NATIONAL AND KAPODISTRIAN UNIVERSITY OF ATHENS
MEDICAL SCHOOL

A study of the function of hnRNP A3 in the myeloid lineage and innate immunity

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May 2019

Acknowledgments

I would like to thank first and foremost Dr. Kontoyiannis for this great opportunity to work in his lab on such an interesting project and for his support and guidance throughout the way.

I must also give my gratitude to Margarita Chatzimike for her constant support and immense help that she generously offered me this past year as well as for the patience she showed throughout all the difficulties.

Special thanks to Dr. Fotis Ioakeimidis and Dr. Margarita Andreadou for their help with the bacterial infection models and many thanks to all the members of the lab, including Dr. Sofia Gargani, Dr. Marannia Saridaki and Giorgos Giagkas, for their advice and assistance whenever I needed them.

I would like also to thank the Transgenics Units at B.S.R.C.Alexander Fleming, for providing the mutant mice and Dr. Sofia Grammenoudi, head of the flow cytometry facility, for her assistance with the flow cytometry experiments.

Finally, I want to thank my parents, Dimitris and Aggeliki, as well as my sister Georgia for supporting me in every way imaginable and for always having faith in me.

Contents

Abstract

The innate immune response against pathogens involves the activation of pro-inflammatory processes that need to be resolved once the threat is removed. The successful regulation of these responses relies on the production and posttranscriptional regulation of mRNAs encoding cytokines and RNA-binding proteins are major components of this posttranscriptional control. In this project, we studied the role of the RNA-binding protein hnRNP A3 under inflammatory conditions. Mice lacking hnRNP A3 in myeloid-lineage cells, which include many cells of the innate immune system, were also infected with bacterial pathogens. At the molecular level, activated macrophages from these mice displayed upregulation of both pro-inflammatory and anti-inflammatory markers. The results of this study indicate that hnRNP A3 is a downregulator of macrophage activation.

Περίληψη

Η αντίδραση της φυσικής ανοσίας σε παθογόνα περιλαμβάνει την ενεργοποίηση προ-φλεγμονωδών αντιδράσεων οι οποίες πρέπει να λυθούν όταν η απειλή δεν είναι πια παρούσα . Η επιτυχής ρύθμιση αυτών των αντιδράσεων βασίζεται στην παραγωγή και μετα-μεταγραφική ρύθμιση των mRNAs που εκφράζουν κυτταροκίνες. Οι πρωτεΐνες που δεσμεύουν RNA είναι βασικά συστατικά του μετα-μεταγραφικού ελέγχου. Σε αυτή την εργασία μελετήθηκε ο ρόλος της hnRNP A3 στη φλεγμονή. Ποντίκια με έλλειψη της hnRNP A3 στα κύτταρα της μυελώδους σειράς, που περιλαμβάνουν πολλά κύτταρα του συστήματος φυσικής ανοσίας, μολύνθηκαν επίσης με βακτηριακά παθογόνα. Σε μοριακό επίπεδο, ενεργοποιημένα μακροφάγα από αυτά τα ποντίκια παρουσίασαν αύξηση τόσο στους προ-φλεγμονώδεις, όσο και στους αντι-φλεγμονώδεις δείκτες. Τα αποτελέσματα αυτής της μελέτης υποδεικνύουν ότι η hnRNP A3 είναι αρνητικός ρυθμιστής της ενεργοποίησης των μακροφάγων.

Introduction

Immunity

The role of the immune system is to defend the organism against the threat of pathogens, whether these are microbes (viruses, bacteria, fungi) or larger parasites. Depending on the nature of the pathogen and the site of infection, different immune responses are activated. The different immune responses are divided into two categories: the innate immunity and the adaptive immunity. The main difference between the two types is their specificity for pathogens; the adaptive immune system has a much higher specificity for a particular pathogen[1,2]. It has also the ability to "remember" pathogens that have infected the organism in the past and quickly and effectively deal with them on a subsequent infection. The two types of immune responses are not independent of each other and in fact interact quite often.

Innate immunity

The innate immune system is the first line of defense against a pathogen. The role of the innate immune system is to prohibit infection from foreign pathogens, recognize and neutralize pathogens that have successfully infected the organism and activate the cells of the adaptive immune system. Physical and chemical barriers stop pathogens from entering the organism[1]. The skin and other epithelial surfaces act as the aforementioned physical barriers. These surfaces are covered in a mucus layer consisted of mucin and other glycoproteins, that prohibit pathogens from adhering on the epithelium, as well as anti-microbial substances such as defensins[1].

The cells of the innate immune system have the ability to recognize pathogens but not with the specificity of the cells of the adaptive immune system. They rely on the recognition of molecules that are common among the various pathogens but absent in the host. These molecules are called pathogen-associated molecular patterns (PAMPs) and include components of cell wall (lipopolysaccharide (LPS), teichoic acids, zymosan, muramyl dipeptide (MDP), chitin etc.), bacterial DNA, bacterial peptides (iEDAP), viral components (poly I:C) etc[1]. Special receptors, called pattern recognition receptors (PRRs), that are expressed in the host's cells can recognize and bind PAMPs. PRRs can be found on the cell membrane, intracellularly or in a soluble form in the blood. There are multiple types of PRRs with the most prominent being the Toll-like receptors (TLRs)[1]. There are multiple TLRs and each one recognizes a different PAMP. Binding of a PAMP on a cellular PRR stimulates the cell to secrete cytokines and other signal molecules that will lead to an inflammatory response at the site of the infection[1].

Immune cells

Both types of immune responses are mediated mainly by **leukocytes**, also known as white blood cells. Like all blood cells, leukocytes are produced in the bone marrow by pluripotent hematopoietic stem cells (HSCs) and their various progenitors and subgroups are characterized by individual or combination of surface proteins specific to each group (Table 1)[1]. For example, HSCs are characterized by the lack of lineage specific markers (Lin^-) and the expression of markers Sca1 and cKit. HSCs give rise to two distinct lineages, the myeloid lineage and the lymphoid lineage (Figure 1). The myeloid progenitors give rise to the cells of innate immunity as well as the erythrocytes and megakaryocytes. Lymphocytes, which are one of the main types of cells of adaptive immunity, originate from the lymphoid line. The various cell types that contribute in immunity are presented in Figure 1.

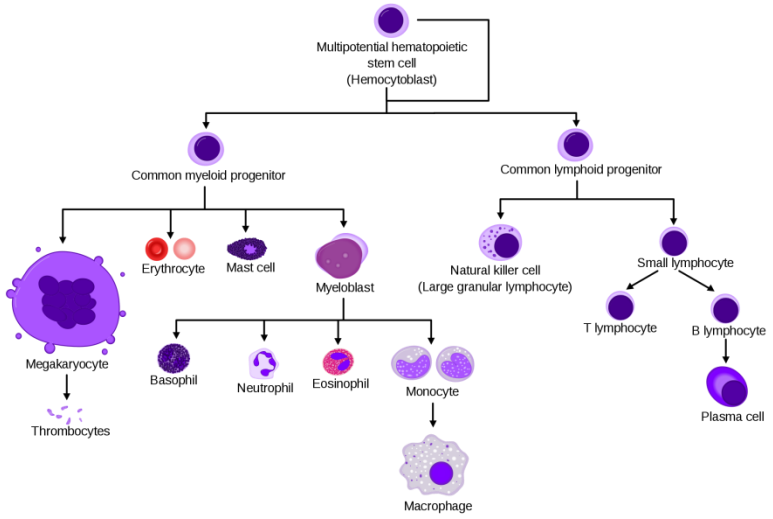


Figure 1: The development of the different blood cells. Hematopoietic stem cells (HSCs) are the progenitors of all blood cells. This cells differentiate to common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs). CMPs give eventually rise to megakaryocytes, erythrocytes, mast cells and myeloblasts. Myeloblasts further differentiate to granulocytes and monocytes/macrophages. CLPs give rise to natural killer cells and to T and B lymphocytes.

Type of progenitor	Surface marker
Hematopoietic stem cell	$Lin^- Sca1^+ cKit^+$
Common lymphoid progenitors	$Lin^- Sca1^+ cKit^{low}$
Myeloid progenitors	$Lin^- Sca1^+ cKit^-$
Common myeloid progenitors	$Lin^- Sca1^+ cKit^- CD34^+ CD16/32^-$
Megakaryocyte/erythrocyte progenitors	$Lin^- Sca1^+ cKit^- CD34^- CD16/32^-$
Granulocyte-macrophage progenitors	$Lin^- Sca1^+ cKit^- CD34^+ CD16/32^+$

Table 1 The main surface markers used to characterize blood cell progenitors. During the various stages of differentiation, expression of certain surface proteins turns on and off. Detection of the various combinations of expression allows for characterization of the main progenitor subgroups.

Innate immunity

i. **Mononuclear phagocytes:** these are long-lived phagocytic cells. Their main function is to engulf particles, whether these are generated by pathogens or dead cells, internalize and dissolve them[1,2]. They can be found in organs and tissues where they

will encounter such particles. **Monocytes** are the mononuclear phagocytic cells that circulate in the blood. The tissue-specific cells of this lineage are the **macrophages** and they can develop either from migrating monocytes or from precursors in the yolk sac during embryonic development[2,3]. Macrophages in different tissues perform different functions and thus have different morphology. Another cell group that originates from monocytes are the **dendritic cells.** These are antigen-presenting cells that act as a bridge between the two types of immunity. They uptake and process antigens and then migrate to lymphoid tissues, where they present the antigen on their surface to lymphocytes, leading to their activation[1].

ii. **Polymorphonuclear granulocytes:** these cells contain granules used to fight off pathogens. They are subdivided into three categories based on their staining with hematoxylin and eosin[2]. **Neutrophils,** which are stained a neutral pink, are the most abundant and the first type of immune cell to respond to an infection[1]. They engulf microorganisms and digest them intracellularly. **Eosinophils** are stained bright red and release the contents of their granules extracellularly. They participate in the immune reaction against pathogens too large to be phagocytosed[1]. **Basophils** are stained dark blue and like eosinophils, they release the contents of their granules extracellularly. They are involved in allergic reactions[1]. A fourth group of granulocytes are the **mast cells**. These cells are located in mucosal and epithelial tissue throughout the body and their function and morphology are similar to that of the basophils', although they develop from a different lineage[1,2].

iii. **Natural killer (NK) cells:** these cells are also lymphocytes and their function is to identify and kill virus-infected or cancerous cells. NK cells can identify infected or cancerous cells[1] and then induce apoptosis. Simultaneously, they help to activate other immune cells[1]. Although they are lymphocytes, they are considered part of the innate immunity, since they don't recognize specific foreign antigens like the rest of the adaptive immune cells.

Adaptive immunity

i. **T cells:** these are lymphocytes that mature in the thymus gland. They are divided in 3 main subgroups of T cells. The **T-helper cells** (Th cells) interact with other immune cells and support their functions[1,2]. The population of T cells that recognize and kill infected cells, are called **T-cytotoxic cells** (Tc cells)[1,2]. The third subgroup is the **T-regulatory cells** (T-reg cells). They are immunosuppressive cells and play an important role in the prevention of autoimmune diseases[1].

ii. **B cells:** these lymphocytes mature in the bone marrow. Each B cell expresses receptors specific for a particular antigen on its surface. Binding of the surface receptor to its target antigen induces the proliferation of this particular B cell and its' differentiation into **plasma cells**[1,2]**.** Plasma cells secrete antibodies that bind to their target antigen leading to its neutralization (i.e. inhibiting a virus from infecting a cell) or in its phagocytosis. Activated B cells that remain after the successful resolution of the infection are called **memory cells**[2].

Type of cell	**Surface markers**
Monocytes/Macrophages	$CD11b^+$ $F4/80^+$ $CD11c^-$ $GR1^-$

Granulocytes	$CD11b^+ GR1^+$
Dendritic cells	$CD11b^+ CD11c^+$
Granulocytic myeloid suppressor cells	$CD11b^+ F4/80^- GR1^+ Lys6C^+$
Monocytic myeloid suppressor cells	$CD11b^+ F4/80^- GR1^- Lys6C^+$
B cells	$B220^+$
Th cells	$CD3^+ CD4^+$
Tc cells	$CD3^+ CD8^+$
Treg cells	$CD3^+ CD25^+$
Activated T cells	$CD3^+ CD69^+ CD44^+$
Naive T cells	$CD3^+ CD62L^+$

Table 2 The main surface markers used to characterize immune cells. The various immune cell groups can be characterized based on the expression of certain surface proteins. Here are presented the main proteins that are used for identification of these groups.

Inflammation

Inflammation is the physiological response to infection and is characterized clinically by swelling, redness, heat and local pain. The first step of inflammation is dilation of the blood vessels, leading to increased blood supply and causing the redness and heat at the area[1]. Next there is an increase in capillary permeability and subsequent exudation of blood proteins that are required to control the inflammation. It's this accumulation of blood proteins that causes the swelling. Additionally, leukocytes are recruited to the site of infection by chemotaxis[1]. The types of cells that are recruited, their number and their time of arrival depend on the type of antigen and the site of infection. Generally, neutrophils are the first cells to arrive and are the predominant population for several days. Mononuclear macrophages arrive at early stages, followed by Tc cells and B cells at later stages[1].

Macrophages-Responses to inflammation

Macrophages play a vital role during inflammation. As mentioned, their main function is to phagocytose pathogens or dead cells. But they are also vital regulators of inflammation. They present antigens to lymphocytes causing their activation and at the same time produce pro-inflammatory cytokines that attract more immune cells at the site of inflammation. At the same time macrophages contribute to the resolution of inflammation and the restoration of the tissue. Macrophage polarization refers to how macrophages are activated at a given point in space and time[4]. Polarization is not fixed and is dependent on the microenvironment of the macrophage and more specifically by the stimuli produced by Th cells. As a result of these stimuli and the stimulation from PAMPs, transcription factors are activated and epigenetic changes occur resulting in activation of certain genes. Combined with post-transcriptional regulation this events lead to polarization towards mainly two phenotypes.

