, Qin L, Niessen N, Baines KJ, McDonald VM, Scott HA, et al. Relationship of sputum mast cells with clinical and inflammatory characteristics of asthma. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2020.
31. Irani AA, Schechter NM, Craig SS, DeBlois G, Schwartz LB. Two types of human mast cells that have distinct neutral protease compositions. *Proceedings of the National Academy of Sciences of the United States of America*. 1986;83(12):4464-8.
32. Irani AM, Goldstein SM, Wintroub BU, Bradford T, Schwartz LB. Human mast cell carboxypeptidase. Selective localization to MCTC cells. *Journal of immunology (Baltimore, Md : 1950)*. 1991;147(1):247-53.
33. Beil WJ, Pammer J. In situ detection of the mast cell proteases chymase and tryptase in human lung tissue using light and electron microscopy. *Histochemistry and cell biology*. 2001;116(6):483-93.
34. van der Haegen A, Kunzle F, Gerber V, Welle M, Robinson NE, Marti E. Mast cells and IgE-bearing cells in lungs of RAO-affected horses. *Veterinary immunology and immunopathology*. 2005;108(3-4):325-34.
35. Dacre KJ, McGorum BC, Marlin DJ, Bartner LR, Brown JK, Shaw DJ, et al. Organic dust exposure increases mast cell tryptase in bronchoalveolar lavage fluid and airway epithelium of heaves horses. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2007;37(12):1809-18.
36. Ekmann A, Rigdal ML, Gröndahl G. Automated counting of nucleated cells in equine synovial fluid without and with hyaluronidase pretreatment. *Veterinary clinical pathology*. 2010;39(1):83-9.
37. Berger JT, Voynow JA, Peters KW, Rose MC. Respiratory carcinoma cell lines. MUC genes and glycoconjugates. *American journal of respiratory cell and molecular biology*. 1999;20(3):500-10.
38. Waern I, Lundequist A, Pejler G, Wernersson S. Mast cell chymase modulates IL-33 levels and controls allergic sensitization in dust-mite induced airway inflammation. *Mucosal immunology*. 2013;6(5):911-20.
39. Balzar S, Chu HW, Strand M, Wenzel S. Relationship of small airway chymase-positive mast cells and lung function in severe asthma. *American journal of respiratory and critical care medicine*. 2005;171(5):431-9.
40. Kunzle F, Gerber V, Van Der Haegen A, Wampfler B, Straub R, Marti E. IgE-bearing cells in bronchoalveolar lavage fluid and allergen-specific IgE levels in sera from RAO-affected horses. *Journal of veterinary medicine A, Physiology, pathology, clinical medicine*. 2007;54(1):40-7.
41. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *The New England journal of medicine*. 2002;346(22):1699-705.

42. Dougherty RH, Sidhu SS, Raman K, Solon M, Solberg OD, Caughey GH, et al. Accumulation of intraepithelial mast cells with a unique protease phenotype in T(H)2-high asthma. *The Journal of allergy and clinical immunology*. 2010;125(5):1046-53.e8.
43. Woodruff PG, Boushey HA, Dolganov GM, Barker CS, Yang YH, Donnelly S, et al. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104(40):15858-63.
44. Hosoki K, Ying S, Corrigan C, Qi H, Kurosky A, Jennings K, et al. Analysis of a Panel of 48 Cytokines in BAL Fluids Specifically Identifies IL-8 Levels as the Only Cytokine that Distinguishes Controlled Asthma from Uncontrolled Asthma, and Correlates Inversely with FEV1. *PloS one*. 2015;10(5):e0126035.
45. Yang T, Li Y, Lyu Z, Huang K, Corrigan CJ, Ying S, et al. Characteristics of Proinflammatory Cytokines and Chemokines in Airways of Asthmatics: Relationships with Disease Severity and Infiltration of Inflammatory Cells. *Chinese medical journal*. 2017;130(17):2033-40.
46. Ferrari CR, Cooley J, Mujahid N, Costa LR, Wills RW, Johnson ME, et al. Horses With Pasture Asthma Have Airway Remodeling That Is Characteristic of Human Asthma. *Veterinary pathology*. 2018;55(1):144-58.
47. Haslam PL, Baughman RP. Report of ERS Task Force: guidelines for measurement of acellular components and standardization of BAL. *The European respiratory journal*. 1999;14(2):245-8.
48. Lam S, LeRiche JC, Kijek K. Effect of filtration and concentration on the composition of bronchoalveolar lavage fluid. *Chest*. 1985;87(6):740-2.
49. Afford SC, Stockley RA, Kramps JA, Dijkman JH, Burnett D. Concentration of bronchoalveolar lavage fluid by ultrafiltration: evidence of differential protein loss and functional inactivation of proteinase inhibitors. *Analytical biochemistry*. 1985;151(1):125-30.
50. Heutinck KM, ten Berge IJ, Hack CE, Hamann J, Rowshani AT. Serine proteases of the human immune system in health and disease. *Molecular immunology*. 2010;47(11-12):1943-55.
51. Pejler G, Abrink M, Ringvall M, Wernersson S. Mast cell proteases. *Advances in immunology*. 2007;95:167-255.
52. Pejler G, Ronnberg E, Waern I, Wernersson S. Mast cell proteases: multifaceted regulators of inflammatory disease. *Blood*. 2010;115(24):4981-90.
53. Reber LL, Marichal T, Galli SJ. New models for analyzing mast cell functions in vivo. *Trends in immunology*. 2012;33(12):613-25.
54. Han X, Jorgensen JL, Brahmandam A, Schlette E, Huh YO, Shi Y, et al. Immunophenotypic study of basophils by multiparameter flow cytometry. *Archives of pathology & laboratory medicine*. 2008;132(5):813-9.
55. Galli SJ, Tsai M, Wershil BK. The c-kit receptor, stem cell factor, and mast cells. What each is teaching us about the others. *The American journal of pathology*. 1993;142(4):965-74.
56. Hochdorfer T, Winkler C, Pardali K, Mjosberg J. Expression of c-Kit discriminates between two functionally distinct subsets of human type 2 innate lymphoid cells. *European journal of immunology*. 2019;49(6):884-93.

57. Lewis A, Wan J, Baothman B, Monk PN, Suvarna SK, Peachell PT. Heterogeneity in the responses of human lung mast cells to stem cell factor. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*. 2013;43(1):50-9.
58. Shimizu Y, Ashman LK, Du Z, Schwartz LB. Internalization of Kit together with stem cell factor on human fetal liver-derived mast cells: new protein and RNA synthesis are required for reappearance of Kit. *Journal of immunology (Baltimore, Md : 1950)*. 1996;156(9):3443-9.
59. Simoes J, Sales Luis J, Tilley P. Contribution of lung function tests to the staging of severe equine asthma syndrome in the field. *Research in veterinary science*. 2019;123:112-7.

## **APPENDIX**

**Table 3.1. Defined experimental groups.**

| <b>Experimental Group</b>                                                | <b>History &amp; Physical exam</b>                                                                                                                                                                                  | <b>CBC/fibrinogen</b>                        | <b>BALF cytology</b>                                                                                         |
|--------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------|--------------------------------------------------------------------------------------------------------------|
| <b>Healthy</b><br><i>Main population</i><br><i>UT-CVM</i>                | Normal                                                                                                                                                                                                              | Normal                                       | <10% neutrophils,<br>≤1% eosinophils,<br>≤2%<br>metachromatic<br>cells, and<br>≤50%<br>lymphocytes           |
| <b>Asthma</b><br><i>Main population</i><br><i>UT-CVM</i>                 | History, clinical signs,<br>and physical exam<br>supportive of lower<br>airway inflammation;<br>no changes to or<br>initiation of<br>corticosteroids<br>(systemic or<br>intranasal) within 2<br>weeks of enrollment | Not consistent<br>with systemic<br>infection | ≥10% neutrophils,<br>≥5% mast cells,<br>and/or ≥5%<br>eosinophils<br><br>*severe asthma,<br>>25% neutrophils |
| <b>Mastocytic<br/>Asthma</b><br><i>Sub-population</i><br><i>NCSU-CVM</i> | History, clinical signs,<br>and physical exam<br>supportive of lower<br>airway inflammation;<br>no corticosteroids<br>administered within 2<br>weeks of BAL                                                         | Not performed                                | ≥2% mast cells<br>≤6% neutrophils<br>≤1% eosinophils                                                         |

