

Retinoid X receptor suppresses a metastasis-promoting transcriptional program in myeloid cells via a ligand-insensitive mechanism

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Edited by Bert W. O'Malley, Baylor College of Medicine, Houston, TX, and approved August 18, 2017 (received for review January 18, 2017)

Retinoid X receptor (RXR) regulates several key functions in myeloid cells, including inflammatory responses, phagocytosis, chemokine secretion, and proangiogenic activity. Its importance, however, in tumor-associated myeloid cells is unknown. In this study, we demonstrate that deletion of RXR in myeloid cells enhances lung metastasis formation while not affecting primary tumor growth. We show that RXR deficiency leads to transcriptomic changes in the lung myeloid compartment characterized by increased expression of prometastatic genes, including important determinants of premetastatic niche formation. Accordingly, RXR-deficient myeloid cells are more efficient in promoting cancer cell migration and invasion. Our results suggest that the repressive activity of RXR on prometastatic genes is mediated primarily through direct DNA binding of the receptor along with nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) corepressors and is largely unresponsive to ligand activation. In addition, we found that expression and transcriptional activity of RXR α is down-modulated in peripheral blood mononuclear cells of patients with lung cancer, particularly in advanced and metastatic disease. Overall, our results identify RXR as a regulator in the myeloid cell-assisted metastatic process and establish lipid-sensing nuclear receptors in the microenvironmental regulation of tumor progression.

retinoid X receptor | metastasis | premetastatic niche | myeloid cell | NCoR

Retinoid X receptor (RXR) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors that bind various lipophilic hormones and lipid metabolites. Sensing the lipid milieu by RXR, in concert with other nuclear receptors, plays critical roles in physiology, including the regulation of organogenesis, lipid metabolism, and skin homeostasis (1). RXR is able to bind vitamin A derivatives 9-cis-retinoic acid and 9-cis-13,14-dihydroretinoic acid as well as fatty acids such as docosahexanoic acid and phytanic acid (1, 2). The receptor has three isoforms, RXR α , RXR β , and RXR γ , which appear to be functionally interchangeable in tissues in which more than one isoform is expressed (3). RXR has a unique integrative function in nuclear receptor signaling because of its ability to form homodimers as well as heterodimers with several other members of the superfamily, such as PPAR, LXR, RAR, and VDR. RXR and its heterodimers have a profound effect on the function of myeloid cells by linking cellular metabolism and immune function (4, 5). Specific activation of RXR has been shown to up-regulate chemokine expression, promote phagocytosis of apoptotic cells, and attenuate antiviral responses in myeloid cells (6–8). To further delineate the function of the receptor, we recently mapped the genomic binding sites of RXR in macrophages

and identified a network of RXR-bound enhancers that control angiogenic genes including *Vegfa*, *Hbegf*, *Lita*, and *Hipk2* (9). Activation of these enhancers by RXR induced a proangiogenic transcriptional program and phenotype (9). Interestingly, we found that half of the 5,200 RXR-occupied genomic sites were transcriptionally inactive in macrophages (9). This suggested that the receptor may also possess extensive regulatory functions in myeloid cells that are distinct from ligand recognition and activation. These observations provided a rationale to investigate whether RXR influences tumor progression through regulating the phenotype of recruited or tissue-resident myeloid cells.

Results

RXR Deletion in Myeloid Cells Promotes Metastasis to the Lung. In myeloid cells, RXR α shows the highest expression and RXR β is expressed at a lower level, while RXR γ is not expressed (5). Specific deletion of RXR α in myeloid cells was achieved by crossing mice bearing lox-P-targeted RXR α (RXR α ^{fl/fl}) with mice carrying the lysozyme-M Cre (LysM-Cre) recombinase transgene. To exclude the potential compensatory effects of RXR β in the absence of RXR α resulting from the functional

Significance

Metastasis formation from malignant tumors is the leading cause of cancer-related deaths. There is an increasing body of evidence indicating that immune cells in distant organs actively contribute to this process by establishing a tissue environment that is hospitable for cancer cells. In this study, we show that deletion of retinoid X receptor (RXR), a cellular sensor of vitamin A metabolites, specifically in the myeloid lineage of the immune system, leads to an enhanced metastasis rate. We also demonstrate that RXR inhibits the expression of a number of genes that encode proteins involved in the promotion of metastasis formation. Surprisingly, our results suggest that this activity of RXR is independent of the presence of its activators.

