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Regulation of monocyte functional heterogeneity by *miR-146a* and Relb

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SUMMARY

Monocytes serve as a central defense system against infection and injury but can also promote pathological inflammatory responses. Considering the evidence that monocytes exist in at least two subsets committed to divergent functions, we investigated whether distinct factors regulate the balance between monocyte subset responses *in vivo*. We identified a microRNA (miRNA), *miR-146a*, which is differentially regulated both in mouse (Ly-6C^{hi}/Ly-6C^{lo}) and human (CD14^{hi}/CD14^{lo}CD16⁺) monocyte subsets. The single miRNA controlled the amplitude of the Ly-6C^{hi} monocyte response during inflammatory challenge whereas it did not affect Ly-6C^{lo} cells. *miR-146a*-mediated regulation was cell-intrinsic and depended on Relb, a member of the non-canonical NF- κ B/Rel family, which we identified as a direct *miR-146a* target. These observations not only provide novel mechanistic insights into the molecular events that regulate responses mediated by committed monocyte precursor populations but also identify novel targets to manipulate Ly-6C^{hi} monocyte responses while sparing Ly-6C^{lo} monocyte activity.

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Accession Numbers

The microarray data newly generated in this study are available on the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/gds>) under the accession number GSE32364.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and Figures.

INTRODUCTION

Monocytes are the circulating precursors of several types of macrophages and dendritic cells (Geissmann et al., 2010). They confer protection of injured or infected tissue but also propagate chronic diseases (Auffray et al., 2009; Qian and Pollard, 2010; Shi and Pamer, 2011). At least two CD11b⁺ CD115⁺ monocyte populations exist in mice: (i) Ly-6C^{hi} (Gr-1⁺ CCR2⁺ CX3CR1^{lo}) cells respond to pro-inflammatory cues such as CCL2 (or MCP-1), migrate to inflamed sites and draining lymph nodes and can differentiate into antigen-presenting dendritic cells (Cheong et al., 2010) and orchestrate inflammatory functions (Swirski et al., 2007; Tacke et al., 2007); and (ii) Ly-6C^{lo} (Gr-1⁻ CCR2⁻ CX3CR1^{hi}) cells patrol the resting endothelium (Auffray et al., 2007), can be recruited to tissue after the onset of inflammation, and participate in granulation tissue formation (Nahrendorf et al., 2007). Ly-6C^{hi} monocytes recirculate into the bone marrow where they can convert into Ly-6C^{lo} monocytes (Varol et al., 2007). Monocyte heterogeneity is conserved at least in part in mice and humans: mouse Ly-6C^{hi} monocytes share phenotypic and functional features with human CD14^{hi} cells, whereas mouse Ly-6C^{lo} monocytes resemble human CD14^{lo} CD16⁺ cells (Cros et al., 2010).

Infection (Shi and Pamer, 2011), injury (Nahrendorf et al., 2007), atherosclerosis (Swirski et al., 2007; Tacke et al., 2007); cancer (Movahedi et al., 2010) and other pathophysiological conditions alter monocyte subset ratios. Changes of ratios can occur rapidly (e.g., hours after pathogenic infection), be long lasting (e.g. in chronic inflammatory disorders), and typically result in the selective amplification of pro-inflammatory Ly-6C^{hi} cells. Human studies have underscored the relevance of studying monocyte subsets because an imbalance in their relative proportion is linked to several diseases (Ziegler-Heitbrock, 2007). The factors that regulate the balance between monocyte subset responses are largely unknown. The identification of such factors is potentially useful as it may offer new vantage points for tailoring immune responses to a desired phenotype.

RESULTS

Mir-146a Is A Candidate Regulator Of Monocyte Functional Heterogeneity

MicroRNAs (miRNAs) regulate target genes at the post-transcriptional level and can control distinct functional properties in cell types that are closely related ontogenically. miRNAs are known to regulate the development and function of various immune cell types (O'Connell et al., 2010) but have to date not been investigated in the context of monocyte heterogeneity. Here we compared the expression levels of 380 miRNAs in sorted monocyte subsets (Fig S1a) and defined significant genes as those with at least 2-fold differential expression and a $p < 0.05$ (Student's t-test). The approach identified 9 miRNAs, which were highly expressed either in Ly-6C^{hi} (*miR-20b*, *-135a*, *-424*, *-702*) or in Ly-6C^{lo} monocytes (*miR-146a*, *-150*, *-155*, *-342*, *-29b*) (Fig 1a).

Independent assays indicated ~2 orders of magnitude higher expression of *miR-146a* in Ly-6C^{lo} monocytes when compared to hematopoietic stem cells (HSC), granulocyte/macrophage progenitors (GMP), macrophage/dendritic cell progenitors (MDP) and Ly-6C^{hi} monocytes in steady-state (Fig 1b). Thus, monocytes express *miR-146a* only at a late maturation stage and selectively in the Ly-6C^{lo} subset. Steady-state dendritic cell populations expressed *miR-146a* at intermediate levels (Fig 1b).

miRNAs and their respective target genes are often mutually exclusively expressed in a given tissue (Farh, 2005). In keeping with previous observations that *miR-146a* suppresses NF- κ B-dependent inflammatory pathways (Taganov et al., 2006), we confirmed with two independent genome-wide profiling methods that splenic *miR-146a*^{lo} Ly-6C^{hi} monocytes

showed increased inflammatory signatures (Swirski et al., 2009) and expressed components of the NF- κ B signaling cascade at higher levels than their *miR-146a*^{hi} Ly-6C^{lo} counterparts (Fig 1c, S1b). Also, splenic and blood *miR-146a*^{lo} Ly-6C^{hi} monocytes stimulated with lipopolysaccharide (LPS) produced more TNF α , IL-6 and IL-1 β inflammatory cytokines than *miR-146a*^{hi} Ly-6C^{lo} cells (Fig 1d and S1c).