**Table 3.2. Individual horse's clinical data.**

|          | Diagnosis | Clinical Signs                                            | Rebreathing Exam                                          | Sex | Age (yrs)   | Breed        |
|----------|-----------|-----------------------------------------------------------|-----------------------------------------------------------|-----|-------------|--------------|
| Horse 1  | sEAS      | Chronic cough                                             | Delayed recovery                                          | M   | 20          | Pony         |
| Horse 2  | sEAS      | Chronic cough, intermittent nasal discharge               | NSF                                                       | M   | 13          | Not reported |
| Horse 3  | sEAS      | Chronic cough                                             | Tracheal rattle                                           | M   | 13          | MFT          |
| Horse 4  | sEAS      | Chronic cough, intermittent nasal discharge               | Crackles, wheezes                                         | M   | 13          | ASH          |
| Horse 5  | sEAS      | Chronic cough                                             | Crackles, wheezes                                         | G   | 12          | Pony         |
| Horse 6  | sEAS      | Increased respiratory effort                              | Wheezes                                                   | M   | 14          | TWH          |
| Horse 7  | sEAS      | Chronic cough, exercise intolerance                       | Wheezes                                                   | G   | 19          | Not reported |
| Horse 8  | sEAS      | Chronic cough                                             | NSF                                                       | G   | 20          | Arab         |
| Horse 9  | sEAS      | Increased respiratory rate at rest                        | Wheezes                                                   | G   | 14          | TWH          |
| Horse 10 | sEAS      | Chronic cough, intermittent nasal discharge               | Crackles, wheezes                                         | M   | 20          | Arab         |
| Horse 11 | sEAS      | Wheezes and increased respiratory effort at rest          | Wheezes                                                   | G   | 10          | TWH          |
| Horse 12 | sEAS      | Chronic intermittent cough and increased respiratory rate | Wheezes, occasional crackle, increased respiratory effort | G   | 19          | H            |
| Horse 13 | sEAS      | Chronic intermittent cough                                | Wheezes and worsened cough                                | G   | 12          | QH           |
| Horse 14 | sEAS      | Cough, increased respiratory effort                       | Worsened respiratory effort and cough                     | G   | 17          | QH           |
| Horse 15 | mEAS      | Increased respiratory rate in barn                        | NSF                                                       | M   | 18          | QH           |
| Horse 16 | mEAS      | Cough, intermittent nasal discharge                       | Cough upon tracheal palpation, delayed recovery           | G   | 13          | QH           |
| Horse 17 | mEAS      | None observed                                             | Delayed recovery                                          | M   | 22          | QH           |
| Horse 18 | mEAS      | Chronic cough                                             | Wheezes                                                   | G   | 14          | MFT          |
| Horse 19 | mEAS      | Exercise intolerance, intermittent nasal discharge        | Delayed recovery, nasal discharge                         | G   | 11          | PF           |
| Horse 20 | Ctr       | None                                                      | NSF                                                       | M   | 8           | TWH          |
| Horse 21 | Ctr       | None                                                      | NSF                                                       | M   | 18          | QH           |
| Horse 22 | Ctr       | None                                                      | NSF                                                       | M   | 19          | QH           |
| Horse 23 | Ctr       | None                                                      | NSF                                                       | M   | 16          | QH           |
| Horse 24 | Ctr       | None                                                      | NSF                                                       | M   | 14          | MFT          |
| Horse 25 | Ctr       | None                                                      | NSF                                                       | M   | 17          | QH           |
| Horse 26 | Ctr       | None                                                      | NSF                                                       | M   | 26          | APH          |
| Horse 27 | Ctr       | None                                                      | NSF                                                       | M   | 16          | TWH          |
| Horse 28 | Ctr       | None                                                      | NSF                                                       | G   | 11          | TWH          |
| Horse 29 | Ctr       | None                                                      | NSF                                                       | M   | 14          | QH           |
| Horse 30 | Ctr       | None                                                      | NSF                                                       | G   | 12          | TB           |
| Horse 31 | maEAS     | None                                                      | NSF                                                       | M   | 6           | QH           |
| Horse 32 | maEAS     | Abdominal lift                                            | NSF                                                       | M   | 18          | QH           |
| Horse 33 | maEAS     | None                                                      | NSF                                                       | M   | unkno<br>wn | unknown      |
| Horse 34 | maEAS     | Abdominal lift, nasal discharge                           | NSF                                                       | M   | unkno<br>wn | unknown      |
| Horse 35 | maEAS     | None                                                      | NSF                                                       | G   | 9           | TBx          |
| Horse 36 | maEAS     | Nostril flare                                             | Delayed recovery                                          | M   | unkno<br>wn | unknown      |
| Horse 37 | maEAS     | None                                                      | NSF                                                       | M   | 3           | unknown      |
| Horse 38 | maEAS     | Cough, abdominal lift                                     | NSF                                                       | M   | 3           | unknown      |

\*sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, Ctr= Healthy, maEAS= Mastocytic Asthma; M= Mare, G= Gelding; NSF= No significant findings; MFT= Missouri Fox Trotter, ASH= American Saddle Horse, TWH= Tennessee Walking Horse, H= Holsteiner, QH= Quarter Horse, PF= Paso Fino, TB= Thoroughbred, APH= American Paint Horse, TBx= Thoroughbred cross.

**Table 3.3. Bronchoalveolar lavage fluid cytology results.**

BALF characteristics for the four defined groups: TNCC, Neutrophil %, Lymphocyte %, Macrophage %, Eosinophil %, and Mast cell %. Data are expressed as median [IQR].

sEAS, n=14; mEAS, n=5; Ctr, n=11; maEAS, n=8.

| Group        | TNCC<br>(cell/ $\mu$ L)  | %                                     |                                      |                                      |                    |                      |
|--------------|--------------------------|---------------------------------------|--------------------------------------|--------------------------------------|--------------------|----------------------|
|              |                          | N                                     | L                                    | M                                    | E                  | MC                   |
| <b>sEAS</b>  | 500<br>[257 – 3775]      | 74.5 <sup>*†</sup><br>[55.25 – 89.75] | 14.5 <sup>*†‡</sup><br>[1.75 – 29.0] | 10.0 <sup>*†</sup><br>[6.75 – 18.25] | 0                  | 0                    |
| <b>mEAS</b>  | 400<br>[250 – 725]       | 12.0<br>[6.5 – 19.0]                  | 50 <sup>*</sup><br>[41.5 – 58.5]     | 36<br>[25.5 – 44.5]                  | 1.0<br>[0.0 – 3.0] | 0<br>[0.0 – 2.0]     |
| <b>Ctrl</b>  | 250<br>[200 – 300]       | 4.0 <sup>*</sup><br>[0.0 – 9.0]       | 32 <sup>‡</sup><br>[26 – 40.0]       | 66 <sup>*</sup><br>[55 – 73]         | 0                  | 0                    |
| <b>maEAS</b> | 291.5<br>[169.5 – 355.8] | 2.35 <sup>†</sup><br>[1.18 – 5.03]    | 47.65 <sup>†</sup><br>[22 – 58.73]   | 46 <sup>†</sup><br>[28.2 – 70.5]     | 0.0<br>[0.0 – 0.7] | 5.0<br>[4.18 – 5.75] |

\*sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, maEAS= Mastocytic Asthma,

Ctrl= Healthy; TNCC= Total Nucleated Cell Count (Average recorded); N= neutrophil,

L= lymphocyte, M= macrophage, E= eosinophil, MC= mast cell. Note: Medians in the same column with the same superscript symbol differ significantly from each other.

