2014

Study of Role of Ribosomal Protein L13a in Resolving Inflammation

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STUDY OF ROLE OF RIBOSOMAL PROTEIN L13A IN RESOLVING INFLAMMATION

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Submitted in partial fulfillment of requirements for the degree
DOCTOR OF PHILOSOPHY IN REGULATORY BIOLOGY
at the
CLEVELAND STATE UNIVERSITY
MAY 2014

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DEDICATION

I dedicate this thesis to the following most important people in my life without whom it would've been impossible to move on:

To my baby Shoubhit, who has blessed me with motherhood and made me a better person.

To Shuvojit, who has been a true friend, philosopher and guide in my life. Thank you for your patience and for being there when I've needed you the most.

To Maa, who has been my role model ever since and for insisting that I continue a career in science.

To Baba, for being the sweetest and most pampering dad I've ever known, for supporting all my decisions in life.

Last, but not the least, Dada for being an encouraging and supportive brother and for teaching me how to be efficient, dedicated and honest in one's profession.

ACKNOWLEDGEMENT

There are several people whom I wish to thank for helping me throughout the course of my Ph.D. It has been only for their help, co-operation support and motivation that I have achieved this Doctoral degree.

I want to begin by thanking my husband, Shuvojit, for being such an inspiration in both my personal and professional life. For standing by my side during those tough times, for mentoring and guiding me, for discussing science with me, providing new ideas for experiments and helping me realize my potentials.……I thank you for everything. I thank my baby Shoubhit, whose mere presence is like sunshine in my life. His ever smiling face makes me forget all my worries and brings out the best in me. Thank you Maa and Baba, for bringing me into this world and giving me the best possible education, for being such wonderful parents from whom I could learn so much and for being my strength always. You were always so encouraging about me pursuing a higher education in science abroad and today I am really glad that I have been able to live up to your expectations and make you proud. Not to forget my brother, sister-in-law, Sohitri and their little angel Sree, and my in-laws for believing in me, respecting and supporting my choices. Thank you all for being my family.

To my advisor, Dr. Mazumder for being such a great mentor, advisor and guide. His candid and welcoming nature has never made me think twice before approaching him with any problem. His belief in me as a graduate student has been a great contributor in molding the scientist in me. I am truly grateful to him for his help, support, patience and understanding which have played a major role in helping me complete this doctoral

degree. Thank you BarsanDa for being so optimistic about my capabilities, it has been a great source of inspiration for me throughout my Ph.D.

I'd like to offer my sincere gratification to my Advisory Committee Members, Dr. Weyman, Dr, Komar and Dr. Baldwin for their immense help, valuable comments and useful suggestions during the course of my research. Presenting and discussing my work with them has helped me come up with different approaches and looking at my dissertation research from an unbiased point of view. Dr. Baldwin, you have been great in helping me interpret the histology results and suggesting new directions. You have been ever welcoming whenever I was there in your lab with experimental samples. I thank you all sincerely for your time and cooperation and for agreeing to serve on my Thesis Committee.

I'd also like to extend my gratitude toward Dr. Roman Kondratov for agreeing to serve as the Internal Reader on my Thesis Committee and for providing useful suggestions and helping me with my animal studies. I also thank Dr. Aimin Zhou for taking time off his busy schedule and agreeing to serve as an External Reader of my thesis.

To all my lab members: Dr. Abhijit Basu, Ravinder and Varsha for their help and co-operation and for sharing reagent. Dr. Basu has been a great mentor in guiding me through the basics of the molecular biology experiments. I thank Ravinder for looking after my animals and helping me with my experiments. I also want to thank my previous lab members Dr. Sujan Chaudhuri and Keyur Vyas for teaching me basic lab experimentation techniques when I'd just begun my Ph.D career and Priyanka and for

being a nice lab mate and companion. It has been a real pleasure and a great experience to have worked with all past and current Mazumder lab members.

I would like to thank Nina in Dr. Baldwin's lab who is one of the nicest people I've ever known; for her tremendous help with the immunostaining studies. She has always welcomed me into her lab with a wide smile on her face and has taken care of my slides in my absence.

There are several other people in my department who I wish to offer my gratitude: To Michele and all the stockroom staff members for allowing timely delivery of all my research reagents. To Beth and all the Animal Facility technicians for taking care of my animals. To all the other faculties, fellow students and labs in the department for sharing reagents and just for being there and making me feel welcomed in this department. I thank you all, without your support and cooperation, making this significant achievement wouldn't have been smooth.

STUDY OF ROLE OF RIBOSOMAL PROTEIN L13A IN RESOLVING INFLAMMATION

DARSHANA PODDAR

ABSTRACT

Inflammation is an obligatory attempt of the host immune system to protect the body against infection. However, unregulated synthesis of pro-inflammatory products can have detrimental effects. Though mechanisms which contribute to inflammation are well appreciated, those that resolve inflammation are poorly understood. Therefore, understanding the molecular basis of such pathways will provide an entirely novel approach to treat and prevent inflammatory diseases. Transcript-selective translational control can regulate the expression of a set of inflammatory genes. We have identified one such mechanism in a novel animal model which relies on the abrogation of ribosomal protein L13a-dependent translational silencing by creating macrophage-specific L13a-knockout mice where resolution of inflammation is severely compromised. We have used these knockout mice to study two different kinds of inflammation: LPS induced systemic inflammation and dextran sodium sulphate (DSS) induced experimental colitis. Upon LPS induced endotoxemia, these mice displayed high mortality rates and severe symptoms of inflammation such as infiltration of immune cells in the peritoneum and major organs leading to tissue destruction. These animals also exhibited high serum levels of TNF-α, blood urea nitrogen (BUN), aspartate aminotransferase (AST) and several other markers of inflammation. Macrophages from these knockouts showed unregulated synthesis of several chemokines (e.g., CXCL13, CCL22, CCL8 and CCR3) and increased polysomal abundance of these mRNAs due to the abrogation of their

translational silencing. Upon DSS induced colitis, these knockout mice demonstrated higher susceptibility to colitis displaying reduced survival, significant weight loss, enhanced rectal bleeding and diarrhea. Histopathology analysis of tissue sections from the knockouts showed disruption of epithelial crypts in the colon with infiltration of macrophages in colon and spleen sections. Additionally, elevated levels of several chemokines and cytokines were found to be associated with the serum and colons of the DSS administered knockout animals. Therefore, based on these observations, we hypothesize that L13a-dependent translational silencing has evolved as an endogenous defense mechanism in monocytes and macrophages against uncontrolled inflammatory response and disruption of this pathway can severely impair the resolution phase of inflammation.

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CHAPTER I

INTRODUCTION

1.1. Inflammation and overview of inflammatory response

Inflammation is an adaptive response generated by the immune system upon stimulation by destructive conditions and inflammatory assaults like infection and tissue injury (Majno and Joris, 2004, Medzhitov, 2008). Despite the cause, an inflammatory response is initiated with the goal of restoring tissue homeostasis. Inflammation governs diverse physiological and pathological processes. Though research over the past couple of decades have highlighted the importance of inflammation in the context of pathogenesis of several inflammatory diseases, it is essential to understand and appreciate the physiological functions of inflammation as well. It is generally considered that a regulated and balanced inflammatory response is beneficial to the host; but at the same time a dysregulated response can result in an autoimmune attack and prove to be lethal for the host. At the initial stage, an inflammatory condition is induced by infection or tissue injury which activates the primary mediators which are the body's immune cells. The activation of these immune cells is followed by coordinated and precise recruitment

of these cells (plasma cells, lymphocytes and leukocytes) to the sites of infection or injury (Majno and Joris, 2004, Medzhitov, 2008). Microbial (bacterial and viral) infections are recognized by the body's innate immune receptors like the Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs) and the cytosolic RIG –I (retinoic acid inducible gene 1) like receptors (RLRs) (Medzhitov, 2008, Barton, 2008). This elementary recognition of infection is carried out by the tissue resident macrophages and mast cells which subsequently results in the synthesis of various pro-inflammatory molecules like cytokines, chemokines, vasoactive amines, eicosanoids etc., to mediate the inflammatory response. This is then followed by selective extravasation of the neutrophils from the bloodstream to the distressed tissue sites where they release the toxins from their granules to kill the pathogen (Medzhitov, 2008, Nathan, 2006). The toxins cannot distinguish between the foreign pathogen and the somatic cells thus leading to substantial tissue damage (Nathan, 2002). If a favorable acute inflammatory response is able to eliminate the foreign pathogen, then it is followed by the resolution phase where there occurs restoration of tissue homeostasis and extensive tissue repair by the tissue resident and recruited macrophages (Serhan and Savill, 2005). However, if an acute response fails to remove the pathogen, then an adaptive response is generated where the T cells and the macrophages come into play leading to a chronic inflammatory state. A chronic inflammatory state can also be triggered in response to persistent self antigens or due to malfunctioning of the tissue itself or due to loss of homeostatic equilibrium of some biological processes that are not directly related to host defense or tissue repair (Figure 1) (Medzhitov, 2008).

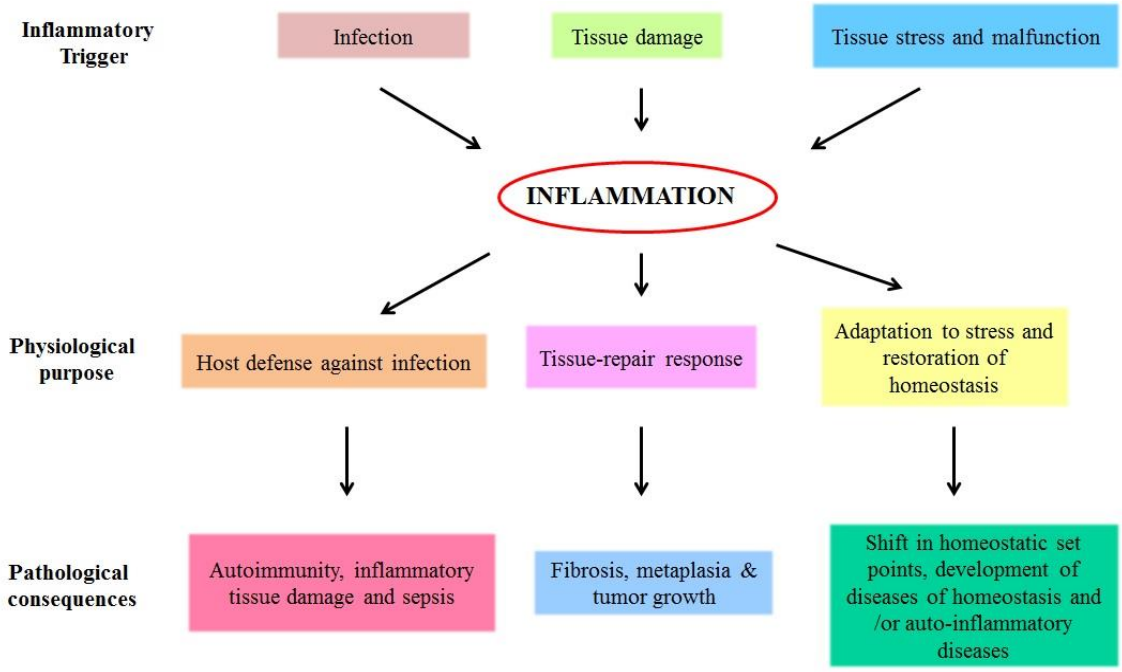

Figure 1. Causes, physiological and pathological outcomes of inflammation. Depending on the stimulus, inflammatory response can have different physiological purpose and pathological outcomes. Of the three possible inflammatory triggers, only infection-stimulated inflammation can be associated with an immune response. Tissue damage and stress on the other hand can promote a chronic inflammatory state and autoimmune diseases. Figure 1 has been adapted from figure 1 of "Origin and physiological roles of inflammation." Medzhitov, R. 2008. Nature. 454: 428-435.

1.2. Macrophage-mediated inflammation and disease

Macrophages are primitive cells existing in the metazoans and primarily identified by their phagocytic nature. They are present in all tissues where they demonstrate anatomical and functional diversity (Wynn et al., 2013). Macrophages can be described as terminally differentiated mononuclear phagocytic cells derived from the circulating monocytes that originated in the bone marrow but they may have different lineages as well (Wynn et al., 2013). Macrophages can be classified on the basis of their

inflammatory states which include the classically activated and the alternatively activated macrophages (AAMs) designated as M1 and M2 respectively. Activation of these two sets of macrophages depends on response to interferon-gamma (IFN-γ) and toll-like receptors (TLRs) or to interleukin-4 (IL-4) and IL-13 respectively (Gordon, 2003, Sica and Mantovani, 2012). Macrophages participate in a variety of biological processes in an organism starting from development, tissue homeostasis and wound healing to immune responses to microbial invasions. The tissue resident macrophages act as guards to regulate tissue homeostasis and this process may also lead to recruitment of macrophages from circulating monocytes, spleen and bone marrow or local proliferation (Jenkins et al., 2011, Schulz et al., 2012). However, sometimes wound healing and tissue homeostasis may not be restored due to residual persistent inflammatory assault and in that scenario macrophages can be leading causes of several inflammatory diseases like cancer, obesity, diabetes, atherosclerosis, inflammatory bowel diseases etc.

During tissue damage after invasion by foreign pathogens, Ly6C+ inflammatory peripheral blood monocytes are recruited from circulation to the sites of tissue injury where they differentiate into macrophages. These macrophages then secrete several pro-inflammatory molecules including tumor necrosis factor alpha (TNF-α), IL-1 and nitric oxide (NO) which then activate several downstream anti-pathogenic defense mechanisms (Wynn et al., 2013). These tissue recruited macrophages also secrete other cytokines like IL-12 and IL-23 which help in the expansion of T_H1 and T_H17 cell subsets to further control the infection and eliminate the pathogen (Sica and Mantovani, 2012). Although synthesis of pro-inflammatory products is an obligatory attempt of the macrophages and monocytes to mediate inflammation, uncontrolled accumulation of these molecules may

be toxic to the host. Release of the cytokines and chemokines by the macrophages and the reactive oxygen species synthesized by the T cells during tissue injury often leads to considerable damage to the surrounding tissue (Nathan and Ding, 2010). If this inflammatory macrophage response is not checked in a timely manner, it can be causal in the initiation and progression of several chronic inflammatory and autoimmune diseases (Sindrilaru et al., 2011, Krausgruber et al., 2011). In order to neutralize the excessive tissue damage response, these activated macrophages sometimes undergo apoptosis or even change to a suppressive phenotype to promote tissue healing (Murray and Wynn, 2011b). Therefore, it can be said that switching of macrophages from an inflammatory state to an anti-inflammatory or suppressive sub-type and back to a pro-inflammatory phenotype can be vital in the pathogenesis or resolution of many chronic inflammatory diseases (Figure 2).

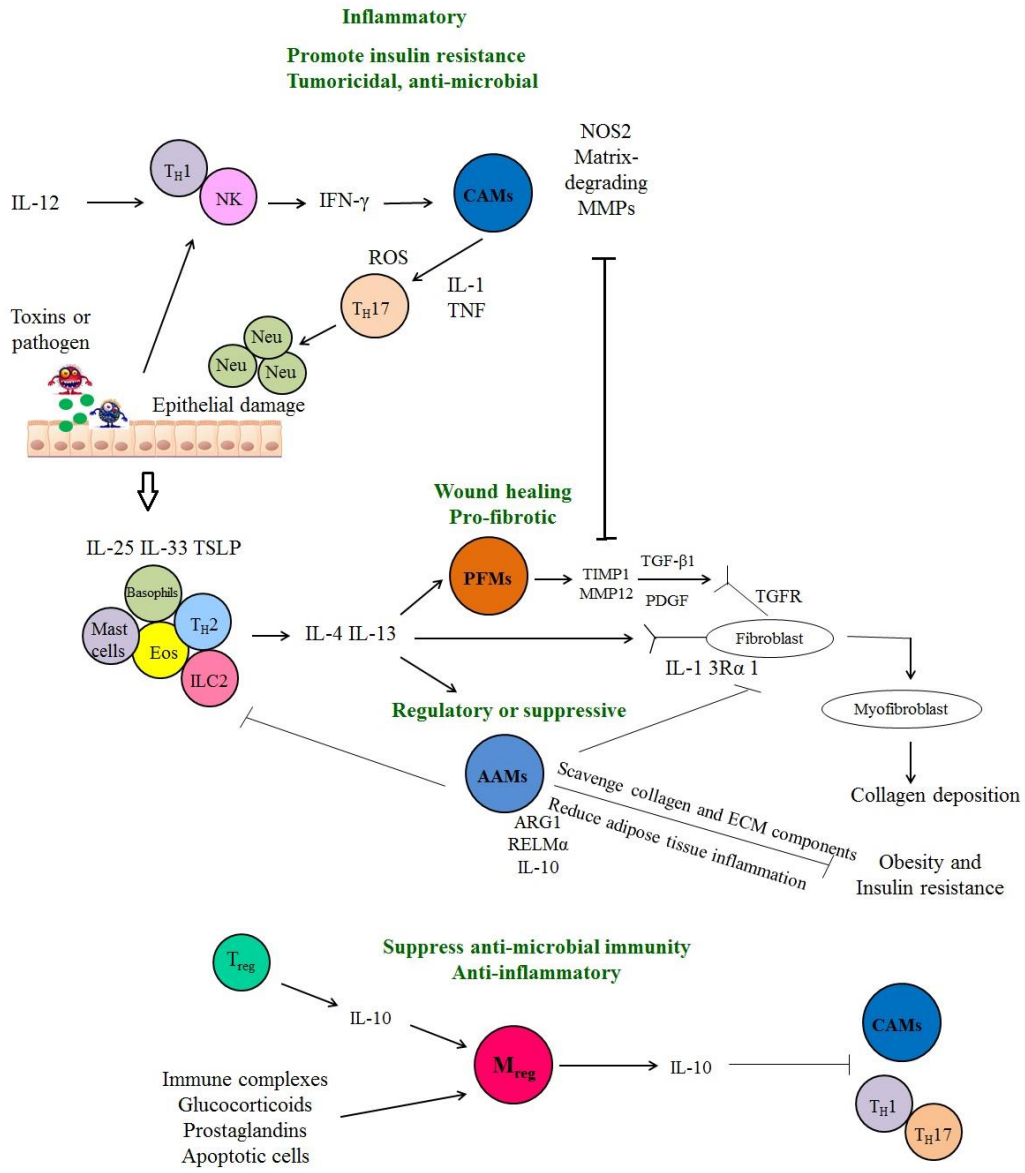

Figure 2. Macrophages that have unusual activation profiles have essential functions in regulating disease progression and resolution. Macrophages are highly fexible cells that exhibit different activation states (CAMs, PFMs, AAMs and M_{regs}) based on different environmental stimuli. During a pathogenic attack or associated tissue damage, the local resident macrophages assume an "inflammatory phenotype" and are then known as Classically Activated Macrophages (CAMs). These macrophages are activated by IFN-γ and then activate the STAT1 and NFκB signaling pathways. This in turn increases the production of several inflammatory cytokines like IL-1, IL-6, TNF-α and reactive oxygen or nitrogen species. These molecules then

promote anti-tumorigenic and anti-microbial immunity and also insulin resistance and obesity. On the contrary, some of the epithelium-derived sentinels produce type 2 cytokines like IL-4 and IL-13 which activate the "alternative" state of the macrophages (AAMs) which are primarily engaged in wound healing and promoting fibrosis and insulin sensitivity. They also activate the wound healing pro-fibrotic macrophages (PFMs) which produce several growth factors such as TGF-β1, VEGF and PDGF which activate myofibroblasts and help in collagen synthesis. AAMs also synthesize a variety of Immunoregulatory proteins like arginase 1 (ARG1), RELM-α and IL-10 which monitor the magnitude and span of an immune response. The AAMs also scavenge collagen and other extra-cellular components (ECM) therefore aiding in ECM remodeling. Thus where CAMs are responsible for eliciting an immune defense response, the AAMs are primarily involve in immune suppression and restoring tissue homeostasis. The regulatory T cells (T_{regs}) secreting IL-10 and the engulfment of apoptotic debris selectively increases the population of regulatory macrophages (M_{regs}) that suppresses inflammation and facilitates anti-tumorigenic and anti-microbial defense. Figure 2 has been adapted from Figure 4 of "Macrophage biology in development, homeostasis and disease." Wynn, T. A., Chawla, A. and Pollard, J. W. 2013. Nature. 496: 445-455.

1.2.1. Role of macrophages in inflammatory diseases

Macrophages play important roles in the pathogenesis of several inflammatory diseases like cancer, asthma, atherosclerosis, rheumatoid arthritis, inflammatory bowel diseases, endotoxemia and fibrosis. Previously it was believed that macrophages are anti-tumorigenic but recent studies have implicated the role of macrophages in tumor initiation, progression and metastasis (Qian and Pollard, 2010). Macrophages synthesize several pro-inflammatory cytokines like TNF-α, IFN-γ and IL-6 which sustain the chronic inflammatory response thereby facilitating tumor initiation and progression (Balkwill and Mantovani, 2012). Asthma is a chronic inflammatory disease characterized by inflammation in the airway tract, airway obstruction and lung remodeling. The pulmonary macrophages produce a variety of cytokines like IL-4, IL-13 and IL-33 which

stimulate pulmonary smooth muscle contractions and degradation of the extra-cellular matrix (ECM) leading to pathogenesis of asthma (Gordon, 2003). Atherosclerosis is a process that initiates with the activation of the resident macrophages of the vessel wall triggering the adhesion of leukocytes to the endothelium. These adhered leukocytes then extravasate through transendothelial space in response to a chemotactic gradient. These processes are regulated by the coordinated action of many different chemokines and their cognate receptors (Zernecke and Weber, 2010, Golledge, 2013, Mukhopadhyay, 2013). Macrophages secrete several inflammatory cytokines like TNF-α, IL-12, IL-18 and IL-23 that play critical roles in triggering autoimmune diseases like rheumatoid arthritis (Murray and Wynn, 2011a). Macrophage derived IL-23 has been studied to promote end-stage joint inflammation whereas TNF-α has been shown to promote polyarthritis via macrophage and dendritic cell secreted IL-12 and IL-18. Macrophages have been shown to play essential roles in contributing to the pathogenesis of inflammatory bowel diseases (IBDs). IBDs are thought to be caused by an imbalance between the commensal bacteria and the host's immune system leading to mis-functioning of the immune cells and infiltration of macrophages and other leukocytes into the colon mucosa. Studies have shown that a group of $TLR2^{+}$ $CCR2^{+}$ $CX3CR1^{int}$ $Ly6C^{hi}$ $GR1^{+}$ macrophages induce colonic inflammation by producing large quantities of TNF-α (Platt et al., 2010). Recent finding also suggest that the inflammatory cytokines and reactive oxygen species generated in the colon can transform the anti-inflammatory macrophages to the pro-inflammatory dendritic cell like cells which secrete large amounts of IL-12, IL-23 and nitric oxide synthase thereby promoting colitis (Rivollier et al., 2012). On the contrary, recruited monocytes and tissue resident macrophages also help in clearing out the

apoptotic cells and debris thereby allowing wound healing and epithelial cell repair. Thus macrophages and dendritic cells are key players of inflammation associated with colitis.

1.3. Monocyte and macrophage recruitment during infection and inflammation: a role of the chemokines

In this thesis, we have focused on anti-inflammatory role of ribosomal protein L13a-mediated translational silencing pathway. The expression of several chemokines and their cognate receptors has been identified to be regulated by this pathway (36). Therefore, it is essential to have a basic understanding about different classes of inflammatory monocytes and their mechanisms of recruitment to sites of infection. Monocytes are a subgroup of circulating leukocytes that can differentiate into a wide variety of tissue macrophages and dendritic cells (Auffray et al., 2009, Shi and Pamer, 2011). Monocytes have the ability to differentiate in response to signals from chemokines/cytokines or microbial components and are important mediators of host microbial defense (Auffray et al., 2009, Serbina et al., 2008). Monocytes and macrophages have been implicated in a variety of inflammatory diseases including atherosclerosis (Woollard and Geissmann, 2010), cancer, endotoxemia, IBDs, etc. The ability of monocytes to be chemo-attracted and recruited to the inflamed tissues is fundamental to their function in triggering an immune response and driving inflammatory diseases (Shi and Pamer, 2011).

Monocytes can be categorized into different subsets depending on the expression of the chemokine receptors and surface molecules. In mice, CD11b and Ly6C positive monocytes express high levels of the chemokine receptor 2 (CCR2) and are referred to as

$Ly6C^{hi}$ inflammatory monocytes. These monocytes are present in the peripheral blood and are immediately recruited to the regions of infection and inflammation (Serbina et al., 2008). There are a second major class of monocytes which express high levels of CX3CR1 and low levels of CCR2 and Ly6C (Geissmann et al., 2003). This subset of monocytes is less frequent in the circulating blood and their main function is to adhere and migrate along the luminal surface of blood vessels (Auffray et al., 2007). Human monocytes on the other hand are classified on the basis of (cluster of differentiation) CD14 or CD16 expression (Ziegler-Heitbrock, 2007). Though the different classes of monocytes appear to be different in humans and mice, their differentiation and role in immune defense have been found to be similar (Belge et al., 2002, Ingersoll et al., 2010).

The chemokine ligands and receptors act as important chemo-attractants for recruiting monocytes and macrophages to sites of tissue infection. CC-chemokine ligand – 2 (CCL2, also known as MCP-1) and CCL7 (also known as MCP-3) are responsible for binding to CCR2 and henceforth recruitment of the $Ly6C^{hi}$ inflammatory monocytes from circulation (Tsou et al., 2007). The $Ly6C^{hi}CCR2^{+}$ monocytes differentiate into classically activated macrophages (CAMs) and induce inflammation. Unlike the CAMs which are pro-inflammatory, the alternatively activated macrophages (AAMs) which are induced by IL-4 and IL-13 are less inflammatory and have secretory and functional capacities (Gordon, 2003) (Fig. 2). Where the CAMs promote metabolic inflammation and insulin resistance, the AAMs aid in damping inflammation and protects from diet-induced obesity (Odegaard and Chawla, 2011) (Fig. 2). Many types of infections induce the production of CCL2 resulting in high expression of CCL2 in the serum or within injured tissues. CCL7 expression is also induced in cases of bacterial infection, which helps in

trafficking of the Ly6C^{hi} monocytes. Other chemokines such as CCR1 and CCR5 are also expressed on the surface of many monocytes which bind to the ligands CCL1 (also known as macrophage inflammatory protein 1 alpha or MIP-1α) and CCL5 and support chemotaxis (Charo and Ransohoff, 2006). Apart from their roles in refreshing macrophages and dendritic cells in the steady state, monocytes can be directly recruited to the inflamed tissues where they carry out anti-microbial activity.

1.4. Resolution of inflammation and its mediators

Accelerated but regulated synthesis of pro-inflammatory cytokines and chemokines by the macrophages and monocytes is an obligatory attempt by the immune system to protect the host from invading pathogens. The key to controlling inflammation lies in understanding and appreciating the mechanisms and molecules which have evolved to restrict inflammation. This is absolutely necessary for treating inflammatory diseases. Lipoxins are autocoids that are activated under various conditions and act on both neutrophils and monocytes. They possess anti-inflammatory functions in the aspect that they prevent neutrophil infiltration and therefore act as local stop signals (Takano et al., 1998, Takano et al., 1997). Subsequent studies have also suggested that lipoxins inhibit peripheral mononuclear (PMN) cell recruitment therefore controls inflammation and reduces fibrosis (Takano et al., 1998, Takano et al., 1997, Serhan, 2002). Another class of anti-inflammatory molecules consists of glucocorticoids which stimulate macrophage phagocytic activity to eliminate apoptotic white blood cells. A third class involves the resolvins which originate from the precursor polyunsaturated fatty acids (PUFAs) and act as important mediators to resolve inflammation. Using secreted materials from inflammatory macrophages and subjecting them to LC-MS/MS techniques, it has been

shown that resolvins can be biosynthesized from ω-3 fatty acids (Serhan, 2007). They act by reducing neutrophil infiltration without causing immunosuppression, but at the same time promoting microbial killing (Spite et al., 2009) and stimulating macrophages to clear out the apoptotic cells.

1.5. Translation control and regulation of inflammation

Regulation of gene expression at the level of translation can provide essential means of controlling the briskness of a response and its reversibility (Mazumder et al., 2010). Regulation of gene expression can be either universal (regulating the global translation of transcripts) or transcript selective (regulating the expression of a limited group of messages). The latter case usually involves binding of RNA binding complexes to either the 3' or 5' untranslated regions (UTRs) of the target messenger RNAs thereby repressing their translation (Mazumder et al., 2003b, Sonenberg and Hinnebusch, 2009). These RNA-binding proteins (RBPs) include heterogeneous nuclear ribonucleoproteins (hmRNP) and other pre-mRNA/mRNA-binding proteins (mRNP) that bind to RNA through special RNA binding domains (Glisovic et al., 2008). The greater lengths of the 3'-UTRs as compared to the 5'-UTRs makes the former more susceptible to regulation. Emerging studies suggest that it is mandatory for the immune system to rapidly activate the synthesis of the inflammatory mediators in response to a pathogenic attack. Simultaneously, the inflammatory response needs to be attenuated after removal of the pathogen to promote resolution. Translation control provides a crucial leverage in this aspect by making use of the pre-existing mRNAs. Since un-regulated synthesis of pro-inflammatory products can be detrimental for the host leading to neoplastic growth, endogenous defense mechanisms have evolved to limit inflammation.

Translational control can regulate the expression of several pro-inflammatory cytokines. TNF-α is a pro-inflammatory cytokine that is secreted by the lymphocytes, activated macrophages and mast cells in response to a systemic inflammatory response. Reports suggest that TNF-α can recruit leukocytes to the sites of inflamed tissue via activation of cell adhesion molecules (Tsang et al., 2005). Inflammatory genes like TNF-α and cyclooxygenase 2 (COX2) contain AU rich elements (AREs) in its 3'-UTR which can regulate the stability of the parent transcripts by recruiting several protein factors that bind to them (Mazumder et al., 2010). The presence of these AREs therefore prevent the overexpression of TNF-α and COX2 and helps in restoring homeostasis. Regulation via the AREs require the recruitment and subsequent binding of complex RNA binding proteins which bind to the ARE sequences of the target mRNAs. These ARE binding proteins (AREBPs) include heterogeneous nuclear ribonucleoprotein (hnRNP)-A1, HuR, T cell intracellular Ag (TIA)-1, TIA-1–related protein (TIAR) and TTP (Mazumder et al., 2010). TIA-1 and TIAR have been studied to play important functions in regulating the expression of not only TNF-α gene (Piecyk et al., 2000, Gueydan et al., 1999) but also COX2 gene (Dixon et al., 2003). Another inflammatory gene called the matrix metalloproteinase (MMP)-13 plays important role in remodeling of the extra-cellular matrix (ECM) and its expression is increased during chronic inflammatory diseases like cancer. MMP-13 expression has been studied to be silenced by the binding of TIAR (Yu et al., 2003). Thus the AU rich elements and various other 3'-UTR translation control mechanisms (which have not been discussed and are beyond the scope of this thesis) provide efficient regulation of expression of several inflammatory genes thereby controlling physiological inflammation. In this aspect, I shall discuss in detail one

recently identified novel mechanism of translational regulation which has possibly evolved as an endogenous mechanism to limit inflammation. This pathway is called the Gamma-Interferon Activated Inhibitor of Translation mechanism or the GAIT pathway.