M1 macrophages

Also called classically activated macrophages (CAMs), these macrophages exhibit a pro-inflammatory phenotype. They have anti-microbial and anti-tumoral activity. One of the main markers of M1 polarization is the increased expression of the inducible NO synthase (iNOS), an enzyme that synthesizes nitric oxide (NO) from L-arginine. NO plays various roles in inflammation. It can act as a toxic agent against microbes. Additionally it is a major

immunoregulatory factor. It mediates NK cell functions[5], inhibits mast cell activity[6] and depending on its concentration, it can have either a stimulatory or inhibitory effect on neutrophils[7]. M1 macrophages express many pro-inflammatory cytokines like TNF, IL-6, IL-1β and IL-12 as well as a plethora of chemokines (CCL2, CXCL10,CXCL11 etc.) that further exacerbate the inflammatory response at the site of inflammation[8,9] (Fig.2). Additionally, increased production of reactive oxygen species (ROS) from M1 macrophages seems to aid their phagocytic functions[10,11].

One of the main stimuli toward M1 polarization is interferon-γ (IFN-γ) produced by mainly by Th1 cells. IFNγ binds to its receptor (formed by the IFNGR1 and IFNGR2 chains) which recruits Janus kinase 1 and 2 (JAK1 and JAK2, respectively) which in turn activate signal transducer and activator of transcription1 (STAT1) and interferon regulatory factors (IRFs) like IRF-1 and IRF-8[4,9]. As a result of this cascade, certain pro-inflammatory genes are activated. Most PAMPs polarize macrophages towards the M1 phenotype. LPS is the one studied most extensively. It binds TLR4 and induces gene activation through MyD88 and MaL/Tirap-dependent pathways and the NF-kB pathway. The IFNγ and LPS gene profiles display a degree of overlap but are distinct from each other. Additionally, the combination of IFNγ+LPS induces a gene expression profile distinct from the gene profiles of the individual stimuli[9].

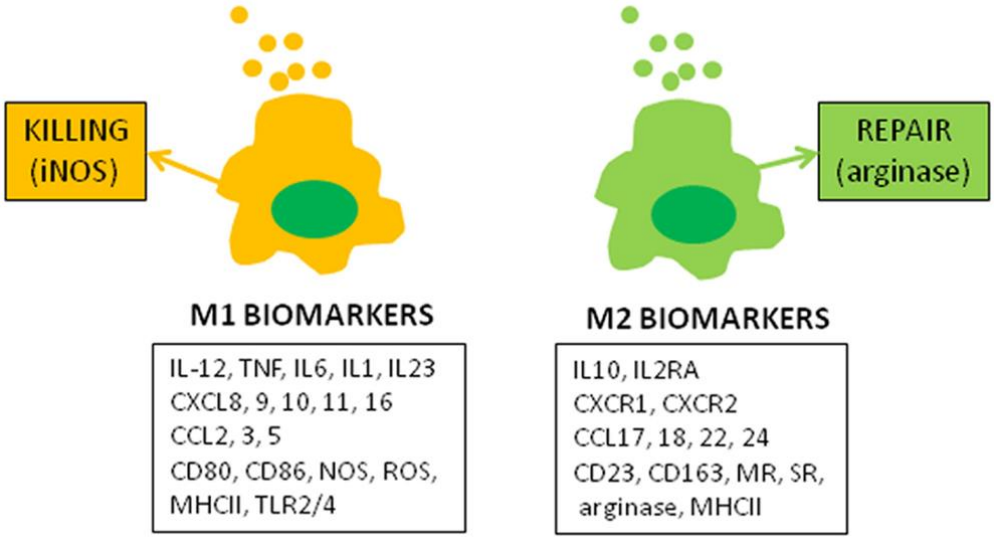


Figure 2: the most important M1 and M2 markers[12]. M1 macrophages have anti-microbial functions and are characterized by increased expression of nitric oxide synthase (iNOS) as well as of pro-inflommatory cytokines (IL-12, TNF, IL-6, IL-1, IL-23) and chemokines (CCL2, CCL3, members of the CXCL family). M2 macrophages have anti-inflammatory functions and are associated with tissue repair. Increased expression of arginase, anti-inflammatory cytokines (IL-10), certain chemokine receptors (CXCR1, CXCR2) and certain chemokines of the CCL family characterize these macrophages.

M2 macrophages

These macrophages are also called alternatively activated macrophages (AAM) and exhibit an anti-inflammatory phenotype. They are associated with the resolution of inflammation, tissue repair, wound healing, asthma, allergies and defense against parasites. These diverse functions are a result of various sub-types of M2 macrophages. A main marker of the M2 phenotype is increased expression of type I arginase (Arg1). This enzyme produces ornithine and urea using L-arginine as a substrate. Ornithine production promotes cell

proliferation and tissue repair through generation of polyamines and collagen. M2 macrophages are also characterized by increase production of anti-inflammatory cytokines, especially IL-10 and TGFβ[11]. Other M2 markers include Ym1, Fizz1, Egr2 and Mrc1[13].

The main stimulus that leads macrophages towards M2 polarization is interleukin-4 (IL-4), which is produced by Th2 cells, eosinophils, basophils and macrophages themselves, and binds on two types of receptors[9]. The Type I receptor consists of the IL-4Rα chain and the common gamma chain (γC), while the type II receptors is a complex of IL-4Rα with IL-13Rα1[14]. Binding of IL-4 to the type I receptor phosphorylates JAK1/3, which in turn phosphorylates tyrosine. Phosphorylated tyrosine lead to phosphorylation of STAT6 and IRS-2. Phosphorylated STAT6 units homodimerize, translocate to the nucleus and initiate gene transcription[14]. Phosphorylation of IRS-2 leads to activation of PI3-K, AKT and NF-κB-driven gene transcription[14]. Binding to the type II receptor also phosphorylates JAK1 as well as JAK2/TYK2 leading to activation and homodimerization of STAT6, STAT3 and STAT1 and then nuclear translocation and gene transcription[14].

Post-transcriptional regulation

During the initial stages of inflammation, macrophages acquire the M1 phenotype. When the threat has been neutralized, they acquire the M2 phenotype in order to restore homeostasis. The balance between the two states is very important for the organism's homeostasis and disruption of this balance could lead to serious health problems[15]. Thus, macrophage polarization must be tightly controlled.

The control of gene expression that is required during polarization could be achieved by two ways. Firstly, epigenetic changes could allow for transcription of certain genes and inhibition of expression of other genes. Indeed, TLR stimulation induces histone modifications that lead to pro-inflammatory gene expression[15]. Alternatively, regulation of the expression of RNA transcripts could affect gene expression in a short time span and in a reversible way[15]. Given the rapid phenotypic changes that are required during macrophage polarization, this seems to be the ideal way to regulate gene expression. In contrast, the modification of histones is a complex process that cannot be rapidly readjusted.

Post-transcriptional regulation is achieved through four main processes: splicing, editing, decay and translation[16]. Splicing refers to the process of removal of introns from pre-mRNAs and joining of the remaining exons[17]. Alternative splicing can give rise to different mRNAs originating from the same pre-mRNA. RNA is also subjected to various modifications such as the addition of a 5' 7-methylguanosine cap, the addition of a poly-adenosine tail (poly (A) tail) at the 3' terminal and various other nucleoside modifications.[17] The cap and the poly (A) tail contribute to the stability of the mRNA and the cap is also necessary for the initiation of translation[17]. Removal of either the cap or the poly (A) tail leads to degradation from exonucleases[17]. Another mechanism of RNA decay is through endonuclease degradation. In order for translation to occur, the mRNAs must translocate from the nucleus to the cytoplasm and then the ribosome must be assembled.

RNA Binding proteins

RNA binding proteins (RBPs) play crucial role in all of these processes. More than 1,000 RBPs have been identified so far in the human genome and as the name suggests, these proteins bind to RNA on specific sequences[18]. A lot of RBPs can bind AU-rich cis-elements (AREs) that are found at the 3'UTR regions of many mRNAs, contributing to their stability[19]. Aside from their RNA-recognition domains, most RBPs are capable for protein-protein interaction with various enzymes and factors and some have some catalytic functions themselves[16]. Thus, binding of an RBP on an RNA can result to recruiting other proteins creating a bigger RNA-protein complex. These complexes are dynamic structures and their composition and subsequently their function can change depending on the state of the cell[16].

RBPs have many diverse roles in innate immunity. Some RBPs, like CIRP and the ADAR enzymes, are components of PPRs in the cell surface (CIRP) or intracellularly (ADAR enzymes)[20,21]. Moreover, some RBPs can be activated by the signaling pathways activated by TLRs and then regulate the same pathways[16]. Several inflammatory mRNAs have been found to contain ARE sequences[22] bringing their expression under the control of RBPs. The degradation of inflammatory mRNAs is the most prominent way of control. Some endonucleases like regnase-I are deactivated by phosphorylation after TLR activation[16]. Lack of further stimuli re-activates regnase-I and it proceeds to inhibit pro-inflammatory mRNAs[16]. Tristetraprolin (TTP) is another RBP that positively regulates mRNA decay by recruiting decapping and deadenylation complexes[23]. Deletion of TTP leads to increased stability of pro-inflammatory mRNAs resulting in sensitivity to acute inflammation[15,24]. Regulation of translation plays also a major role in the regulation of the immune responses. RBPs like T-cell intracellular antigen-1 and –R (TIA-1 and TIAR) are involved in translation and act as translational silencers of pro-inflammatory mRNAs by binding on U-rich areas in their 3'-UTR region[25,26]. TIA-1 knock-out mice display sensitivity to LPS-induced endotoxemia[26]. Other major regulators of inflammation are the Human antigen R (HuR) and the hnRNP family.

The HuR protein

HuR (also known as Elavl1) is a RBP that binds AU- and U-rich sequences of RNA. This protein appears to act both as stabilizer and destabilizer of mRNAs, with its role often depending on its interaction with other proteins[27–29]. Deletion of HuR can cause major defects during or after embryogenesis, as well as in the maturation of T cells[30,31]. Moreover, it has been shown to downregulate inflammation. Lack of HuR leads to an exacerbated inflammatory response with an increase in expression of pro-inflammatory cytokines such as TNFa and IL-6[32]. On the other side, overexpression of HuR suppresses the expression of pro-inflammatory reactions[32,33]. It appears that interaction with other RBPs is crucial for these functions of HuR[33].

The hnRNP family

The family of hetero-nuclear ribonucleoproteins (hnRNPs) is a family of RBPs consisting of more than 20 members. Members of this family have been shown to be involved in transcription, splicing, telomere formation, mRNA translocation and translation. They have been categorized based on their structure and their RNA-binding domains (RBDs) composition.

There are four main RBDs that are found in hnRNPs: the RNA recognition motif (RRM), the quasi-RRM, the K-Homology (KH) domain and the RGG box. Additionally, auxiliary domains enriched in glycine, proline or acids are in close proximity to those RBDs and are responsible for protein-protein interactions. The composition and combination of these domains define the RNA specificity of each hnRNP.

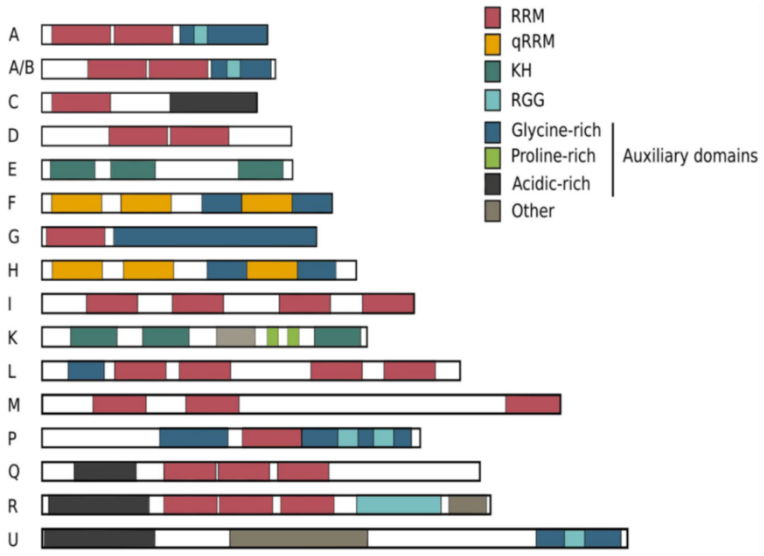


Figure 3. The hnRNP family and its RNA-binding domains The members of the family are named alphabetically from hnRNP A1 to hnRNP U. They are built up of four RNA-binding domains (RBDs). Multiple members carry the same RBDs, explaining their shared RNA-binding properties. *RRM* RNA recognition motif, *qRRM* quasi-RNA recognition motif *KH* K-homology domain, *RGG* RNA-binding domain consisting of Arg-Gly-Gly repeats. [36]

The hnRNP A/B subfamily

The hnRNP A/B subfamily contains four main members: hnRNP A1, A2/B1, A3 and A0. The members of these subfamily exhibit high levels of sequence and structure similarity, and are characterized by two RRM domains in tandem at the N-terminus followed by a RGG box with a glycine-rich auxiliary domain (GRD) at the C-terminus[34]. This structure enables the hnRNP A/B proteins to interact with other proteins and nucleic acids[35].