Author contributions: M.K., Z.C., I.S., M.M., and L.N. designed research; M.K., Z.C., P.B.-K., M.E., A.P., S.P., P.B., P.T., Z.K., and B.D. performed research; M.K., Z.C., G.N., P.B.-K., M.E., S.P., A.H., and Z.K. analyzed data; and M.K. and L.N. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The data reported in this paper have been deposited in the NCBI Sequence Read Archive database, <https://www.ncbi.nlm.nih.gov/sra> (accession no. SRP095164).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1700785114/-DCSupplemental.

redundancy of RXR isotypes (3, 10), we crossed LysM-Cre $\text{RXR}^{\text{fl/fl}}$ with $\text{RXR}^{\beta-/-}$ mice. Given the low fertility and viability of the resulting LysM-Cre $\text{RXR}^{\text{fl/fl}}$ $\text{RXR}^{\beta-/-}$ mice (11), we transplanted their bone marrow to syngeneic C57BL/6J mice. This way, we were able to generate age-matched groups of mice bearing LysM-Cre $\text{RXR}^{\text{fl/fl}}$ $\text{RXR}^{\beta-/-}$ and LysM-Cre $\text{RXR}^{\text{fl/fl}}$ $\text{RXR}^{\beta-/-}$ bone marrow (hereafter referred to as $\text{RXR}^{\text{fl/fl}}$ and $\text{RXR}^{\text{fl/fl}}$, respectively).

To investigate the effect of RXR α deletion in the myeloid infiltrate on tumor progression, we s.c. implanted $\text{RXR}^{\text{fl/fl}}$ and $\text{RXR}^{\text{fl/fl}}$ mice with Lewis lung carcinoma (LLC), a murine model of non-small cell lung carcinoma showing abundant infiltration of myeloid cells from early stages. Primary tumor growth remained unaltered (Fig. 1A), and the immune cell infiltration in $\text{RXR}^{\text{fl/fl}}$ mice was largely unaltered with a significantly lower abundance of M1-like $\text{CD11b}^+\text{Ly6G}^-\text{Ly6C}^-\text{MHC-II}^{\text{high}}$ macrophages (SI Appendix, Fig. S1). However, the frequency of M2-like $\text{MHC-II}^{\text{low}}$ macrophages and other immune cell populations was not affected by RXR α deficiency (SI Appendix, Fig. S1). Although primary tumor growth was not affected, we observed a higher frequency of animals with spontaneous lung metastases, as well as a higher number of metastatic nodules in the $\text{RXR}^{\text{fl/fl}}$ knock-outs compared with controls (Fig. 1B and C). Thus, we further investigated the role of myeloid cell-expressed RXR α in lung colonization by using an experimental metastasis model. Consistent with the results obtained in the LLC model, we found that B16-F10 melanoma cells formed a higher number of macroscopic and microscopic metastases in the lungs of $\text{RXR}^{\text{fl/fl}}$ mice after i.v. injection (Fig. 2A–C). $\text{RXR}^{\text{fl/fl}}$ lungs contained a considerably higher frequency of micrometastases with diameters less than 100 μm , demonstrating that extravasation followed by micrometastasis formation in the lung is more efficient in the presence of RXR α -deficient