1.5.1. IFN-γ–activated inhibitor of translation pathway: the regulator of IFN-γ–induced inflammation

Recent findings from our laboratory and from a group of other investigators have identified an IFN-γ induced delayed translational silencing pathway that is active in cells of myeloid origin (primarily monocytes and macrophages, but also in other lymphocytes) and regulates the expression of several inflammatory genes (Vyas et al., 2009, Mazumder and Fox, 1999, Sampath et al., 2003, Mazumder et al., 2003a, Sampath et al., 2004, Kapasi et al., 2007, Arif et al., 2009, Keene, 2007). In this mechanism, the GAIT complex, which is a multi-subunit RNA binding complex assembles and binds to the cis-acting 29 nucleotide long putative hairpin structure GAIT element present in the 3'-UTR of its target mRNA (Mazumder and Fox, 1999, Sampath et al., 2003). The GAIT complex comprises of four protein constituents: ribosomal protein L13a, glutamyl-prolyl-tRNA synthetase (EPRS), NS1-associated protein 1 (NSAP1) and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (Fig. 3) (Mazumder et al., 2003a, Sampath et al., 2004). In this pathway, there occurs phosphorylation dependent release of EPRS from the amino-acyl tRNA multisynthetase complex during the initial hours of IFN-γ induction (Sampath et al., 2004, Arif et al., 2009) followed by release of phosphorylated L13a from the large 60S ribosomal subunit (Mazumder et al., 2003a). The assembly of the GAIT complex occurs in two distinct steps: in the initial period (within the first 4 hours) of IFN-γ induction, EPRS gets phosphorylated and released from the tRNA multi-synthetase

complex and forms an inactive pre-GAIT complex with NSAP1. In the second step after another 12 hours, L13a is phosphorylated and released from the 60S subunit and joins GAPDH and then binds to the pre-GAIT complex to form the functionally active GAIT complex which then assembles on the GAIT element (Fig. 3). In the course of this mechanism, phospho-L13a binds to translation initiation factor eIF4G and blocks the recruitment of the eIF3 bound 40S pre-initiation complex thereby preventing the formation of the 48S initiation complex and inhibiting translation (Kapasi et al., 2007).

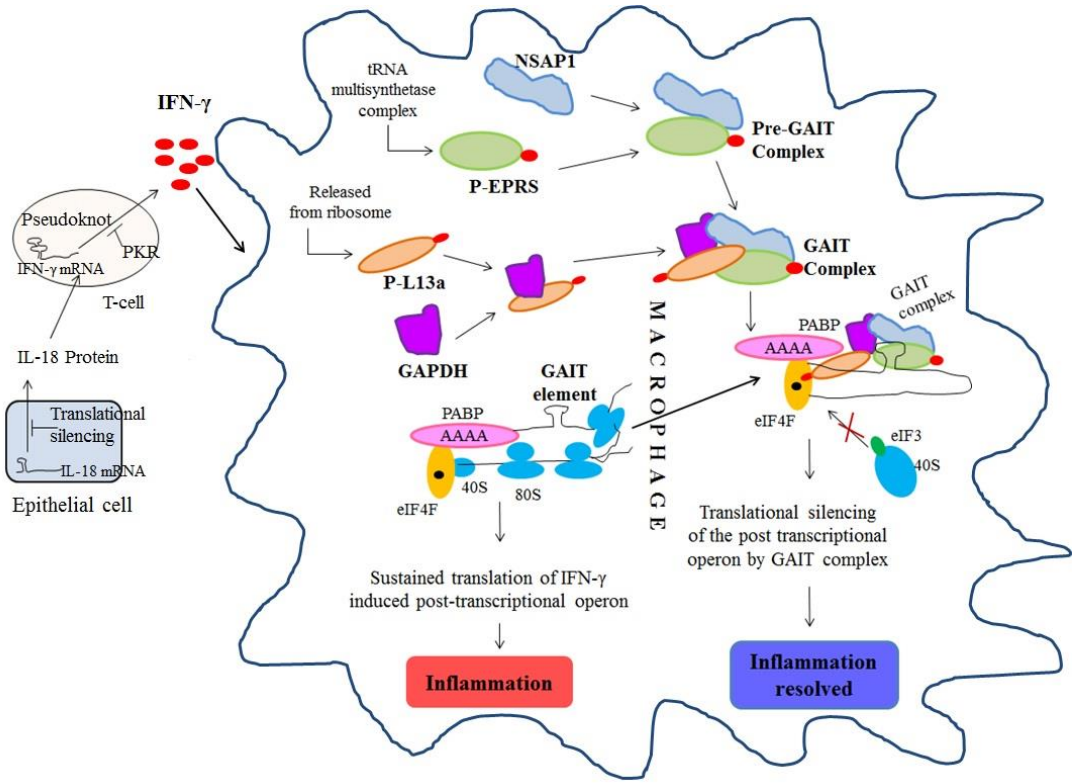

Figure 3. Control of IFN-γ axis and the GAIT model of translational silencing. IL-18 is an epithelial cell derived factor which induces the production of IFN-γ by the T cells. The synthesis of both IL-18 and IFN-γ are regulated by specific sequence elements present in the 5'-UTRs of their prescursor transcripts (left). IFN-γ then induces the activation of the GAIT silencing pathway in the macrophages (right). The GAIT complex is composed of four different proteins, L13a, EPRS, NSAP-1 and GAPDH (refer to the text for full names). The silencing pathway is

initiated by the phosphorylation depended release of EPRS from the aminoacyl tRNA multi-synthetase complex followed by release of phosphorylated L13a from the 60S ribosomal subunit. (the phosphate groups are shown in red). The binding of the functional GAIT complex to the GAIT element in the 3'-UTRs of the target mRNAs (members of the post-transcriptional operon) encoding for inflammatory proteins directly silences their expression by blocking the recruitment of the eIF3 bound 40S pre-initiation complex. Translational silencing of a cohort of inflammatory proteins by the GAIT complex permits resolution of inflammation. Figure 3 has been adapted from Figure 4 of "Translation control: a multifaceted regulator of inflammatory response." Mazumder, B., Li, X. and Barik, S. 2010. J. Immunol. 184: 3311–3319.

1.5.2. L13a-mediated GAIT pathway acts as a post-transcriptional regulon, of inflammatory gene expression

Though the concept of operon was believed to exist strictly in the prokaryotic system, recent studies have implicated the existence of post-transcriptional operons in the eukaryotic system where a small ensemble of genes performing similar functions can be regulated by a single mechanism. This mechanism relies on the recognition of specific structural elements present in the target transcripts by a single RNA-binding complex (Keene, 2007, Keene and Tenenbaum, 2002). Based on this emerging notion, further studies from our laboratory, taking a genome-wide approach identified a cohort of mRNAs encoding proteins having a role in inflammation, to be direct target of the L13a-dependent GAIT translational silencing pathway (Vyas et al., 2009). Most of these targets were identified as chemokine ligands and receptors. Chemokines and their cognate receptors have essential roles in attracting and recruiting immune cells to the sites of infection, which has been studied to be crucial in the pathogenesis of several inflammatory diseases. Therefore, regulation of these inflammatory molecules may serve

as an endogenous defense against pathological outcomes of uncontrolled inflammation. Many of these target mRNAs were validated independently and were found to contain a characteristic and functional GAIT element in their 3'-UTRs (Vyas et al., 2009). Our additional studies also confirmed the absolute requirement of ribosomal protein L13a in the successful operation of the GAIT silencing pathway but fortunately, depletion of L13a in monocytic cells did not affect the overall protein synthesis activity of the ribosome (Chaudhuri et al., 2007).

Based on the foregoing, we had decided to test the consequence of depletion of L13a in cells of myeloid origin on physiological inflammation. The rationale behind using myeloid-derived cells was that macrophages and monocytes are the central components and key effector cells in an immune response. For this purpose, we have generated the macrophage-specific knockout mice of L13a (designated as $L13a^{flox/flox}LysMCre^{+}$ mice in this thesis) and have used this animal model to study uncontrolled inflammation. We have challenged these knockout animals together with their wild type controls (designated as $L13a^{flox/flox}$ mice throughout this thesis unless indicated differently) to different inflammatory assaults like lipopolysaccharide (LPS) and dextran sodium sulfate (DSS) to induce inflammation and have studied their associated response. The results discussed in this thesis are direct outcomes of these initiatives taken. This thesis covers two major studies, the LPS induced endotoxemia model and the DSS-induced experimental colitis model which have been the two primary projects in the course of my Ph.D dissertation research. The studies have been discussed in detail in the following two chapters of this thesis (Chapters II and III respectively).

CHAPTER II

AN EXTRARIBOSOMAL FUNCTION OF RIBOSOMAL PROTEIN L13A IN MACROPHAGES RESOLVES INFLAMMATION

2.1. ABSTRACT

Inflammatory response is an imperative role of the immune system to protect the host against invading pathogens. Synthesis of pro-inflammatory molecules by the immune cells mediates the inflammatory response. However, uncontrolled inflammation can prove to be lethal for the host. Although a lot of studies focus on mechanisms that lead to inflammation, pathways that resolve inflammation are poorly understood. Creating macrophage-specific knockout mice of ribosomal protein L13a, which relies on the abrogation of the endogenous translation control of several chemokines and their cognate receptors, we have tested the physiological consequence of depletion of L13a in resolving inflammation. Upon challenging with inflammatory assaults like LPS, these animals have displayed higher susceptibility to LPS-induced endotoxemia, showing poor survival rates, widespread tissue damage in the lung and liver, infiltration of macrophages and neutrophils in the major organs (e.g., lungs and kidneys) and in the peritoneum. These

knockout animals have also shown elevated levels of several cytokines and chemokines (e.g., IL-1β, IL-6, MCP-1, MIP-1α, RANTES and KC) in their serum upon LPS challenge. Macrophages from these knockout animals have shown unregulated synthesis of several chemokines (e.g., CXCL13, CCL22, CCL8 and CCR3). The macrophages also failed to show RNA binding GAIT (Gamma-interferon Activated Inhibitor of Translation) complex formation on the 3'-untranslated regions of the target mRNAs. Additionally, the macrophages have also shown higher polysomal abundance of these mRNAs thereby indicating a defect in their translational control. Altogether, these observations document that the absence of L13a in the macrophages can abrogate the endogenously imposed translational silencing pathway and cause sustained synthesis of a group of inflammatory proteins. This unregulated synthesis of pro-inflammatory molecules could lead to unresolved inflammation and our knockout mice serves as a novel model to study uncontrolled inflammation in a mammalian host.

2.2. INTRODUCTION

Inflammation is an adaptive response that is generated by the immune system to toxic stimuli and conditions such as invasion by foreign pathogens or tissue injury (Majno et al. 2004, Medzhitov 2008). Thus, inflammatory gene expression and synthesis of pro-inflammatory molecules play a pivotal role of the immune system to protect the host from infections. The pro-inflammatory molecules comprises of a variety of inflammatory mediators such as chemokines, cytokines, vasoactive amines, eicosanoids which are secreted by the monocytes, macrophages, neutrophils and other key immune cells (Medzhitov 2008). This initial recognition of infection is crucial in eliciting an immune response which helps in removal of foreign pathogens and setting up the host's defense system (Serbina et al. 2008, Serbina and Pamer 2008). It is generally considered that a regulated inflammatory response is constructive innate immune response, but unrestricted synthesis of pro-inflammatory products can have adverse and detrimental effects (Medzhitov 2010). Thus endogenous mechanisms have evolved to limit the cytokine storm to permit the resolution of inflammation (Poddar et al. 2013). Although studies understanding the pathways that contribute to inflammation are well appreciated, those that have evolved to conclude it remain unclear. Identification and characterization of such pathways and anti-inflammatory molecules can serve as prime targets for generating novel therapeutic drugs against inflammation. Our laboratory has identified one such pathway in a novel animal model which is dependent on the abrogation of L13a mediated translational silencing mechanism by creating macrophage specific knockout mice of L13a ($L13a^{flox/flox}LysMCre^{+}$). The termination of inflammation is markedly jeopardized

in this new animal model (Poddar et al. 2013). Such uncontrolled inflammation is coherent with earlier set of studies done in our laboratory discovering a translational silencing pathway in cells of myeloid origin when induced by interferon-gamma and requires active participation of ribosomal protein L13a (Mazumder and Fox 1999, Mazumder et al. 2001, Mazumder et al. 2003, Mazumder and Seshadri 2003, Kapasi et al. 2007, Vyas et al. 2009). To describe the mechanism briefly, when monocytes and macrophages are treated with interferon-gamma (IFN-γ), after 16 hours of treatment, a multi-protein complex, known as the GAIT (Gamma Activated Inhibitor of Translation) complex becomes active and assembles on the GAIT element (a cis-acting element) present in the 3'-UTR (untranslated region) of target mRNAs and inhibits their translation. The formation of the GAIT complex requires the phosphorylation dependent release of ribosomal protein L13a from the 60S large subunit (Mazumder and Sampath et al. 2003). Further studies from our laboratory also showed that phosphorylated L13a, as a part of the GAIT complex can bind to translation initiation factor and scaffolding protein, eIF4G and prevent the recruitment of the eIF3 bound 40S subunit, hence blocking the formation of the 48S initiation complex and inhibit translation initiation of GAIT element containing target mRNAs (Kapasi et al. 2007). Our subsequent studies using cell culture models have also suggested that depletion of L13a in monocytic cells can abrogate the L13a-dependent translational silencing of its target mRNAs without affecting the total protein synthesis (Chaudhuri et al. 2007). In addition to our studies, research from other laboratories has also suggested that production and accumulation of inflammatory molecules can be regulated by exercising control at their pre-existing mRNA levels (Mazumder et al. 2010).

Macrophages and monocytes are key players of an immune response and have a protective role against various inflammatory assaults, thus help maintaining tissue homeostasis (Murray et al. 2011). These cells in co-ordination with IFN-γ aid in the secretion and production of several other chemokines and cytokines which altogether trigger an innate immune response to kill the invading micro-organisms. Using a genome-wide screening approach, we have identified that the L13a-dependent GAIT mediated silencing pathway can act as a post-transcriptional operon and regulate the synthesis of a cohort of inflammatory genes encoding mostly chemokine ligands and receptors such as CCL11, CCL22, CCL8, CCL21, CXCL13, CCR3, CCR4 and CCR6 (Vyas et al. 2009). Further detailed studies from our laboratory have also identified functional GAIT elements in the 3' UTRs of these mRNAs which can recruit the L13a containing GAIT complex (Vyas et al. 2009). Recent studies indicate that chemokines and their receptors act as endogenous signals that help to recruit macrophages neutrophils etc. to the sites of inflammation (Charo and Ransohoff 2006). Based on our previous observations and available literature in the field we hypothesize that L13a dependent translational silencing mechanism has evolved as an endogenous defense mechanism to protect against uncontrolled inflammation. This led us to anticipate that depletion of L13a in the macrophages could result in sustained synthesis of many inflammatory genes and thus promote unresolved inflammation due to repudiation of the translational silencing pathway. To test this at the physiological level, we have generated the macrophage-specific knockout (KO) mice of L13a using the Cre-LoxP system of recombination (Nagy 2000), in which the termination of inflammation is severely compromised. We have challenged these animals with inflammatory stimuli like lipopolysaccharide (LPS),

monitored and studied their response and compared them to those of the similarly treated controls to get an in-depth understanding of the role played by L13a-mediated translational silencing pathway in resolving physiological inflammation. The rationale for using LPS as an inflammatory assault is that LPS triggers production of cytokines by macrophages, causes systemic inflammation and is also a potent inducer of IFN-γ in cells of myeloid origin (Le et al. 1986, Mattern et al. 1999, Dai et al. 2008). In this chapter, I will show and discuss results obtained from the LPS-induced endotoxemia study. In all the experiments discussed henceforth, we have used the $L13a^{flox/flox}$ mice as experimental controls and $L13a^{flox/flox}LysMCre^{+}$ mice as the KOs unless otherwise mentioned (treated or untreated as will be mentioned specifically for the respective experiments). Upon challenging the animals with LPS, we have observed higher sensitivity of the KOs to LPS-induced endotoxemia showing reduced survival rates, severe organ damage, tissue infiltration of immune cells, infiltration of macrophages and granulocytes in the peritoneal cavity, overproduction of several chemokines and cytokines and unregulated expression of several GAIT target proteins which can be attributed to the probable loss of L13a-mediated translational silencing. In addition, the macrophages harvested from the LPS treated L13a KO mice have shown greater polysomal abundance of some of the GAIT target mRNAs and failed RNA binding complex formation on these mRNAs. Thus, our observations demonstrate a novel extra-ribosomal anti-inflammatory function of ribosomal protein L13a and our macrophage-specific L13a knockout mice serve as an innovative animal model to study uncontrolled inflammation in a mammalian host. In this chapter of the thesis I will present and discuss in detail the observations made and results

obtained from the LPS-induced endotoxemia study using the KO mice model that exhibits disruption of L13a-catalyzed translational silencing pathway in the macrophages.

2.3. MATERIALS AND METHODS

2.3.1. Generation of macrophage-specific L13a knockout mice.

The targeting construct of the L13a gene was designed by introducing LoxP sites upstream of exon 2 and downstream of exon 8 so that exons 2 to 8 of the L13a gene could be conditionally targeted. A Neomycin (Neo) cassette flanked by LoxP and FRT sites was also introduced downstream of exon 8 as a marker for selection of positive embryonic stem (ES) cell clones. ES cells from C57BL/6 mice were transfected with the L13a targeting construct. Recombinant ES (rES) clones were selected by genotyping for the presence of the 1^{st} LoxP site with Lox1/SDL2 primer pair. The primer sequences are listed in Table 1 of this thesis. Cre-dependent depletion of L13a was confirmed by infecting the positive ES cell clones with adenovirus expressing Cre recombinase under the control of the CMV promoter. (Ad-CMV-Cre). Two different rES cell clones were selected for injection into the blastocytes. The blastocytes were then transferred into a surrogate mother mouse and the chimeras were generated. The blastocyte injection and transfer into surrogate mother mouse was done at a contract facility, ingenious Targeting Laboratory (Ronkonkoma, NY). The chimeras were identified by presence of mixed fur coat color. The male chimeras were then crossed with wild type (WT) female C57BL/6 mice for germline transmission. The Neo cassette was removed by crossing these animals with $ACTFLP^{+}$ mice in the C57BL/6 background (Flipper mice expressing the FLP transgene under the control of the actin promoter). The F4 generation mice which were homozygous for the L13a floxed allele ($L13a^{flox/flox}$) and Neo deletion were confirmed by

genotyping with Lox1/SDL2 and NDEL3/Anti-AT2 primer pairs. (Primer sequences listed in Table 1). Lung fibroblasts were isolated from the L13a$^{flox/flox}$ mice and infected with Ad-CMV-Cre followed by immunoblotting with anti-L13a antibody (Ab), to test for the Cre-dependent depletion of L13a gene. Finally, the macrophage-specific L13a KO mice (L13a$^{flox/flox}$LysMCre^{+}) were generated by crossing the L13a$^{flox/flox}$ mice with LysMCre^{+} mice (bought from The Jackson Laboratories, Bar Harbor, ME), where the expression of the Cre transgene is driven by the Lysogen M promoter which is myeloid cell-specific. These KOs were identified by genotyping and the macrophage-specific depletion of L13a gene was confirmed by immunoblotting with anti-L13a Ab.

2.3.2. Animal housing, treatments and induction of endotoxemia.

All animals were housed, and bred according to the rules of National Institute of Health (NIH) and the Institutional Animal Care and Use Committee (IACUC) of Cleveland State University. They were supplied with regular drinking water and fed normal chow diet. For all experiments discussed henceforth, animals aged between 8-12 weeks and of matched genders were used unless otherwise mentioned specifically. All injections were done intra-peritoneally (i.p.). Peritonitis was induced by i.p. injection of 1.5 ml of 4% thioglycollate solution. Systemic inflammation of endotoxemia was induced by i.p. injection with LPS (Sigma-Aldrich, St. Louis, MO) at a dose of 15 mg/kg of body weight. For survival analysis, a sub-lethal dose of 20 mg/kg of body weight was used. For most experiments except for measuring the serum cytokine levels, LPS injection was done for 24 hours unless otherwise indicated specifically.

2.3.3. Isolation of peritoneal macrophages, total leukocytes and peripheral blood monocytes.

For peritoneal macrophages, mice were injected with either LPS for 24 and 48 hours or thioglycollate for 48 or 72 hours (as will be mentioned specifically for respective experiments). After the said time periods, animals were euthanized and peritoneal lavage was harvested by flushing with ice-cold PBS. Cells were then pelleted and resuspended in PBS and counted with a cell counter before proceeding with respective experiments. Total leukocytes were isolated from the spleen by crushing the spleen through a cell strainer followed by centrifugation and lysis of the red blood cells (RBCs) from the cell pellet with RBC Lysis Buffer (eBioscience) following the manufacturer's protocol. The mononuclear cells were obtained from blood by layering over Ficoll-Hypaque Gradient followed by centrifugation, using established methods (Kanof et al. 2001).

2.3.4. Flow Cytometry

10^6 cells per sample were counted and used for staining. Cells were incubated with rat-anti-mouse CD16/CD32 Fc Block (BD Pharmingen) to block non-specific Fcγ receptors. Cells were then stained with the following antibodies: FITC (fluorescein isothiocyanate) conjugated rat-anti-mouse CD11b IgG2b (BD Pharmingen), APC (allophycocyanin) conjugated rat-anti-mouse F4/80 IgG2b (Abd Serotec), PE (phycoerythrin) conjugated Ly6G IgG2a (BD Pharmingen), FITC conjugated rat anti-mouse Mac2 IgG2a (Cedarlane Laboratories) and APC conjugated Ly6C (eBioscience). Cells were also stained with corresponding isotype control antibodies which included PE conjugated IgG2b (eBioscience) and FITC conjugated IgG2a (eBioscience), to subtract

background staining. For peritoneal cells and peripheral blood monocytes, the cells were gated for leukocytes, for total leukocytes, cells were gated for granulocytes based on their respective scatters. Flow cytometry analysis was done using the FACSCantoII cytometer and FACSDiva (BD Biosciences) and FlowJo (TreeStar) softwares.

2.3.5. Quantification of cytokines and chemokines

Serum levels of TNF-α and IFN-γ, and IFN-γ levels secreted by the total leukocytes, and chemokines secreted by the primary macrophages were measured by ELISA using commercially available kits (R&D Systems). For detection of chemokines secreted by macrophages, peritoneal macrophages from each animal were harvested and then plated (1×10^5 cells /well) in triplicate in RPMI 1640 media supplemented with 10% serum and antibiotics. Macrophages were selected by overnight adherence. Cells were then washed with PBS and incubated in fresh RPMI media with or without IFN-γ (500U/mL, from R&D Systems) for different time intervals. Conditioned media was collected and subjected to ELISA. Serum levels of IL-1β, MCP-1, KC, RANTES, IL-6 and MIP-1α were determined using MultiPlex Mouse Quantikine ELISA array in a commercial facility (Quansys Biosciences, Logan, UT).

2.3.6. Tissue histological analysis

Tissues (lungs and kidneys) were harvested from mice followed by fixation in 10% neutral buffered formalin (Sigma-Aldrich) for 16 hours and stored in PBS overnight prior to paraffin embedding and sectioning. For Ym1 staining, tissues were fixed in methanol (a solution containing 60% methanol, 30% glacial acetic acid and 10% water). 5μM sections were cut from each block and stained with hematoxylin and eosin (H&E).

Paraffin embedded tissue sections were deparaffinized and antigen retrieval was done by incubating with Trilogy solution (Richard Allan Scientific) in a steamer for 30 minutes. For macrophage staining, rat-anti-mouse anti-Mac2 antibody (Cedarlane Laboratories) and rat-anti-rabbit anti-Ym1 antibody (Wako Chemicals, USA) were used. Biotin-SP conjugated appropriate secondary antibodies (Jackson ImmunoResearch) were used.

2.3.7. Polysomal profiling and analysis

Polysomal analysis was done with the lysates made from the peritoneal macrophages harvested from the control and KO mice injected with LPS (15 mg/kg of body weight) for 24 hours. Lysates were prepared using 0.5 ml of polysome lysis buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1mg/mL cycloheximide, 50 U of recombinant RNasin [Promega, Madison, WI] and 0.1% Igepal-CA630 [Sigma, St. Louis, MO]. Ten optical density units of the Peritoneal macrophage extracts from 4 controls and 4 KOs were pooled together and then carefully layered over 5 to 50% linear sucrose gradients prepared in the polysome buffer (as mentioned above without the RNasin and Igepal-CA630) and centrifuged at 17,000 rpm in a Beckman SW32.1 Ti rotor for 18 h at 4°C. Gradients were fractionated using an ISCO gradient fractionation system supplied with a UA-6 detector using an upward displacement method. Lighter ribonucleoprotein (RNP) fractions, 40S, 60S, 80S, and heavy polysome fractions were monitored by constant UV absorption profiles at A_{264}, and 12 tubes of 1.2 mL fractions were collected. Total RNA was isolated from each fraction using Trizol (Invitrogen, CA) and then subjected to reverse transcriptase (RT)-PCR with specific primers against CXCL13, CCL22, CCR3 and actin. PCR products were then resolved on 1.5% agarose gels.

2.3.8. Determination of in-vivo interaction of L13a with different chemokine mRNAs and 60S ribosomal subunit.

The interaction of L13a with cellular mRNAs and the 60S ribosomal subunit was determined using our previously standardized protocols (Mazumder et al. 2003). Briefly, macrophage lysates containing 500μg of total protein were subjected to immunoprecipitation reaction using 10μL of affinity purified anti-L13a antibody. The L13a bound RNA was isolated using Trizol reagent (Invitrogen). For mRNAs, reverse transcription was done using oligodT primers and random primer was used for the ribosomal subunit. Specific primer pairs for CCL22, CXCL13 and CCR3 were used. (detail of primer sequences have been included in Table 1).

2.3.9. Determination of GAIT-element mediated translational silencing activity in the mouse macrophages.

The translational silencing activity of the mouse macrophages was reconstituted using a cell-free in-vitro translation system of rabbit reticulocyte lysate. A chimeric luciferase reporter RNA containing a 29 nucleotide WT GAIT element in its 3'–UTR was generated using in-vitro transcription with the corresponding DNA construct. 100 ng of the reporter RNA was used in the in-vitro translation reaction with rabbit reticulocyte lysates (Promega) in the presence of ^{35}[S] methionine. 4 μg of lysates prepared from peritoneal macrophages of saline and LPS treated control and KO mice were used to check translational silencing activity. IFN-γ treated U937 cell lysates for 8 and 24 hours were used as controls for this experiment. An aliquot of the translation reaction mixture was resolved by SDS-PAGE (7% polyacrylamide) followed by autoradiography.

2.3.10. Antibodies for immunoblot analysis

Anti-L13a antibody was previously raised against a peptide NVEKKIDKYTEVLKTHG near the C terminus of human L13a gene (Mazumder et al. 2003). This antibody recognizes a specific band both for human and mouse L13a between 28 and 21 kDa. Anti-L28 and anti-actin antibodies were purchased from Santa Cruz Biotechnology and Sigma-Aldrich, respectively. Anti-Cre recombinase antibody was bought from Abcam.

2.3.11. Statistical analysis

The log-rank (Mantel-Cox) test was used to determine the differences in the survival rates between the LPS treated controls and knockouts. All results presented are mean ± SD. The statistical significance and p values between two groups were calculated using two-tailed Student's t test. All statistical analysis were performed using the GraphPad Prism 5.0 software.

2.4. RESULTS

2.4.1. Generation of L13a$^{flox/flox}$LysMCre^{+} mice which show macrophage-specific depletion of L13a.

Since macrophages and monocytes are the prime effector cells of inflammatory and innate immune responses, we have generated the macrophage-specific knockout mice of L13a using the Cre-Lox system of recombination (Nagy 2000), in order to evaluate the role of L13a-dependent translational silencing in controlling physiological inflammation. The design of the targeting construct is shown in Fig. 4A. Lox P sites were inserted in the targeting construct upstream of exon 2 and downstream of exon 8 of the mouse L13a genomic sequence. A Neomycin cassette was also introduced into the targeting construct as a marker flanked by two LoxP, FRT (flippase recognition target) sites to facilitate its removal. Embryonic stem (ES) cells from C57BL/6 mice were transfected with the L13a targeting construct and the recombinant embryonic stem (rES) cells harboring the Lox P allele were identified by PCR genotyping with Lox1/SDL2 primer pair (Fig. 4B). Before injecting into the blastocytes of surrogate mother mice, the Cre-dependent depletion of L13a protein in the rES cells was confirmed by infecting with Adenovirus expressing Cre under the control of the CMV promoter (ad-CMV-Cre, Vector Biolabs, PA) followed by immunoblotting with anti-L13a antibody (Fig. 4C). The male chimeric mice were identified by presence of mixed fur coat color (data not shown) and then crossed with wild type C57Bl/6 female mice for germ line transmission. The neomycin gene from L13a$^{flox\text{-}neo/+}$ mice was removed by crossing with ACTFLPe^{+} mice in a C57BL/6 background (The Jackson Laboratory). The F4 mice (L13a$^{flox/flox}$) homozygous for the

L13a floxed and Neo deletion allele were confirmed by PCR genotyping with Lox1/SDL2 and NDEL3/Anti AT2 primer pairs (Fig. 4D). To confirm the Cre-dependent excision of L13a in the F4 mice, lung fibroblasts were isolated from the the L13a$^{lox/flox}$ mice followed by infection with Ad-CMV-Cre, followed by immunoblot analysis with anti-L13a antibody (Fig. 4E). The complete absence of the L13a band in Ad-CMV-Cre treated L13a$^{flox/flox}$ mice fibroblasts lysates confirmed the successful generation of the F4 mice and its potential to generate the Macrophage-specific L13a KO mice upon future crossing with appropriate Cre mice.

