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-6Clo) and human (CD14hi/CD14loCD16+) monocyte subsets. The single miRNA controlled the amplitude of the Ly-6Chi monocyte response during inflammatory challenge whereas it did not affect Ly-6Clo cells. miR-146a-mediated regulation was cell-intrinsic and depended on Relb, a member of the noncanonical NF-κB/Rel family, which we identified as a direct miR-146a target. These observations not only provide mechanistic insights into the molecular events that regulate responses mediated by committed monocyte precursor populations but also identify targets for manipulating Ly-6Chi monocyte responses while sparing Ly-6Clo monocyte activity.

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: 1), Ly-6C hi (Gr-1+CCR2+ CX3CR1lo) cells respond to proinflammatory 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 2), Ly-6C lo (Gr-1–CCR2–CX3CR1hi) 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-6Chi monocytes share phenotypic and functional features with human CD14hi cells, whereas mouse Ly-6Clo monocytes resemble human CD14loCD16+ 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 proinflammatory 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 posttranscriptional 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 (Figure 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 nine miRNAs, which were highly expressed either in Ly-6Chi (miR-20b, -135a, -424, -702) or in Ly-6Clo monocytes (miR-146a, -150, -155, -342, -29b) (Figure 1A).

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

miRNAs and their respective target genes are often mutually exclusively expressed in a given tissue (Farh et al., 2005). In keeping with previous observations that miR-146a suppresses NF-κB-dependent inflammatory pathways (Taganov et al., 2006), we confirmed with two independent genome-wide profiling methods that blood miR-146ahi Ly-6Clo monocytes showed increased inflammatory signatures (Swirski et al., 2009) and expressed components of the NF-κB signaling cascade at higher levels than their miR-146ahi Ly-6Clo counterparts (Figures 1C and S1B). Also, splenic and blood miR-146ahi Ly-6Clo monocytes stimulated with lipopolysaccharide (LPS) produced more TNFα, IL-6, and IL-1β inflammatory cytokines than miR-146ahi Ly-6Clo cells (Figures 1D and S1C).

The elevated miR-146a expression in Ly-6Clo cells and the inflammatory profile of Ly-6Clo cells reported above were likely not due to a premature activation artifact induced by the isolation procedure because IκBα protein levels were similar in both monocyte subsets ex vivo (Figure S1D) and NF-κB subunit p65 only became detectable in the nucleus of Ly-6Clo cells upon in vitro challenge (Figure 1E). The cause for constitutive (NF-κB...
-independent) miR-146a expression in Ly-6C\textsuperscript{hi} 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-\textalpha. miR-146a was induced only in Ly-6C\textsuperscript{hi} monocytes, in response to all stimuli, and reached levels matching those in Ly-6C\textsuperscript{lo} cells (Figure S1E).

In vivo LPS challenge studies confirmed the in vitro findings (Figure S1F), miR-146a expression in Ly-6C\textsuperscript{lo} cells increased within 4 hr after LPS challenge and reached levels equivalent to those found in Ly-6C\textsuperscript{lo} cells after 16 hr (Figure 1F). Thus, miR-146a expression is constitutive in Ly-6C\textsuperscript{lo} monocytes and inducible in Ly-6C\textsuperscript{hi} monocytes. LPS-stimulated Ly-6C\textsuperscript{lo} monocytes were CD11c\textsuperscript{+} MHC II\textsuperscript{high} (Ly-6C\textsuperscript{lo}) and thus distinct from Ly-6C\textsuperscript{lo} monocytes (Figure 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 downregulated experimentally. To constitutively overexpress miR-146a we reconstituted mice with HSC transduced to coexpress EGFP and miR-146a (Figures S2A and S2B). miR-146a overexpression did not alter monocyte numbers or subset ratios in steady state (Figure S2C); however, upon *Listeria monocytogenes* (Lm) infection (Shi and Pamer, 2011), it prevented the unfolding of a full-fledged TNF-\textalpha-producing Ly-6C\textsuperscript{hi} monocyte response (Figures 2A and 2B).

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 (Figures S2D and S2E). LNA treatment did not alter monocyte subset ratios in steady state (Figure S2F) but it increased the number of TNF-\textalpha-producing Ly-6C\textsuperscript{lo} monocytes at Lm infected sites (Figures 2C and 2D).