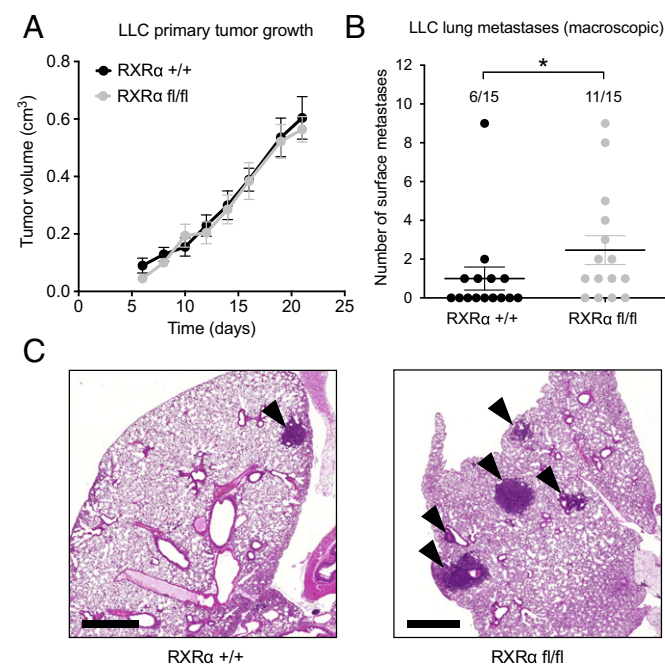


Fig. 1. RXR α deletion in myeloid cells enhances Lewis lung carcinoma metastasis. (A) Subcutaneous LLC tumor growth in mice with myeloid cell-specific deletion of RXR α ($\text{RXR}^{\text{fl/fl}}$) and control ($\text{RXR}^{\text{fl/fl}}$) mice ($n = 5$ –7/group). (B) Number of macroscopic lung metastases in $\text{RXR}^{\text{fl/fl}}$ and $\text{RXR}^{\text{fl/fl}}$ mice ($*P < 0.05$, Mann-Whitney test, $n = 15$ /group, data combine two independent experiments). (C) Representative images of hematoxylin and eosin-stained mid-coronal lung sections from $\text{RXR}^{\text{fl/fl}}$ and $\text{RXR}^{\text{fl/fl}}$ mice. Arrowheads indicate metastatic nodules. (Scale bar, 1 mm.) Graphs on A and B show mean \pm SEM.

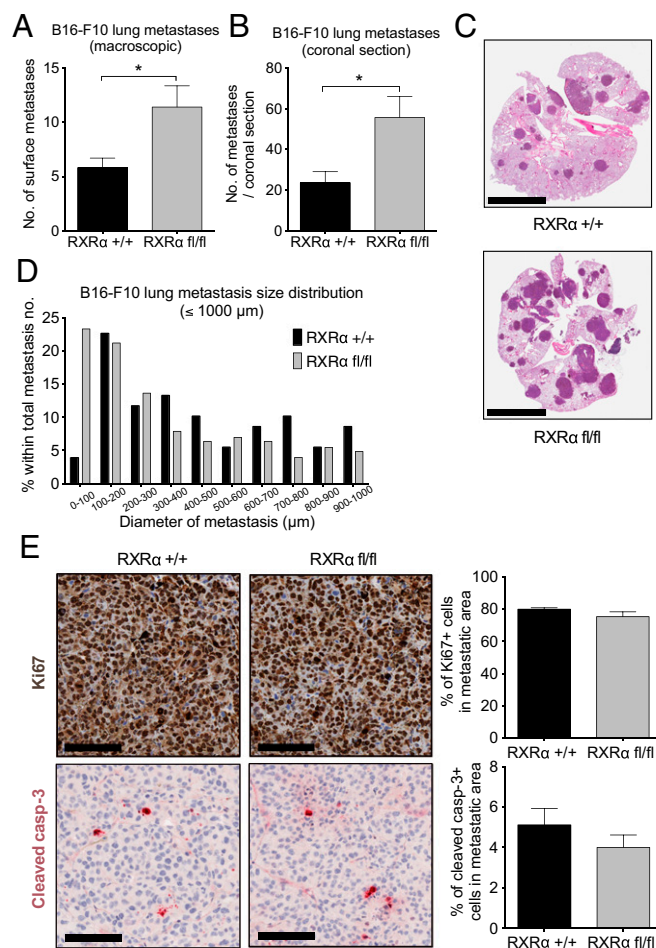


Fig. 2. RXR α deletion in myeloid cells results in enhanced lung colonization by B16-F10 melanoma cells. (A) Number of macroscopic lung metastases in $\text{RXR}^{\text{fl/fl}}$ and $\text{RXR}^{\text{fl/fl}}$ mice ($*P < 0.05$, unpaired Student's t test, $n = 10$ –13/group, data combine two independent experiments). (B) Number of microscopic lung metastases on midcoronal lung sections in $\text{RXR}^{\text{fl/fl}}$ and $\text{RXR}^{\text{fl/fl}}$ mice ($*P < 0.05$, unpaired Student's t test, $n = 8$ /group). (C) Representative images of hematoxylin and eosin-stained midcoronal lung sections from $\text{RXR}^{\text{fl/fl}}$ and $\text{RXR}^{\text{fl/fl}}$ mice. (Scale bar, 6 mm.) (D) Size distribution of B16-F10 melanoma lung metastases with a diameter $\leq 1,000 \mu\text{m}$ in $\text{RXR}^{\text{fl/fl}}$ and $\text{RXR}^{\text{fl/fl}}$ mice. Metastasis diameter data are pooled from eight lungs/group. (E) Representative images and quantification of Ki67 and cleaved caspase-3 stainings on midcoronal lung sections with B16-F10 melanoma metastases from $\text{RXR}^{\text{fl/fl}}$ and $\text{RXR}^{\text{fl/fl}}$ mice (Scale bar, 100 μm). ($P > 0.05$, unpaired Student's t test, $n = 5$ /group). Bar graphs on A, B, and E show mean \pm SEM.

