UNIVERSITY OF CRETE

SCHOOL OF MEDICINE

PhD THESIS

"Regulation of microRNAs during activation of macrophages"

Christina Doxaki

Department of Medicine - University of Crete
Supervisor: Prof. Christos Tsatsanis

Heraklion 2015

ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

«Ρύθμιση των microRNAs κατά την ενεργοποίηση των μακροφάγων»

Χριστίνα Δοξάκη

Τμήμα Κλινικής Χημείας – Βιοχημείας
Ιατρική Σχολή –Πανεπιστήμιο Κρήτης

Ηράκλειο 2015

Τριμελής Συμβουλευτική Επιτροπή

Αριστείδης Ηλιόπουλος: Καθηγητής Κυτταρικής Μοριακής Βιολογίας, Τμήμα Ιατρικής,
Πανεπιστήμιο Κρήτης

Χαράλαμπος Σπηλιανάκης: Επίκουρος Καθηγητής Βιολογίας, Τμήμα Βιολογίας,
Πανεπιστήμιο Κρήτης

Χρήστος Τσατσάνης: Αναπληρωτής Καθηγητής Κλινικής Χημείας, Τμήμα Ιατρικής,
Πανεπιστήμιο Κρήτης

Επταμελής Συμβουλευτική Επιτροπή

Κατερίνα Βαπορίδη: Επίκουρος Καθηγήτρια Εντατικής Ιατρικής, Τμήμα Ιατρικής,
Πανεπιστήμιο Κρήτης

Αχιλλέας Γραβάνης: Καθηγητής Φαρμακολογίας, Τμήμα Ιατρικής,
Πανεπιστήμιο Κρήτης

Αριστείδης Ηλιόπουλος: Καθηγητής Κυτταρικής Μοριακής Βιολογίας, Τμήμα Ιατρικής,
Πανεπιστήμιο Κρήτης

Σωτήριος Καμπράνης: Επίκουρος Καθηγητής Βιοχημείας, Τμήμα Ιατρικής,
Πανεπιστήμιο Κρήτης

Ιωσήφ Παπαματθαιάκης: Ομότιμος Καθηγητής Βιολογίας, Τμήμα Βιολογίας,
Πανεπιστήμιο Κρήτης

Χαράλαμπος Σπηλιανάκης: Επίκουρος Καθηγητής Βιολογίας, Τμήμα Βιολογίας,
Πανεπιστήμιο Κρήτης

Χρήστος Τσατσάνης: Αναπληρωτής Καθηγητής Κλινικής Χημείας, Τμήμα Ιατρικής,
Πανεπιστήμιο Κρήτης

Ευχαριστίες...

Η παρούσα διατριβή, αποτέλεσμα πολυετούς ενασχόλησης μου στους πάγκους των εργαστηρίων εκπονήθηκε στα πλαίσια απόκτησης του διδακτορικού τίτλου στο εργαστήριο κλινική χημείας υπο την επίβλεψη του καθηγητή Χρήστου Τσατσάνη.

Η εργαστηριακή εμπειρία χτίστηκε απο πολλή δουλειά, πολλή σκέψη, πολλές μικρές χαρές, πολλές εκπλήξεις, αμέτρητα ερωτηματικά, πολλές δημιουργικές ασάφειες, πολύωρες συζητήσεις, αρκετά δάκρυα και απογοητεύσεις. Κάποιοι ανθρωποι στάθηκαν δίπλα μου σε όλο αυτό, στήριξαν την επιλογή μου να ενασχοληθω με την βασική έρευνα και δεν μένει πια παρά να τους ευχαριστήσω απο τα βάθη της καρδιάς μου.

Ο καθηγητής Παματθαιάκης και η ερευνήτρια Νίκη Κρετσόβαλη ήταν αυτοι που για πρώτη φορά με υποδέχτηκαν στην Κρήτη στο εργαστήριο τους και μου έδωσαν τα εφόδια και τη δύναμη να ξεκινήσω.

Ο άνθρωπος που με στηριξε , με πίστεψε, με καθοδήγησε, μου εδωσε τη δυνατότητα να κυνηγησω τα όνειρα μου, μου άνοιξε τους ορίζοντες μου, έβαζε προθεσμίες για το καλό μου, με αντιμετώπιζε με ηρεμία και χαμόγελο και συνολικά με εξασφαλιζε με την ακέραιη στάση του ηθικά και οικονομικά είναι ο καθηγητής Χρήστος Τσατσάνης και γι αυτό το λόγο του οφείλω πολλά.

Ο καθηγητής Χαράλαμπος Σπηλιανάκης είναι επίσης ένας άνθρωπος σημαντικός για μένα διότι με στήριξε, μου μιλησε ειλικρινά, αφιέρωσε πολύτιμο χρόνο στα σκοτεινά δωμάτια του confocal και υποχρεώθηκε σε πολύ "troubleshooting" για πολλά αποτυχημένα FISH.

Όλα αυτά τα χρόνια, είχα φίλους καλούς σε εργαστήρια κοντινά, σε αντικρυστά παράθυρα που έμειναν δίπλα μου στην ατελείωτη περίοδο μιας διδακτορικής διατριβής…

Όλα αυτά τα χρόνια, γνώρισα άτομα, τα οποία με συμβούλευαν, μου εδιναν δυναμη, με εκαναν να αποδράσω με εκδρομες και ορειβασίες και με στηριζαν με κάθε τρόπο που μπορούσαν…

Όλα αυτά τα χρόνια, συνεργάστηκα με πολλά άτομα, γέλασα, εκλαψα, προβληματίστικα, τσακώθηκα (αν και ευτυχώς για λίγο και με λίγα) ...

Δεν έχει νόημα να τους ονοματίσω, ο καθένας ξέρει..

έχει νόημα να τους ευχαριστήσω ομως διοτι χάρη σε αυτούς αποκόμισα ανεκτίμητη γνώση και εμπειρία, αυτογνωσία και ωρίμανση.

List of contents

Summary

Endotoxin tolerance occurs to protect the organism from hyperactivation of innate immune responses, primarily mediated by macrophages. Regulation of endotoxin tolerance occurs at multiple levels of cell responses and requires significant changes in gene expression. During macrophage activation, induced expression of miR-155 and miR-146a contributes to the regulation of the inflammatory response and endotoxin tolerance. Herein, we demonstrate that expression of both miRNAs is co-ordinately regulated during endotoxin tolerance by a complex mechanism involving mono-allelic inter-chromosomal association, alterations in histone methyl marks and transcription factor binding. Upon activation of naïve macrophages, Histone3 was tri-methylated at lysine4 (H3K4me3) and NFkBp65 was bound on both miR-155 and miR-146a gene loci. However, at the stage of endotoxin tolerance both miR gene loci were occupied by C/EBPβ, NFkBp50 and the repressive Histone3 marks H3K9me3. DNA fluorescence in situ hybridization (DNA-FISH) experiments revealed mono-allelic inter-chromosomal co-localization of miR-155 and miR-146a gene loci at the stage of endotoxin tolerance, while RNA-DNA-FISH experiments showed that the co-localized alleles were silenced, suggesting a common repressive mechanism. Genetic ablation of Akt1, which is known to abrogate endotoxin tolerance, abolished induction of loci co-localization and C/EBPβ binding, further supporting that this mechanism occurs specifically in endotoxin tolerance. This thesis demonstrates that two miRNAs are co-ordinately regulated via gene co-localization at the three dimensional chromatin space, similar transcriptional machinery and Histone3 methylation profile, contributing to the development of endotoxin tolerance. Further insight into the role of AKT in regulation of M1/M2 polarization, revealed the essential role of these microRNAs in macrophage phenotype. Akt1 ablation promotes miR-155 expression in LPS-stimulated macrophage. Measuring miR-155 in Akt2-depleted macrophages revealed that Akt2 ablation had the opposite effect, reducing miR-155 expression in both resting and LPS-activated macrophages. Therefore, down-regulation of miR-155 in Akt2-defiecient macrophages results in up-regulation of its target C/EBPβ and, consequently, in the induction of Arg1, a hallmark of M2 macrophage polarization. Akt2 deficiency

resulted, however, in a significant upregulation of miR-146a, which mediates M1 phenotype suppression and assure endotoxin tolerance. miR-146a transfection in WT macrophages was able to inhibit iNOS induction while miR-146a suppression in Akt2-depleted mice resulted in upregulation of iNOS expression. The physiological and clinical significance of these miRs in sepsis was supported by further data in humans. Critically ill patients with impaired immune responses (CARS syndrome) are associated with increased miR-155 and miR-146 expression. In vivo transferring of these miRs by using amphoteric liposomes seems to be highly promising, underlining miR-155 and miR-146 as potential novel molecular biomarkers of macrophage sensitivity and CARS syndrome and tools for therapeutic purposes.

Περίληψη

Η ανοσολογική ανοχή είναι απαραίτητη για την προστασία του οργανισμού από την υπερ-ενεργοποίηση της ανοσολογικής απόκρισης και διαμεσολαβείται κυρίως μέσω μακροφάγων. Η ρύθμιση της ανοσολογικής ανοχής συντελείται σε πολλαπλά επίπεδα και απαιτεί σημαντικές αλλαγές στη γονιδιακή έκφραση. Κατά τη διαδικασία της ενεργοποίησης των μακροφάγων, η επαγόμενη έκφραση του miR-155 και miR-146a συμβάλλει στη ρύθμιση της φλεγμονώδους απόκρισης και της ανοσολογικής ανοχής. Στην παρούσα διατριβή, αποδεικνύεται ότι η έκφραση και των δύο miRNAs συν-ρυθμίζεται κατά τη διάρκεια της ανοσολογικής ανοχής μέσω ενός πολύπλοκου μηχανισμού που περιλαμβάνει χρωμοσωμικές αλληλεπιδράσεις, μεταβολές στο πρότυπο μεθυλίωσης των ιστονών και μεταβολές στην πρόσδεση μεταγραφικών παραγόντων. Κατά την ενεργοποίηση των μακροφάγων, παρατηρείται τρι-μεθυλίωση της λυσίνης 4 στην ιστόνη H3 (H3K4me3) και πρόσδεση του NFkBp65 στους υποκινητές των γονιδίων miR-155 και miR-146a. Ωστόσο, κατά το στάδιο της ανοσολογικής ανοχής παρατηρείται τρι-μεθυλίωση της ιστόνης H3 στην λυσίνη 9 (H3K9me3) και πρόσδεση των μεταγραφικών παραγόντων C/EBPβ και NFkBp50.

DNA-FISH πειράματα αποκάλυψαν την αλληλεπίδραση των γονιδιακών τόπων του miR-155 και miR-146a στο ένα αλλήλιο, στο στάδιο της ανοσολογικής ανοχής, ενώ πειράματα RNA-DNA-FISH έδειξαν ότι όταν αυτοί οι γονιδιακοί τόποι συνεντοπίζονται, δεν παρατηρείται μεταγραφή των γονιδίων τους, αποδεικνύοντας έναν κοινό μηχανισμό σίγησης. Στην περίπτωση των AKT1-/- ποντικών, στα οποία καταργείται το φαινόμενο της ανοσολογικής ανοχής, δεν παρατηρείται συνεντοπισμός των γονιδιακών τόπων, υποστηρίζοντας περαιτέρω ότι αυτός ο μηχανισμός συμβαίνει ειδικά κατά το στάδιο της ανοχής των μακροφάγων.

Επομένως, δύο miRNAs συν-ρυθμίζονται μέσω αλληλεπίδρασης των γονιδιακών τους τόπων, μέσω κοινών μεταγραφικών παραγόντων και παρόμοιου προφίλ μεθυλίωσης της ιστόνης H3, συμβάλλοντας στην ανάπτυξη της ανοσολογικής ανοχής. Επιπλέον, η ανάλυση του ρόλου της AKT κινάσης στα διάφορα στάδια ενεργοποίησης των μακροφάγων και στον M1 / M2 φαινότυπο ανέδειξε τη σημαντική συμβολή αυτών των microRNAs. Η απαλοιφή του γονιδίου Akt1 προήγαγε την έκφραση του miR-155 στα LPS-διεγερμένα μακροφάγα. Η μέτρηση των επιπέδων του miR-155 σε Akt2 - / - μακροφάγα αποκάλυψε το αντίθετο αποτέλεσμα. Ως εκ τούτου, η μείωση της έκφρασης του miR-155 στα Akt2 - / - μακροφάγα

είχε ως αποτέλεσμα την επαγωγή του στόχου του, του C/EBPβ και, κατά συνέπεια, την επαγωγή της Arg1, πρωτείνης που εκφράζεται στον M2 φαινότυπο. Αντιστοίχως, η απαλοιφή της Akt2 οδήγησε σε σημαντική αύξηση της έκφρασης του miR-146a, το οποίο παίζει κρίσιμο ρόλο στην καταστολή του φαινοτύπου M1 και στην εξασφάλιση της ανοσολογικής ανοχής. Η επαγωγή του miR-146a σε WT μακροφάγα ήταν ικανή να αναστείλει την επαγωγή της iNOS ενώ η καταστολή του miR-146a σε Akt2 - / - μακροφάγα είχε σαν αποτέλεσμα της αύξηση της έκφρασης της iNOS. Η κλινική σημασία των ευρημάτων αυτών στη σήψη και την ανοσολογική ανοχή υποστηρίχθηκε από περαιτέρω πειραματικά δεδομένα σε ανθώπινα δείγματα. Σε βαρέως πάσχοντες ασθενείς με μειωμένη ανοσολογική απόκριση (σύνδρομο CARS), όπως αυτή χαρακτηρίζεται από τη μειωμένη ανταπόκριση –παραγωγή κυτταροκινών στο πλάσμα έπειτα απο την ex vivo επώαση του περιφερικού αίματος με LPS, παρατηρήθηκε αύξηση της έκφρασης του miR-155 και miR-146. Η in vivo μεταφορά αυτών των Mirs με τη βοήθεια λιποσωμάτων ήταν ο επόμενος στόχος που επετεύχθη, πράγμα που καθιστά αυτά τα δύο Mirs πιθανούς μοριακούς δείκτες του συνδρόμου CARS και εργαλεία για θεραπευτικούς σκοπούς.

Significance Statement

Endotoxin tolerance occurs to protect the organism from hyperactivation of innate immune responses, primarily mediated by macrophages. Development of endotoxin tolerance requires significant changes in gene expression, transcriptional machineries and histone modifications to ensure temporal gene regulation. Coordinate transcriptional regulation of genes can occur when gene loci come in proximity and share common regulatory components. miR-155 and miR-146a are small non-coding RNAs that regulate multiple cellular functions and play an essential role in the development of endotoxin tolerance. In this thesis, it is shown that these two miRNAs are coordinately regulated via mono-allelic inter-chromosomal-association, sharing the same transcription factors and obtaining similar histone methyl marks at the stage of endotoxin tolerance. These findings support an additional level of gene regulation in endotoxin tolerant macrophages, which ensures timely gene expression and limitation of energy and resource expenditure in the cell. Moreover, the results firstly demonstrate that gene loci harboring microRNAs are co-localized at the three-dimensional chromatin space to ensure simultaneous regulation. Further insight into the role of AKT in regulation of M1/M2 polarization, highlighted the essential role of miR-155 and miR-146a in determination of macrophage phenotype. The physiological significance of the findings was supported further by data in human samples. miR-155 and miR-146 expression is associated with CARS syndrome. In fact, this is of great clinical significance rendering these micrornas potential novel molecular markers of macrophage sensitivity and CARS syndrome. Finally, in vivo transferring of miRs by using amphoteric liposomes seems to be highly promising, as they may be used for therapeutic purposes.

Introduction

1.Innate immunity

Immune responses are crucial for the protection of an organism against exogenous or endogenous threats, such as pathogens and cancer respectively. Through evolution vertebrates have developed a complicated recognition system in order to effectively guard themselves, which involves both fast acting innate and slowly activated adaptive immune responses. Innate immunity is an evolutionarily ancient and universal system that is directed by cells with capacity to recognize pathogen patterns and eliminate infection, thus provides the first line of host defense [1]. Such cells are macrophages, neutrophils, eosinophils, basophils and NK cells, or non – professionally immune cells such as epithelial cells. The innate immunity has no memory compared to adaptive or acquired immunity, which is characterized by antigenic specificity, diversity, memory, and self/non-self recognition and is mediated mainly via B and T lymphocytes [1]. The network of cells, especially dendritic cells (DCs) and macrophages, are well equipped with various pathogen-associated molecular pattern recognition receptors (PRRs) or damage associated molecular pattern receptors to recognize these wide variety of microbes, dusts, and pollutants and thus trigger the initiation events of inflammation (figure 1)[2],[3]. PRRs recognize relatively invariant highly conserved molecular patterns expressed by microorganisms, the pathogen-associated molecular patterns (PAMPs) and endogenous danger signals, damage-associated molecular patterns (DAMPs). The best-known examples of PAMPs include lipopolysaccharide (LPS) of gram-negative bacteria; lipoteichoic acids of gram-positive bacteria; peptidoglycan; lipoarabinomannan of mycobacteria; double-stranded RNA, which is produced by most viruses during the infection cycle; and ß-glucans and mannans found in fungal cell walls. Several families of PRRs have been characterized so far, including Toll-like receptors, RIG-I-like receptors, NOD-like receptors, and C-type lectin receptors [4]. These PRRs are expressed not only in macrophages and DCs but also in various nonprofessional immune cells such as epithelial cells [4, 5]. The mammalian Toll-like receptors (TLRs) are important signaling receptors in innate host defense. There are

at least twelve TLRs in mammalian species that differ from one another in their expression pattern, their ligand specificities, the signaling pathways they utilize, and the cellular responses they induce. Some of the TLRs can recognize more than one ligand, and again, these ligands can be structurally unrelated to each other. Available evidence suggests that TLRs respond also to endogenous molecules, most of which are released from dead cells (DAMPs), suggesting that TLRs can survey danger signals and are associated with sterile inflammation and have important roles in the pathogenesis of inflammatory diseases [4, 5]. Engagement of TLRs activates multiple signaling cascades leading to the induction of genes involved in innate immune responses. Binding of ligands followed by dimerization of TLRs recruits adapter proteins such as myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor protein-inducing IFN-b (TRIF), TIR-associated protein (TIRAP), and TRIF-related adaptor molecule (TRAM). MyD88 associates with IL-1R-associated kinases (IRAKs), TNFR-associated factor 6 (TRAF6), while TRIF activates interferon related factors (IRFs). Both pathways result in activation of nuclear factor NF-kB [6]. A well studied example is Toll Like Receptor 4 (TLR4), that is known to bind the Lipopolysaccharide (LPS) which is present on the Gram negative bacteria cell wall [3, 7]. Once LPS is recognized by TLR-4, ligand binding to this receptor triggers the formation of an intracellular complex between IRAK-1, IRAK-4, MyD88, and TRAF-6 [7] [3, 8-12]. This complex seems to play an important role in the internal signaling provoked by LPS stimulation and it could be the first step in the activation of the MAPK and NFκB pathways [13-15].

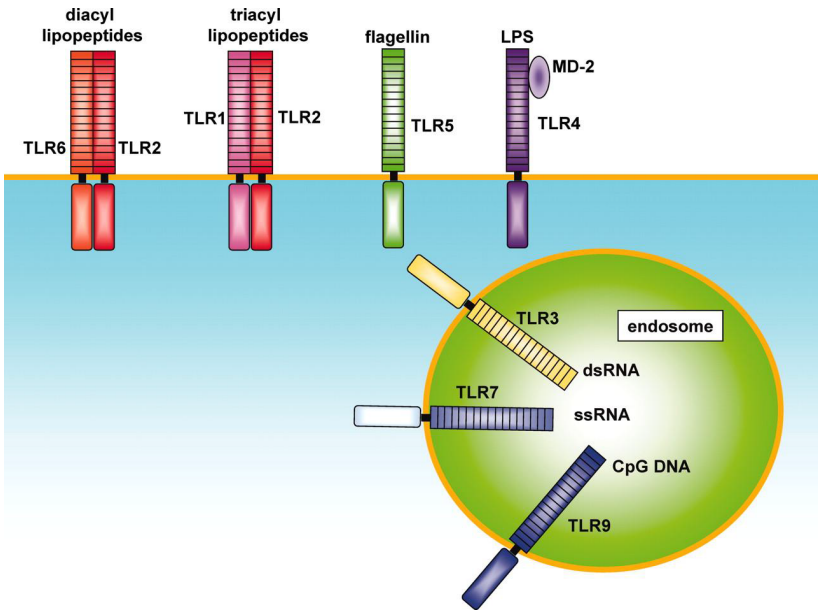


Figure 1. TLRs and their ligands. TLR2 is essential in the recognition of microbial lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in CpG DNA recognition. TLR3 is implicated in the recognition of viral dsRNA, whereas TLR7 and TLR8 are implicated in viral-derived ssRNA recognition. TLR5 recognizes flagellin. Thus, the TLR family members recognize specific patterns of microbial components.

Macrophage activation phenotype

Macrophages have remarkable plasticity and extended flexibility that allows them to efficiently respond to environmental signals and change their phenotype respectively. These changes can give rise to different populations of cells with distinct functions. It has been recognized that these changes can be roughly categorized in classical activation (M1) and alternative macrophage activation (M2) [16]. In general, classically activated macrophages are "effector" macrophages, activated through Toll-like receptors, interferon-γ (INF-γ), LPS and tumor necrosis factor (TNFα) [16] (figure 2). These cytokines prime the cells towards high production of iNOS and reactive oxygen species (ROS), IL-12β secretion and high capacity to present antigen, leading to a type I response [16]. These cells exhibit enhanced microbicidal or tumoricidal capacity, increased secretion of cytokines and mediators, and higher expression of co-stimulatory molecules [17].

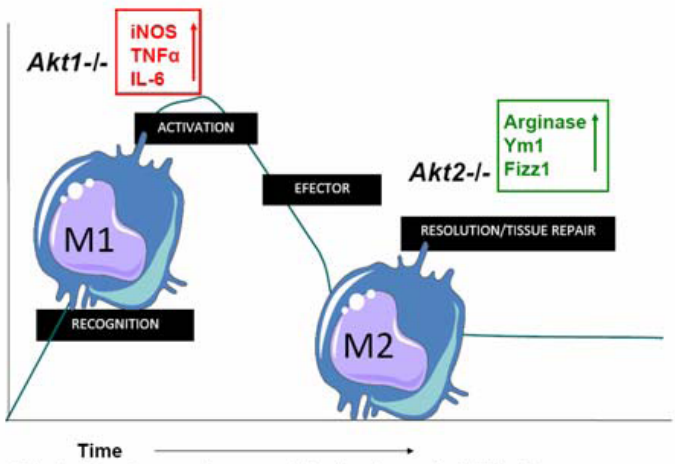


Figure 2.Polarization of macrophages and the involvement of AKT isoforms

Thus, M1 macrophages contribute to the development of inflammation and tissue injury. On the other hand, alternatively activated macrophages are activated mostly by interleukin-4 (IL-4) or IL-13. PAMPs that are expressed by helminths and parasites as well as metabolic pathways of obesity and insulin resistance may also drive the alternative activation of macrophages [18, 19]. M2 is characterized by high levels of arginase-1 (Arg-1), found-in-inflammatory-zone-1 (Fizz1), chitinase-3-like-3 (Ym1), and macrophage galactose C-type lectin 1 and 2 (MGL1, 2) expression [20]. M2 macrophages participate in the resolution of inflammation, and are known to be beneficial in the outcome of several inflammatory diseases [16, 21, 22] (Figure 3). In M2 macrophages, arginase-1 antagonizes iNOS for the same substrate, L-arginine, but produces urea instead of nitric oxide, compromising by this way the generation oxidative stress [16, 21]. In general, the M2 phenotype is characterized by anti-parasitic activity, tissue remodeling, wound healing and tumor sustained progression through its immunoregulatory activity (Tumor Associated Macrophages).

Another population of macrophages is the ‘tolerant macrophages”. Tolerance is defined as the reduced capacity of a cell to respond to LPS activation after an initial exposure to this stimulus ensuring that macrophages will not over-react to sustained TLR stimuli [10]. Endotoxin tolerance is characterized by a decreased production of cytokines in response to the proinflammatory stimulus. These cells combine potent phagocytic activity with impaired capability for Ag presentation [11]. Recent findings have shown that tolerant macrophages share many similar characteristics with M2 macrophages and represent a distinct state of M2 polarization [23] .

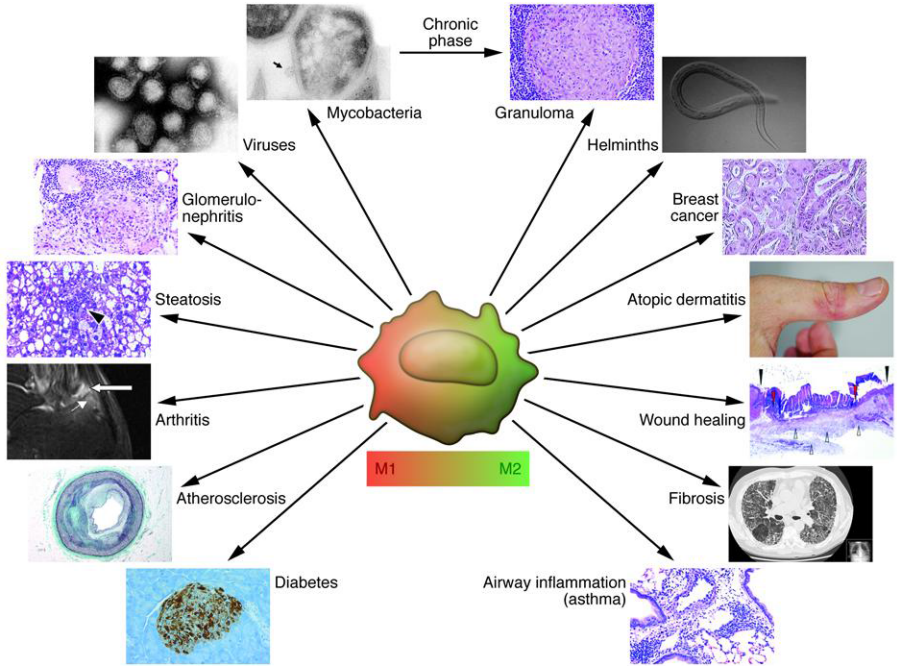


Figure 3. Schematic representation of macrophage plasticity and polarization in pathology. Dynamic changes occur over time with evolution of pathology: for instance, a switch from M1 to M2 macrophage polarization characterizes the transition from early to chronic phases of infection. Images adapted from reference [24].

Regulation of macrophage activation

Apart from the signaling cascades elicited by receptor stimulation, there is a group of signaling pathways and regulatory genes which tune the polarization program [25]. NFkB p65 subunit, STAT-1 phosphorylation and IRF-5 upregulation as well as suppression of suppressor of cytokine signaling SOCS1 have been shown to promote M1 activation [14, 21, 26-29]. For example, forced expression of IRF5 in M2 macrophages drove M1-specific cytokines, chemokines, and costimulatory molecules production and led to a potent Th1 response. Moreover, induction of M1-markers was impaired in irf5−/− macrophages[27]. Several other genes have been found to specifically alter the ability of macrophages to undergo alternative activation. The elements identified so far belong to the same families that control classical activation, such as the NF-kB member p50, the IRF family member IRF4, the C/EBP family member C/EBPβ controlled by Creb, the STAT member STAT6 and NF-

kB competitor PPAR- γ [30-33]. Noncoding RNAs such as miR-155 have also been shown to regulate macrophage polarization although their role in macrophage polarization need to be further defined. In figure 4 the major transcription factor pathways that control M1 and M2 activation are depicted.

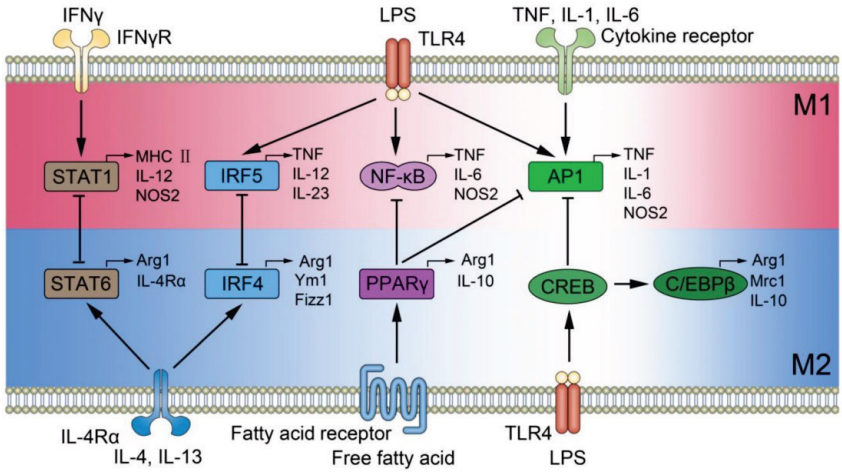


Figure 4. Signal pathways of macrophage polarization. The figure illustrates several mechanisms underlying macrophage polarization and shows the feedback regulation between M1 and M2 signal pathways. The feedback regulation between M1 and M2 are implemented by STAT1-STAT6, IRF5-IRF4, NF-κB-PPARγ, AP1-CREB, and AP1-PPARγ, and they play essential roles in the initiation, development, and cessation of inflammatory diseases [34].

Evidence shows that not only pathway-specific transcription factors and receptors, but also cytosolic enzymes, and functionally distant genes can be regulators and contribute to tuning and determining the macrophage activation profile. Within the different signaling complexes, the PI3K pathway and its downstream mediators are emerging as central for the elicitation and control of M1 and M2 macrophages. The PI3K/Akt pathway do regulate macrophage activation pathways by regulating cytokine and TLR4 receptor signaling [35] and by modulation of IKK phosphorylation and NF-kB suppression[1]. However, the potential distinct function Akt isoforms in macrophage differentiation remains elusive. Recently, our work has shown that Akt1 and Akt2 kinase isoforms have a major role in macrophage polarization.

The role of Akt Kinases in macrophage activation.