The hnRNP A/B proteins are some of the most abundant hnRNPs in a cell and have been associated with various functions. Like many other RBPs, they recognize and bind ARE sequences[36]. Additionally, multiple hnRNP A/B proteins participate in mRNA transport by binding through the combined activity of their RRMs on a 21-nucleotide-long element, called the A2-response element (A2RE), that is present in several mRNAs[34,37]. Other cellular functions that the hnRNP A/B proteins have also been associated with are telomere formation, alternative RNA splicing, regulation of transcription and translation[35,38–41]. Their significant role in gene regulation means that dysregulations in their expression could lead to various forms of cancer. Increase of expression of hnRNP A1 has been associated with lung cancer and more specifically with increase in tumor proliferation[42,43]. Similarly, knockdown of hnRNP A2/B1 decreases cell invasion of breast cancer cells[44]. In addition, several oncogenes have been identified as direct targets of hnRNP A1[36]. Many members of this family have also been associated with neurodegenerative diseases. In rare cases, patients with frontotemporal lobar

degeneration (FLD) have been found to carry mutations in hnRNP A1 and hnRNP A2/B1 that cause massive self-aggregation of the proteins[45]. More frequently, mutations in target mRNAs lead to increased binding and accumulation of members of this subfamily[36,46]. Autoantibodies for several hnRNP A/B proteins have also been detected in the serum of patients with rheumatoid arthritis[47].

hnRNP A3: structure, functions and role in diseases

HnRNP A3 is one of the less studied members of the hnRNP A/B subfamily. It was first discovered in *Xenopus* in 1993[48]. Four isoforms of hnRNP A3 have been detected at 38, 39, 41 and 41,5 kDa[49]. As a member of the hnRNP A/B subfamily, it has two RRM domains at the N-terminus and a RGG box with a glycine-rich domain at the C-terminus. This glycine-rich region is phosphorylated through the SAPK2a/p38 pathway and also exhibits a high degree of similarity with the other members of the family. More specifically, it is evolutionarily closer to hnRNP A1, with their RRMs having up to 85% overlap, but its GRD exhibits higher resemblance with hnRNP A2's GRD[48,50].

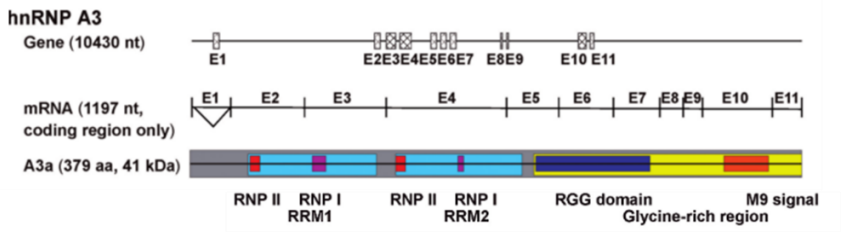


Figure 4 Structure of hnRNP A3 (E: exon)

Like its structure, hnRNP A3's functional role is similar to those of the other hnRNP A/B members. It binds A2REs, contributing in the shuttling of RNAs[49,50]. It has also been shown that hnRNP A3 binds to single-stranded telomeric repeats, acting as a negative regulator of telomere length maintenance[41,51]. Additionally, it binds AREs on many mRNAs, including some pro-inflammatory mRNAs, and thus plays a role in regulation of mRNA degradation[19,52]. Another biological mechanism that hnRNP A3 has been associated with is age-related regulation of gene expression. It binds age-related increase elements (AIEs) which are stretches of dinucleotide repeats in the 3'-UTR region[53].

Dysregulation of the functions of hnRNP A3 have been linked with multiple diseases. Like other members of the hnRNP A/B subfamily, It has been shown that hnRNP A3 participates in the inclusion bodies in the brains of patients with neurodegenerative diseases such as frontotemporal lobar degeneration (FLD) and amyotrophic lateral sclerosis (ALS)[46,54]. More specifically, hnRNP A3 seems to bind to GGGGCC repeats on the mutated *C9orf72* leading to accumulation of hnRNP A3 both in the cytoplasm and the nucleus[46,54]. Overexpression of hnRNP A3 has been linked to colorectal and non-small lung cell cancer[43,55]. Additionally, hnRNP A3 has been shown to bind in RNA-dependent way to APOBEC3B (apolipoprotein B mRNA-editing enzyme, catalytic subunit3B),a cytosine deaminase whose dysregulation leads to hypermutations and cancer in various tissues[56]. HnRNP A3 is also one of the hnRNP A/B members that has been identified as an autoantigen in rheumatoid arthritis patients[47].

Aims of the study

HnRNP A3 is one of the least researched members of the hnRNP protein family and thus information on its function is scarce. Previous experiments in Dr. Kontoyiannis' lab, using mass spectrometry and subsequent immunoprecipitation in the presence of RNase, have confirmed that HuR directly binds hnRNP A3 after M1 macrophage activation. HuR is a known regulator of inflammation so this interaction raised questions about hnRNP A3's role in inflammation and macrophage activation. Does hnRNP A3 play any role in macrophage physiology and activation? Is it a regulator of inflammation? If yes, does it downregulate inflammation like HuR or does it have a different role? In this study*, in vitro* and *in vivo* methods were used to answer these questions and to shed some light on the functions of this protein.

Materials & Methods

Cell lines

RAW 264.7 macrophages which had the hnRNP A3 gene removed using the CRISPR/Cas9 method by Margarita Chatzimike in our lab, were cultured in DMEM + 10%FBS and were polarized towards the M1 state with LPS (100ng/μl), LPS (100ng/μl) combined with IFNγ (10ng/μl) and towards the M2 state with IL-4 (10ng/μl).

Mice

Mice of the C57BL strain were used in this project. Embryonic stem cells provided by EUCOMM were used to create knock-out lines. Conditional and complete hnRNP A3 knock-out mice had been created as follows: a cassette was inserted between the first and the second exon of the hnRNP A3 gene through homologous recombination on the embryonic stage (Figure 5). This cassette consists of a FRT cleavage site, an en2 splicing acceptor, an internal ribosome entry site, the lacZ gene, a poly-A sequence, a loxP site, followed by the antibiotic resistance genes hBact and neo, and then another FRT and loxP site. A third loxP is also inserted between exons 7 and 8. This genotype is referred to as tm1a. In the presence of the FLP recombinase, cleavage occurs at the FRT site. This genotype is referred to as tm1c or flox and leads to expression of hnRNP A3. Cre recombinase cleaves at the loxP sites leading to removal of exons 2 to 7 and thus deletion of functional hnRNP A3. Expression of Cre under the control of lysozyme leads to Cre expression only on the cells of the myeloid lineage and thus to a conditional hnRNP A3 knock-out. Treatment of the tm1a genotype with Cre leads to the tm1b genotype which expresses LacZ and acts as a reporter genotype.

Genotyping was performed using the PCR method and the primers used were as follows:

Tm1a and tm1c

Primer1: 5'-ATTTCTGCATTGCTGGGAATC-3'

Primer2: 5'-TCTACTAAGCCAAGGACAACTAAC-3'

Tm1d

Primer1: 5'-ATTTCTGCATTGCTGGGAATC-3'

Primer2: 5'-TCTACTAAGCCAAGGACAACTAAC-3'

Primer3: 5'-ATTCCTCTGGTCAGGCGGTA-3'

Cre

Cre-F: 5'-ATT-ACC-GGT-CGA-TGC-AAC-GAG-T-3'

Cre-R: 5’-CAG-GTA-TCT-CTG-ACC-AGA-GTC-A-3’

LysMCre

LysMCre-F: 5’-CTT-GGG-CTG-CCA-GAA-TTT-CTC-3’

LysMCre-R: 5’-CAG-GTA-TGC-TCA-GAA-AAC-GCC-T-3’

LacZ

LacZ-F: 5’-GATCCCGTCGTTTTACAACGTCGT-3’

LacZ-R: 5’-GAACTTCAGCCTCCAGTACAGCGC-3’

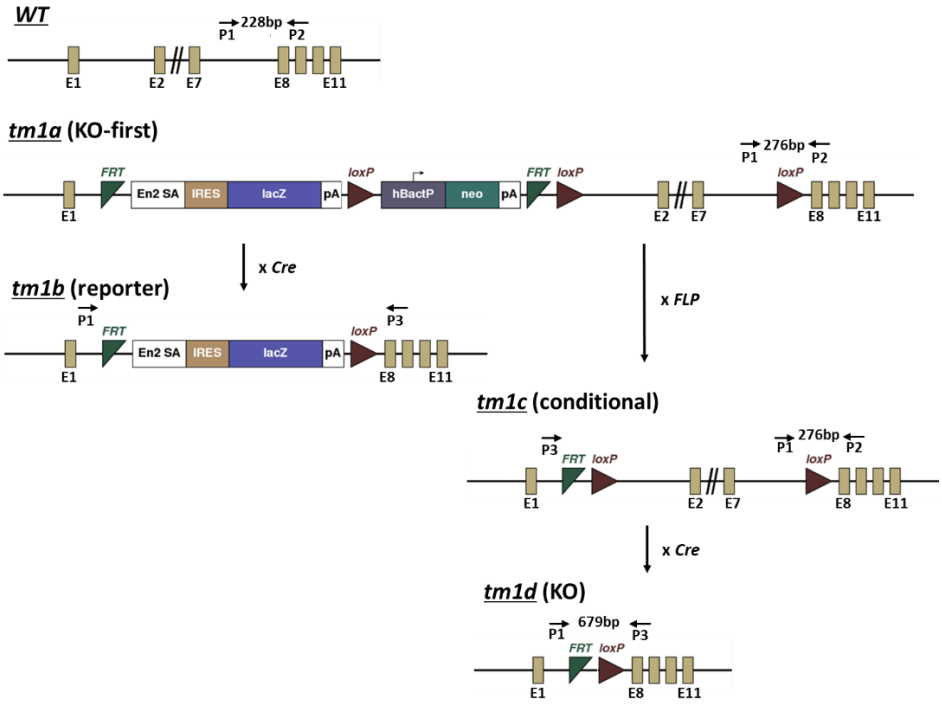


Figure 5 A representation of the process leading to complete and conditional hnRNP A3 knock-out mice. Also presented are the primers used for genotyping: *P1* primer1, *P2* primer2, *P3* primer3

DNA extraction for genotyping

Tail or ear biopsies are acquired from at least 10-days old mice. 500µl of Tail Lysis Buffer + 3µl Proteinase K (10mg/ml) are added and the samples are left at 55°C overnight. Then, after good vortexing, 139µL 6M NaCl are added. Mixing and centrifugation at 13000 rpm for 10min at room temperature follow. The supernatant is transferred in a new tube and 1 volume of cold ethanol 95% (EtOH) is added. Mixing and centrifugation follow, as before. The pellet is rinsed by adding 75% EtOH and centrifuging as before. After the pellet is left to dry, it is dissolved in approximately 50µl ddH_2O (volume depends on pellet size).

Bone Marrow Derived Macrophages

Bone Marrow was extracted from mice femurs and pelvis bones by flushing them with 1xPBS. After treatment with Gey's solution for 5min to remove the erythrocytes, the remaining cells were plated in Petri dishes with complete RPMI (RPMI 1640 – 10, 10% L-medium from L929 cells containing M-CSF (the percentage depends on the batch), 5% Fetal Bovine Serum(FBS), 1% streptavidin, 1% ampicillin and 2mM glutamine. On day 3 fresh complete RPMI is added without removing the original medium. On day 6 the supernatant is removed and fresh complete RPMI is added. After 8 days the bone marrow progenitors differentiate into macrophages and are plated depending on the experiment, without L-medium.

L-medium was produced as follows:

L-medium from L929 cells

- Day 1.

Thaw L929 fibroblasts onto 10cm^2 TC dish in 10 ml of *DMEM-10%FBS (+P/S +L-glut)*

- Day 2 (90% confluency) .

Split 1:4 in TC dishes with 8ml DMEM-10%FBS. (Split protocol: discard supernatant, wash with 1x PBS, remove PBS, add 0,8ml tripsin, incubate 37oC for 5min, add 2-4ml DMEM +FBS, collect cells in 15ml tube, spin 1200rpm 5min, resuspend and seed)

- Day 4 (90% confluency):

Freeze in FBS 10%DMSO 1 plate and return to stock -80^o C. Split the rest 3 1:6 in 10cm TC dishes (=18 plates).

- Day 6 (70%-80% confluency):

Aspirate supernatant and feed them with 11 ml *RPMI-5%FBS* in each plate.

- Day 8:

After 48h collect the supernatants (11x18= 198ml) and store at 4oC. (batch #1). Add fresh 9 ml *RPMI-5%FBS*

- Day 10:

After 48h collect the supernatants (batch #2) (11x18= 198ml) and mix with batch #1 (=400ml total). Pass through a 0.45μM filter and aliquot (5x50ml falcon 8x15ml falcon) and store at -80^{0}C for UP TO 6 months.

- The filtered supernatants is the L-medium

Test L-medium (by experience the L-medium is used at about 7,5%-10%):

- Collect BM from 1 mice.
- Seed 5x10^6 cells per petri dish with 5%, 7,5%, 10% 12,5% 15% and 20% L-medium
- Procced with differentiation protocol for each concentration of L-medium.
- After differentiation A) inspect the dishes in the microscope. B) Collect the cells and count. Determine the yield C) test for NO production with Griess D) stain for live dead.