myeloid cells (Fig. 2D). Since survival and proliferation of extravasated cancer cells at the distant organ is a critical early step of metastasis, we also examined the effect of myeloid cell RXR α on the apoptotic and mitotic rate of metastatic cancer cells. Staining of B16-F10 lung metastases with Ki67, a marker of cell proliferation, did not show major differences between $\text{RXR}^{\text{fl/fl}}$ and $\text{RXR}^{\text{fl/fl}}$ lungs (Fig. 2E). The apoptosis rate, as assessed by staining of cleaved caspase-3, was not significantly different in metastases of $\text{RXR}^{\text{fl/fl}}$ lungs either (Fig. 2E). This suggested that myeloid-specific RXR α deficiency does not affect the proliferation or survival of metastatic cancer cells in the lung, and the enhanced metastasis rate in $\text{RXR}^{\text{fl/fl}}$ mice originates principally from enhanced lung colonization. Altogether, these results indicate that deletion of RXR α in the myeloid compartment generates a lung microenvironment that is more permissive toward metastasis formation.

answer this, we used our previously published RNA-seq data from BMDMs treated with a highly selective, synthetic RXR agonist LG268 (9). The short-term ligand activation (30/60/120 min) enabled us to exclude secondary transcriptional changes after activation of the receptor. By examining the ligand response of RXR-repressed genes identified in pulmonary myeloid cells, we found that only a small fraction of them (14.1%) showed gene expression change in response to RXR activation (8/2, 29/3, 58/25 up-/down-regulated from 695 after 30, 60, 120 min of LG268 stimulation, respectively) (Fig. 4A). In the previously described prometastatic gene set, only *Hbegf*, *Pdgfa*, and *Pdgfb* showed significant up-regulation on ligand stimulus (Fig. 4B). This suggested that the majority of RXR-repressed genes in myeloid cells (including genes with prometastatic effect) cannot be modulated by RXR agonists. This was further supported by the observation that the cancer cell migration-promoting activity of iBMDMs was not altered after pretreatment with selective RXR agonist LG268 (Fig. 4C).

RXR Colocalizes with NCoR/SMRT Corepressors to Mediate Repression of Prometastatic Genes. Unliganded RXR homodimers and heterodimers bind the promoters and/or enhancers of their target genes in association with corepressors nuclear receptor corepressor (NCoR) or silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). Using our previously published RXR ChIP-seq dataset from BMDMs (9), we determined which genes show RXR binding in the proximity of their transcription start site (± 100 kb) at the genome-wide level. Intriguingly, gene ontology analysis of these RXR-bound genes revealed a significant overrepresentation of genes involved in the regulation of cell migration and activation of the immune response (SI Appendix, Fig. S3). This suggested that the major genomic targets of RXR binding (and possibly repression) in the steady state are genes that can be functionally linked to cell migration and inflammation. Next, we focused on genes that are repressed by RXR in pulmonary myeloid cells and examined whether RXR binding sites can be found in the vicinity of them. We could detect RXR binding sites within 100 kb of the transcription start sites in 66% of RXR-repressed genes. This suggested that direct DNA binding by the receptor accounts for its repressive effect by the majority of repressed genes. As