The second approach to suppress miR-146a expression used recently described mice with targeted deletion of the miR-146a gene (Boldin et al., 2011) (Figure S2G). miR-146a/\textsuperscript{–}\textsuperscript{–} mice contained both monocyte subsets thus Ly-6C\textsuperscript{hi} \rightarrow Ly-6C\textsuperscript{lo} monocyte conversion should not require miR-146a. Also, miR-146a knockdown did neither alter the ratio (Figure 2E) nor the phenotype (Figure S2H) of monocyte subsets in 8-week-old mice. To compare miR-146a/\textsuperscript{–}\textsuperscript{–} 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/\textsuperscript{–}\textsuperscript{–} (CD45.2) and wild-type (EGFP\textsuperscript{+} CD45.2) cells (Figure S2I). The absence of miR-146a strongly amplified TNF-\textalpha-producing Ly-6C\textsuperscript{hi} peritoneal monocytes in response to LPS challenge (Figures 2F and 2G). Ly-6C\textsuperscript{lo} monocytes mediate immune defense in early phase of Lm infection (Shi and Pamer, 2011). Accordingly, Lm-infected miR-146a/\textsuperscript{–}\textsuperscript{–} mice contained reduced numbers of viable Lm 24 hr postinfection when compared to Lm-infected wild-type mice (Figure 2H). Amplification of the Ly-6C\textsuperscript{lo} monocyte response in absence of miR-146a was confirmed in a model of sterile peritonitis induced by thioglycollate (Figure 2I).

**Cell-Intrinsic Mir-146a-Mediated Regulation of the Ly-6C\textsuperscript{hi} Monocyte Response**

The experiments above involved indiscriminate alteration of miR-146a expression in all hematopoietic cells. We reasoned that injection of miR-146a/\textsuperscript{–}\textsuperscript{–} GMP into wild-type mice would permit to track miR-146a/\textsuperscript{–}\textsuperscript{–} monocytes in a wild-type environment because miR-146a is only upregulated upon progenitor cell maturation. Specifically, we coadministered equal numbers of miR-146a/\textsuperscript{–}\textsuperscript{–} (CD45.2 EGFP\textsuperscript{+}) and wild-type (CD45.2 EGFP\textsuperscript{+}) GMP into nonirradiated wild-type (CD45.1) mice, which were subsequently challenged with LPS intraperitoneally (i.p.) (Figure S2J). Wild-type and miR-146a/\textsuperscript{–}\textsuperscript{–} hematopoietic progenitor cells show comparable clonogenic potential (Boldin et al., 2011; Figure S2K) and the transferred cells' progeny contained monocytes and neutrophils, as expected. miR-146a/\textsuperscript{–}\textsuperscript{–} monocytes recruited to the peritoneal cavity outnumbered their wild-type counterparts (Figures 2J and 2K) and were Ly-6C\textsuperscript{hi} (Figure 2L); in marked contrast, miR-146a/\textsuperscript{–}\textsuperscript{–} neutrophils—that do not upregulate miR-146a in vivo—mounted a response that was similar to their wild-type counterparts (Figure 2K). Thus miR-146a should regulate Ly-6C\textsuperscript{lo} monocytes at least in part in a cell-intrinsic manner.

**miR-146a Controls Ly-6C\textsuperscript{lo} 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 (Figure S3A), the absence of miR-146a did not detectably alter inflammatory cytokine production by Ly-6C\textsuperscript{lo} and Ly-6C\textsuperscript{hi} monocytes on a per-cell basis (Figures 3A and 3B). However, LPS challenge increased the percentage of miR-146a/\textsuperscript{–}\textsuperscript{–} Ly-6C\textsuperscript{lo} monocytes undergoing cell division in bone marrow (Figures 3C and 3D) and to a lower extent in the spleen and peritoneal cavity (Figure 3D). The absence of miR-146a did not affect proliferation of Ly-6C\textsuperscript{lo} monocytes (Figure S3B). Cocultures of miR-146a/\textsuperscript{–}\textsuperscript{–} and wild-type cells also indicated a proliferative advantage for bone marrow miR-146a/\textsuperscript{–}\textsuperscript{–} Ly-6C\textsuperscript{lo} monocytes (Figures S3C and S3D).

In addition, coinjection of bone marrow miR-146a/\textsuperscript{–}\textsuperscript{–} (EGFP\textsuperscript{+} CD45.2) and control (EGFP\textsuperscript{+} CD45.2) Ly-6C\textsuperscript{lo} monocytes into LPS-treated wild-type (CD45.1) mice showed higher accumulation of miR-146a/\textsuperscript{–}\textsuperscript{–} cells at the site of inflammation within only 6 hr (Figure 3E). The chemokine CCL2 controls Ly-6C\textsuperscript{lo} monocyte migration to inflamed sites (Shi and Pamer, 2011). Interestingly, miR-146a/\textsuperscript{–}\textsuperscript{–} blood Ly-6C\textsuperscript{lo}—but not Ly-6C\textsuperscript{lo}—monocytes expressed the cognate receptor CCR2 at higher levels than their wild-type counterparts (Figures 3F and S3E) and migrated more efficiently toward a CCL2 gradient in vitro (Figure 3G).