The macrophage specific knockout mice of L13a (L13a$^{flox/flox}$LysMCre^{+}) were generated by crossing L13a$^{flox/flox}$ with LysMCre^{+} mice (The Jackson Laboratories). In the LysMCre^{+} mice, the expression of the Cre transgene is regulated by the Lysogen M promoter which is active only in cells of myeloid origin. These knockout animals were confirmed initially by genotyping from DNA isolated from tail snips with Lox1/SDL2 and Cre-specific primers (Fig. 5A). The macrophage specific depletion of L13a was tested by the immunoblot analysis of lysates obtained from macrophages, liver and kidneys using anti-L13a antibody. The absence of L13a protein was immunologically detected in the macrophages; however its expression in the other organs like liver and kidneys remained unaltered (Fig. 5B). Throughout the study, we have used the L13a$^{flox/flox}$ mice as our control and the L13a$^{flox/flox}$LysMCre^{+} mice as our L13a KO mice unless otherwise indicated.

The KO mice progeny obtained from crossing of L13a$^{flox/flox}$ mice with LysMCre^{+} followed normal Mendelian Genetics of distribution with no observed embryonic lethality. The newborn KO mice were identical to the controls. Under unchallenged

conditions, no visible symptoms of pathology such as reduced survival, high breath rate, difference in food or water intake, weight loss, retarded motor activity, growth, fertility or visible change in major organs was observed in the macrophage-specific L13a KOs. These observations suggest that depletion of L13a in the macrophages does not cause any serious defect in animal development.

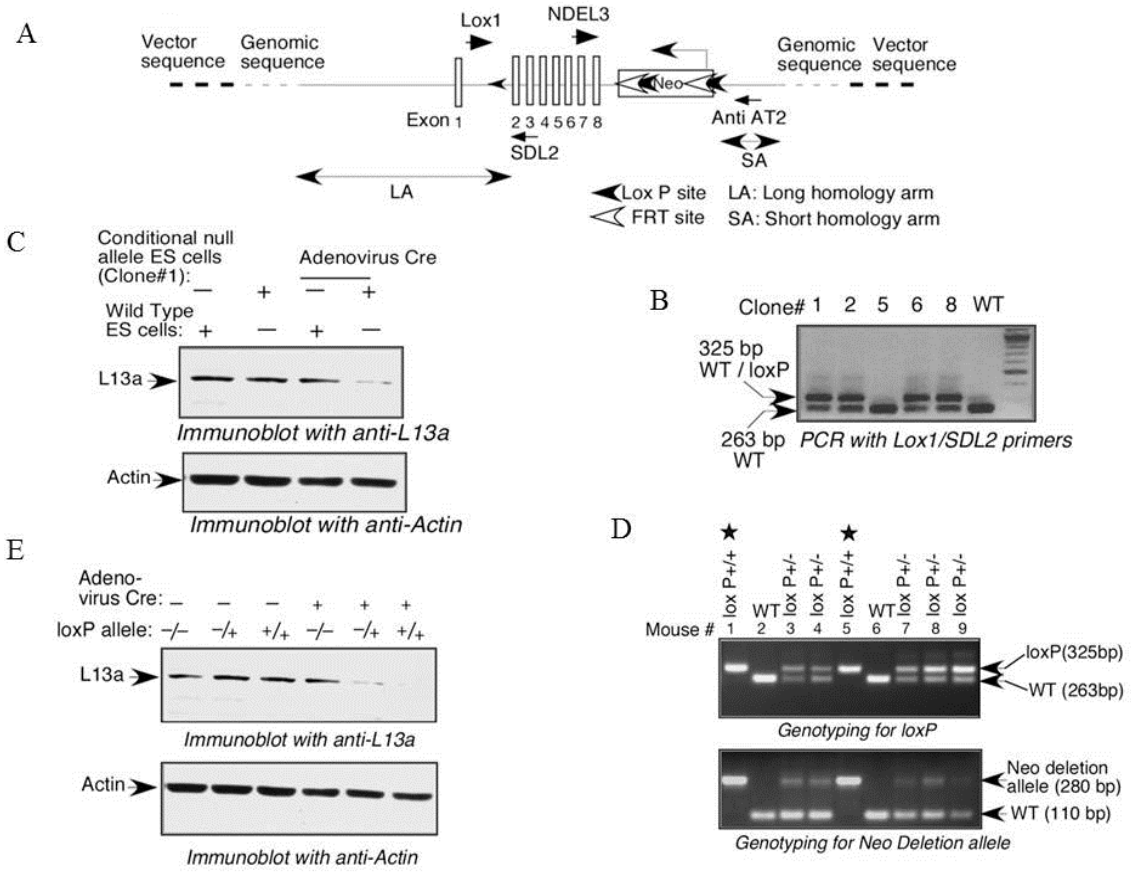

Figure 4. Generation of F4 mice homozygous for the L13a floxed allele. (A) Design of the targeting construct. The positions of the LoxP and FRT sites, Neomycin cassette, the long and the short homology arms are shown. (B) Identification of the mouse recombinant ES cells harboring the conditional null allele. The recombinant ES cells were genotyped using the Lox1/SDL2 primers and identified on the basis of a doublet band at 263/325 bp. (C) Confirmation of Cre-dependent depletion of L13a protein in the ES cells. The recombinant ES cells were infected with Ad-CMV-Cre and the Cre dependent depletion was confirmed by immunoblotting with anti-L13a antibody. (D) Identification of the F4 mice homozygous for the LoxP and Neo deletion allele.

Tail DNA samples of the F4 generation pups were screened by PCR with the Lox1/SDL2 primer pair for the LoxP allele and the NDEL2/anti-AT2 primer pair for the Neo deletion allele. The presence of the 280 bp band shows the deletion of the Neo cassette in both the alleles. The stars denote two representative animals in a litter which were genotypically confirmed to be homozygous for LoxP and Neo deletion allele. (E) Fibroblasts were isolated from the lungs of the F4 mice harboring the conditional null allele and were infected with Ad-CMV-Cre to test the Cre-dependent excision of L13a. This was confirmed by immunoblotting with anti-L13a Ab.

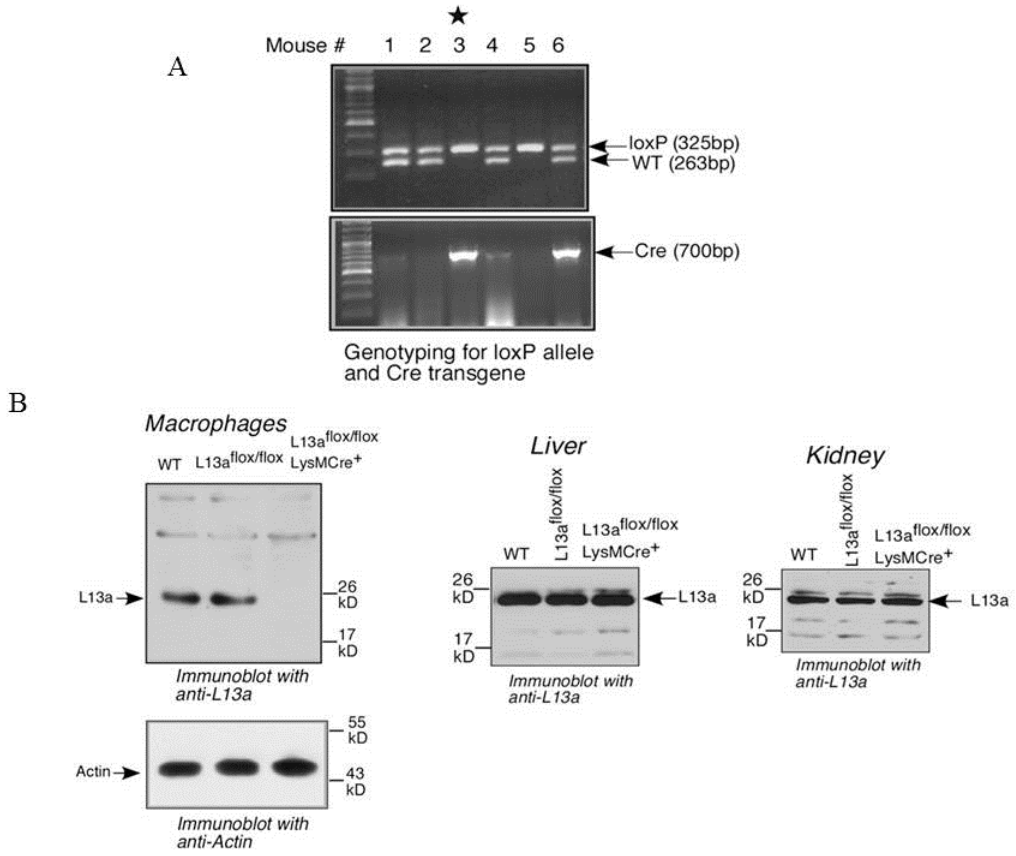

Figure 5. Generation of the macrophage-specific L13a KO mice. (A) The macrophage-specific L13a KO mice were generated by mating L13a$^{flox/flox}$ mice with the L13a$^{flox/+}$LysMCre$^{+/-}$ mice and then genotyping the tail DNA from the progeny with Lox1/SDL2 primer pair for the LoxP band and Cre-specific primer pairs (The Jackson Laboratories) for the presence of the Cre transgene. The star denotes one animal from one representative litter which was successfully genotyped to be homozygous for the LoxP allele (presence of the 325 bp band) and the Cre transgene (presence of the 700 bp band). This mouse has been designated as the macrophage-specific KO mouse of L13a. (B) Confirmation of the macrophage-specific depletion of L13a.

Lysates were made from peritoneal macrophages, liver and kidneys harvested from $L13a^{flox/flox}LysMCre^{+}$, $L13a^{flox/flox}$ and wild type mice and then subjected to immunoblot analysis with anti L13a antibody. The blot from the macrophage lysates was reprobed with anti-Actin antibody.

2.4.2. Macrophage specific L13a KO mice show higher susceptibility to LPS-induced endotoxin challenge.

Previous studies from our laboratory have shown that L13a-dependent translational silencing, induced by IFN-γ in monocytes and macrophages can act as a post-transcriptional operon and control the expression of a cohort of inflammatory genes (Mazumder and Seshadri 2003, Vyas et al. 2009). In our study we have used LPS induced endotoxemia model to determine the inflammatory response in the knockout animals. The relevance of this model is based on the ability of LPS to induce IFN-γ in-vivo and synthesis of pro-inflammatory cytokines by cells of myeloid lineage (Le et al. 1986, Mattern et al. 1999, Dai et al. 2008). IFN-γ mediated activation of immune cells is essential in eliciting an inflammatory response. So we hypothesized that if the L13a-dependent silencing of the cohort of inflammatory gene is absent in the KO animals, they would be more prone to inflammation and hence LPS-induced pathological outcomes would be more severe in the knockouts. In order to test this, we treated both control and L13a KO mice with a sub-lethal dose of LPS (20 mg/kg of body weight) and monitored their survival over a period of 120 hours post LPS administration. The KO animals displayed significantly reduced survival rates as compared to the controls (48% versus 78%) (Fig. 6A). A Kaplan-Meier survival curve analysis shows the statistical significance of the data (Fig.6A). Treatment with LPS is known to cause lethargy and a decrease in

the ambulatory activity in mice (Ogimoto et al. 2006, Grossberg et al. 2011). Therefore we treated our animals with a sub-lethal dose of LPS (15 mg/kg of body weight) and observed their ambulatory activities up to 25 hours post LPS challenge using a device equipped to measure the number of brakes in sequential laser beams in two dimensions. A rapid reduction in the ambulatory activities was observed in the KO group only upon LPS challenge and not with saline (Fig. 6B). We also measured other parameters of inflammation like blood urea nitrogen (BUN), aspartate amino-transferase (AST, a liver enzyme that increases drastically during systemic inflammation) and tumor necrosis factor-alpha (TNF-α) in the mice serum upon LPS administration and all of them were found to be significantly elevated in the KO mice (Fig. 6C) Together these results suggest that macrophage-specific abrogation of L13a in the KO mice leads to increased sensitivity to endotoxemia.

2.4.3. Macrophage specific depletion of L13a causes increased serum level of cytokines upon LPS challenge.

Since we had already observed elevated levels of BUN, AST and TNF-α, we decided to test for other markers of inflammation in the serum of the KO mice. We tested the serum levels of some other inflammatory cytokines e.g., IL-1β, IL-6, MCP-1, MIP-1α, RANTES and KC in the LPS treated KO mice and all of them were found to be significantly elevated in comparison to the control mice (Fig. 7). These results further document that macrophage-specific depletion of L13a causes increased susceptibility to endotoxic challenge.

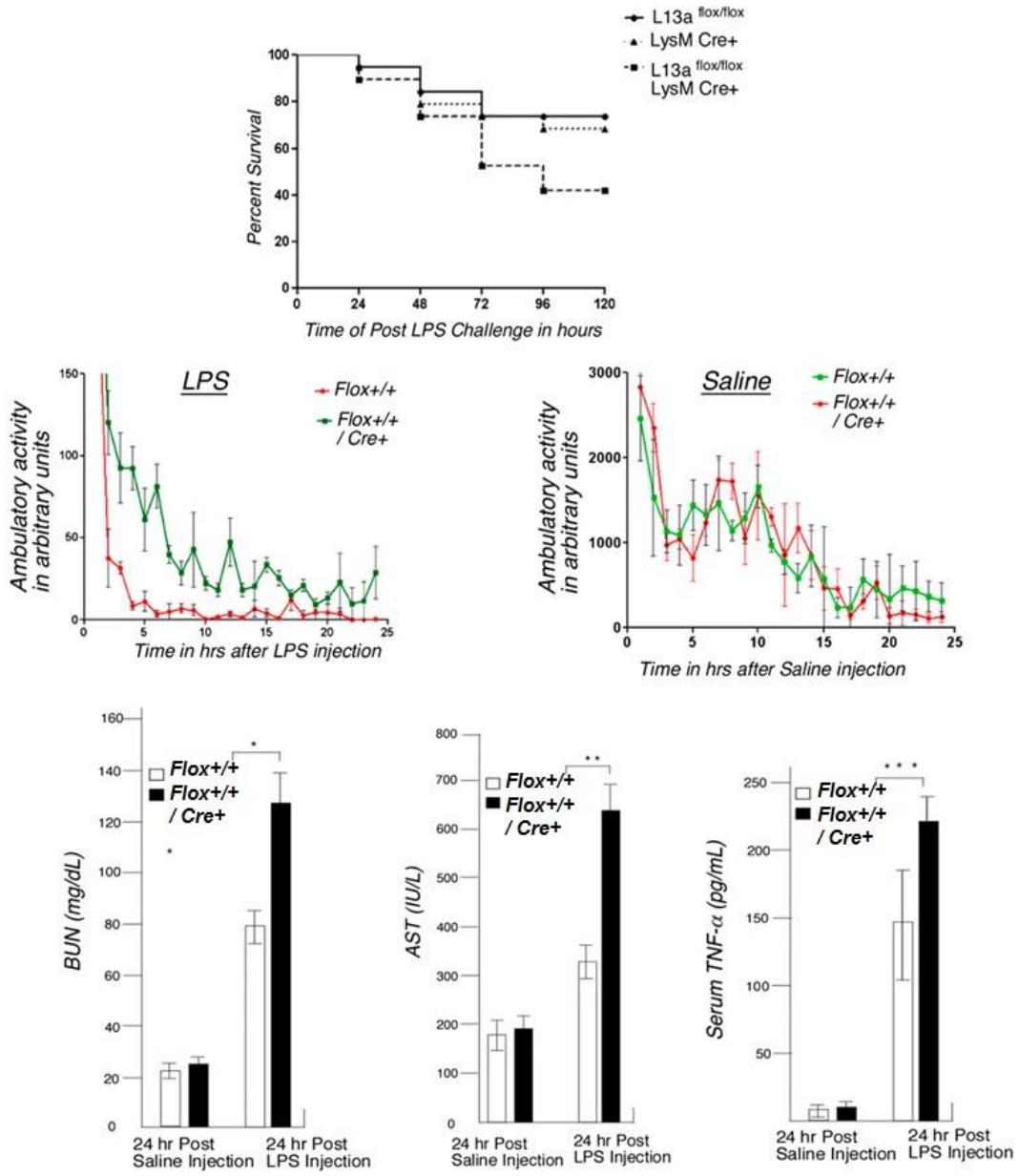

Figure 6. Macrophage-specific L13a KO animals show enhanced susceptibility to endotoxemia. (A) Increased mortality of the KO mice in response to endotoxin challenge. Age and sex matched control, KO and LysMCre^{+} mice were challenged with a sub-lethal dose of LPS (20 mg/kg of body weight) and their survivals were monitored over a period of 120 hours. The survival rates were plotted on a Kaplan-Meier survival curve, n=19 in each group and p=0.042 (log-rank test) (B) Reduced ambulatory activity of the KO mice upon endotoxin challenge. Both control and KO mice were challenged with LPS (15 mg/kg of body weight) and their motor activities were monitored for 25 hours post LPS challenge using a device equipped to measure

brakes in the sequential laser beams in two dimensions. The results are plotted on an arbitrary scale. Results are mean ± SD (n=8) p=0.0049, unpaired two-tailed Student t test. (C) KO mice show elevated serum levels of markers of inflammation. Both control and KO animals were administered with LPS (15 mg/kg of body weight) and their serum levels of BUN, AST and TNF-α were measured after 24 hours of LPS challenge. Results are mean ± SD. (n=4), *p= 0.011, **p = 0.004, ***p = 0.032 paired two-tailed Student t test.

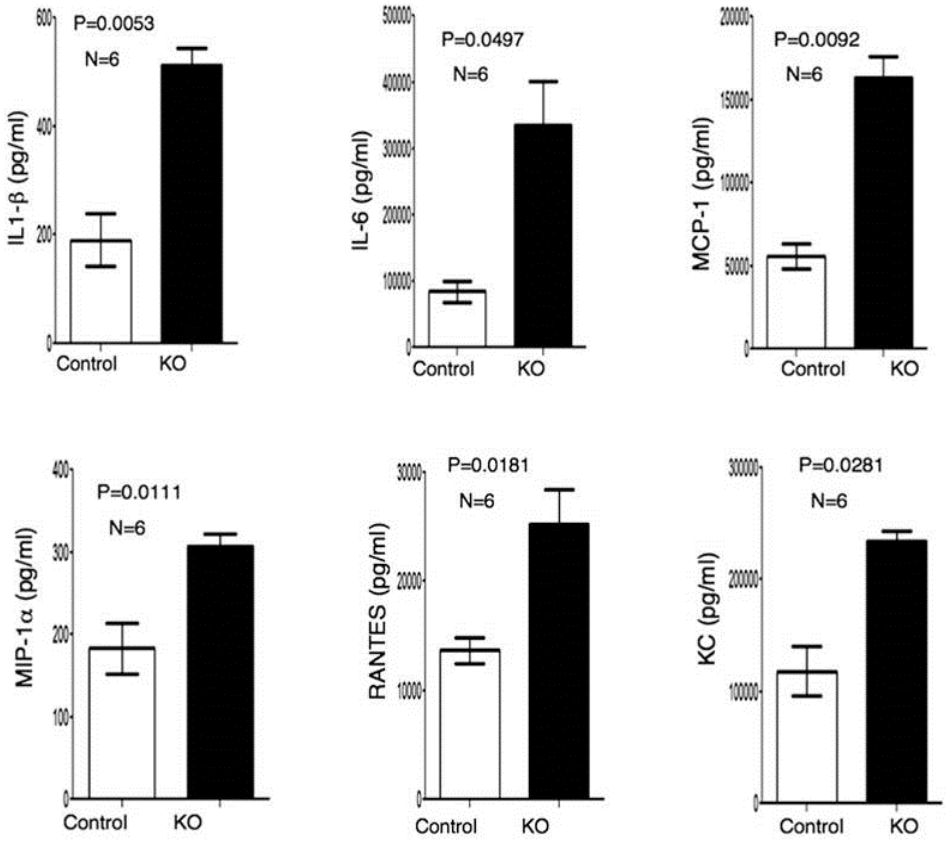

Figure 7. Increased serum levels of inflammatory cytokines in the KO mice upon endotoxin challenge. Control and KO mice were injected with a sub-lethal dose of LPS (15mg/kg of body weight) for 12 hours. Blood was then collected through cardiac puncture; serum was isolated and subjected to a cytokine/chemokine ELISA array in a commercial facility (Quansys Biosciences). The results are mean ± SD and p values have been calculated using paired two-tailed Student t test.

2.4.4. Myeloid specific depletion of L13a causes enhanced macrophage infiltration in the peritoneum and expansion of leukocyte population in the spleen.

Infiltration of leukocytes in the peritoneal tissue is a characteristic feature of inflammation and requires signals generated by chemokines and chemokine receptors (Medzhitov 2010, Shi and Pamer 2011). We reasoned that the macrophage specific deficiency of L13a in the KO animals could lead to greater influx of leukocytes in the tissues and peritoneum by removing the endogenous translational silencing of the chemokines and chemokine receptors. To assess the inflow of mononuclear cell population in the peritoneum, we employed fluorescence activated cell staining (FACS) analysis of the peritoneal cells isolated from control and KO mice upon thioglycollate induced peritonitis and LPS induced endotoxemia. We stained the peritoneal cells with markers specific for macrophages like CD11b and F4/80 and Gr-1 for granulocytes,. We then quantified the population of Gr-1/CD11b, F4/80/CD11b and F4/80/Gr-1 double positive cells upon 24 and 48 hours of LPS and thioglycollate administration to test for macrophage and neutrophil infiltration. Thioglycollate treatment of KO mice for 48 h showed significant enhancement of Gr1-CD11b and F4/80-Gr1 double-positive cell relative to control mice (Fig. 8A). In contrast, since LPS is a far more inflammatory agent than thioglycollate, LPS treatment for 24 h showed significant enhancement of the double positive cells in all three categories (Fig. 8B and 8C). No significant differences in the double positive cells in all three categories were observed with peritoneal cells harvested from saline injected animals (data not shown). Quantification of the results from four

different experiments with statistical analysis for saline, thioglycollate and LPS treatment have been shown in Fig. 8C.

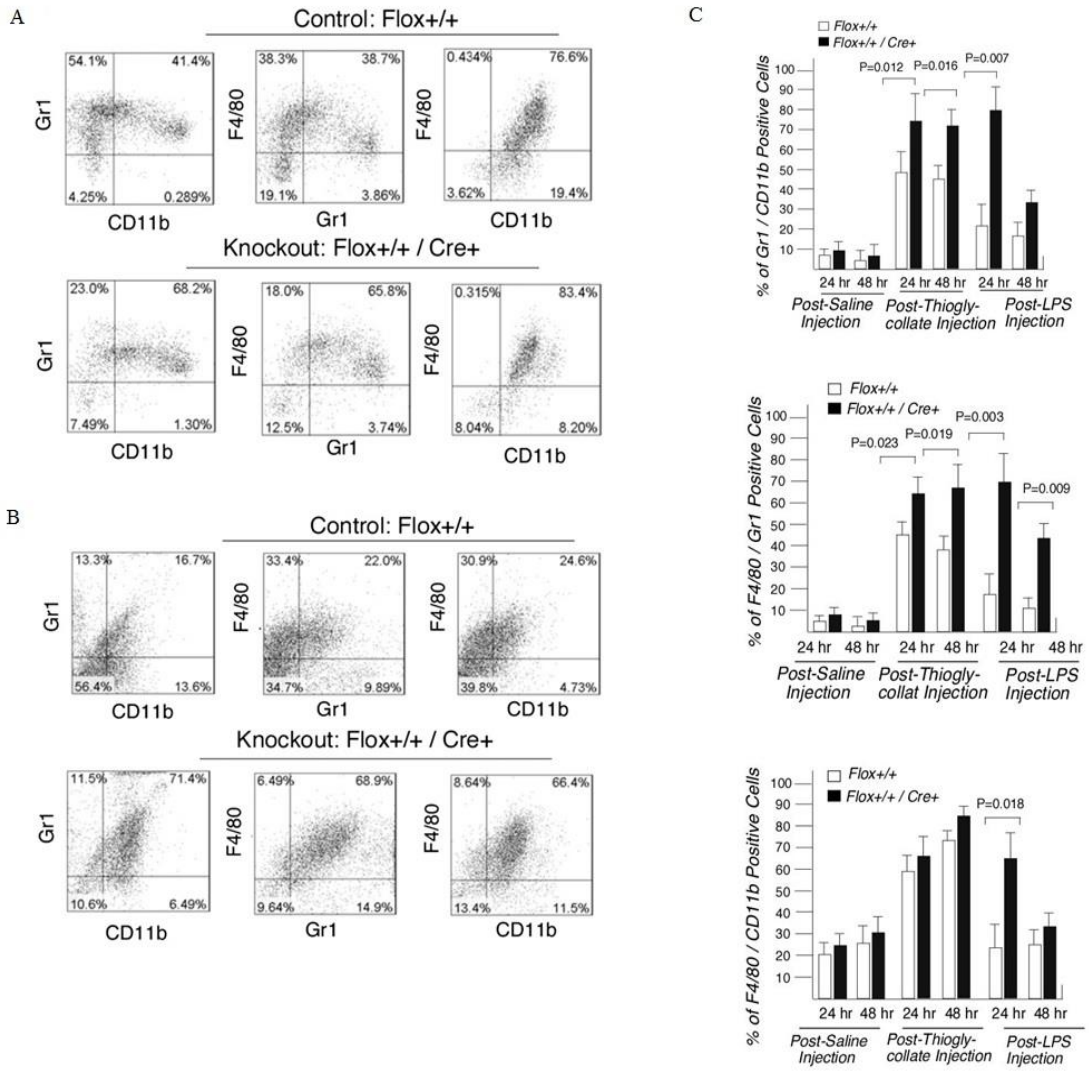

Figure 8. Endotoxin challenge causes increased infiltration of immune cells in the peritoneum. Control and KO mice were injected with thioglycollate for 48 hours and LPS (15 mg/kg) for 24 hours followed by isolation of their peritoneal lavage. Peritoneal cells were stained with F4/80, CD11b and Gr-1 antibodies and subjected to flow cytometry analysis. Increased infiltration of Gr-1/CD11b, F4/80/Gr-1 and F4/80/CD11b double positive cells were found in the peritoneum of the KO mice upon 48 hours of thioglycollate treatment (A) and 24 hours of LPS challenge (B). (C) Statistical analysis of the data obtained from 4 animals in each group for saline, thioglycollate and LPS at 24 and 48 hours post injection are shown. Results are mean ±

S.D., n=4, the corresponding P values are shown at the top of the bars, calculated using paired two-tailed Student's t-test.

Galectin-3, also known as Mac2 is a carbohydrate binding protein expressed on the surface of highly inflammatory monocytes and macrophages (Dong et al. 1997, Mosser et al. 2008). To assess the inflammatory macrophage infiltrates we determined the population of Mac2 positive cells in the peritoneum of LPS treated mice by FACS analysis. On treatment of our KO mice with LPS for 24 hours, we have found enhanced infiltration of F4/80/Mac2 and Gr-1/Mac2 double positive cells in the peritoneum, relative to the control animals (Fig. 9A and B). The quantitative representation of the results has been included in the right hand side sub-figure C. Negligible populations of the double positive cells in both controls and knockouts were detected upon saline treated conditions (data not shown).

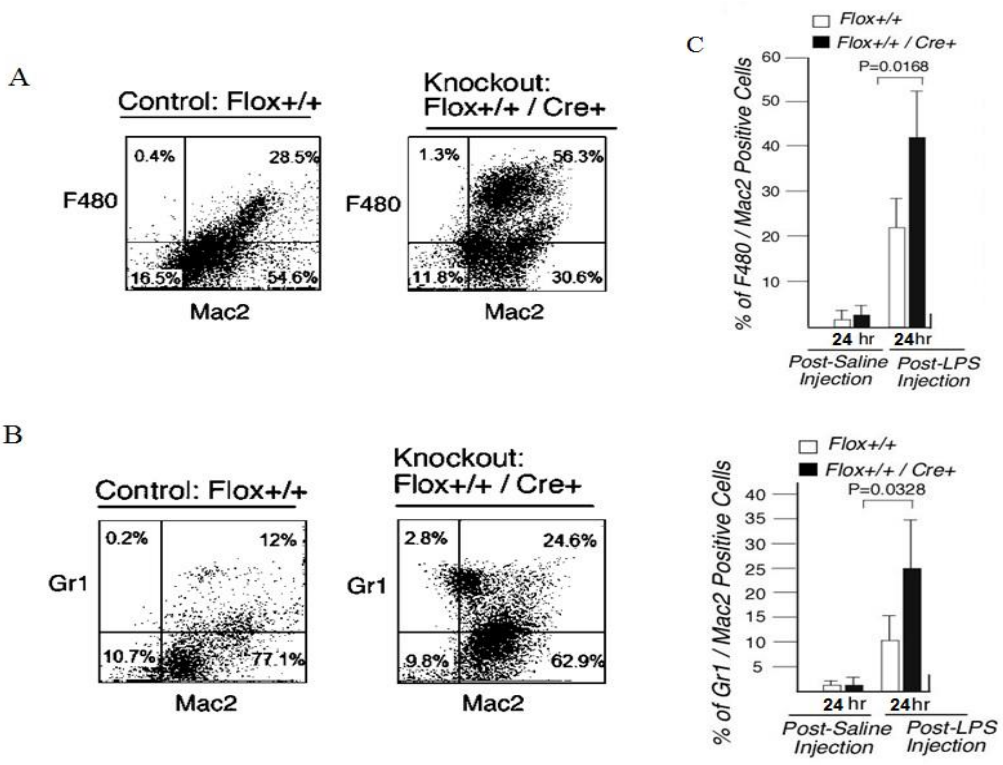

Figure 9. Increased infiltration of inflammatory macrophages in the peritoneum of L13a KO mice upon endotoxin challenge. Control and KO mice were injected with a sub-lethal dose of LPS (15mg/kg). 24 hours post injection peritoneal cells were harvested and stained with Mac2, Gr-1 and F4/80 antibodies. Higher population of F4/80/Mac2 (A) and Gr-1/Mac2 (B) double positive cells was found in the peritoneum of the KO animals 24 hours post LPS challenge. (C) Quantifications of the FACS analyzed data are shown on the right hand side panel. Results are mean ± SD, n=4, the corresponding P values are shown at the top of the bars and calculated using paired two-tailed Student's t-test.