Taking in to consideration all the above tests, determine the best concentration

Peritoneal macrophages

Live mice are injected intraperitoneally with 4% thioglycollate to induce aseptic inflammation. Three days later the mice are sacrificed and the peritoneum is washed with 1xPBS to collect the macrophages. The macrophages can then be plated in complete RPMI,

Protein extraction

Cells that have been stimulated for 24h with LPS, LPS+IFNγ and IL-4 are lysed with RIPA buffer. Then the extracts are sonicated 3 times at 40% of full power using a sonicator to further lyse the nuclei. The protein extracts are collected after centrifugation at 13.000rpm for 5min at 4°C and discarding the pellet.

Bradford assay

The protein concentration of the extracts is quantified using the Coomassie Brilliant Blue G-250 dye. This dye allows for colorimetric calculation of the protein concentration by binding with proteins and changing color from brown to blue. The protein samples are diluted 1:10 in a 96-well plate. The Coomassie reagent is added bringing the final volume to 20 µl. Using serial dilutions, a standard curve is created with bovine serum albumin (BSA) (starting concentration=1mg/ml) in dublo. The product of the reaction can be measured at 595nm and using the standard curve the proteins extracts are quantified.

Coomassie staining

Based on the Bradford assay calculations, samples are prepared for SDS-PAGE electrophoresis; the appropriate volume of extract is mixed with Laemli sample buffer (final concentration 1x), heated at 57^{o}C for 5min, spinned at max speed for a few seconds and then loaded on the electrophoresis gel. The sample buffer charges all the protein molecules negatively and combined with the heating step disrupts their secondary structures. The gel is composed of polyacrylamide and it consists of the stacking and the running part. The electrophoresis takes place initially at 80V and then at 120V (once the proteins start to enter the running gel) for about an hour using an electrophoresis apparatus and 1x Running buffer. During that time the various proteins are separated based on their molecular weight; smaller proteins move faster through the porous gel and thus are at the lowest part of the gel. After the electrophoresis the gel is stained with Coomassie Blue R-250 for 10 min. Then the gel is placed in destain buffer overnight (O/N) to remove excess dye. Coomassie Blue R-250 binds to the proteins on the gel thus allowing their visualization. The gel is then photographed and analyzed using the ImageJ software. This method allows for more accurate quantification of the protein extracts compared with the Bradford method.

Western blot

The western blot's first step is an SDS-PAGE electrophoresis as was described in the previous method. After the electrophoresis the negatively charged proteins on the gel are transferred on a nitrocellulose membrane with the use of electrical current (300mA, 2h). A

transfer machine is used and 1xTransfer buffer, 20%Methanol. Then the membrane is washed with 1xTBS-0,1%Tween (TBS-T), blocked with 5% milk in TBS-T and incubated with an antibody O/N. The next day, two membrane is washed and then incubated with the horseradish peroxidase-conjugated secondary antibody for 1h. After another round of washes, ECL is added on the membrane and the product if the reaction is visualized on the Chemidoc. If another antibody must be used on the same membrane then the membrane must be stripped and blocked again. The antibodies used were: rabbit anti-hnRNP A3 (Abcam), mouse anti-GAPDH (AMBION)

RNA isolation

Cells that have been stimulated for 6h with LPS (100ng/μl), IFNγ+LPS (10ng/μl and 100ng/μl, respectively) or IL-4 (10ng/μl) are lysed with Trizol reagent. Chloroform is added to the solution and after mixing and centrifugation at 14.000rpm for 5min at 4°C, the aquatic phase is transferred on a new tube. The RNA is precipitated with 1 volume of ice cold isopropanol and centrifugation at 14.000rpm for 5min at 4°C. Then the supernatant is discarded and the pellet is washed with 70% ethanol and centrifugation at 14.000rpm for 5min at 4°C. The supernatant is once again discarded and it is dissolved in water.

cDNA synthesis

Approximately 1μg of RNA is mixed with oligodT primers and ddH_2O and placed at 70°C for 5min. Then M-MLV reverse transcriptase, its buffer and deoxynucleotide triphosphate (dNTPs) are mixed with the first mixture and are incubated at 37°C for 1h.

qPCR

The synthesized cDNA was diluted 1:20. 2μl of the diluted cDNA were mixed with EvaGreen mixture, and the appropriate primers. Duplicates were prepared for each sample. The reactions were performed with Rotor-Gene 6000 and the results were analyzed with the Rotor-Gene 6000 software.

Gene	Forward primer	Reverse primer
B2M	5'-TTCTGGTGCTTGTCTCACTGA-3'	5'-CAGTATGTTCGGCTTCCCATTC-3'
TNF-a	5'-CACGCTCTTCTGTCTACTGA-3'	5'-ATCTGAGTGTGAGGGTCTGG-3'
Arginase	5'-CCAGAAGAATGGAAGAGTCAGTGT-3'	5'-GCAGATATGCAGGGAGTCACC-3'
IL-6		
IL-12b	5'-TGT CCT CAG AAG CTA ACC AT-3'	5'-CCA GTC CAC CTC TAC AAC AT-3'
CCL2	5'-AGC ACC AGC ACC AGC CAA CT-3'	5'-TTC CTT CTT GGG GTC AGC AC-3'
IL-1b	5'-GCAACTGTTCCTGAACTCAACT-3'	5'-ATCTTTTGGGGTCCGTCAACT-3'
Fizz1	5'-CTGCCCTGCTGGGATGACT-3'	5'-CATCATATCAAAGCTGGGTTCTCC-3'
Mrc1	5'-ACTGCGTGGTTATGAAAGGC-3'	5'-TGAGCGACGAGTACAAGATGC-3'
INOS	5'-ATG GCT TGC CCC TGG AAG TT-3'	5'-TGA TGG ACC CCA AGC AAG AC-3'

Table 3 The genes that were studied with qPCR and the primers used for each.

Flow cytometry

Blood, spleen and bone marrow were isolated from mice and their immune cell populations were analyzed using flow cytometry. The isolated blood and spleen cells are initially blocked with Fc block mix but not the bone marrow cells. The mix of antibodies is then added in FACS buffer each sample for 30min. Unstained and single-stain samples are prepared as well. After washes with FACS buffer the mix of secondary antibodies is added for another 30min. After washes with FACS buffer, the cells are resuspended in 1xPBS and are ready for flow cytometer. The data were analysed using the FloJo v10 software.

The antibodies used were:

Blood

- GR1 (Ly-6G) – FITC (Biolegend)
- CD11c-PE-Cy5 (Biolegend)
- CD3 - APC-Cy7 (Biolegend)
- CD11b (Mac-1) - A647 (Biolegend)
- B220 – biotin (Biolegend), streptavidin-PE (BD)

Spleen

1)

- GR1 (Ly-6G) – FITC (Biolegend)
- CD11c-PE-Cy5 (Biolegend)
- CD3 - APC-Cy7 (Biolegend)
- CD11b (Mac-1) - A647 (Biolegend)
- B220 – biotin (Biolegend), streptavidin-PE (BD)

2)

- CD62L-Biotin (eBioscience)
- CD25-PE (Biolegend)
- CD69-PE-Cy7 (Pharmigen)
- CD44-APC (eBioscience)
- CD4-Alexa 700 (Biolegend)
- CD8-APC-Cy7 (BD)

3)

- CD11b-APC-Cy7 (eBioscience)
- F4/80-PE-Cy7 (Biolegend)
- Ly6C-biotin (Pharmigen)
- GR1-FITC (Biolegend)
- CD54-APC (Biolegend)

Bone marrow

- CD34-FITC (BD)
- Sca-1-PE (eBioscience)
- Lin Biotin, streptavidin-APC (BD)
- ckit-PE-Cy7 (Biolegend)
- CD16/32-PE-Cy5,5 (eBioscience)

Griess assay

A method to study the M1 polarization is by measuring the production of NO. $4x10^4$ cells/96 well are plated and stimulated towards the M1 state with LPS (100ng/µl) and IFNγ+LPS (10ng/µl and 100ng/µl, respectively) for 24h. 100ml of the supernatant are transferred in a new 96-well plate. In a separate set of wells standard curve in dublo with culture medium using a nitrite standard is prepared. Then 100µl are of Griess reagent are added in each well. This reagent can, in the presence of NO2-, a byproduct of NO metabolism, produce a dye product which can be detected spectrometrically at 540nm. Using the standard curve the concentration of NO can be calculated.

Arginase activity assay

Increase of the activity of the arginase enzyme is one of the main characteristics of the M2 state. Cells that have been stimulated with IL-4 for 24h are lysed with 30µl of lysis buffer and left on a rocking platform for 15min. The cells are then placed at 56°C for 7min to activate arginase. 30 µl of L-arginine 0,5M, arginase's substrate, are added to the lysate. After incubation at 37°C for 1h the reaction is stopped with an acid mix of H_3PO_4, H_2SO_4 and H_2O (1:3:7). Urea standards are created as well. A-isonitropropiophenone 6% (a-ISPP) in 100% ethanol is added on the samples and then paraffin oil is added to prevent evaporation. The samples are placed then at 90°C for 30min and then at 4°C for another 30min. The result of this last reaction is colored product which can be measured at 540 nm.

ELISA

To measure the cytokine levels produced during polarization the ELISA method was used. 96-well plates are first incubated with the capture antibody O/N at 4°C. Then blocking buffer (1xPBS, 1%BSA) was used to block the plate for at least one hour. The medium of cells stimulated with LPS, LPS+IFNγ or IL-4 is collected after 24h. Each sample is diluted in triplicate replicas and additionally a standard curve of known concentration is prepared in dublo. The plate is left to incubate O/N at 4°C. The next day the detection antibody was added and was left to incubate for 2h at room temperature. Then streptavidin was added for 45min. O-phenylenediamine dihydrochloride (OPD), streptavidin's substrate, is added resulting in the production of a colored product. The reaction is stopped with acid before saturation. Then the absorbance can be read at 450nm. It should be noted that between each step up until the addition of OPD the plate is washed three times with wash buffer (1xPBS, 0,05% Tween-20). The ELISA kits that were used were: TNFα (Peprotech), IL-6 (Peprotech), IL-1β (eBioscience) and CCL2 (Peprotech).

In vivo endotoxemia

Lys-Cre;$A3^{f/f}$ mice were injected intraperitoneally with 350μg LPS/25g of body weight. $A3^{f/f}$ mice were used as control. Body temperature was monitored for 8h post-injection and mortality was monitored for 6 days post-injection. The experiment was repeated with 300μg LPS/g of body weight and once again the rate of mortality was monitored for 6 days post-injection.

Citrobacter rodentium infection

Citrobacter rodentium is a bacterium that infects the large intestine of mice and causes inflammation. Lys-Cre;$A3^{f/f}$ mice and control mice were infected with the bacterium ($1x10^9$ colony forming units (C.F.U.s)) via oral gavage. On days 3 and 6 post-infection the mice were weighted and feces were collected. The feces were weighted and homogenized in 1xPBS and then were serially diluted six times. The last five dilutions were plated in LB agar plates in dublo and the plates were left O/N in an incubator. The next day the bacterial colonies were counted. On day 9 the mice were weighed and their feces were processed as on the previous days. Then the mice were sacrificed and their liver and spleen were collected. These organs were homogenized and then plated on MacConkey plates and were left O/N in the incubator. The next day the number of colonies was measured.

Listeria monocytogenes infection

Listeria monocytogenes is an intracellular foodborne pathogen that can infect mammals and cause inflammation in various organs. Lys-Cre;$A3^{f/f}$ mice and their controls were infected with $5x10^6$ CFUs of *Listeria* via intraperitoneal injection and their mortality was monitored for 12 days post-infection.

Reagent	Recipe
Tail Lysis Buffer (500ml)	25ml Tris 1M pH 8 100ml EDTA 0,5M 10ml NaCl 5M 50ml SDS 10%
Gey's solution (1L)	**200ml Solution A (1000ml filtered)** 35g $(NH_4)Cl$ 1,85g KCl 1,5g Na_2HPO_4 0,12g KH_2PO_4 5g glucose 50mg Phenol Red **50ml Solution B (100ml autoclaved)** 0,42g $MgCl_2H_2O$ 0,14g $MgSO_47H_2O$ 0,34g $CaCl_2$ **50ml Solution C (100ml autoclaved)** 2,25g $NaHCO_3$ **700ml H_2O for injection**

Thioglycolate 4% (100ml)	4g in 100ml ddH_2O Boil to dissolve Sterilize
10xPBS (1L)	Mix 80g NaCl and 2g KCl Mix 14,4g Na_2HPO_4 and 2,4g KH_2PO_4 Mix all together pH≈7,4 (do not adjust) Autoclave
RIPA buffer	50mM Tris pH 7,5 150mM NaCl 1mM EDTA 10% glycerol 1% NP-40 0,25% sodium deoxycholate Add PMSF (1mM) and protease inhibitors (1x) right before using
Coomasie Brilliant Blue G-250	Mix 100mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol Add 100ml 85% phosphoric acid and mix Add 850ml ddH_2O Filter
2xLaemli sample buffer	0,125M Tris-HCl pH 6.8 4% SDS 20% Glycerol 10% β-Merc-OH ~0,05% Bromophenol Blue
Polyacrylamide gel	**10% Running gel (10ml)** 4ml ddH_2O 3,3ml 30% acrylamide 2,5ml 1,5M Tris-HCl pH 8,8 100µl 10% SDS 100µl 10%APS 10µl TEMED **4% Stacking gel (6ml)** 3,6ml ddH_2O 0,8ml 30% acrylamide 1,5ml 0,5M Tris-HCl pH 6,8 60µl 10% SDS 60µl 10%APS 6µl TEMED
Electrophoresis Running Buffer (10x) 2L	30,3g Tris 144g glycine 100ml 10% SDS ddH_2O up to 2L
10x Transfer Buffer (2L)	60,6g Tris 28,8g glycine 2ml 10% SDS ddH_2O up to 2L
Mild stripping buffer (100ml)	1ml 10% SDS 1,5g glycine 1ml Tween

	Adjust pH to 2,2
Coomassie Brilliant Blue R-250	45ml methanol 10ml acetic acid 0,25g coomassie blue dye 45ml ddH_2O Filter
Destain buffer	5% acetic acid 10% methanol
10xTBS (1L)	80g NaCl 24,2g Tris base ddH_2O up to 1L
FACS buffer	1x PBS 2% FBS 0.01% sodium azide
Griess reagent	400 mg of sulfanilamide 100 mg of Napthyl-ethylenediamide Dichydrochloride 10 ml ddH_2O-mix 1.2 ml 85% Phosphoric Acid in 8.8 ml ddH_2O-Add to the above 20 ml of ddH_2O Vortex to dissolve
Arginase lysis buffer	25 mM Tris-HCl pH 7.5 0,1% Triton X-100 5mM $MnCl_2$
OPD	7mg OPD in 20ml in citrate phosphate buffer 10µl 30%H_2O_2
Citrate phosphate buffer	

Table 4 Chemical reagents table. The reagents used in these experiment and their recipes

Results

HnRNP A3 is expressed mainly in immune related tissues

To examine the expression of hnRNP A3 in various tissues, tissues were isolated from C57BL/6 mice for protein isolation and analysis of expression by western blot. HnRNP A3 was found to be highly expressed in lung, spleen, thymus, brain and testis and moderately expressed in liver, pancreas, eyes and skin (Fig6a). Limited or no expression was observed in the rest of the analysed tissues. This initial experiment shows that hnRNP A3 is not uniformly expressed in all body tissues but is highly expressed in immune, reproductive and nervous related tissues.