These observations indicate that miR-146a controls the expansion of Ly-6C\textsuperscript{lo} monocytes during acute inflammatory conditions in part through elevated proliferation of Ly-6C\textsuperscript{lo} 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\textsuperscript{lo} and Ly-6C\textsuperscript{lo} monocytes either
at 2 hr or 8 hr after *Lm* challenge (Figure S4A and Supplemental Information), identified the transcription factor Relb (Figure 4A). Experimental evidence also indicates that Relb is a *miR-146a* target. First, ectopic *miR-146a* expression in resting Ly-6Chi monocytes in vivo reduced *Relb* transcript levels (Figure 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 (Figure 4C). Third, immunofluorescence microscopy with a validated anti-Relb Ab (Figure S4B) showed efficient nuclear translocation of Relb protein at 30 min after LPS challenge in both wild-type and miR-146a−/− animals; however at 6 hr cytoplasmic Relb levels were recovered more prominently in the miR-146a−/− cells (Figure 4D). Fourth, flow cytometry analysis confirmed that Relb protein levels remained higher in miR-146a−/− Ly-6C+ monocytes upon LPS challenge (Figure 4E).

**Modulation of Relb Expression Affects the Ly-6CH Monocyte Response**

To investigate whether modulation of Relb affects the monocyte response, we generated both Relbhi EGFPhi CD45.1 HSC (that expressed Relb from a cDNA sequence that could not be regulated by miR-146a) and control Relbnorm EGFPhi CD45.2 HSC, which were adoptively transferred at a 1:1 ratio into LPS-treated CD45.1 recipient animals (Figure S4C). Relb overexpression did not alter HSC expansion (Figure S4D) but amplified the monocyte response in vivo (Figure 4F) and thus recapitulated the phenotype observed for miR-146a−/− Ly-6C+ monocytes.

We also injected LPS-treated CD45.1 mice either with miR-146a−/− shRelb EGFPhi HSC (that expressed a miR30-hairpin based shRNA to silence Relb to the levels found in challenged wild-type monocytes) or with miR-146a−/− EGFPhi HSC (that expressed a control EGFP vector) (Figures 4G and S4E). Relb silencing did not alter HSC expansion (Figure S4F) but decreased miR-146a−/− Ly-6C+ monocyte responses in vivo (Figure 4H). These data indicate that miR-146a can control Ly-6C+ 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 ENST000000221452, Figure S4G). miR-146a, and most notably miR-146a*, were detected at higher levels in human CD16+ CD14+ monocytes 6 hr post-LPS challenge (Figures 4J, S4J, and S4K). miR-146a* was also detected in mouse
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 hr (early) and 8 hr (late) postinflammatory challenge. Data are normalized to Ly-6C<sup>hi</sup> monocytes at 2 hr (n = 3).

(B) Relative Relb mRNA expression in steady-state Ly-6C<sup>hi</sup> monocytes that overexpress miR-146a (miR-146ahi) or not (miR-146anorm) (n = 3).

(C) Luciferase reporter assay for miR-146a–dependent regulation of Relb 3' UTR. Luciferase activity was measured in NIH 3T3 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<sup>−/−</sup> Ly-6C<sup>hi</sup> monocytes at 0, 0.5 and 6 hr after LPS challenge. (Images are representative of n = 7–21 cells analyzed per condition). Scale bar represents 10 µm. Quantification shows cytoplasmic versus nuclear fluorescence signal ratios.

(E) Flow cytometry evaluation of intracellular Relb protein expression levels in wild-type or miR-146a<sup>−/−</sup> Ly-6C<sup>hi</sup> blood monocytes in steady-state or 6 hr after LPS challenge (n = 5).

(F) Tracking of EGFP<sup>+</sup> monocytes reconstituted with a Relb-overexpressing (Relb<sup>hi</sup>) or control (Relb<sup>norm</sup>) vector in the peritoneal cavity 7 days 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<sup>+</sup> cells measured by real-time PCR in miR-146a<sup>−/−</sup> Ly-6C<sup>hi</sup> monocytes (n = 3).

(H) Accumulation in the peritoneal cavity of EGFP<sup>+</sup> miR-146a<sup>−/−</sup> monocytes transfected either with a shRelb or control construct 7 days after transfer into LPS challenged recipients.

(I) Differential miR-146a* expression in CD14<sup>+</sup>(CD16<sup>−</sup>) and CD16<sup>+</sup>(CD14<sup>−</sup>) monocytes from three healthy donors (HD) ex vivo.