Research done in other laboratories in this field has suggested that the spleen can serve as a reservoir for the leukocytes for their brisk recruitment to the sites of infection (Swirski et al. 2009). Therefore we measured the population of splenic leukocytes in response to 48 hours of LPS treatment. In this experiment, spleens were harvested from LPS challenged animals followed by RBC lysis of the cell suspensions to isolate total leukocytes. These cells were then stained with F4/80, CD11b and GR-1 antibodies and subjected to flow cytometry analysis. Consistent with our expectations, we found increased population of Gr-1/CD11b, F4/80/CD11b and F4/80/Gr-1 double positive cells in the KO mice in comparison to the control (Fig. 10A). Quantification of the data has been shown in fig. 10B. Taken together, these results show that the macrophage specific depletion of L13a causes higher infiltration of immune cells (macrophages and neutrophils) in the peritoneum and increased abundance of leukocytes in the spleen in response to inflammatory stimuli.

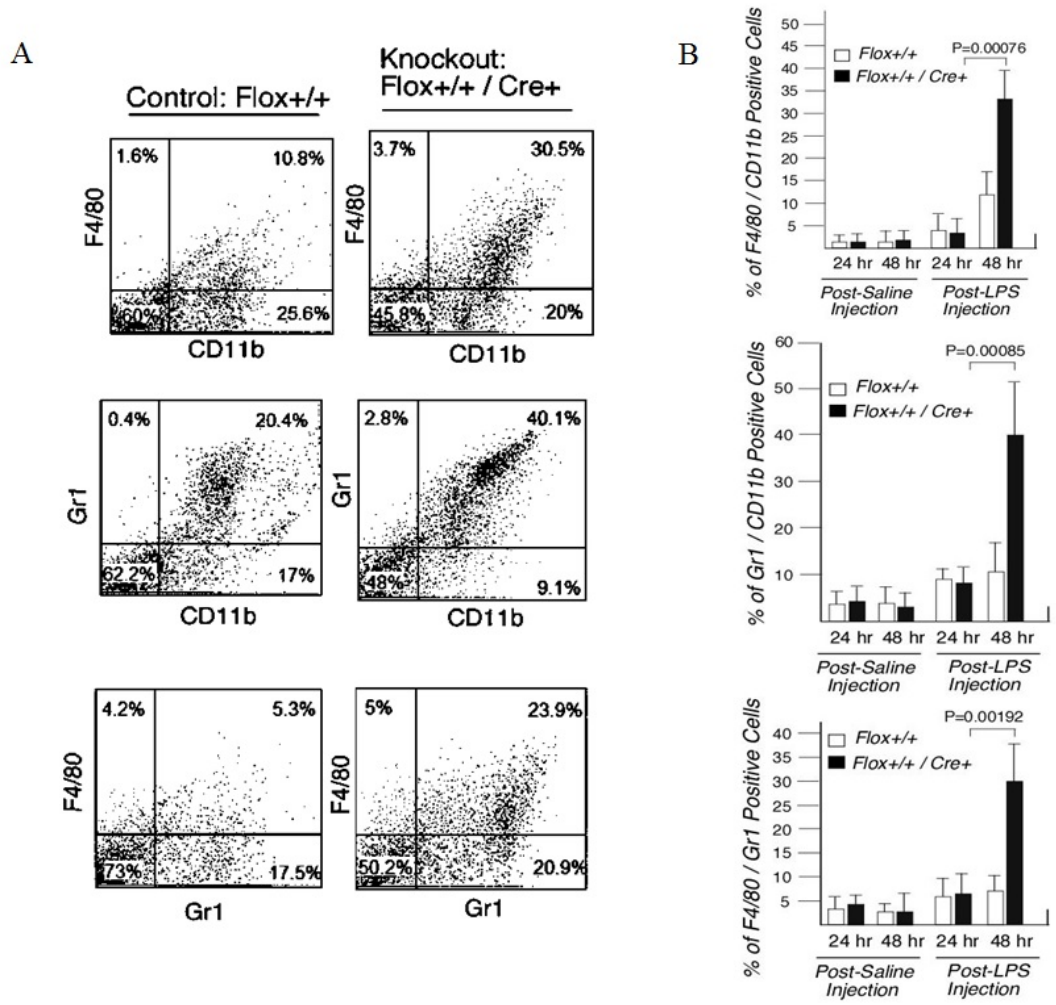

Figure 10. Higher abundance of splenic leukocytes in macrophage-specific L13a KO mice in response to LPS treatment. Control and KO mice were injected with LPS (15 mg/kg). After 48 hours, animals were euthanized and spleens harvested. Total leukocyte population was isolated from the splenocytes after RBC lysis. Cells were then stained with F4/80, CD11b and Gr-1 antibodies and subjected to flow cytometry analysis. Higher abundance of F4/80/CD11b, Gr-1/CD11b and F4/80/Gr-1 double positive cells were observed in the splenic leukocyte population of the experimental KO mice. (B) Quantifications of the FACS data. Results are mean ± S.D., n=4, the corresponding P values are shown at the top of the bars, P values calculated using paired two-tailed Student's t-test.

2.4.5. Increased abundance of Ly6G^{hi}/Ly6C^{hi} splenic leukocytes and Ly6G^{hi} circulatory monocytes in the KO mice due to absence of L13a dependent silencing mechanism.

Ly6G is a marker for granulocytes whereas Ly6C^{hi} is a marker for the inflammatory monocytes. Ly6C^{+} bone marrow macrophages are released into the peripheral blood from where they are directly recruited to the sites of inflammation in the tissues. Most of these inflammatory monocytes then differentiate into macrophages which is important for the clearance and resolution of inflammation (Gordon and Taylor 2005). We have assessed the Ly6C^{hi} monocyte population both in the splenic leukocytes as well as in circulation. The total leukocyte population was harvested from the spleen using same method as described previously. They were then stained with Ly6G and Ly6C antibodies and analyzed by flow cytometry. Peripheral blood monocytes were isolated from blood of LPS treated controls and knockouts using established protocols with Ficoll-Hypaque gradient centrifugation (Kanof et al. 2001). We have observed Ly6G^{hi}/Ly6C^{hi} double positive leukocytes populations in the spleen (Fig 11A) as well as Ly6C^{hi} peripheral blood monocytes in the circulation (Fig.11B) of the LPS treated KO mice to be considerably enhanced. Quantitative representations of the data are included on the right panel of the figure. Therefore, our results suggest that the macrophage specific L13a deficiency leads to increased recruitment of inflammatory neutrophils and monocytes into the tissues as well as in circulation to resolve excessive physiological inflammation in the KO animals.

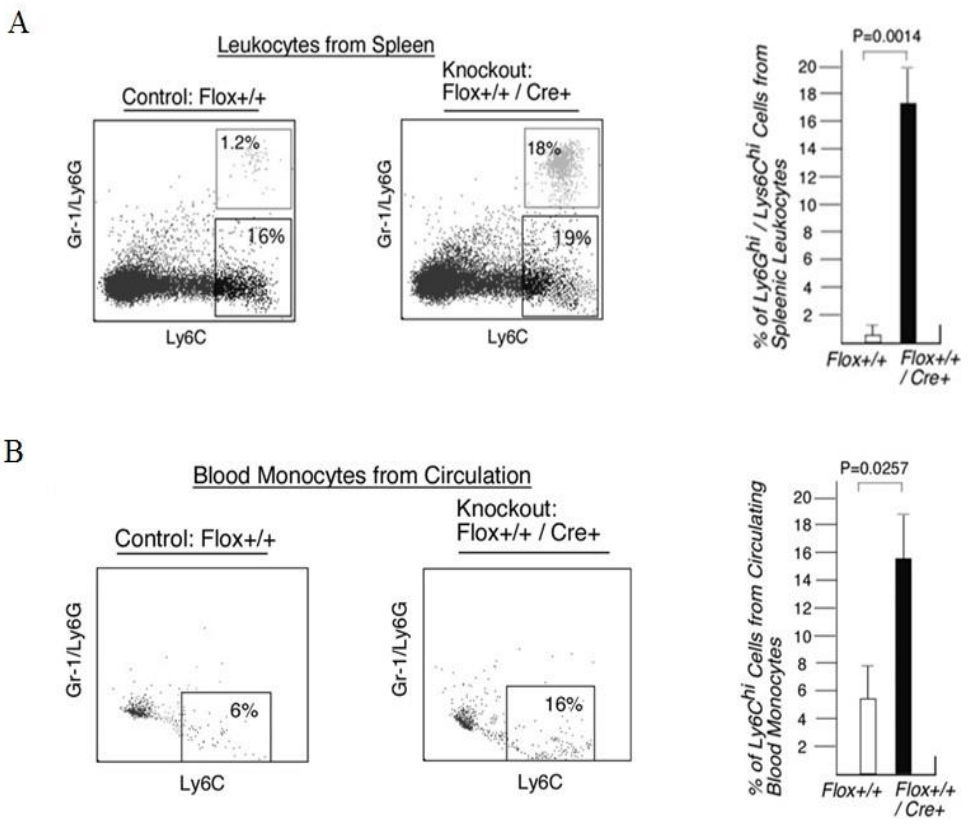

Figure 11. Increased abundance of Ly6G^{hi}/Ly6C^{hi} splenic leukocytes and Ly6G^{hi} circulatory monocytes in the KO mice upon endotoxin challenge. Mice were injected with LPS (15mg/kg) for 24 hours. (A) Total leukocyte population was isolated from the spleen after RBC lysis and the Ly6G^{hi}/Ly6C^{hi} percentage of double positive cells were determined by FACS analysis. (B) Increased abundance of Ly6C^{hi} inflammatory circulating monocytes was observed in circulating blood of the LPS challenged KO animals. Blood was collected by cardiac puncture followed by isolation of peripheral blood mono nuclear cells (PBMCs) by Ficoll-Hypaque gradient centrifugation (C) Upper and lower panels show the statistical representation of the data. Results are mean ± SD. P values have been calculated using paired two-tailed Student's t test.

2.4.6. L13a deficiency in myeloid lineage enhances endotoxemia induced tissue damage.

One of the essential features of endotoxic shock is severe tissue damage in the organs in the form of disseminated intravascular coagulation. These injuries are characterized by widespread blood coagulation and vessel hemorrhage, particularly in the kidney and lungs (Parillo 1993). Systemic inflammation caused by LPS often translates into the infiltration of immune cells in the tissues (Puneet et al. 2010, Raetz et al. 2002). Therefore, we investigated whether macrophage-specific depletion in L13a could cause greater tissue damage and immune cell infiltrates following LPS administration. Lungs and kidneys were harvested from 24 hours LPS injected control and knockout mice, fixed, paraffin embedded, sectioned and stained with H&E, Mac2 and Ym1. Mac2 and Ym1 are markers for macrophages. Histopathological analysis of the lungs by H&E staining revealed extensive RBC accumulation in bronchiolar and alveolar spaces in the KO mice. Whereas, the RBCs were mostly intravascular and confined to the alveolar walls in the control animals (Fig. 12A). Immunohistochemical staining of the lungs also showed significantly more $Mac2^{+}$ macrophages (Fig. 12B, upper panel) lining the blood vessels in the lung and adherent to vascular endothelium. Fig. 12B lower panel shows quantitation of the results. These data show greater invasion and adhesion of the macrophages to the vessel walls of KO mice lungs in response to endotoxemia which is a hallmark of severe inflammation. Analysis of the kidney sections showed enhanced infiltration of both $Mac2^{+}$ (Fig. 12C) and $Ym1^{+}$ macrophages (Fig. 12D) in the kidney glomeruli of LPS treated KOs. Statistical representation of the data is shown on the right of the figures (right panels). In summary, these results demonstrate that absence of L13a

in the macrophages leads to increased infiltration of these cells in the tissues which is consistent with the enhanced outcome of LPS induced endotoxic shock in the knockouts.

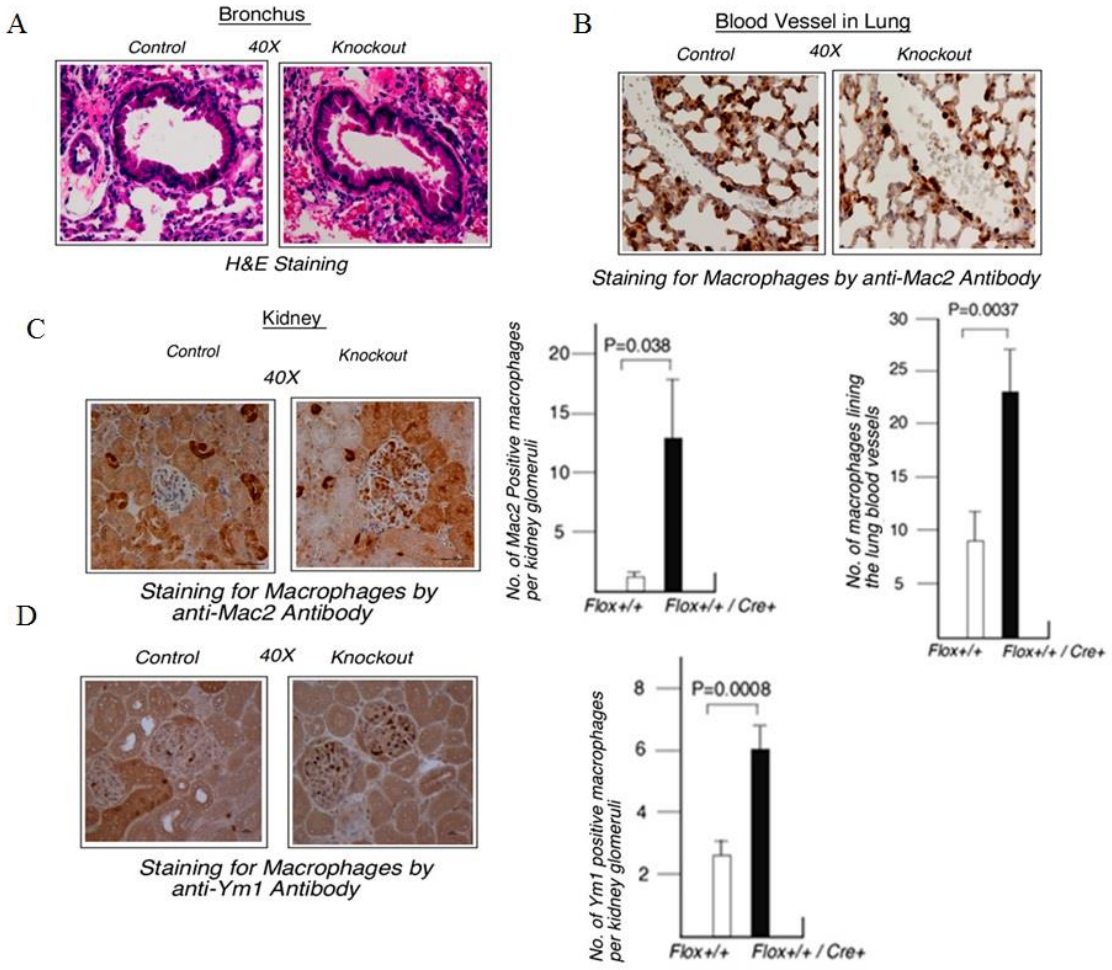

Figure 12. Infiltration of immune cells in the major organs of KO mice upon LPS challenge. (A) H&E staining of the bronchus and adjacent alveoli of lung. In the control mice RBCs are intravascular with more open alveolar spaces, whereas in the KO mice they have penetrated into the alveolar spaces. (B) Adhesion of macrophages to the blood vessels in the lung. Mac2 staining for macrophages shows more number of $Mac2^+$ macrophages lining the lung blood vessels of the L13a KO mice. Quantification of the data is shown on the lower panel. (n=5). (C) and (D). Infiltration of macrophages in the kidney glomeruli. Kidney sections were stained with anti-Mac2 and anti Ym1 antibodies. In both cases increased presence of $Mac2^+$ and $Ym1^+$ macrophages were found in the kidney glomeruli of the KO mice. All tissues were fixed in 10% neutral buffered formalin except for Ym1 where tissues were fixed in methanol (See Materials and Methods

section). Statistical representation of the results is shown on the right panels. A total of 70 kidney glomeruli from 6 controls and 6 knockouts were counted for quantitative representation of Mac2 and Ym1 staining results. All results are mean ± SD. P values were calculated using paired two-tailed Student's t test.

2.4.7. Disruption of the L13a mediated silencing pathway in the macrophages results in unregulated synthesis of the GAIT target proteins in vivo.

To delineate the underlying molecular mechanisms defining the uncontrolled inflammation in our L13a KO mouse models, we decided to investigate whether the expression of the target proteins of the L13a-mediated translational silencing mechanism was up-regulated upon LPS and thioglycollate treatment. For this purpose, animals were injected with thioglycollate for 72 hours after which peritoneal macrophages were harvested. Ex-vivo cultures of peritoneal macrophages were then subjected to 0, 8 or 24 hours of IFN-γ (500U/mL) treatment. The conditioned media from these cultures were subjected to ELISA to check the steady state levels of few GAIT target chemokines, CCL22, CXCL13 and CCL8. The levels of all three chemokines were found to have increased significantly at the 24 hour time point in the KOs as compared to the controls (Fig. 13A). On the contrary, no significant differences in the levels of proteins were observed after 8 hours of IFN-γ treatment (Fig. 13A). This can be explained on the basis of activation of the GAIT silencing complex after 24 hours of IFN-γ treatment which inhibits any further synthesis of the target chemokines in the control mice macrophages, but not in the knockouts. Bone marrow derived macrophages from control and KO mice were cultured ex-vivo with or without IFN-γ for 8 and 24 hours respectively and the culture supernatants were used to determine the levels of CCL22, CXCL13 and CCL8 by

ELISA. Same results were obtained from the bone marrow derived macrophages with significant increase in the level of the chemokines after 24 hours of IFN-γ treatment (Fig. 14). In our L13a KO mouse model, LPS is a potent inducer of IFN-γ and pro-inflammatory cytokines in cells of myeloid lineage (Le et al. 1986, Mattern et al. 1999, Dai et al. 2008). Therefore, we tested the capability of the macrophages harvested from LPS treated KO mice to build up a steady state level of the GAIT target proteins. Consistent with our previous results, we found higher levels of CCL22, CXCL13 and CCL8 in the conditioned medium of 24 hour ex-vivo culture (Fig. 13B). We then investigated whether the increase in the steady state levels of the chemokines were due to increased production of IFN-γ in our KO mouse system. Therefore we determined the levels of IFN-γ in the serum and the total leukocyte populations obtained from LPS treated mice. No significant difference was found between the controls and knockouts upon measuring the levels of IFN-γ produced in the serum and ex-vivo cultures of total leukocytes harvested at different time points (Fig. 13C). Taken together, these observations document that the depletion of L13a in the macrophages abrogates the silencing mechanism imposed on the inflammatory chemokines that leads to their greater steady state levels in the conditioned medium. This might contribute to enhanced physiological response to endotoxin challenge.

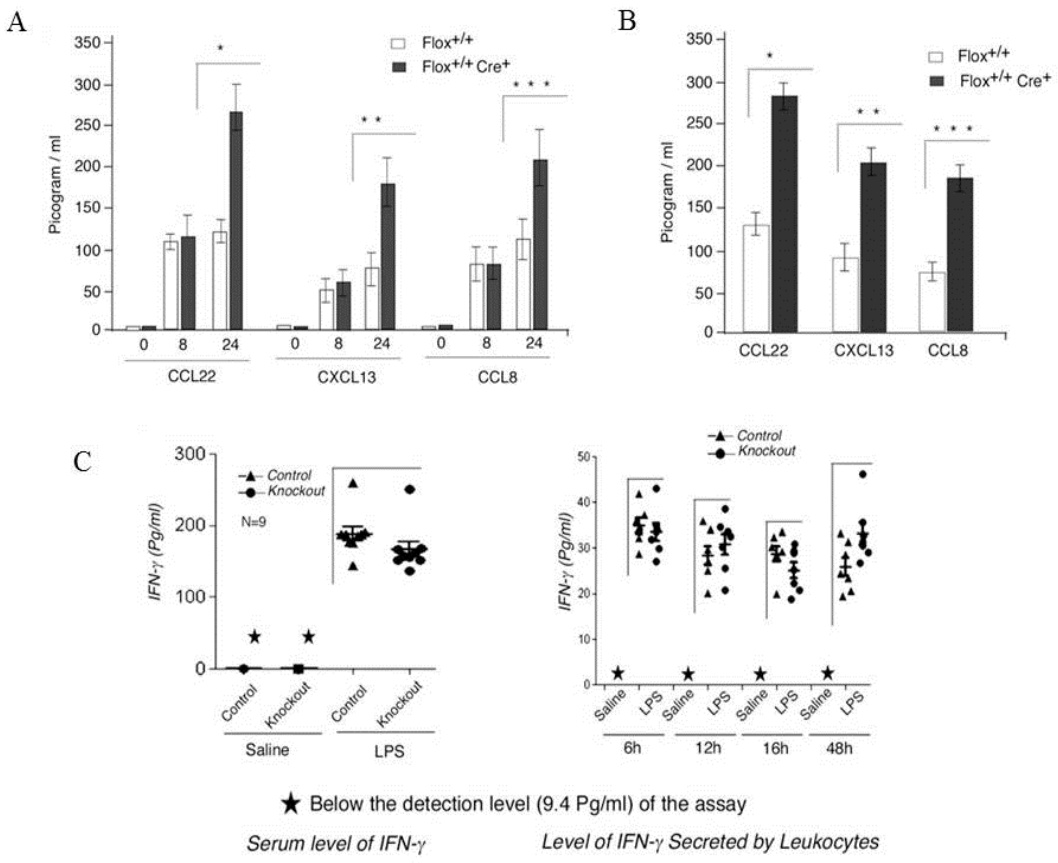

Figure 13. Depletion of L13a in the macrophages causes sustained synthesis of chemokine ligands CCL22, CXCL13 and CCL8. (A) Ex-vivo analysis of peritoneal macrophages from KO mice showed elevated synthesis of CCL22, CXCL13 and CCL8 upon 24 hours of IFN-γ treatment (500U/ml). No difference in the protein levels was seen at 8 hours of IFN-γ treatment. Results are mean ± SD. (n=5/group) *p = 2.5 X 10^{-5}, ** p = 5.2 X 10^{-6}, ***p = 1.6 X 10^{-6}, two-tailed Student t test. (B) Peritoneal macrophages harvested from LPS treated KO mice showed higher accumulated levels of the same chemokine ligands. Steady state levels have been measured by ELISA from culture supernatants. Results are mean ± SD. (n=5/group) *p = 9.8 X 10^{-6}, **p = 1.9 X 10^{-4}, ***p = 2.7 X 10^{-6}, two-tailed Student t test. (C) Ability to induce IFN-γ upon LPS administration is similar in both control and KO mice. Serum levels of IFN-γ upon LPS and saline injection (left panel). Control and KO mice were injected with saline or LPS (15mg/kg) and their serum levels of IFN-γ was measured by ELISA (n=9). No significant difference between controls and KOs were observed. Levels of IFN-γ in the total leukocytes (right panel). Control and KO mice were injected with saline or LPS (15mg/kg). 3 hours post injection, total leukocytes were isolated after RBC lysis and cultured for various times. IFN-γ was

measured by ELISA from culture supernatants. No significant difference was observed between controls and KOs. Results are mean ± SD. (n=5/group).

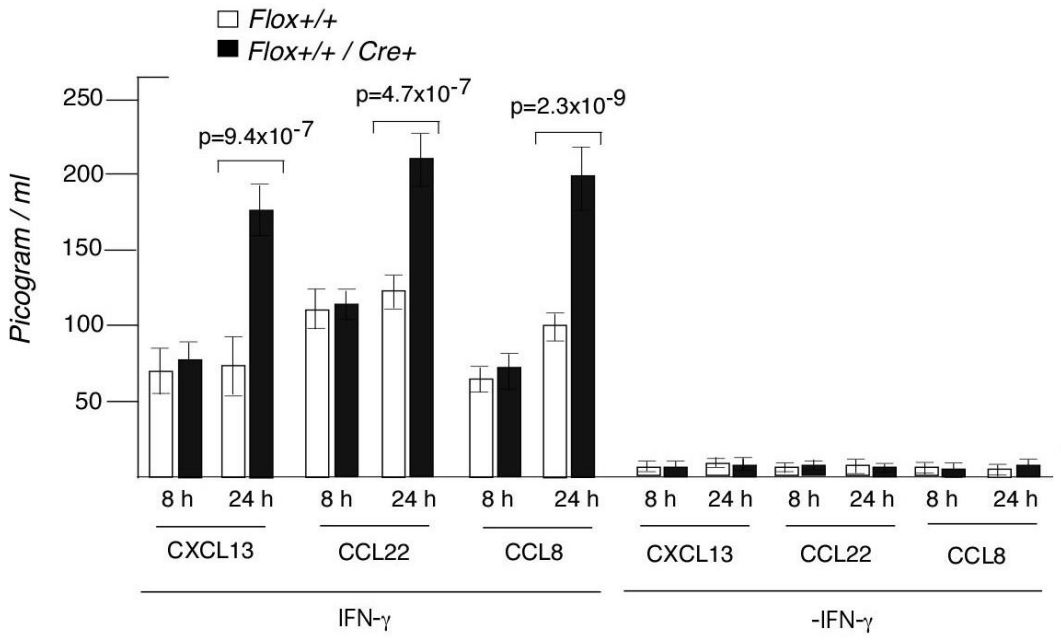

Figure 14. Ex vivo studies using bone marrow derived macrophages of the KO mice show high accumulation of chemokine ligands in response to 24 hr of IFN-γ treatment. Mouse bone marrow cells were differentiated into macrophages by culturing in presence of L929 cell conditioned media. The bone marrow derived macrophages were treated with IFN-γ (500 U/ml) for 8 and 24 hours. The chemokine ligands CXCL13, CCL22 and CCL8 were measured by ELISA from the conditioned medium after different periods of IFN-γ or vehicle treatment. Results are mean ± s.d, n=4, the corresponding P values were shown at the top of the bar, same P values were obtained using paired two-tailed Student's t-test and repeated measures 2 way ANOVA.

2.4.8. Deficiency of L13a in the macrophages abrogates the translational silencing of the GAIT target chemokine ligands and receptors.

As a direct mechanistic test of our hypothesis that depletion of L13a in the macrophages can abrogate the endogenously imposed translational silencing pathway and

cause increased production of the GAIT target proteins, we determined the polyribosomal abundance of some of the GAIT target mRNAs in the LPS administered control and KO mice. Testing the polyribosomal abundance is a widely accepted method to check the translation efficiencies of mRNAs (Vyas et al. 2009, Brown et al. 2001, Arava et al. 2003). Untranslated messages will co-sediment with the lighter free fractions whereas actively translated messages will be polyribosome bound and therefore appear with the heavy polyribosomal fractions. Lysates of peritoneal macrophages isolated from LPS challenged control and KO mice were subjected to a 5-50% sucrose gradient centrifugation to separate the translationally active pool of mRNAs from the translationally inactive one. RNA was isolated from each fraction followed by RT PCR with specific primers against CCL22, CXCL13 and CCR3, some of the GAIT target mRNAs. Results showed these mRNAs to be primarily associated with the lighter/free fractions in the control mice (Fig. 15A left panel), whereas there was a significant shift of these mRNAs to the heavier polysomal fractions in the KO mice as compared to the control actin mRNA (Fig. 15A right panel). Translational efficiencies of the target mRNAs were quantitatively expressed by the ratio of band intensities of the corresponding mRNAs and actin in polysomal and non-polysomal fractions (Fig. 15B). This demonstrates that the absence of the L13a mediated silencing pathway in the macrophages can elevate the translational status of pro-inflammatory chemokines whose synthesis is very low under normal basal conditions. Similar results were obtained with bone marrow derived macrophages treated with IFN-γ ex-vivo for 24 hours followed by polysomal profiling analysis (data not shown).

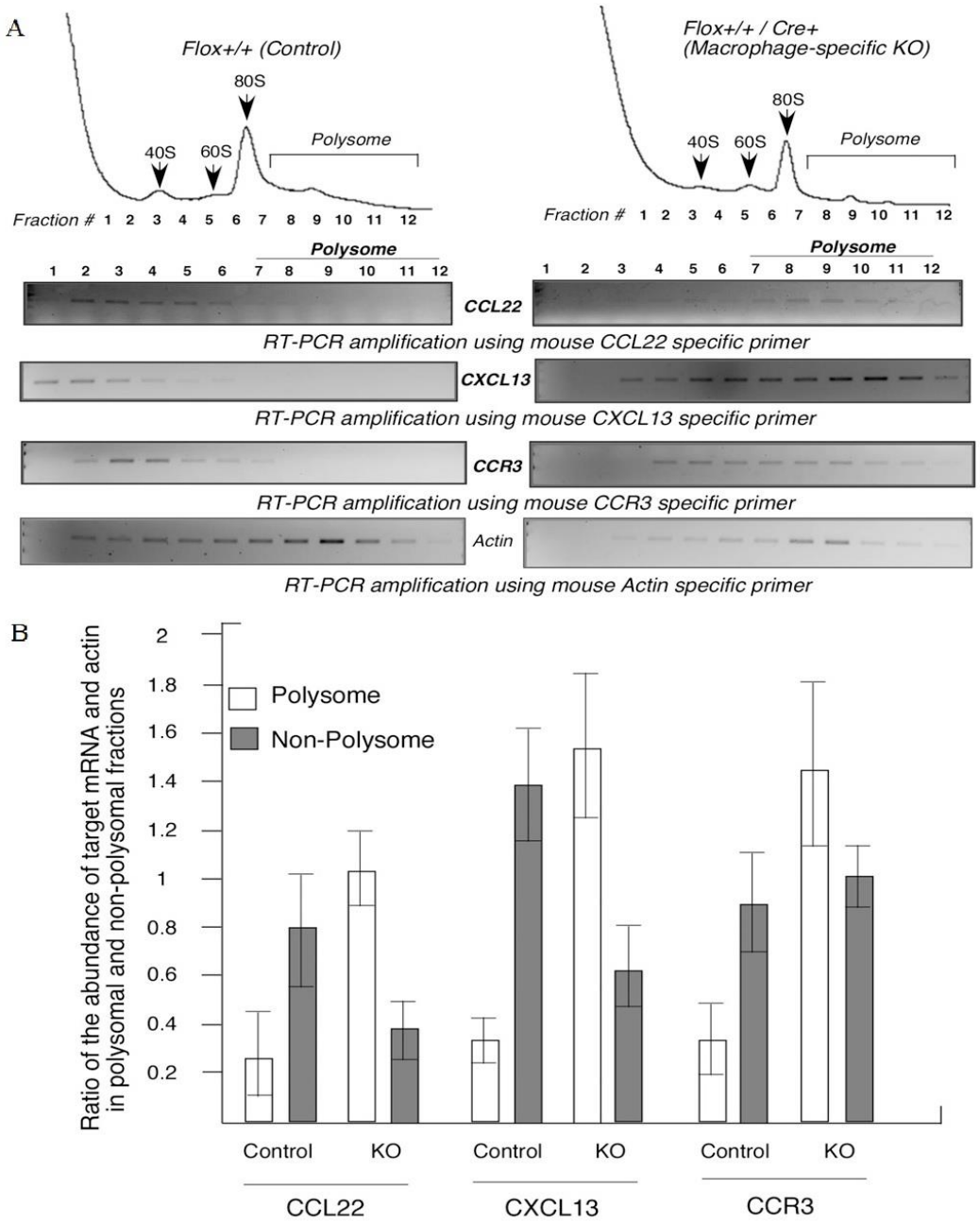

Figure 15. L13a is important for the regulation of expression of CCL22, CXCL13 and CCL8 mRNAs in animal model. (A) Increased polyribosomal abundance of the GAIT target proteins in the L13a KO mice. Peritoneal macrophages were harvested from control and KO mice 24 hours after LPS treatment (15mg/kg). Lysates were made in polysome lysis buffer (see Materials and Methods section of this chapter for buffer compositions) and layered on top of a 5-50% sucrose density gradient and subjected to ultracentrifugation for 18 hours at 17000 rpm at 4^0C. Total RNA was isolated from each fraction followed by RT-PCR using specific primers

against CCL22, CXCL13 CCL8 and actin. Significant shift to the heavier polysomal fractions and increased polysomal abundance in the KO mice was seen only for the GAIT target proteins and not actin. (B) Quantification of results from (A). Ratios of target mRNA versus actin in polysomal and non-polysomal fractions were measured by calculating intensities of the corresponding bands.