**Table 3.4. Bronchoalveolar lavage fluid characteristics for individual horses.**

Fluid volume recovered, % volume recovered, TNCC, neutrophil %, lymphocyte %, macrophage %, eosinophil %, and mast cell % for individual horses in each group. Data is represented as the median [IQR] for each group.

| Horse               | mLs Fluid Recovered | % Volume Recovered  | TNCC (cell/ $\mu$ L)  | N                  | L                  | % M              | E               | MC                |
|---------------------|---------------------|---------------------|-----------------------|--------------------|--------------------|------------------|-----------------|-------------------|
| <b>sEAS</b>         |                     |                     |                       |                    |                    |                  |                 |                   |
| 1                   | 23                  | 11.5                | 13,000                | 70                 | 22                 | 8                | 0               | 0                 |
| 2                   | 62                  | 31                  | 900                   | 33                 | 40                 | 27               | 0               | 0                 |
| 3                   | 120                 | 60                  | 49,000                | 98                 | 1                  | 1                | 0               | 0                 |
| 4                   | 52                  | 26                  | 2,000                 | 57                 | 26                 | 17               | 0               | 0                 |
| 5                   | 24                  | 12                  | 250                   | 81                 | 5                  | 14               | 0               | 0                 |
| 6                   | 78                  | 39                  | 200                   | 32                 | 32                 | 36               | 0               | 0                 |
| 7                   | 19                  | 9.5                 | Unable to determine   | 97                 | 0                  | 3                | 0               | 0                 |
| 8                   | 30                  | 15                  | 5,550                 | 89                 | 4                  | 7                | 0               | 0                 |
| 9                   | 58                  | 29                  | 300                   | 50                 | 28                 | 22               | 0               | 0                 |
| 10                  | 70                  | 35                  | 1,150                 | 79                 | 14                 | 7                | 0               | 0                 |
| 11                  | 40                  | 40                  | 500                   | 69                 | 15                 | 16               | 0               | 0                 |
| 12                  | 60                  | 60                  | 200                   | 92                 | 2                  | 6                | 0               | 0                 |
| 13                  | 46                  | 46                  | 300                   | 87                 | 1                  | 12               | 0               | 0                 |
| 14                  | 81                  | 81                  | 350                   | 59                 | 33                 | 8                | 0               | 0                 |
| <b>Median [IQR]</b> | 55 [28.5 – 72]      | 27.5 [14.25 – 36.0] | 500 [257 – 3775]      | 83.0 [57.0 – 94.0] | 10 [1 – 26.5]      | 6.5 [4.5 – 16.5] | 0               | 0                 |
| <b>mEAS</b>         |                     |                     |                       |                    |                    |                  |                 |                   |
| 15                  | 70                  | 35                  | 1,000                 | 11                 | 66                 | 23               | 0               | 0                 |
| 16                  | 120                 | 60                  | 400                   | 20                 | 44                 | 36               | 0               | 0                 |
| 17                  | 90                  | 45                  | 250                   | 2                  | 51                 | 42               | 5               | 0                 |
| 18                  | 65                  | 32.5                | 450                   | 18                 | 50                 | 28               | 1               | 3                 |
| 19                  | 100                 | 50                  | 250                   | 12                 | 39                 | 47               | 1               | 1                 |
| <b>Median [IQR]</b> | 78 [67.5 – 95]      | 39.0 [33.8 – 47.5]  | 250 [150 – 425]       | 12.0 [4.5 – 17.0]  | 50 [34.5 – 56.5]   | 36 [28.5 – 54.5] | 1.0 [0.0 – 5.0] | 0 [0 – 3.0]       |
| <b>Ctr</b>          |                     |                     |                       |                    |                    |                  |                 |                   |
| 20                  | 70                  | 35                  | 200                   | 0                  | 25                 | 75               | 0               | 0                 |
| 21                  | 120                 | 60                  | 300                   | 2                  | 26                 | 71               | 0               | 0                 |
| 22                  | 50                  | 25                  | 100                   | 0                  | 28                 | 73               | 0               | 0                 |
| 23                  | 60                  | 30                  | 400                   | 0                  | 34                 | 66               | 0               | 0                 |
| 24                  | 60                  | 30                  | 250                   | 8                  | 44                 | 47               | 0               | 0                 |
| 25                  | 90                  | 45                  | 300                   | 4                  | 32                 | 64               | 0               | 0                 |
| 26                  | 80                  | 40                  | 200                   | 5                  | 46                 | 49               | 0               | 0                 |
| 27                  | 70                  | 35                  | 400                   | 1                  | 25                 | 74               | 0               | 0                 |
| 28                  | 40                  | 16                  | 200                   | 5                  | 29                 | 66               | 0               | 0                 |
| 29                  | 120                 | 60                  | 300                   | 9                  | 34                 | 57               | 0               | 0                 |
| 30                  | 88                  | 44                  | 250                   | 4                  | 40                 | 55               | 0               | 1                 |
| <b>Median [IQR]</b> | 70 [60 – 90]        | 35.0 [30.0 – 45.0]  | 250 [200 – 300]       | 1.0 [1.0 – 3.0]    | 30 [24 – 44]       | 68 [56 – 75]     | 0               | 0                 |
| <b>maEAS</b>        |                     |                     |                       |                    |                    |                  |                 |                   |
| 31                  | 155                 | 51.7                | 118                   | 1.7                | 40                 | 54               | 0.7             | 3.6               |
| 32                  | 160                 | 53.3                | 145                   | 2.7                | 45.3               | 47               | 0               | 5                 |
| 33                  | 150                 | 50                  | 300                   | 3                  | 59.3               | 33               | 0               | 4.7               |
| 34                  | 160                 | 53.3                | 283                   | 5.7                | 62                 | 26.6             | 0.7             | 5                 |
| 35                  | 200                 | 66.7                | 313                   | 1                  | 50                 | 45               | 0               | 4                 |
| 36                  | 110                 | 36.7                | 788                   | 9                  | 57                 | 25               | 2               | 7                 |
| 37                  | 220                 | 73.3                | 370                   | 0                  | 16                 | 79               | 0               | 5                 |
| 38                  | 160                 | 53.3                | 243                   | 2                  | 16                 | 76               | 0               | 6                 |
| <b>Median [IQR]</b> | 160 [151.3 – 190]   | 53.3 [50.4 – 63.4]  | 291.5 [169.5 – 355.8] | 2.35 [1.18 – 5.03] | 47.65 [22 – 58.73] | 46 [28.2 – 70.5] | 0.0 [0.0 – 0.7] | 5.0 [4.18 – 5.75] |

\*sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, maEAS= Mastocytic Asthma,  
Ctr= Healthy; TNCC= Total Nucleated Cell Count (Average recorded); N= neutrophil,  
L= lymphocyte, M= macrophage, E= eosinophil, MC= mast cell.

**Table 3.5. Relative gene expression in BALF cell pellets.**

Relative gene expression level of CD117, tryptase, chymase, BMP7, Tgfβ1, and Tgfβ1/BMP7. Data is represented at the median [IQR] for each group.

| Group        | CD117                 | Tryptase              | Chymase               | BMP7                  | Tgfβ1                 | Tgfβ1/BMP7            | BMP4                  | ACVR1                 | BMP7                  |
|--------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| <b>Ctrl</b>  | 1.09<br>[0.70 – 1.73] | 0.94<br>[0.60 – 2.01] | 1.88<br>[0.33 – 2.46] | 1.12<br>[0.42 – 1.93] | 1.06<br>[0.81 – 1.22] | 1.12<br>[0.61 – 2.07] | 0.99<br>[0.8 – 1.37]  | 0.94<br>[0.81 – 1.09] | 0.97<br>[0.85 – 1.16] |
| <b>mEAS</b>  | 1.03<br>[0.41 – 2.90] | 1.38<br>[0.30 – 2.50] | 0.48<br>[0.13 – 1.44] | 1.12<br>[0.38 – 3.99] | 1.43<br>[1.2 – 1.83]  | 1.57<br>[0.40 – 3.82] | 0.82<br>[0.57 – 1.21] | 0.91<br>[0.78 – 1.06] | 1.01<br>[0.87 – 1.29] |
| <b>sEAS</b>  | 0.38<br>[0.27 – 1.50] | 0.30<br>[0.20 – 1.36] | 0.92<br>[0.65 – 1.97] | 6.07<br>[0.52 – 9.23] | 1.08<br>[0.97 – 1.57] | 0.21<br>[0.12 – 2.33] | 0.73<br>[0.6 – 1.58]  | 0.84<br>[0.43 – 1.18] | 1.48<br>[0.72 – 1.99] |
| <b>maEAS</b> | 2.32<br>[1.31 – 3.85] | 1.82<br>[1.21 – 2.14] | 0.18<br>[0.08 – 0.54] | 0.74<br>[0.27 – 0.82] | 1.42<br>[1.27 – 1.5]  | 1.92<br>[1.73 – 5.65] | 0.98<br>[0.52 – 1.06] | 1.13<br>[0.87 – 1.42] | 1.51<br>[1.08 – 1.76] |