Akt, also known as protein kinase B (PKB), is a family of three serine/threonine protein kinases, Akt1, Akt2 and Akt3, important to control cell survival, proliferation, differentiation and intermediary metabolism [36, 37]. The first two are being expressed in most tissues including myeloid cells while the third is expressed primarily in neuronal tissues. In general, Stimulation of macrophages with the TLR4 agonist LPS activates PI-3K and its downstream targets, including Akt kinases. Thus, activation of the PI-3K-Akt pathway suppresses LPS-activated MAPK and NF-kB signaling cascades in monocytes and dendritic cells, resulting in decreased production of TNF-a and other cytokines [38, 39]. Moreover, inhibition of Glucogen Synthase Kinase-3 (GSK3) in vivo, a kinase that is phosphorylated and inactivated by Akt, results in resistance to endotoxin shock [40] suggesting that inhibition of Akt will render mice more sensitive to LPS. Consistent with these observations, Akt overexpression in lymphocytes prevents sepsis-induced apoptosis and improves survival in a cecal ligation and puncture (CLP) model of polymicrobial sepsis [41]. Moreover, whereas macrophages deficient of the p85a subunit of PI-3K are hyperresponsive, macrophages deficient of PTEN, a phosphatase that antagonizes PI-3K, are hyporesponsive to signals associated with macrophage activation [42]. Akt isoforms perform similar as well as unique functions within cells as were revealed by knock-out mouse models. Knock out mice of Akt-1 exhibit global growth defects (growth retardation) and reduced life duration, knockout of Akt-2 results to insulin resistance and development of diabetes and Akt-3 knock out seems to be implicated in the development of brain and neuronal survival [43, 44]. Despite their high homology, Akt isoforms regulate distinct physiological functions via mechanisms including distinct tissue distribution of the Akt isoforms, differential activation of the Akt isoforms by different PI3K kinases, and isoform specific subcellular compartmentalization [45]. PI3 kinase is the major upstream regulator and activator of Akt [46]. PI3K catalyzes the formation of a lipid second messenger, PIP3 from PIP2. Then proteins that contain PH domains such as Akt kinases can bind to PIP3 and can be activated. Activation of Akt is a complex process that can be divided in three steps. First, Akt must translocate to the cell membrane through the

interaction of its PH domain with PIP3 [47]. Binding to PIP3 induces a conformational change in Akt (unfolding) which facilitates its further activation through two additional independent phosphorylation events, in two specific sites (Thr309 and Ser474) by upstream serine/threonine kinases, the PDK-1 and mammalian target of rapamycin (mTOR) complex 2 (mTORC2/Rictor) [47]. Both phosphorylation events are essential for the full activation of Akt [48]. Once upon activation, Akt dissociates from the plasma membrane and phosphorylates many substrates in the cytoplasm and the nucleus. Most of the substrates contain conserved AKT phosphorylation sites (RXRXXS/T) [49]. There are also several proteins such as protein phosphatase-2A (PP2A) and the PHLPP-1 (PH-domain Leucine-rich repeat protein phosphatase-1) [50] that are direct or indirect inhibitors of Akt. Apart from these, there are also several inhibitors of the upstream molecules that indirectly inhibit the activation of Akt and the insulin signaling in general. The most common is SHIP2 (SH2-containing inositol polyphosphate 5-phosphatase-2) and phosphatase PTEN. Moreover, several natural and synthetic inhibitors of PI3K, wortmannin and LY294002 respectively, reduce the level of PIP3 and prevent the phosphorylation and activation of Akt. Another study reported that Akt1 inhibition resulted in enhanced clearance and reduced intracellular growth of Salmonella typhimurium and Mycobacterium tuberculosis [51]. The precise mechanism by which Akt isoforms regulate macrophage activation is not known.

Inhibition of inflammatory response

The innate immune response is activated rapidly (within hours) compared with adaptive immunity. Exposure of macrophages to endotoxin triggers a robust response resulting in the transcription of numerous genes that contribute to the inflammatory response. If inflammation is not resolved promptly, it progresses into a chronic state in which the persistence of immune cells infiltrating the tissue is associated with its destruction and fibrotic alteration. Also, overproduction of cytokines by immune cells to overwhelm pathogens can lead to sepsis and can be fatal [13]. Therefore, immune responses, physiologically, are tightly regulated to avoid sustained activation and in many cases self-restricted via negative feedback signaling loops. Inhibition of Toll-like receptor signaling is a major mechanism in

suppression of immune response. For instance, in the case of TLR4, continual stimulation with endotoxins shifts responsive cells into a transient anergic and tolerant state, under which restimulation with LPS is unable to transduce the early pro-inflammatory features, a phenomenon termed Endotoxin Tolerance [10]. This altered status is believed to be the net result of an accordingly altered transcriptional profile. Tolerant macrophages are characterized by the downregulation of pro-inflammatory cytokines, the upregulation of anti-inflammatory cytokines (and cytokine antagonists such as IL-1Ra) and decreased antigen presentation capability, as conferred by the downregulation of MHC-II molecules and CIITA. Thus, at the stage of endotoxin tolerance, a different set of genes is expressed, that includes anti-inflammatory cytokines and negative regulators of TLR signaling (Figure 5). It seems that activation of TLR signaling pathway leads essentially to its own dampening. This involves either the downregulation of key effector molecules in the TLR signaling pathway, such as the TLR4 receptor [12], or the upregulation of negative regulators such as SOCS-1 [9], A20 [8] and interleukin-1 receptor-associated kinase- M (IRAK-M), mitogen and stress activated protein kinase (MSK) 1 and 2, TGF-b-activated kinase 1 (TAK1) and the Src homology 2 domain-containing protein tyrosine phosphatase-1 and -2 (SHP-1, -2)[52]. Regulation is also performed at a post transcriptional level, with the modulation of miRNA expression via Akt kinases [14].

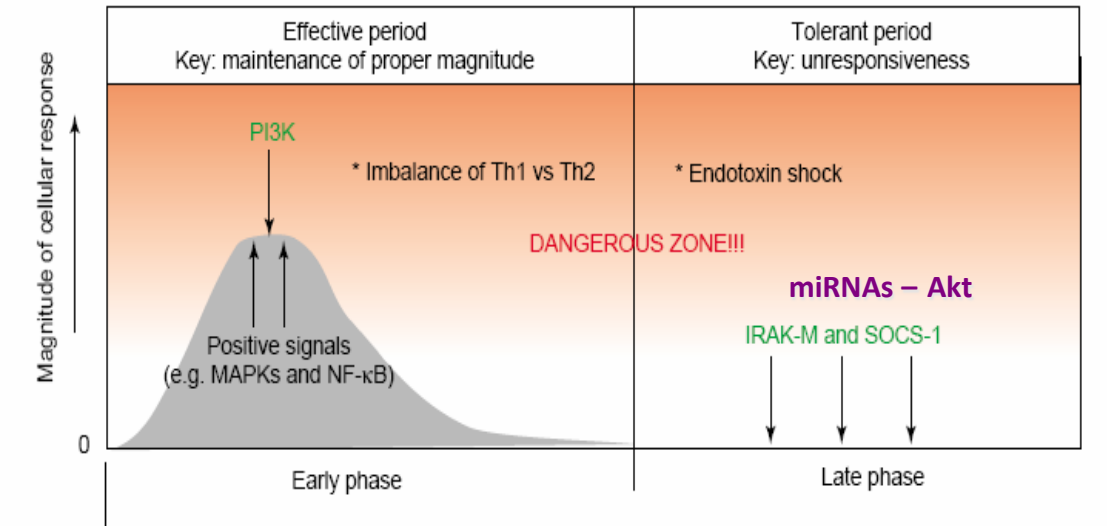


Figure 5. Magniture of cellular response; at the stage of endotoxin tolerance, a different set of genes is expressed, that includes anti-inflammatory cytokines and negative regulators of TLR signaling.

MiRNA regulation of macrophage activation

MicroRNAs (miRs) are small non-coding RNAs of about 19–22 nucleotides that regulate multiple cellular functions via post-translational control of mRNA expression, including ones involved in innate and adaptive immune responses. MiRs bind to the 3' untranslated region of target mRNA and direct their post-transcriptional repression. In detail, miRs are transcribed by RNA polymerase II as part of capped and polyadenylated primary transcripts that are then cleaved by the Drosha ribonuclease III enzyme to produce an approximately 70-nt stem-loop precursor microRNA, which is further cleaved by the cytoplasmic Dicer ribonuclease to generate the mature microRNA and antisense microRNA products [53]. The mature microRNA is incorporated into a RNA-induced silencing complex, which recognizes target mRNAs through base pairing with the microRNA and most commonly results in translational inhibition or destabilization of the target mRNA [53] (Figure 6). Several miRs such as miR-146a, miR-221, miR-125b, miR181c, miR-155 and let-7e, miR-98 have been identified to regulate TLR4 signaling and induce or maintain endotoxin tolerance [54-56]. Among these miR-155, miR-21 and miR-146a, and let-7e appear to have a central role (26-28). MiR-155, rapidly upregulated by NF-kB, is best characterized as a pro-inflammatory miRNA because it enhances the production of inflammatory cytokines in macrophages and other immune cell types. MiR-155 has been reported to increase TNF-a production by stabilizing the TNFa transcript thus promoting M1 response and to targets the IL-13 receptor which promotes alternative macrophage activation [57]. Interestingly, a recent study has shown that miR-155 delivery in alternatively activated macrophages is sufficient to reprogram these cells toward a

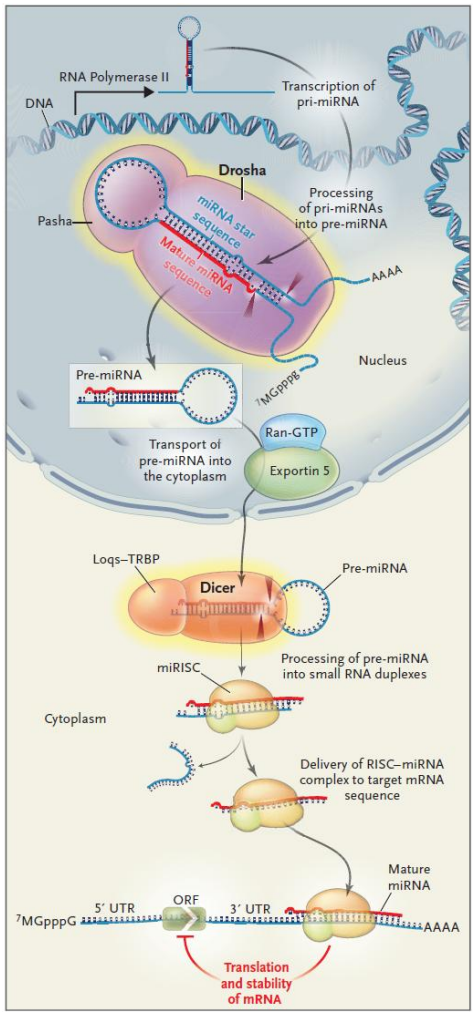


Figure 6. miRNA biosynthesis

more pro-inflammatory M1 phenotype [58]. MiR-155 has been also shown to target key molecules involved in TLR signal transduction TGF-beta-activated kinase 1 (TAK1)-binding protein (TAB)2, and SHIP1 [59]. SHIP1 negatively regulates the PI3K/AKT1 pathway, therefore, SHIP1 repression by miR-155 may increase AKT1 signaling and promote alternative macrophage activation [60]. The aforementioned data suggest that miR-155 induction by inflammatory signaling sustains or even amplifies classical proinflammatory macrophage activation [61]. MiR-146a as a potent inhibitor of inflammation blocks TLR signaling by targeting IRAK1 and TRAF6, proteins that are important components of the myeloid differentiation primary-response protein 88 (MyD88)-dependent pathway for NF-κB activation downstream of TLR2, TLR4, TLR5, TLR7–TLR8 and TLR9. Mice with a targeted deletion of the miR-146a locus were found to be hypersensitive to bacterial challenge to produce excessive amounts of proinflammatory cytokines in response to endotoxin and succumb to septic shock faster than the wildtype littermates [62]. MiR-146 deficient mice also display severe tissue inflammation increased basal cytokine production, as well as elevated titers of autoantibodies—all classical signs of autoimmunity. Furthermore, aged knockout mice develop tumors in their secondary lymphoid organs and undergo myeloproliferation, suggesting that miR-146a regulates proliferation in immune cells [63]. Furthermore, miR-146a is necessary for the *in vivo* suppressor function of Tregs [64] and it is found elevated in tolerant “M2-like” macrophages [65]. MiR-146a has target sites in additional, though less well-validated targets, which include IRAK2, STAT1, IRF5, SMAD4 and CXCR4.

Other miRNAs that are important in controlling innate immune responses and macrophage activation include miR-125b, let-7e, miR-223, miR-9, miR-21, miR-187 and miR-147 [66]. MiR-125b expression is upregulated in macrophages in response to proinflammatory stimuli and finely tunes TNF-a production, NF-kB activation, and IFN-g signaling in macrophages [60]. miR-125b has been shown to directly regulate TNF-α 3’-UTR. Enforced expression of miR-125b drives macrophages to M1 activation, whereas anti–miR-125b treatment decreases CD80 surface expression [24]. MiR-125b can also sustain pro-inflammatory cell activation by targeting the transcription factor IFN regulatory factor (IRF)4 [67], which promotes alternative

macrophage activation. Therefore, miRNA-mediated post-transcriptional regulation is critical for innate immune cell response to microbial infection. Chen et al. have shown that in human biliary epithelial cells (cholangiocytes), miRNA let-7i (one of the isoform of miR-let-7) is downregulated in response to C. parvum or LPS, whereas TLR4 is upregulated [68]. Their observation suggests that let-7i regulates TLR4 expression in vitro. In mouse peritoneal macrophages, the induction of let-7e expression in response to LPS decreases cell surface expression of TLR4, the mRNA of which contains a let-7 target site. In this case, LPS signals activate Akt1, and let-7e is upregulated in an Akt1-dependent manner. Thus, Akt1−/− macrophages have exhibited increased responsiveness to LPS in culture and consistently Akt1−/− mice do not develop endotoxin tolerance in vivo. Overexpression of let-7e in Akt1−/− macrophages can restore tolerance to LPS in culture and in animals, indicating that Akt1 regulates the response of macrophages to LPS by controlling miRNA expression [14]. Both miR-155 and miR-146a have attracted particular attention, as they are responsive to a plethora of inflammatory stimuli including TLR ligands, TNFα, IL-1b, type I and type II interferons or RANKL in different cell types, including macrophages [69-76]. Contribution of miR-146a and miR-155 in endotoxin tolerance has been demonstrated in vivo in mouse models and in human disease. miR-146a was indispensable for the development of endotoxin tolerance [77, 78]. Contribution of these miRNAs in endotoxin shock and tolerance can also occur through their secretion in exosomes [79]. In humans, miR-146a appears to be important in the development of innate immune tolerance observed in leukemic patients [80]. Expression of both miRs has been associated with inflammatory diseases including rheumatoid arthritis [81, 82], systemic lupus erythematosus [83], nephropathy [84], atherosclerosis [85], infertility [86], periodontitis [87] and type 2 diabetes [88]. miRs are regulated at transcriptional and post-transcriptional level [89]. miR-155 is transcribed within the BIC gene while miR-146a appears to be intergenic [70]. Analysis of the gene promoters of these two miRNAs revealed that both are regulated by NFkB [51, 70, 90], suggesting a common transcriptional regulatory mechanism.

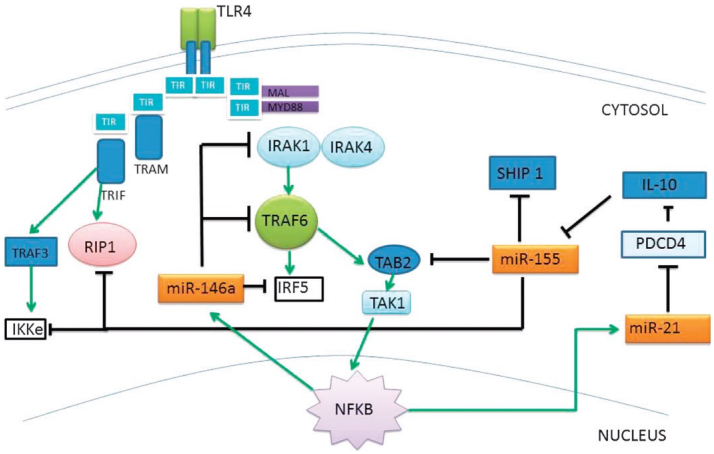


Figure 7. Regulation of TLR signaling by microRNAs.

Epigenetic control of macrophage activation/inactivation

The term epigenetics refers to modifications that do not modify the genetic code but instead control how information encoded in DNA is expressed in a tissue and context-specific manner [91]. Epigenetic components and mechanisms are normally interceded by posttranslational modifications (such as phosphorylation, acetylation, methylation etc) of histones and other chromatin proteins that bind DNA, by methylation and hydroxyl methylation of CpG DNA motifs, and by non-coding RNA [92], [93], [94]. These epigenetic marks have been considered to be stable, possibly transmissible to offspring, and to underlie steady differentiation into various tissues and cell types that express distinctly different patterns of gene expression despite containing identical DNA sequences and genomes. Lately it has become clear that epigenetic chromatin marks are dynamically regulated in response to environmental stimuli. Despite the fact that epigenetic marks are dynamically regulated, they are normally more stable than the quickly fluctuating post-translational modifications of upstream 'traditional' signaling proteins. Subsequently, epigenetic changes that continue to carry on after the original stimulus has ended, provide a mechanism for extending temporary short-lived signals into a more stable and constant cellular response lasting quite a few hours or days (or longer). A model that has been developed is that the 'epigenetic

landscape' of a cell which includes the total patterns of DNA methylation, chromatin modifications, and proteins pre-bound to gene regulatory regions (promoters and enhancers) determines the accessibility for binding and therefore the genomic localization of signaling transcription factors that are activated by acute signals. [91]. Consequently, the pattern of gene expression in accordance to an external stimulus is formed by the developmental history of a cell and previous environmental exposures that have shaped the epigenetic landscape. The epigenetic landscape, in turn, could be reformed in response to acute stimulation and polarizing stimuli. Such remodeling of the epigenetic landscape helps to eventually integrate signals and guides reprogramming of cells to change their gene expression pattern in response to subsequent stimuli. This gene-specific regulation occurs at the chromatin level and includes nucleosome remodelling and covalent histone modifications. Histone methylation, as a mechanism for modifying chromatin structure, is associated w ith gene regulation. Analysis of the epigenetics of macrophage polarization until now has primarily focused on post-translational modification of histones, with partial analysis of ATP-dependent nucleosome remodeling. There is a huge amount of histone modifications, which can be generally divided into positive and negative marks that promote or suppress transcription, respectively. Table 5 shows the most broadly studied marks relevant for macrophage activation / inactivation (polarization). Whereas trimethylation of histone 3 lysine 4 (H3K4) is associated with active gene transcription, trimethylation of H3K9, H3K27 and H3K79 are linked to silencing of gene expression. [95-97]. These histone marks are 'written' and 'erased' by enzymes called chromatin regulators. The pattern of histone marks forms a sort of 'code' that is 'read' by supplementary chromatin regulators and transcriptional co-activators / co-repressors to determine the rates of transcription initiation and elongation. Therefore, the equilibrium of positive and negative histone marks at gene promoters and enhancers determines transcription rates. The current chromatin state, defined by well-established combinations of histone marks, determines basal transcription level, the extent and kinetics by which a gene locus responds to extracellular stimulus[94]. A key concept is that gene loci relevant for polarized macrophage phenotypes exist in three broad states [2], [98], [91, 99]. First, there is a repressed state characterized by the

presence of negative marks such as histone 3 lysine 9 trimethylation (H3K9me3) and H3K27me3, absence of positive marks, and a closed chromatin conformation. These genes are refractory to fast induction by activating stimuli. Second, there is a poised state characterized by the existence of activating histone marks (H3K4me3, H3K9, 14-Ac), chromatin conformation that is partially open, and in some cases, a pre-bound RNA polymerase II that is paused near the transcription start site. Transcription at poised genes is controlled by concurrent presence of the repressive histone marks such as H3K9me3 and H3K27me3, co-repressor complexes, and partially closed chromatin that requires additional positive histone marks and ATP-dependent nucleosome remodeling to be fully accessible to transcription factors. Lastly, there is a third active state that is characterized by active histone marks, an open chromatin conformation, and ongoing transcription. A complex biological process such as endotoxin tolerance requires significant changes in gene expression via specific transcription factors and histone modifications to ensure temporal gene regulation [100, 101]. Histone acetylation is induced in response to TLR stimulation in macrophages, and is involved in the expression of multiple pro-inflammatory cytokine genes. Furthermore, trimethylation of H3K4 on cytokine gene promoters was also shown to be induced in M1 macrophages in response to TLR stimulation, indicating that a change in histone modification is induced in the course of M1 macrophage activation leading to chromatin remodeling and inflammatory gene expression (figure 8) [2]. mPolycomb repressive complex 2 (PRC2) composed of Ezh2, Suz12 and Eed mediate the methylation of H3K27. On the other hand demethylases that are known to act on H3K27 by catalyzing trimethylation of H3K27me3 to monomethylation H3K27me1 and harbor a Jumonji-C (JmjC) domain are Jmjd3 (also known as Kdm6b), UTX and UTY, [88–90]. Importantly, the expression of Jmjd3 is induced by TLRs in macrophages via an NF-

	Function	Location	Writer	Eraser
H3K4me3	+	P	MLL	KDM5B
H3K9,14-Ac	+	P,E	HAT (CBP/p300)	HDAC3
H4K5,8,12, 16-Ac	+	P,E	HAT (CBP, MOF)	HDAC
H3K27-Ac	+	E,P	HAT (CBP)	HDAC1,2
H3K4me1	+	E	MLL	LSD1/KDM1A
H3K9me3	–	E,P	G9a	JMJD2
H3K27me3	–	P,E	EZH2 (PRC2)	JMJD3, UTX

[a]Ac, acetylation; CBP, CREB binding protein; E, enhancer; H, histone; K, lysine; KDM, lysine demethylase; me, methylation; MLL, mixed lineage leukemia; P, promoter; PRC2, polycomb related complex 2.

Figure 8. Histone marks that promote or suppress transcription

kB-dependent pathway. In view of the fact that H3K27 trimethylation is implicated in the silencing of gene expression, it has been postulated that Jmjd3 is involved in the fine-tuning of macrophage activation toward M1 by regulating a set of genes such as Bmp2 and Hox [[102, 103]]. Expression of the M2 marker genes like Arg1, Ym1, Fizz1, MR and IL-13 was severely impaired in Jmjd3 deficient bone marrow macrophages cultivated in the presence of M-CSF which induces M2 polarization. This observation indicates that Jmjd3 is crucial for the expression of M2 marker genes in bone marrow macrophages. Conversely, HDAC3 acts as a brake on IL-4-induced M2 polarization by deacetylating putative enhancers of IL-4-induced M2 genes [104]. Consequently, Jmjd3 acts as a demethylase to induce M2 macrophage polarization, although recent studies show a demethylase-independent role in controlling chromatin remodeling [96]. Thus, both histone methylation and acetylation are important for M2 polarization. In general chromatin immunoprecipitation sequencing analysis shows that trimethylation of H3K27 is enriched in the promoter regions close to the transcription start sites in bone marrow macrophages. M2 marker genes, such as Ym1, Mrc1 and Arg1, were not trimethylated at H3K27 either in the presence or in the absence of Jmjd3, suggesting that these genes are not directly controlled by Jmjd3 through histone modification. Conversely, H3K27 trimethylation of transcription factors such as Irf4 and CEBPb was differentially regulated between wild-type and Jmjd3 deficient macrophages. Development of endotoxin tolerance in macrophages requires global changes in gene expression, which is reflected in global changes of H3 methylation. [6, 105-107]. A genome-wide analysis recently has shown the loss of H3K4me3 marks from the class of genes that they are not inducible in tolerant macrophages selected genes upon development of endotoxin tolerance [6, 105]. However, no information is yet available on histone modification changes of miRNA genes in the process of endotoxin tolerance.

Chromatin interactions

Accumulating evidence is consistent with the fact that the subnuclear localization of chromatin is not random. It seems that each chromosome is localized in a limited and specific space, called a 'territory', and DNA sequences within a chromosome are organized into euchromatin or heterochromatin. As cellular differentiation proceeds, changes in transcriptional activity are often coupled with changes in subnuclear localization of chromosomes. For example, silent genes in developing B and T cells are repositioned in the nucleus at pericentromeric heterochromatin.

Additionally, in some cases, large chromosomal loops containing active genes extend outside of the defined chromosomal territories. In detail, genes contributing to a

particular cellular function or cell fate can be coordinately regulated through long-range chromatin interactions [108-115].

According to this mechanism, genes present on the same or different chromosomes come in close proximity and utilize the same transcription factory by looping out of

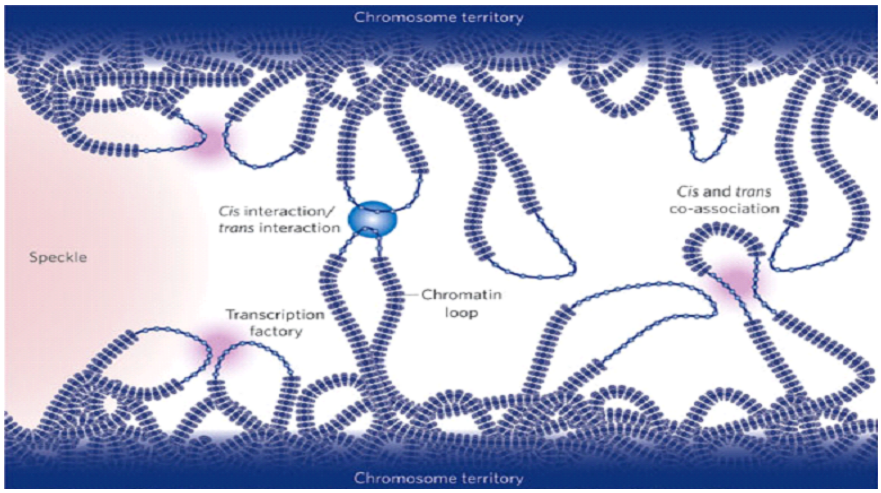


Figure 9. Genes present on the same or different chromosomes come in close proximity and utilize the same transcription factory by looping out of their chromosome territories

their chromosome territories (Figure 9). Thus, genes are dynamically organized into shared nuclear subcompartments, which ensure a common regulation of their transcription. An example of such association is this of the Th2 gene locus with the loci harboring the Th2 cytokines IL-4, IL-5 and IL-13, which are located on different chromosomes [115]. These associated gene loci utilize common transcription

factors to secure timely and coordinate gene regulation, necessary for massive changes in gene expression that occur under conditions such as macrophage activation and endotoxin tolerance. For the repositioning of specific genetic loci to regions with active or silent transcription, transcriptional regulatory elements such as locus control regions, enhancers or insulators are responsible.

Collectively, large chromosomal loops containing protein genes for a particular cellular function or cell fate extend outside of the defined chromosomal territories leading to dynamic intra- and interchromosomal interactions and subsequently to co-ordinate regulation of these loci. Similarly to protein coding genes, recent results have provide a novel insight into the 3D regulation of MIR transcription, highlighting the existence of 'transcription factories' in the cell-defined chromatin space for co-transcribing MIRs and protein-coding genes [116]. Besides this, Papantonis et al have shown that miR-155 participates in NF-kB factories where TNF-a –responsive genes - hosting miRNAs are co-associated and transcribed [117]. However, it is not known whether chromatin interactions between gene loci harboring microRNAs take place. In addition, whether intercromosomal associations facilitate expression or silencing of genes during the development of endotoxin tolerance in macrophages remains unknown.

Clinical significance of Endotoxin tolerance

Endotoxin tolerance is a phenomenon that renders cells or organisms exposed to low concentrations of endotoxin (LPS) to enter into a transient unresponsive state and become refractory to a further endotoxin challenge. Several studies have used in vitro and in vivo models of endotoxin tolerance in order to reveal the molecular mechanism. Randow et al showed that Il-10 could lead to deactivation of macrophages in response to LPS [118] while miR-146a, mimicking LPS induced cross-tolerance, contributes to controlling TNF-a production and is critical for the in vitro monocytic cell based endotoxin tolerance [65, 73]. However, the endotoxin tolerant state is not restricted to sepsis and appears to be a general paradigm of immunosuppression, as it has been observed for a number of pathologies such as hepatic and renal ischemia, coronary occlusion, acute coronary syndromes, cystic

fibrosis and even cancer [15, 119, 120], [121]. Several studies have elucidated the mechanistic basis of immunosuppression across different diseases. Patients suffering from gram-negative septicaemia [122] and acute coronary syndrome [120], show a rapid increase in the negative regulator IRAK-M expression while TNFa induction was poor in circulating monocytes. In cystic fibrosis patients, studies demonstrated an endotoxin tolerant state in their circulating monocytes, elucidating the significant role of TREM-1 [119]. In cancer, tumor associated macrophages (TAMs) show an immunosuppressive phenotype similar to endotoxin tolerance. TAMs show increased production of anti-inflammatory cytokines, IL-10 and TGFb and downregulation of inflammatory cytokines like IL-12p40 and TNFa upon ex vivo LPS stimulation.