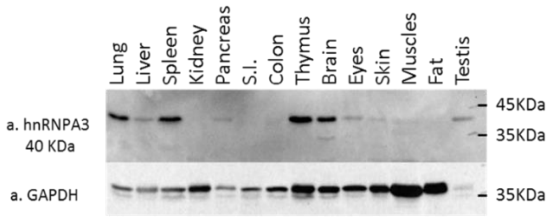


Figure 6 hnRNP A3 is expressed in specific cell tissues. Western blot of hnRNP A3 in various tissues of a C57/BL6 mouse (courtesy of Margarita Chatzimike). HnRNP A3 is highly expressed in lung, spleen, thymus, brain and testis. Moderate expression of the protein is observed in liver, pancreas, eyes and skin.

HnRNP A3 expression is increased upon macrophage activation

The next step was to study the expression of hnRNP A3 in macrophages, before and after activation. To do that, mice were injected with 4% thioglucolate and peritoneal macrophages were isolated 3 days later. These macrophages were activated towards the M1 state using LPS and IFN-γ, and towards the M2 state using IL-4. Analysis by western blot showed that hnRNP A3 is expressed in non-activated macrophages (M0 state). Moreover, upon activation towards either the M1 or M2 state, hnRNP A3 protein expression is highly increased (Fig6b). It is confirmed then that not only is hnRNP A3 expressed in macrophages but also its expression is increased after actication, indicating that itplays a role in the macrophage physiology.

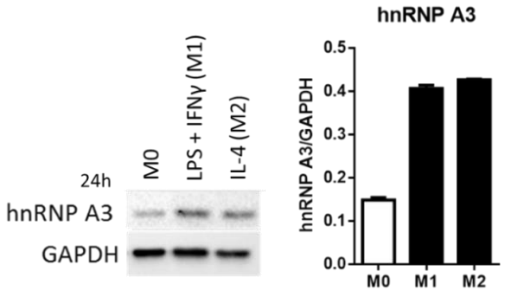
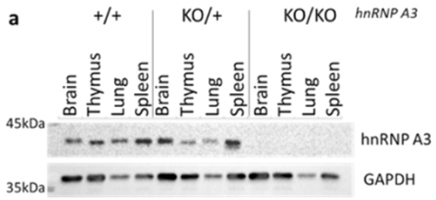


Figure 7 Expression of hnRNP A3 in macrophages is increased after activation Western blot of hnRNP A3 in peritoneal macrophages and graphic representation of the blot. HnRNP A3 is expressed in non-activated macrophages and activation leads to increase in expression. Increase of expression does not appear to differ between the two states of activation (M1 and M2)

Complete hnRNP A3 KO mice displayed a complex developmental phenotype

Complete hnRNP A3 KO mice were created with the aim to study *in vivo* the effects of hnRNP A3 deletion. To test the lack of hnRNP A3 in these mice, brain, spleen, thymus and lung were removed from them, as well as from wild type and heterozygous mice. The tissues were subsequently analyzed by western blot and the deletion of hnRNP A3 was confirmed (Fig 7a) . All the homozygous males were able to mate and plug their mate but were unable to fertilize the females. Homozygous females were successfully fertilized and gave normally birth. The complete hnRNP A3 KO mice had no detectable levels of hnRNP A3 expression and display a complex developmental phenotype.



b

Genotype	Expected	Observed
Wt	69	54
hnRNP A3 KO/+	27	42
	X-square	**11,5942**
	p-value	**0.0007**

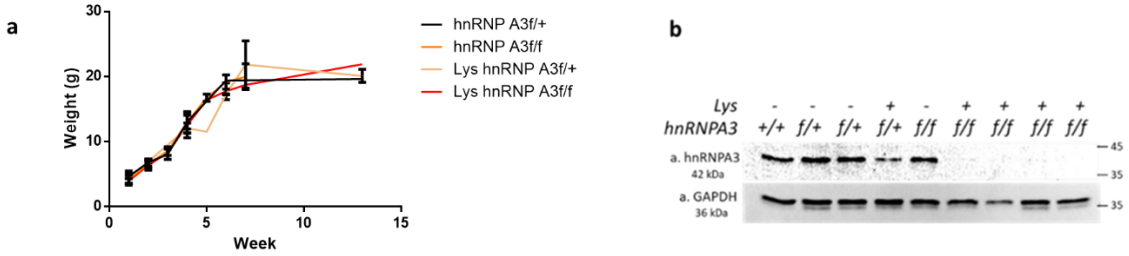
c

Genotype	Expected	Observed
Wt	27,25	44
hnRNP A3 KO/+	58	63
hnRNP A3 KO/KO	30,75	9
	x2	**26,11105**
	p-value	**<0.0001**

Figure 8 Complete deletion of hnRNP A3 leads to a complex developmental phenotype a) Western blot of hnRNP A3 in various tissues from complete KO mice, WT mice and heterozygous mice confirms the lack of hnRNP A3 in the former. b) x-square table for matings for heterozygous mice. c) x-square table for matings for homozygous mice.

Macrophages from conditional hnRNP A3 KO mice did not express hnRNP A3

Conditional myeloid KO mice for hnRNP were created to focus on the role of this protein in the immune system. These mice did not have any apparent morphological defects and their weights were normal (Fig8a). BMDMs were isolated from these mice and their control and were analyzed for hnRNP A3 expression by western blot. No hnRNP A3 expression was detected on BMDMs from conditional KO mice (Fig8b).



c

Genotype	Expected	Observed
Wt	8,625	4
LyshnRNP A3+/+	8,625	3
hnRNP A3 f/+	24	19
Lys hnRNP A3 f/+	24	23
hnRNP A3 f/f	26,875	37
LyshnRNP A3 f/f	26,875	33
	X-square	**12,44235**
	p-value	**0.0292**

Figure 8 Myeloid-specific deletion of hnRNP A3 in mice and its phenotypical effects. a) Graph representing the body weight of conditional KO mice (Lys hnRNP A3$^{f/f}$) and their control mice over time. b) Western blot of hnRNP A3 in BMDMs from conditional KO mice and their controls confirms the lack of hnRNP A3 in the former. c) x-square table for births of conditional KO mice.

Immunophenotyping using flow cytometry didn't display any defects in myelopoiesis and differentiation

Next, the possible role of hnRNP A3 in myelopoiesis was studied. Bone marrow was extracted from LysMCre-hnRNPA3$^{fl/fl}$, LysMCre-hnRNPA3$^{fl/+}$ and hnRNPA3$^{fl/f}$, stained and analyzed using flow cytometry. Gating for Lin- cells lead to identification of the progenitor population. Lin- cells were then gated using the cKit and Sca1 markers. Sca^{+} cKit^{+} cells were characterized as hematopoietic stem cells (HSCs), Sca1^{+} cKit^{-} cells were characterized as common lymphoid progenitors (CLPs) and Sca1^{-} cKit^{+} were characterized as myeloid progenitors. The latter population was gated using the CD34 and CD16/32 markers. CD16/32^{+} CD34^{+} cells were characterized as granulocyte-monocyte progenitors (GMPs). CD16/32^{-} CD34^{+} were characterized as common myeloid progenitors (CMPs) while CD16/32^{-} CD34^{-} cells were characterized as megakaryocyte-erythrocyte progenitors (MEPs). No significant changes appeared between the three groups for any of the seven cell populations (Fig.9b). So lack of hnRNP A3 does not affect myelopoiesis.

Next, blood and spleen were removed from LysMCre-hnRNPA3$^{fl/fl}$ and hnRNPA3$^{fl/fl}$ to test if the major immunce cell populations were affected by the deletion of hnRNP A3. The tissues were stained as mentioned in Material and Methods and analysed using flow cytometry. The isolated cells were gated for CD11b. CD11b is a marker that characterizes cell of the myeloid lineage so CD11b$^-$ are lymphocytes. Lymphocytes were subsequently gated for B220 and CD3 to distinguish between T cells (CD3$^+$ B220$^-$) and B cells (B220$^+$ CD3$^-$). Lymphocytes were gated for GR1. GR1$^+$ cells were characterized as granulocytes and GR1$^-$ were characterized as monocytes/dendritic cells. Dendritic cells were distinguished from monocytes/macrophages by gating for the dendritic cell marker CD11c. CD11c$^-$ cells were characterized as monocytes/macrophages and CD11c$^+$ cells were characterized as dendritic cells. The main immune cell populations didn't display any differences between the two groups, in the blood or the spleen (Fig10b,c).

Additionally, the spleen was studied also for the main T cell subgroups (Fig11). Cells were gated for CD4 and CD8, the main markers for Th and Tc cells, respectively. Both of these subgroups were then gated for CD69 and CD44 to distinguish between activated T cells (CD69$^+$ CD44$^+$, CD69$^+$ CD44$^-$) and naïve T cells (CD69$^-$ CD44$^-$). CD69$^-$ CD44$^+$ were further gated for CD62L and CD25 to distinguish between Treg cells (CD62L$^-$ CD25$^+$) and naive Treg cells (CD62L$^+$ CD25$^+$, CD62L$^+$ CD25$^-$). No significant differences were observed between test and control mice.

A third staining was performed on the spleen tissues, this time focusing on the main myeloid cell subgroups (Fig12). Gating for CD11b lead to the characterization of myeloid cells. Subsequent gating for F4/80 lead to identification of the macrophage population (CD11b$^+$ F4/80$^+$). CD11b$^+$ F4/80$^-$ cells were gated for GR1 and Lys6C. Lys6C$^+$ GR1$^+$ cells were characterized as granulocytic myeloid suppressor cells. Lys6C$^+$ GR1$^-$ were characterized as monocytic myeloid suppressor cells. Lys6C$^-$ GR1$^+$ were characterized as granulocytes. Once again, no significant differences appeared between test and control mice.

Using flow cytometry to phenotype for the major immune groups and their progenitors lead to the conclusion that myeloid deletion of hnRNP A3 does not affect myelopoiesis and immune cell maturation and activation.

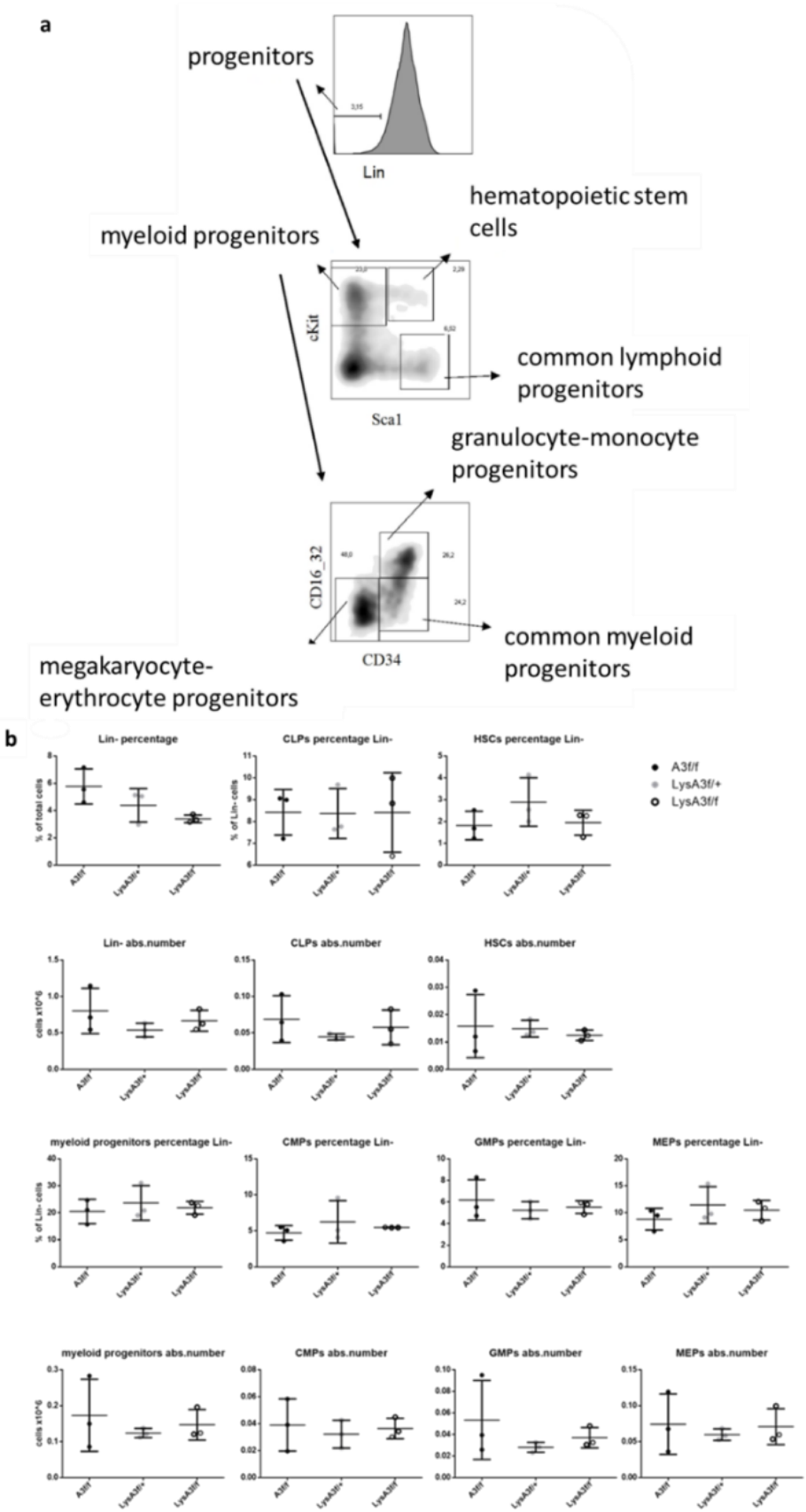


Figure 9 Bone marrow immunophenotyping for LysMCre-hnRNPA3$^{fl/fl}$, LysMCre-hnRNPA3$^{fl/+}$ and hnRNPA3$^{fl/fl}$ mice. a) The gating strategy that was used. Representative dot plots are shown. b) The number of each cell groups presented as percentages and absolute number of cells