The elevated *miR-146a* expression in Ly-6C^{lo} cells and the inflammatory profile of Ly-6C^{hi} cells reported above were likely not due to a premature activation artifact induced by the isolation procedure because I κ B α protein levels were similar in both monocyte subsets *ex vivo* (Fig S1d) and NF- κ B subunit p65 only became detectable in the nucleus of Ly-6C^{hi} cells upon *in vitro* challenge (Fig 1e). The cause for constitutive (NF- κ B-independent) *miR-146a* expression in Ly-6C^{lo} cells will require additional investigation.

Differential *Mir-146a* Expression In Monocytes In Steady-State And Inflammation

We addressed the regulation of *miR-146a* expression in monocyte subsets upon *ex vivo* challenge with either LPS, heat killed *Listeria monocytogenes* (HKLM) or TNF α . *miR-146a* was induced only in Ly-6C^{hi} monocytes, in response to all stimuli, and reached levels matching those in Ly-6C^{lo} cells (Fig S1e). *In vivo* LPS challenge studies confirmed the *in vitro* findings (Fig S1f). *miR-146a* expression in Ly-6C^{hi} cells increased within 4 h after LPS challenge and reached levels equivalent to those found in Ly-6C^{lo} cells after 16 h (Fig 1f). Thus, *miR-146a* expression is constitutive in Ly-6C^{lo} monocytes and inducible in Ly-6C^{hi} monocytes. LPS-stimulated Ly-6C^{hi} monocytes were CD11c⁺ MHC II^{high} (Ly-6C^{hi}) and thus distinct from Ly-6C^{lo} monocytes (Fig S1g).

Mir-146a Controls Monocyte Subset Ratios During Inflammatory Reactions

To investigate the role of *miR-146a* in monocytes *in vivo*, we generated mice in which *miR-146a* expression was either up- or down-regulated experimentally. To constitutively over-express *miR-146a* we reconstituted mice with HSC transduced to co-express EGFP and *miR-146a* (Fig S2a–b). *miR-146a* overexpression did not alter monocyte numbers or subset ratios in steady-state (Fig S2c); however, upon *Listeria monocytogenes* (*Lm*) infection (Shi and Pamer, 2011), it prevented the unfolding of a full-fledged TNF α -producing Ly-6C^{hi} monocyte response (Fig 2a,b).

To suppress *miR-146a* expression *in vivo* we used two independent approaches. The first one involved systemic delivery of anti-miRNA locked nucleic acid (LNA) formulations (Fig S2d,e). LNA treatment did not alter monocyte subset ratios in steady-state (Fig S2f) but it increased the number of TNF α -producing Ly-6C^{hi} monocytes at *Lm* infected sites (Fig 2c,d).

The second approach to suppress *miR-146a* expression used recently described mice with targeted deletion of the *miR-146a* gene (Boldin et al., 2011) (Fig S2g). *miR-146a*^{-/-} mice contained both monocyte subsets thus Ly-6C^{hi}→Ly-6C^{lo} monocyte conversion should not require *miR-146a*. Also, *miR-146a* knockdown did neither alter the ratio (Fig 2e) nor the phenotype (Fig S2h) of monocyte subsets in 8 wk old mice. To compare *miR-146a*^{-/-} and wild-type monocyte responses as they developed in the same environments, we reconstituted wild-type (CD45.1) mice with equal numbers of *miR-146a*^{-/-} (CD45.2) and wild-type (EGFP⁺ CD45.2) cells (Fig S2i). The absence of *miR-146a* strongly amplified TNF α -producing Ly-6C^{hi} peritoneal monocytes in response to LPS challenge (Fig 2f, g). Ly-6C^{hi} monocytes mediate immune defense in early phase of *Lm* infection (Shi and Pamer, 2011). Accordingly, *Lm*-infected *miR-146a*^{-/-} mice contained reduced numbers of viable *Lm* 24 h post infection when compared to *Lm*-infected wild-type mice (Fig 2h). Amplification of the

Ly-6C^{hi} monocyte response in absence of *miR-146a* was confirmed in a model of sterile peritonitis induced by thioglycollate (Fig 2i).

Cell-Intrinsic *Mir-146a*-Mediated Regulation Of The Ly-6C^{hi} Monocyte Response

The experiments above involved indiscriminate alteration of *miR-146a* expression in all hematopoietic cells. We reasoned that injection of *miR-146a*^{-/-} GMP into wild-type mice would permit to track *miR-146a*^{-/-} monocytes in a wild-type environment because *miR-146a* is only upregulated upon progenitor cell maturation. Specifically, we co-administered equal numbers of *miR-146a*^{-/-} (CD45.2 EGFP⁻) and wild-type (CD45.2 EGFP⁺) GMP into non-irradiated wild-type (CD45.1) mice, which were subsequently challenged with LPS i.p. (Fig S2j). Wild-type and *miR-146a*^{-/-} hematopoietic progenitor cells show comparable clonogenic potential (Boldin et al., 2011; Fig S2k) and the transferred cells' progeny contained monocytes and neutrophils, as expected. *miR-146a*^{-/-} monocytes recruited to the peritoneal cavity outnumbered their wild-type counterparts (Fig 2j, k) and were Ly-6C^{hi} (Fig 2l); in marked contrast, *miR-146a*^{-/-} neutrophils—which do not upregulate *miR-146a* *in vivo*—mounted a response that was similar to their wild-type counterparts (Fig 2k). Thus *miR-146a* should regulate Ly-6C^{hi} monocytes at least in part in a cell-intrinsic manner.