\*sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, maEAS= Mastocytic Asthma,

Ctrl= Healthy

**Table 3.6. Normalized cytokine concentrations in BALF supernatant.**

Median normalized cytokine concentrations of IFN $\gamma$ , CXCL8, and TNF $\alpha$  measured via multiplex bead immunoassay in healthy (n=11), mild/moderate asthma (n = 5), and severe asthma (n = 14). sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, Ctr= Healthy. Raw concentrations (pg/ml) are normalized to BALF volume recovered (ml) and multiplied by 100. Data are expressed as median [IQR].

| <b>Group</b> | <b>IFN<math>\gamma</math><br/>Normalized</b> | <b>CXCL8<br/>Normalized</b> | <b>TNF<math>\alpha</math><br/>Normalized</b> |
|--------------|----------------------------------------------|-----------------------------|----------------------------------------------|
| <b>sEAS</b>  | 84.92*<br>[41.07 –<br>230.1]                 | 34.44*<br>[26.7 – 70.12]    | 17.37*<br>[5.88 – 87.54]                     |
| <b>mEAS</b>  | 54.67<br>[29.4 – 195.3]                      | 13.19<br>[9.21 – 52.0]      | 8.23<br>[4.71 – 10.51]                       |
| <b>Ctr</b>   | 30.11*<br>[23.42 –<br>42.16]                 | 5.16*<br>[2.29 –<br>10.62]  | 2.22*<br>[1.14 – 3.8]                        |

Note: Medians in the same column with the same superscript symbol differ significantly from each other.

**Table 3.7. Individual horse's normalized cytokine concentrations.**

Cytokine concentrations normalized to BALF volume returned of IFN $\gamma$ , CXCL8, and TNF $\alpha$  measured via multiplex bead immunoassay in healthy (n=11), mild/moderate asthma (n = 5), and severe asthma (n = 14). Data is represented at the median [IQR] for each group. sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, Ctr= Healthy.

| Horse               | IFN $\gamma$<br>([pg/mL / mL] * 100) | CXCL8<br>([pg/mL / mL] * 100) | TNF $\alpha$<br>([pg/mL / mL] * 100) |
|---------------------|--------------------------------------|-------------------------------|--------------------------------------|
| <b>sEAS</b>         |                                      |                               |                                      |
| 1                   | 353.22                               | 202.87                        | 397.13                               |
| 2                   | 51.11                                | 27.19                         | 7.94                                 |
| 3                   | 817.73                               | 70.58                         | 92.96                                |
| 4                   | 40.54                                | 40.37                         | 2.75                                 |
| 5                   | 87.83                                | 31.21                         | 5.96                                 |
| 6                   | 33.47                                | 25.23                         | 5.65                                 |
| 7                   | 811.53                               | 204.79                        | 107.63                               |
| 8                   | 189.07                               | 69.97                         | 85.73                                |
| 9                   | 126.76                               | 34.43                         | 30.91                                |
| 10                  | 41.24                                | 34.46                         | 21.49                                |
| 11                  | 97.18                                | 42.15                         | 13.25                                |
| 12                  | 82                                   | 32.57                         | 23.38                                |
| 13                  | 45.83                                | 21.52                         | 1.74                                 |
| 14                  | 26.02                                | 23.02                         | 10.60                                |
| <b>Median [IQR]</b> | 84.92<br>[41.07 – 230.1]             | 34.44<br>[26.7 – 70.12]       | 17.37<br>[5.88 – 87.54]              |
| <b>mEAS</b>         |                                      |                               |                                      |
| 15                  | 41.24                                | 35.06                         | 10.21                                |
| 16                  | 17.57                                | 12.01                         | 4.52                                 |
| 17                  | 54.67                                | 13.19                         | 4.9                                  |
| 18                  | 285.55                               | 68.94                         | 10.82                                |
| 19                  | 106.07                               | 6.42                          | 8.23                                 |
| <b>Median [IQR]</b> | 54.67<br>[29.4 – 195.3]              | 13.19<br>[9.21 – 52.0]        | 8.23<br>[4.71 – 10.51]               |
| <b>Ctr</b>          |                                      |                               |                                      |
| 20                  | 30.11                                | 2.29                          | 1.14                                 |
| 21                  | 17.57                                | 1.33                          | 0.67                                 |
| 22                  | 42.16                                | 2.52                          | 1.6                                  |
| 23                  | 48.12                                | 20.05                         | 3.8                                  |
| 24                  | 35.13                                | 10.7                          | 7.35                                 |
| 25                  | 23.42                                | 10.62                         | 2.22                                 |
| 26                  | 26.35                                | 1.58                          | 1                                    |
| 27                  | 31.49                                | 9.69                          | 5.93                                 |
| 28                  | 52.7                                 | 3.15                          | 2                                    |
| 29                  | 17.57                                | 7.4                           | 3.03                                 |
| 30                  | 23.95                                | 5.16                          | 3.52                                 |
| <b>Median [IQR]</b> | 30.11<br>[23.42 – 42.16]             | 5.16<br>[2.29 – 10.62]        | 2.22<br>[1.14 – 3.8]                 |

**Table 3.8. Raw cytokine concentrations for each horse.**

Cytokine concentrations of IL-5, IL-17A, IL-2, IL-4, IFN $\gamma$ , CXCL8, and TNF $\alpha$  measured via multiplex bead immunoassay in healthy (n=11), mild/moderate asthma (n = 5), and severe asthma (n = 14). Numbers in italics indicate this value was the software assigned lowest detectable concentration for the individual analyte. Data is represented at the median [IQR] for each group. sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, Ctr= Healthy.