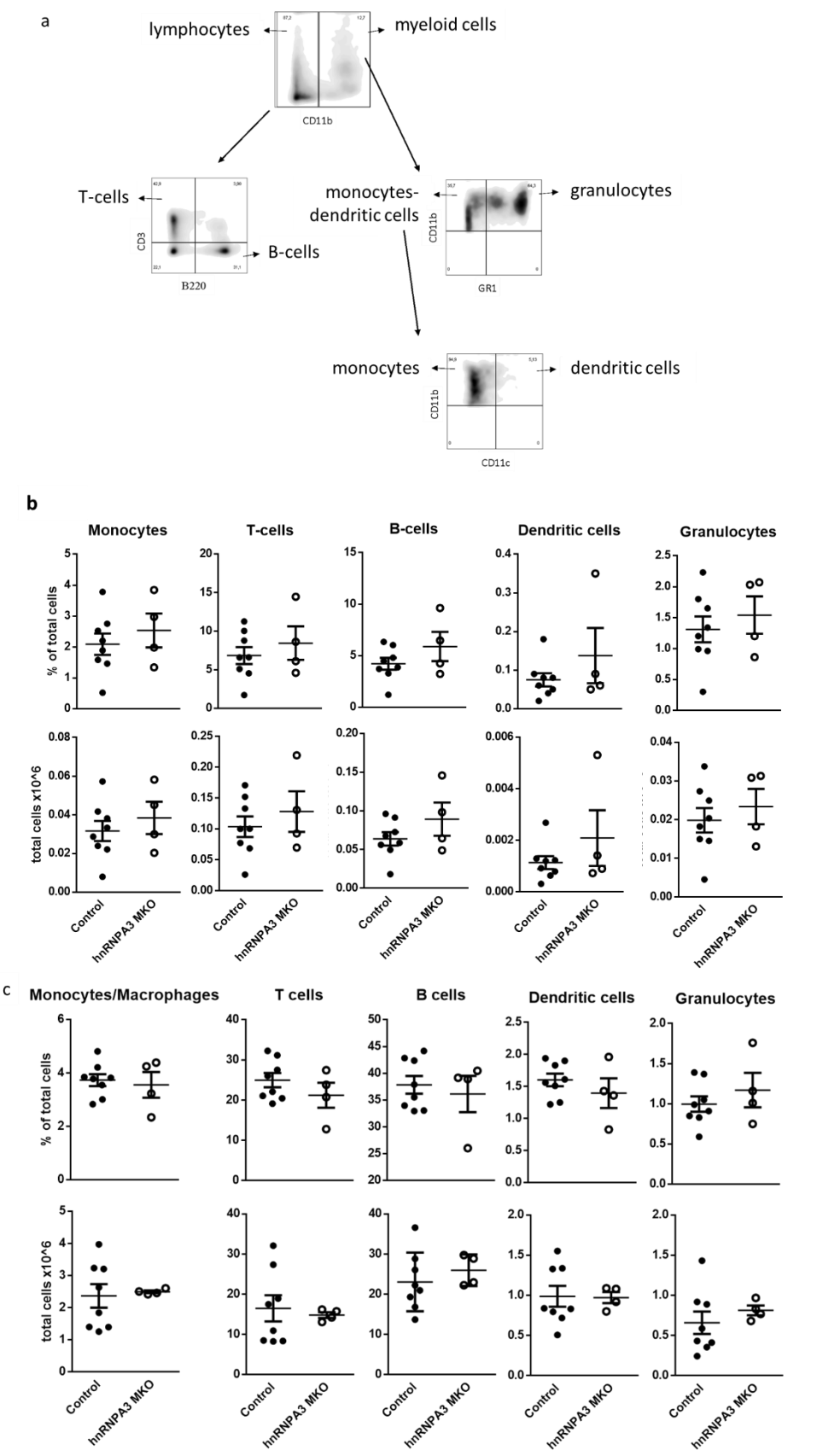


Figure 10 Blood and spleen immunophenotyping for LysMCre-hnRNPA3$^{fl/fl}$ and control mice. a) The gating strategy that was used. Representative dot plots are shown. b) Immune cells in blood: the number of each cell groups presented as percentages and absolute number of cells. c) Immunce cells in spleen: as for b

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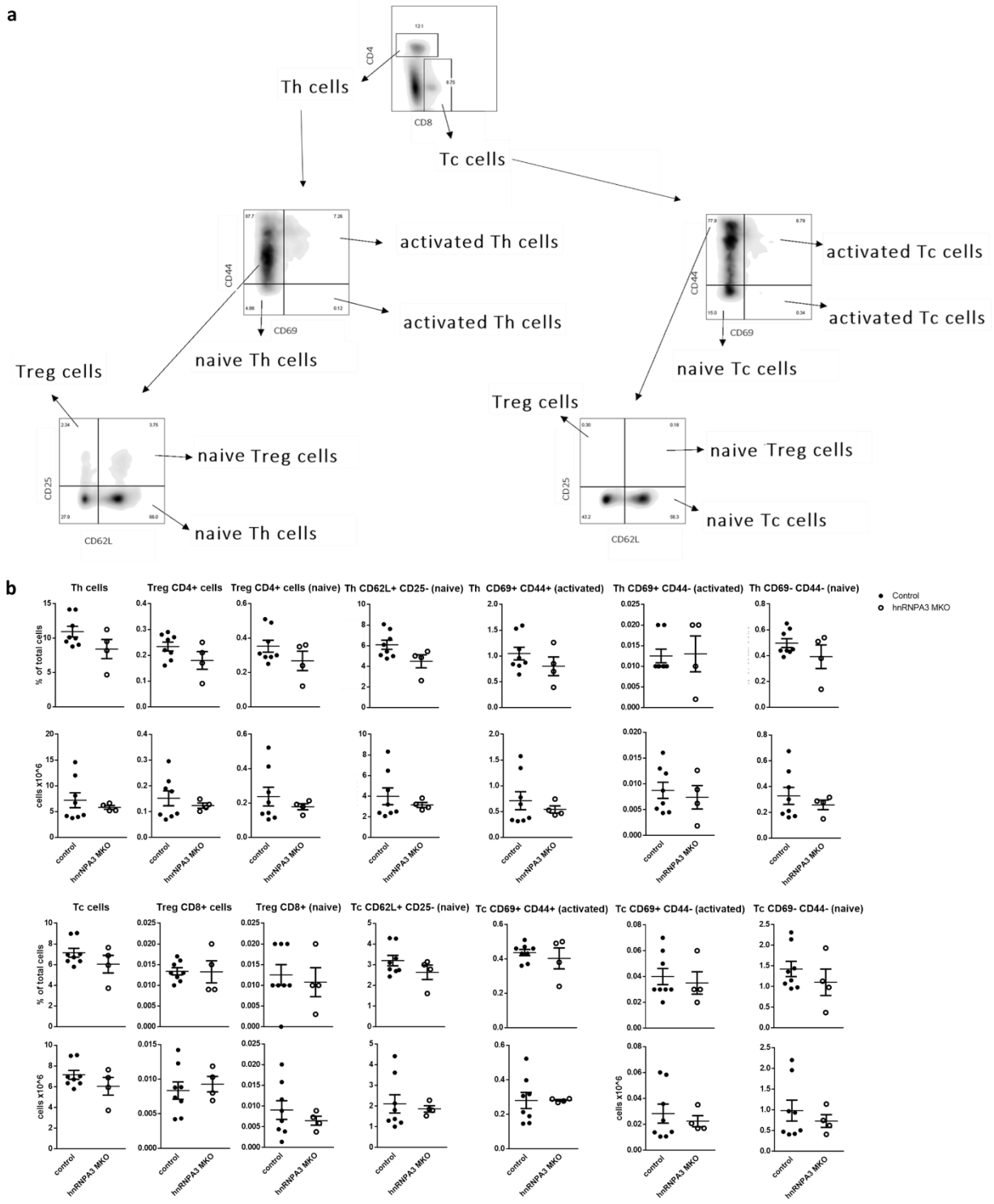

Figure 11 Spleen immunophenotyping for the major T cell groups for LysMCre-hnRNPA3$^{fl/fl}$ and control mice. a) The gating strategy that was used. Representative dot plots are shown. b) The number of each cell groups presented as percentages and absolute number of cells.

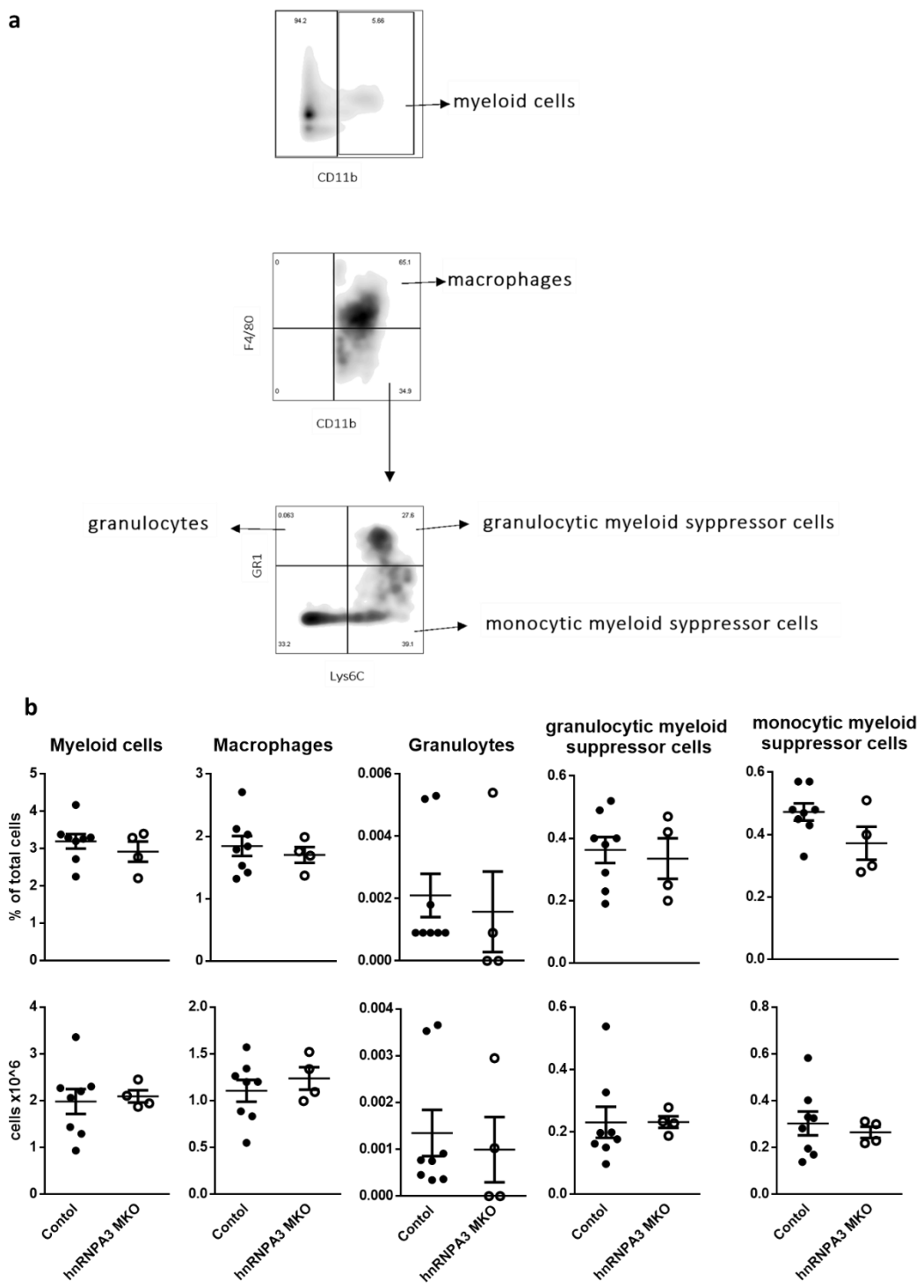

Figure 12 Spleen immunophenotyping for major lymphoid cell groups for LysMCre-hnRNPA3$^{fl/fl}$ and control mice. a) The gating strategy that was used. Representative dot plots are shown. b) The number of each cell groups presented as percentages and absolute number of cells.