Mir-146a Controls Ly-6C^{hi} Monocyte Proliferation And Trafficking In Inflammatory Conditions

In contrast to previous descriptions for other cell types (Nahid et al., 2009; Boldin et al., 2011), including macrophages (Fig S3a), the absence of *miR-146a* did not detectably alter inflammatory cytokine production by Ly-6C^{hi} and Ly-6C^{lo} monocytes on a per-cell basis (Fig 3a,b). However, LPS challenge increased the percentage of *miR-146a*^{-/-} Ly-6C^{hi} monocytes undergoing cell division in bone marrow (Fig 3c, d) and to a lower extent in the spleen and peritoneal cavity (Fig 3d). The absence of *miR-146a* did not affect proliferation of Ly-6C^{lo} monocytes; Fig S3b. Co-cultures of *miR-146a*^{-/-} and wild-type cells also indicated a proliferative advantage for bone marrow *miR-146a*^{-/-} Ly-6C^{hi} monocytes (Fig S3c, d).

In addition, co-injection of bone marrow *miR-146a*^{-/-} (EGFP⁻ CD45.2) and control (EGFP⁺ CD45.2) Ly-6C^{hi} monocytes into LPS-treated wild-type (CD45.1) mice showed higher accumulation of *miR-146a*^{-/-} cells at the site of inflammation within only 6 h (Fig 3e). The chemokine CCL2 controls Ly-6C^{hi} monocyte migration to inflamed sites (Shi and Pamer, 2011). Interestingly, *miR-146a*^{-/-} blood Ly-6C^{hi}—but not Ly-6C^{lo}—monocytes expressed the cognate receptor CCR2 at higher levels than their wild-type counterparts (Fig 3f, S3e) and migrated more efficiently toward a CCL2 gradient *in vitro* (Fig 3g).

These observations indicate that *miR-146a* controls the expansion of Ly-6C^{hi} monocytes during acute inflammatory conditions in part through elevated proliferation of Ly-6C^{hi} monocytes—predominantly in the bone marrow—and increased trafficking to inflamed sites.

Relb Is A *Mir-146a* Target

We aimed to find endogenous *miR-146a* target genes that contribute to altering the monocyte response. The screening approach, which compared the expression profiles of *miR-146a*-predicted target genes in Ly-6C^{hi} and Ly-6C^{lo} monocytes either at 2 h or 8 h after *Lm* challenge (Fig S4a and supplementary information), identified the transcription factor *Relb* (Fig 4a). Experimental evidence also indicates that *Relb* is a *miR-146a* target. First, ectopic *miR-146a* expression in resting Ly-6C^{hi} monocytes *in vivo* reduced *Relb* transcript levels (Fig 4b). Second, NIH-3T3 cells transfected with a luciferase reporter

plasmid expressing *Relb* 3' UTR (ENSMUST00000049912) containing a potential *miR-146a* binding sequence showed reduced luciferase activity upon *miR-146a* overexpression. The phenotype was rescued by mutating the seed sequence (Fig 4c). Third, immunofluorescence microscopy with a validated anti-Relb Ab (Fig S4b) showed efficient nuclear translocation of Relb protein at 30 min after LPS challenge in both wild-type and *miR-146a*^{-/-} Ly-6C^{hi} monocytes; however at 6 h cytoplasmic Relb levels were recovered more prominently in the *miR-146a*^{-/-} cells (Fig 4d). Fourth, flow cytometry analysis confirmed that Relb protein levels remained higher in *miR-146a*^{-/-} Ly-6C^{hi} monocytes upon LPS challenge (Fig 4e).

Modulation Of Relb Expression Affects The Ly-6C^{hi} Monocyte Response

To investigate whether modulation of Relb affects the monocyte response, we generated both Relb^{hi} EGFP^{hi} CD45.1 HSC (which expressed Relb from a cDNA sequence that could not be regulated by *miR-146a*) and control Relb^{norm} EGFP^{hi} CD45.2 HSC, which were adoptively transferred at a 1:1 ratio into LPS-treated CD45.1/2 recipient animals (Fig S4c). Relb overexpression did not alter HSC expansion (Fig S4d) but amplified the monocyte response *in vivo* (Fig 4f) and thus recapitulated the phenotype observed for *miR-146a*^{-/-} Ly-6C^{hi} monocytes.

We also injected LPS-treated CD45.1 mice either with *miR-146a*^{-/-} shRelb EGFP^{hi} HSC (which expressed a *miR30*-hairpin based shRNA to silence Relb to the levels found in challenged wild-type monocytes) or with *miR-146a*^{-/-} EGFP^{hi} HSC (which expressed a control EGFP vector) (Fig 4g, S4e). Relb silencing did not alter HSC expansion (Fig S4f) but decreased *miR-146a*^{-/-} Ly-6C^{hi} monocyte responses *in vivo* (Fig 4h). These data indicate that *miR-146a* can control Ly-6C^{hi} monocyte fate in response to acute inflammatory challenge via *Relb* targeting.

miR-146a And Relb Expression In Human Monocytes

The human Relb 3'UTR contains a binding site for the alternative processing isoform *miR-146a-3p* (*miR-146a**) instead of the "canonical" *miR-146a-5p* isoform (*miR-146a*) (Transcript ENST00000221452, Fig S4g). *miR-146a*, and most notably *miR-146a**, were detected at higher levels in human CD16⁺(CD14^{lo}) monocytes than in their CD14⁺(CD16⁻) counterparts *ex vivo* (Fig 4i, S4h,i), and were selectively induced in CD14⁺ (CD16⁻) monocytes 6 h post LPS challenge (Fig 4j and S4j,k). *miR-146a** was also detected in mouse monocytes (Fig S4l). These data are in line with previous findings that CD14^{lo}CD16⁺ monocytes resemble Ly6C^{lo} cells and respond less well to LPS in comparison to CD14⁺CD16⁺ and CD14⁺CD16⁻ monocytes, which resemble mouse Ly-6C^{hi} monocytes (Cros et al., 2010). Furthermore, human CD14⁺ monocytes challenged with LPS decreased *Relb* mRNA levels (Fig 4k), although treatment with a LNA to suppress *miR-146a** induction (Fig S4m) was sufficient to prevent Relb downregulation (Fig 4k, l).