mentioned earlier, NCoR and SMRT corepressors are the most common interacting partners of RXR in mediating transcriptional repression. To determine whether RXR binding is associated with NCoR or SMRT binding at RXR-repressed genes, we combined our RXR ChIP-seq data with public NCoR and SMRT ChIP-seq datasets from BMDMs (17). Interestingly, 59% of RXR binding sites detected in the vicinity of RXR-repressed genes showed overlap with both NCoR and SMRT, while 12% and 7% showed overlap with only NCoR or only SMRT, respectively. In addition, NCoR and SMRT occupancy in the proximity of RXR-repressed genes was higher at genomic sites where RXR was also present, suggesting that the presence of RXR facilitates the binding of these corepressors (Fig. 5A). When we examined the genomic loci of the members of RXR-repressed prometastatic gene set identified in pulmonary myeloid cells, we detected overlapping RXR/NCoR/SMRT binding in the close proximity of 11 of the 18 genes (Fig. 5B). It has been shown previously that nuclear receptor-mediated transcriptional repression largely required either NCoR alone or both NCoR and SMRT, only a small fraction of genes showing repression specifically by SMRT (18). Accordingly, deletion of the dominant corepressor NCoR in iBMDMs resulted in the up-regulation of *Il1a*, *Il1b*, *Pdgfb*, *Sema4d*, *Igf1*, and *Ctss*, confirming the requirement of NCoR for the repression of these genes (Fig. 5C). Lack of de-repression by several RXR/NCoR/SMRT-bound genes in NCoR-deficient iBMDMs suggested the existence of compensatory mechanisms emerging in the absence of NCoR, as reported previously (19).

RXR Signaling Is Down-Modulated in Peripheral Blood Mononuclear Cells of Patients with Non-Small Cell Lung Cancer. Finally, we set out to determine whether any alterations in RXR signaling can be observed in patients with cancer. Deletion of RXR α in mice results in embryonic lethality caused by abnormal development of the heart (1). Similarly, DNA sequence data from the Exome Aggregation Consortium showed that loss-of-function mutations in genes coding any of the RXR isotypes are highly improbable, with a probability of loss-of-function intolerance of 94%, 100%, and 94% for RXR α , RXR β , and RXR γ , respectively (20). Thus, since loss-of-function mutations of RXR are predicted to be very rare, we aimed to investigate whether any changes in RXR expression can be observed in myeloid cells of patients with cancer that could potentially influence its repressive activity. As expression data from tissue-resident or metastasis-associated myeloid cells is difficult to obtain in the case of patients with cancer, we decided to analyze gene expression changes in peripheral blood mononuclear cells (PBMCs). PBMCs have been described to exhibit distinct gene expression changes in a number of malignancies (21–23). We chose to analyze the gene expression of PBMCs from patients with non-small cell lung cancer (NSCLC) and cancer-free control patients, using the largest publicly available dataset including samples from 137 patients with NSCLC and 91 cancer-free control patients (22). Intriguingly, we found that PBMCs from patients with NSCLC showed a significantly lower expression of RXR α (Fig. 6A). In addition, the mean expression level of RXR α exhibited decreasing kinetics during disease progression, showing the lowest expression in patients with advanced and metastatic disease (stage III–IV) (Fig. 6A). RXR β and RXR γ isotypes displayed lower average expression than RXR α in PBMCs and did not show any changes in patients with NSCLC (Fig. 6A). These data showed that RXR α , the dominant RXR isotype expressed in myeloid cells, was down-regulated in individuals with NSCLC, and its decreased expression was not compensated for by the up-regulation of other RXR isotypes. Next, we wanted to determine whether down-regulation of RXR α in patients with lung cancer is associated with its altered transcriptional activity. RXR's activity can be assessed by examining the regulation of the target genes of its