While endotoxin tolerance has been thought of as a protective mechanism against septi

c shock and ischaemia, its incidence is associated with high risks of secondary infections. It has been noted that people who succumb from sepsis die after the end of the initial proinflammatory stimulus, probably from a second infection. Similarly, in acute pulmonary syndromes and cystic fibrosis, endotoxin tolerance relates to an increased susceptibility to nosocomial infectious. During sepsis or other major inflammatory stresses, a carefully orchestrated balance between pro-inflammatory and anti-inflammatory mediators within the host organism is necessary in order to counteract and restore homeostasis from an inflammatory state (Figure 10). The excessive production of different or redundant proinflammatory mediators (TNF-a, IL-1, IL-6, and IL-8) leads to a hyper-inflammatory status and characterizes Systemic inflammatory response syndrome

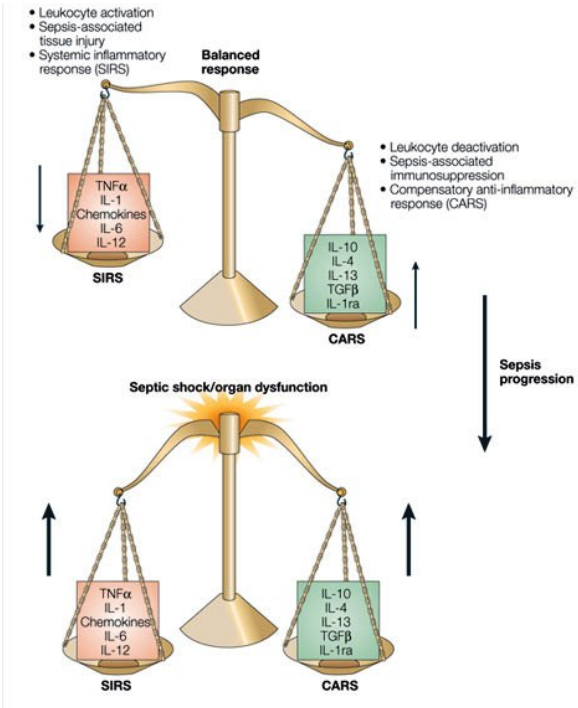


Figure 10. SIRS and CARS syndrome

(SIRS). This potential destructive effect of excessive proinflammatory response has to be reversed by the development of a compensatory anti-inflammatory response syndrome (CARS). This systemic deactivation of the immune response is characterized by cutaneous anergy, reduction of lymphocytes, decreased cytokine response of monocytes to stimulation, decreased numbers of HLA antigen presenting receptors on monocytes, expression of anti-inflammatory substances, including IL-4, IL-10, IL-11, IL-13, soluble tumor necrosis factor receptors, interleukin-1 receptor antagonists and transforming growth factors. However, a deregulation of the balance between SIRS and CARS response can be lethal either through uncontrolled inflammation or failure to defend against infectious organisms. In fact, the balance of SIRS and CARS determines a patient's prognosis after an insult. Moreover, many of the new medications meant to inhibit the proinflammatory mediators may lead to deleterious immunosuppression. For this reason there have been several studies with the aim to further understand the correlation of CARS response and patient outcome and find CARS biomarkers as a possible tool for prognosis and therapy. Despite the progress in revealing the pathogenesis of CARS syndrome, the mechanisms that regulate this syndrome (CARS) remain incompletely understood, while a biomarker for prognosis and therapy is not found yet. The role of micro RNAs (miRNAs) in modulating immune responses is appeared to be catalytic as they are involved in innate and adaptive immune signaling pathways. Significantly, changes in miRNA expression have been implicated in various disease states such as cardiovascular, autoimmune, neurodegenerative, liver and inflammatory diseases, introducing the idea of miRNAs as potential biomarkers for diseases. Many studies have been performed to characterize the expression of various miRNAs both intracellularly and extracellularly, advocating their potential role as biomarker for prognosis and therapy. Diagnostic value of miRNAs was firstly noted in cancer and in acute stage of diseases, such as drug induced liver injury. Recent studies demonstrate that miR-150 and serum miR-146a and miR-223 might serve as potential biomarkers for sepsis[123]. miR-155 and miR-146a are regulated by PI3K/Akt1 pathway and control the magnitude of inflammatory responses. TLR signalling strongly induces miR-155 and miR-146a, enhancing or resolving the proinflammatory response, respectively,

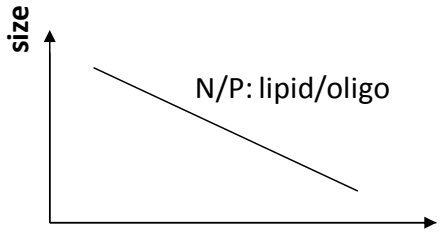
while ectopic expression of miR-146a/b inhibited Il-6 secretion from primarly human fibroblast [14, 22]. Furthermore, the expression of miR-155 and miR-146a has been associated with inflammatory diseases including Rheumatoid arthritis [81, 82], Systemic Lupus Erythematosus [83], nephropathy [84], atherosclerosis [85], periodontitis [87] and type 2 diabetes [88]. Subsequently, miR-155 and miR146a dysregulation correlates with clinical manifestation an d inflammation, and therefore may have potential role as biomarkers reflecting the severity of the inflammatory response. Despite the ongoing studies, plasma remains one of the best sources for measurement inflammatory mediators enabling a rapid characterization of patient's inflammatory status and correlation with mortality. As predictor tool of the poor outcome, studies have focused on the anti-inflammatory cytokine levels profile and mainly on the role of IL10, where high levels of it are correlated with three or more organ dysfunctions and mortality. Even though the causes of CARS can be diverse, patients share common features that are underlined by impaired immune responses. The endotoxin tolerant state appears to be a general paradigm of immunosuppression across different diseases. Studies elucidating the molecular mechanism of endotoxin tolerance may provide clues in understanding the mechanistic basis of immunosuppression in other pathologies and give the possibility to manipulate macrophage responses by targeting molecules that affect the tolerant state.

Delivery technologies of oligonucleotides for therapeutic use in vivo - Liposomal formulations

RNA interference (RNAi) represents a highly promising approach for the selective silencing of genes involved in the pathogenesis of inflammatory disease. RNAi by oligonucleotides is a post-transcriptional pathway in which double-stranded RNA (dsRNA) triggers the degradation of complementary mRNA in the cytoplasm of eukaryotic cells. However, RNAi has only rarely been used in vivo in inflammatory diseases, despite its potential for contributing to the understanding of disease mechanisms and the development of novel therapeutics. This is due to the fact that there is a lack of an effective delivery of oligonucleotides to immune cells and/or

sites of inflammation. Numerous approaches for systemic and targeted delivery technologies have been tested including the use of neutral liposomes [124] or atelocollagen [125] in experimental allergic dermatitis, cationic liposomes in experimental arthritis [126], and antibody-targeted stabilized nanoparticles in experimental colitis [127]. Approaches based on cationic liposomes dominate today due to the easy and reversible complex formation between oligonucleotides and carrier and the efficient delivery of their cargo into cells. However, cationic carrier systems bind oligonucleotides at their surface, leading to their serum aggregation and rapid interaction with endothelial surfaces. This fact limits their in vivo application, because of the limited penetration of such carriers into tissues and of tolerability problems. Unlike cationic liposomes, amphoteric particles generated from Novosom is a novel class of liposomes that are fully charge-reversible particles, do not aggregate in the serum, penetrate beyond endothelia and accumulate in the liver, spleen, and in sites of inflammation. These amphoteric liposomes allow delivery of active substance (siRNA, antisense, decoy, etc.) inside the cell either by local or systemic administration. The liposomal formulation is able to change surface charge properties (zeta potential) with changes in solution pH. The charge switch at acidic pH in fusion with the cell membrane during endocytosis uptake, allowing escape of the nanocarriers into the cytoplasm to deliver the therapeutic load. More precisely, these formulations are negatively charged under physiological conditions. However, as the pH drops down to 5 or 4 during endocytosis, the vector surface becomes neutral and eventually positively charged which allows the release of the oligonucleotide cargo into the cytosol. This unique property offers the advantage of stable and aggregate-free travel within the bloodstream. This provides a known and controllable mechanism for endosomal escape, a required feature for siRNA delivery. The product properties that play significant role on the efficacy of the liposome are the following:

- Size particle
- PI (Polydispersity index)
- Lipid concentration L



- Oligo concentration P
- Lipid integrity

So far, there are siRNA delivery data in liver, inflammation and oncology. *Andreakos et al. have found that* Nov038 is well tolerated, devoid of immune-stimulatory effects, and efficacious in mediating systemic oligonucleotide delivery to sites of inflammation [128]. In mice with collagen-induced arthritis, Nov038 enabled the therapeutic administration of CD40-ASO and improved established disease via rapid downregulation of CD40, inhibition of DC antigen presentation, and reduction in collagen-specific T cell responses, as well as decreased levels of TNF-a, IL-6, and IL-17 in arthritic joints. They found that this amphoteric carrier Nov038 penetrates beyond endothelia and accumulates in sites of inflammation. The uniqueness of this system is its amphoteric, charge- reversible character, achieved by the combination of a novel, weakly cationic lipid, MoChol, which provides cationic charge at low pH, and CHEMS, which is another pH-sensitive but anionic amphiphile, allowing delivery of active substance inside the cell either by local or systemic administration.

Materials and Methods

Animals

C57BL/6 mice were purchased from the Hellenic Pasteur Institute (Athens, Greece). $Akt1^{-/-}$ and $Akt1^{+/+}$ mice [129, 130] were housed at the University of Crete School of Medicine, Greece. All procedures described below were approved by the Animal Care Committee of the University of Crete School of Medicine, Heraklion, Crete, Greece and from the Veterinary Department of the Region of Crete (Heraklion, Crete, Greece).

Cell culture

The murine macrophage cell line RAW264.7 and primary murine thioglycollate elicited peritoneal macrophages were cultured as previously described [131] in macrophage complete medium (DMEM: Dulbecco's Modified Eagle Medium) (GIBCO, Life technologies Corp, Carlsbad, CA) supplemented with 10% (v/v) FBS, 10 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. RAW264.7 cells were tested by FISH analysis and found to be diploid at least on chromosomes 7, 8, 11, 16 and 17, and were kindly provided by Prof. P. Tsichlis, Tufts Medical School, Boston, US. E.coli-derived LPS (100 ng/ml) (O111:B4; catalogue no. L2630; Sigma-Aldrich) was used as described at the results section. Cells were harvested and supernatants were collected at the indicated time points of LPS incubation and stored at -80 °C until assayed for cytokine levels. Cell pellets were washed in PBS and stored at -80 °C for total RNA extraction and subsequent analysis.

ELISA

Cytokine concentration in serum for TNFa, and IL-10 was determined by ELISA at the indicated time points using ELISA kits (R&D Systems), according to the manufacturer's instructions.

Transfection

Transient transfection using the Lipofectamine reagent (RNAimax, Life technologies Corp, Carlsbad, CA) was performed as per the manufacturer's instructions and as described previously [73, 132, 133]. In short, a unique RNAi molecule was used in each well prior to transfection and combined with diluted lipofectamine to form complexes. Cells were added directly to the complexes and transfection was performed while cells were attaching on to the well. $1x10^5$ macrophages per well were transfected with either 30nM of siRNA (small interfering RNA) for C/EBPβ (designed in Cenix Bioscience, Dresden, Germany) or negative control dsRNA in 24-well tissue-culture plates in a volume of 500 µL serum-free/antibiotic-free DMEM culture media. Cells were incubated at 37°C for 24 hours and then the medium was replaced with the one prior to transfection. Transfection efficiency and biological effect was assessed 48 or 72h hours post-transfection. For siAKT2 experiments, cells were transfected with either 30 nM small interfering RNA (siRNA) for Akt2 designed at Cenix Bioscience or with negative-control dsRNA. For miR-146a experiments, transfection was carried out with 30 nM miR-146a mimic, and nontargeting controls that were purchased from Ambion (Life Technologies).

In Vitro Induction of Endotoxin Tolerance

An LPS-tolerance cell model using either the murine macrophage cell line RAW 264.7 or primary thioglycollate elicited peritoneal macrophages was adapted from methods previously described with minor modifications [73]. Briefly, cells were cultured in complete culture medium (DMEM: Dulbecco's Modified Eagle Medium) (GIBCO, Life technologies Corp, Carlsbad, CA) supplemented with 10% (v/v) FBS, 10 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin) for 1 day and viability was monitored by trypan blue staining and was found to be approximately 99%. Cells (300x 10^3 cells/ml) were seeded in 12 or 24-well tissue-culture plates in a volume of 1 ml fresh complete medium and incubated with LPS (100 ng/ml) for 18 h. After two washes with tissue culture grade 1XPBS, cells were cultured in complete culture media in a CO_2 incubator for 2 hours at 37 °C. In case of re-stimulation, LPS (100 ng/ml, final concentration) was added for 2h. Supernatants and cell pellets were harvested and stored at -80 °C until assayed.

RNA and miRNA isolation and quantitative PCR

RNA fro RAW264.7 macrophages or from primary thioglycollate-elicited peritoneal macrophages was isolated using the Trizol reagent (Life technologies Corporation, Carlsbad, CA). One µg of total RNA following DNAse treatment was used for cDNA synthesis (TAKARA, Japan). The following oligonucleotides were used in semi-quantitative RT-PCR approach: Pri-miR-155; Fwd: 5'-ACCCTGCTGGATGAACGTAG-3'; Rev: 5-'CATGTGGGCTTGAAGTTGAG-3'; Pri-miR-146a; Fwd:5'-

CACGGACCTGAAGAACACTGG-3'; Rev: 5'-AGAAATGAAATTAGAACACACATCAATCC-3'; C/EBPβ; Fwd:5'- GGGGTTGTTGATGTTTTTGGTT- 3'; Rev: 5'-TCACTTTAATGCTCGAAACGGA-3'; RPS9; Fwd:5'-GCTAGACGAGAAGGATCCCC-3'; Rev: 5'-CAGGCCCAGCTTAAAGACCT-3'; Hprt1; Fwd:5'-CTGGTGAAAAGGACCTCTCG-3'; Rev: 5'-TGAAGTACTCATTATAGTCAAGGGCA-3'. Ribosomal Protein S9 (RPS9) and Hprt1 served as control housekeeping genes. Annealing was carried out at 60°C for 30 sec, extension at 72°C for 30 sec, and denaturation at 95°C for 15 sec for 40 cycles in a 7500 Fast Real-Time PCR System (Life technologies Corp, Carlsbad, CA/ Applied Biosystems). The amplification efficiencies were the same as the one of RSP9 or Hprt1 as indicated by the standard curves of amplification, allowing us to use the following formula: fold difference = 2^-(DCtA– DCtB), where Ct is the cycle threshold. Reactions were performed in triplicate for statistical evaluation. To isolate micro-RNAs from RAW264.7 or thioglycollate-elicited peritoneal macrophages, total RNA was isolated as described above. For cDNA synthesis and qPCR of specific miRNAs, the following TaqMan MicroRNA Assays (Life technologies Corp, Carlsbad, CA) were used: mmu-miR-155; UUAAUGCUAAUUGUGAUAGGGGU; hsa-miR-146a; UGAGAACUGAAUUCCAUGGGUU; SnoRNA135; CUAAAAUAGCUGGAAUUACCGGCAGAUUGGUAGUGGUG. SnoRNA135 served as housekeeping miRNA. Annealing and extension was carried out at 60°C for 30 sec and denaturation at 95°C for 15 sec for 40 cycles in a 7500 Fast Real-Time PCR System (Life technologies Corp, Carlsbad, CA).

Ex-vivo LPS-stimulated blood cytokine production in human peripheral blood samples

Peripheral blood samples from critically ill patients admitted in the ICU between June and October 2009, were incubated with 100ng/ml LPS for 4h at 37o C. Cytokine production in plasma of IL-8, IL-1β, IL-6 and IL-10 was measured by elisa before or after the ex vivo LPS incubation. RNA was extracted from peripheral blood leucocytes of critically ill patients by using trizol and miR-155 and miR-146 expression was evaluated by realtime PCR with Taqman technology (as described above).

DNA and DNA-RNA fluorescence in situ hybridization

Cells attached onto poly-L-lysine-coated glass coverslips were incubated for 3 min in Cytoskeletal (CSK) buffer (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM Pipes, 0.5% TritonX-100), fixed with 4% paraformaldehyde/1×PBS for 10 min, washed three times with 1xPBS and stored in 70% ethanol at −20°C overnight. Hybridization was carried out in 50% formamide, 2×SSC, 10% dextran sulphate, 1 μg mouse Cot-1 (Invitrogen) and 100 ng fluorescently labelled BAC probe and after a 5 min denaturation of genomic DNA at 73°C coverslips were incubated for 16 hours at 37°C. Slides were washed three times for 5 min each in 2×SSC and mounted in Prolong Gold antifade reagent supplemented with DAPI (Invitrogen). For the generation of fluorescently-labeled DNA probes 2μg Bacterial Artificial Chromosome (BAC) DNA [(miR-146a ID for BAC clone: RP23-390G17) (miR-155 ID for BAC clone: RP24-278G19)], [(let-7e ID for BAC clone: RP24-308G19), [(TNF-α ID for BAC clone: RP23-446C22) were labeled using a Nick translation kit (Roche/cat. no 11745808910) supplemented with either 0.025 mM Spectrum Orange dUTP

(Vysis), 0.025 mM Spectrum Green dUTP (Vysis) or 0.025 mM OBEA dCTP-647 (Invitrogen). The following primers were utilized in PCR reactions to confirm the presence of each gene of interest in the BAC clones: MiR-146a; Fwd 5'-GGCCTTCAGAGTTTGTTCCA- 3'; Rev 5'-GGCCTCATCTGGAGAGTCTG- 3'; MiR-155; Fwd 5'-TTGCTGAAGGCTGTATGCTG- 3'; Rev 5'-ATCCAGCAGGGTGACTCTTG- 3'; Let7e; Fwd 5'-AAAGAAACAAGAAGACGGAC- 3'; Rev 5'- ATCCCTTAGAGAAGACAATCTG- 3' and TNF-α; Fwd 5'-TTCCTCCTTATCTCTCATGC- 3'; Rev 5'-TGACTAAACATCCTTCGTCG- 3'. To detect DNA and RNA by FISH, cells attached onto poly-L-lysine-coated coverslips were incubated for 3 min in CSK buffer (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM Pipes, 0.5% TritonX-100, 1 mM EGTA and 2 mM vanadyl-ribonucleoside complex), fixed with 4% paraformaldehyde/1×PBS for 10 min, washed three times with 70% ethanol and stored in 70% ethanol at −20°C overnight. Hybridization was performed with biotinylated probes for 16 hours at 37°C after a 5 min denaturation at 73°C. The coverslips were washed at 37°C sequentially with 2×SSC/50% formamide, 2×SSC, 1×SCC and 4×SSC. RNA signals were amplified using the Renaissance TSA™ Biotin System (PerkinElmer). The biotinylated DNA probes were prepared as follows: 1000bp genomic region containing the pri-miR-155 or pri-miR-146a RNA sequence was PCR amplified and cloned in a PCRII-TOPO TA cloning vector (Life Technologies). Nick-translated biotin-labeled cloned DNA was used as a probe for the detection of the miR RNA signals. The following pairs of primers were used for the amplification of the miR loci: Pri-miR-155; 5'ACCCTGCTGGATGAACGTAG 3'; 5' CATGTGGGCTTGAAGTTGAG3'; Pri-miR-146a; 5'AGCACTGTCAACCTGACACA 3'; 5' GGACCAGCAGTCCTCTTGAT 3'.

Microscopy and image analysis

FISH signals were examined on a Leica SP8 confocal microscope unit and image stacks were captured on a CCD camera with a step of 250 nm. The distances of DNA-FISH signals were further analyzed using the Volocity image analysis software (Improvision) by two independent investigators. Photomicrographs represent merged confocal images but statistical analysis for locus proximity has been performed for images of cells with preserved three-dimensional structure. As co-localized we considered DNA FISH signals that were either touching (adjacent pixels) or overlapping (overlapping pixels).

Chromatin immunoprecipitation

Chromatin from $15x10^6$ RAW264.7 cells or primary peritoneal thioglycollate elicited macrophages was prepared by fixation of the cell culture with 1/10th volume of Formaldehyde-containing buffer (11% Formaldehyde, 100mM NaCl, 1mM EDTA, 0.5mM EGTA, 50mM Hepes pH 8.0) and incubation for 10 min at room temperature (RT). For quenching the crosslinking, glycine was added for 5 minutes at RT to a final concentration of 125 mM directly in the culture media. Scraping and transfer of cells to a 15 mL conical tube and two washes with ice cold 1xPBS (supplemented with 1mM PMSF) followed, and the cell pellet was incubated with 10ml cell lysis buffer [5mM PIPES (pH 8.0), 85mM KCl, 0.5% NP-40, 1mM PMSF, complete protease inhibitors] for 10 min on ice. The cells were lysed with 800 µl SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris–HCl pH 8.1) for 10 min on ice. Chromatin was sonicated to an average length of DNA 500-1000 bp. Immunoprecipitation was

performed with the equivalent of 3-4x106 cells per sample, diluted 10 times with ChIP dilution buffer (0.01% SDS, 1.1% TritonX-100, 1.2mM EDTA, 16.7mM Tris–HCl pH 8.0, 167mM NaCl supplemented with protease inhibitors) and 5 μg of each antibody. Samples were rotated at 4oC overnight (1% of chromatin input was kept). Next day each sample was mixed with 20 μL of magnetic beads and was rotated for 2 h at 4oC. Immunoprecipitated material was incubated for 5 min with each of the following buffers: low-salt Wash Buffer A (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.0, 150mM NaCl supplemented with protease inhibitors), high-salt Wash Buffer B (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris- HCl pH 8.8, 500mM NaCl supplemented with protease inhibitors), Buffer C [20mM Tris HCl (pH 8.0), 250mM LiCl, 1mM EDTA, 0.5% NP-40, 0.5% Na-Deoxycholate, 0.5 PMSF supplemented with Protease inhibitors] and once with TE, pH 8.0. After the last wash, samples were incubated with proteinase K (200 mg/ml), 0.5% SDS in TE for 2 h at 55oC and then were incubated overnight at 65oC for the reversion of formaldehyde cross-links. DNA was purified with phenol/chloroform extraction, and was precipitated with ethanol (1% glycogen and 10% CH3COONa). Immunoprecipitated DNA was resuspended in 40 μL of 100mM Tris-HCL pH 7.5. 5% of the immunoprecipitated DNA was used in qPCR analysis and data have been normalized using the formula: 100*2^(Adjusted input – Ct IP). The antibodies that were used in the ChIP experiments were the following: RNA Polymerase II N-20 (sc899, Santa Cruz Biotechnology); NFκB p65 antibody-c-20x (sc 372x, Santa Cruz Biotechnology); NFκB p50 antibody-c-19 (sc 1190, Santa Cruz Biotechnology); C/EBPβ antibody-c-19 (sc 150x, Santa Cruz Biotechnology); Anti-Histone H3 antibody (D2B12)/#4620 Cell Signaling (chip-formulated); Anti-Histone H3 (tri

methyl K4) antibody (ab8580, Abcam); Anti-Histone (tri methyl K9) antibody (ab6001, Abcam); Anti-Histone (tri methyl K27) antibody (Cat. No. 07-449, Upstate). The following primer pairs were used for the amplification of the miR-155 and miR-146a promoter regions in the ChIP experiments: MiR-146a: Fwd 5'-ACTCCGTCTTGCAACGAACT- 3'; Rev 5'-TCCCTTCTCAATTCCCTCCT - 3'; MiR-155: Fwd 5'TTTCGAGCCGGAGGTTCCA- 3' Rev 5'-CGGCGACCCTTTTATAGCCC - 3'.

Statistical analysis

All values were expressed as mean ± SD. Comparison of results between different groups was performed by non-parametric analysis (Mann-Whitney and t-test where applicable), using GraphPadInStat (GraphPad Software, San Diego, CA). A P value of<0.05 was considered significant.

Results

miR-146a and miR-155 are coordinately regulated in endotoxin tolerance

Several miRs (such as miR-146a, miR-221, miR-125b, miR-181c, miR-155 and let-7e, miR-98) have been identified to regulate TLR4 signaling and induce or maintain endotoxin tolerance. Among these, miR-155 and miR-146a have been implicated in both macrophage activation and development of endotoxin tolerance. miRNA arrays have revealed that both miRs are being upregulated from different inflammatory signals. LPS, IL-1b and TNFa all induce the expression of these two miRNAs in macrophages (Figure 1). Since this induction was observed, I questioned whether there is a possible common transcriptional regulatory mechanism that affects the expression of these miRs. I analyzed the mechanism that controls expression of miR-155 and miR-146a in response to LPS in naïve and endotoxin tolerant macrophages using the RAW264.7 cell line and mouse primary macrophages. I found that both mature and immature (pri-miR) miR-155 and miR-146a were induced upon LPS stimulation of naïve macrophages while in endotoxin–tolerant macrophages their expression was induced at much lower levels. Chromatin immunoprecipitation (ChIP) analysis revealed that their genomic loci are regulated by the same transcription factors, namely NFκB and C/EBPβ. FISH

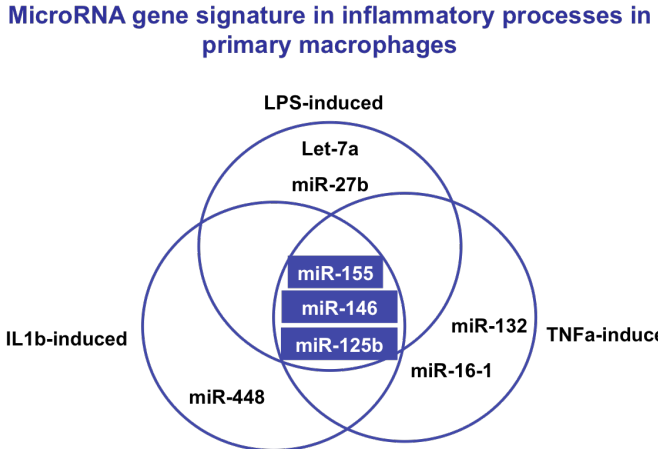


Figure 1. miRNA arrays in primary macrophages revealed that miR-155, miR-146a and miR-125b are being upregulated independent of the inflammatory stimulus; LPS, IL-1b and TNFa all induce the expression of these miRs.

experiments showed a monoallelic inter-chromosomal association between miR-155 and miR-146a gene loci at the stage of endotoxin tolerance, which conferred gene silencing. Genetic ablation of Akt1, which was used as genetic tool to abrogate endotoxin tolerance, changed miR155 and miR146a expression, altered NFkB/p65 and C/EBPβ binding on their promoters and inhibited the induction of co-localization of miR-155 and miR-146a gene loci, further highlighting the significance of this association. These results demonstrate a coordinated mechanism regulating the expression of miR-155 and miR-146a genes at the stage of endotoxin tolerance.

1. **Induction of miR-155 and miR-146a expression by LPS is reduced at the stage of endotoxin tolerance.**

LPS stimulation activates macrophages and induces expression of both miR-155 and miR-146a. Time-course analysis of miR-155 and miR-146a expression upon LPS treatment of RAW264.7 macrophages revealed that pri-miR-155 and pri-miR-146a reached a peak between 2 to 4 hours and then their levels declined (Figure 2A). The mature forms of the same miRNAs were induced simultaneously; miR-155 peaked at 18 hours and declined thereafter reaching the levels of miR-146a, suggesting that there is an additional, post-transcriptional level of regulation (Figure 2B). When macrophages became endotoxin tolerant, following an established protocol according to which they become hypo-responsive to a subsequent LPS stimulation (Figure 3 and [73]), mature

miR-155 and miR-146a levels were not further increased (Figure 2C), while their transcripts were only moderately induced (Figure 2D). These findings suggested the potential

coordinated transcriptional regulation of miR-155 and miR-146a gene expression at the stage of endotoxin tolerance.

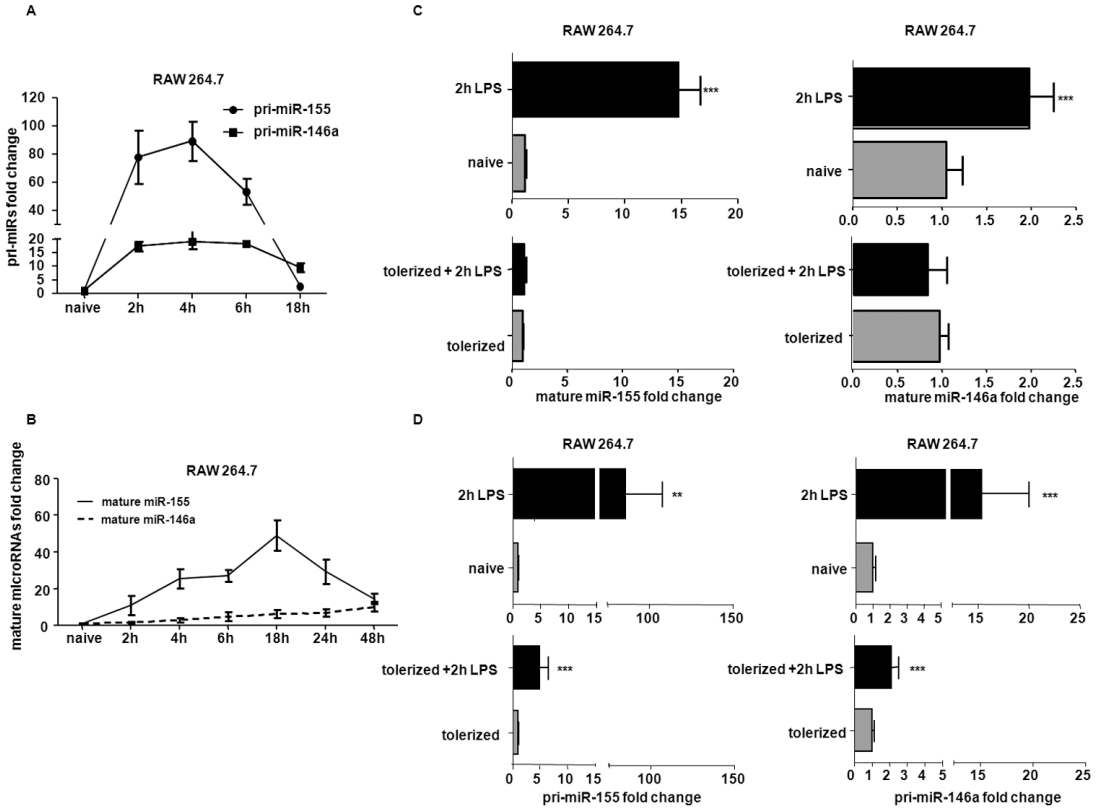


Figure 2. miR-155 and miR-146a are not further induced by LPS at the stage of endotoxin tolerance.

(A) The microRNAs transcripts for pri-miR-155 and pri-miR-146a were measured in stimulated RAW264.7 macrophages with LPS for the indicated time course. (B) The mature forms of miR-155 and miR-146a were measured in stimulated RAW264.7 macrophages with LPS for the indicated time course (C) Steady state RNA levels of mature miR-155 and miR-146a were measured in naïve, activated for 2h with LPS, endotoxin tolerant and LPS-restimulated for 2h tolerant RAW264.7 macrophages (**D)** Pri-miR-155 and pri-miR-146a levels were measured in naïve, activated for 2h with LPS, endotoxin tolerant and LPS-restimulated for 2h tolerant RAW264.7 macrophages Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to unstimulated or tolerant macrophages.

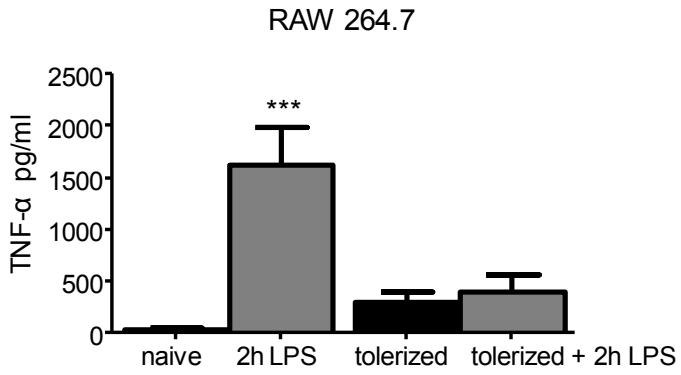


Figure 3. Induction of endotoxin tolerance.

TNF-α was measured in culture supernatants from naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant RAW264.7 macrophages. Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to unstimulated or tolerant macrophages.

2. Transcriptional regulation and H3 methylation status of miR-155 and miR-146a genes in naïve and endotoxin tolerant macrophages.

To evaluate the status of the transcriptional machinery of miR-155 and miR-146a genes at the endotoxin tolerant state of macrophages, we assessed the binding of RNA Polymerase II to their respective regulatory sequences using chromatin immunoprecipitation (ChIP) assays. RNA Polymerase II binding was augmented following 2h LPS activation of naïve macrophages and was reduced in tolerant or LPS-restimulated tolerant macrophages (Figure4), in accordance with the reduced transcription of these miRs. Accordingly, trimethylation of K4 of histone H3 (H3K4me3), a mark associated with transcriptionally active chromatin was increased upon LPS stimulation of naïve macrophages on both miR-155 and miR-146a promoters (Figure 4B). H3K4me3 levels dropped upon LPS restimulation of tolerant macrophages, supporting a negative regulation of miR expression at that stage.