(J) Induction of miR-146a* in CD14<sup>+</sup>(CD16<sup>−</sup>) monocytes 6 hr post-LPS challenge (same donors as in I; n = 3 technical replicates per group).

(K) Percent change of Relb mRNA expression in CD14<sup>+</sup>(CD16<sup>−</sup>) monocytes of HD5 6 hr post-LPS challenge in presence of a scrambled or anti-miR-146a LNA (n = 3).

(L) Immunofluorescence staining of Relb protein in CD14<sup>+</sup>(CD16<sup>−</sup>) monocytes analyzed ex vivo (a) or treated as in (K) Images are representative of n = 11–19 cells analyzed per condition. Scale bar represents 10 µm. Quantification shows cytoplasmic versus nuclear fluorescence signal ratios. Data are presented as mean ± SEM (*p < 0.05, **p < 0.001, ***p < 0.0001, Student’s t test).

See also Figure S4.
monocytes (Figure S4L). These data are in line with previous findings that CD14<sup>+</sup>CD16<sup>+</sup> monocytes resemble Ly6C<sup>+</sup> cells and respond less well to LPS in comparison to CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>+</sup> monocytes, which resemble mouse Ly-6C<sup>hi</sup> monocytes (Cros et al., 2010). Furthermore, human CD14<sup>+</sup> monocytes challenged with LPS decreased Relb mRNA levels (Figure 4K), although treatment with a LNA to suppress miR-146a* induction (Figure S4M) was sufficient to prevent Relb downregulation (Figures 4K and 4L).

**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<sup>hi</sup>—but not the Ly-6C<sup>lo</sup>—monocyte response: premature miR-146a induction aborts Ly-6C<sup>hi</sup> 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<sup>lo</sup> 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-κB-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 noncanonical NF-κB 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., 1998; 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 noncanonical NF-κB pathway activator CD40L also controls Ly-6C<sup>hi</sup> 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<sup>lo</sup> monocytes constitutively express miR-146a in accordance with their noninflammatory properties (Nahrendorf et al., 2007; Auffray et al., 2009). Nevertheless, miR-146a<sup>−/−</sup> Ly-6C<sup>lo</sup> 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<sup>lo</sup> cells; yet, other regulatory mechanisms may keep Ly-6C<sup>lo</sup> cells in check in absence of miR-146a. The study of Ly-6C<sup>lo</sup> 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<sup>hi</sup> monocyte responses while preserving Ly-6C<sup>lo</sup> cells.

Previous work has identified miR-146a<sup>−/−</sup> 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<sup>−/−</sup> monocytes. Challenged miR-146a<sup>−/−</sup> and wild-type Ly-6C<sup>hi</sup> monocytes may produce the same amount of cytokines on a per-cell basis because miR-146a upregulation is induced after the initial burst of inflammatory cytokine production (4–24 hr versus 0–8 hr, respectively). Yet, miR-146a<sup>−/−</sup> Ly-6C<sup>hi</sup> 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<sup>−/−</sup> 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<sup>hi</sup> 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<sup>hi</sup> monocyte responses while sparing Ly-6C<sup>lo</sup> monocyte activity.

**EXPERIMENTAL PROCEDURES**

**Mouse and Human Samples**

The studies used 6- to 12-week-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, Flow Cytometry, and Cell Sorting**

Cell staining and cell sorting was performed as described in Supplemental Experimental Procedures.

**Gene Expression Arrays and analysis**

Gene expression studies were performed in accordance to MIAME guidelines and are described in Supplemental Experimental Procedures.

**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 days (or 7 days when indicated). *Lm* bacteria (strain EGDe, ATCC) were expanded in Brain Heart Broth (Fluka) and given intravenously at 3 x 10<sup>8</sup> colony forming units. Thiglycollate was given i.p. as a 4% solution in 1 ml RMPI.

**In Vitro Challenges**

Isolated cells (5–6 x 10<sup>3</sup>) were plated in complete medium (RPMI, Cellgro Mediatech), 10% FCS (Stem Cell Technologies), 100 U/ml Pen/strep, and 2 mM L-Glu (both Cellgro Mediatech) in round bottom 96-well plates. Stimulations included LPS (100 ng/ml, Sigma), rmTNFα (50 ng/ml, Peprotech), and HKLM (5 x 10<sup>8</sup> heat-killed Lm/ml, Invivo Gen). Luminox cytokine assays (R&D Biosciences) were analyzed on a Luminox 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 ± SEM.

**ACCESSION NUMBERS**

The microarray data generated in this study have been deposited to the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under accession number GSE32392.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.celrep.2012.02.009.
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