DISCUSSION

This study provides functional evidence that *miR-146a* and Relb differentially regulate monocyte subsets. Following inflammatory challenge, modulation of *miR-146a* expression tunes the amplitude of the Ly-6C^{hi}—but not the Ly-6C^{lo}—monocyte response: premature *miR-146a* induction aborts Ly-6C^{hi} cell amplification whereas lack of *miR-146a* induction leads to expansion and increased recruitment of these cells. *miR-146a* in monocytes targets Relb, which expression levels tune the amplitude of Ly-6C^{hi} monocyte responses.

Recent work has identified *miR-146a* as a negative regulator of the canonical NF-κB inflammatory cascade by targeting Traf6 and Irak1/2 (O'connell et al., 2010) and as a tumor

suppressor gene by decreasing transcription of NF- κ B-targeted genes (Boldin et al., 2011; Zhao et al., 2011). The present study extends the role of *miR-146a* to the control of Relb, which is mostly implicated in the non-canonical NF- κ B pathway (Vallabhapurapu and Karin, 2009). Relb has sizable effects on mononuclear phagocytes as it controls dendritic cell development in humans (Platzer et al., 2004) and mice (Burkly et al., 1995; Cejas et al., 2005; Wu et al., 1998), and the generation of monocyte-derived osteoclasts (Vaira et al., 2008). In accordance with the present study, the non-canonical NF- κ B pathway activator CD40L also controls Ly-6C^{hi} monocyte expansion (Lutgens et al., 2010). Of note, *miR-146a* can regulate proinflammatory gene expression by controlling RelB-dependent reversible chromatin remodeling (El Gazzar et al., 2011).

Ly-6C^{lo} monocytes constitutively express *miR-146a* in accordance with their non-inflammatory properties (Nahrendorf et al., 2007; Auffray et al., 2009). Nevertheless, *miR-146a*^{-/-} Ly-6C^{lo} cells did not mount an inflammatory response that was notably higher than their wild-type counterparts. It is possible that *miR-146a* does not play a significant role in Ly-6C^{lo} cells; yet, other regulatory mechanisms may keep Ly-6C^{lo} cells in check in absence of *miR-146a*. The study of Ly-6C^{lo} cells that bear defects in several candidate factors (e.g., *miR-146a* and other miRNAs) may serve to address this question. Either way, the present findings indicate that selective targeting of the *miR-146a* pathway should control Ly-6C^{hi} monocyte responses while preserving Ly-6C^{lo} cells.

Previous work has identified that *miR-146a*^{-/-} macrophages produce higher levels of inflammatory cytokines than their wild-type counterparts (Boldin et al., 2011); however, we could not recapitulate these findings in *miR-146a*^{-/-} monocytes. Challenged *miR-146a*^{-/-} and wild-type Ly-6C^{hi} monocytes may produce the same amount of cytokines on a per-cell basis because *miR-146a* up-regulation is induced after the initial burst of inflammatory cytokine production (4–24 h vs 0–8 h, respectively). Yet, *miR-146a*^{-/-} Ly-6C^{hi} monocytes will contribute more cytokine production at target sites not only because more of these cells are recruited but also because they can give rise locally to *miR-146a*^{-/-} macrophages, which exhibit heightened inflammatory functions.

The findings presented here place *miR-146a* and Relb as key regulators of monocyte subset population dynamics. *miR-146a* and Relb preferentially control Ly-6C^{hi} monocytes, which are cells that selectively expand in many chronic inflammatory conditions. Targeting of *miR-146a* or Relb may serve to suppress adverse inflammatory Ly-6C^{hi} monocyte responses while sparing Ly-6C^{lo} monocyte activity.

EXPERIMENTAL PROCEDURES

Mouse and human samples

The studies used 6–12 wk old mice. The institutional subcommittee on research animal care at Massachusetts General Hospital approved the animal studies. Human blood was obtained from healthy volunteers and collected in heparinized collection tubes in accordance to a protocol approved by the Committee on microbiological safety at Harvard Medical School.

Monoclonal Antibodies (Mabs), Flow Cytometry And Cell Sorting

Cell staining and cell sorting was performed as described in supplemental methods.

Gene expression arrays and analysis

Gene expression studies were performed in accordance to MIAME guidelines and are described in supplemental methods.

In Vivo Challenges

LPS from *Escherichia coli* (serotype O55:B5, Sigma) was given at 0.4 mg/kg in PBS daily i.p. for 4 d (or 7 d when indicated). *Lm* bacteria (strain EGDe, ATCC) were expanded in Brain Heart Broth (Fluka) and given i.v. at 3×10^3 CFU. Thioglycollate was given i.p. as a 4% solution in 1 ml RMPI.

In Vitro Challenges

$5\text{--}6 \times 10^4$ isolated cells were plated in complete medium (RPMI, Cellgro Mediatech Inc.), 10% FCS (Stem Cell Technologies), 100 U/ml Pen/strep, 2 mM L-Glu (both Cellgro Mediatech Inc.) in round bottom 96-well plates. Stimulations included LPS (100 ng/ml, Sigma), rmTNF α (50 ng/ml, Peprotech) and HKLM (5×10^8 /ml, heat killed *Lm*, Invivo Gen). Luminex cytokine assays (R&D Biosciences) were analyzed on a Luminex FlexMap 3D (Agilent) instrument.