In agreement with this observation, trimethylation of K9 of H3 (H3K9me3), found in silenced chromatin, was increased in the tolerant state, and then dropped upon LPS restimulation (Figure 4C). These findings show that both miRs obtain the activation mark H3K4me3 at the initial activation stage, while at the stage of endotoxin tolerance both miR promoters were occupied by the repressive mark H3K9me3. Upon LPS-restimulation of endotoxin tolerant macrophages, the ratio H3K4me3 to H3K9me3 did not change whereas both active and repressive histone methylation marks were reduced.

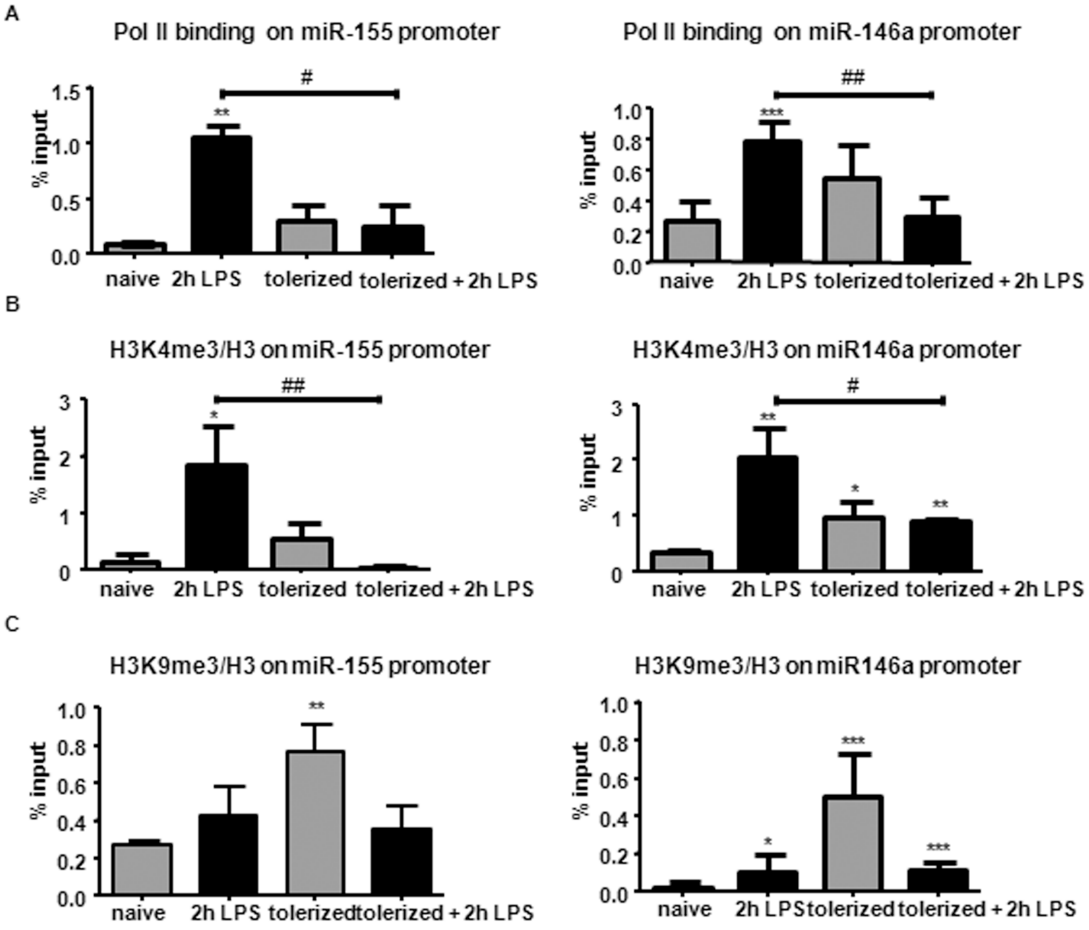


Figure 4. H3 methylation status of miR-155 and miR-146a genes in naïve and endotoxin tolerant macrophages.

Cell lysates from naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant RAW264.7 macrophages were used in ChIP experiments with a monoclonal antibody directed to **(A)** RNA Pol II, **(B)** H3K4me3, **(C)** H3K9me3. Precipitated DNA was amplified using primer pairs against a region of the miR-155 and miR-146a promoters. All H3K modifications were normalized against total levels of H3. Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to unstimulated macrophages, *# p, 0.05, ##* p, 0.01 compared to cells activated with LPS for 2h.

To identify transcription factors likely involved in the regulation of both miR-155 and miR-146a transcription, we analyzed their promoter regions, based on published data [70, 134, 135] and on relevant software (Genomatix, Rvista, Patch) (Figure 5A). Both regulatory sequences included putative NFκB and C/EBPβ binding sites. ChIP experiments revealed that binding of p65, the activation component of the NFκB complex, increased upon LPS stimulated macrophages and not in LPS-tolerant macrophages. However p50, the inhibitory component of the NFκB complex, was bound on both promoters at the stage of endotoxin tolerance, but not upon LPS-treatment of naïve macrophages (Figure 5B, C). C/EBPβ, a transcription factor associated with both transcriptional activation and suppression, including silencing of miR-155 [136, 137], was bound on the promoter of both miRNA genes in the naïve state. C/EBPβ binding was reduced upon LPS activation only on the miR-155 promoter and it occupied both miR-155 and miR-146a promoters in tolerant and restimulated tolerant macrophages (Figure 5D). Knock-down of C/EBPβ in RAW264.7 cells (Figure 6A) resulted in up-regulation of pri-miR-155 and pri-miR146a in endotoxin-tolerant macrophages, indicating that C/EBPβ participates in transcriptional suppression complexes at the stage of LPS-tolerance (Figure 5E). Furthermore, C/EBPβ depletion resulted in increased TNF-α secretion upon LPS stimulation, supporting the role of C/EBPβ as a negative transcriptional regulator (Figure 6B).

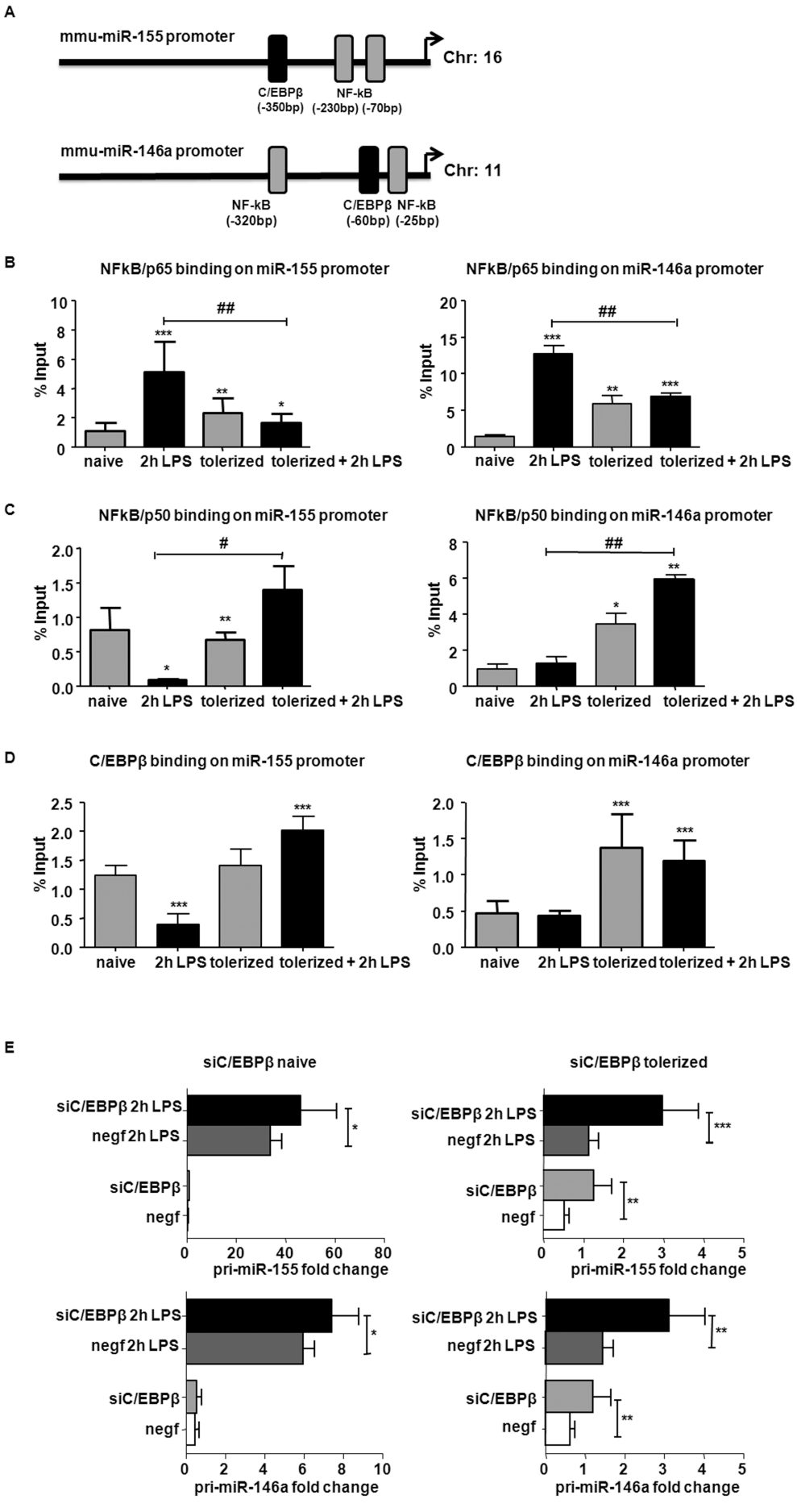

A
mmu-miR-155 promoter
Chr: 16
C/EBPβ (-350bp)
NF-kB (-230bp) (-70bp)
mmu-miR-146a promoter
Chr: 11
NF-kB (-320bp)
C/EBPβ (-60bp) NF-kB (-25bp)
B
NFkB/p65 binding on miR-155 promoter
NFkB/p65 binding on miR-146a promoter
% Input
naive
2h LPS
tolerized
tolerized + 2h LPS
C
NFkB/p50 binding on miR-155 promoter
NFkB/p50 binding on miR-146a promoter
D
C/EBPβ binding on miR-155 promoter
C/EBPβ binding on miR-146a promoter
E
siC/EBPβ naive
siC/EBPβ tolerized
siC/EBPβ 2h LPS
negf 2h LPS
siC/EBPβ
negf
pri-miR-155 fold change
pri-miR-146a fold change

Figure 5. Transcriptional regulation of miR-155 and miR-146a genes in naïve and endotoxin tolerant macrophages.

miR-155 and miR-146a promoters include similar transcription factor binding sites. Schematic representations of miR-155 (upper) and miR-146a (lower) gene loci on mouse chromosomes 16 and 11 are shown, respectively. Putative binding sites for NF-kB (gray) and C/EBPβ (black) transcription factors are shown (boxes). **(B-D)** Cell lysates from naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant RAW264.7 macrophages were used in ChIP experiments with a monoclonal antibody directed to **(B)** NFκB/p65 and **(C)** NFκB/p50 **(D)** C/EBPβ. Precipitated DNA was amplified using primer pairs against a region of the miR-155 and miR-146a promoters. Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to unstimulated macrophages. , *# P, 0.05, ##* p, 0.01 compared to cells activated with LPS for 2h. **(E)** Pri-miR-155 and pri-miR-146a RNA levels were measured in naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant RAW264.7 macrophages upon transfection with siRNAs for C/EBPβ or control siRNA (siNegF). Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to transfected macrophages with siNegF.

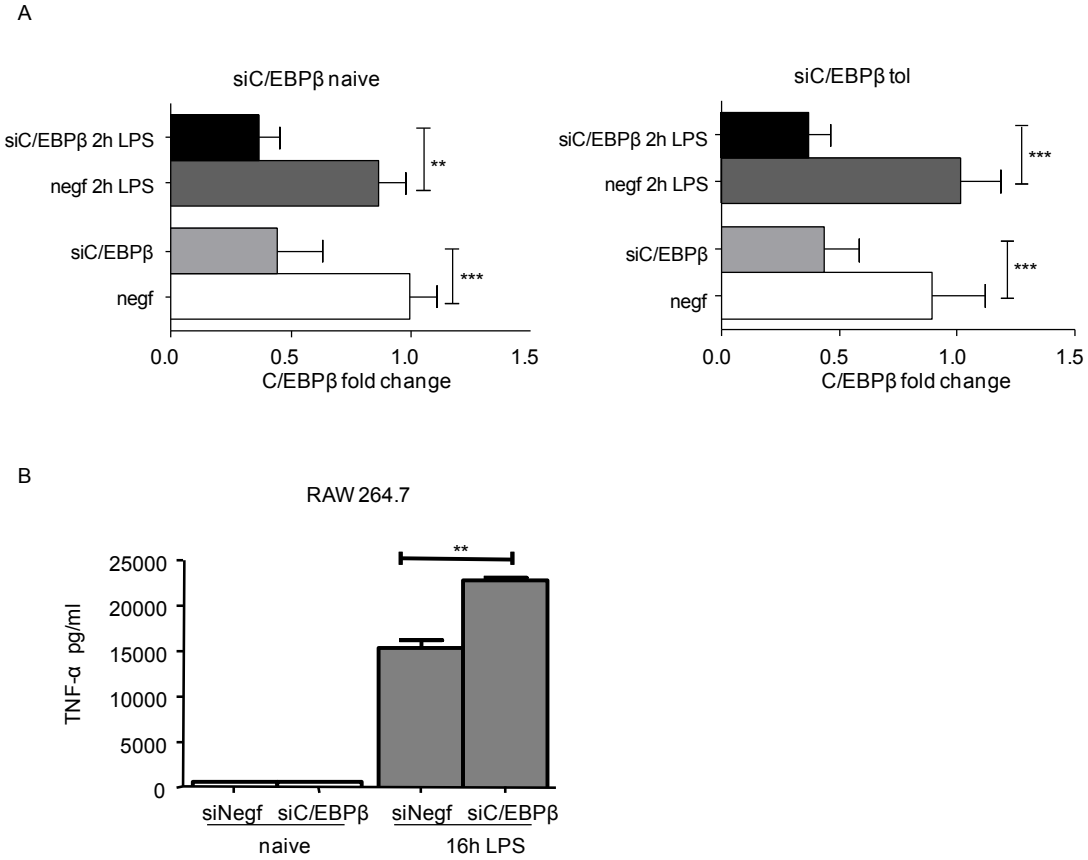


Figure 6. Suppression C/EBPβ in stimulated RAW264.7 cells resulted in increased TNF-α secretion.

A. C/EBPβ levels were measured in naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant RAW264.7 macrophages upon transfection with siRNAs for C/EBPβ or control siRNA (siNegF), in order to validate the efficacy of the siC/EBPβ. Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to transfected macrophages with siNegF. B. TNF-α was measured in culture supernatants from naïve and 16h LPS-stimulated RAW264.7 macrophages in which siRNAs targeting C/EBPβ or control siRNA (siNegF) was transfected. Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to macrophages transfected with siNegF.

3. Monoallelic co-localization of miR-155 and miR-146a gene loci in endotoxin tolerant macrophages.

Gene transcription can be coordinately regulated via the same transcription complex when two gene loci are found in close proximity [114]. As both miRNAs were regulated by the same transcription factors and responded similarly to LPS at the stage of endotoxin tolerance, we examined whether miR-155 and miR-146a gene loci come in close proximity. For this purpose, we analyzed the subnuclear localization pattern of the gene loci for miR-155/bic and miR-146a, which are located on mouse chromosomes 16 and 11 respectively, by DNA fluorescence in situ hybridization (DNA-FISH). Assessment of the percentage of cells bearing co-localized FISH signals of the two miR loci based on the distance between the two FISH signals. The results showed that the aforementioned loci came into proximity upon endotoxin tolerance in RAW264.7 macrophages and this co-localization became even more prominent in LPS-restimulated tolerant macrophages (Figure 7A), suggesting interchromosomal association. The observed differences in the percentage of cells harboring co-localized miR-155 and miR-146a alleles were not due to differences in the cell volume, as this was not found to decrease at the LPS tolerant stage (Figure 8A). The observed co-localization of the two loci was mono allelic in the conditions analyzed (Figure 7A), indicating that only one of the two alleles of each miRNA locus participates in this association. Analyzing the subnuclear localization pattern of gene loci for let-7e, a micro-

RNA known to participate in macrophage activation and endotoxin tolerance, no co-localization with miR-155 gene locus was observed, indicating that the association of miR-155 and miR-146a is specific rather than coincidental (Figure 8B). In addition, FISH analysis of miR-155, miR-146a and TNFα, a cytokine gene known to be silenced at the stage of endotoxin tolerance, did not reveal co-localization of the TNFα locus with any of the miRNA gene (data not shown), suggesting that associations may be restricted to certain groups of genes.

While bi-allelic gene expression can be considered the default state, several cases have been reported where genes are transcribed only from one of the two alleles, while the other remains in a heterochromatic inactive state [138-141]. To determine the allelic expression profile of miR-155 and miR-146a, we performed RNA-DNA FISH experiments in naïve, 2h LPS-stimulated, endotoxin tolerant and LPS-re-stimulated endotoxin tolerant RAW264.7 macrophages, detecting either the miR-155 transcript (RNA) and the primary miR-155 gene locus (DNA) or the primary miR-146a transcript (RNA) and the miR-146a gene locus (DNA) (Figure 8C). We measured the percentage of cells expressing miR-155 or miR-146a from one or both alleles. We found that the monoallelic to bi-allelic expression ratio of miR-155 and miR-146a was increased upon induction of endotoxin tolerance. The percentage of cells with bi-allelic expression of miR-155 and miR-146a was significantly reduced from 25% to 2% and 20% to 4.5%, respectively (Figure 8D, E). To examine whether the inter-chromosomal association of the two miR loci occurred in transcribed or silenced alleles, we performed RNA-DNA FISH experiments simultaneously detecting the miR-155 or miR-146a primary transcript together with the miR-155 and miR-146a gene loci (Figures 7B, 7C). The results showed that co-localization occurred primarily between alleles that were not transcribed, which was more prominent when endotoxin tolerant cells were re-

stimulated with LPS (Figures 7B, 7C). This observation indicated that the close proximity of miR-155 and miR-146a gene loci resulted in silencing of miRNA transcription.

The reduction of miRNA expression in endotoxin tolerant cells was grater than what can be accounted from silencing one allele, suggesting that transcription from the non-co-localized allele may also be suppressed and remain in a state of low expression. At the stage of endotoxin tolerance binding of the transcriptional repressors C/EBPβ and NFkB p50 was increased, while binding of the transcriptional activator NFkB p65 was decreased. Accordingly, in endotoxin tolerant cells the ratio of p65/p50 bound on miR-155 locus decreased 11 fold and the same ratio decreased 6-fold on miR-146a locus (Figure 9A). Therefore the NFkB complexes in endotoxin tolerant cells favored the inhibitory p50 isoform, suggesting that the alleles that are not co-localized were also affected. The transcriptional repressor C/EBPβ was increased 3-fold on both promoters at the stage of endotoxin tolerance (Figure 5D), when compared to LPS-stimulated cells, also suggesting that this change may also affect the alleles that are not co-localized. Thus, changes on transcription factor binding observed at the stage of endotoxin tolerance may not be restricted to the co-localized allele but also affect the non-co-localized allele, allowing reduced levels of expression.

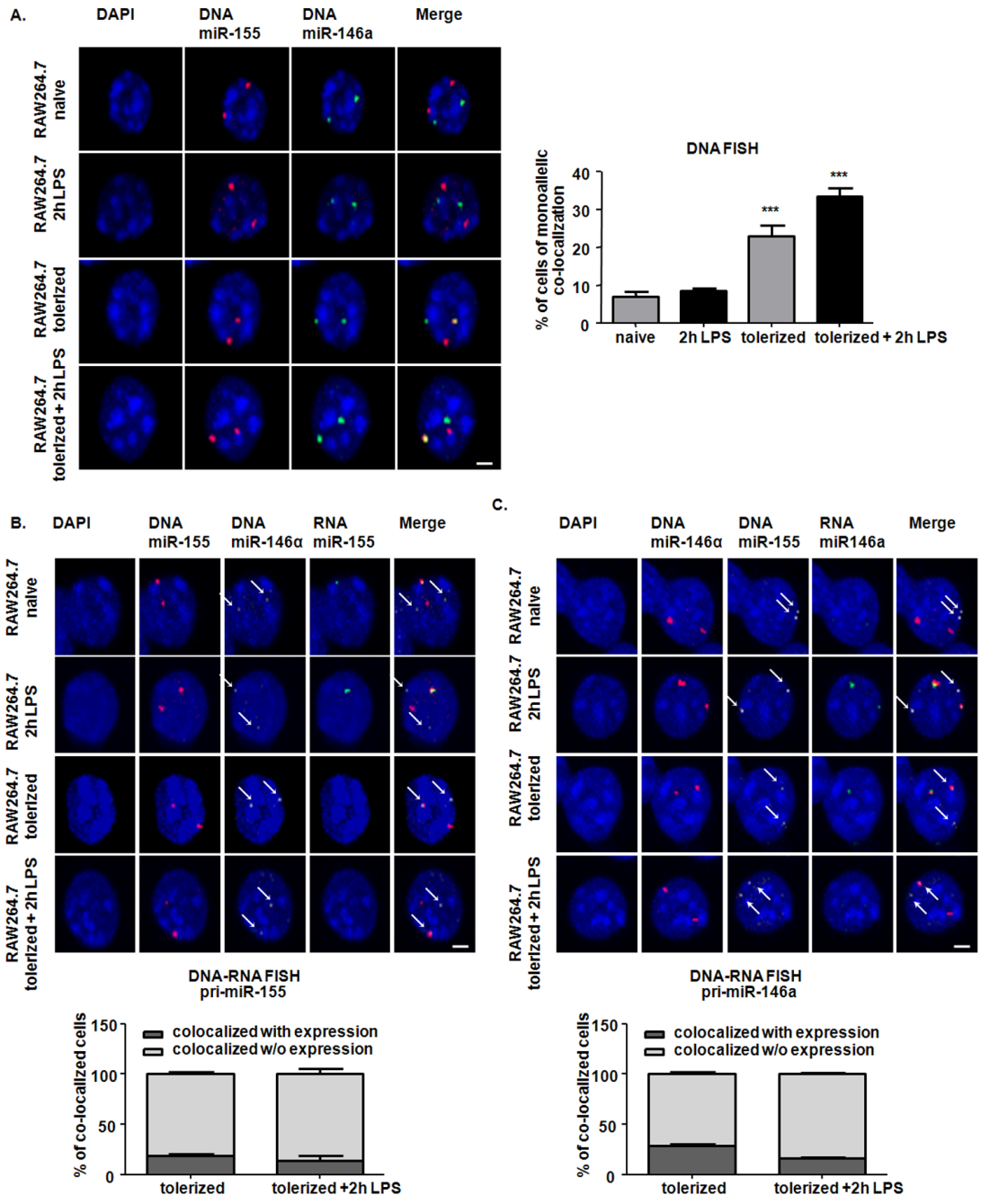


Figure 7. Monoallelic co-localization of miR-155 and miR-146a gene loci.

DNA FISH experiments were performed in naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant RAW264.7 macrophages for the detection of miR-155 and miR-146a gene loci. Graphs depict the percentage of cells harboring miR-155 and miR-146a gene loci co-localization. The measurements were performed in three-dimensionally preserved cell nuclei and 1000 cells were scored totally in 3 independent experiments for each state; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to unstimulated macrophages. Scale bar 2μM. **(B, C)** RNA-DNA FISH experiments performed in naïve and endotoxin tolerant RAW 264.7 macrophages with a subsequent exposure to LPS for the simultaneous detection of either the

nascent miR-155 gene transcript and the miR-155 and miR-146a gene loci or the nascent miR-146a gene transcript and miR-155 and miR-146a gene loci. Arrows have been added to highlight miR-146a staining. Scale bar 2μM. Graphs depict the percentage of endotoxin tolerant and re-stimulated macrophages with miR-155 and miR-146a gene loci co-localization, monitoring in parallel the transcription status of the locus. The measurements were performed in three-dimensionally preserved cell nuclei and 500 cells were scored in total in 3 independent experiments for each state.

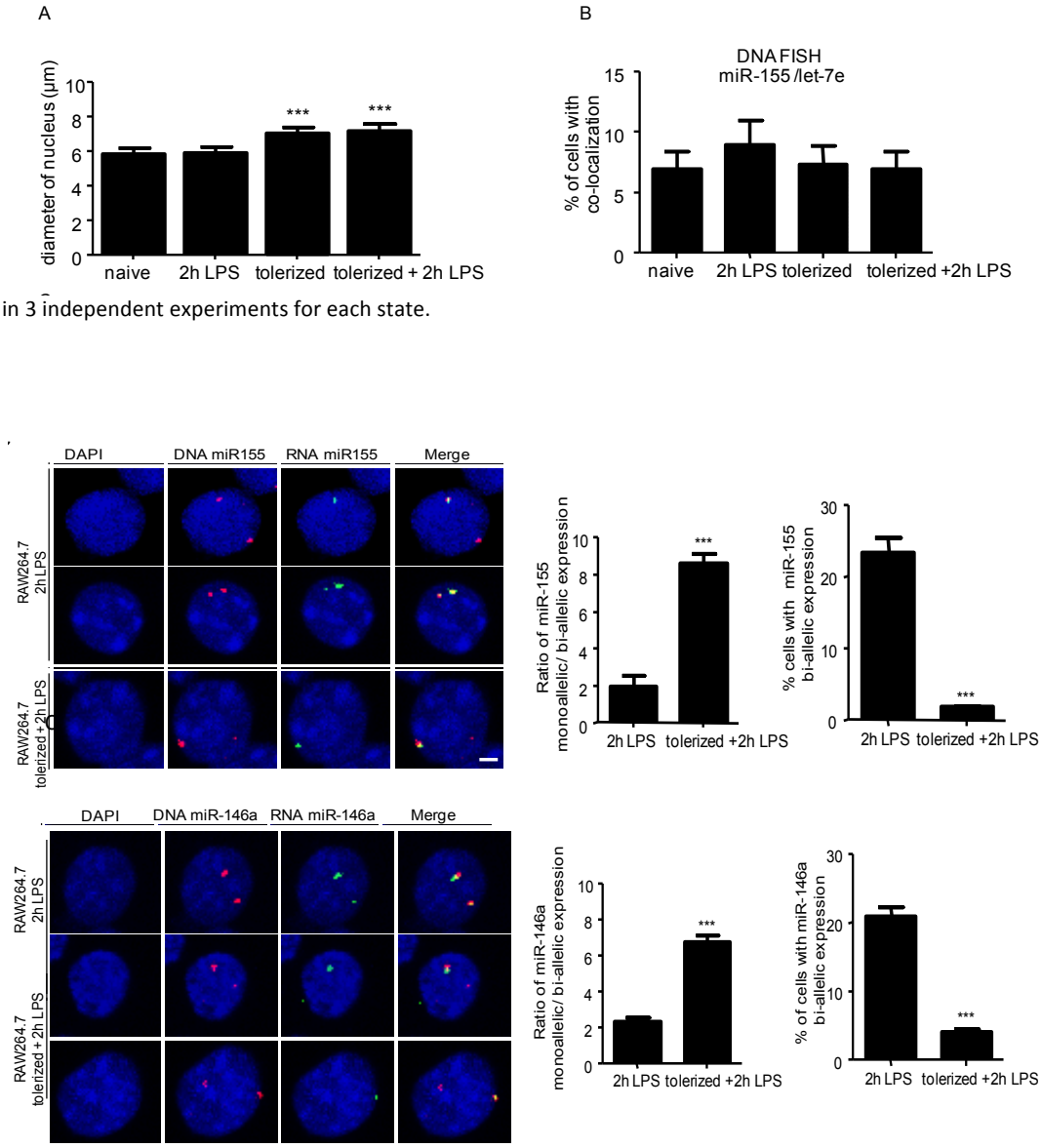


Figure 8. Allelic expression profile of miR-155 and miR-146a.

A. For the DNA-FISH experiments presented in Figure 3A for each cell scored (total number of cells: 1000), we measured the longest diameter of each individual cell. The measurements were performed in three-

dimensionally preserved cell nuclei using the Volocity software. The average diameter of the cells we have scored was 5.7±0.5 μm for naïve and 2h LPS stimulated macrophages and 7.4±0.5 μm for tolerant and re-stimulated endotoxin tolerant macrophages. Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to unstimulated macrophages. **B**. DNA FISH experiments were performed in naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant RAW264.7 macrophages for the detection of miR-155 and let-7e gene loci. The graph depicts the percentage of cells harboring miR-155 and let7e gene loci co-localization. The measurements were performed in three-dimensionally preserved cell nuclei and 500 cells were scored totally in 2 independent experiments for each state; Scale bar 2μM. **C**. RNA-DNA FISH experiments performed in naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant RAW264.7 macrophages to simultaneously detect either the nascent miR-155 gene transcript and miR-155 gene locus or the nascent miR-146a gene transcript and miR-146a gene locus. The percentage of cells with either mono- or bi-allelic expression was measured in three-dimensionally preserved cell nuclei and 500 cells were scored in total in 2 independent experiments for each state. The graphs depict the monoallelic/ bi-allelic expression ratio and the percentage of cells with bi-allelic expression of miR-155 (D) and miR-146a (E) in LPS stimulated naïve and tolerant RAW264.7 macrophages.