HnRNP A3-MKO mice displayed no sensitivity to the LPS-induced endotoxemia model

The LPS-induced endotoxemia model is a model that is frequently used to study the inflammatory responses. Thus it was chosen to study the effects of myeloid deletion of hnRNP A3 on inflammation. Mice were initially injected with 350μg LPS /25g of body weight. By day 6 37.5% of the control mice had survived versus 20% of the hnRNP A3 MKO mice, with p-value=0,6 (Fig13a). Additionally, monitoring of body temperature didn't display significant changes between the two groups (Fig13b). The dosage was deemed slightly higher than IC50 since more than 50% of control mice died. A second experiment was performed with dosage of 300μg LPS/ 25g of body weight. In this experiment 60% of control mice survived versus 80% of hnRNP A3 MKO mice, with p-value=0,3 (Fig13c).

These experiments showed that hnRNP A3-MKO mice do not appear to be more sensitive to this model of sepsis, compared to control mice. Interestingly, this appears to contrast the sensitivity that HuR-MKO mice display at the same model[33].

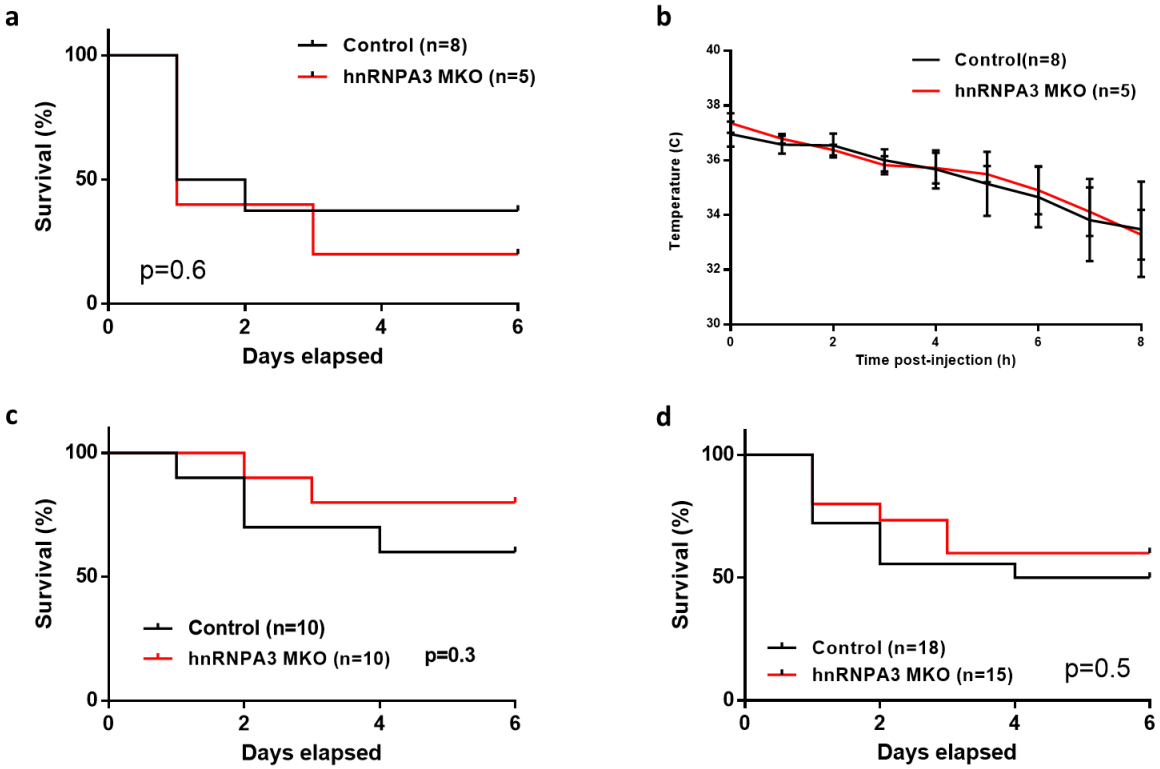

Figure 13 Myeloid deletion of hnRNP A3 does not increase sensitivity to LPS-induced endotoxemia a) Kaplan-Meier distribution of control and MKO mice that survived endotoxemia induced by 350μg LPS/25g of body weight. Group numbers and P value are shown. b) Body temperature graph for control and MKO mice injected with 350μg LPS/25g of body weight. Group numbers c) Kaplan-Meier distribution of control and MKO mice that survived endotoxemia induced by 300μg LPS/25g of body weight. Group numbers and P value are shown. d) Kaplan-Meier distribution of control and MKO mice that survived endotoxemia (combined data). Group numbers and P value are shown.

HnRNP A3 MKO mice display signs of increased inflammation after *Citrobacter rodentium* infection

After it was observed that hnRNP A3 MKO mice do not display sensitivity to LPS-induced endotoxemia, it was decided to infect mice with a living pathogen. *Citrobacter rodentium* was chosen as it is a well-studied bacterium that causes intestinal inflammation. Control and hnRNPA3 MKO mice were infected orally with *C.rodentium*. The animals were monitored the next days for signs of diarrhea and weight loss and feces were collected on days 3, 6, 9 and 14. On day 14 the animals were sacrificed and their spleen, liver and colon were collected, the first two to count their microbial load and the latter for histological analysis.

None of the animals lost weight (Fig14a) or displayed diarrhea. The hnRNP A3 MKO mice displayed slightly increased bacterial load in their feces compared to their control, especially on D9, before falling at similar levels on D14 (Fig14b). The bacterial loads in spleen and liver were similarly at the same levels (Fig14c, d). Histological analysis performed by Dr. Fotis Ioakeimidis showed increased inflammation in the colon of hnRNP A3 MKO mice (Fig 14g). More specifically, colons of hnRNP A3 MKO displayed increased crypt length and scored higher for intestinal inflammation (submucosal inflammation, damaged epithelium, infiltration) compared to control mice (Fig 14 e,f). These results indicate that hnRNP A3 MKO mice have difficulty to successfully battle *C.rodentium* leading to increased inflammation.

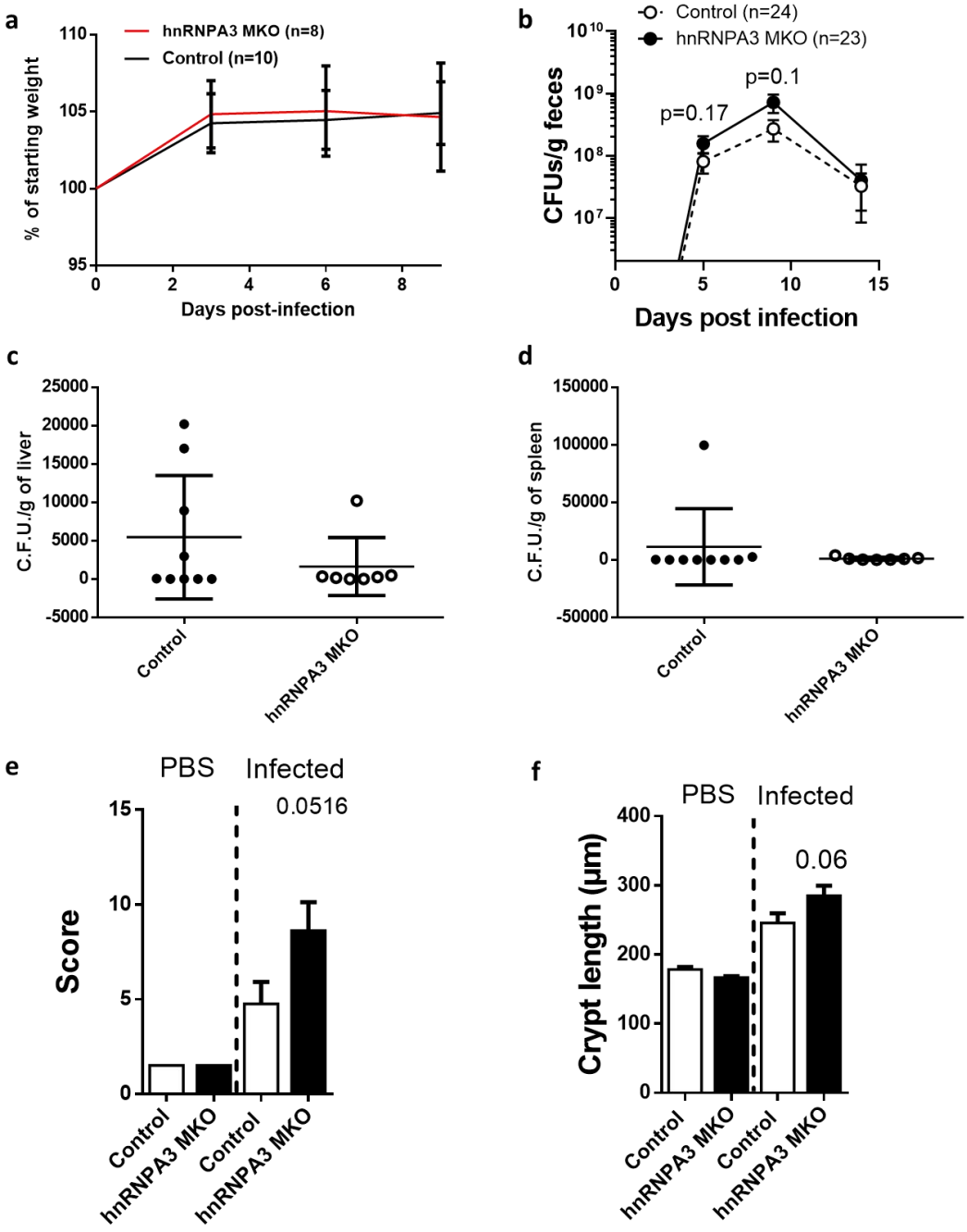
a
hnRNPA3 MKO (n=8)
Control (n=10)
% of starting weight
Days post-infection
b
Control (n=24)
hnRNPA3 MKO (n=23)
CFUs/g feces
p=0.17
p=0.1
Days post infection
c
C.F.U./g of liver
Control
hnRNPA3 MKO
d
C.F.U./g of spleen
Control
hnRNPA3 MKO
e
PBS
Infected
0.0516
Score
Control
hnRNPA3 MKO
Control
hnRNPA3 MKO
f
PBS
Infected
0.06
Crypt length (µm)
Control
hnRNPA3 MKO
Control
hnRNPA3 MKO
g
Uninfected
Control
Infected
Control
M-hnRNPA3ko

Figure 14 hnRNP A3 MKO mice exhibit sensitivity to *C.rodentium* compared to control mice. a) The weight change of of control and hnRNP A3 MKO mice presented as a percentage of their starting weight. b) The bacterial load in feces of control and hnRNP A3 MKO mice in the course of 14 days post-infection. P-values are presented for days 5 and 9. The bacterial load in c) liver and d) spleen at D9 of the experiment. Plot shows individual and mean values (± SEM). e) Bar plot representing the histomorpological scores of collected colons. p-value is presented above the plot. Control (n=14), hnRNP A3 MKO (n=14) f) Bar plot representing crypt length. p-value is presented above the plot. Scoring performed by Dr. Fotis Ioakeimidis Control (n=15), hnRNP A3 MKO (n=14). g) Photographs of colonic sections stained with H&E. Photo courtesy of Dr. Fotis Ioakeimidis

HnRNP A3 MKO mice display sensitivity to *Listeria monocytogenes* infection

Since Intracellular pathogens activate different molecular pathways than extracellular bacteria like *C.rodentium,* infection with the intracellular bacterium *Listeria monocytogenes* was next used to study the effects of myeloid deletion of hnRNP A3. HnRNP A3 MKO and control mice that were injected intraperitoneally with $5x10^6$ C.F.U.s of *L.monocytogenes*.. 33.3% of hnRNP A3 MKO mice died during the experiment versus 0% of the control mice (Fig15). Although the groups were small and thus the result not statistically significant, it appears that myeloid deletion of hnRNP A3 leads to increased sensitivity to *L.monocytogenes* infection.

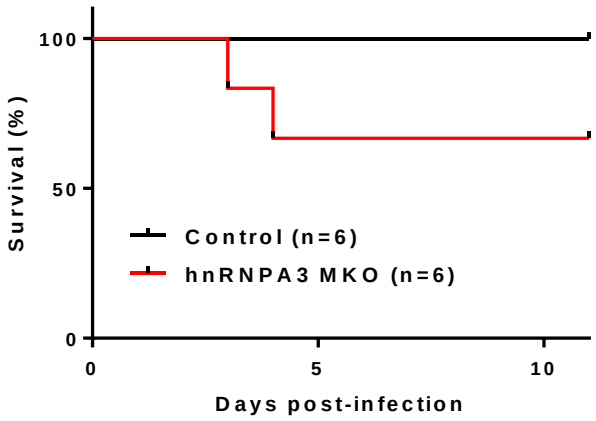

Figure 15 The Kaplan-Meier distribution of control and MKO mice that survived infection with $5x10^6$ C.F.U.s of *L.monocytogenes*. Group numbers are shown.