Statistical Analysis

Results were analyzed with Prism 4.0 (GraphPad). P-values were determined using Student's *t* tests. A p-value < 0.05 was taken as statistically significant and higher significance is indicated in the figure legends. All graphs show mean \pm SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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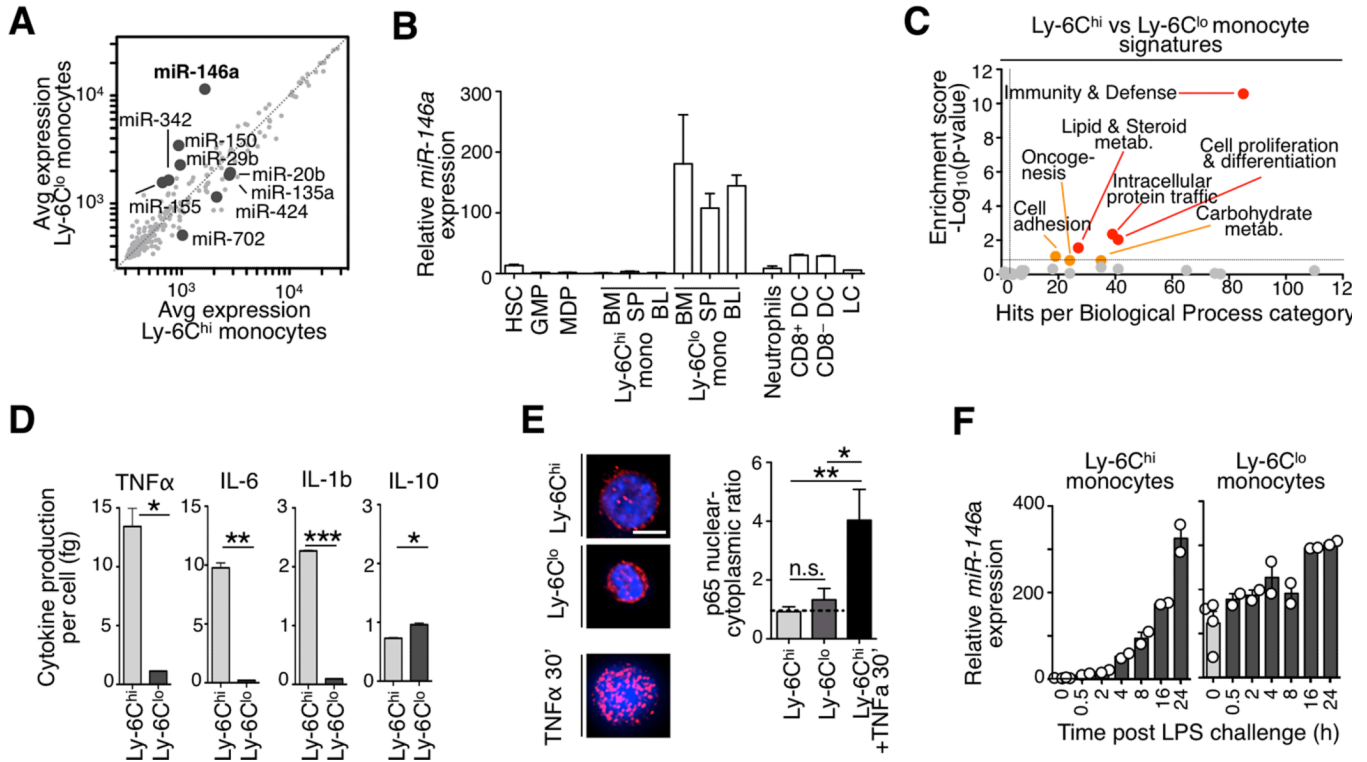


Figure 1. Mouse monocyte subsets show distinct miR-146a and inflammatory profiles
a) Microarray analysis of miRNA expression in Ly-6C^{hi} vs. Ly-6C^{lo} splenic monocytes. Genes with >2-fold change among subsets and p<0.05 are highlighted (n=4 biological replicates).
b) Relative *miR-146a* expression in various hematopoietic cell types. Expression is relative to splenic Ly-6C^{hi} monocytes (n=3 animals for all cell populations except for spleen monocytes, n=7).
c) Analysis of differentially expressed genes in Ly-6C^{hi} vs. Ly-6C^{lo} blood monocytes using the Panther database of biological functional categories.
d) Quantification of TNF α , IL-6, IL-1b and IL-10 production by splenic monocytes 8 h after LPS challenge (n=3–4).
e) p65 immunofluorescence staining in sorted monocyte subsets. Ly-6C^{hi} monocytes stimulated for 30' with TNF α prior to fixation served as a positive control for effective nuclear translocation. Scale bar: 10 μ m
f) Time-course analysis of *miR-146a* levels after LPS challenge. Expression is relative to Ly-6C^{hi} monocytes at time 0 h (n=2–4).
 Data are presented as mean \pm SEM. (* p<0.05, ** p<0.01, *** p<0.001, Student's t-test).