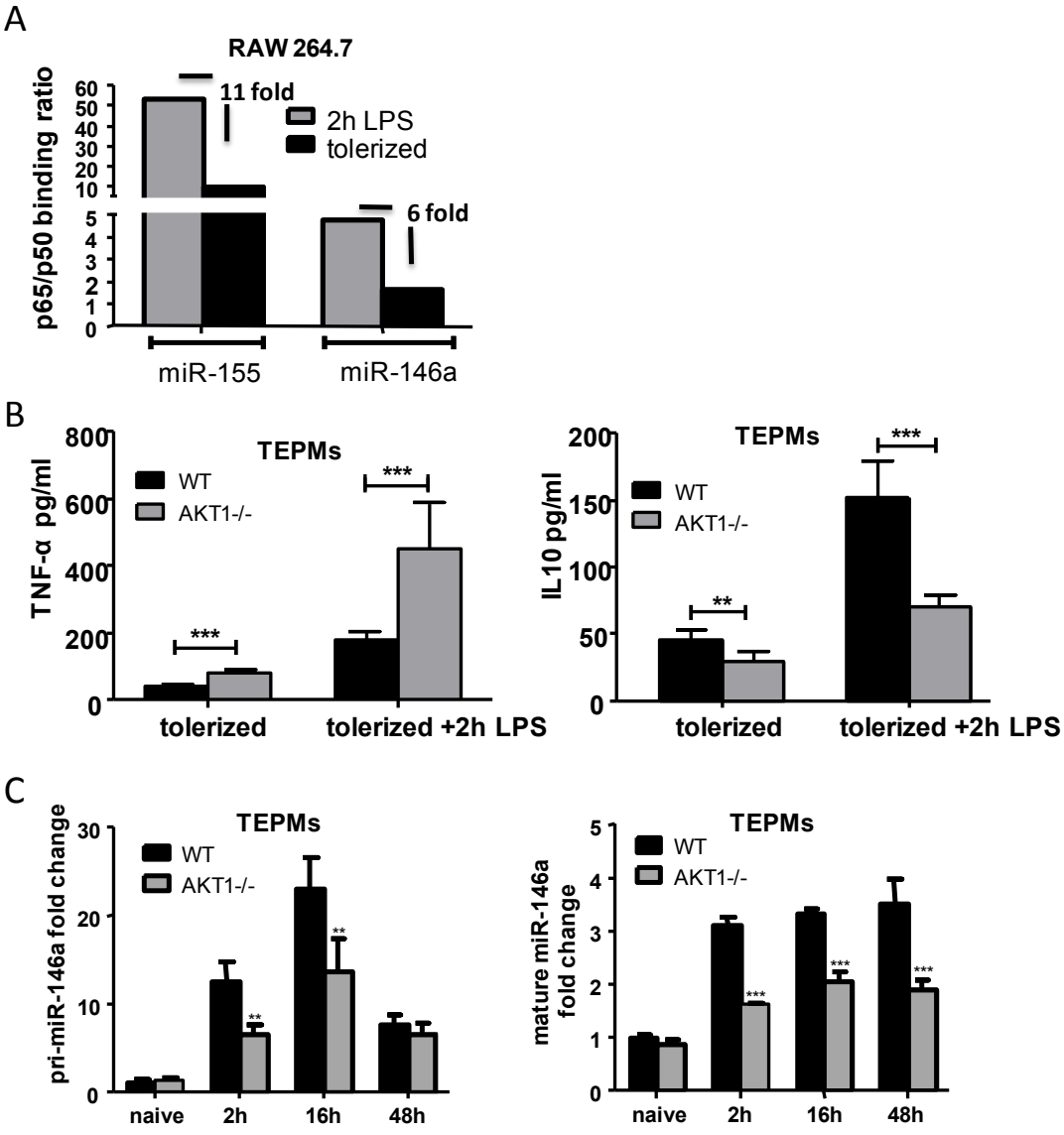


Figure 9. miR-146a levels are lower in Akt1$^{-/-}$ macrophages

A. Ratio of p65/p50 binding in miR-155 and miR146a promoter locus at the stage of LPS stimulation and endotoxin tolerance . B. TNF-α and IL-10 were measured in culture supernatants from endotoxin tolerant and LPS-restimulated primary Thioglycollate Elicited Peritoneal Macrophages (TEPMs) from WT and Akt1$^{-/-}$ mice. Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p , 0.001 compared to WT TEPMs macrophages. C. The microRNA transcript for pri-miR-146a and mature miR-146a were measured in stimulated primary Thioglycollate Elicited Peritoneal Macrophages (TEPMs) from WT and Akt1$^{-/-}$ mice for the indicated time points.

4. Co-localization of miR-155 and miR-146a gene loci is not induced in Akt1$^{-/-}$ macrophages

To extend the aforementioned observations to primary cells, we used thioglycollate-elicited and LPS stimulated peritoneal macrophages (TEPMs) from C57BL/6 mice. Re-stimulation of tolerized wild-type TEPMs resulted in reduced expression of both the mature (Figure 10A, left panel) and the microRNA transcripts (Figure 10B, right panel) of miR-155 and miR-146a (Figure 10A, B). Similar to RAW264.7 cells, in TEPMs co-localization of the two miRNA loci at the stage of endotoxin tolerance was associated with transcriptional silencing since the co-localized alleles did not express their miRNA transcripts (Figure 10C).

When Akt1$^{-/-}$ TEPMs were used as genetic tool for abrogation of endotoxin tolerance [Reference [14] and (Figure 9B)], pri-miR-155 was more potently upregulated in response to secondary LPS stimulation, compared to wild type macrophages (Figure 10C). Furthermore, LPS- re-stimulated endotoxin tolerant wild type macrophages displayed enhanced induction of pri-miR-146a compared to Akt1$^{-/-}$ macrophages (Figure 10D). The overall levels of pri- and mature miR-146a were lower in Akt1$^{-/-}$ macrophages, possibly due to the fact that they do not develop endotoxin tolerance (Figure 9B).

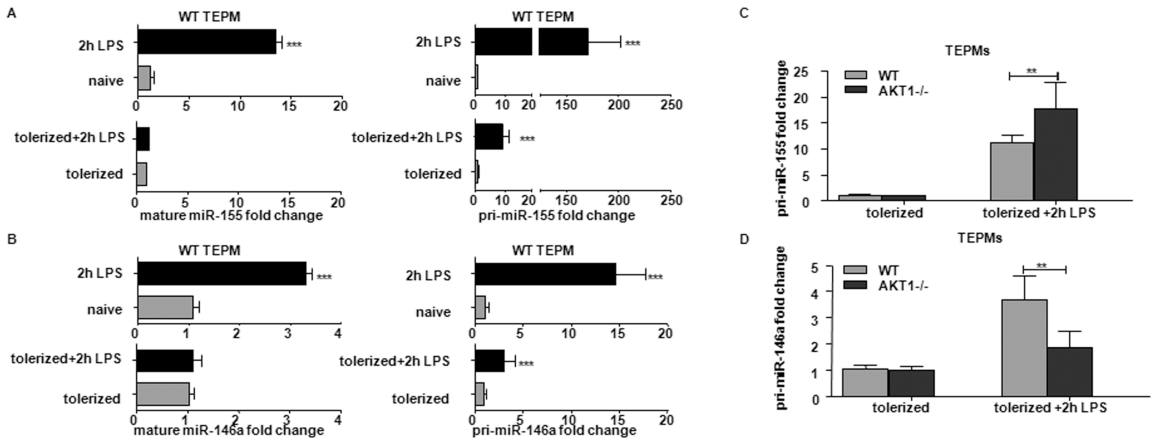


Figure 10. Differential expression profile of miR-155 and miR-146a in TEPMS of Akt1$^{-/-}$mice.

Expression of mature and microRNA transcripts of miR-155 **(A**) and miR-146a **(B)** was measured in naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant primary thioglycollate

elicited peritoneal macrophages (TEPMs) from wild type (WT) mice. Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001. **(C)** RNA-DNA FISH experiments performed in 2h LPS activated and endotoxin tolerant TEPMs macrophages for the simultaneous detection of either the nascent miR-155 gene transcript and the miR-155 and miR-146a gene loci or the nascent miR-146a gene transcript and miR-155 and miR-146a gene loci. Graph depict the percentage of LPS-activated and endotoxin tolerant macrophages with miR-155 and miR-146a gene loci, monitoring in parallel the transcription status of the locus. The measurements were performed in three-dimensionally preserved cell nuclei and 500 cells were scored in total in 3 independent experiments for each state. **(D, E)** Expression of pri- miR-155 and pri-miR-146a was measured in WT and Akt1$^{-/-}$ macrophages at the stage of endotoxin tolerance and following secondary LPS stimulation. Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001.

To verify that the transcriptional machinery regulating transcription of these miRNAs is the same in primary macrophages as in RAW264.7 cells, we performed ChIP experiments to detect the recruitment of NFκBp65 and C/EBPβ on miR-155 and miR-146a regulatory elements. NFκBp65 was bound on both promoters upon LPS activation of primary naïve macrophages, which was abrogated in LPS-re-stimulated endotoxin tolerant cells. In Akt1$^{-/-}$ macrophages, NFκBp65 was still recruited to the promoters of miR-155 and miR-146a genes (Figure 11A, B left panel), in accordance with their active transcription state. C/EBPβ was bound on both promoters of endotoxin tolerant and re-stimulated tolerant wild type macrophages but not in Akt1$^{-/-}$ cells (Figure 11C, D right panel), further confirming its contribution in suppressing the expression of these miRs. According to the different expression profile of pri-miR-155 and pri-miR-146a and altered NFkB/p65 and C/EBPβ binding on their promoters in Akt1$^{-/-}$ macrophages, no increase in the co-localization of miR-155 and miR-146a gene loci was observed. This finding further supports the significance of this association at the endotoxin tolerant stage (Figure 12A, B).

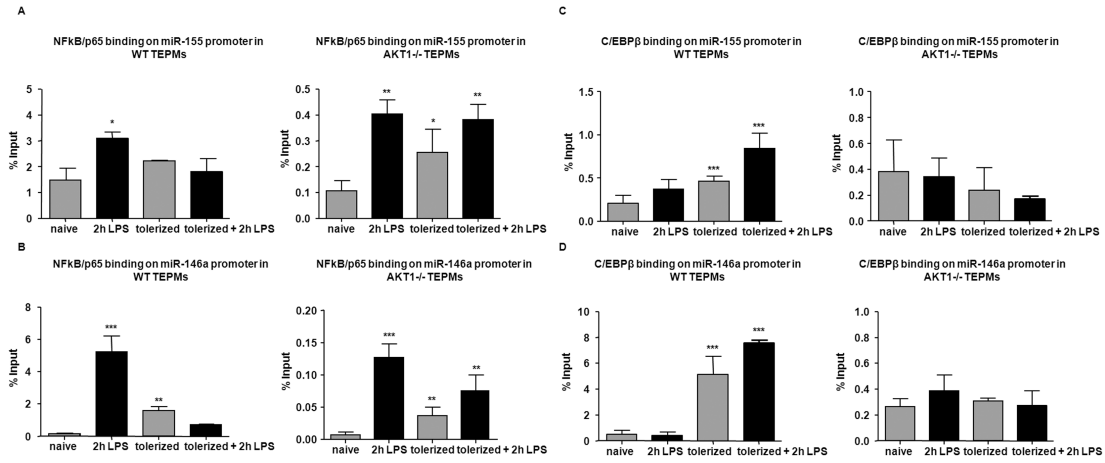


Figure 11. Differential regulation of NFkB and C/EBPβ in TEPMs from Akt1$^{-/-}$ mice

Cell lysates from naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant TEPMs from WT or Akt1$^{-/-}$ mice were used in ChIP experiments using monoclonal antibodies directed to NFκBp65 **(A,B)** and C/EBPβ **(C,D)**. Precipitated DNA was amplified using primer pairs against regions of the miR-155 and miR-146a promoters. Results represent 3 independent experiments; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to unstimulated macrophages.

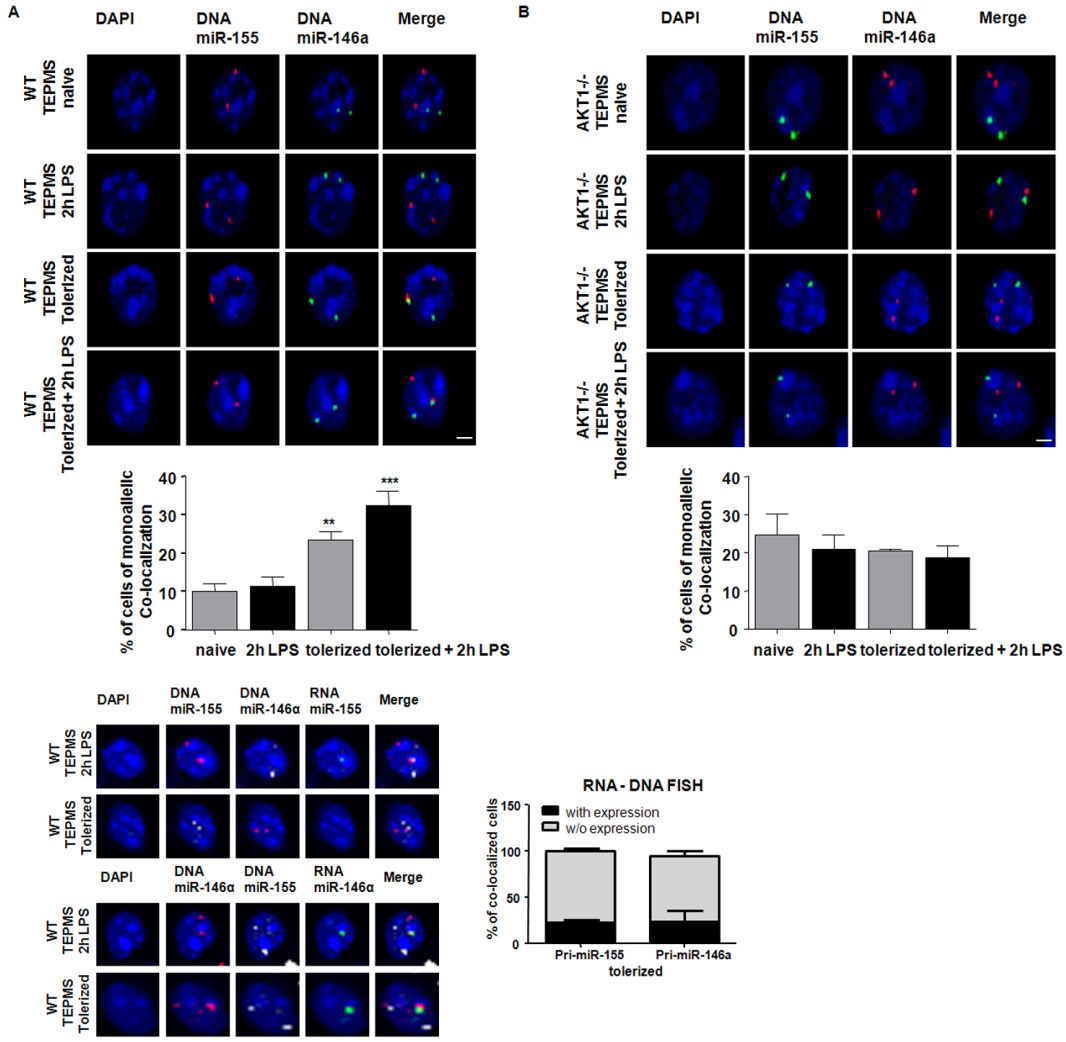


Figure 12. Co-localization of miR-155 and miR-146a is not induced in Akt1$^{-/-}$ macrophages

(A, B). DNA-FISH experiments were performed in naïve, activated for 2h with LPS, endotoxin tolerant and LPS-re-stimulated for 2h tolerant TEPMs from WT **(A)** or Akt1$^{-/-}$ **(B)** mice for the detection of miR-155 and miR-146a gene loci. Graphs depict the percentage of TEPMs harboring co-localized miR-155 and miR-146a alleles. The measurements were performed in three-dimensionally preserved cell nuclei and 500 cells were scored in total in 2 independent experiments for each state; (±SD) *p, 0.05, **p, 0.01, ***p, 0.001 compared to unstimulated macrophages.

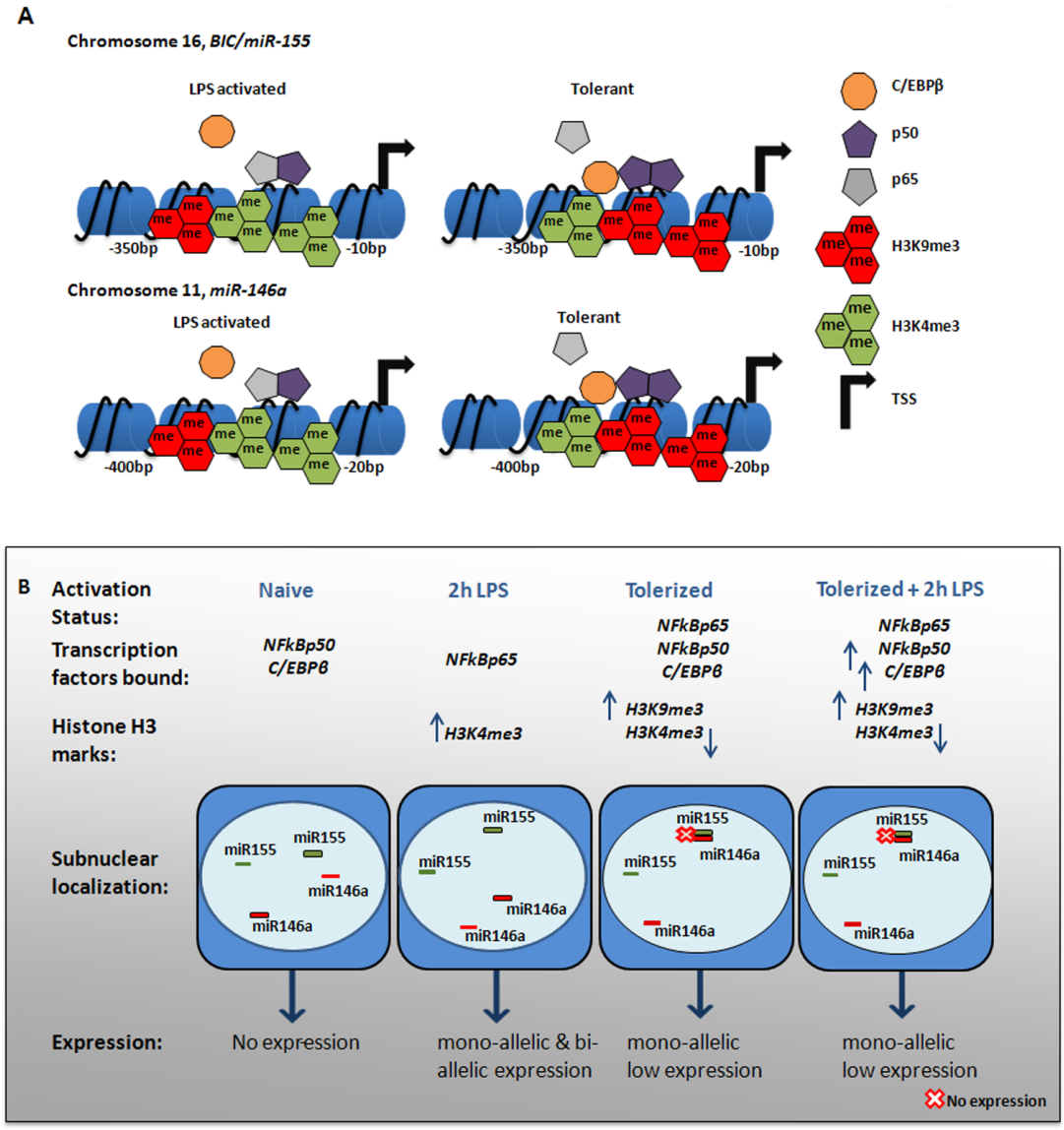


Figure 13. miR-155 and miR-146a share common regulatory events during macrophage activation and development of endotoxin tolerance. A. Schematic representation of transcription factor binding and histone modification at the activation and endotoxin tolerance stages. B. At the naïve state, C/EBPβ and p50 were bound on the promoter of both miRNA genes. Upon LPS treatment, the transcription is induced from one or both alleles, which are marked with H3K4 tri-methylation, and NFκB-p65 was recruited while C/EBPβ binding was reduced. At the stage of endotoxin tolerance one of the two alleles of each gene came into proximity conferring transcriptional silencing. C/EBPβ and NfkB-p50 occupied both miR-155 and miR-146a promoters in tolerant and re-stimulated tolerant macrophages, which were primarily marked by H3K9 tri-methylation.

Regulation of microRNAs by Akt isoforms and their role in macrophage polarization

Akt1 controls macrophage response to LPS by regulating microRNAs

Akt kinases play central role in innate immunity as they are activated by TLRs and cytokine receptors and contribute to the inflammatory response. Akt1 and Akt2 kinases were recently found that hold a key role in the regulation of macrophage activation phenotype. The role of Akt1 in the regulation of microRNA expression in LPS stimulated macrophages was examined further. LPS-treated Akt1− /− macrophages expressed higher cytokine production (figure 14), failed to induce let-7e (data not shown) and expressed higher levels of miR-155 and pri-miR-155 (figure 15, figure 16). In silico analysis and transfection studies revealed that let-7e controls TLR4 while miR-155 targets SOCS1 and that these miRNAs are under the control of Akt1. Given the increased response of Akt1− /− macrophages to LPS and reduced induction of SOCS1 (figure 17), we questioned whether ablation of Akt1 interferes with the development of endotoxin tolerance. When macrophages are exposed to an initial LPS stimulus they initiate tolerance mechanisms and upon re-stimulation they fail to effectively produce pro-inflammatory cytokines. BMDMs were, therefore, tolerized with an initial LPS treatment for 6 hours, culture media were changed and cells were exposed to a second LPS stimulus for different time periods. The results showed that while Akt1+/+ macrophages became tolerant to subsequent LPS stimulation and produced low amounts of pro-inflammatory cytokines, Akt1−/− macrophages continued to produce pro-inflammatory cytokines in response to the second LPS stimulus. Therefore, Akt1− /− macrophages were

hyperresponsive to LPS and failed to develop endotoxin tolerance in culture and in vivo (figure 18). The molecular mechanism is described in more detail. In mouse peritoneal macrophages, the induction of let-7e expression in response to LPS decreases cell surface expression of TLR4, the mRNA of which contains a let-7 target site. In this case, LPS signals activate Akt1, and let-7e is upregulated in an Akt1-dependent manner. Our transfection studies have revealed that let-7e represses TLR4 and that miR-155 represses SOCS1, two proteins critical for LPS-driven TLR signaling, which are thought to regulate endotoxin sensitivity and tolerance. Thus, Akt1−/− macrophages have exhibited increased responsiveness to LPS in culture and consistently Akt1−/− mice do not develop endotoxin tolerance in vivo. Overexpression of let-7e in Akt1−/− macrophages can restore tolerance to LPS in culture and in animals, indicating that Akt1 regulates the response of macrophages to LPS by controlling miRNA expression [14].

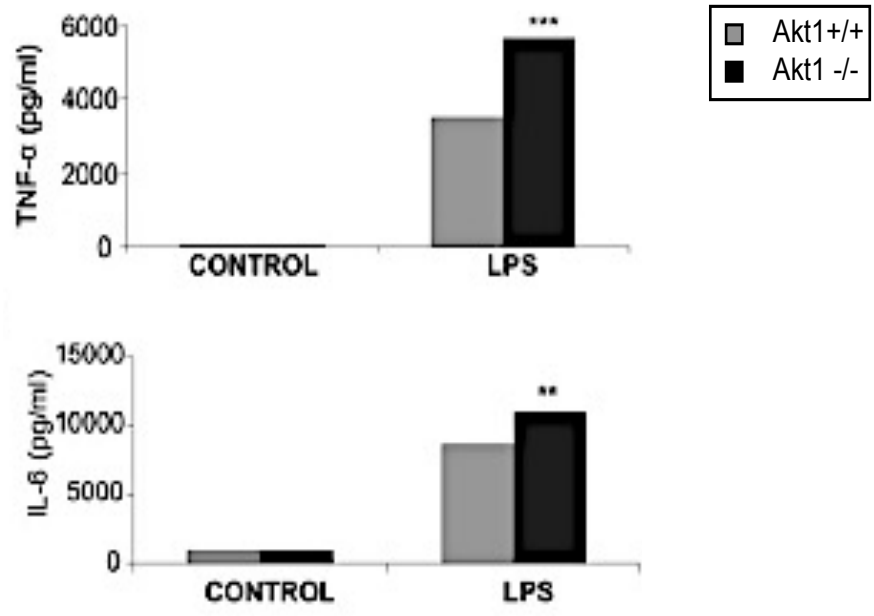


Figure 14. LPS-treated Akt1− /− macrophages expressed higher cytokine production
Primary macrophages from Akt1+/+ and Akt1− /− mice were stimulated for 4 hours with LPS. TNF-a and IL-6

expression was analyzed by elisa. Results are representative of three independent experiments (±SD); **p<0.01***p<0.001.

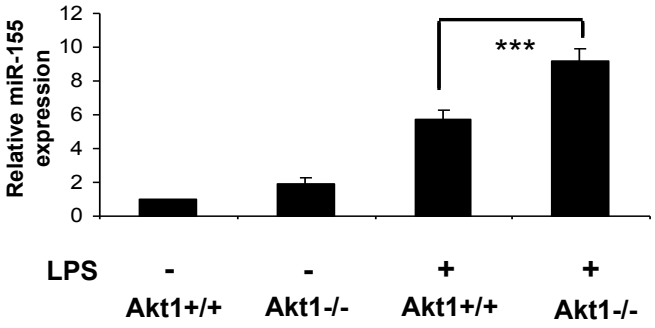


Figure 15. LPS-treated Akt1− /− macrophages expressed higher levels of miR-155

Primary macrophages from Akt1+/+ and Akt1− /− mice were stimulated for 4 hours with LPS and miR-155 expression was analyzed by real time RT-PCR. Results are representative of three independent experiments (±SEM); ***p<0.001.

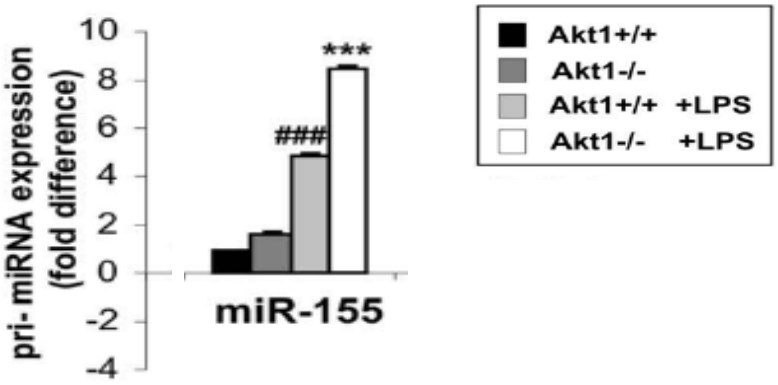


Figure 16. Akt1 regulates miR-155 at the transcriptional level

Expression of the pri-miR-155 was measured in LPS-stimulated Akt1+/+ and Akt1− /− primary BMDMs. ***p<0,001 compared to LPS-stimulated Akt1+/+ macrophages; ###p<0.001 compared to unstimulated AKT1+/+ macrophages.

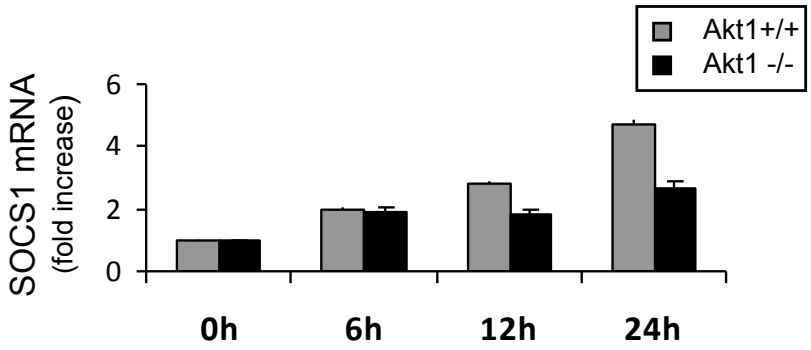


Figure 17. Akt1 regulates the expression of SOCS1

Primary thioglycollate-elicited peritoneal macrophages from Akt1− /− and Akt1+/+ mice were treated with LPS for 6, 12 or 24 hours. The LPS- induced expression levels of SOCS1 mRNA were measured by Real-Time PCR. Results represent 3 independent experiments (±SEM). **p<0.01, ***p<0.001 compared to unstimulated cells.

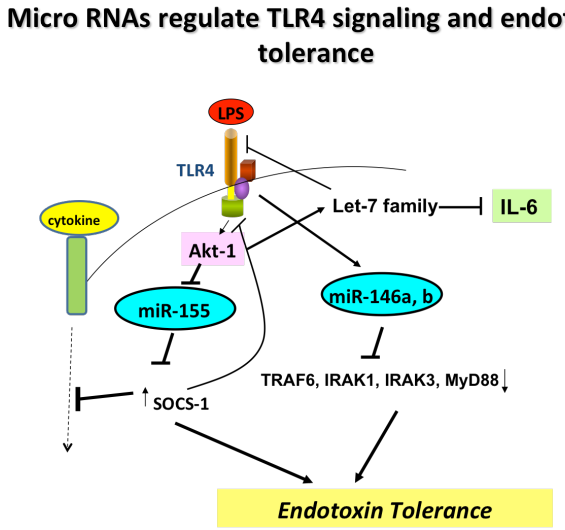


Figure 18. AKT1 kinase regulates endotoxin tolerance via microRNAs

Akt2 ablation results in reduced levels of miR-155 and increased expression of its target C/EBPβ, a key regulator of M2 polarization.

The M2 phenotype in Akt2−/− macrophages was due to increased C/EBPβ expression, mediated by down-regulation of miR-155.

Further insight into the molecular mechanism of miR-155 and miR-146a regulation upon different stages of macrophage activation, revealed the essential role of Akt kinases in determining M1/M2 polarization via these microRNAs. Activated macrophages are described as classically activated or M1 type and alternatively activated or M2 type, depending on their response to pro-inflammatory stimuli and the expression of genetic markers. It is known that M1 macrophages stimulated with LPS express high levels of TNF-α, IL-6, and iNOS, produce high levels of the iNOS catalytic product, NO [21], and are prominent in the acute phase of inflammation whereas LPS-stimulated M2 macrophages produce reduced levels of all these molecules. M2 macrophages are characterized by high levels of arginase-1 (Arg-1), found-in-inflammatory zone-1 (Fizz1), chitinase-3–like- 3 (Ym1), and macrophage galactose C-type lectin 1 and 2 (MGL1, MGL2) and participate in the resolution of inflammation.