| Horse       | IL-5<br>pg/mL | IL-17A<br>pg/mL | IL-2<br>pg/mL | IL-4<br>pg/mL | IFN $\gamma$<br>pg/mL | CXCL8<br>pg/mL | TNF $\alpha$<br>pg/mL |
|-------------|---------------|-----------------|---------------|---------------|-----------------------|----------------|-----------------------|
| <b>sEAS</b> |               |                 |               |               |                       |                |                       |
| 1           | 0.5           | 0.49            | 0.44          | 16.35         | 81.24                 | 46.66          | 91.34                 |
| 2           | 0.92          | 0.49            | 0.46          | 16.35         | 31.69                 | 16.86          | 4.92                  |
| 3           | 28.38         | 0.49            | 20.66         | 120.22        | 981.27                | 84.69          | 111.55                |
| 4           | 0.5           | 0.49            | 0.44          | 16.35         | 21.08                 | 20.99          | 1.43                  |
| 5           | 0.5           | 0.49            | 0.55          | 16.35         | 21.08                 | 7.49           | 1.43                  |
| 6           | 2.2           | 0.49            | 0.46          | 16.35         | 26.11                 | 19.68          | 4.41                  |
| 7           | 0.5           | 0.49            | 0.46          | 16.35         | 154.19                | 38.91          | 20.45                 |
| 8           | 1.6           | 0.49            | 0.46          | 16.35         | 56.72                 | 20.99          | 25.72                 |
| 9           | 1.46          | 0.49            | 2.48          | 16.35         | 73.52                 | 19.97          | 17.93                 |
| 10          | 1.05          | 0.49            | 0.44          | 16.35         | 28.87                 | 24.12          | 15.04                 |
| 11          | 0.5           | 0.49            | 0.44          | 16.35         | 38.87                 | 16.86          | 5.3                   |
| 12          | 3.02          | 0.49            | 0.86          | 19.98         | 49.2                  | 19.54          | 14.03                 |
| 13          | 0.5           | 0.49            | 0.44          | 16.35         | 21.08                 | 9.9            | 0.8                   |
| 14          | 1.9           | 0.49            | 0.44          | 16.35         | 21.08                 | 18.65          | 8.59                  |
| <b>mEAS</b> |               |                 |               |               |                       |                |                       |
| 15          | 0.8           | 0.49            | 0.44          | 16.35         | 28.87                 | 24.54          | 7.15                  |
| 16          | 0.63          | 0.49            | 0.44          | 16.35         | 21.08                 | 14.41          | 5.42                  |
| 17          | 6.44          | 0.49            | 0.91          | 31.96         | 49.2                  | 11.87          | 4.41                  |
| 18          | 11.24         | 0.49            | 2.54          | 105.48        | 184.96                | 44.81          | 7.03                  |
| 19          | 0.5           | 0.49            | 0.44          | 16.35         | 106.07                | 6.42           | 8.23                  |
| <b>Ctr</b>  |               |                 |               |               |                       |                |                       |
| 20          | 0.5           | 0.49            | 0.44          | 16.35         | 21.08                 | 1.6            | 0.8                   |
| 21          | 0.5           | 0.49            | 0.44          | 16.35         | 21.08                 | 1.6            | 0.8                   |
| 22          | 0.99          | 0.49            | 0.44          | 16.35         | 21.08                 | 1.26           | 0.8                   |
| 23          | 3.02          | 0.49            | 0.44          | 16.35         | 28.87                 | 12.03          | 2.28                  |
| 24          | 0.5           | 0.49            | 0.44          | 16.35         | 21.08                 | 6.42           | 4.41                  |
| 25          | 0.86          | 0.49            | 0.44          | 16.35         | 21.08                 | 9.56           | 2                     |
| 26          | 0.5           | 0.49            | 0.44          | 16.35         | 21.08                 | 1.26           | 0.8                   |
| 27          | 0.5           | 0.49            | 0.44          | 16.35         | 22.04                 | 6.78           | 4.15                  |
| 28          | 0.5           | 0.49            | 0.44          | 16.35         | 21.08                 | 1.26           | 0.8                   |
| 29          | 0.5           | 0.49            | 0.44          | 16.35         | 21.08                 | 8.88           | 3.63                  |
| 30          | 3.02          | 0.49            | 0.44          | 28.99         | 21.08                 | 4.54           | 3.1                   |

**Table 3.9. Cytokine percent recovery of spiked samples.**

| Analyte                       | Expected (pg/mL) | Observed (pg/mL) | % Recovered |
|-------------------------------|------------------|------------------|-------------|
| <b>IL-5</b>                   | 3.66             | 4.49             | 122.68      |
|                               | 14.65            | 15.06            | 102.80      |
|                               | 58.59            | 62.36            | 106.43      |
|                               | 234.38           | 238.25           | 101.65      |
|                               | 937.5            | 866.24           | 92.40       |
|                               | 3750             | 3336.18          | 88.96       |
| <b>IL-17A</b>                 | 3.66             | 2.37             | 64.75       |
|                               | 14.65            | 5.65             | 59.04       |
|                               | 58.59            | 37.58            | 64.14       |
|                               | 234.38           | 143.81           | 61.36       |
|                               | 937.5            | 543.24           | 57.95       |
|                               | 3750             | 2023             | 53.95       |
| <b>IL-2</b>                   | 3.66             | 3.84             | 104.92      |
|                               | 14.65            | 16.67            | 113.79      |
|                               | 58.59            | 67.74            | 115.62      |
|                               | 234.38           | 254.92           | 108.76      |
|                               | 937.5            | 1058.77          | 112.94      |
|                               | 3750             | 3955.77          | 105.49      |
| <b>IL-4</b>                   | 73.24            | 130.78           | 178.56      |
|                               | 292.97           | 435.57           | 148.67      |
|                               | 1172             | 1469             | 125.34      |
|                               | 4688             | 5494             | 117.19      |
|                               | 18750            | 17736            | 94.59       |
|                               | 75000            | 71106            | 94.81       |
| <b>IFN<math>\gamma</math></b> | 122.07           | 133.4            | 109.28      |
|                               | 488.28           | 513.67           | 105.2       |
|                               | 1953             | 1942.76          | 99.48       |
|                               | 7812             | 7485.76          | 95.82       |
|                               | 31250            | 27647.76         | 88.47       |
|                               | 125000           | 103586.76        | 82.87       |

**Table 3.9. continued**

| Analyte                       | Expected (pg/mL) | Observed (pg/mL) | % Recovered |
|-------------------------------|------------------|------------------|-------------|
| <b>CXCL8</b>                  | 14.65            | 18.78            | 128.19      |
| <b>(IL-8)</b>                 |                  |                  |             |
|                               | 58.89            | 54.56            | 93.12       |
|                               | 234.38           | 201.51           | 85.98       |
|                               | 937.5            | 844.48           | 90.08       |
|                               | 3750             | 3449             | 91.97       |
|                               | 15000            | 8979             | 59.86       |
| <b>TNF<math>\alpha</math></b> | 0.98             | 2.82             | 287.75      |
|                               | 3.91             | 4.99             | 127.62      |
|                               | 15.62            | 18.54            | 118.69      |
|                               | 62.5             | 66.74            | 106.78      |
|                               | 250              | 270.67           | 108.27      |
|                               | 1000             | 1056             | 105.6       |

**Table 3.10. Taqman Gene Expression Assay IDs.**

| <b>Gene Target</b>                    | <b>Assay ID</b>           |
|---------------------------------------|---------------------------|
| $\beta$ -actin                        | Ec04176172_gH             |
| CD117                                 | Ec03469720_m1             |
| Tryptase                              | Ec03468172_m1             |
| Chymase                               | ARMFXWU (custom)          |
| Carboxypeptidase A3                   | APU629N, ARH6ATU (custom) |
| Transforming growth factor- $\beta$ 1 | Ec03468030_m1             |
| Bone morphogenetic protein-7          | Ec04320876_m1             |
| Bone morphogenetic protein-4          | Ec03470252_s1             |
| ACVR1                                 | Ec07023073_s1             |
| BMP $\alpha$ 2                        | Ec06959500_m1             |

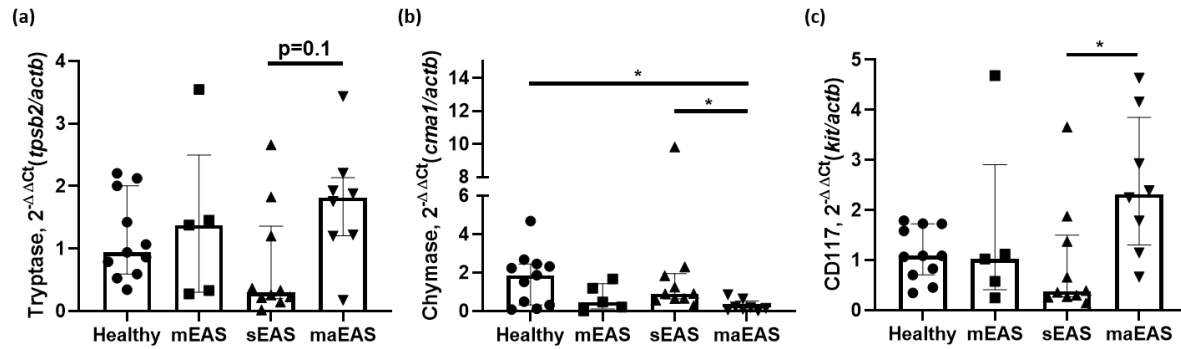


**Figure 3.1. Equine airway mast cells decrease chymase expression in mastocytic asthma.** Relative gene expression level of (a) tryptase (*tpsb2*), (b) chymase (*cma1*), and (c) CD117 (*kit*) in BALF cell pellets in healthy (n = 11), mild/moderate asthma (n = 5), severe asthma (n = 10), and mastocytic asthma (n = 8). All target genes were normalized to the house keeping gene  $\beta$ -actin (*actb*). Displayed figures represent the median and IQR. sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, Ctr= Healthy, maEAS= Mastocytic Asthma.