To directly test the abrogation of the L13a-dependent translational silencing pathway in the macrophages from the KO mice we reconstituted the translational silencing ex-vivo. For this we used a luciferase reporter construct RNA with the authentic GAIT element from the ceruloplasmin 3'-UTR and cloned in the 3' UTR of the luciferase reporter, so that its expression is under the regulation of the GAIT pathway mediated translational control. We have used the T7 gene 10 RNA without the GAIT element as the control for this reaction. Both of these RNAs were to in-vitro-translation reaction using cell-free rabbit reticulocyte lysates. Macrophage lysates from saline and LPS treated control and knockout mice were used to test the translational silencing activity in mouse macrophages. U937 cell lysates treated with IFN-γ for 8 hours and 24 hours served as experimental controls. Results from the ex-vivo study showed that treatment only with LPS (and not saline) can induce the translational silencing activity in the WT mouse macrophages. However, this silencing activity was found to be absent in the KO mice macrophages even upon LPS treatment. This result could be due to the absence of L13a in the macrophages which might abrogate the formation of the functional GAIT complex (Fig. 16). Translational silencing of the luciferase reporter construct was observed when lysates from 24 hours of IFN-γ treated U937 cells was used. Together, this study indicates that macrophage-specific deficiency of L13a can disrupt the endogenously imposed translational silencing mechanism in-vivo.

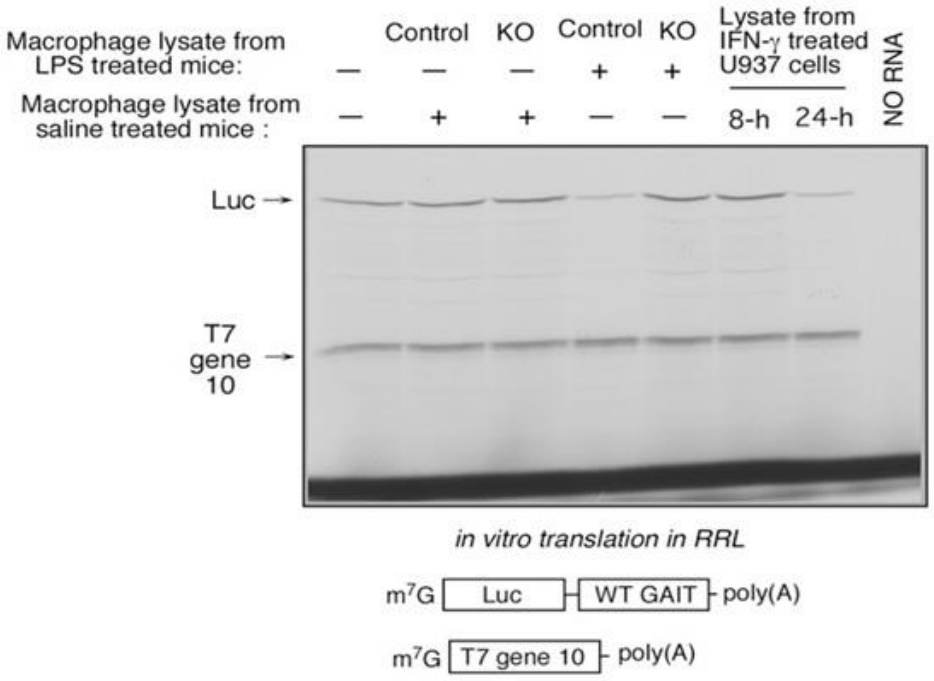

Figure 16. LPS treatment activates the GAIT mediated translational silencing mechanism in animals. Design of the GAIT element containing reporter luciferase RNA and the control T7 gene 10 RNA are shown in the bottom panel. An authentic GAIT element from the ceruloplasmin 3'-UTR was cloned in the 3'UTR of the luciferase reporter gene. These RNAs were in-vitro translated in rabbit reticulocyte lysates in the presence or absence of lysates prepared from macrophages harvested from saline or LPS injected control or KO mice. Lysates from IFN-γ treated U937 cells for 8 and 24 hours served as positive controls. The translation reaction mixture was subjected to SDS-PAGE followed by autoradiography to see the translation products. Translation silencing of the Luciferase gene was seen only in the LPS injected control mouse macrophages and not in the KO mice macrophages in-spite of LPS treatment.

The ability of the L13a-containing GAIT complex to silence translation of its target mRNAs relies on its binding to functional GAIT elements in the 3'-UTR (Mazumder et al. 2003, Vyas et al. 2009). Therefore we investigated the in-vivo interaction of these target mRNAs with L13a. The chemokines are present at very low undetectable basal

levels under normal un-induced conditions, but LPS being a potent in-vivo inducer of IFN-γ can trigger the synthesis of several of the GAIT target chemokines. We have tested the induction by LPS using peritoneal macrophages from LPS and saline treated WT mice. RT-PCR analysis of total RNA isolated from the macrophages of LPS and saline treated mice showed the presence of CCL22 and CCR3 mRNAs only upon LPS treatment (Fig. 17A). To further test whether L13a indeed associates with these mRNAs in vivo, macrophages were isolated from LPS treated mice and then subjected to immunoprecipitation with anti-L13a and control anti-L28 antibody. L28 is another protein of the large 60S ribosomal subunit and served as a control in this experiment. RNA was extracted from the immunoprecipitates and subjected to RT-PCR with specific primers against CCL22 and CCR3. Both the GAIT target mRNAs were only detected in the immunoprecipitates with anti-L13a antibody and not with the anti-L28 immunoprecipitates (Fig. 17B), thereby confirming that L13a actually binds to the GAIT target mRNAs in-vivo. Our previous studies using cell culture models have shown the regulated release of L13a upon IFN-γ treatment (Mazumder et al 2003). Since LPS is a potent inducer of IFN-γ in-vivo, we determined the association of L13a with the 60S large ribosomal subunit in the peritoneal macrophages isolated from LPS treated mice. Lysates of macrophages harvested from saline and LPS treated wild type mice were immunoprecipitated with anti-L13a antibody and RNA isolated from the immunoprecipitates were subjected to RT-PCR with 28S rRNA specific primers, because 28S is the building block for 60S ribosomal subunit assembly. The results revealed less association of L13a with the 60S subunit in the macrophages isolated from LPS treated mice as compared to the saline treated mice (Fig. 17C). Taken together, these results

demonstrate the in-vivo operation of the L13a-mediated GAIT silencing pathway and that macrophage specific deficiency of L13a abrogates the naturally imposed silencing mechanism on the GAIT target mRNAs. This highlights the physiological importance of this mechanism as an endogenous defense against runaway inflammation.

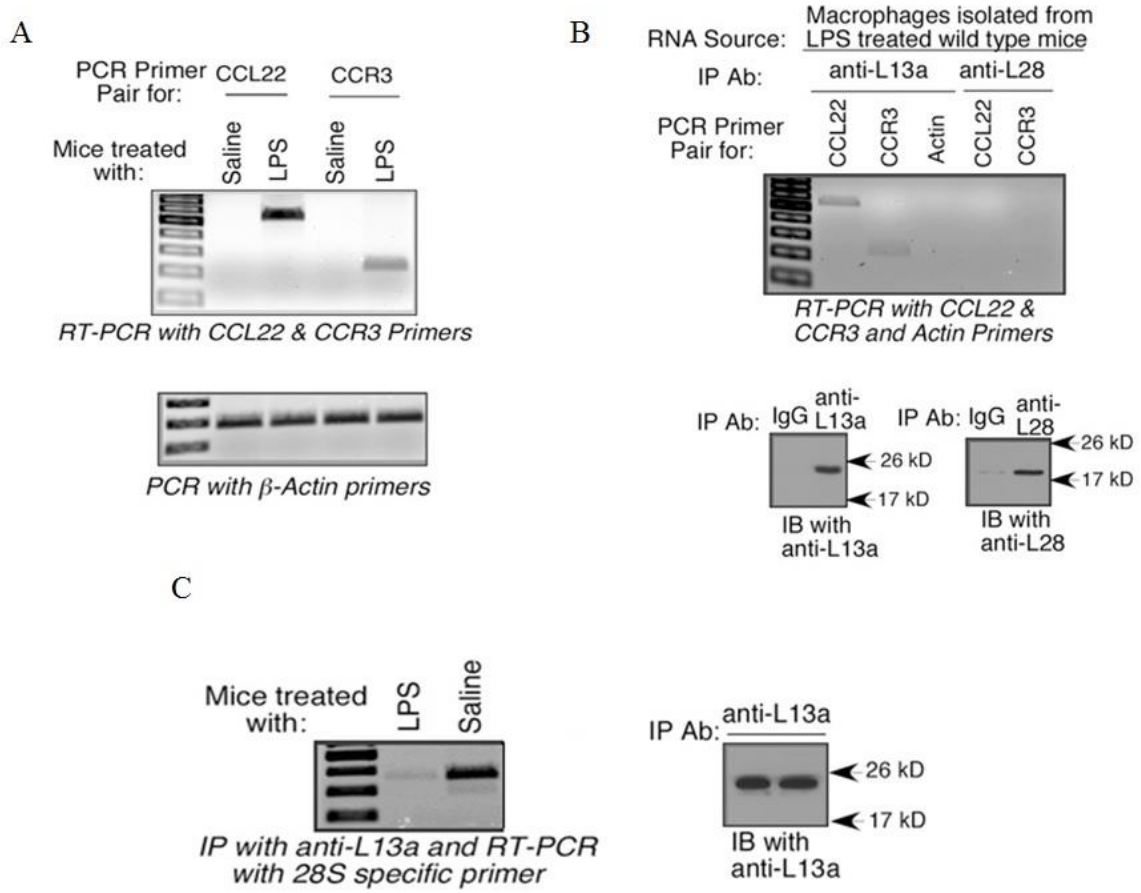

Figure. 17. in-vivo association of L13a with target mRNAs and release of L13a from the large ribosomal subunit. (A) RT-PCR of the total RNA isolated from macrophages of LPS-treated mice shows induction of CCL22 and CCR3 mRNAs. Macrophages were harvested from saline or LPS treated mice, total RNA was isolated from them and then subjected to RT-PCR with specific primers against CCL22 and CCR3. The lower panel shows RT-PCR results with control actin specific primers (B) Lysates were prepared from macrophages harvested from LPS injected wild type mice. These lysates were then subjected to immunoprecipitation with anti-L13a

and control anti-L28 antibodies. RNA was extracted from the immunoprecipitates followed by RT-PCR with specific primers against CCL22 and CCR3 (upper panel). The efficiency of immunoprecipitation was confirmed by immunoblot analysis (lower panel) with the respective antibodies. (C) Reduced association of L13a with the 60S large ribosomal subunit in macrophages harvested from LPS treated mice. Macrophages harvested from LPS or saline treated mice were subjected to immunoprecipitation using anti-L13a antibody. An aliquot of the immunoprecipitates were then subjected to RT-PCR with mouse 28S rRNA-specific primer left panel). The other aliquot was subjected to immunoblot analysis with anti-L13a antibody (right panel).

2.5. DISCUSSION

The elementary discovery of this study is that extra-ribosomal L13a possesses significant anti-inflammatory role and thus can act as a physiological attenuator of endotoxin-triggered inflammation. In this study we have used a novel animal model, the macrophage-specific knockout mice of L13a, to identify the defensive role played by ribosomal protein L13a-mediated GAIT translational silencing pathway in resolving uncontrolled inflammation induced by endotoxic shock. Upon targeted abrogation of the translational silencing pathway by depletion of L13a in the macrophages, the mice displayed severe symptoms of inflammation in response to endotoxin challenge. Increased susceptibility was associated with higher mortality rates, reduced activity and widespread tissue damage in the LPS treated knockouts. The knockouts also showed reduced motor activity, clinical symptoms of inflammation with high levels of several markers of inflammation like BUN, AST and TNFα and other cytokines in the serum. Additionally, infiltration of macrophages in the lungs and kidneys together with intra-vascular congestion in the lungs was observed in the knockouts upon LPS administration. The exaggerated inflammatory response seen upon LPS treatment could be attributed to the overproduction of some of the GAIT target chemokines (e.g., CCL22, CXCL13, CCR3 and CCL8) in the macrophages harvested from the LPS administered knockout mice. This is in agreement with our previous observations from in-vitro studies which suggested that disruption of the L13a mediated GAIT silencing pathway can cause sustained synthesis of a group of pro-inflammatory proteins by removing the endogenously imposed regulation. Most fascinatingly, our study also indicates that

treatment with LPS can induce the L13a mediated translational silencing pathway and diminish the association of L13a with the 60S ribosomal subunit in the macrophages of the wild type control animals. In summary, these observations demonstrate that macrophage specific deficiency of L13a can abrogate the naturally imposed translational silencing of the GAIT target mRNAs. Thus, our studies have identified the novel moonlighting function of L13a as an endogenous defense mechanism to resolve runaway inflammatory response caused by endotoxin challenge.

Past research from our laboratory using in-vitro cell culture models of monocytes had revealed the capability of ribosomal protein L13a mediated translational silencing pathway to regulate the expression of a cohort of mRNAs encoding inflammatory proteins predominantly chemokines and chemokine receptors. Using RNA folding programs, it was also found that specific segments in the 3'UTRs of the target mRNAs contained stem loop like structures, conformationally and functionally similar to the classical GAIT element found in the 3'-UTR of ceruloplasmin mRNA. Subsequent experiments also showed their role in translational silencing driven by the L13a containing RNA binding complex (Vyas et al. 2009). These observations are in agreement with the concept that folding identities between elements present in different transcripts can lead to translational regulation of multiple transcripts by a single RNA binding complex, thereby supporting the idea of a post-transcriptional operon in the eukaryotic system (Morris et al. 2010, Keene 2007). Our experiments using polysomal analysis of macrophages isolated from LPS treated control and KO mice showed higher polysomal abundance of a group of mRNAs synthesizing pro-inflammatory chemokines. To our knowledge this is the first study reporting the importance of a ribosomal-protein

dependent translational silencing pathway in regulating physiological inflammation. For the scope of this study, we have just tested the translational regulation on a few target mRNAs such as CCL22, CXCL13, CCL8 and CCR3, though several other targets identified from cell culture studies (Vyas et al. 2009) are yet to be tested in our KO mice model.

Post-transcriptional mechanisms have evolved which regulate the synthesis of inflammatory proteins at the level of their mRNAs to control inflammation such as activation and chemo-attraction of T lymphocytes, infiltration of neutrophils, natural killer cells negotiated cellular toxicity, adhesion of monocytes and macrophages and their survival in damaged tissues to initiate repair (Mazumder et al. 2010, Anderson 2010). The pathophysiological importance of these regulatory mechanisms have been investigated using genetically modified mouse models having impairments in the AU-rich sequence of TNF-α 3'UTR (Kontoyiannis et al. 1999) and RNA binding proteins like TTP (Taylor et al. 1996), TIA-1 (Piecyk et al. 2000) and AUF-1 (Lu et al. 2006). All of these studies revealed higher inflammatory responses due to overexpression of TNF-α and associated diseased pathology caused by the altered stability of the TNF-α mRNA even under unstimulated conditions. However, our macrophage-specific KO mice of L13a did not show any developmental defects or significant differences from the controls under unchallenged conditions. Disease pathology was observed in the KOs only upon LPS administration. It is important to mention here that LPS being an in-vivo inducer of IFN-γ also activates the L13a dependent translational silencing pathway in mice which in turn targets LPS-induced messenger RNAs encoding for inflammatory proteins. These results are consistent with the concept of termination of inflammation by a negative

feedback response (Nathan and Ding 2010) and distinguishes the L13a dependent translational silencing from other post-transcriptional mechanisms to regulate inflammation.

Chemokines and their receptors play a critical function in chemoattracting and recruiting mononuclear cells to the sites of inflammation in the tissues. This is a fundamental step in innate immune responses as it aids in tissue repair and clearing out of the pathogens. But like all biological processes, immune response needs to be controlled at a certain checkpoint otherwise excessive response might be detrimental for the host. Our studies show that the deficiency of L13a mediated translational silencing results in unregulated and overproduction of many chemokines such as CCL22, CXCL13, CCL8 and CCR3. All of these chemokines have important functions in various aspects of inflammation (e.g., regulating the networking of activated T cells (Chang et al. 1997) and regulatory T cells (Riezu-Boj et al. 2011) to sites of inflammation by CCL22, the role of CXCL13 in B cell homing (Gunn et al. 1998) and its connection with early onset of lupus erythematosus (Proost et al. 1996), activation of a large group of immune cells by CCL8 (Dajani et al.), advancement of LPS induced lung inflammation (Li et al. 2011) and ulcerative colitis (Manousou et al. 2010) by CCR3. Our results showing elevated levels of these chemokines in response to endotoxemia are in accordance with the above-mentioned previous studies in literature. Another important facet of our study is the increased abundance of both Mac2+ and Ym1+ macrophages in the kidney glomeruli of the LPS treated knockouts. Classically activated macrophages express Mac2 whereas Ym1 expressing macrophages are the alternately activated ones which have prime roles in wound healing and repair. Therefore connecting our results with the available

knowledge we can say that a fibrotic response is started at the beginning of acute inflammation.

In humans, haploinsufficiency of many ribosomal proteins like S19, S17, S7, S15, L35A, L11 and L36 can lead to serious defects resulting in genetic diseases like Diamond-Blackfan anemia (Narla and Ebert 2010). In mice, deficiency of L38 leads to impairment in tissue patterning (Kondrashov et al. 2011). For many other ribosomal proteins, their deficiencies result in anomalies in ribosome biogenesis (Narla and Ebert 2010). On the contrary, our previous in-vitro studies using human U937 monocytic cells had shown that depletion of RPL13a does not affect ribosome biogenesis and overall protein synthesis activity of the ribosome (Chaudhuri et al. 2007). However, according to our knowledge, the LPS-induced endotoxemia study provides the first evidence of L13a dependent translational silencing mechanism to simultaneously control the expression of several inflammatory genes, thereby resolving physiological inflammation caused by endotoxin challenge. We hypothesize that any abnormality in this pathway may promote the development of several other inflammatory diseases and targeting this pathway by small molecules can provide effective means of designing novel therapeutic strategies.

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TABLE I: LIST OF PRIMERS FOR PCR

PRIMER NAME	PRIMER SEQUENCE
Lox1 (F)	5’ AGGTTCTGCTTGAGCATCTGAG 3’
SDL2 (R)	5’ CCGTCAGGATGCCTACTACCCAG 3’
NDEL3 (F)	5’ GGGTGAATCCTTGCTCACGGGAT 3’
Anti AT2 (R)	5’ GCTCTTGTATCTTGGTGCTGACTT 3’
Mouse 28S (X00525)	
Forward	5’ GTTGTTGCCATGGTAATCCTGCTCA 3’
Reverse	5’ CAGAAGCAGGTCGTCTACGAATGGT 3’
Mouse-Actin (NM_007393)	
Forward	5’ GTCCCTCACCCTCCCAAAAGC 3’
Reverse	5’ AGGTAAGGTGTGCACTTTTAT 3’
Mouse CCL22 (NM_009137)	
Forward	5’ TTCTTGCTGTGGCAATTCAGACCT 3’
Reverse	5’ CAGGTCCTCCTCCCTAGGACAGTT 3’
Mouse CXCL13 (NM_018866)	
Forward	5’ CTTGTAAAACGCAGGCTTCCACA 3’
Reverse	5’ GGGTCACAGTGCAAAGGAATATA 3’
Mouse CCR3 (NM_009914)	
Forward	5’ TTCTACCGGCCCTCACATAC 3’
Reverse	5’ ATCCAGAGAGCACCTCCTGA 3’

CHAPTER III

EXTRA-RIBOSOMAL L13A PLAYS A CRITICAL ROLE IN PREVENTING DSS-INDUCED COLITIS

3.1. ABSTRACT

Inflammatory bowel disease (IBD) is a chronic inflammatory disease affecting the gastro-intestinal mucosa. The exact etiology of IBD is yet to be studied in detail, but both genetic and environmental factors (commensal microflora, diet etc.) have been shown to contribute significantly to the pathogenesis of IBD. The intestinal microbiota and inflammatory cell infiltrates play a predominant role in the pathogenesis of acute colitis. Recently studies on L13a-mediated GAIT (Gamma–interferon Activated Inhibitor of Translation) silencing pathway has been implicated as the endogenous defense mechanism against several inflammatory diseases like endotoxemia and atherosclerosis. Further dissection into the molecular pathways has revealed that the presence of L13a in macrophages critical for resolution of inflammation. The goal of our study is to test the role of L13a-mediated silencing pathway as a physiological defense in dextran sodium

sulfate (DSS)-induced experimental colitis progression using murine model of macrophage-specific L13a knockout. The study was initiated by introducing acute colitis through administration of DSS in drinking water of the animals. Their activities and disease parameters were monitored and recorded during the course of the study and then compared to those of similarly treated controls to evaluate the protective role played by L13a in the macrophages. Upon DSS treatment, the macrophage-specific L13a KO mice showed more susceptibility to colitis displaying high mortality rates, drastic weight loss, enhanced rectal bleeding, diarrhea and shortening of colons. Histopathology analysis of tissue sections from the KO animals has shown erosion of epithelial crypts in the colon with very high infiltration of macrophages in both the colon and spleen sections. Additionally, elevated levels of several chemokines and cytokines were found to be associated with the serum (e.g., MDC, TARC, eotaxin and IL-6) and colon culture supernatants (IL-1β, IL-6, GM-CSF, eotaxin, KC, MIP-1α and RANTES) of the DSS administered knockouts, some of which are the previously validated targets of the L13a-dependent translational silencing pathway. In consistence with this result we have also found increased polysomal abundance of several GAIT target mRNAs in the colons of DSS fed knockouts suggesting a defect in translation control of those inflammatory genes in the colons upon depletion of L13a from the macrophages. These findings suggest that L13a mediated translational silencing pathway in macrophages contributes significantly to protect against inflammation and tissue damage in acute colitis.

3.2. INTRODUCTION

Immune-related disorders including inflammatory bowel diseases (IBD), cancer, arthritis, etc. have increased in occurrence over the last 50 years. IBD are chronic inflammatory disorders affecting the gastrointestinal tract along with the colon and small intestine and are marked by chapters of relapse and remission (Ponder and Long, 2013). There are two major forms of IBD: Crohn's Disease (CD) and Ulcerative Colitis (UC). The pathological features of UC are primarily localized to the colonic mucosa with localized inflammation and immune cell infiltration whereas CD affects the entire gastrointestinal tract involving inflammatory lesions and ulcerations (Coskun et al., 2013). The clinical symptoms of IBD include diarrhea, rectal bleeding, weight loss and anemia resulting from intestinal inflammation. Over the last few decades the incidence and prevalence of IBD has been increasing across both pediatric and adult populations. Presently, the annual occurrence of CD is highest in the United States (20.2 per 100,000, per person years); whereas the annual occurrence of UC is highest in Europe (24.3 per 100,000 per person years). However, the predominance of both UC and CD are highest in Europe (505 and 322, per 100,000 per person years respectively) and much lower elsewhere worldwide (Ponder and Long, 2013).

The human oral-gastrointestinal tract is colonized by trillions of microorganisms, predominantly bacteria and most of these microorganisms reside in the distal intestine (Kamada et al., 2013). The variety of bacterial species can vary between individuals and largely depends on a variety of factors (Gill et al., 2006, Honda and Littman, 2012, Hayashi et al., 2013). Commensal bacteria constitute the major microbiota environment

in the gut and contribute to the host physiology in many ways like digestion fermentation of carbohydrates, nutrient acquisition and protection against invading pathogens (Kamada et al., 2013, Li et al., 2011). In this mutually beneficial relationship the host supplies nutrients and niches for the gut microbiota. The host immune responses generated by the commensal populations helps in determining the distribution and architecture of the gut microbiota. Thus, a complex interplay between the host immune system and the commensal bacteria maintains the intestinal homeostasis and the integrity of the mucosal barrier. Any abnormal interaction between the intestinal mucosal system and the luminal microbiota may disrupt this barrier and elicit a pro-inflammatory immune response and cause sustained inflammation leading to IBD. The exact cause of IBD is unknown but it involves an intricate communication among genetic and environmental factors that generates an improper mucosal immune response (Coskun et al., 2013, Kaser et al., 2010, Sartor, 2006). Genetic linkage mapping and genome wide association studies have revealed several risk granting loci with considerable overlap in genetic risk factors between CD and UC (Gregersen and Olsson, 2009, Van Limbergen et al., 2009, Cho, 2008, Zhernakova et al., 2009). The intestinal microbiota plays a critical role in determining the host immune composition under normal physiological conditions and thus acts as an essential environmental factor in IBD as the target for inflammatory response (Kaser et al., 2010).

The dextran sodium sulfate (DSS) model has been widely used to induce experimental colitis in mice, which mimics IBD and then study the contribution of the innate immune mechanisms in the pathogenesis of colitis (Wirtz et al., 2007). Feeding mice for several days with DSS supplemented in drinking water induces acute colitis. DSS-induced colitis

causes a robust inflammatory response characterized by weight loss, liquid diarrhea, high death rates and marked loss of goblet cells leading to severe epithelial injury. This injury is characterized by increased permeability of the epithelial cells, disruption of crypt morphology and infiltration of immune cells in the colons (Wirtz et al., 2007, Ekstrom, 1998). The inflated immune response is generated principally by the invasion of the lamina propria with innate immune cells (e.g., macrophages, neutrophils, dendritic cells, natural-killer T cells etc.) which causes an overproduction of pro-inflammatory cytokines and thereby promoting an inflammatory cascade leading to damage of the intestinal epithelial cell barrier (Coskun et al., 2013). Macrophages and monocytes are key players of immune response and have pathogenic and protective roles against various inflammatory assaults (Murray and Wynn, 2011). Macrophages are one of the most abundant leukocytes in the intestines of all mammalian species. Mucosal macrophage numbers in different parts of the intestines appear to correlate to the relative microflora population (Mowat and Bain, 2011). The intestine is continuously exposed to numerous antigens and some of them might be potentially harmful. As principle effector cells of the immune system, macrophages serve a protective role against these agents. Such protective phagocytic activity can also be directed toward normally harmless antigens which can potentially disrupt the balance between the intestinal commensal bacteria and the luminal microbiota thereby leading to IBD (Mowat and Bain, 2011). Thus, macrophages can be central to the pathogenesis of such diseases.

Previous studies from our laboratory have shown an indispensable role of ribosomal protein L13a in the translational silencing of a cohort of mRNAs encoding inflammatory proteins in macrophages. Majority of these target proteins are chemokines and chemokine

receptors (e.g., CCL1, CCL3, CCL8, CCL11, CCL21, CXCL13, CCL22, CCL25, CCL27, CCL28, CCR2, CCR3, CCR4, CCR5, CCR6 and CCR7) (Vyas et al., 2009). This led us to hypothesize that this L13a-dependent translational silencing could function as an endogenous defense mechanism against diseases caused by uncontrolled inflammatory response in macrophages. Results from our laboratory also revealed significant molecular detail about this translational control mechanism. Our work has identified a multi-subunit RNA binding complex, Interferon-Gamma Activated Inhibitor of Translation (GAIT) (Sampath et al., 2004) that binds to the 29 nucleotide long GAIT element present in the 3' untranslated region (UTR) of the target mRNA (Mazumder et al., 2003a) and silences translation by preventing the formation of 48S initiation complex (Kapasi et al., 2007). IFN-γ mediated release of phosphorylated L13a from the 60S large ribosomal subunit has been identified as a critical step for the assembly of the GAIT complex (Sampath et al., 2003). Together these studies show that L13a dependent translational silencing is an extra-ribosomal function of this ribosomal protein. Using the newly generated macrophage-specific knockout mice of L13a we have shown that extra-ribosomal function of RP-L13a is absolutely essential for resolving inflammation in LPS-induced endotoxemia (Poddar et al., 2013). This part of the study had been discussed in detail in chapter 2 of this dissertation. Studies using the LPS-induced endotoxemia model and also high-fat-diet-induced atherosclerosis model *(the respective study where I had a significant contribution, which has been published but has not been included in this dissertation)* (Basu et al., 2014) revealed the critical role of L13a in resolving physiological inflammation. As aforementioned, the cytokine storm resulting from infiltration of macrophages and other immune cells into the colon mucosa can cause

tissue damage and can be important regulators in the pathogenesis of IBD. Therefore we hypothesize that the presence of L13a in the macrophages can also serve a protective role against inflammation caused during colitis. To test this hypothesis we took advantage of our pre-existing model of macrophage-specific L13a knockout mice and subjected them to DSS containing drinking water in order to induce colitis. During the course of the disease, we have monitored the different pathological features and symptoms in the animals to assess inflammation. We have also compared the inflammatory responses in the knockout animals to those of the respective control mice to better understand the consequence of the absence of L13a in progression of colitis. In the current chapter we are going to discuss the methodology and outcomes of this study in detail. Simultaneously, we have also tried to dissect the molecular mechanism associated with the anti-inflammatory role of L13a in experimental colitis and other intestinal diseases like inflammatory bowel diseases (IBDs).