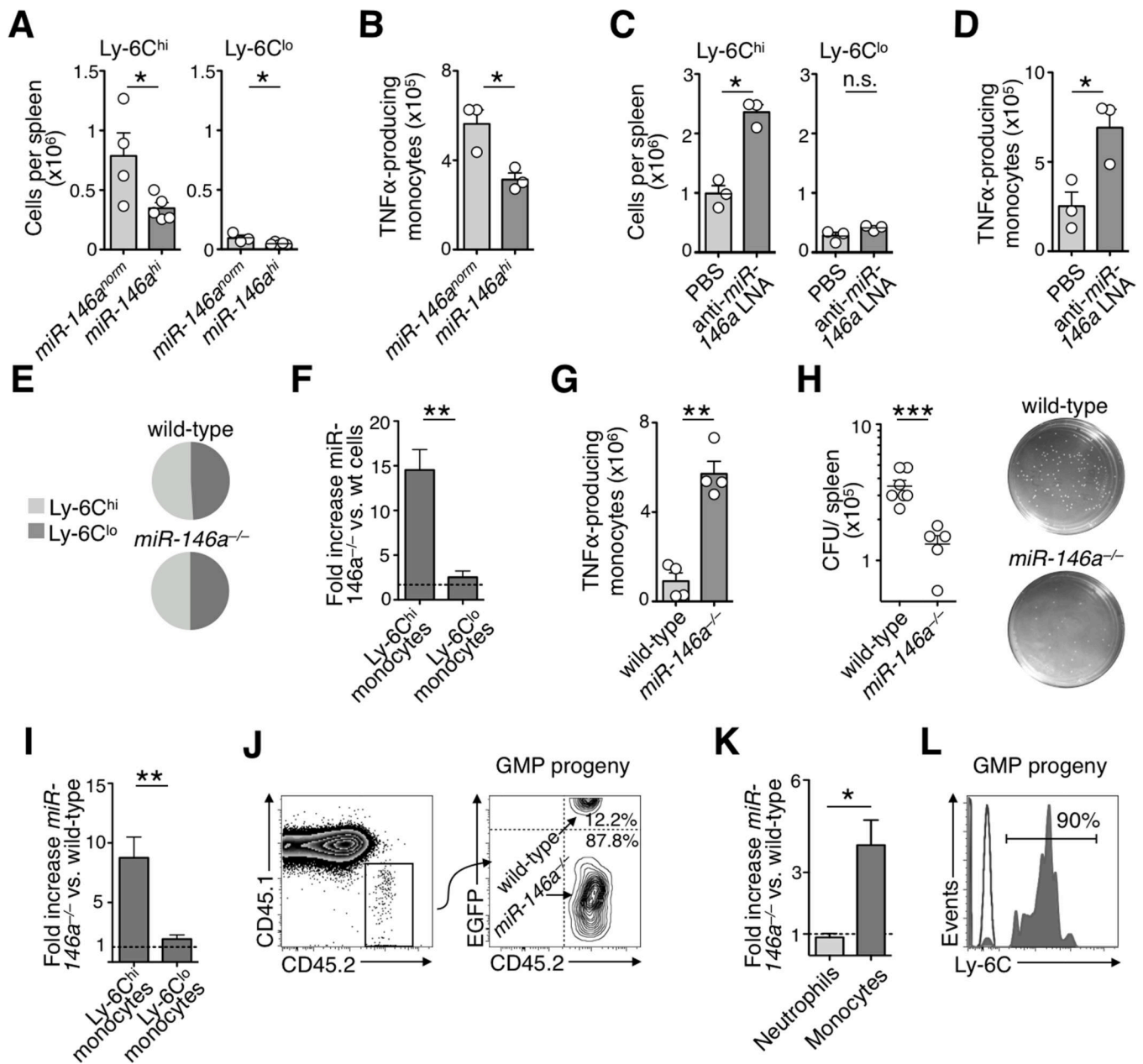


Figure 2. Effects of ectopic miR-146a expression or miR-146a silencing on the Ly-6C^{hi} monocyte response

a) Monocyte counts in spleen of mice reconstituted with *miR-146a*-expressing (*miR-146a^{hi}*) or control (*miR-146a^{norm}*) vector and challenged with *Lm* (n=3–5 from 2 independent experiments).

b) Number of TNFα-producing monocytes after *ex vivo* re-stimulation (same mice as in a).

c) Monocyte counts in spleen of mice that received either anti-*miR-146a* LNA or PBS and were challenged with live *Lm* for 24 h (n=3).

d) Number of TNFα-producing monocytes after LNA treatment (same mice as in c).

e) Ly-6C^{hi}/Ly-6C^{lo} monocyte ratios in wild-type (1.03±0.03) and *miR-146a^{-/-}* (1.05±0.04) mice in steady-state (mean±SEM).

- f)** Fold increase of Ly-6C^{hi} and Ly-6C^{lo} *miR-146a*^{-/-} monocytes in peritoneal cavity compared to their wild-type counterparts in bone marrow chimeras 4 d after peritoneal LPS injection (n=4 from 2 independent experiments).
- g)** Number of TNF α -producing wild-type or *miR-146a*^{-/-} monocytes (same mice as in f).
- h)** Colony forming unit (CFU) assay to quantify viable *Lm* from the spleen of wild-type (n=6) and *miR-146a*^{-/-} (n=5) mice 24 h after infection.
- i)** Fold increase of Ly-6C^{hi} and Ly-6C^{lo} *miR-146a*^{-/-} monocytes compared to their wild-type counterparts in bone marrow chimeras 24 h after peritoneal thioglycollate injection (n=3).
- j)** Tracking of EGFP⁺ wild-type and EGFP⁻ *miR-146a*^{-/-} CD45.2 GMP progeny. Right dot plot shows CD45.2 Lin⁻ CD11b⁺ CD115⁺ donor GMP-derived monocyte (representative of 4 independent experiments).
- k)** Fold increase of *miR-146a*^{-/-} neutrophils and monocytes (GMP donor-derived) compared to their wild-type counterparts in the peritoneal cavity 4 d after LPS challenge (n=4 from 2 independent experiments).
- l)** Ly-6C expression by donor GMP-derived monocytes (same mice as in k).
- Data are presented as mean \pm SEM. (* p<0.05, ** p<0.01, *** p<0.001, Student's t-test).