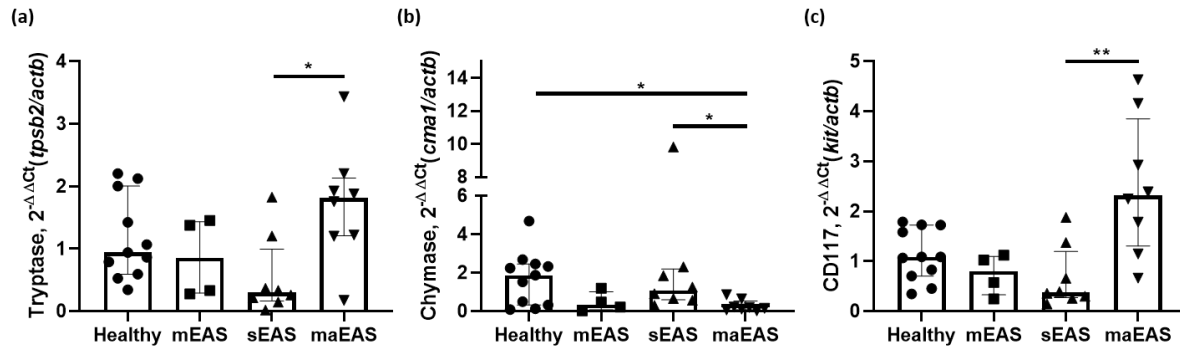


**Figure 3.2. Protease expression in BALF cell pellets, excluding those horses on corticosteroids.** Relative gene expression level of (a) tryptase (*tpsb2*), and (b) chymase (*cma1*), and (c) CD117 (*ckit*) in BALF cell pellets in healthy (n = 11), mild/moderate asthma (n = 4), severe asthma (n = 8), and mastocytic asthma (n = 8). One horse in the mEAS group, and 2 in the sEAS group had been on corticosteroids with continued clinical signs. Excluding these horses did not change overall results. All target genes were normalized to the house keeping gene  $\beta$ -actin (*actb*). Displayed figures represent the median and IQR. sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, Ctr= Healthy, maEAS= Mastocytic Asthma.

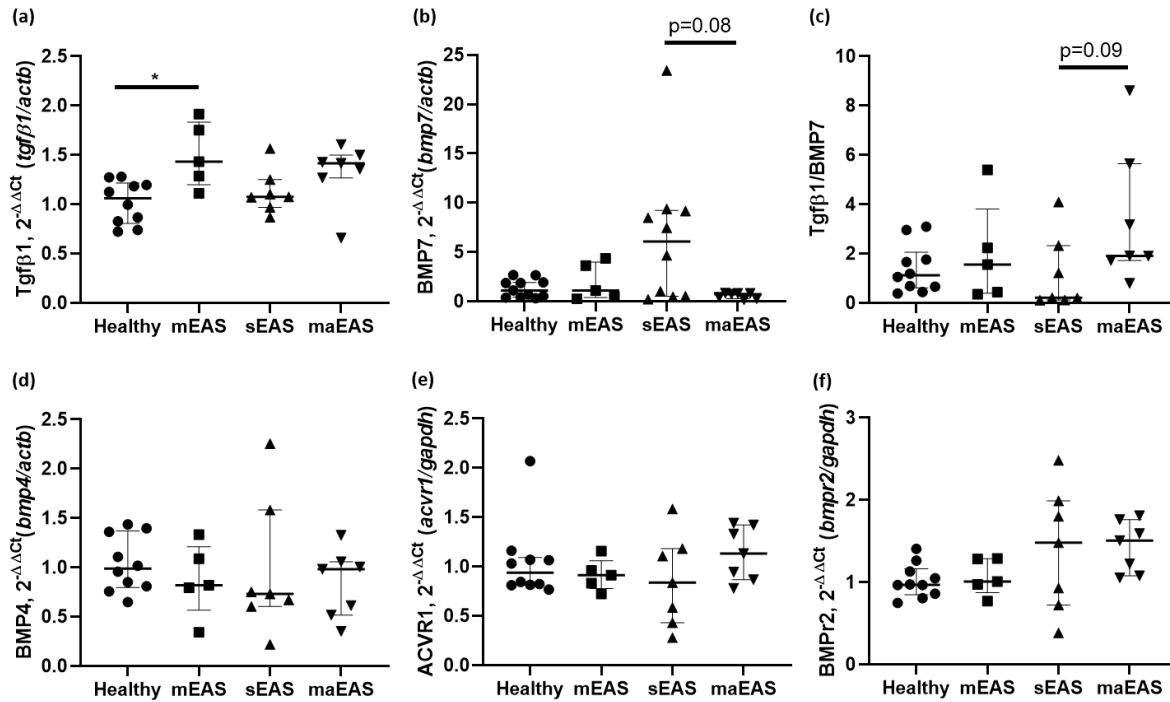


**Figure 3.3. Tgfb $\beta$ 1 is elevated in mild/moderate asthma and BMP7 is variably expressed in severe asthma.** Relative gene expression level of (a) *tgfb $\beta$ 1*, (b) *bmp7*, (c) *tgfb $\beta$ 1/ bmp7*, (d) *bmp4*, (e) *acvr1*, (f) *bmpr2* in BALF cell pellets. Sample size was reduced as targets were analyzed due to remaining sample input available. BMP7 sample size was healthy, n = 11; mEAS, n = 5; sEAS, n = 10; and maEAS, n = 7. Further analytes had reduced sample size for healthy, n= 10; and sEAS, n = 7. Displayed figures represent the median and IQR. sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, maEAS= Mastocytic Asthma.

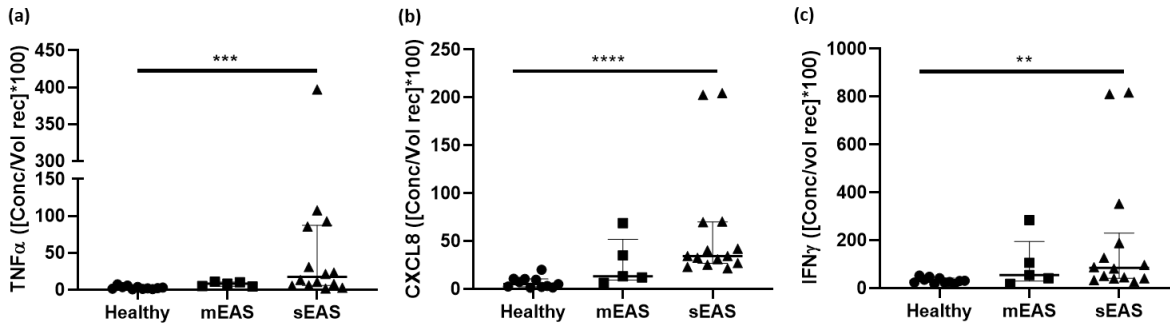


**Figure 3.4. Asthmatic horses have elevated pro-inflammatory cytokines.**

Normalized cytokine concentrations of (a) TNF $\alpha$ , (b) CXCL8/IL-8, and (c) IFN $\gamma$  measured via multiplex bead immunoassay in healthy (n=11), mild/moderate asthma (n = 5), and severe asthma (n = 14). Displayed figures represent the median and IQR. sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, Ctr= Healthy.