3.3. MATERIALS AND METHODS

3.3.1. Mice

Macrophage-specific L13a knockout mice ($L13a^{flox/flox}LysMCre^{+}$) were generated in the laboratory by crossing $L13a^{flox/flox}$ mice with LysMCre mice, where the expression of the Cre gene is under the control of the Lysogen M (LysM) promoter which is myeloid cell specific, in the C57BL/6 background. The details of the conditional KO mice generation has been outlined in our previously published manuscript (Poddar et al., 2013) and also in chapter 2 of this dissertation. Macrophage-specific L13a conditional KOs were harbored, fed and bred in the animal facility of Cleveland State University following approved IACUC protocols. All animals were specific pathogen-free and were maintained in pathogen-free conditions and under normal conditions of temperature and light. The animals were fed normal laboratory chow diet and water as required. All control mice used for this study are $L13a^{flox/flox}$ and the KOs are $L13a^{flox/flox}LysMCre^{+}$.

3.3.2. Induction of colitis

Age and sex matched 8-12 weeks old control ($L13a^{flox/flox}$) and KO ($L13a^{flox/flox}LysMCre^{+}$) mice were transferred to fresh cages before each respective experiment and were supplied with 3% DSS (w/v) (molecular weight 36,000-50,000 Da, MP Biomedicals, OH) continuously for 9 days followed by 1 day of regular drinking water. For DSS-induced colitis survival study, mice were administered 2.5% DSS water for 14 days followed by regular drinking water for 7 days. For DSS induced colitis recovery study the mice were administered 3% DSS water for 7 days to induce colitis

followed by 4 weeks of regular drinking water for their recovery. For analysis of cytokines from colon sections, the mice were administered 3% DSS water for 7 days only followed by 1 day of drinking water. During the course of experiments the mice were monitored daily for changes in body weights, rectal bleeding and diarrhea to assess the disease severity. In all experiments, both controls and KOs have received similar treatments (DSS water in this study) and their differential responses have been measured. In experiments where normal drinking water data have been included, both controls and KOs received regular drinking water for same periods of time.

3.3.3. Pathological scoring and assessment of colitis

The hallmarks of colitis like rectal bleeding and diarrhea were monitored in the DSS administered animals daily during the course of the experiment and were given appropriate scores on a scale of 0-4. Scoring was done by an individual blinded to the treatment. For rectal bleeding, scoring was done as follows: 0 = no blood, 2 = prominent traces of blood and 4 = liquid blood around the anus with 1 and 3 having respective intermediate scores. The scoring for stool consistency was done as follows: 0 = normal fecal pellet, 2 = loose fecal pellet and 4 = liquid diarrhea. For measurement of shortening of colon lengths, colons were harvested from mice after induction of colitis and were lined along a measuring scale and their lengths determined. Representative colons from DSS fed control and KOs was photographed. The percentage of epithelial crypt disruption was assessed by observing each H&E stained colon section under the microscope by a person blinded to the treatment and recording the percentage of crypt erosion accordingly, where 0% denotes absolutely normal crypt architecture and 100%

denotes complete loss of epithelial crypt morphology. A total of 30 colon sections from 10 controls and 10 KOs were assessed.

3.3.4. Tissue histology and immunostaining

For histological analysis, colons and spleens were harvested from DSS administered animals, fixed in methanol (a mixture of 60% methanol, 30% glacial acetic acid and 10% water) overnight and paraffin embedded. 5μM sections were cut and the paraffin-embedded tissue sections were deparaffinized with Trilogy (Cell Marque, Austin, TX) in a steamer for 30 min. The colon sections were then stained with H&E, Gomori Trichrome stain (Richard Allan Scientific, Catalog # 87021) and PAS (Poly Scientific R&D Corp., NY, Catalog # cy008) following manufacturer's protocol. Colon tissues were also stained for macrophages with purified anti-Mac2 Ab (rat anti-mouse; Cedarlane Laboratories) followed by incubation with Biotin-SP–conjugated appropriate secondary antibodies (Jackson ImmunoResearch). The modified H&E staining to view plasma cell and eosinophil infiltration was done as follows: Colon sections were incubated in xylene solution (two changes for 10 minutes each) followed by 100%, (two changes for 10 minutes each) 90% and 70% ethanol (1 minute each) followed by rinsing with distilled water. The sections were then stained with hematoxylin followed by brief rinsing in water, 0.5% acid alcohol mixture (a mixture of 0.5 ml of hydrochloric acid, 69.8 ml of isopropanol and 29.7 ml of water) and bluing solution (0.2% ammonia water solution) and finally stained with 50% Eosin Y solution for 2 minutes. After final dehydration with ethanol, the colon sections were mounted with mounting medium and viewed under the microscope.

3.3.5. Quantification of cytokine and chemokine expression

The cytokine and chemokine profiles were assessed both from the colons and serum of DSS administered control and knockout animals. After 7 days of DSS water treatment, the animals were euthanized and their colons were harvested. The distal part of the colons was used for creating colon lysates for ELISA, whereas the proximal colons were used for ex-vivo culture. Colon lysates were prepared as follows: colon samples were transferred to liquid nitrogen for quick freezing followed by chopping of frozen tissues with razors. 500µL of protease inhibitor (Sigma, Catalog # P2714) was added to the samples and chopped further in liquid phase. 1 ml of 1.5% triton X-100 (Sigma, Catalog # T8787-250ML) was added followed by incubation of the tissue samples at 4ºC on a shaker. Lysates were finally collected by centrifugation at 12000g for 10 minutes. Protein concentrations were determined and equal amounts of protein were used for ELISA to determine levels of IL-1β, IL-6, eotaxin, KC, MIP-1α and TARC. The proximal colons were chopped with sterile razors into minute pieces and were cultured overnight in DMEM media supplemented with 10% serum and antibiotics in a 37ºC incubator. The colon culture supernatants were used for ELISA to determine levels of IL-1β, IL-6, GM-CSF, IL-10, TNF-α, IFN-γ, eotaxin, KC, MDC, MIP-1α, RANTES and TARC. Blood was isolated from DSS treated animals by cardiac puncture. Serum was then isolated and serum levels of IL-6, eotaxin, MDC and TARC were determined by ELISA. The levels of all cytokines and chemokines were determined using cytokine/chemokine ELISA array in a commercial facility (Quansys Biosciences, Logan, UT).

3.3.6. Polysomal fraction analysis

Polysomal analysis was done from colon lysates of control and KO mice fed with DSS water for 7 days. Colon lysates were prepared by quick freezing of colon tissues in liquid nitrogen and finely chopping the frozen tissues with a sterile scalpel, followed by homogenizing the tissue in a Dounce homegenizer in 1 ml of polysome lysis buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1mg/mL cycloheximide, 50U of recombinant RNasin [Promega, WI], 0.1% Igepal-CA630 [Sigma, St. Louis, MO] and 10mM of sodium orthovanadate [Sigma-Aldrich, MO, USA]). Twelve optical density units of the colon extracts were carefully layered over 5 to 50% linear sucrose gradients prepared in the polysome buffer (as mentioned above without the RNasin, sodium orthovanadate and Igepal-CA630) and centrifuged at 17,000 rpm in a Beckman SW32.1 Ti rotor for 18 h at 4°C. Gradients were fractionated using an ISCO gradient fractionation system supplied with a UA-6 detector using an upward displacement method. Lighter ribonucleoprotein fractions, 40S, 60S, 80S, and heavy polysome fractions were monitored by constant UV absorption profiles at A_{254}, and 24 tubes of ~750 μL fractions were collected. Total RNA was isolated from each fraction using Trizol (Invitrogen, CA) and then subjected to reverse transcriptase (RT)-PCR with specific primers against CXCL13, CCL22, CCR3 and actin. PCR products were resolved on 1.5% agarose gels. Please refer to Table 1 for details of primer sequences.

3.3.7. Blood agar plating

Blood was isolated from 3% DSS (w/v) water fed animals by cardiac puncture in tubes containing 0.1M EDTA. Serum was isolated by centrifuging at 4000 rpm for 5

minutes at room temperature. The serum from each animal was diluted with sterile PBS in 1:8 ratio and was plated on blood agar plates (Remel Inc., Lenexa, KS). The plates were incubated at 37°C for 24 hours after which colony formation was studied.

3.3.8. Statistical analysis

The log-rank test (Mantel–Cox) was used to determine the statistical significance of the differences in survival rates between DSS fed controls and knockouts. Results have been represented as mean ± SD. All other statistical significance of the differences between groups was determined by a two-tailed Student t test. All statistical analysis was performed using GraphPad Prism 5.0 software.

3.4. RESULTS

3.4.1. Macrophage-specific knockout mice of L13a show increased susceptibility to experimental colitis.

In chapter 2 we have discussed (and also published data in the form of a manuscript) that macrophage-specific knockout (KO) mice of L13a showed higher susceptibility to LPS induced endotoxemia due to the depletion of L13a in the macrophages, which also leads to abrogation of the endogenously imposed silencing mechanism. In the DSS-induced experimental colitis study we have used the same genetically modified murine models (macrophage-specific L13a KO mice) and have subjected them to an inflammatory assault in the form of DSS water to assess their physiological response. Control and knockout mice were subjected to free access to 3% DSS in drinking water for 9 days followed by 1 day of regular drinking water (Hayashi et al., 2013). The mice were monitored daily for clinical symptoms of colitis involving weight loss, rectal bleeding and diarrhea and respective scores were given. The details of the clinical scoring method have been mentioned in the Materials and Methods section of this chapter. In comparison to the controls the L13a KO mice showed significant body weight loss (~10% weight loss in the controls and ~22% weight loss in the KOs) on the 10^{th} day post DSS treatment (Fig. 18A) together with high clinical scores for rectal bleeding (Fig 18B) and diarrhea (Fig. 18C), the two major clinical symptoms associated with IBD. No significant decrease of body weights were observed in mice administered with regular drinking water for the same time (data not shown) and both groups of animals consumed

equivalent amounts of food and DSS water over the course of the experiment (data not shown). On the 10th day, all animals were euthanized; their colons were harvested and photographed. Shortening of colons, an essential feature associated with colitis was found to be more evident in the knockouts after 9 days of DSS treatment as shown in Fig. (18D, left panel). No significant difference in the colon lengths were observed in the regular drinking water fed animals (Fig. 18D, left panel). Fig. 18D (right panel) shows the quantitative representation of the data.

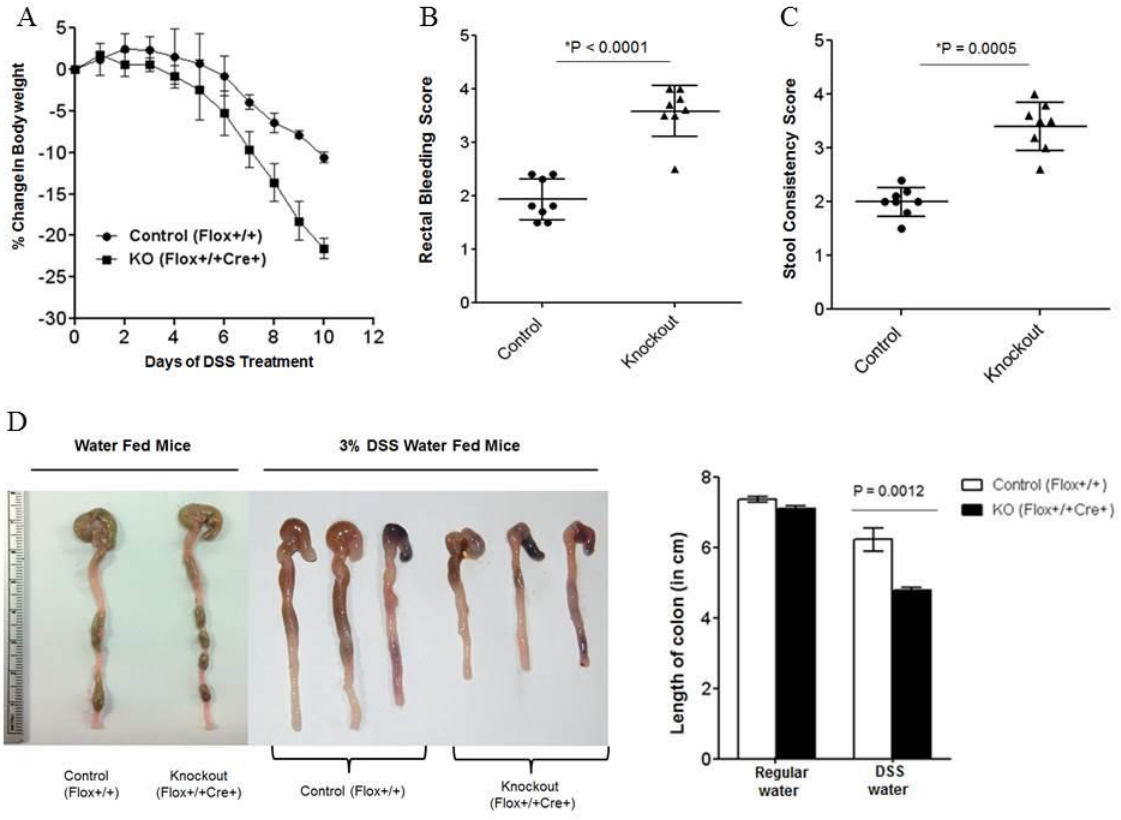

Figure 18. Macrophage-specific KO mice of L13a are more susceptible to DSS-induced colitis. Both groups of control and KO mice were given free access to 3% DSS containing drinking water for 9 days after which they were supplied with regular drinking water for 1 day. During the 9 day study, the animals were monitored daily for weight loss, gross rectal bleeding and stool consistency and relative scores were given. (A) The change in body weights during the

course of DSS administration is shown. (n = 10 controls and 11 knockouts). (B) The gross rectal bleeding scores as seen by blood around the anus are plotted. (n=8/group). Scores were given as follows: 0 – no blood, 1 – slight traces of blood, 2 – prominent traces of blood and 4 – liquid blood around anus. (C) The feces from the DSS treated mice were given stool consistency scores as follows: 0 – normal fecal pellet, 2 – loose fecal pellet and 4 – liquid frank diarrhea. (n = 8/ group). (D) On the 10^{th} day, all DSS treated animals were euthanized and their colons were harvested and photographed. The colons from three control and three KO mice from the DSS treatment group and one control and one KO animal from the saline treated group have been shown. The right hand panel shows a quantitative representation of the data. n=4/group for saline treatment and n=8/ group for DSS treatment. Results are mean ± SD. All p values have been calculated using paired two-tailed Student t test.

3.4.2. Macrophage-specific L13a knockout mice show reduced survival upon DSS challenge.

We had observed significant loss of body weights in the knockouts upon DSS treatment. Loss of body weights was also demonstrated by the controls, but to a much lower extent. Therefore we wanted to test whether depletion of L13a could translate the excessive loss of body weights to reduced survival rates in the knockouts. So we supplied 2.5% DSS in drinking water to both groups of control and knockout animals for 15 days followed by 9 days of regular drinking water. They were monitored for 24 days in total. In this experiment a lower dose of DSS was used because we were keeping the animals to the DSS treatment over a longer period of time. According to our expectation, the knockouts showed much reduced survival rates as compared to the controls (31.25% survival in the KOs compared to 81.25% survival in the controls) (Fig. 19). This suggests that presence of L13a in macrophages plays an important role in determining the susceptibility to colitis and hence survival.

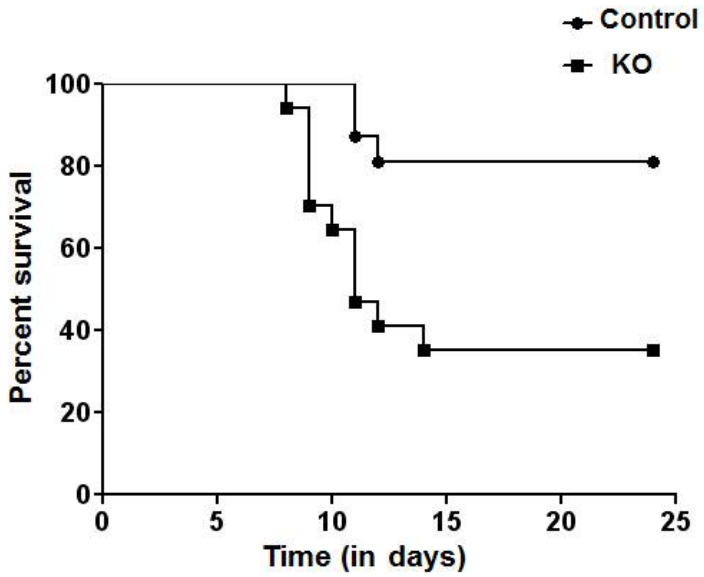

Figure 19. Macrophage-Specific KO Mice of L13a show reduced survival upon DSS Treatment. Animals were fed with 2.5% DSS in drinking water for 14 days followed by 7 days of regular drinking water. They were monitored for 24 days in total for survival. N = 16 in each group out of which 13 controls (81.25% survival) and only 5 knockouts (31.25% survival) were found to be alive at the end of the study. P = 0.0049 Log-rank (Mantel-Cox) Test.

3.4.3. Increased inflammation in the colon mucosa in experimentally-induced colitis upon L13a depletion.

Mounting evidence from the histopathological data in the LPS-induced endotoxemia study (discussed in chapter 2) (Poddar et al., 2013) suggested that absence of L13a in the macrophages could lead to greater tissue damage. In case of inflammatory bowel disease, injury to the colon architecture is a widely accepted phenomenon. Since higher susceptibility of the mice to colitis upon depletion of L13a was observed, we wanted to further investigate and assess the symptoms at the tissue level. For this purpose the colon morphology of the animals at the stage of post DSS administration was studied in detail.

Colons were harvested from DSS and normal drinking water fed animals, fixed and then sectioned for histological analysis. Hematoxylin and eosin (H&E) staining of colon tissue sections obtained from the normal drinking water fed mice (Fig. 20A) did not show any disease morphology with intact crypt and mucosal architecture and normal distribution of leukocytes within the lamina propria thereby indicating that under normal untreated conditions there is no difference between the controls and knockouts. H&E staining picture from one representative animal in each group is shown. On the contrary, after 9 days of DSS treatment, the knockouts demonstrated striking differences from the controls. Severe inflammation in the mucosa, muscularis and submucosa with entire loss of epithelial crypts, and surface epithelia together with immune cell infiltrates (as seen by the appearance of several tiny purple spots) was found in KO mice colons (Fig. 20B). Whereas very little to no appreciable disruption of epithelial crypt morphology was seen in the DSS treated control mice colon sections. A quantitative representation of the H&E data in terms of percentage of epithelial crypt disruption in each colon sections is shown (Fig. 20B right panel).

To understand in detail the changes going on at the level of the epithelial crypt morphology, we have used Periodic acid Schiff (PAS) staining. PAS staining is used to detect the polysaccharides such as glycogen, glycoproteins and mucin in cells. Goblet cells or epithelial cells are mucin producing cells and their integrity can be better understood using PAS staining. This reagent stains the epithelial cells purple to magenta on a contrasting background and the amount of loss of the magenta implies loss of goblet cells. Using this stain we have seen only slight perturbation of the crypt architecture in the DSS treated controls, whereas in the knockouts there was much more erosion of the

epithelial crypts with high infiltration of the immune cells as seen with H&E (Fig. 20C). To focus on the changes going on at the muscularis level in the colons, we have used yet another staining called the Gomori Trichrome Staining (Fig. 20D). This particular stain stains the muscle and cytoplasmic regions light red to red, normal connective tissue as light green and collagen as dark green. The nuclei are shown as dark red spots. Difference in tissue damage between control and KO is evident in this picture, where the epithelial crypts have completely effaced in the KO colons. There is also more collagen that has replaced the normal connective tissue in the KO mice colons (more dark green appearance in the lamina propria and the disrupted crypts). The muscularis also appeared to be more extended or thickened in some regions of the KO mice colons (Fig. 20E) thereby showing exaggerated inflammation. Taken together, these observations suggest that depletion of L13a from the macrophages can lead to greater colon tissue damage in inflammatory bowel disease.

3.4.4. Depletion of L13a in the macrophages results in greater infiltration of immune cells in the colon walls in colitis.

Upon induction of systemic inflammation in the L13a KO animals, we had observed extensive tissue damage with widespread infiltration of macrophages and other immune cells in various tissues (discussed in chapter 2 of this dissertation). Macrophages are immune cells primarily concerned with phagocytic removal of pathogens, infected and apoptotic cells in our body. They are recruited to sites of tissue injury to play a protective role. Since DSS induced experimental colitis in mice leads to severe injury to the colon epithelial lining, we have investigated whether the elevated inflammatory response in the

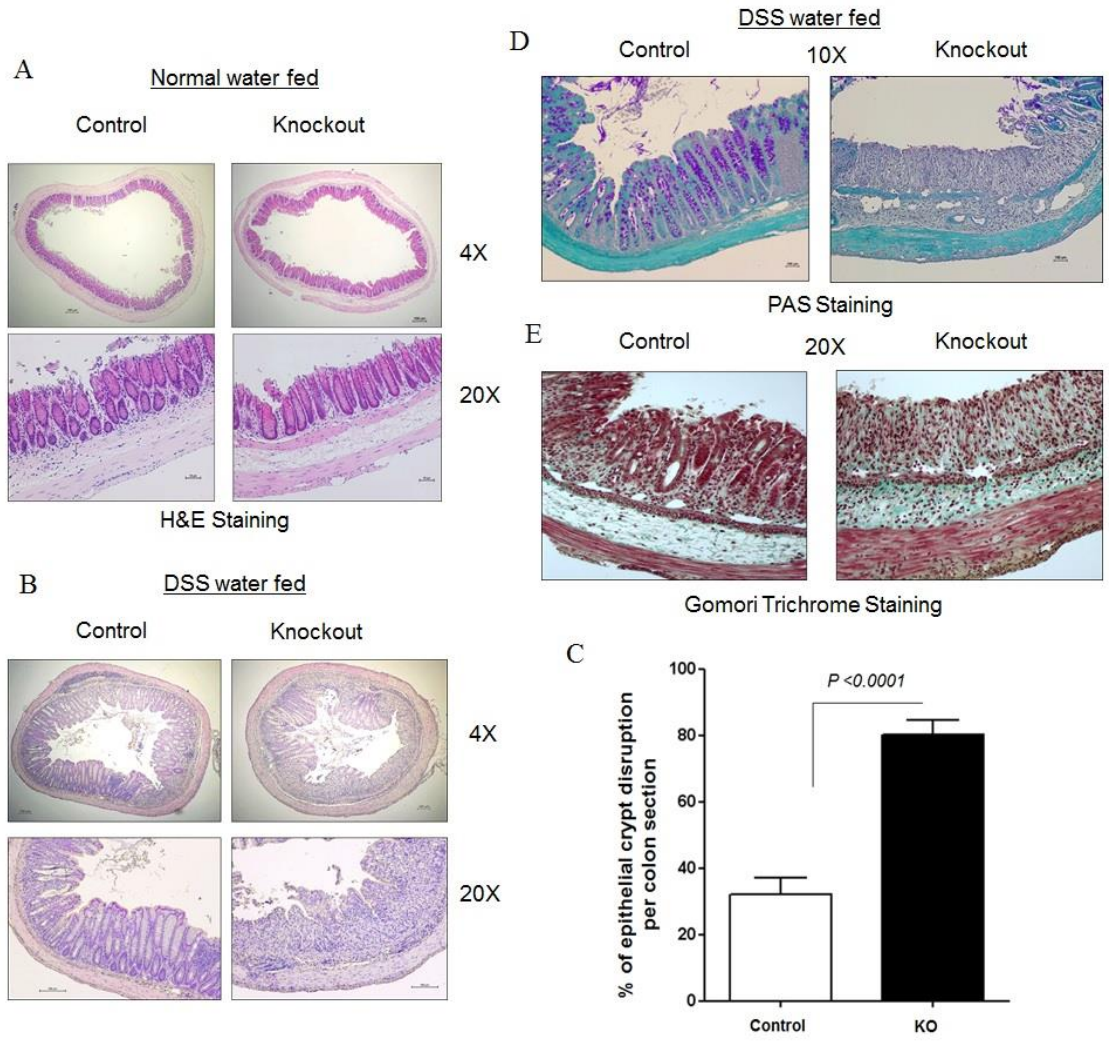

Figure 20. Enhanced injury to epithelial-crypt morphology in the macrophage-specific L13a KO mice during colitis. (A) H&E staining of the distal colon sections from normal drinking water fed mice show no disease morphology. Mice were fed with normal drinking water for 10 days after which they were sacrificed and their colons were harvested, fixed and then stained with hematoxylin and eosin to study the crypt morphology. Pictures shown at 4X (upper panel) and 20X (lower panel) magnification and on 100 and 10μ scales respectively. N = 3 in each group. Pictures from one representative animal in each group are shown. (B) H&E stained distal colon sections from DSS treated mice. Both control and KO mice were fed 3% DSS in drinking water for 9 days and normal water for 1 day after which they were euthanized, colons were harvested, fixed and then stained. A total of 9 controls and knockouts were used. Pictures from one representative mouse colon section from each group are shown at 4X and 20X magnifications on a 100 μ scale. Statistical representation of the data from DSS fed animals is shown focusing on

the percentage of colon disruption after DSS treatment. 27 colon sections from 9 mice in each group have been calculated. (C) PAS staining of DSS treated control and KO mice confirm loss of epithelial architecture. Colons sections harvested from animals subjected to 9 days of DSS water treatment were stained with PAS. N=4 in each group. Pictures at 10X magnification from one representative animal in each group are shown on a 100 μ scale. (D) Gomori Trichrome staining of colon sections from 3% DSS water fed control and KO mice was done to view the disruption of the muscularis layer and leukocyte infiltration. N= 4 in each group. Pictures of one representative animal in each group at 20X magnification are shown.

KOs was also accompanied by increased macrophage infiltrates in the colon mucosa. For this purpose, colons were harvested from regular drinking water (as vehicle) and 3% DSS water fed control and KO mice and immunostained with anti-Mac2 antibody to view infiltration of macrophages in the tissue. Colon sections from vehicle treated mice did not show any significant presence of immune cells. No disease morphology was observed with negligible distribution of macrophages just around the crypt region of both control and KO mice (Fig. 21A). On the contrary the DSS fed KO mice colons showed conspicuous differences from those of the controls in terms of increased number of Mac2 positive macrophages associated with the crypts, basal membrane and lamina propria. The lamina propria and the basal muscularis layer in the KO mice colons also appeared to be more swollen and there was erosion of the normal crypt morphology than those in the control mice (Fig. 21B). Modified H&E staining of the colons was done to check for plasma cell and eosinophil invasion. This suggested eosinophil and plasma cell infiltration in the colon tissues of the DSS fed KOs (Fig. 21C). Based on these results we can assume that since macrophage infiltration is an immediate response to mucosal injury that leads to development of acute inflammation, absence of L13a in the macrophages of the KO mice leads to increased destructive activity of these cells that can result in

uncontrolled inflammation. Also, since eosinophilia occurs in response to signals from certain chemokines (e.g., CCL11), therefore, it is possible that accumulation of eosinophils and plasma cells in the colon tissues harvested from the KO mice could be an indication of increased disease phenotype due to inability to resolve inflammation.

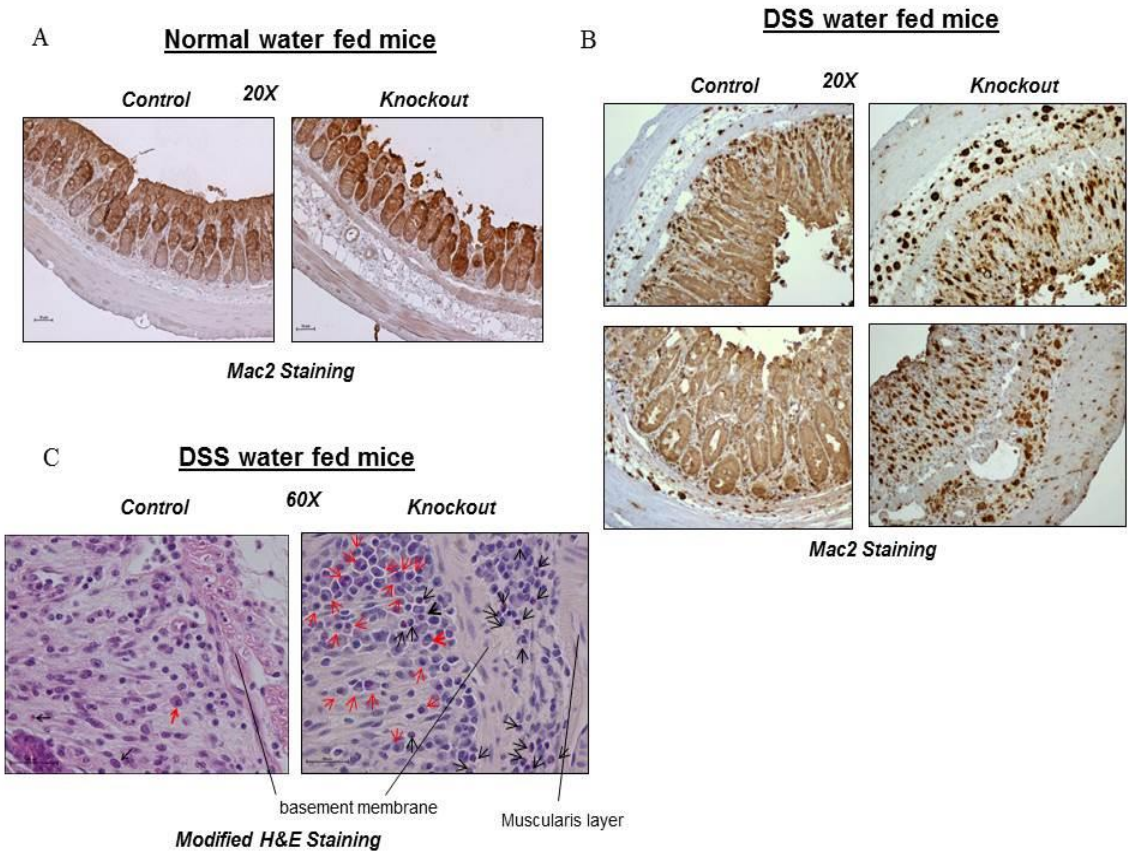

Figure 21. Infiltration of immune cells in the colon mucosa of the L13a KO mice. (A) Colon sections from water fed control and KO mice show no significant difference in the presence of macrophages. Mice were fed with normal drinking water for 10 days after which they were sacrificed, their colons harvested and stained with Mac2. Pictures from one representative animal from each group are shown at 20X magnification. (B) Colon sections from DSS water fed KO mice show greater infiltration of Mac2 positive macrophages in the lamina propria. Colons were harvested and stained similarly as mentioned above for water fed controls. More number of Mac2 positive macrophages was found in the lamina propria of the KO mice colons. A total of 6 controls and 6 KOs were used. Pictures of colon sections from 2 different mice from each group

are shown at 20X magnification. (C) DSS treatment leads to infiltration of plasma cells and eosinophils in the lamina propria and sub-mucosa. Colons harvested from DSS treated mice were subjected to modified H&E staining to view the plasma cells and eosinophil infiltration. The plasma cells are indicated with red arrows whereas the eosinophils are indicated with black arrows. N = 3 in each group. Picture from one representative animal from each group is shown at 60X magnification on a 100μ scale.