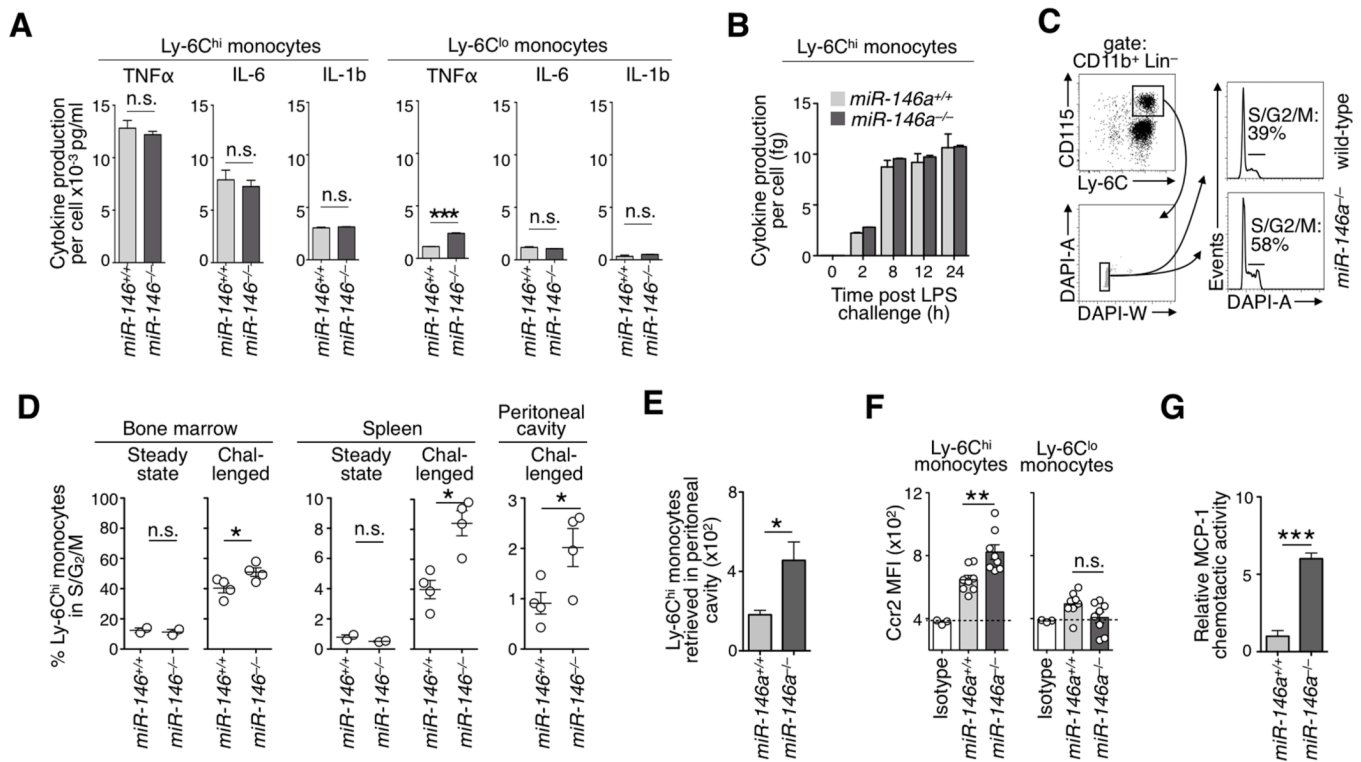


Figure 3. Altered proliferation and trafficking of *miR-146a*^{-/-} Ly-6C^{hi} monocytes during inflammation

a) Cytokine production of sorted wild-type and *miR-146a*^{-/-} monocyte subsets after *in vitro* LPS stimulation. Cytokine production is expressed per cell (n=2-3).

b) Time course TNF α production by Ly-6C^{hi} monocytes upon LPS challenge *in vitro* (n=2).

c) Gating strategy for DAPI staining of bone marrow Ly-6C^{hi} monocytes. Histograms show data for LPS stimulated wild-type or *miR-146a*^{-/-} animals.

d) Quantification of cell cycle status in wild-type and *miR-146a*^{-/-} animals in steady state (n=2) or after 4 consecutive days of LPS injection i.p. (n=4) in bone marrow, spleen and peritoneal cavity.

e) Number of donor wild-type and *miR-146a*^{-/-} EGFP⁺ CD45.2 Ly-6C^{hi} monocytes retrieved in the peritoneal cavity 6 h after transfer into LPS-treated CD45.1 recipient mice (n=4).

f) Flow cytometry-based cell surface CCR2 mean-fluorescence intensity (MFI) in wild-type and *miR-146a*^{-/-} blood monocytes (n=8).

g) *In vitro* chemotactic activity of wild-type (EGFP⁺) and *miR-146a*^{-/-} (EGFP⁻) Ly-6C^{hi} monocytes toward MCP-1 (n=4).

Data are presented as mean \pm SEM. (* p<0.05, ** p<0.01, *** p<0.001, Student's t-test).