HnRNP A3 knock-out RAW264.7 macrophages display upregulation of M1 markers

To further elucidate the molecular mechanisms that are affected by lack of hnRNP A3, major markers of macrophage activation were studied. Initially, hnRNP A3-KO RAW264.7 macrophages were activated with LPS or LPS+IFNγ. Nitrite oxide production was measured using the Griess method and major M1 cytokines TNFα and IL-6 were measured using ELISA assays. No increased production of NO was observed from these macrophages but TNFα and IL-6 were upregulated compared to control (Fig 16b, c). This indicates that hnRNP A3 has an inhibitory role in the production of M1 cytokines.

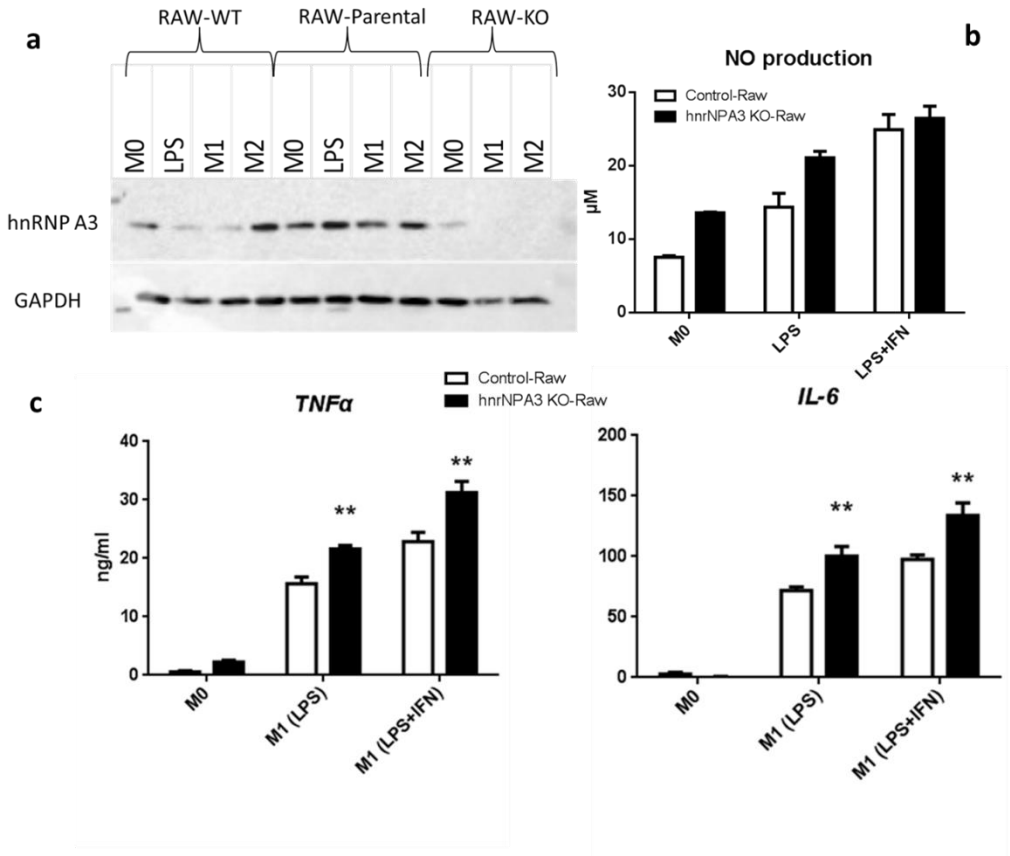

Figure 16 hnRNP A3 KO RAW264.7 macrophages display upregulated M1 markers a) Western blot for RAW264.7 wilt type, parental and hnRNP A3 KO cell extracts. b) Levels of NO secreted from activated (with LPS or LPS+IFNγ) and non-activated RAW264.7 control and hnRNP A3 KO macrophages. Data are shown as mean values ±SEM. c) Levels of secreted TNFα and IL-6 from activated (with LPS or LPS+IFNγ) and non-activated RAW264.7 control and hnRNP A3 macrophages. Data are shown as mean values ±SEM.**p-value≤0,01

HnRNP A3 deletion leads to an increase of M1 marker protein synthesis

After the initial *in vitro* experiments with the RAW264.7 macrophages, macrophages from mice were used to study M1 markers. Bone-marrow derived macrophages from hnRNP A3 MKO mice and their control were activated towards the M1 state with LPS+IFNγ and major M1 markers were measured using ELISA assays and qPCR. Measurement of NO production using the Griess method displayed no significant difference between the two groups. Similarly, lack of hnRNP A3 does not to significantly the RNA levels of M1 markers. Interestingly, hnRNP A3 KO macrophages produced major cytokines, such as TNFα and IL-6, at higher protein levels (Fig17). These results support the previous evidence that hnRNP A3 can act as a downregulator of M1 activation.

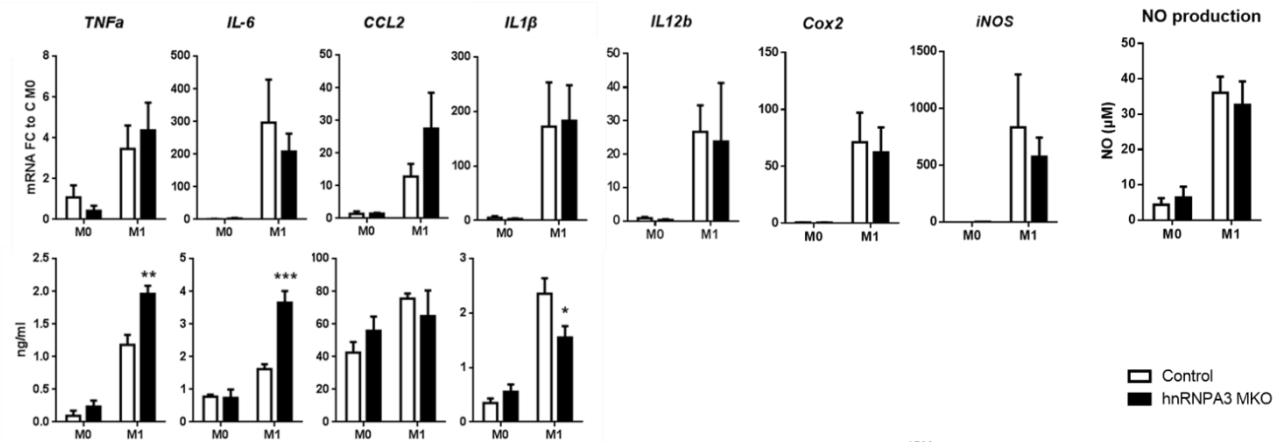

Figure 17 Protein levels of major M1 markers are increased in activated hnRNP A3 BMDMs compared to control. Top: Bar graphs depict differences in M1 mRNAs in control and hnRNP A3 macrophages, in M0 and M1 states. Data are fold change +SEM relative to control values (inactivated control macrophages). Top far right: Levels of NO secreted from activated and non-activated control and hnRNP A3 KO macrophages. Data are shown as mean values ±SEM. Bottom: Levels of secreted M1 cytokines from activated and non-activated control and hnRNP A3 macrophages. Data are shown as mean values ±SEM.*p-value≤0,05 **p-value≤0,01 ***p-value≤0,001

Deletion of hnRNP A3 leads to upregulation of M2 markers.

Differences in the production of M2 markers were studied as well. Control and hnRNP A3-KO BMDMs were activated towards the M2 state with IL-4. Initial experiments to measure the activity of arginase did not display significant differences between control and hnRNP A3 KO macrophages. The sample size for this experiment was small and the experiment must be replicated. Interestingly, the mRNA levels of the enzyme were significantly higher in the hnRNP A3-KO macrophages compared to the control and other M2 markers displayed a tendency for upregulation in the absence of hnRNP A3 (Fig18). This could imply a role for hnRNP A3 as a downregulator of the M2 state as well.

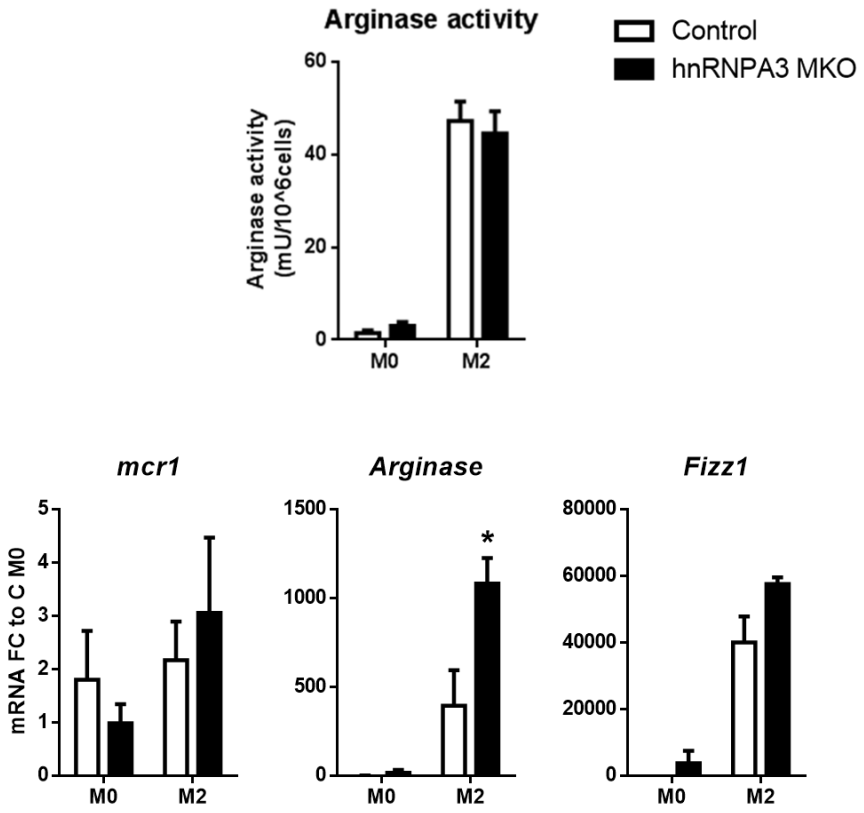

Figure 18 Lack of hnRNP A3 in BMDMs leads to upregulation of M2 markers. Top: Bar graphs depict levels of urea produced by arginase in control and hnRNP A3 KO macrophage, at the M0 and M2 states. Bottom: Bar graphs depict differences in M1 mRNAs in control and hnRNP A3 macrophages, in M0 and M2 states. Data are fold change +SEM relative to control values (inactivated control macrophages). *p-value≤0,05

Discussion

HnRNP A3 is a RBP and a member of the hnRNP family. It is known to be involved in mRNA decay and shuttling but generally, little is known about its functions. This protein was shown to directly interact with HuR, a RBP known for its pleiotropic functions, in LPS-activated macrophages. So it was decided that it merits for further investigation into its role in inflammation and macrophage polarization.

The role of hnRNP A3 in macrophage physiology and activation was confirmed by observing upregulation of its protein levels after M1 or M2. Complete deletion of hnRNP A3 lead to a complex phenotype which included male infertility but myeloid deletion of hnRNP A3 did not cause any apparent morphologic defects or defects in myelopoiesis and immune cell maturation. Additionally, further experiments with hnRNP A3 MKO mice did not reveal any sensitivity to disease models like LPS-induced endotoxemia, although HuR MKO mice display sensitivity in similar models[18]. However, infection with bacterial pathogens *C.rodentium* and *L.*monocytogenes lead to exacerbated intestinal inflammation and increased mortality of these mice, respectively. To better understand these reactions, bone marrow-derived macrophages were activated and levels of cytokines were measured. M1 activated hnRNP A3 KO macrophages did not display significant changes in RNA levels, but at the protein level TNFα and IL-6 were increased while IL-1β was decreased. A similar phenotype was observed in M1 activated hnRNP A3 KO RAW264 macrophages. The apparent differences between RNA and protein levels could indicate a role of hnRNP A3 in translational regulation. It should also be noted that HuR KO macrophages display increased secretion of TNFα and IL-6 and decreased secretion of IL-1β. Interestingly, M2 activated hnRNP A3 KO macrophages display an upregulation of M2 markers as well, a phenotype contrary to that of the HuR KO macrophages. So it appears that hnRNP A3 act as a downregulator of both M1 and M2 state.

These results could explain the outcomes of the in vivo experiments in a few ways. The bacterial loads of both species were increased in hnRNP A3 MKO mice. This would suggest that lack of hnRNP A3 somehow negatively affects macrophages ability to battle infection. A successful response against these bacteria is heavily dependent on activation of the inflammasome[57,58,59]. IL-1β, a product of inflammasome activation, is significantly decreased in the absence of hnRNP A3. It is likely that lack of hnRNP A3 leads to a defect in the activation of the inflammasome and thus to an unsuccessful clearance of the pathogens. Alternatively, the upregulation of M2 markers could suppress the inflammatory responses responsible for the destruction of bacteria as well as the induction of septic shock from LPS. Further experiments with these pathogens must be performed to confirm these findings as well as further studies on the cytokine secretion of activated hnRNP A3 KO macrophages.

In future experiments, the complete hnRNP A3 knock-out mice will be further studied. These mice displayed a complex developmental phenotype including male infertility and thus were not the focus of this study. Further analysis will help illuminate the functions of hnRNP A3. Additionally, the identification of hnRNP A3's RNA targets in activated macrophages using sequencing will provide more information on its role in macrophage activation. Moreover, co-immunoprecipitation with HuR will identify the common targets of the two proteins. Finally,

to further decipher the association between the two RBPs mutant mice that lack both HuR and hnRNP A3 in macrophages can be generated and be tested for their sensitivity to pathologic responses.

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