3.4.5. Cytokine and chemokine analysis in the colonic tissue of DSS administered mice.

Infiltration of leukocytes into tissues is a hallmark of inflammation and chemokines and cytokines can serve as internal cues to attract various immune cells to sites of inflammation and tissue injury. Our previous observations had confirmed the infiltration of macrophages, eosinophils and plasma cells in the colons of DSS treated KOs. To gain further insight into the activation levels of several chemokines and cytokines, we have performed a chemokine/cytokine ELISA array with both colon culture supernatants as well as colon tissue homogenates obtained from DSS administered control and KO animals. The results obtained are shown in Fig. 22. The colon culture supernatants showed elevated levels of several inflammatory cytokines and chemokines such as, IL-1β, IL-6, GM-CSF, IL-10, TNF-α, IFN-γ, eotaxin (CCL11), KC (CXCL1), MDC (CCL22), MIP-1α (CCL3), RANTES (CCL5) and TARC (CCL17) (Fig. 22A) while the colon tissue homogenates showed increased levels of IL-1β, IL-6, eotaxin, KC, MIP-1α and TARC (Fig. 22B). The levels of the abovementioned cytokines and chemokines in the colons of normal drinking water fed animals were found to be below detection levels of the assays and hence have been omitted. Together, these results indicate that DSS

treatment leads to an increased expression of these pro-inflammatory proteins which aid in recruiting the macrophages to the more inflamed parts of the colons in the KO mice upon DSS treatment.

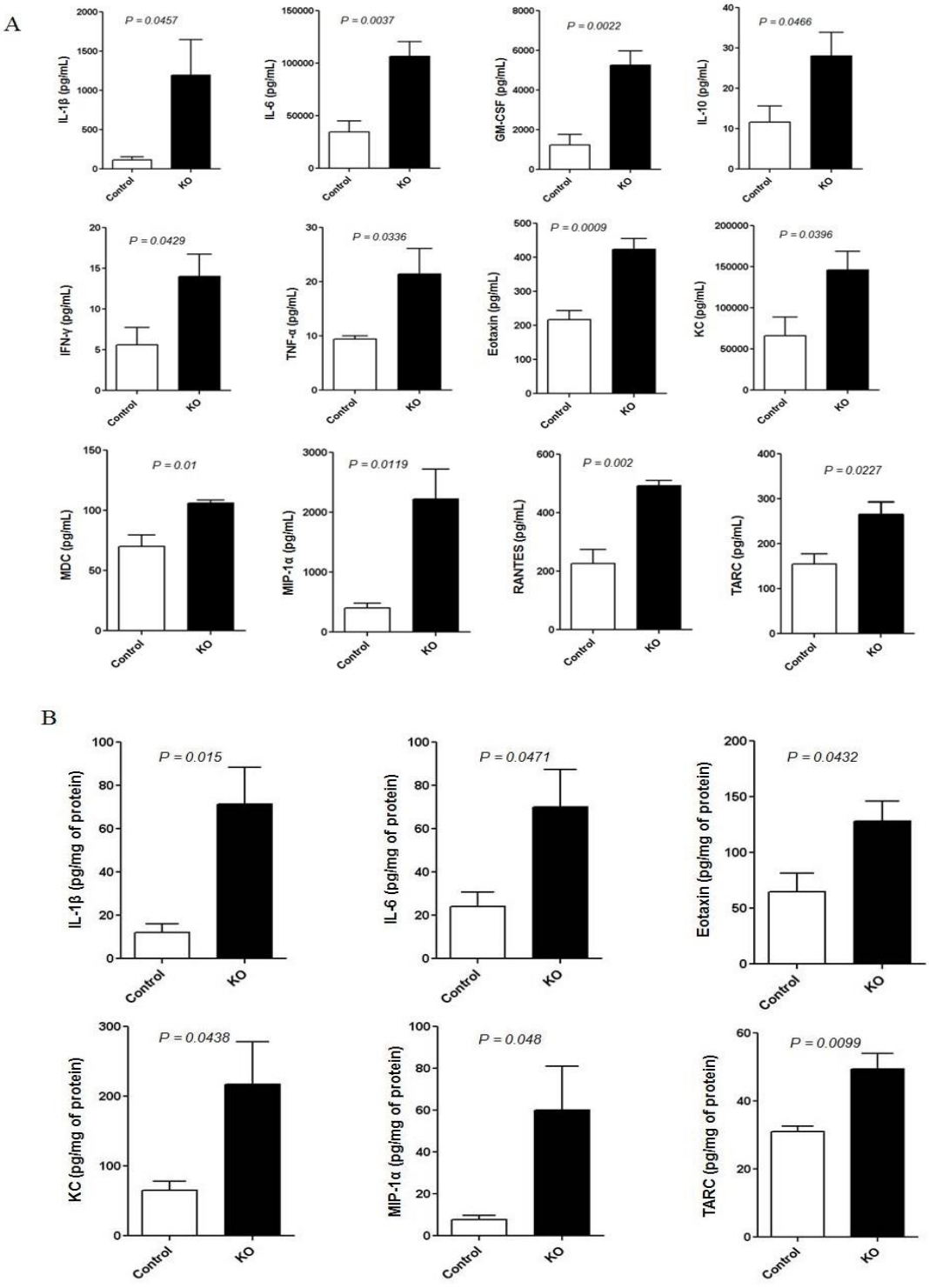

Figure 22. Elevated cytokine and chemokine expression in the colons of DSS treated knockout mice. Control and knockout mice were given free access to 3% DSS in drinking water for 7 days after which they were euthanized and their colons harvested. (A) A part of the colon from each mouse was cultured overnight in DMEM after which the colon culture supernatants were harvested and then used for ELISA to determine the levels of IL-1β, IL-6, GM-CSF, IL-10, TNF-α, IFN-γ, eotaxin, KC, MDC, MIP-1α, RANTES and TARC. (B) The other portion of the colon from each animal was homogenized to obtain tissue lysates. Equal amount of protein concentrations of total lysates were used for ELISA to determine levels of of IL-1β, IL-6, eotaxin, KC, MIP-1α and TARC. Lower limit of detection (LLD) of assays are as follows: IL-1β = 2.1 pg/ml, IL-6 = 0.2 pg/ml, IL-10 = 1.9 pg/ml, IFN-γ = 0.3 pg/ml, TNF-α = 1.7 pg/ml, GM-CSF = 0.2 pg/ml, eotaxin = 1.2 pg/ml, KC = 0.4 pg/ml, MDC = 1.1 pg/ml, MIP-1α = 1.4 pg/ml, RANTES = 0.4 pg/ml and TARC = 3.3 pg/ml. N =5 in each group. Results are mean ± SD. The p values were determined using the paired two-tailed Student t test.

3.4.6 Disruption of the L13a in the macrophages abrogates the translational silencing of GAIT target proteins in DSS-induced colitis.

Colon cytokine/chemokine profile analysis showed elevated levels of several chemokines and cytokines some of which are direct targets of the L13a dependent translational silencing pathway (eg., eotaxin, MIP1-α, and MDC). To assess whether there was a direct involvement of the GAIT target proteins in promoting severity of the disease in DSS-triggered colitis, we determined the translation status of these proteins in the colons harvested from DSS treated animals. Testing the polyribosomal abundance is a widely accepted method to check the translation efficiencies of mRNAs (Vyas et al., 2009, Brown et al., 2001, Arava et al., 2003). Untranslated messages will co-sediment with the lighter free fractions whereas actively translated messages will be polyribosome bound and therefore appear with the heavy polyribosomal fractions. Colon lysates were

prepared from DSS administered control and KO mice and were subjected to a 5-50% sucrose gradient centrifugation to separate the translationally active and translationally repressed pools of mRNAs. RNA was isolated from each fraction followed by RT PCR with specific primers against CCL22, CXCL13 and CCR3, some of the GAIT target mRNAs. Table 1 contains details of primer sequences. Results showed these mRNAs to be primarily associated with the lighter/free fractions in the control mice (Fig. 23 left panel), whereas there was a significant shift of these mRNAs to the heavier polysomal fractions in the KO mice (Fig. 23 right panel). This result demonstrates that the absence of the L13a mediated silencing pathway in the macrophages can elevate the translational status of pro-inflammatory chemokines whose synthesis is very low under normal basal conditions. Such dysregulation of expression of pro-inflammatory genes can possibly contribute to increased disease pathology in DSS induced colitis.

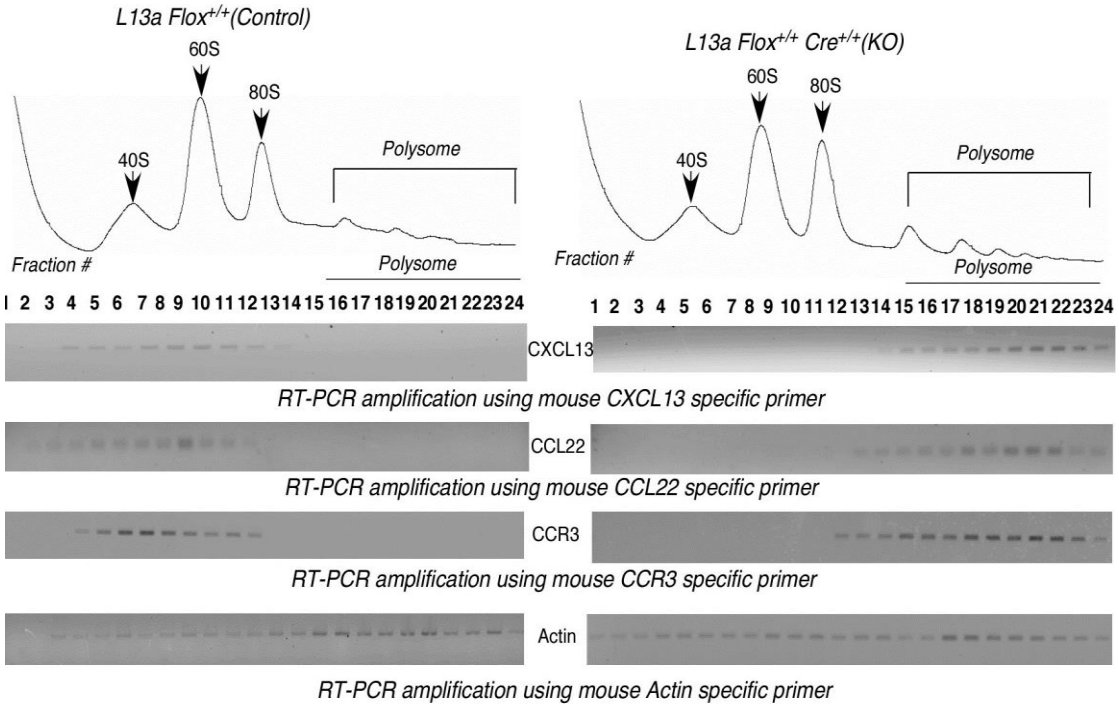

Figure 23. L13a deficiency in macrophages disrupts translational regulation of CCL22, CXCL13 and CCR3 mRNAs upon DSS treatment. Increased polyribosomal abundance of the GAIT target proteins in the L13a KO mice. Colons were harvested from control and KO mice after 7 days of 3% DSS administration. Lysates were made in polysome lysis buffer (see Materials and Methods section of this chapter for buffer compositions) and layered on top of a 5-50% sucrose density gradient and subjected to ultracentrifugation for 18 hours at 17000 rpm at 4^0C. Total RNA was isolated from each fraction followed by RT-PCR using specific primers against CCL22, CXCL13 CCL8 and actin. Significant shift to the heavier polysomal fractions and increased polysomal abundance in the KO mice was seen only for the GAIT target proteins and not actin.

3.4.7. Absence of L13a in macrophages can lead to systemic inflammation resulting in splenomegaly upon DSS treatment.

Enlargement of the spleen occurs due to systemic inflammatory reaction which also occurs during DSS-induced acute colitis and can be correlated to the disease severity (Axelsson et al., 1998, Morteau et al., 2000, Da Silva et al., 2006). Before discussing the experimental details, we would like to discuss the microscopic anatomy of the spleen in brief that would help us to better interpret the results discussed in this section. The spleen has two specific regions – the red pulp and the white pulp. In rodents, the red pulp area is a site of hematopoiesis in fetal and neonatal animals, whereas innate immune responses to blood borne antigens are carried out by the white pulp region. The red pulp is made up of a three dimensional meshwork of the splenic cords (containing the reticular fibers and associated macrophages) and venous sinuses. The red pulp macrophages are actively phagocytic and remove old and damages erythrocytes. The white pulp is composed of the periarteriolar lymphoid sheath (PALS), the follicles and the marginal zone. This region

contains the lymphocytes, macrophages, dendritic cells, plasma cells, arterioles and the capillaries. The marginal zone is a unique region of the spleen situated at the interface of the red pulp and the PALS and follicles.

The LPS-induced endotoxemia study had demonstrated that depletion of L13a in macrophages leads to enhanced susceptibility to systemic inflammation. Using this knowledge, we wanted to test whether abrogation of L13a mediated translational silencing pathway in the macrophages could take the DSS-induced inflammation a step further to affect the entire system. Since the spleen acts as a reservoir of immune cells, we have investigated whether deficiency of L13a in the macrophages can also lead to splenomegaly (enlargement of the spleen) in DSS-induced experimental colitis. Spleens from DSS fed L13a KO animals were found to be much enlarged, with an increase in both size and weight as compared to the DSS fed control animals (Fig. 24A). A quantification of the data has been shown in Fig. 24B. To better understand the phenomenon that lead to the pronounced enlargement of the spleens only in the knockouts, we have carried out an immunostaining of the spleen sections with anti-Mac2 antibody. Mac2 staining revealed that there was an increase both in size and number of macrophages in the white pulp area of the spleen, close to the periarteriolar lymphoid sheath (PALS) (Fig. 24C). A probable explanation could be that the enlarged macrophages could be tingible body macrophages which are predominantly found in germinal centers of secondary lymphoid tissues and contain debris from ingested lymphocytes phagocytized apoptotic cells. This could indicate that the macrophages in the white pulp region in the KO mice spleens have become highly activated due to absence of the silencing pathway in them, thereby phagocytizing more number of injured

cells and thus increasing in size. These observations can lead us to hypothesize that absence of the anti-inflammatory function of L13a in the macrophages can exaggerate the DSS-induced inflammatory response and elevate it to a systemic response whose effects are visible in the spleen.

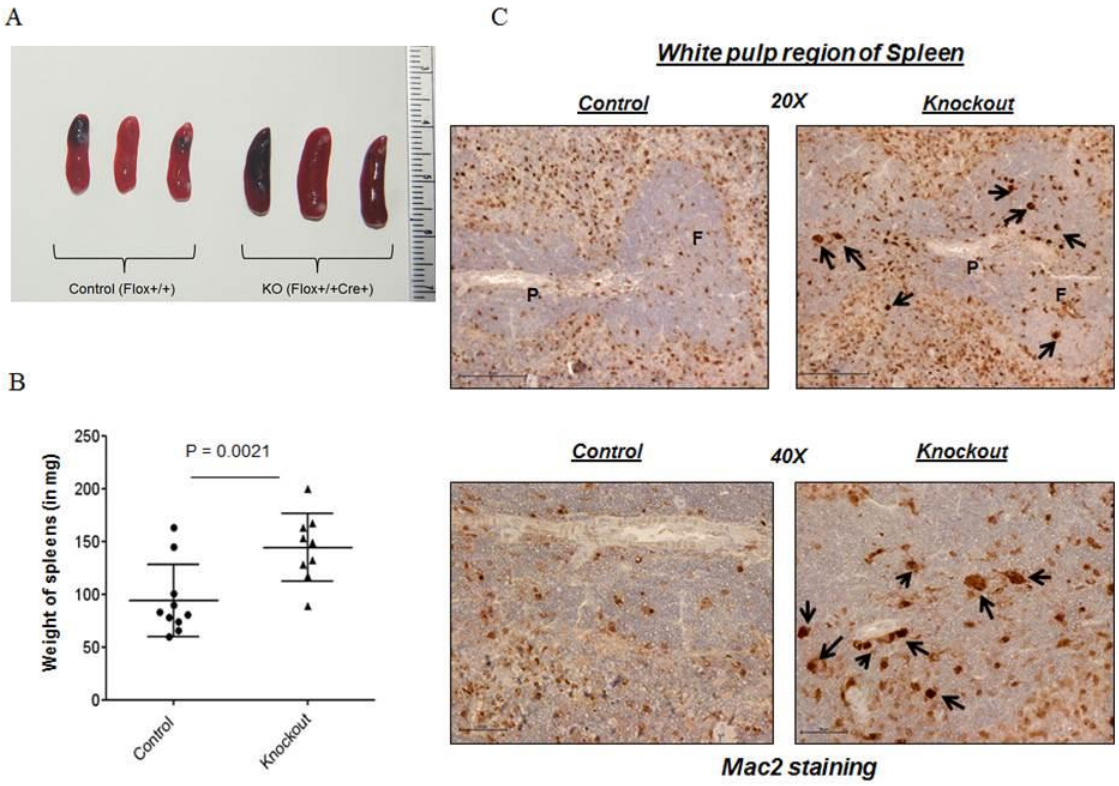

Figure 24. Enlargement of spleens and macrophages within the spleen in the macrophage-specific L13a KO mice upon DSS treatment. (A) Increase in spleen size in the DSS treated KO mice. 3% DSS water fed control and KO mice spleens were harvested and then photographed. Picture from 3 representative animals from each group has been shown. (B) Quantification of the difference in spleen size between DSS treated controls and knockouts. N = 10 controls and 9 knockouts. P value was calculated using paired two-tailed Student t test. (C) Increased infiltration of macrophages with increase in cell size was observed in the KO mice spleens upon DSS treatment. Spleen sections from DSS treated mice were stained with anti-Mac2 antibody. Increase in the number of Mac2 positive macrophages together with an increase in their cell size was observed in the white pulp. Enlarged macrophages are pointed with black arrows. 20X

magnification picture show the marginal zone including the white and red pulp area of the spleen). P indicates the periarteriolar lymphoid sheath (PALS) region and F indicates the follicles. 40X magnification picture focus on the white pulp region on a 10 μ scale.

3.4.8. Absence of L13a in the macrophages prevents resolution of inflammation in DSS-induced colitis.

A series of in-vitro studies performed in our lab to delineate the detailed mechanism of L13a mediated GAIT silencing pathway identified that this particular pathway is active during the later time points of interferon gamma (IFN-γ) treatment of myeloid cells, thereby preventing excess accumulation of inflammatory products and permitting resolution of inflammation (Mazumder and Fox, 1999, Mazumder et al., 2001, Mazumder et al., 2003a, Mazumder et al., 2003b, Kapasi et al., 2007, Vyas et al., 2009). Our hypothesis behind generating the conditional KO mice of L13a was to test the physiological consequence of uncontrolled inflammation due to the abrogation of the endogenously imposed translational silencing. To directly test our hypothesis in the context of the colitis disease model, we have employed the DSS-induced acute colitis recovery study. In this experiment, we have induced colitis in the mice by feeding them with DSS water for a short period (7 days) followed regular drinking water over a longer period of time (4 weeks). In the course of the experiment, we have monitored the changes in body weights of the controls versus the knockouts and have compared their abilities to recover from colitis and regain normal activities. The result in terms of percent change in body weights is shown in Fig. 25. Both controls and KOs showed decrease in their body weights with induction of colitis till day 11 with more loss seen in the knockouts (22% as

opposed to 15% in the controls). The experiment was initiated with 8 controls and 8 knockouts. By day 12 of the experiment, only two controls and 5 knockouts were found to be dead, thereby confirming our previous observations that the L13a KOs are more susceptible to DSS treatment. The animals were monitored for a period of 35 days in total and at the end of the study 5 controls and only 2 knockouts were found to have survived and recovered from the effects of DSS. These were the same animals which had survived after 12 days and had continued to live till the end of the experiment. Moreover, the macrophage-specific L13a KOs took a longer time to recover from colitis though complete recovery in terms of gain in body mass was not observed within the time frame of the experiment (Fig. 25A). After the span of the experiment (35 days), the survived animals were euthanized, their colons were harvested, sectioned and stained with PAS for study the recovery at the tissue level. Epithelial cells lined intestinal crypts were found to have regenerated to a more extent in the controls than in the knockouts (Fig 25B). Also, a substantial amount of fibrosis was still evident in the colon sections from the colitis recovered KOs. These observations suggest that though the knockouts might have shown a certain degree of recovery in terms of regaining body masses and resuming normal, regular activities, but at the level of tissue regeneration, recovery was considerably delayed in comparison to the controls. Taken together these observations demonstrate that presence of L13a in the macrophages is a critical factor in the execution of the resolution of inflammation and perhaps plays a protective role against excessive inflammation in DSS-induced experimental colitis.

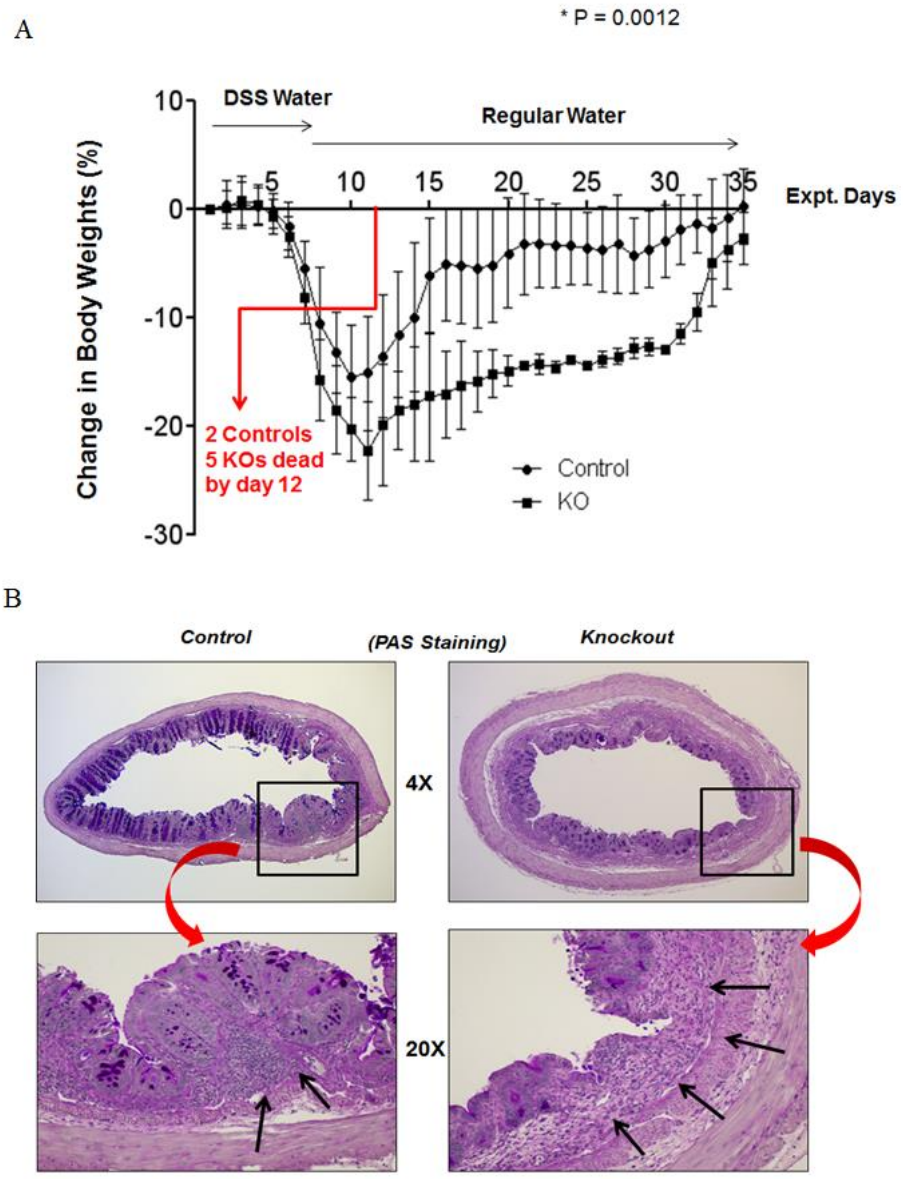

Figure 25. Delayed recovery of L13a KO mice from DSS-induced colitis. (A) Control and L13a knockout mice were administered 3% DSS in drinking water for 7 days followed by 4 weeks of regular drinking water treatment, allowing them to recover. The animals were monitored daily for changes in body weights for the entire length of the study for 35 days. At the end of the study, 5 control mice and only 2 KO mice were found to be alive and have recovered from colitis from a total of 8 mice in each group with which the study was initiated. The KO mice showed more loss of body weights and also took more time to regain their normal body masses and recover from the effects of DSS induced inflammation. (B) PAS staining of colon sections from Kos show more fibrosis. Colons were harvested from colitis survived control and KO mice

and sections were subjected to PAS staining. PAS stained colon section pictures from one representative animal from each group has been shown at 4X (upper panel) and 20X (lower panel) magnifications respectively. Black arrows indicate regions of fibrosis. Results are mean ± SD. P value calculated using unpaired two-tailed Student's t test.

3.4.9. Macrophage-specific depletion of L13a amplifies the DSS-induced inflammatory response to septicemia.

In the model of DSS induced colitis the macrophage-specific KO mice of L13a showed damage in other organs in addition to the hallmark symptoms of colitis. As discussed earlier we had observed splenomegaly and infiltration of macrophages in the white pulp region of the spleen, which is normally devoid of them (Fig. 24). Therefore, we wanted to investigate whether the DSS-induced colonic inflammation could be amplified to a systemic one. So, we analyzed the serum levels of the pro-inflammatory cytokines and found significantly elevated levels of IL-6, eotaxin, MDC and TARC in the serum of the DSS treated knockout mice as compared to the controls (Fig. 26A). Eotaxin (CCL11) and MDC (CCL22) are direct targets of the L13a mediated GAIT translational silencing pathway. No cytokines or chemokines were detected in the serum of regular water fed controls and knockouts (data not shown). Our previous observations from the endotoxemia study (discussed in Chapter 2) had shown increased expression of a few of the same GAIT target chemokines upon LPS challenge (Poddar et al., 2013). One of the pathogenic causes of colitis is imbalance between the commensal microflora and the intestinal immune response (Kaser et al., 2010, Sartor, 2006). This could potentially lead to the disruption of the epithelial barrier and the subsequent release of the gut bacteria into the bloodstream, hence resulting in septic shock or septicemia. To directly test the presence of live bacteria in the serum samples, we have plated diluted serum samples

from DSS treated mice on blood agar plates to observe bacterial colony formation. A total number of 9 controls and 8 knockouts were used for the experiment. Out of 8 KOs, 4 showed formation of multiple colonies whereas only 1 out of the 9 controls showed significantly less colony formation (Fig. 26B). Pictures from four representative animals from each group have been shown. Absolutely no colonies were observed from serum of water fed mice (data not shown). Therefore we hypothesize that the absence of L13a magnifies the DSS induced inflammatory response to a systemic immune response, causing increased production of the pro-inflammatory chemokines thereby making the animals more sensitive to the DSS challenge.

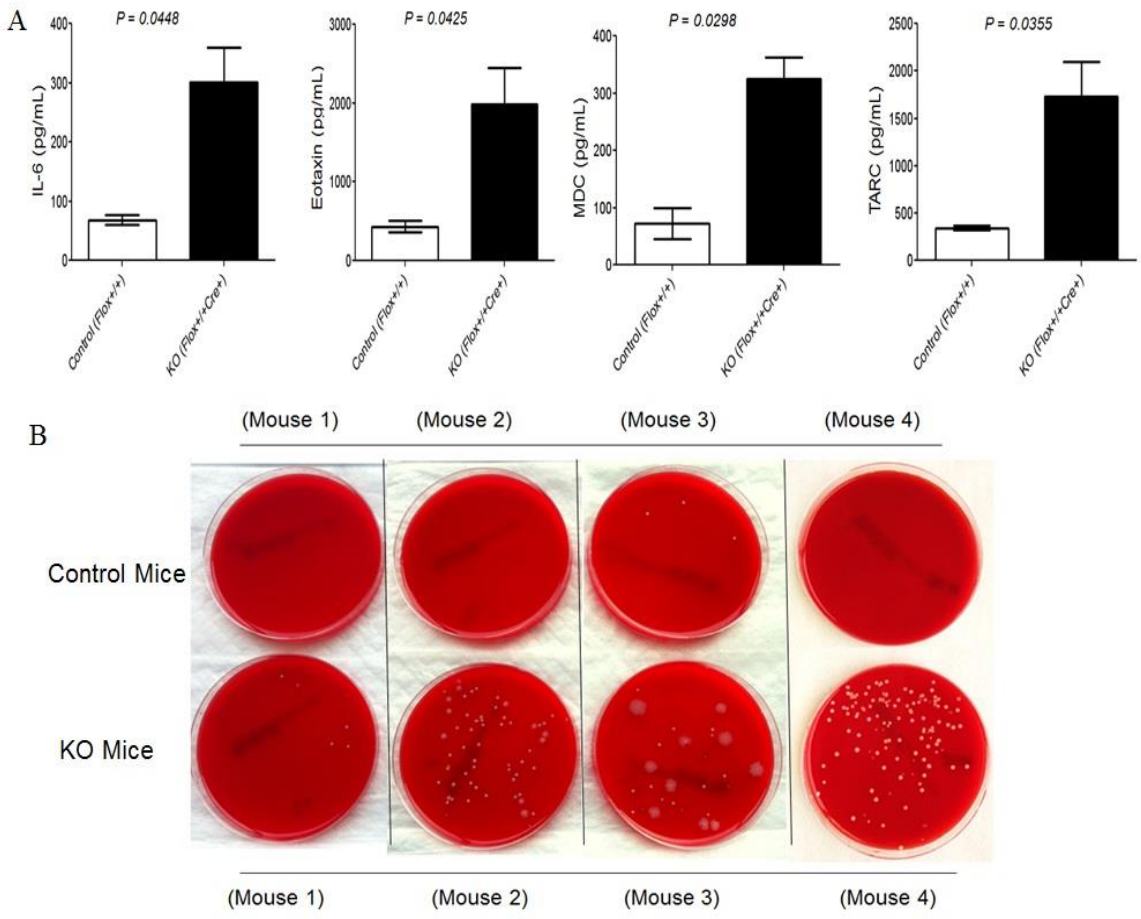

Figure 26. DSS induced inflammation results in septicemia in the absence of L13a mediated translation silencing in the macrophages. Mice were administered with 3% DSS supplemented in drinking water for 9 days after which they were switched to regular drinking water for a day. On the 10^{th} day, blood was collected through cardiac puncture and serum was isolated from these animals. (A) The serum samples were analyzed by ELISA to quantify levels of cytokines. The KO mice serum showed elevated levels of IL-6, MDC, eotaxin and TARC. Results are mean ± SD. P values were calculated using paired two-tailed Student's t tests. (B) Serum samples were diluted with PBS and then plated on blood agar plates. They were incubated at 37^0C for 24 hours after which representative plates were photographed. N = 9 controls and 8 KOs. Picture shown from 4 representative mice in each group.