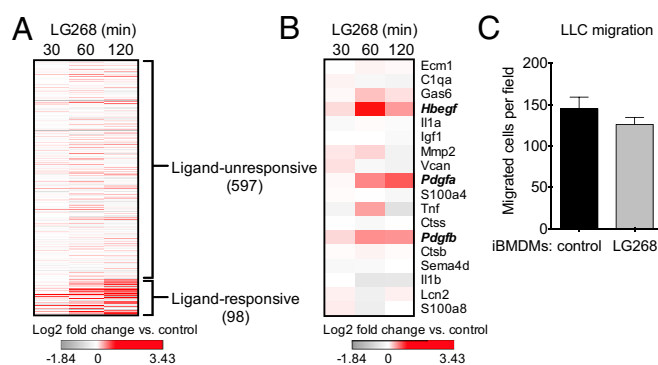


Fig. 4. RXR-mediated repression of prometastatic genes is predominantly ligand-insensitive. (A) Heat map showing expression changes in response to synthetic RXR agonist LG268 in BMDMs among RXR-repressed genes identified in pulmonary myeloid cells (adjusted $P < 0.01$, Wald test, $n = 2$ /time point). (B) Heat map showing expression changes in response to synthetic RXR agonist LG268 in BMDMs among selected RXR-repressed prometastatic genes identified in pulmonary myeloid cells. Genes with significant expression changes are shown in bold italics (adjusted $P < 0.01$, Wald test, $n = 2$ /group). (C) Number of migrated LLC cells in the presence of control or LG268-pretreated iBMDMs in Transwell migration assay ($P > 0.05$, unpaired Student's t test, $n = 3$ /group).

(15, 25, 26). Similarly, versican, an extracellular matrix proteoglycan with multifaceted roles in premetastatic niche formation, was also up-regulated in RXR $\alpha^{\text{fl/fl}}$ pulmonary myeloid cells (25, 26). In the Lewis lung carcinoma model, tumor-derived versican activated myeloid cells through TLR2 to induce an inflammatory microenvironment in the premetastatic lung (15). In contrast, in the MMTV-PyMT model of breast carcinoma, versican secreted by CD11b⁺Ly6C^{high} myeloid cells in premetastatic lungs stimulated mesenchymal to epithelial transition of cancer cells, leading to enhanced metastatic colonization (27). Another characteristic feature of premetastatic niche is tissue remodeling and degradation of the extracellular matrix that favors extravasation of circulating cancer cells (25, 26). Analogously, RXR $\alpha^{\text{fl/fl}}$ myeloid cells in the lung up-regulated several proteases capable of matrix remodeling, including MMP2, cathepsin B, and cathepsin S. As a consequence, RXR-deficient iBMDMs had an enhanced capacity to promote cancer cell invasion through an extracellular matrix layer.

Administration of selective RXR agonists have a well-established beneficial effect in solid tumors because of their capacity to induce differentiation and/or apoptosis of cancer cells (28). Nevertheless, the influence of RXR modulation both on the stromal compartment of primary tumor microenvironment and on premetastatic niches remains to be determined. Our findings suggest that the majority of genes differentially expressed in RXR α -deficient pulmonary myeloid cells do not respond to RXR activation. Therefore, it appears unlikely that endogenous or synthetic RXR agonists can alter the repressive activity of the receptor on prometastatic regulators. To therapeutically exploit RXR's ability to control the expression of several key regulators in metastasis, RXR-specific inverse agonists could be used.

Inverse agonists have the ability to stabilize the nuclear receptor–corepressor complex and enhance transcriptional repression (29). Although RXR-specific inverse agonists are not yet available, similar compounds have already been developed and used successfully in preclinical studies for several other nuclear receptors including RAR, LXR, and ROR (30–33). This class of RXR-specific ligands could enhance the repressive effect of RXR on prometastatic regulators, potentially rendering the lung microenvironment less permissive toward metastatic colonization.

Materials and Methods

Details of mice and bone marrow transplantation, cell lines and tumor models, histology, flow cytometry and cell sorting, RNA-seq, ChIP-seq analysis, immortalization of mouse bone marrow-derived macrophages, cancer cell migration and invasion assays, real-time quantitative PCR, microarray analysis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and statistical analysis are described in the *SI Appendix*. All experiments were carried out in accordance with the institutional ethical guidelines at the University of Debrecen (license no.: 21/2011/DE MAAB), following the European regulations.

ACKNOWLEDGMENTS. We thank Ms. T. Cseh, Mr. M. Peloquin, and Mr. J. Shelley for their excellent technical assistance. We thank Dr. M. Komatsu and members of the L.N. laboratory for discussions and comments on the manuscript. We are grateful to I. Schulman for providing LysM-Cre NCoR^{fl/fl} bone marrow. L.N. and P.T. are supported by “NR-NET” ITN PITN-GA-2013-606806 from the EU-FP7 PEOPLE-2013 program. B.D. is supported by an American Heart Association postdoctoral fellowship (17POST33660450). L.N. is supported by the Hungarian Scientific Research Fund (Grants OTKA K100196, K111941, and K116855) and by the Sanford Burnham Prebys Medical Discovery Institute.

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