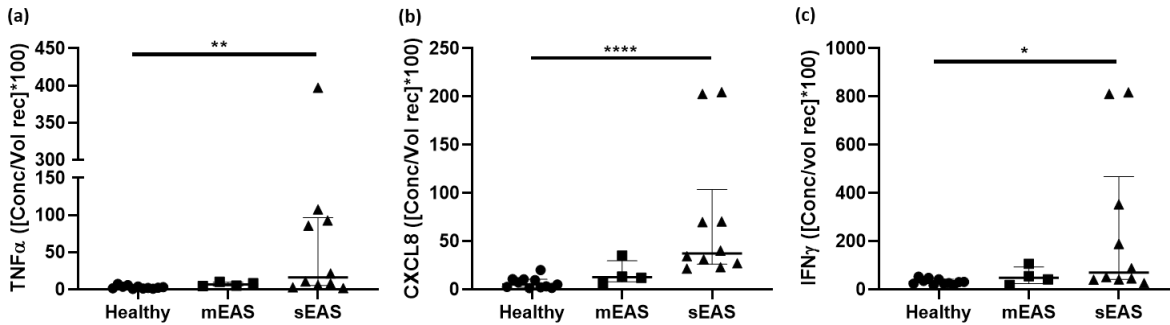


**Figure 3.5. Normalized cytokine concentrations of horses, excluding those on corticosteroids.** Normalized cytokine concentrations of (a) TNF $\alpha$ , (b) CXCL8/IL-8, and (c) IFN $\gamma$  measured via multiplex bead immunoassay in healthy (n=11), mild/moderate asthma (n = 4), and severe asthma (n = 10). One horse in the mEAS group, and 4 in the sEAS group had been on corticosteroids with continued clinical signs. Excluding these horses did not change overall results. Displayed figures represent the median and IQR. sEAS= Severe Asthma, mEAS= Mild/Moderate Asthma, Ctr= Healthy.

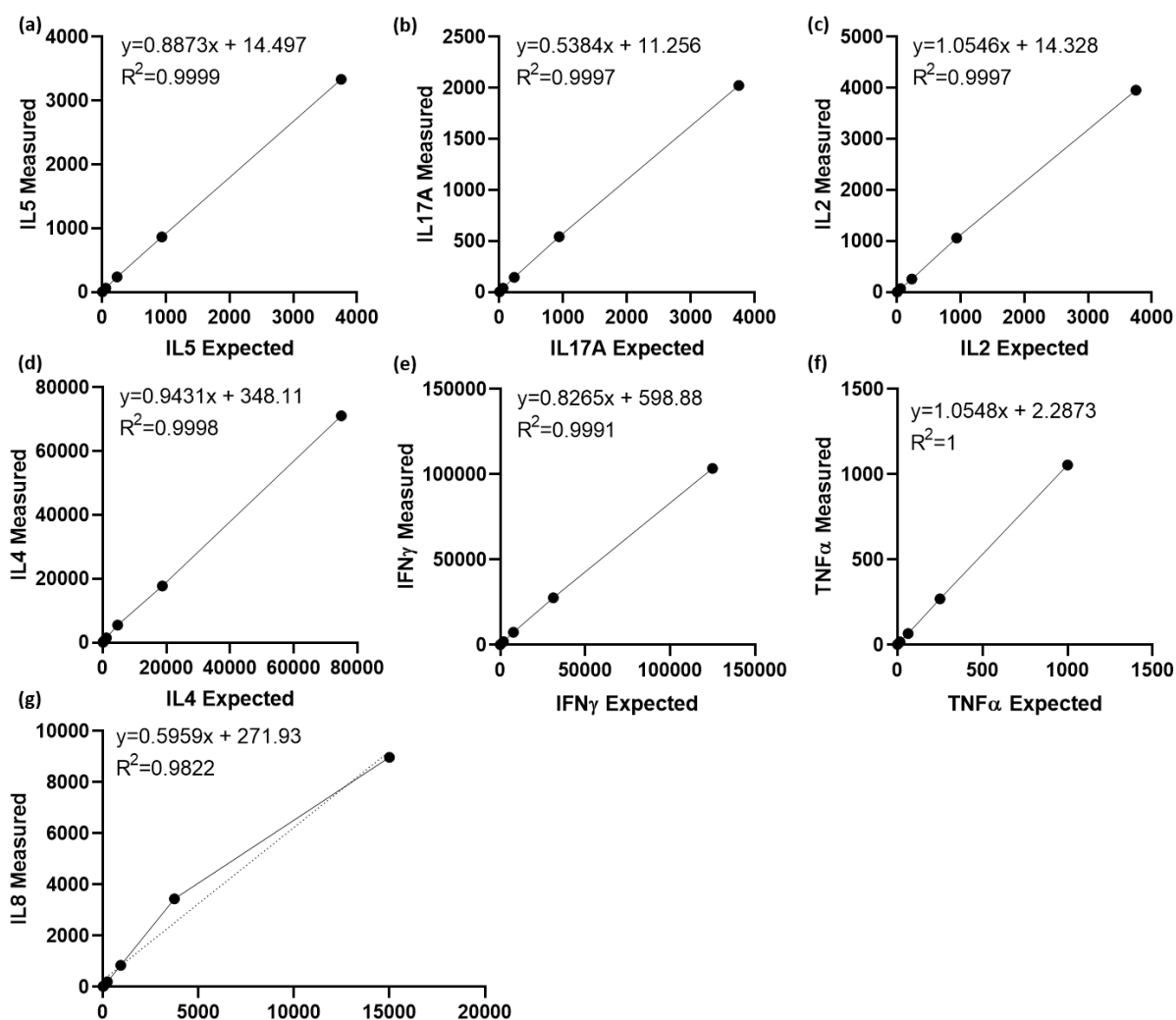


**Figure 3.6. Dilutional linearity of individual cytokines.** Dilutional linearity as depicted by expected versus measured with linear regression analysis for all cytokines of interest: (a) IL-5, (b) IL-17A, (c) IL-2, (d) IL-4, (e) IFN $\gamma$ , (f) TNF $\alpha$ , (g) CXCL8/IL8. All cytokines show high linear dilution of spiked samples. The kit cytokine standard was used to re-capitulate the standard curve by using the top standard, initially reconstituted in water, to further dilute in BALF supernatant. The points depicted are based on the kit's standard reference range for each analyte.

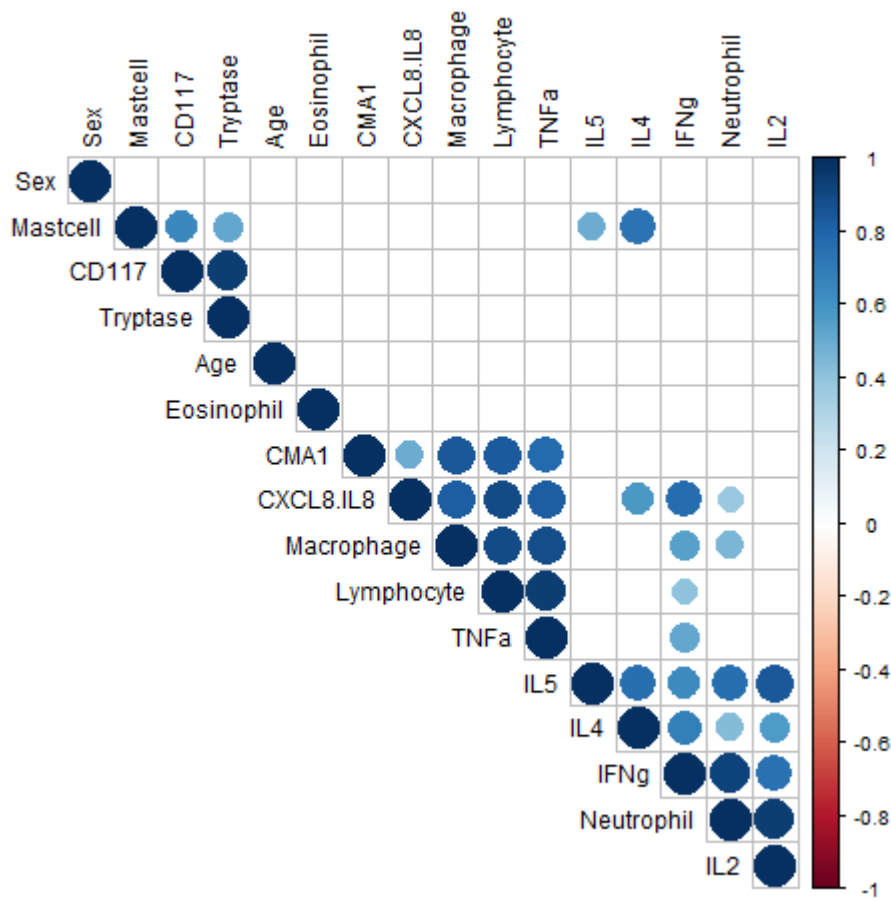


**Figure 3.7. Correlation data.** Pairwise correlation of variable. Only significant ( $p < 0.05$ ) correlations are shown. The size of the circle represents the strength of the correlation, and the color represents directionality. The variables are hierarchically clustered based on their correlations.