3.5. DISCUSSION

In this study, using macrophage-specific knockout mice of L13a, we have examined the protective anti-inflammatory role of ribosomal protein L13a against DSS induced experimental colitis (mouse model of IBD). These knockout animals were previously generated in our laboratory by crossing the $L13a^{flox/flox}$ mice with $LysMCre^{+}$ mice (Poddar et al., 2013) and serves as a novel model to study consequences of unregulated inflammation. In the DSS induced IBD model, colitis was induced by feeding the mice with 3% DSS containing water. In response, the macrophage-specific L13a knockouts demonstrated higher susceptibility to colitis and displayed more severe clinical symptoms of colitis such as reduced survival rates, drastic loss of body weights, high diarrhea and rectal bleeding and pronounced shortening of colons lengths. In comparison with the $L13a^{flox/flox}$ control mice, at the microscopic tissue level, the KO mice showed complete loss of crypt architecture causing disruption of epithelial crypts and surface epithelia and muscularis layer upon DSS administration. In addition we have found infiltration of macrophages in the colons of the knockout mice to be significantly higher than the $L13a^{flox/flox}$ littermates. Assessing the cytokine/chemokine expression profiles in the DSS treated animals showed much enhanced levels of IL-1β, IL-6, GM-CSF, IL-10, TNF-α, IFN-γ, eotaxin, KC, MDC, MIP-1α, RANTES and TARC in the colon conditioned media and IL-1β, IL-6, eotaxin, KC, MIP-1α and TARC in the colon homogenates of the DSS treated knockouts only. Apart from the colons, DSS induced inflammation was found to have spread to other organs like the spleen in the macrophage-specific L13a knockout mice which showed significant enlargement of spleens and infiltration of macrophages in

the white pulp region of the spleens which are definite signs systemic inflammation. In addition high serum levels of IL-6, MDC, eotaxin and TARC were observed in the DSS treated knockouts thereby suggesting that depletion of L13a in the macrophages can lead to an elevated inflammatory response thereby making the knockout mice more prone to DSS triggered colitis.

Previous studies from our laboratory had identified ribosomal protein L13a dependent GAIT-mediated translational silencing pathway to play a critical role in controlling the expression of several genes encoding inflammatory proteins mainly chemokine ligands and receptors (Vyas et al., 2009). This, according to our hypothesis acts as a major pathway in resolving physiological inflammation According to this mechanism, induction of monocytes and macrophages with IFN-γ leads to the formation of a multi-protein RNA binding complex known as the GAIT (Gamma-interferon Activated Inhibitor of Translation) complex which assembles on the cis-acting GAIT element present in the 3'UTR of target mRNAs and inhibits their translation (Mazumder and Fox, 1999, Mazumder et al., 2001, Mazumder et al., 2003b). This process also requires active participation of ribosomal protein L13a after its phosphorylation dependent release from the 60S subunit of the ribosome (Mazumder et al., 2003a, Kapasi et al., 2007). In the colitis study we have found significant increase in the levels of eotaxin (CCL11) and MDC (CCL22) both in the colons as well as the serum of the DSS treated KOs. Both of these chemokines are direct targets of the GAIT-mediated silencing pathway (Vyas et al., 2009), thereby implicating a possible role of this pathway in controlling DSS induced colonic inflammation. The colon extracts from DSS fed KOs have also shown increased polysomal abundance of few GAIT target messages like CCL22, CXCL13 and CCR3

(Fig. 6) thus confirming that the deficiency of L13a in the macrophages can disrupt the endogenous translational silencing of these inflammatory proteins which might contribute significantly to the inflammation in DSS induced IBD.

It is already known that chemokine receptors and ligands can generate intracellular signals in response to inflammation to recruit mononuclear cells to sites of infection in the tissues (Medzhitov, 2010, Shi and Pamer, 2011). MIP-1α (macrophage inflammatory protein 1 alpha) also known as CCL3 and RANTES, also known as CCL5 are members of the C-C subfamily of chemokines which are particularly chemotactic for cells of monocyte linage and lymphocytes (Cook, 1996) and have been reported to have important roles in inducing inflammation. The expression of MIP-1α can be triggered in macrophages in response to systemic inflammation by endotoxin (LPS) challenge and in monocytes by their binding to intracellular cell adhesion molecule I (ICAM-1) (Lukacs et al., 1994). In our mouse IBD model, we have found enhanced levels of both RANTES and MIP-1α and increased macrophage infiltrates in the colons of the KOs. RANTES receptor CCR5 is strongly expressed on macrophages and monocytes (Mueller and Strange, 2004) and could thus lead to enhanced trafficking of these cells to the colons thereby promoting colitis in the knockouts. CCR3, the receptor of MIP-1α is a direct target of the L13a dependent translational silencing pathway and was observed to be upregulated upon depletion of L13a in the macrophages of the DSS treated knockout animals. This could have a possible role in increasing expression of its ligand MIP-1α, which in a positive feedback mechanism leads to greater recruitment of the macrophages in the colonic mucosa of the DSS treated knockout mice.

In addition to chemokine expression, IL-1β expression was also found to be increased in the macrophage-specific L13a knockout mice upon DSS administration. IL-1β is a pro-inflammatory cytokine and has been demonstrated to play an essential role in intestinal inflammation associated with colitis and also promoted IBD triggered diarrhea (Elson et al., 1995, Sartor, 1994). High doses of IL-1β can influence organ damage by inducing epithelial cell necrosis and obstructing this endogenous activity of IL-1β can alleviate both acute and chronic IBDs. (Sartor, 1994, Cominelli et al., 1992; Cominelli et al., 1990). Therefore, the increased expression levels of IL-1β can be correlated to the higher disease pathogenicity observed in the knockout mice in response to DSS. Significantly elevated levels of another cytokine, IL-6 was found in the colons and in the serum of the knockouts upon DSS treatment which can be correlated to enhanced susceptibility of these animals to colitis. IL-6 is a multi-functional cytokine with implicated roles in immune responses, cell survival, apoptosis and proliferation (Kishimoto, 2005) and has also been reported as a significant contributor of DSS induced inflammation in colitis. It is a NF-κB dependent pro-tumorigenic cytokine which is produced by the lamina propria myeloid cells and promotes colitis associated cancer (CAC) (Grivennikov et al., 2009).

In the DSS induced experimental colitis model, we have found increased invasion of the colon mucosa of the DSS treated KOs by the $Mac2^{+}$ macrophages. $Mac2^{+}$ macrophages are of the pro-inflammatory nature and could be responsible to inducing inflammation in the colons in colitis. There are studies which report that a group of $TLR2^{+}$ $CCR2^{+}$ $CX3CR1^{int}$ $Ly6C^{hi}$ $GR1^{+}$ macrophages induce colonic inflammation by producing large quantities of TNF-α (Platt et al., 2010). Recent findings also suggest that the inflammatory cytokines and reactive oxygen species generated in the colon can

transform the anti-inflammatory macrophages to the pro-inflammatory dendritic cell like cells which secrete large amounts of IL-12, IL-23 and nitric oxide synthase thereby promoting colitis (Rivollier et al., 2012). $CD14^{+}$ macrophages that secrete IL-23 and TNF-α have been found to be important in promoting Crohn's disease (Kamada et al., 2008). Therefore the increased abundance of inflammatory macrophages in the muscularis, lamina propria and surface epithelia of the DSS treated knockouts could be causal in the increasing the susceptibility of these mice to colitis.

We have discussed previously that L13a mediated translational silencing pathway can regulate the expression of several inflammatory chemokines and cytokines, thus playing an essential role in controlling physiological inflammation. In the previous study using endotoxemia model, we had demonstrated that genetic depletion of L13a from the macrophages can result in higher susceptibility to systemic inflammation (Poddar et al., 2013). In this study we have tested the extra-ribosomal anti-inflammatory function of L13a dependent translational silencing pathway in the context of DSS induced experimental colitis. Our results indicate that disruption of the endogenously imposed translational silencing mechanism in the macrophages influences colitis associated pathogenesis and also leads to greater infiltration of these cells in the colonic mucosa. Colonic infiltration of immune cells and disruption of the crypt morphology in the intestinal epithelial cells are important contributors of inflammatory bowel diseases. In the present colitis study, we have experimentally confirmed the increased translational status of some GAIT target mRNAs (e.g., CCL22, CXCL13 and CCR3) in the colons of the DSS treated knockout animals by testing their polysomal abundance. This mechanism could potentially contribute to the increased susceptibility of our knockout mice to colitis.

Till date, GAIT silencing pathway has been studied to operate only in cells of myeloid origin but our results show similar translational regulation of inflammatory genes in the colons upon induction with DSS. Whether DSS triggers synthesis of IFN-γ in-vivo, is an aspect which still needs further exploration. At this point, we are exactly not certain about the mechanism via which the L13a dependent GAIT silencing pathway operates to provide protection against colitis, but our observations indicate a definitive role of several of the GAIT target molecules in increasing the severity of colitis. Additionally, our results also suggest that the absence of this endogenous pathway can exaggerate the colitic response to spread to the entire system leading to septicemia. Taken together, we can say that L13a mediated translational silencing pathway could have evolved as an endogenous defense mechanism to protect against inflammation triggered by various inflammatory stimuli. Modulating specific targets in this pathway can serve as novel means to design future therapeutic strategies against acute and chronic inflammatory diseases.

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CHAPTER IV

DISCUSSION

Inflammation is an obligatory response of the host immune system to protect from invasion by foreign pathogens. Therefore it is critical for the immune cells to synthesize a variety of pro-inflammatory molecules (including chemokines, cytokines, reactive amines, eicosanoids, reactive oxygen and nitrogen species) to mediate the inflammatory response and clear out the pathogens. Where a controlled and regulated inflammatory response is appreciated and beneficial for the host, an unregulated inflammatory response can result in an autoimmune attack thereby proving to be detrimental for the host itself. Macrophage mediated inflammatory response has been implicated in the pathogenesis of several inflammatory diseases like atherosclerosis (Tabas, 2010), Alzheimers, multiple sclerosis (Amor et al., 2010), cancer (Sica et al., 2008), arthritis (Kelly and Ramanan, 2007), endotoxemia (Poddar et al., 2013) and inflammatory bowel diseases (Heinsbroek and Gordon, 2009). It is essential to restrict the uncontrolled synthesis of inflammatory molecules by the immune cells to resolve inflammation. Thus we hypothesize that endogenous defense mechanisms have evolved as crucial checkpoints of inflammation and restore homeostasis. Recent findings from our laboratory had identified one such

mechanism called the Gamma-Interferon Activated Inhibitor of Translation (GAIT) pathway involving active participation of ribosomal protein L13a. In this thesis, we have discussed study where we have tested the importance of the L13a catalyzed GAIT silencing pathway in resolving physiological inflammation. We have used the recently generated macrophage-specific knockout mice model of ribosomal protein L13a (62) to investigate the physiological consequence of abrogating the endogenously imposed silencing mechanism in the macrophages. Our hypothesis behind this experimental design was that depletion of L13a in the macrophages would disrupt the translational silencing of several inflammatory genes (which have been identified as direct targets of the GAIT silencing pathway) resulting in unresolved inflammation in response to inflammatory stimuli. We have used LPS mediated endotoxin challenge and DSS induced experimental colitis as two different inflammatory assaults to investigate the role of L13a dependent translational silencing pathway in providing protection against systemic inflammation (Poddar et al., 2013, and also outlined in detail in chapter 2 of this thesis) and experimental colitis (described in chapter 3 of this thesis).

Our studies using the LPS-induced endotoxemia model and DSS induced experimental colitis have revealed the essential extra-ribosomal role of L13a in the macrophages as an endogenous defense against endotoxin induced systemic inflammation (Poddar et al., 2013) and DSS induced colitis (manuscript under preparation). Since the latter mimics inflammatory bowel diseases in humans, therefore the protective role of L13a in the macrophages can be extended to include various types of IBDs like Crohn's disease and Ulcerative Colitis (UC). LPS is a potent in-vivo inducer of IFN-γ in cells of myeloid origin (Le et al., 1986) and thus can induce IFN-γ stimulated

L13a catalyzed GAIT silencing pathway in the mouse macrophages. Upon treatment with sub-lethal dose of LPS, the macrophage-specific knockout mice of L13a displayed higher susceptibility to endotoxemia showing reduced survival rates, retarded motor activity and high levels of serum TNF-α, BUN and AST (Fig. 6 of chapter 2), common inflammatory markers whose expression is elevated in LPS induced systemic shock (Lu et al., 2006). In the DSS study, treatment of animals with 3% DSS for a week led to enhanced sensitivity of the knockouts to colitis. The DSS administered knockouts displayed clinical symptoms of colitis such as shortening of colons, reduced survival, loss of body weights, higher pathology scores to a greater extent than the DSS treated controls (Figures 18 and 19 of chapter 3). Assessment of colons from the DSS treated animals also showed severe injury to the colon epithelial crypt architecture in the knockout mice. The DSS fed controls also showed some loss of surface epithelia, but to a far lesser extent than the knockouts (Figure 20 of chapter 3). These data suggest that the depletion of L13a in the macrophages increases the severity of inflammatory response in DSS induced colitis just the same as LPS induced systemic inflammation.

Studies over the past few decades have focused on the importance of cytokine and chemokine cascades in acting as endogenous cues to attract and recruit immune cells to sites of inflammation (Shi and Pamer, 2011, Medzhitov, 2010). Chemokines like MIP-1α (macrophage inflammatory protein 1 alpha) and RANTES can act as major chemoattractants for various lymphocytes and cells of myeloid lineage (Cook, 1996) and MIP-1α expression can also be triggered in macrophages in response to LPS challenge (Lukacs et al., 1994). Cytokines like IL-1β has been demonstrated to have essential roles on mediating endotoxic shock response (Lu et al., 2006) and intestinal inflammation

promoting IBD induced diarrhea (Sartor, 1994). IL-6 is a multi-functional cytokine with implicated roles in inflammatory response, cell proliferation, apoptosis and DSS induced colonic inflammation (Kishimoto, 2005). All of these abovementioned chemokines and cytokines (MIP-1α, RANTES, IL-1 and IL-1β) were found to be elevated in the serum of the LPS treated knockouts and in the colons of the DSS administered macrophage-specific L13a knockout mice (Figure 7 of chapter 2 and figure 23 of chapter 3). Our results also indicated the increased level of IL-6 in the serum of the DSS treated KOs (Figure 26 of chapter 3). Our data thereby suggest that the production of several cytokines and chemokines which are not direct targets of the GAIT silencing pathway can be increased upon depleting L13a from the macrophages. Although this might not be a direct consequence of L13a deficiency in the macrophages and just a downstream outcome, nevertheless, the increased levels of these cytokines may contribute toward increasing the severity of inflammation. Such actions could potentially contribute towards the pathogenesis of colitis and septic shock.

Infiltration of leukocytes in the peritoneal tissue is a characteristic trait of inflammation and occurs in response to signals generated by chemokines and cytokines (Shi and Pamer, 2011, Medzhitov, 2010). Injection with LPS showed increased infiltration of macrophages and granulocytes in the peritoneum of the KOs (Figures 8 and 9 of chapter 2). A population of these macrophages were found to be Mac2 positive thus suggesting their pro-inflammatory nature. Spleen acts as a reservoir of immune cells and initiates their precise recruitment to the inflamed tissues upon tissue injury following a microbial attack (Swirski et al., 2009). Our results have shown higher population of CD11b/F4/80, Gr-1/F4/80 and CD11b/Gr-1 double positive macrophages in the splenic

leukocyte population also (Figure 10 of chapter 2). Further analysis has suggested the increase in population of $Ly6C^{hi}$ inflammatory monocytes in the peripheral blood of the LPS treated knockouts (Figure 11 of chapter 2). The $Ly6C^{hi}$ monocytes are immediately recruited to the sites of infection where they differentiate into the classically activated macrophages (CAMs) and induce inflammation (Shi and Pamer, 2011). This implies that disruption of endogenous translational silencing of the inflammatory genes leads to their sustained synthesis which triggers greater migration and abundance of the pro-inflammatory leukocytes in the peripheral tissues. These results are further supported by our additional experiments which have shown extensive tissue damage. The macrophage-specific L13a knockout mice have shown intravascular congestion and infiltration of Mac2 positive macrophages in the lungs and increased infiltration of both Mac2 and Ym1 positive macrophages in the kidney glomeruli upon LPS challenge (Figure 12 of chapter 2). Ym1 expressing macrophages are primarily involved in wound healing (Mosser and Edwards, 2008) and there can be switching between pro and anti-inflammatory macrophages depending of the severity of inflammation. When the generated inflammatory response peaks, then the $Ym1^{+}$ macrophages rush there to initiate a fibrotic response and facilitate wound repair. Greater invasion of the colon mucosa by inflammatory macrophages were also evident in the DSS administered knockout mice (Figure 21 of chapter 3) as ascertained with Mac2 staining. Infiltration of the colon mucosa with leukocytes is a hallmark of colonic inflammation and contributes toward the pathogenesis of IBDs. Taken together our results demonstrate that disruption of the GAIT silencing pathway by knocking out L13a in the macrophages is like deactivation of a

critical checkpoint of inflammation which contributes to the severity of disease progression.

Previous studies from our laboratory using in-vitro cell culture models had identified a cluster of genes encoding for chemokines and chemokine receptors under the translational regulation of the GAIT pathway (Vyas et al., 2009). Further analysis had identified the characteristic GAIT element in the 3'-UTRs of these mRNA encoding inflammatory proteins. Our subsequent studies also revealed that the regulation of this cohort of target mRNAs is mediated through the binding of L13a-dependent RNA binding complex to the GAIT element harbored in the 3'-UTR (Vyas et al., 2009). Our findings using the macrophage-specific knockout murine model for LPS induced endotoxemia and DSS-induced colitis showed increased synthesis and increased translational efficiencies of several of the GAIT target chemokines (Figures 13 and 15 of chapter 2 and Figure 23 of chapter 3). Our experimental findings using lysates made from the macrophages of LPS injected animals and lysates made from the colons of DSS administered mice in in-vitro-translation assays (data not shown) also confirm that the sustained synthesis of the GAIT target inflammatory proteins is due to abrogation of the translational silencing mechanism due to genetic deficiency of L13a in the macrophages (Figure 16 of chapter 2 and data not shown). These results altogether demonstrate that depletion of L13a in the macrophages abrogate the endogenously imposed translation regulation of several inflammatory genes thereby leading to their continued production. This could possibly cause the severity of inflammation and higher susceptibility of the macrophage-specific L13a knockouts to LPS-induced septic shock and DSS-induced colitis.

Post-transcriptional control of many genes implicated in inflammation has been reported and is a subject of interest in the context of our research. These genes are involved in several cellular events connected to inflammation such as activation and recruitment of T cells, incursion of neutrophils, natural killer (NK) cells mediated cellular toxicity, adherence of monocytes and survival of macrophages in damaged tissues (Mazumder et al., 2010, Anderson, 2010). The pathophysiological significance of such regulatory mechanisms have been studied using genetically modified murine models of the TNF-α gene with genetically impaired or deleted AU-rich sequences (AREs) (Kontoyiannis et al., 1999). The AREs present in the 3'-UTRs of several inflammatory mRNAs regulate their stability by recruiting certain RNA binding complexes and hence regulating their translation. Such RNA binding proteins which can modulate the expression of TNF-α have been studied to be TTP (Taylor et al., 1996), TIA-1 (Piecyk et al., 2000) and AUF-1 (Lu et al., 2006). All of these studies have shown overexpression of TNF-α due to the loss of the AU-rich elements and those mice have displayed increased disease pathology even under unchallenged conditions. However, in our study, no such significant differences were observed between the knockouts and their wild type littermate controls under normal unchallenged conditions.

Macrophages have been studied to be crucial in the pathogenesis of several inflammatory diseases including atherosclerosis (Tabas, 2010). However, the endogenous defense mechanism which have evolved to protect against atherosclerosis have been poorly appreciated. Very recently published studies from our laboratory have identified the L13a-catalyzed GAIT translational silencing mechanism in acting as an endogenous defense mechanism against atherosclerosis (Basu et al., 2014). (This was one of my

secondary projects in the course of my Ph.D. research, where I had active participation and I am also the second author in the published manuscript resulting from this study). As our studies using the LPS-induced endotoxemia model had suggested that presence of L13a in the macrophages can act as a physiological attenuator of inflammation, therefore we hypothesized that the same pathway could offer protection against uncontrolled inflammation in atherosclerosis. To test our hypothesis, we used the same genetically engineered murine models of L13a, i.e., the macrophage-specific knockout mice of L13a and crossed them with mice in the ApoE-/- background to generate macrophage-specific KO mice of L13a in the ApoE knockout background. We induced atherosclerosis in these animals by subjecting them to prolonged feeding of high-fat diet, our rationale being that absence of L13a would increase the susceptibility of the knockouts to high-fat diet induced atherosclerosis.

Our results showed that the L13a knockout mice in the ApoE-/- background suffered from more severe atherosclerosis than the controls in the same background. The knockouts also displayed higher atherosclerotic plaque formation in both the aortic sinus and the entire aorta (Basu et al., 2014). The increase in atherosclerotic lesion formation was not due to higher cholesterol uptake by the macrophages or higher levels of plasma cholesterol in the high-fat diet fed knockout mice. The lesion size was also found to be enlarged in the knockouts. The deficiency of L13a in the macrophages in the knockout mice led to infiltration of macrophages and neutrophils in the initimal plaque regions together with disruption of the smooth muscle cells (SMC) layer in the media. Breakdown and thinning of the SMC layer are characteristic features of atherosclerosis (Campbell et al., 2012). Current finding in the field suggest that certain matrix-degrading

metalloproteinases synthesized by macrophages can contribute toward the damage of the smooth muscle cell layer and facilate their migration toward the initima layer (Newby, 2006). Our research till date has not identified any matrix metalloproteinase as direct targets of the L13a mediated GAIT silencing pathway. On the other hand, we have pinpointed CCL3 as one of the targets of the translational silencing mechanism. Research from other laboratories has suggested that CCL3 can be actively involved in governing neutrophil migration into the atherosclerotic plaques (de Jager et al., 2013). In agreement with the previous studies, our results showed increased steady state levels of CCL3 in the plasma of the high-fat diet fed knockouts mice (Basu et al., 2014).

Findings from other laboratories have indicated the importance of Gr-1 positive $Ly6C^{hi}$ inflammatory monocytes have important functions in progression of several inflammatory diseases (Serbina et al., 2008). Also, they have the ability to differentiate into macrophages which subsequently infiltrate into the atherosclerotic plaques (Combadiere et al., 2008, Woollard and Geissmann, 2010). Our results in the atherosclerosis study have demonstrated increased abundance of Gr-1-$Ly6C^{hi}$ inflammatory monocytes in circulation and highly inflammatory Gr-1/Mac2 double positive macrophages in the peritoneum of the high-fat diet fed KO mice in comparison to the controls (Basu et al., 2014). We have also detected increased synthesis of some of the L13a-dependent GAIT pathway target chemokines (CCL11, CCL3 and CCL22) in the cells of the aorta and in circulation of the knockout mice. Macrophages harvested from the high-fat diet fed L13a knockout mice also demonstrated increased polysomal abundance of several of mRNAs (CCL22, CXCL13 and CCR3) which under the regulation of the L13a translation silencing pathway (Basu et al., 2014). These findings

implicate a potential role of the L13a-dependent GAIT mediated translational silencing mechanism in determining severity of atherosclerosis progression.

The high-fat-diet-induced atherosclerotic study in the KO mice therefore identifies an endogenous athero-protective role of ribosomal protein L13a. This additional extra-ribosomal function of L13a is coupled with its role in translational control of a group of genes implicated in inflammation. This is important in the aspect that such coordinated and unanimous action of several molecules grouped together can lead to the pathogenesis of several inflammatory diseases, one of which has been studied to be atherosclerosis. Therefore, targeting of an entire regulatory pathway instead of one individual molecule can provide efficient means of restricting the progression of chronic inflammatory diseases.

Emerging studies in the field have reported the active involvement of several micro-RNAs (miRNAs) in regulating inflammation and contributing to different inflammatory diseases including inflammatory bowel diseases, tumor progression and cancer (Sonkoly and Pivarcsi, 2009, Iborra et al., 2012, Tili et al., 2009, Tili et al., 2008). However, previous findings from our laboratory using in-vitro translation assays with cell-free rabbit reticulocyte extracts and purified recombinant L13a reconstituted the translational silencing mechanism. Also, analysis of the functional GAIT element from several target mRNAs did not reveal any such miRNA binding sites. Therefore we can rule out the possibility of any direct involvement of miRNAs in regulating GAIT pathway mediated translational silencing in our knockout murine models.

Ribosomopathies are a class of diseases occurring due to some defects in ribosome biogenesis and activity (Narla and Ebert, 2010). A disease called Diamond-Blackfan anemia which is characterized by anemia, macrocytosis and reticulocytopenia (Lipton and Ellis, 2009) has been reported to be caused due to mutations in ribosomal proteins RPS19, RPS24, RPS17 and RPL35A (Gazda et al., 2006, Cmejla et al., 2007, Farrar et al., 2008). It has been identified that mutations in RPS19 and RPS25 lead to defects in processing of the 18S rRNA precursor resulting in reduced synthesis of the 40S ribosomal subunit (Choesmel et al., 2007, Flygare et al., 2007). Haploinsufficiencies of specific ribosomal proteins has been linked to irregularities in certain distinct steps in pre-rRNA processing. In this context, recent studies from our laboratory have identified some important residues which is essential for its binding to the ribosome (Das et al., 2013). It has also been suggested that incorporation of ribosomal protein L13a into the 90S pre-ribosome is required for rRNA methylation of the 90S complex (Das et al., 2013). Haploinsufficiency of RPS14 leads to 5q- syndrome (Ebert et al., 2008) whose characteristic traits include macrocytic anemia, elevated platelet levels with slow progression to acute myeloid leukemia (AML). In mice, deficiency of RPL38 has been shown to cause impairment in tissue patterning (Kondrashov et al., 2011). Ribosomal Haploinsufficiency can also result in impaired ribosome biogenesis and build-up of free ribosomal proteins that can bind to MDM2, a repressor of p53. This leads to activation of p53 followed by cell cycle arrest and apoptosis which might progress to anemia (McGowan et al., 2008). Studies also suggest that abnormal maturation of ribosomal subunits can retard translation of the globin genes thereby leading to surplus of free heme and hence anemia (Keel et al., 2008). However, to our knowledge, and based on previous

data from our laboratory, we have not observed any such defects arising due to L13a deficiency other than impairment in rRNA methylation (Chaudhuri et al., 2007).

Our studies therefore identify the essential anti-inflammatory protective role of ribosomal protein L13a mediated translational silencing in controlling physiological inflammation caused by endotoxemia, DSS-induced colitis and high-fat diet induced atherosclerosis. Our gene knockout murine model serves as a novel model to study the uncontrolled inflammation. Any genetic discrepancy in this silencing pathway has the potential to increase the severity of inflammation and contribute to disease pathogenesis. Therefore manipulation of this pathway with small molecule can offer novel therapeutic strategies in future.

CHAPTER V

CONCLUSION

Inflammation is an adaptive response of the immune system to protect the host against invading pathogens. But unresolved inflammation can have adverse effects and lead to an auto-immune attack. Studies over the past several years have focused on the mechanisms contributing to an inflammatory response, but little is known about those which help in controlling inflammation. In our study, we have dissected the physiological importance and the molecular underpinnings of one such recently identified pathways: the L13a-dependent GAIT-mediated translational silencing mechanism. To address the physiological importance of this mechanism in resolving inflammation, we have used the macrophage-specific knockout murine model of ribosomal protein L13a, where the resolution phase of inflammation is severely compromised. We have used this animal model to study the role of L13a in three particular diseases covering acute and chronic inflammation such as LPS-induced endotoxemia, DSS-induce colitis and high-fat diet-induced atherosclerosis. The details of the first two studies have been mentioned in chapters 2 and 3 of this thesis respectively, and only the results of the atherosclerosis study has been included in the discussion section in chapter 4. Our results obtained from

the LPS study show more severe systemic inflammation in the knockout mice in response to inflammation. These include reduced survival rates, high levels of several markers of inflammation, infiltration of macrophages in the kidneys, lungs and peritoneum and abrogation of translational silencing of several chemokine ligands and receptors whose expression is under the control of the L13-dependent translation silencing pathway. Our results from the DSS-induced colitis study support the protective role of L13a in the macrophages against colitis. Significant observations made from this study include severe colitis and disease activity index, higher mortality rates, disruption of epithelial crypt morphology and infiltration of macrophages in the colons of the DSS treated knockouts. Additionally, elevated levels of several pro-inflammatory cytokines and chemokines were found in the colons and serum of the DSS treated knockout animals, thereby demonstrating that the knockouts are more susceptible to DSS-induced colitis. Finally, upon high-fat-diet feeding, the macrophage-specific L13a knockout mice showed higher atherosclerotic plaque formation in the aorta accompanied by destruction of the smooth muscle layer, invasion of macrophages in the intimal layers of the plaques and elevated plasma levels of inflammatory cytokines. This was also followed by increased polysomal abundance of several GAIT target mRNAs in the macrophages harvested from the high-fat-diet fed knockout mice, thus showing a defect in their translation control. Taken together, our results demonstrate that the genetic depletion of L13a in the macrophages can increase the susceptibility to several inflammatory diseases like endotoxemia, atherosclerosis and inflammatory bowel diseases. This occurs possibly due to the abrogation of translational silencing of several inflammatory genes which are under the control of L13a-catalyzed translation silencing mechanism. Therefore, the presence of

L13a in the macrophages can act as a critical checkpoint of physiological inflammation permitting the resolution of inflammation. Further detailed studies about the molecular basis of this pathway could lead to the identification of potential target-mediated modulation of this pathway, which can prevent many acute and chronic inflammatory diseases.

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