Data from our lab have shown that LPS stimulated Akt1−/− macrophages also express high levels of iNOS and produce high levels of NO, TNFα, and IL-6 [22]. However, LPS stimulated Akt2−/− macrophages produce low levels of these proinflammatory mediators (figure 19). The aim of our study was to analyze the role of AKT kinase in polarization of macrophages and in regulation of miRs, giving a further sight in modulating macrophage response. These data suggest that the ablation of Akt1 may give rise to an M1, whereas the ablation of Akt2 may give rise

to an M2 macrophage phenotype. Thioglycollate elicited Akt2−/− peritoneal macrophages expressed high levels of Arg1 at both the mRNA and protein levels and exhibit high Arg1 enzymatic activity. Moreover, LPS treatment induced Arg1 activity in WT and Akt2−/−, but not in Akt1−/− macrophages [22] (figure 20B). Expression of high levels of Arg1 and increased arginase activity is one of the hallmarks of M2 macrophages. Arg1 converts L-arginine to urea and ornithine, competing with iNOS, which converts it into NO. By inhibiting NO production, Arg1 promotes the M2 phenotype and contributes to the suppression of the M1 phenotype. Arginase activity can also be induced by IL-4, which promotes M2 differentiation of macrophages. The expression of Arg1 and the M2 polarization of macrophages are regulated primarily by C/EBPβ and STAT6 [142]. We found that Akt2−/− macrophages express elevated levels of C/EBPβ compared with their respective WT controls or Akt1-deficient cells (figure 20A). On the other hand, STAT6 phosphorylation was not affected by the ablation of either Akt1 or Akt2 in macrophages. These results suggest that the M2 phenotype promoted by Akt2 ablation or knocking down was due to altered C/EBPβ expression[22]. Indeed, chromatin immunoprecipitation (ChIP) experiments showed increased binding of C/EBPβ on Arg1 promoter in Akt2−/− macrophages (Figure 21). C/EBPβ expression is regulated posttranscriptionally by the microRNA miR-155, which targets its 3' UTR [142]. Earlier work from our group had shown that Akt1 ablation promotes miR-155 expression in LPS-stimulated macrophage [14]. Measuring miR-155 in Akt2−/− macrophages revealed that Akt2 ablation had the opposite effect, reducing miR-155 expression in both resting and LPS-activated macrophages (Figure 22A). These results suggest that the down-regulation of miR-155 in Akt2−/− macrophages

results in the up-regulation of its target C/EBPβ and, consequently, the induction of Arg1, a hallmark of M2 macrophage polarization. Introduction of miR-155 into Akt2−/− macrophages suppressed C/EBPβ and Arg1 expression and resulted in increased LPS-induced iNOS expression (figure 22B), suggesting that miR-155 up-regulation can restore the M1 phenotype in these cells. Suppression of miR-155 with antisense-miR-155 augmented C/EBPβ and Arg1 levels in Akt1−/− macrophages and suppressed LPS-induced iNOS expression. Induction or suppression of miR- 155 modulated C/EBPβ, Arg1, and LPS-induced iNOS expression in WT macrophages. Overall, these results indicated that miR-155 was, at least partly, responsible for the differential phenotype observed in Akt1−/− and Akt2−/− macrophages. Collectively, Arg1 is primarily regulated by the transcription factors C/EBPβ and STAT6. Ablation or knockdown of C/EBPβ abrogates Arg1 expression and M2 differentiation. Ablation or knockdown of Akt2 enhances C/EBPβ expression and its binding to the Arg1 promoter, suggesting that the M2 phenotype observed in Akt2−/− macrophages is mediated by C/EBPβ. C/EBPβ is regulated by miR-155, which is differentially regulated by Akt1 and Akt2 in macrophages. The ablation or knockdown of Akt2 inhibits the expression of miR-155, suggesting that the up-regulation of C/EBPβ and Arg1 in Akt2−/− macrophages is, at least partly, due to miR-155 suppression.

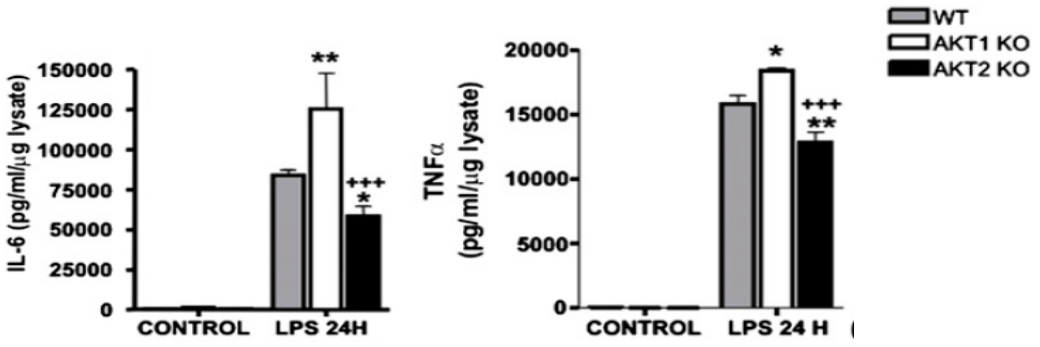


Figure 19. In LPS -treated Akt2−/− macrophages, lower levels of IL-6 and TNF-a are produced.

Primary macrophages from WT and Akt2− /− mice were stimulated for 24 hours with LPS. TNF-a and IL-6 expression was analyzed by elisa. Results are representative of three independent experiments (±SD); *p, <0.05**p<0.01***p<0.001.

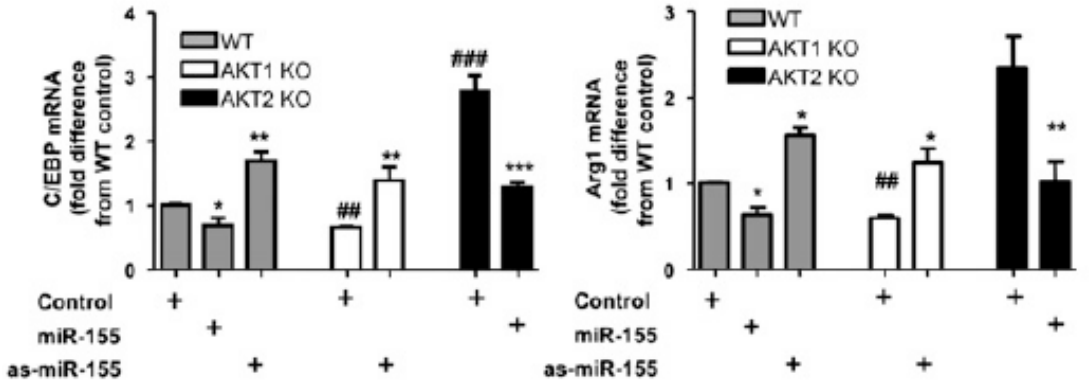


Figure 20. Akt2−/− macrophages expressed high levels of C/EBPβ and Arg1.

Expression of C/EBPβ (D) or Arg1 (E) was measured by realtime RT-PCR in macrophages transfected with miR-155 or as-miR-155 or scrambled RNA (control).

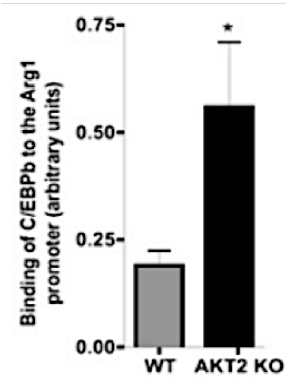


Figure 21. C/EBPβ binds on ArgI promoter.

ChIP of C/EBPβ in WT and Akt2−/− macrophages and PCR quantification using primers flanking the C/EBPβ-binding element of the Arg1 promoter were performed. Results are shown as the ratio of target to input.

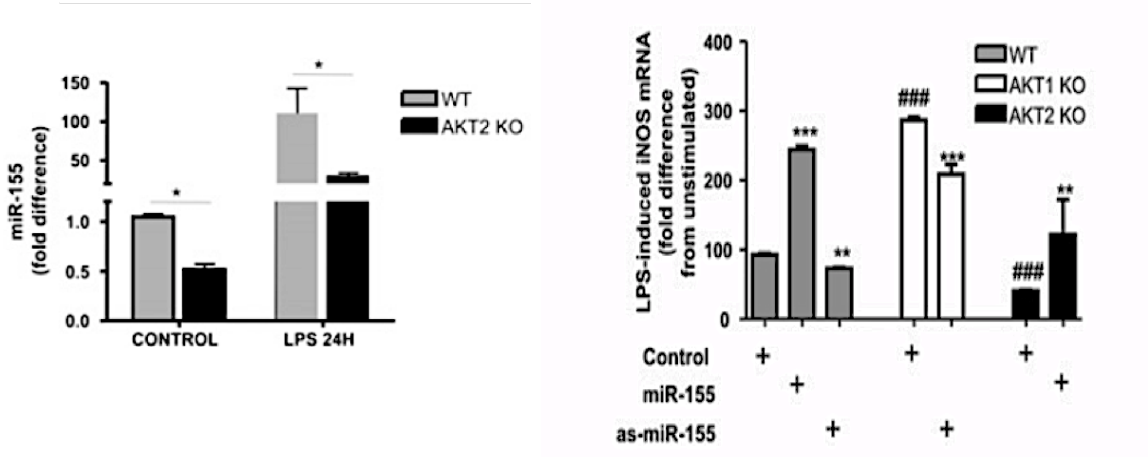


Figure 22. In naïve and LPS -treated Akt2−/− macrophages, miR-155 and iNOS are downregulated

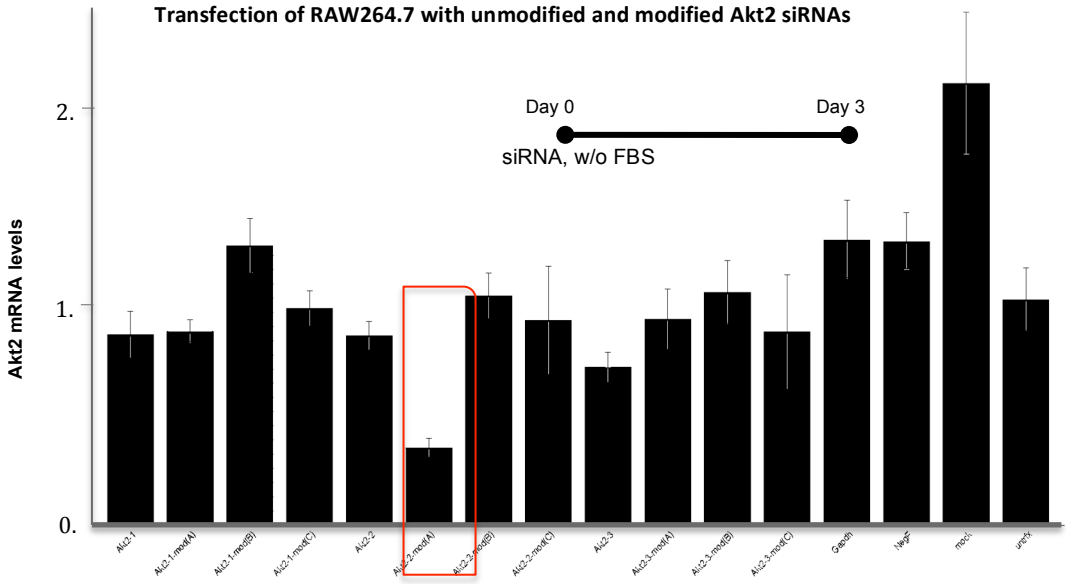
(a) miR-155 expression was evaluated in LPS-stimulated (24 h) and unstimulated WT and Akt2−/− macrophages.

(b) iNOS expression was measured by real-time RT-PCR in macrophages transfected with scrambled RNA (control),miR-155, or asmiR-155 and stimulated with LPS for 6 h.

Akt2 suppression and miR-146a induction promote the M2 macrophage phenotype

As analyzed previously, ablation or knockdown of Akt2 leads to a M2 phenotype by enhancing C/EBPβ expression. Further insight into M2 phenotype is supported by data recently published from our lab [135]. My aim was to further examine the molecular mechanisms involved in the regulation of the macrophage phenotype M2, focusing on the roles of Akt2 and miR-146a that it is already known as suppressor of M1 phenotype and significant regulator of endotoxin tolerance. At first place, in co-operation with Cenix Bioscienece, we designed siRNAs for AKT2 for transfection of RAW264.7 cells and primary macrophages (BMDMs) and validated their efficacy (figure 23). In parallel, we evaluated the expression pattern of M1/M2 polarization marker (IRAK1, TRAF6), knocking down the AKT2 with the siRNA (figure 24). Finally, given the important role of miR- 146a in suppression of excessive macrophage activation and induction of endotoxin tolerance, we transfected RAW264.7 and primary macrophages with mimic miR146a, and analyze the expression of affected genes such as IRAK1, TRAF6, iNOs, TNF-a (figure 25,26). Overall, we found that Akt2 deficiency resulted in a significant upregulation of miR-146a, which is of critical importance in suppressing the M1 phenotype (figure 27). miR-146a transfection in WT macrophages was able to inhibit iNOS induction while miR-146a suppression in Akt2-/- mice resulted in upregulation of iNOS expression. Furthermore, overexpression of miR-146a in WT macrophages induced expression of the transcription factor C/EBPβ (data not shown).

A


Transfection of RAW264.7 with unmodified and modified Akt2 siRNAs
Day 0
Day 3
siRNA, w/o FBS
Akt2 mRNA levels
2.
1.
0.

B

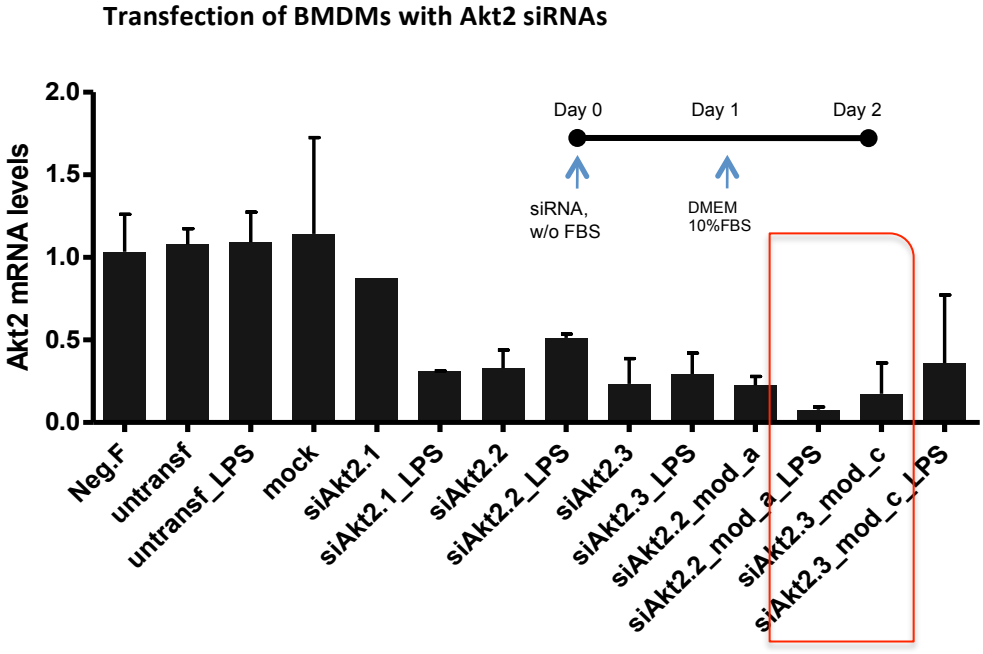

Transfection of BMDMs with Akt2 siRNAs
Day 0
Day 1
Day 2
siRNA, w/o FBS
DMEM 10%FBS
Akt2 mRNA levels
2.0
1.5
1.0
0.5
0.0
Neg.F
untransf
untransf_LPS
mock
siAkt2.1
siAkt2.1_LPS
siAkt2.2
siAkt2.2_LPS
siAkt2.3
siAkt2.3_LPS
siAkt2.2_mod_a
siAkt2.2_mod_a_LPS
siAkt2.3_mod_c
siAkt2.3_mod_c_LPS

Figure 23. Validation of the efficacy and the stability of the siAKT2

(a) Expression of AKT2 was measured by real time PCR in RAW264.7 macrophages that were transfected with different siRNAs for Akt2 (AKT2_1, AKT2_2, AKT2_3), and different modifications of the same siRNA (AKT2_1 modification a/b/c, AKT2_2 modification a/b/c, AKT2_3 modification a/b/c).

(b) Expression of AKT2 was measured by real time PCR in naive and activated with 4h LPS primary BMDMs that were transfected with different siRNAs for Akt2 (AKT2_1, AKT2_2, AKT2_3), and different modifications of the siAKT2 AKT2_2 modification a/b/c)

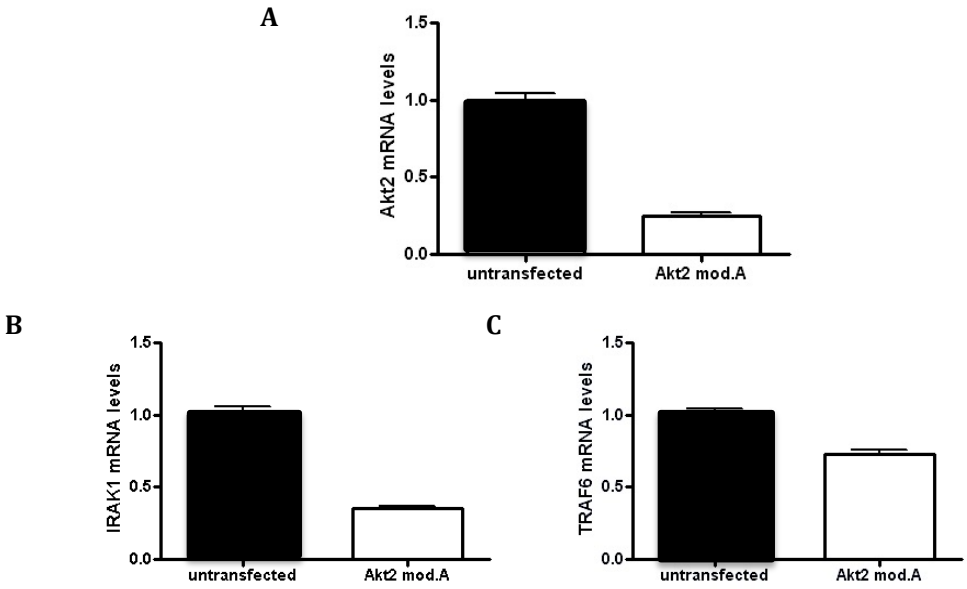


Figure 24. Evaluation of IRAK1 and TRAF6, knocking down the AKT2 with the siRNA

(a) Expression of Akt2 mRNA was measured by real time PCR in RAW264.7 macrophages transfected with the siAKT2_2mod a, evaluating the efficacy of the siRNA.

(b) Expression of IRAK1 mRNA was measured by real time PCR in RAW264.7 macrophages transfected with the siAKT2_2mod a.

(c) Expression of TRAF6 mRNA was measured by real time PCR in RAW264.7 macrophages transfected with the siAKT2_2mod a.

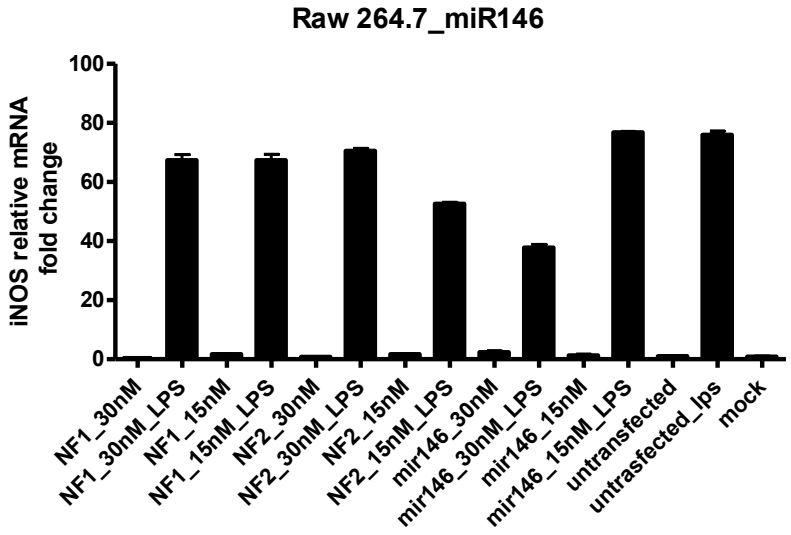


Figure 25. Dose optimization of mimic miR146 in macrophages with different concentrations

(a) Expression levels of INOS were evaluated by real time PCR in naive and activated with LPS (for 4h) RAW264.7 macrophages transfected with different doses (30nM & 15nM) of mimic miR-146a and negative factor as control (NF1&NF2)

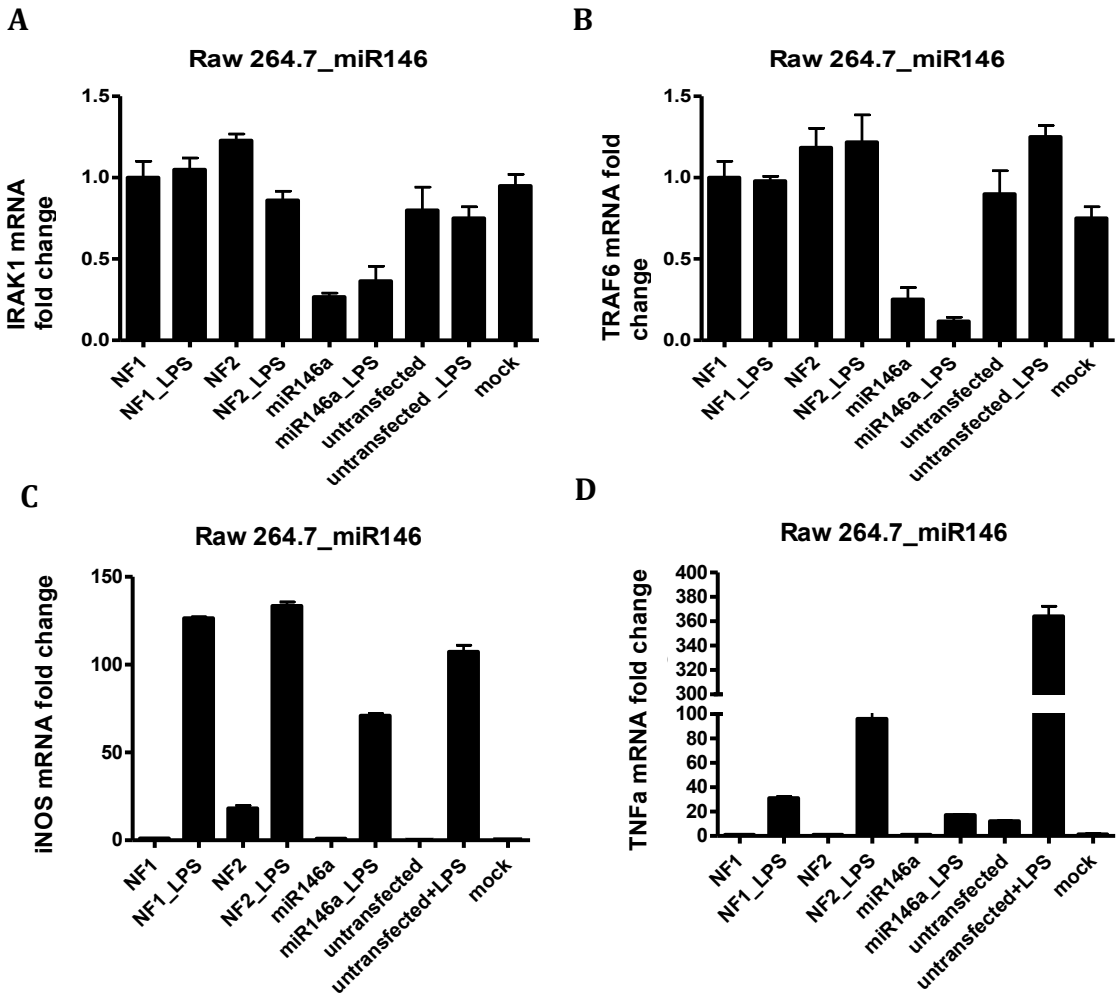


Figure 26. MiR-146a effect on macrophage phenotype

(a) Expression levels of IRAK1 were evaluated by real time PCR in naive and activated with LPS (for 4h) RAW264.7 macrophages transfected with different doses (30nM & 15nM) of mimic miR-146a and negative factor (NF1&NF2)

(b) Expression levels of TRAF6 were evaluated by real time PCR in naive and activated with LPS (for 4h) RAW264.7 macrophages transfected with different doses (30nM & 15nM) of mimic miR-146a and negative factor (NF1&NF2)

(c) Expression levels of INOS were evaluated by real time PCR in naive and activated with LPS (for 4h) RAW264.7 macrophages transfected with different doses (30nM & 15nM) of mimic miR-146a and negative factor (NF1&NF2)

(d) Expression levels of TNFa were evaluated by real time PCR in naive and activated with LPS (for 4h) RAW264.7 macrophages transfected with different doses (30nM & 15nM) of mimic miR-146a and negative factor (NF1&NF2)

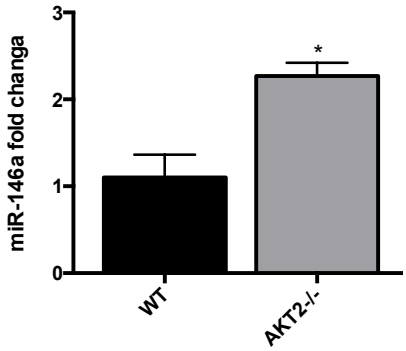


Figure 27. miR-146a levels are higher in Akt2$^{-/-}$ macrophages

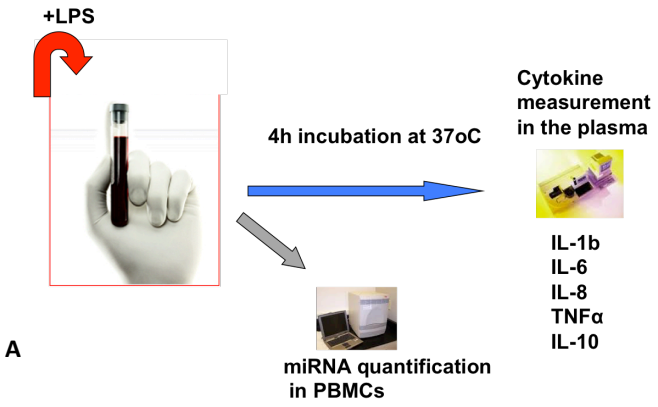
miR-146a expression was evaluated in unstimulated WT and Akt2−/− macrophages *P < 0.05 compared with macrophages from WT mice.

Functional significance of miR-155 and miR-146a in sepsis in humans

Association of miR155 and miR146 expression with LPS responsiveness of peripheral lymphocytes and the development of Compensatory anti inflammatory response syndrome (CARS)

Elucidating the role of miR-155 and miR-146a in macrophage polarization and endotoxin tolerance, it remains clear that these microRNAs participate in regulating immune responses of septic patients or patients with compensatory anti-inflammatory response syndrome (CARS). In fact, the mechanisms that regulate this compensatory anti-inflammatory response syndrome (CARS) remain incompletely understood. Even though the causes of CARS can be diverse, patients share common features that are underlined by impaired immune responses. These critically ill patients often present impaired immune responses as a result of an exaggerated anti-inflammatory response to their initial inflammatory stimulus, resulting in susceptibility to infections and increased mortality. The impairment of patients' immune response can be evaluated by the *ex-vivo* LPS-stimulated blood cytokine production (figure 28A). The LPS responsiveness was evaluated in peripheral blood samples from critically ill patients admitted in the ICU between June and October 2009, including sepsis, trauma, and H1N1-infected patients. Specimens were divided as non-responsive (group A) and responsive (group B) to LPS treatment as measured by cytokine production following exposure to LPS (figure 28B). To further investigate the physiological and clinical significance of these miRs in sepsis, their expression profile was analysed in peripheral blood cells from both groups. The results showed that miR-155 and miR-146 levels were higher in samples of patients whose

peripheral blood did not respond to LPS compared to those that responded, suggesting that increased miR-155 and miR-146a may be causally associated with the development of CARS (figure 29). H1N1 patients, a cohort of patients with severe CARS, had reduced LPS-stimulated blood cytokine production. In addition H1N1-infected patients had higher serum levels of IL-10, both prior and after LPS stimulation, compare to other critically ill patients with reduced LPS-stimulated blood cytokine production, indicating that in H1N1-infected patients, this impairment in immune responses is associated with high levels of IL-10 in the serum (figure 30). In total, miR-155 and miR-146 expression was increased in critically ill patients with impaired immune responses underlying that these miRs may be used as potential novel molecular biomarkers of macrophage sensitivity and CARS syndrome.



B

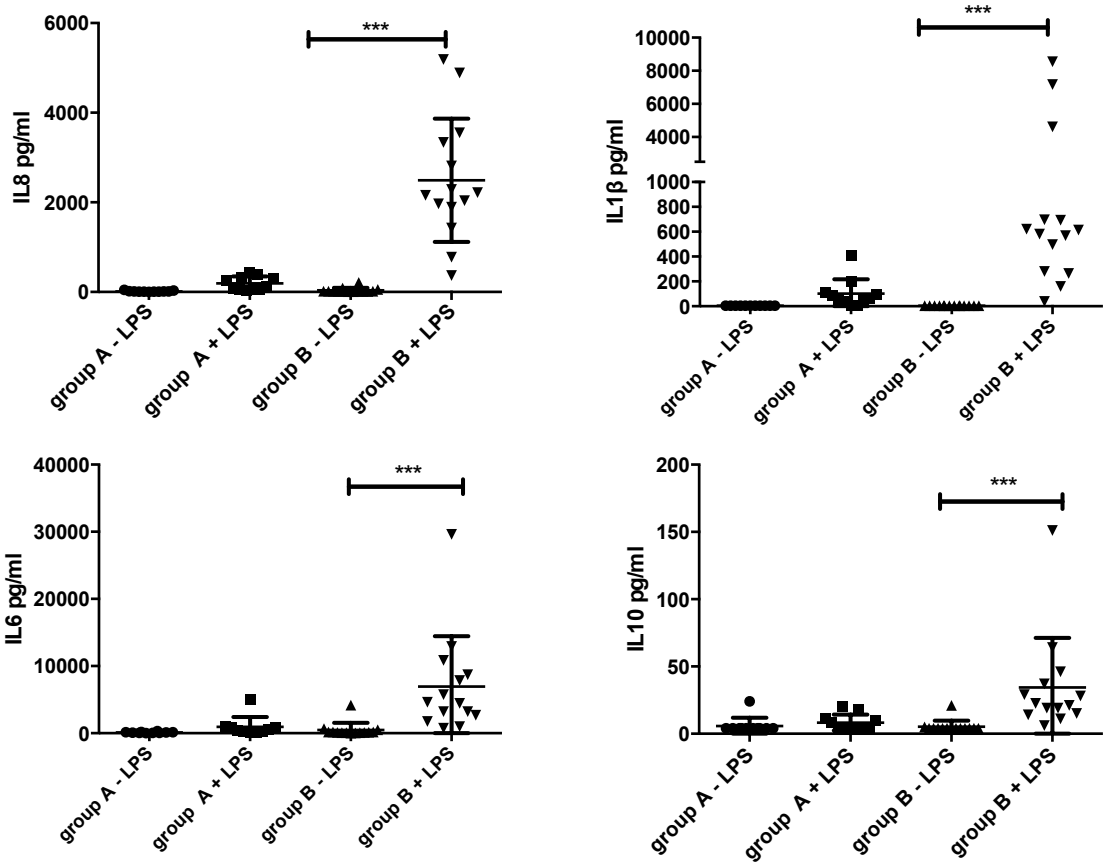


Figure 28. Ex vivo LPS responsiveness of peripheral blood samples from critically ill patients

(A) Schematic presentation of the experiment: Peripheral blood samples from critically ill patients admitted in the ICU between June and October 2009, were incubated with 100ng/ml LPS for 4h at 37o C. (b) Cytokine production in plasma of IL-8, IL-1β, IL-6 and IL-10 was measured by elisa before or after the ex vivo LPS incubation. Specimens were divided as responsive and non-responsive to LPS treatment depending on the analysis of cytokine production.