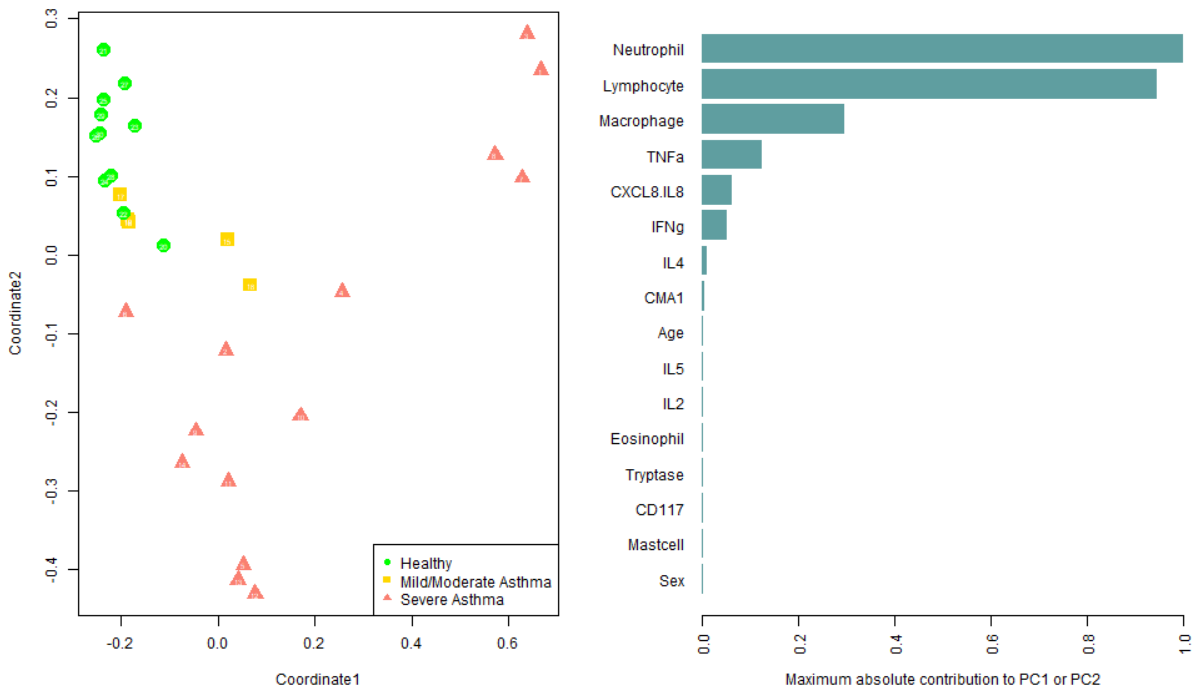


**Figure 3.8. PCA/MDS.** (a) Multidimensional scaling of the samples based on age, sex, cell concentrations, gene expression fold changes, and normalized cytokine concentrations. Each sample is labeled with its anonymous numeric identifier. Asthma status is indicated by gray scale and symbol. (b) The maximum contribution to first two principal components from each variable, which shows the relative importance of each variable in driving the observed clustering pattern.

**CHAPTER 4**  
**CONCLUSIONS AND FUTURE DIRECTIONS**

Mast cells are a more complicated innate immune cell than its generally accepted role in IgE-mediated inflammatory responses. As our knowledge of immunology and the involved cell types advances, there are more cell subtypes and roles being identified. Chronic inflammatory conditions, such as colitis and asthma pose complicated questions in understanding each of their pathology. Chronic inflammatory conditions have many cell types involved, both immune and non-immune, with prolonged exposure to inflammatory milieu that can lead to detrimental consequences, such as fibrosis. In the first section of my dissertation I explain how mast cells and BMPs may be involved in controlling chronic inflammation and fibrosis in disease of the intestine and lung. In the further sections we set out to further investigate these possibilities.

Our focus on the mast cell – BMP axis in the second section was spurred from several observations brought up in the first chapter, but in short was due to previous laboratory findings that suggested a dysregulation of BMPs in a mast cell-deficient colitis model and that BMP<sub>2</sub> is a marker that differentiates mast cells from basophils and other immune cells, therefore BMP ligands, receptors, and signaling may be important in mast cell functions. Conclusions from the second section suggest that BMP<sub>7</sub> reduces TNF $\alpha$  release without effecting degranulation. Additionally, the mast cell may be a source of BMP<sub>7</sub> in an inflammatory environment. Findings that slightly complicated the results were that blockade of specific BMP receptors resulted in further significant reduction in TNF $\alpha$  release. In mast cells it appears that engagement of different BMP receptors may drive what BMP signaling pathway, canonical versus non-canonical, is engaged. Future experiments in order to further investigate canonical versus non-canonical BMP signaling in mast cells will include the use of mast cells

either deficient or overexpress specific BMP receptors. TNF $\alpha$  release may be different based on what BMP ligands are applied and/or what BMP receptors are engaged. Mast cell specific BMP ligand and receptor knockouts are currently being derived in the laboratory that will further the *in vitro* and *in vivo* experimentation. Additionally, confirming in mast cells the BMP ligand-receptor binding affinity should be investigated in order to better understand the interactions.

In the third section my objective was to use a novel technique in investigating mast cell molecular phenotype in equine asthma and correlate with inflammatory cytokines found in BALF. Thanks to collaborator samples we were able to identify differences in mast cell molecular phenotype in mastocytic asthmatic horses. Unfortunately, I was not able to correlate this groups mast cell molecular phenotype with inflammatory cytokines due to differences in BALF collection that would potentially bias cytokine concentrations. Cytokine evaluation showed a strong Th1 response in severely asthmatic horses with elevation in neutrophil recruiting and activating chemokine, CXCL8. I further evaluated Tgf $\beta$ , BMP ligand and receptor expression in BALF cells in the remaining RNA from the BALF cell pellets. An interesting finding was the wide expression of BMP7 in the severely asthmatic group. This finding could not be explained by comparing to Tgf $\beta$  expression, but further investigation would be interesting in correlating BMP7 expression with diagnosis, response to treatment, and lung function evaluation. Additional evaluation of mast cell molecular phenotype and lung function analysis would be interesting based on human studies that see a correlation between mast cell phenotype and lung function. Horses

with asthma may also be a human asthma translational model in which inflammation and fibrosis could be evaluated.

In summary mast cells and BMP7 each pose interesting targets for immune modulation alone and in combination. Continued evaluation of BMP actions in specific immune cell types and in disease is an exciting area of continued investigation especially in light of BMP7's anti-fibrotic effects.

## **VITA**

Jane Seymour Woodrow was born and grew up in Florida. Her undergraduate studies took her to Georgia Institute of Technology in Atlanta, GA where she received a BS in Applied Biology. Following graduation Jane moved back to Florida and pursued a Master of Science in Medical Science at the University of South Florida in Tampa, FL and worked at an equine clinic. During this time Jane was accepted to the DVM program at the University of Florida at Gainesville, FL. After completion of her DVM and MS degree Jane pursued an equine internship at the University of Georgia, followed by a Large Animal Internal Medicine residency at the University of Tennessee in Knoxville, TN with subsequent LAIM Diplomate status achieved. Following the residency Jane started a PhD in the Comparative & Experimental Medicine program at the University of Tennessee in Elizabeth Lennon's laboratory. During the third year of Jane's PhD Elizabeth Lennon's laboratory moved to the University of Pennsylvania in Philadelphia, PA. Jane remained a UT student with Barry Rouse as a co-mentor and went to Philadelphia in order to complete her dissertation in Elizabeth Lennon's laboratory. Jane received her PhD from the CEM Department with a focus in immunology from UT in 2021.