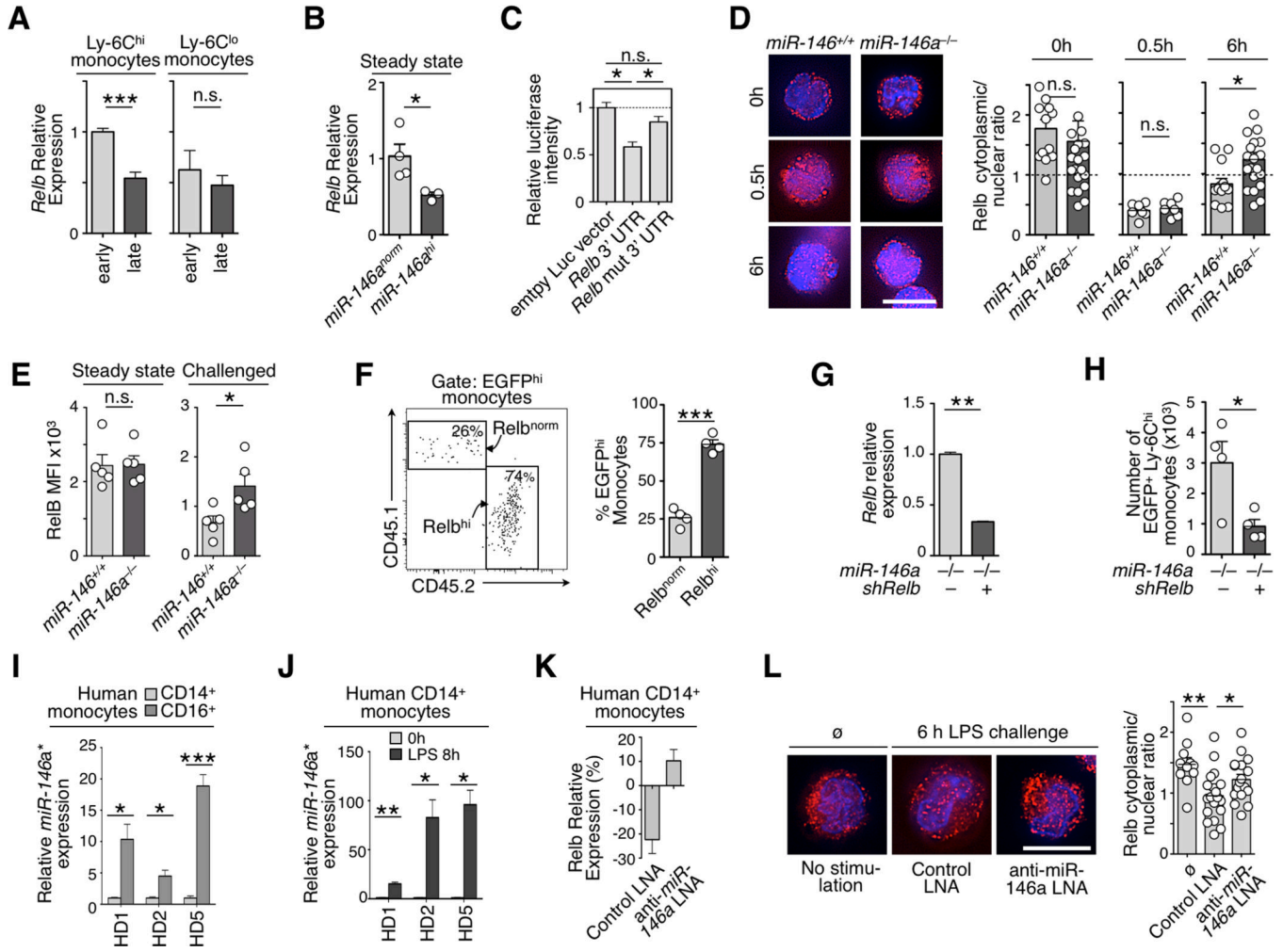


Figure 4. Relb is a miR-146a target in monocytes

a) Relative *Relb* mRNA expression in monocytes subsets recruited to the peritoneal cavity at 2 h (early) and 8 h (late) post inflammatory challenge. Data are normalized to Ly-6C^{hi} monocytes at 2 h (n=3).

b) Relative *Relb* mRNA expression in steady-state Ly-6C^{hi} monocytes that over-express *miR-146a* (*miR-146a^{hi}*) or not (*miR-146a^{norm}*) (n=3).

c) Luciferase reporter assay for *miR-146a*-dependent regulation of *Relb* 3' UTR. Luciferase activity was measured in NIH3T3 cells transfected with control empty vector, *Relb* 3' UTR or a mutated version of the *Relb* 3' UTR.

d) Immunofluorescence staining of Relb protein in wild-type or *miR-146a*^{-/-} Ly-6C^{hi} monocytes at 0, 0.5 and 6 h after LPS challenge. (Images are representative of n=7–21 cells analyzed per condition). Scale bar 10µm. Quantification shows cytoplasmic vs. nuclear fluorescence signal ratios.

e) Flow cytometry evaluation of intracellular Relb protein expression levels in wild-type or *miR-146a*^{-/-} Ly-6C^{hi} blood monocytes in steady-state or 6 h after LPS challenge (n=5).

f) Tracking of EGFP⁺ monocytes reconstituted with a *Relb*-overexpressing (*Relb^{hi}*) or control (*Relb^{norm}*) vector in the peritoneal cavity 7 d after LPS challenge. Gating shows a representative result of the two competing monocyte populations (n=4 animals per group).

g) shRNA-mediated knockdown in EGFP⁺ cells measured by real-time PCR in *miR-146a*^{-/-} Ly-6C^{hi} monocytes (n=3).

- h)** Accumulation in the peritoneal cavity of EGFP⁺ *miR-146a*^{-/-} monocytes transfected either with a *shRelb* or control construct 7 d after transfer into LPS challenged recipients.
- i)** Differential *miR-146a*^{*} expression in CD14⁺ (CD16⁻) and CD16⁺ (CD14⁻) monocytes from 3 healthy donors (HD) *ex vivo*.
- j)** Induction of *miR-146a*^{*} in CD14⁺ (CD16⁻) monocytes 6 h post LPS challenge (same donors as in i; n=3 technical replicates per group).
- k)** Percent change of Relb mRNA expression in CD14⁺ (CD16⁻) monocytes of HD5 6 h post LPS challenge in presence of a scrambled or *anti-miR-146a* LNA (n=3).
- l)** Immunofluorescence staining of Relb protein in CD14⁺ (CD16⁻) monocytes analyzed *ex vivo* (∅) or treated as in k. (Images are representative of n=11–19 cells analyzed per condition). Scale bar 10μm.
- Quantification shows cytoplasmic vs. nuclear fluorescence signal ratios.
Data are presented as mean±SEM. (* p<0.05, ** p<0.001, *** p<0.0001, Student's t-test).