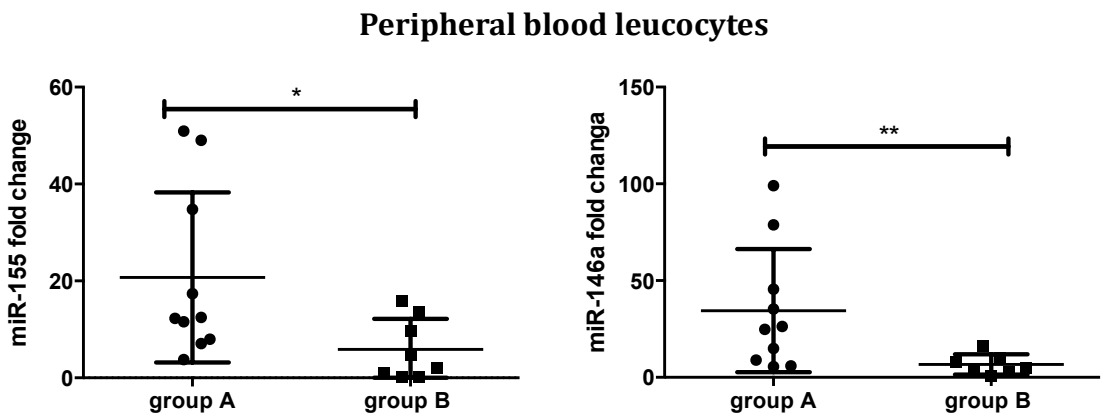


Figure 29. Increased miR-155 and miR-146a expression in non-responsive to LPS group

RNA was extracted from peripheral blood leucocytes of critically ill patients that they devided into two groups depending on their responsiveness to ex vivo LPS (group A and B) and miR-155 and miR-146 expression was evaluated by realtime PCR.

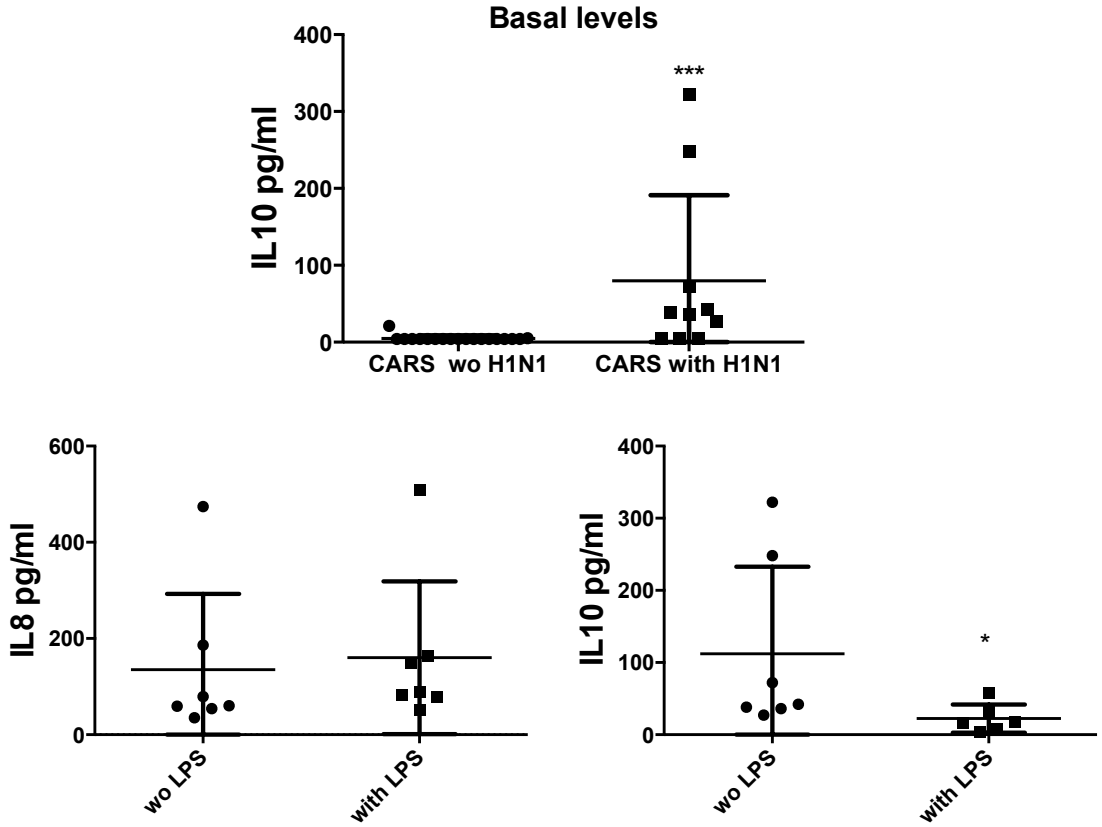


Figure 30. H1N1-infected patients present severe CARS

(a) Il10 cytokine was estimated by elisa in patients with CARS with or w/o infection of H1N1. (b) Peripheral blood samples from H1N1-infected patients were incubated with 100ng/ml LPS for 4h at 37o C. Cytokine production in plasma of IL-8 and IL-10 was measured by elisa before or after the ex vivo LPS incubation

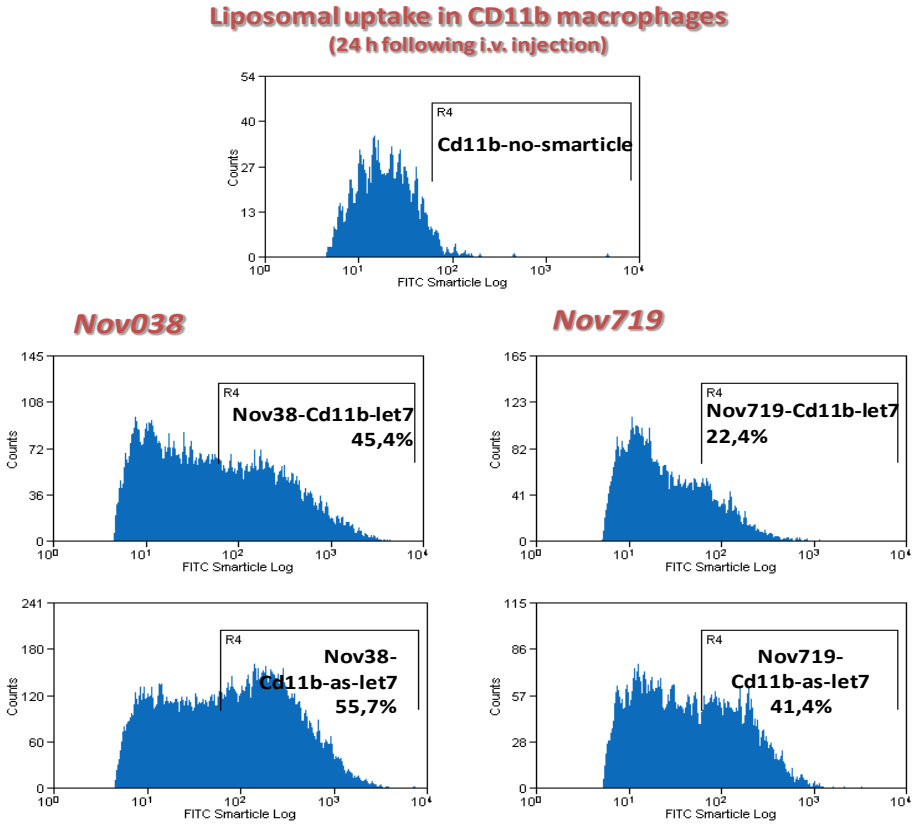
In vivo transfer of nucleic acids for therapeutic purposes

Production of liposomal formulations carrying miRNAs or antagomirs

Given the importance of miR-155 and miR-146a in the regulation of endotoxin tolerance and in modulating macrophage responses in cell culture models, mouse models and in patients, our aim was to investigate potential ways for transferring miRNAs in vivo for therapeutic purposes. Numerous approaches for systemic and targeted delivery technologies have been tested for the development of novel therapeutics. Despite the fact that cationic liposomes have been demonstrated as promising and are commonly used, their limited use in vivo is because of their limited penetration into tissues. Amphoteric particles generated from Novosom is a novel class of liposomes that are fully charge-reversible particles, do not aggregate in the serum, penetrate beyond endothelia and accumulate in the liver, spleen, and in sites of inflammation. The aim of my study was the production of certain liposomal formulations carrying a single or multiple miRNAs & antagomirs based on Novosom technology, the validation of the efficacy of their delivery and the evaluation of their target supression. I generated liposomal formulations such as let7e, as-let7e, miR155, as-miR-155 or the combination of those such as let7e / as-miR-155 or miR155/as-let7e, in order to be evaluated in certain mouse models (for example, injected with a lethal dose of LPS - LPS shock, low dose of LPS - endotoxin tolerance or DDS colitis model). Firstly, my aim was to evaluate different liposomal formulations (Nov038 and Nov719) in macrophages (CD11b) & dendritic cells (CD11c) based on the percentage of cells with the liposomal uptake (figure 31 A and B). After the injection of liposomal formulation intravenously, I collect splenocytes and analyse them by FACS. I observed that the percentage of cells with the liposomal uptake using Nov038 formulation was more efficient than using Nov719 (figure 32). 24 hours following intravenous injection of the Nov038-Let7e formulation in mice, I observed a subsequent suppression of TLR4 expression in FITC-positive splenocytes. Finally, 2 hours after the intravenus injection of the liposomal formulations, intraperitoneal injection of 1mg LPS was performed in mouse and within 6 hours after LPS, spleenocytes were collected for further analysis (figure 33B). Thus, as expected, the

liposomal uptake of Nov038-As-Let7e led to the augmentation of TLR4 expression (figure 33C). In fact, similar experiments based on the in vivo transferring of miR-155 and miR-146a by using amphoteric liposomes is highly promising, underlining miR-155 and miR-146 as potential tools for therapeutic purposes.

A



B

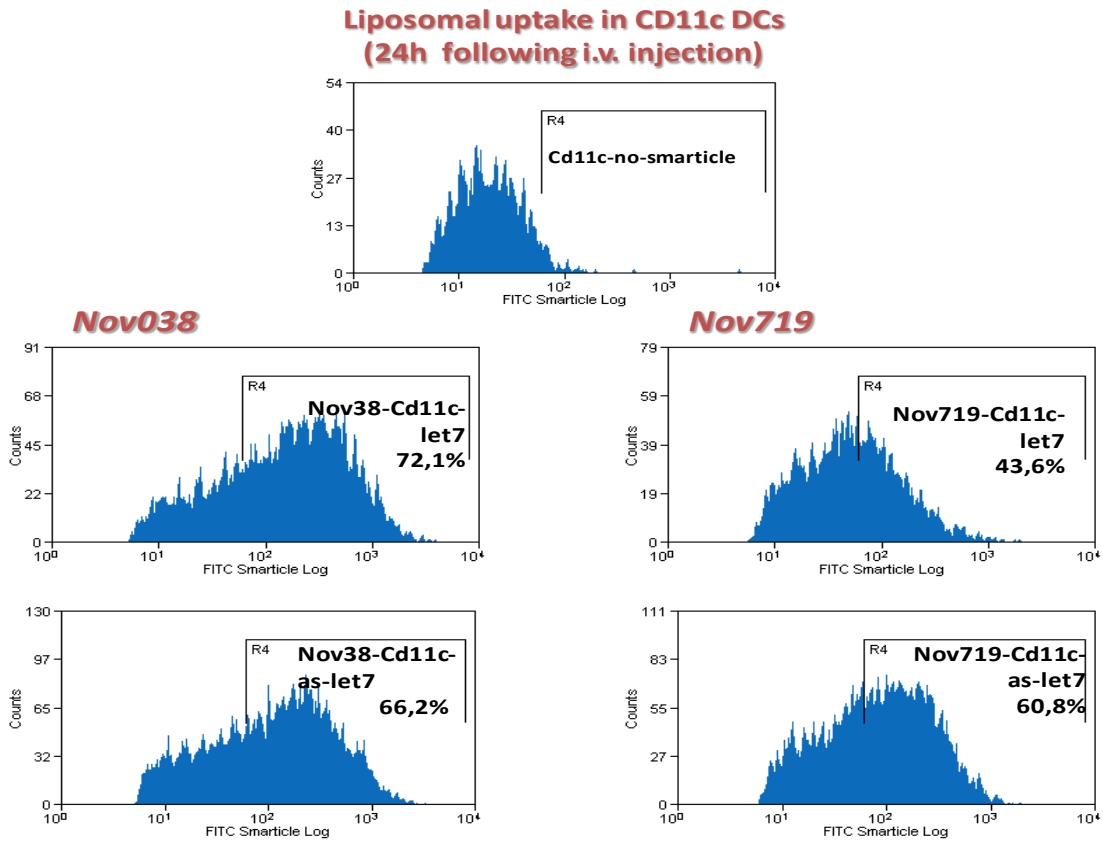


Figure 31. Evaluation of liposomal formulations

24h following intravenous injection of liposomal formulations (Nov038, Nov038-let7e and Nov038-as let7e) (Nov719, Nov719-let7e and Nov719-aslet7e), splenocytes from C57BL/6 mice were collected and the percentage of macrophage cells (CD11b) (A) or dendritic cells (CD11c) (B) with the liposomal uptake was analyzed by FACS.

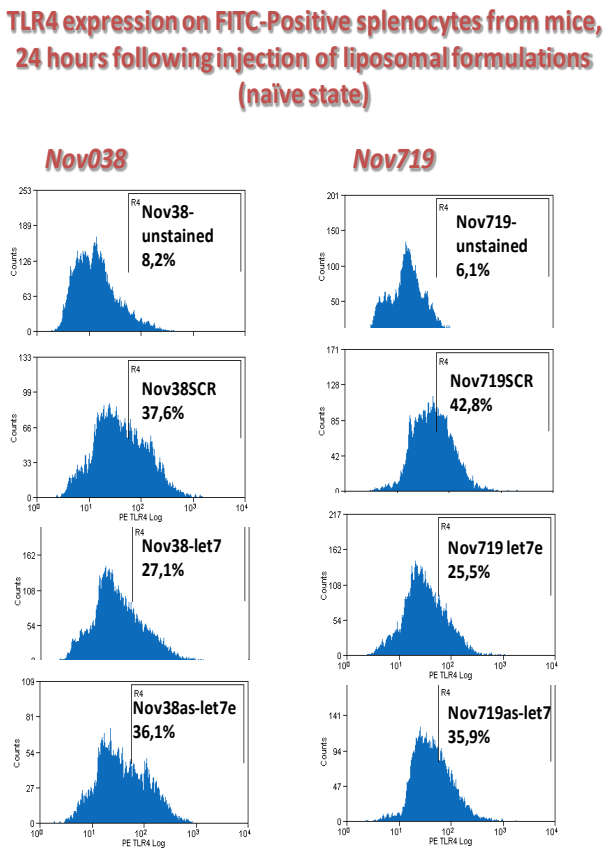
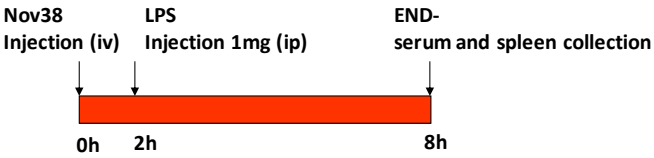


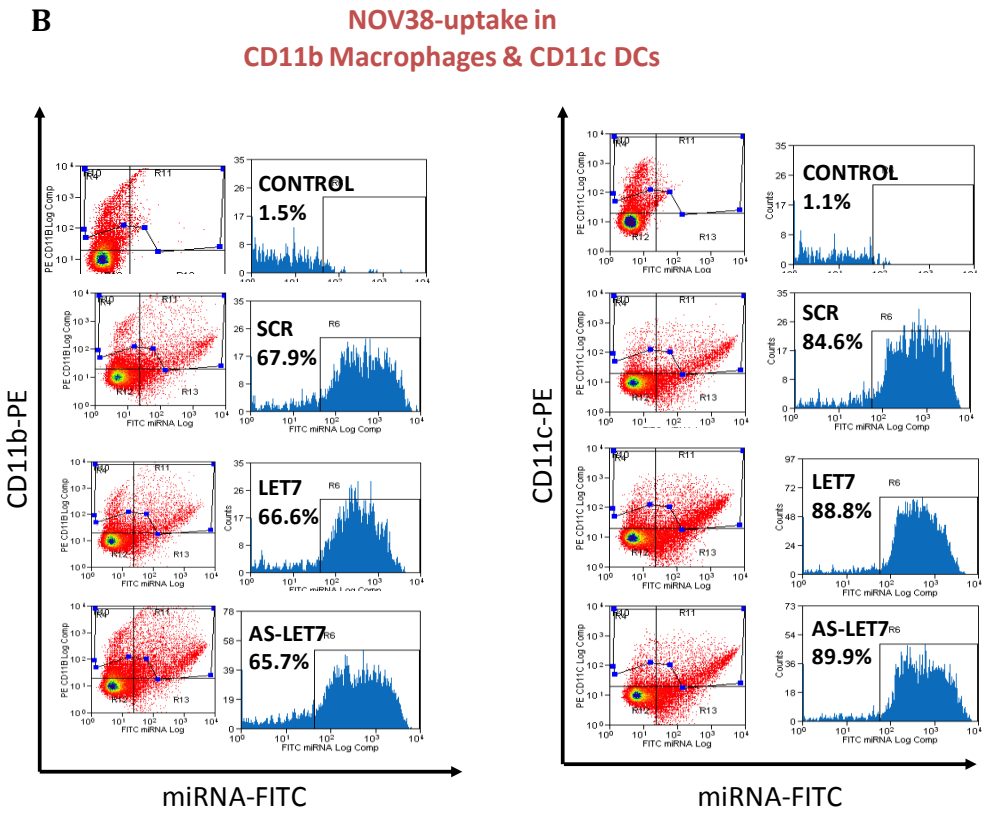
Figure 32. Nov038-Let7e formulation lead to subsequent suppression of TLR4 expression

24h following intravenous injection of liposomal formulations (Nov038, Nov038-scramble, Nov038-let7e and Nov038-as let7e) or (Nov719, Nov719-scramble, Nov719-let7e and Nov719-aslet7e), TLR4 exression was evaluated in FITC-positive splenocytes from C57BL/6 mice by FACS.

A


Nov38
Injection (iv)
LPS
Injection 1mg (ip)
END-
serum and spleen collection
0h
2h
8h

B


NOV38-uptake in
CD11b Macrophages & CD11c DCs
CD11b-PE
miRNA-FITC
CD11c-PE
miRNA-FITC
CONTROL
1.5%
SCR
67.9%
LET7
66.6%
AS-LET7
65.7%
CONTROL
1.1%
SCR
84.6%
LET7
88.8%
AS-LET7
89.9%

c

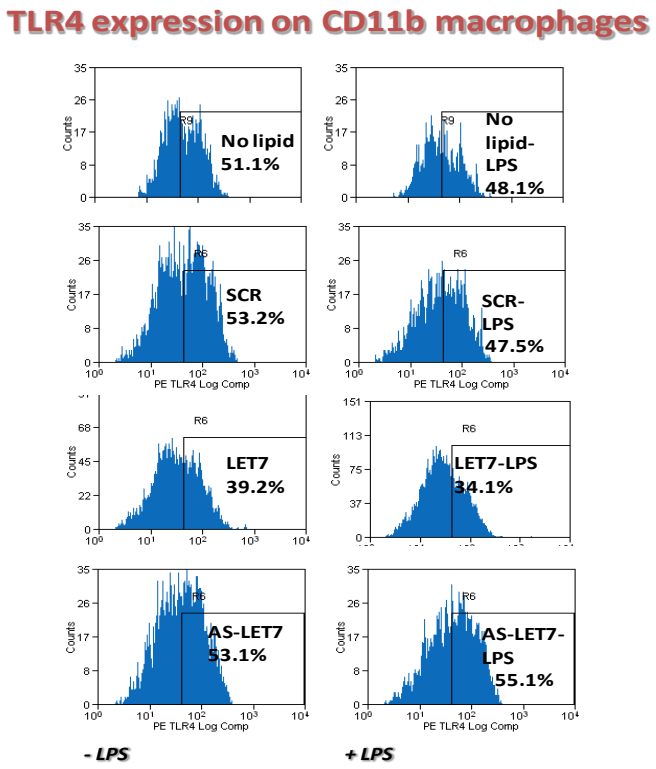


Figure 33. The liposomal uptake of Nov038-As-Let7e led to the augmentation of TLR4 expression upon LPS

(a) Schematic presentation of the following experiment: 2 hours following the intravenus injection of the liposomal formulations (Nov038, Nov038-scramble, Nov038-let7e and Nov038-as let7e) intraperitoneal injection of 1mg LPS was performed and within 6 hours after LPS, spleenocytes from C57BL/6 mice were collected and (b) the percentage of macrophage cells (CD11b) or dendritic cells (CD11c) (A) with the liposomal uptake was analyzed by FACS (c) TLR4 exression was evaluated in macrophage cells (CD11b) by FACS.

Discussion

Endotoxin tolerance occurs to protect the organism from hyper-activation of innate immune responses, rendering macrophages hypo-responsive to subsequent stimuli. During sepsis or other major inflammatory stresses, a carefully orchestrated balance between pro-inflammatory and anti-inflammatory mediators within the host organism is necessary in order to counteract and restore homeostasis. Deregulation of the balance between inflammatory response and tolerance can be lethal either through uncontrolled excessive inflammation leading to sepsis and extensive tissue damage or failure to defend against infectious organisms.

Development of endotoxin tolerance is characterized by significant changes in gene expression, chromatin structure and histone modifications [2]. Such global changes must be coordinated to ensure timely gene expression and limit energy and resource expenditure in the cell. At the signaling level, endotoxin tolerance involves suppression of TLR4 signals via negative regulatory proteins, anti-inflammatory cytokines-mediators and miRNAs. miR-155 and miR-146 play essential role in the process. While mir-155 contributes to the initial activation by targeting negative regulators of TLR signals [14], in the endotoxin tolerance phase it is involved in the negative regulation of macrophage activation partly by targeting TAB2, a mediator of interferon signaling [143]. On the other hand, miR-146a primarily contributes to suppression of activation signals and the development of endotoxin tolerance [73, 132, 133], and cooperates with miR-155 [69]. Herein we demonstrate that at the stage of endotoxin tolerance these two miRNAs are regulated in a coordinated

fashion that involves not only the same transcription factors and histone methylation marks but also an intricate mechanism of monoallelic silencing.

Development of endotoxin tolerance in macrophages requires global changes in gene expression, which is reflected in global changes of H3 methylation. According to these changes, genes that are expressed acquire activatory H3 methyl marks, such as K4me3, and ones that require to be silenced acquire repressive methyl marks on H3, such as K9me3. In the present study we demonstrate that upon initial LPS stimulation both miR genes were activated and acquired H3K4me3 marks [95]. At the endotoxin tolerant state, reduced H3K4me3 and increased H3K9me3 levels were observed at their promoters, indicating a silencing event [144]. This is in agreement with genome-wide analysis showing loss of H3K4me3 marks from selected genes upon development of endotoxin tolerance [6, 105]. Transcription of both miR-155 and miR-146a is regulated by the NFκB transcription complex [70, 74]. Herein, we demonstrated that whereas the p65 subunit of NFκB was bound on their regulatory elements at the initial activation stage, LPS-restimulation of endotoxin tolerant macrophages resulted in recruitment of NFκBp50, which is known to suppress transcription [10]. In accordance with our findings, p50/NFκB has previously been implicated in controlling macrophage inactivation and tolerance [30].

C/EBPβ was also found to bind on both promoters at the stage of endotoxin tolerance and its presence was vital to suppress transcription of these genes. It is involved both in transcriptional activation and suppression and recent evidence has implicated C/EBPβ in the transcriptional silencing of miRNAs such as let-7i, miR-145, miR-155 [137, 145]. Accessibility of promoters to C/EBPβ depends on H3

methylation status at the C/EBPβ binding region, as demonstrated previously [146, 147]. Indeed, H3 methylation was detected in the proximal region of the miR-155 and miR-146a promoters. C/EBPβ has been shown to interact with the SWI/SNF nucleosome-remodeling complex, protein arginine N-methyltransferase-4 (PRMT4) [148], which may potentially affect histone methylation patterns. Moreover, global changes in macrophage enhancer regions on H3K4 methylation marks partly depends on C/EBPs, as demonstrated by global nuclear run-on coupled to deep sequencing analysis [149, 150]. These studies indicate a close association of H3 methylation changes and C/EBPβ binding, which may also apply on miR-155 and miR-146a promoters. Akt signaling regulates miRNA expression in macrophages [14, 131, 132, 137], while it has been demonstrated to regulate C/EBPβ activation either via direct phosphorylation [137], or indirectly via GSK3 [151] or p300/CBP [152]. Interestingly, even though re-stimulation of endotoxin tolerant macrophages with LPS induced binding of p65 and not of CEBPβ in Akt1-/- macrophages, induction of pri-miR-146a was lower in the absence of Akt1. This finding suggests that an alternative transcriptional repressive mechanism exists, which depends on Akt1 and affects miR-146a but not miR-155.

Upon endotoxin tolerance, expression of both mature forms of microRNAs is elevated in comparison to that observed in basal levels or upon 2h LPS activation. The physiological and clinical significance of this finding is confirmed by analysing the expression of miR-155 and miR-146a in peripheral blood cells of septic patients and patients with compensatory anti-inflammatory response syndrome (CARS). According to this syndrome, patients have developed endotoxin tolerance, an impairment of immune responses, responsible for potentially lethal infections and

sepsis. We demonstrated that the critically ill patients with impaired immune responses, as characterized by reduced responsiveness of peripheral blood to LPS, are associated with increased miR-155 and miR-146a expression, while, this impairment in immune response of H1N1-infected patients is mainly associated with high levels of IL-10 in the serum. Therefore, miR155 and miR-146a expression levels observed in peripheral blood cells of patients with impairment of immune responses were elevated. In fact, this finding is, in general, supportive of our main results and it is of great clinical significance rendering these micrornas potential novel biomarkers of CARS syndrome.

Transcriptional regulation of genes can occur in a coordinated manner to ensure simultaneous regulation. For this purpose gene loci come in proximity and are regulated by the same transcriptional machinery [153]. mir-155 has been previously shown to participate in such 'transcription factories' upon TNFα stimulation [117]. Herein, we demonstrated that miR-155 and miR-146a gene loci came into proximity at the stage of endotoxin tolerance. Additionally, we showed that the associated alleles of miR-155 and miR-146a were not transcribed, suggesting a common silencing mechanism. In endotoxin tolerant cells, expression was restricted mainly to one allele and bi-allelic expression was significantly reduced. Further confirmation of the significance of the association between miR-155 and miR-146a comes from the observation that in $Akt1^{-/-}$ macrophages, which fail to develop endotoxin tolerance [14], the association of these gene loci is not induced. Overall, these results demonstrate a coordinated mechanism regulating the expression of miR-155 and miR-146a genes. Upon LPS treatment, the transcription is induced from one or both alleles, which are marked with H3K4 methylation and NFκB-p65 recruitment.

However, at the stage of endotoxin tolerance one of the two alleles of each gene comes into proximity in a silencing environment (Figure 13). Hence, my findings support the importance of coordinated regulation at the three-dimensional chromatin space of gene loci harbouring microRNAs, during endotoxin tolerance. This mechanism may not be restricted to miR-155 and miR-146a but may apply to additional groups of genes that contribute to the same biological condition. Elucidating the molecular mechanisms that orchestrate endotoxin tolerance might lead to modulation of macrophage responses. For this reason further insight into the role of microRNAs in regulating macrophage activation and M1/M2 polarization, revealed the essential role of AKT kinase. Based on the envoromental stimulus, macrophages can possess the classical (M1) phenotype that express high levels of inducible NO synthase (iNOS), generate NO, secrete IL-12b, and are prominent in the acute phase of inflammation or alternative activation (M2) phenotype that participate in the resolution of inflammation and are characterized by high levels of arginase-1 (Arg-1), found-in-inflammatory zone-1 (Fizz1), chitinase-3–like- 3 (Ym1), and macrophage galactose C-type lectin 1 and 2 (MGL1, MGL2). As far as Akt1 is concerned, we observed that Akt1 depleted macrophages were hyperresponsive to LPS and failed to develop endotoxin tolerance in culture and in vivo. *Akt1 ablation induced miR-155 expression in LPS-stimulated macrophage and downregulated its target SOCS1,* a protein with vital role in dampening LPS macrophage activation. *However, measuring miR-155 expression in Akt2-depleted macrophages revealed that miR-155 is reduced in both resting and LPS-activated macrophages. Down-regulation of miR-155 in Akt2-defiecient macrophages resulted in up-regulation of its target C/EBPβ and, consequently, in the induction of Arg1, a hallmark of M2*

macrophage polarization. Overall, ablation or knockdown of Akt2 leads to a M2 phenotype via downregulation of miR-155 and subsequent induction of its target, C/EBPβ. *Moreover, Akt2 deficiency resulted in a significant upregulation of miR-146a, which mediates M1 phenotype suppression and assures endotoxin tolerance. In fact, miR-146a transfection in WT macrophages was able to inhibit iNOS induction while miR-146a suppression in Akt2-depleted mice resulted in upregulation of iNOS expression.* Evidence presented here confirmed that both mir-155 and miR-146a lie under the control of AKT kinase and participate in macrophage polarization and endotoxin tolerance. Understanding the mechanism through which Akt and miRs are regulated and in parallel modulate the inflammatory response, will allow us to selectively target molecules that positively or negatively regulate the inflammatory response. In vivo transferring of miRs by using amphoteric liposomes is indeed highly promising, underlining that miR-155 and miR-146 could be used as potential novel molecular biomarkers of macrophage sensitivity and tools for therapeutic purposes. Besides, MRX34 a double-stranded RNA mimic of the tumor suppressor miR-34, encapsulated in these liposomal nanoparticle formulations is the first microRNA mimic to enter clinical trials against cancer. Given the importance of Akt and miRNAs in inflammation, sepsis and autoimmune diseases, these findings may have significant translational implications.

Future prospects

Endotoxin tolerance is a complex phenomenon as it requires significant changes in gene expression, transcriptional machineries and histone modifications to ensure

temporal gene regulation. In summary, this study focuses on the mechanism of transcriptional regulation of miR-155 and miR-146a during endotoxin tolerance. Both miRNA genes are subjected to endotoxin tolerance, via a complex molecular mechanism characterized by acquisition of specific chromatin features and transcription factors binding at miRNA promoters. Moreover, this study reveals that miR-155 and miR-146 genetic loci become spatially associated upon endotoxin tolerance in a monoallelic manner to mediate coordinated silencing of the two miRNAs. Overall, this study supports the existence of interchromosomal interaction as an additional level of gene regulation in endotoxin tolerant macrophages, which ensures timely gene expression, limitation of energy and resource expenditure in the cell. On the basis of these findings, there are several questions to be further investigated. Firstly, it is unclear whether co-localization of miR-155 and miR-146 in tolerized macrophages is a cause or consequence of silencing. It would be very interesting to block such co-localization (for instance by pharmacological manipulation of the nuclear organization) and afterwards measure the effect on miRNA re-induction, shedding light on the exact role of this co-localization in microRNA regulation. Additionally, it is important to be further investigated whether co-localized miRNAs are also spatially associated with heterochromatic regions or areas marked by repressive histone marks. Moreover, this study reveals that C/EBPβ was bound on both promoters at the stage of endotoxin tolerance and its presence was vital to suppress transcription of these genes. C/EBPβ is involved both in transcriptional activation and suppression and recent evidence has implicated C/EBPβ in the transcriptional silencing of several miRs (let-7i, miR-145, miR-155). Accessibility of promoters to C/EBPβ depends on H3 methylation status at

the C/EBPβ binding region. It has been shown to interact with the SWI/SNF nucleosome-remodeling complex and Protein arginine N-methyltransferase-4 (PRMT4), indicating a close association of H3 methylation changes and C/EBPβ binding [141][148]. Further experiments should be performed elucidating the repressive role of C/EBPβ to miRs upon endotoxin tolerance. Immunostaining of C/EBPβ in colocalized miR loci will give evidence for the putative role of CEBPβ in mediating miRNA association. Moreover, knocking down C/EBPβ and performing DNA FISH with miR probes at the same time will reveal the possibility that its presence is indispensable for the interaction. As far as methylation status is concerned, both miRs obtain the activation mark H3K4me3 at the initial activation stage, while at the stage of endotoxin tolerance both miR promoters were occupied by the repressive mark H3K9me3. Upon LPS-re-stimulation of endotoxin tolerant macrophages, the ratio H3K4me3 to H3K9me3 did not change whereas both active and repressive histone methylation marks were reduced. However, miRNAs are just slightly re-induced after the second LPS stimulus in endotoxin tolerant macrophages. This needs further explanation by validating miRNA re–induction via knocking down specific methyltransferases (i.e PRC2 or G9a). DNA accessibility by FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) has a utility as positive selection for genomic regions associated with regulatory activity. Therefore, FAIRE performed in tolerant versus naïve macrophages will give more information about the chromatin status of miRNA promoters. This study provides evidence that genetic ablation of Akt1, which was used as genetic tool to abrogate endotoxin tolerance, changed miR-155 and miR-146a expression, altered NFkB/p65 and C/EBPβ binding on their promoters and inhibited the induction of co-

localization of miR-155 and miR-146a gene loci, further highlighting the significance of this association. It would be interesting to check whether AKT kinase regulates proteins that participate in the nuclear organization. Further insight into the role of AKT in regulation of M1/M2 polarization, highlighted the essential role of miR-155 and miR-146a in determination of macrophage polarization. Akt kinases, miR-155 and miR-146a seem to be key molecular determinants of macrophage polarization. It was demonstrated that Akt1 depleted macrophages were hyperresponsive to LPS and failed to develop endotoxin tolerance in culture and in vivo via inducing miR-155. However, ablation or knockdown of Akt2 leads to a M2 phenotype via downregulation of miR-155 *and upregulation of miR-146a, which mediates M1 phenotype suppression and assures endotoxin tolerance. Overall,* evidence presented here confirmed that both mir-155 and miR-146a lie under the control of AKT kinase and participate in macrophage polarization and endotoxin tolerance. Understanding further the mechanism through which Akt and miRs are regulated and modulate at the same time the inflammatory response, will allow us to selectively target molecules that positively or negatively regulate the inflammatory response. Cell culture studies by introducing mimic of miR-146a into cancer cell lines derived from patients with autoimmune disorders may lead to dampen macrophage inflammation. Therefore, in vivo transferring of miR-146a by using amphoteric liposomes seems to be highly promising, as it may be used for therapeutic purposes to patients suffering from sepsis or autoimmune disorders. Finally, the finding that miR-155 and miR-146 expression is associated with CARS syndrome in human samples seems to be of great clinical significance. In fact, microRNAs measurement in peripheral blood cells or even in serum, underlies their

usefulness as novel molecular biomarkers of macrophage sensitivity and CARS syndrome.

References

1. Lee, Y.G., et al., *Functional role of Akt in macrophage-mediated innate immunity.* Front Biosci (Landmark Ed), 2011. **16**: p. 517-30.
2. Medzhitov, R. and T. Horng, *Transcriptional control of the inflammatory response.* Nat Rev Immunol, 2009. **9**(10): p. 692-703.
3. O'Neill, L.A. and A.G. Bowie, *The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling.* Nat Rev Immunol, 2007. **7**(5): p. 353-64.
4. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.
5. Hammad, H. and B.N. Lambrecht, *Dendritic cells and airway epithelial cells at the interface between innate and adaptive immune responses.* Allergy, 2011. **66**(5): p. 579-87.
6. Foster, S.L. and R. Medzhitov, *Gene-specific control of the TLR-induced inflammatory response.* Clin Immunol, 2009. **130**(1): p. 7-15.
7. Medzhitov, R., *Recognition of microorganisms and activation of the immune response.* Nature, 2007. **449**(7164): p. 819-26.
8. Heyninck, K. and R. Beyaert, *The cytokine-inducible zinc finger protein A20 inhibits IL-1-induced NF-kappaB activation at the level of TRAF6.* FEBS Lett, 1999. **442**(2-3): p. 147-50.
9. Nakagawa, R., et al., *SOCS-1 participates in negative regulation of LPS responses.* Immunity, 2002. **17**(5): p. 677-87.
10. Biswas, S.K. and E. Lopez-Collazo, *Endotoxin tolerance: new mechanisms, molecules and clinical significance.* Trends Immunol, 2009. **30**(10): p. 475-87.
11. del Fresno, C., et al., *Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: demonstration in isolated monocytes from cystic fibrosis patients.* J Immunol, 2009. **182**(10): p. 6494-507.
12. Medvedev, A.E., K.M. Kopydlowski, and S.N. Vogel, *Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression.* J Immunol, 2000. **164**(11): p. 5564-74.
13. Kobayashi, K., et al., *IRAK-M is a negative regulator of Toll-like receptor signaling.* Cell, 2002. **110**(2): p. 191-202.
14. Androulidaki, A., et al., *The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs.* Immunity, 2009. **31**(2): p. 220-31.
15. Wesche, H., et al., *IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) family.* J Biol Chem, 1999. **274**(27): p. 19403-10.
16. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. **8**(12): p. 958-69.
17. Edwards, J.P., et al., *Biochemical and functional characterization of three activated macrophage populations.* J Leukoc Biol, 2006. **80**(6): p. 1298-307.
18. Lovren, F., et al., *Adiponectin primes human monocytes into alternative anti-inflammatory M2 macrophages.* Am J Physiol Heart Circ Physiol, 2010. **299**(3): p. H656-63.
19. Gordon, S., *Alternative activation of macrophages.* Nat Rev Immunol, 2003. **3**(1): p. 23-35.
20. Herold, S., K. Mayer, and J. Lohmeyer, *Acute lung injury: how macrophages orchestrate resolution of inflammation and tissue repair.* Front Immunol, 2011. **2**: p. 65.

21. Gordon, S. and F.O. Martinez, *Alternative activation of macrophages: mechanism and functions.* Immunity, 2010. **32**(5): p. 593-604.
22. Arranz, A., et al., *Akt1 and Akt2 protein kinases differentially contribute to macrophage polarization.* Proc Natl Acad Sci U S A, 2012. **109**(24): p. 9517-22.
23. Pena, O.M., et al., *Endotoxin tolerance represents a distinctive state of alternative polarization (M2) in human mononuclear cells.* J Immunol, 2011. **186**(12): p. 7243-54.
24. Chaudhuri, A.A., et al., *MicroRNA-125b potentiates macrophage activation.* J Immunol, 2011. **187**(10): p. 5062-8.
25. Martinez, F.O., *Regulators of macrophage activation.* Eur J Immunol, 2011. **41**(6): p. 1531-4.
26. Whyte, C.S., et al., *Suppressor of cytokine signaling (SOCS)1 is a key determinant of differential macrophage activation and function.* J Leukoc Biol, 2011. **90**(5): p. 845-54.
27. Guiducci, C., et al., *PI3K is critical for the nuclear translocation of IRF-7 and type I IFN production by human plasmacytoid predendritic cells in response to TLR activation.* J Exp Med, 2008. **205**(2): p. 315-22.
28. Honda, K. and T. Taniguchi, *IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors.* Nat Rev Immunol, 2006. **6**(9): p. 644-58.
29. Spence, S., et al., *Suppressors of cytokine signaling 2 and 3 diametrically control macrophage polarization.* Immunity, 2013. **38**(1): p. 66-78.
30. Porta, C., et al., *Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor kappaB.* Proc Natl Acad Sci U S A, 2009. **106**(35): p. 14978-83.
31. Satoh, T., et al., *The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection.* Nat Immunol, 2010. **11**(10): p. 936-44.
32. Odegaard, J.I., et al., *Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance.* Nature, 2007. **447**(7148): p. 1116-20.
33. Ruffell, D., et al., *A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair.* Proc Natl Acad Sci U S A, 2009. **106**(41): p. 17475-80.
34. Liu, Y.C., et al., *Macrophage polarization in inflammatory diseases.* Int J Biol Sci, 2014. **10**(5): p. 520-9.
35. Fukao, T. and S. Koyasu, *PI3K and negative regulation of TLR signaling.* Trends Immunol, 2003. **24**(7): p. 358-63.
36. Franke, T.F., et al., *The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase.* Cell, 1995. **81**(5): p. 727-36.
37. Iliopoulos, D., et al., *MicroRNAs differentially regulated by Akt isoforms control EMT and stem cell renewal in cancer cells.* Sci Signal, 2009. **2**(92): p. ra62.
38. Fukao, T., et al., *PI3K-mediated negative feedback regulation of IL-12 production in DCs.* Nat Immunol, 2002. **3**(9): p. 875-81.
39. Guha, M. and N. Mackman, *The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells.* J Biol Chem, 2002. **277**(35): p. 32124-32.
40. Martin, M., et al., *Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3.* Nat Immunol, 2005. **6**(8): p. 777-84.
41. Bommhardt, U., et al., *Akt decreases lymphocyte apoptosis and improves survival in sepsis.* J Immunol, 2004. **172**(12): p. 7583-91.

42. Luyendyk, J.P., et al., *Genetic analysis of the role of the PI3K-Akt pathway in lipopolysaccharide-induced cytokine and tissue factor gene expression in monocytes/macrophages.* J Immunol, 2008. **180**(6): p. 4218-26.
43. Peng, X.D., et al., *Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2.* Genes Dev, 2003. **17**(11): p. 1352-65.
44. Cho, H., et al., *Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta).* Science, 2001. **292**(5522): p. 1728-31.
45. Gonzalez, E. and T.E. McGraw, *The Akt kinases: isoform specificity in metabolism and cancer.* Cell Cycle, 2009. **8**(16): p. 2502-8.
46. Song, G., G. Ouyang, and S. Bao, *The activation of Akt/PKB signaling pathway and cell survival.* J Cell Mol Med, 2005. **9**(1): p. 59-71.
47. Mitsuuchi, Y., et al., *Translocation and activation of AKT2 in response to stimulation by insulin.* J Cell Biochem, 1998. **70**(4): p. 433-41.
48. Cheng, J.Q., et al., *AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas.* Proc Natl Acad Sci U S A, 1992. **89**(19): p. 9267-71.
49. Pearl, L.H. and D. Barford, *Regulation of protein kinases in insulin, growth factor and Wnt signalling.* Curr Opin Struct Biol, 2002. **12**(6): p. 761-7.
50. Naiki, T., et al., *TRB2, a mouse Tribbles ortholog, suppresses adipocyte differentiation by inhibiting AKT and C/EBPbeta.* J Biol Chem, 2007. **282**(33): p. 24075-82.
51. Kluiver, J., et al., *Regulation of pri-microRNA BIC transcription and processing in Burkitt lymphoma.* Oncogene, 2007. **26**(26): p. 3769-76.
52. Hajishengallis, G. and J.D. Lambris, *Microbial manipulation of receptor crosstalk in innate immunity.* Nat Rev Immunol, 2011. **11**(3): p. 187-200.
53. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
54. El Gazzar, M. and C.E. McCall, *MicroRNAs distinguish translational from transcriptional silencing during endotoxin tolerance.* J Biol Chem, 2010. **285**(27): p. 20940-51.
55. Liu, Y., et al., *MicroRNA-98 negatively regulates IL-10 production and endotoxin tolerance in macrophages after LPS stimulation.* FEBS Lett, 2011. **585**(12): p. 1963-8.
56. Quinn, E.M., J. Wang, and H.P. Redmond, *The emerging role of microRNA in regulation of endotoxin tolerance.* J Leukoc Biol, 2012. **91**(5): p. 721-7.
57. Bala, S., et al., *Up-regulation of microRNA-155 in macrophages contributes to increased tumor necrosis factor {alpha} (TNF{alpha}) production via increased mRNA half-life in alcoholic liver disease.* J Biol Chem, 2011. **286**(2): p. 1436-44.
58. Cai, X., et al., *Re-polarization of tumor-associated macrophages to pro-inflammatory M1 macrophages by microRNA-155.* J Mol Cell Biol, 2012. **4**(5): p. 341-3.
59. McCoy, C.E., et al., *IL-10 inhibits miR-155 induction by toll-like receptors.* J Biol Chem, 2010. **285**(27): p. 20492-8.
60. Ruckerl, D., et al., *Induction of IL-4Ralpha-dependent microRNAs identifies PI3K/Akt signaling as essential for IL-4-driven murine macrophage proliferation in vivo.* Blood, 2012. **120**(11): p. 2307-16.
61. Graff, J.W., et al., *Identifying functional microRNAs in macrophages with polarized phenotypes.* J Biol Chem, 2012. **287**(26): p. 21816-25.
62. Boldin, M.P., et al., *miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice.* J Exp Med, 2011. **208**(6): p. 1189-201.

63. Zhao, J.L., et al., *NF-kappaB dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies.* Proc Natl Acad Sci U S A, 2011. **108**(22): p. 9184-9.
64. Lu, L.F., et al., *Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses.* Cell, 2010. **142**(6): p. 914-29.
65. Nahid, M.A., M. Satoh, and E.K. Chan, *Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling.* J Immunol, 2011. **186**(3): p. 1723-34.
66. Squadrito, M.L., et al., *miR-511-3p modulates genetic programs of tumor-associated macrophages.* Cell Rep, 2012. **1**(2): p. 141-54.
67. So, A.Y., et al., *Dual mechanisms by which miR-125b represses IRF4 to induce myeloid and B-cell leukemias.* Blood, 2014. **124**(9): p. 1502-12.
68. Chen, X.M., et al., *A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium parvum infection.* J Biol Chem, 2007. **282**(39): p. 28929-38.
69. Schulte, L.N., A.J. Westermann, and J. Vogel, *Differential activation and functional specialization of miR-146 and miR-155 in innate immune sensing.* Nucleic Acids Res, 2013. **41**(1): p. 542-53.
70. Taganov, K.D., et al., *NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses.* Proc Natl Acad Sci U S A, 2006. **103**(33): p. 12481-6.
71. Kagiya, T. and S. Nakamura, *Expression profiling of microRNAs in RAW264.7 cells treated with a combination of tumor necrosis factor alpha and RANKL during osteoclast differentiation.* J Periodontal Res, 2012. **48**(3): p. 373-85.
72. Kutty, R.K., et al., *Differential regulation of microRNA-146a and microRNA-146b-5p in human retinal pigment epithelial cells by interleukin-1beta, tumor necrosis factor-alpha, and interferon-gamma.* Mol Vis, 2013. **19**: p. 737-50.
73. Nahid, M.A., et al., *miR-146a is critical for endotoxin-induced tolerance: IMPLICATION IN INNATE IMMUNITY.* J Biol Chem, 2009. **284**(50): p. 34590-9.
74. O'Connell, R.M., et al., *MicroRNA-155 is induced during the macrophage inflammatory response.* Proc Natl Acad Sci U S A, 2007. **104**(5): p. 1604-9.
75. Perry, M.M., et al., *Divergent intracellular pathways regulate interleukin-1beta-induced miR-146a and miR-146b expression and chemokine release in human alveolar epithelial cells.* FEBS Lett, 2009. **583**(20): p. 3349-55.
76. Kutty, R.K., et al., *Inflammatory cytokines regulate microRNA-155 expression in human retinal pigment epithelial cells by activating JAK/STAT pathway.* Biochem Biophys Res Commun, 2010. **402**(2): p. 390-5.
77. Chassin, C., et al., *miR-146a mediates protective innate immune tolerance in the neonate intestine.* Cell Host Microbe, 2010. **8**(4): p. 358-68.
78. Banerjee, S., et al., *Morphine induced exacerbation of sepsis is mediated by tempering endotoxin tolerance through modulation of miR-146a.* Sci Rep, 2013. **3**: p. 1977.
79. Alexander, M., et al., *Exosome-delivered microRNAs modulate the inflammatory response to endotoxin.* Nat Commun, 2015. **6**: p. 7321.
80. Jurado-Camino, T., et al., *Chronic lymphocytic leukemia: a paradigm of innate immune cross-tolerance.* J Immunol, 2015. **194**(2): p. 719-27.
81. Pauley, K.M., et al., *Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients.* Arthritis Res Ther, 2008. **10**(4): p. R101.
82. Stanczyk, J., et al., *Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis.* Arthritis Rheum, 2008. **58**(4): p. 1001-9.

83. Wang, G., et al., *Expression of miR-146a and miR-155 in the urinary sediment of systemic lupus erythematosus.* Clin Rheumatol, 2011. **31**(3): p. 435-40.
84. Wang, G., et al., *Elevated levels of miR-146a and miR-155 in kidney biopsy and urine from patients with IgA nephropathy.* Dis Markers, 2011. **30**(4): p. 171-9.
85. Kin, K., et al., *Tissue- and plasma-specific MicroRNA signatures for atherosclerotic abdominal aortic aneurysm.* J Am Heart Assoc, 2013. **1**(5): p. e000745.
86. Tsatsanis, C., et al., *Serum miR-155 as a potential biomarker of male fertility.* Hum Reprod, 2015.
87. Xie, Y.F., et al., *Comparison of microRNA profiles of human periodontal diseased and healthy gingival tissues.* Int J Oral Sci, 2011. **3**(3): p. 125-34.
88. Corral-Fernandez, N.E., et al., *Dysregulated miR-155 expression in peripheral blood mononuclear cells from patients with type 2 diabetes.* Exp Clin Endocrinol Diabetes, 2013. **121**(6): p. 347-53.
89. Doxakis, E., *Principles of miRNA-target regulation in metazoan models.* Int J Mol Sci, 2013. **14**(8): p. 16280-302.
90. Etzrodt, M., et al., *Regulation of monocyte functional heterogeneity by miR-146a and Relb.* Cell Rep, 2012. **1**(4): p. 317-24.
91. Natoli, G., *Maintaining cell identity through global control of genomic organization.* Immunity, 2010. **33**(1): p. 12-24.
92. Margueron, R. and D. Reinberg, *Chromatin structure and the inheritance of epigenetic information.* Nat Rev Genet, 2010. **11**(4): p. 285-96.
93. Mattick, J.S., R.J. Taft, and G.J. Faulkner, *A global view of genomic information--moving beyond the gene and the master regulator.* Trends Genet, 2010. **26**(1): p. 21-8.
94. Zhou, V.W., A. Goren, and B.E. Bernstein, *Charting histone modifications and the functional organization of mammalian genomes.* Nat Rev Genet, 2011. **12**(1): p. 7-18.
95. Santos-Rosa, H., et al., *Active genes are tri-methylated at K4 of histone H3.* Nature, 2002. **419**(6905): p. 407-11.
96. Bernstein, B.E., et al., *Genomic maps and comparative analysis of histone modifications in human and mouse.* Cell, 2005. **120**(2): p. 169-81.
97. Schneider, R., et al., *Histone H3 lysine 4 methylation patterns in higher eukaryotic genes.* Nat Cell Biol, 2004. **6**(1): p. 73-7.
98. Smale, S.T., *Selective transcription in response to an inflammatory stimulus.* Cell, 2010. **140**(6): p. 833-44.
99. Natoli, G., S. Ghisletti, and I. Barozzi, *The genomic landscapes of inflammation.* Genes Dev, 2011. **25**(2): p. 101-6.
100. Strahl, B.D. and C.D. Allis, *The language of covalent histone modifications.* Nature, 2000. **403**(6765): p. 41-5.
101. Jenuwein, T. and C.D. Allis, *Translating the histone code.* Science, 2001. **293**(5532): p. 1074-80.
102. De Santa, F., et al., *The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing.* Cell, 2007. **130**(6): p. 1083-94.
103. De Santa, F., et al., *Jmjd3 contributes to the control of gene expression in LPS-activated macrophages.* EMBO J, 2009. **28**(21): p. 3341-52.
104. Mullican, S.E., et al., *Histone deacetylase 3 is an epigenomic brake in macrophage alternative activation.* Genes Dev, 2011. **25**(23): p. 2480-8.
105. Foster, S.L., D.C. Hargreaves, and R. Medzhitov, *Gene-specific control of inflammation by TLR-induced chromatin modifications.* Nature, 2007. **447**(7147): p. 972-8.

106. Lyn-Kew, K., et al., *IRAK-M regulates chromatin remodeling in lung macrophages during experimental sepsis.* PLoS One, 2010. **5**(6): p. e11145.
107. Jing, J., et al., *Role of macrophage receptor with collagenous structure in innate immune tolerance.* J Immunol, 2013. **190**(12): p. 6360-7.
108. Belmont, A.S., *Large-scale chromatin organization: the good, the surprising, and the still perplexing.* Curr Opin Cell Biol, 2014. **26**: p. 69-78.
109. Hubner, M.R., M.A. Eckersley-Maslin, and D.L. Spector, *Chromatin organization and transcriptional regulation.* Curr Opin Genet Dev, 2013. **23**(2): p. 89-95.
110. Merkenschlager, M. and D.T. Odom, *CTCF and cohesin: linking gene regulatory elements with their targets.* Cell, 2013. **152**(6): p. 1285-97.
111. Hubner, M.R. and D.L. Spector, *Chromatin dynamics.* Annu Rev Biophys, 2010. **39**: p. 471-89.
112. Dekker, J., M.A. Marti-Renom, and L.A. Mirny, *Exploring the three-dimensional organization of genomes: interpreting chromatin interaction data.* Nat Rev Genet, 2013. **14**(6): p. 390-403.
113. Deligianni, C. and C.G. Spilianakis, *Long-range genomic interactions epigenetically regulate the expression of a cytokine receptor.* EMBO Rep, 2012. **13**(9): p. 819-26.
114. Spilianakis, C.G. and R.A. Flavell, *Molecular biology. Managing associations between different chromosomes.* Science, 2006. **312**(5771): p. 207-8.
115. Spilianakis, C.G., et al., *Interchromosomal associations between alternatively expressed loci.* Nature, 2005. **435**(7042): p. 637-45.
116. Chen, D., et al., *Dissecting the chromatin interactome of microRNA genes.* Nucleic Acids Res, 2014. **42**(5): p. 3028-43.
117. Papantonis, A., et al., *TNFalpha signals through specialized factories where responsive coding and miRNA genes are transcribed.* EMBO J, 2012. **31**(23): p. 4404-14.
118. Randow, F., et al., *Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor beta.* J Exp Med, 1995. **181**(5): p. 1887-92.
119. Suganuma, T. and J.L. Workman, *Crosstalk among Histone Modifications.* Cell, 2008. **135**(4): p. 604-7.
120. Cho, Y.W., et al., *PTIP associates with MLL3- and MLL4-containing histone H3 lysine 4 methyltransferase complex.* J Biol Chem, 2007. **282**(28): p. 20395-406.
121. Hulsmans, M., et al., *Interleukin-1 receptor-associated kinase-3 is a key inhibitor of inflammation in obesity and metabolic syndrome.* PLoS One, 2012. **7**(1): p. e30414.
122. Issaeva, I., et al., *Knockdown of ALR (MLL2) reveals ALR target genes and leads to alterations in cell adhesion and growth.* Mol Cell Biol, 2007. **27**(5): p. 1889-903.
123. Wang, J.F., et al., *Serum miR-146a and miR-223 as potential new biomarkers for sepsis.* Biochem Biophys Res Commun, 2010. **394**(1): p. 184-8.
124. Klimuk, S.K., et al., *Enhanced anti-inflammatory activity of a liposomal intercellular adhesion molecule-1 antisense oligodeoxynucleotide in an acute model of contact hypersensitivity.* J Pharmacol Exp Ther, 2000. **292**(2): p. 480-8.
125. Hanai, K., et al., *Potential of atelocollagen-mediated systemic antisense therapeutics for inflammatory disease.* Hum Gene Ther, 2004. **15**(3): p. 263-72.
126. Khoury, M., et al., *Efficient new cationic liposome formulation for systemic delivery of small interfering RNA silencing tumor necrosis factor alpha in experimental arthritis.* Arthritis Rheum, 2006. **54**(6): p. 1867-77.
127. Peer, D., et al., *Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target.* Science, 2008. **319**(5863): p. 627-30.

128. Andreakos, E., et al., *Amphoteric liposomes enable systemic antigen-presenting cell-directed delivery of CD40 antisense and are therapeutically effective in experimental arthritis.* Arthritis Rheum, 2009. **60**(4): p. 994-1005.
129. Mao, C., et al., *Unequal contribution of Akt isoforms in the double-negative to double-positive thymocyte transition.* J Immunol, 2007. **178**(9): p. 5443-53.
130. Maroulakou, I.G., et al., *Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice.* Cancer Res, 2007. **67**(1): p. 167-77.
131. Arranz, A., et al., *Vasoactive intestinal peptide suppresses toll-like receptor 4 expression in macrophages via Akt1 reducing their responsiveness to lipopolysaccharide.* Mol Immunol, 2008. **45**(10): p. 2970-80.
132. Vergadi, E., et al., *Akt2 deficiency protects from acute lung injury via alternative macrophage activation and miR-146a induction in mice.* J Immunol, 2014. **192**(1): p. 394-406.
133. Nahid, M.A., M. Satoh, and E.K. Chan, *Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling.* J Immunol, 2010. **186**(3): p. 1723-34.
134. Chen, D., et al., *Dissecting the chromatin interactome of microRNA genes.* Nucleic Acids Res, 2013. **42**(5): p. 3028-43.
135. Yin, Q., et al., *B-cell receptor activation induces BIC/miR-155 expression through a conserved AP-1 element.* J Biol Chem, 2008. **283**(5): p. 2654-62.
136. Chen, Y., et al., *miR-155 regulates differentiation of brown and beige adipocytes via a bistable circuit.* Nat Commun, 2013. **4**: p. 1769.
137. Sachdeva, M., et al., *Negative regulation of miR-145 by C/EBP-beta through the Akt pathway in cancer cells.* Nucleic Acids Res, 2012. **40**(14): p. 6683-92.
138. Augui, S., E.P. Nora, and E. Heard, *Regulation of X-chromosome inactivation by the X-inactivation centre.* Nat Rev Genet, 2011. **12**(6): p. 429-42.
139. Chess, A., *Mechanisms and consequences of widespread random monoallelic expression.* Nat Rev Genet, 2012. **13**(6): p. 421-8.
140. Rodriguez, I., *Singular expression of olfactory receptor genes.* Cell, 2013. **155**(2): p. 274-7.
141. Yang, P.K. and M.I. Kuroda, *Noncoding RNAs and intranuclear positioning in monoallelic gene expression.* Cell, 2007. **128**(4): p. 777-86.
142. Gray, M.J., et al., *Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPbeta.* Gene, 2005. **353**(1): p. 98-106.
143. Ceppi, M., et al., *MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells.* Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2735-40.
144. Lachner, M., et al., *Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins.* Nature, 2001. **410**(6824): p. 116-20.
145. O'Hara, S.P., et al., *NFkappaB p50-CCAAT/enhancer-binding protein beta (C/EBPbeta)-mediated transcriptional repression of microRNA let-7i following microbial infection.* J Biol Chem, 2010. **285**(1): p. 216-25.
146. Wang, F., et al., *Dynamic CCAAT/enhancer binding protein-associated changes of DNA methylation in the angiotensinogen gene.* Hypertension, 2014. **63**(2): p. 281-8.
147. Pham, T.H., et al., *Dynamic epigenetic enhancer signatures reveal key transcription factors associated with monocytic differentiation states.* Blood, 2012. **119**(24): p. e161-71.

148. Kowenz-Leutz, E., et al., *Crosstalk between C/EBPbeta phosphorylation, arginine methylation, and SWI/SNF/Mediator implies an indexing transcription factor code.* EMBO J, 2010. **29**(6): p. 1105-15.
149. Kaikkonen, M.U., et al., *Remodeling of the enhancer landscape during macrophage activation is coupled to enhancer transcription.* Mol Cell, 2013. **51**(3): p. 310-25.
150. Romanoski, C.E., et al., *Exploiting genomics and natural genetic variation to decode macrophage enhancers.* Trends Immunol, 2015.
151. Piwien-Pilipuk, G., et al., *Growth hormone regulates phosphorylation and function of CCAAT/enhancer-binding protein beta by modulating Akt and glycogen synthase kinase-3.* J Biol Chem, 2001. **276**(22): p. 19664-71.
152. Guo, S., et al., *Insulin suppresses transactivation by CAAT/enhancer-binding proteins beta (C/EBPbeta). Signaling to p300/CREB-binding protein by protein kinase B disrupts interaction with the major activation domain of C/EBPbeta.* J Biol Chem, 2001. **276**(11): p. 8516-23.
153. Williams, A., C.G. Spilianakis, and R.A. Flavell, *Interchromosomal association and gene regulation in trans.* Trends Genet, 2010. **26**(4): p. 188-97.