Myeloid cell-derived inducible nitric oxide synthase suppresses M1 macrophage polarization

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Here we show that iNOS-deficient mice display enhanced classically activated M1 macrophage polarization without major effects on alternatively activated M2 macrophages. eNOS and nNOS mutant mice show comparable M1 macrophage polarization compared with wild-type control mice. Addition of N6-(1-iminoethyl)-L-lysine dihydrochloride, an iNOS inhibitor, significantly enhances M1 macrophage polarization while S-nitroso-N-acetylpenicillamine, a NO donor, suppresses M1 macrophage polarization. NO derived from iNOS mediates nitration of tyrosine residues in IRF5 protein, leading to the suppression of IRF5-targeted M1 macrophage signature gene activation. Computational analyses corroborate a circuit that fine-tunes the expression of IL-12 by iNOS in macrophages, potentially enabling versatile responses based on changing microenvironments. Finally, studies of an experimental model of endotoxin shock show that iNOS deficiency results in more severe inflammation with an enhanced M1 macrophage activation phenotype. These results suggest that NO derived from iNOS in activated macrophages suppresses M1 macrophage polarization.
Macrophages play an important role in inflammation and host defense against various pathogens and therefore are an essential component of innate immune responses. Activated macrophages are defined as classically activated or M1 type and alternatively activated or M2 type1–4. In responses to Toll-like receptor (TLR) ligands and IFN-γ, macrophages undergo classical M1 activation, whereas macrophages will polarize to alternative M2 activation on stimulation with IL-4 and IL-13 (refs 5,6). The M1 phenotype is characterized by the induction of proinflammatory mediators such as TNF-α, IL-6 and iNOS5–7. M1 cells promote Th1 and Th17 immune responses and contribute to a strong microbicidal and tumoricidal activity. In contrast, M2 macrophages are characterized by reduced responsiveness to TLR ligands and IFN-γ, resulting in the induction of low level of proinflammatory mediators and in the upregulation of arginase 1 (Arg1) and IL-10 (refs 2,6). Although the molecular mechanisms that regulate M1 and M2 macrophage polarization are not fully understood, it appears that IRF5 is a key transcription factor for M1 macrophages while IRF4 is important for M2 macrophages2,8. Increasing evidence suggests that M1 cells are involved in the pathogenesis of various autoimmune inflammatory diseases, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases and asthma9,10. Thus, a more complete understanding of the molecular mechanisms involved in the regulation of M1 innate immune responses should provide insights into the pathogenesis and treatment of these and possibly other inflammatory diseases. Although the activation programme for M1 macrophage differentiation has been well established, the intrinsic suppressive programme for M1 macrophage differentiation has not been fully understood.

Nitric oxide (NO), one of the smallest known bioactive products of mammalian cells, is critical to numerous physiological processes including host defense against pathogens, vasodilation and neurotransmission11,12. Three distinct isoforms of NO synthase have been identified, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS)13. nNOS and eNOS both are calcium-dependent and are primarily expressed in neurons and endothelial cells. Induction of iNOS varies depending on cell types and species14. The TLR ligands and inflammatory cytokines including IFN-γ can induce iNOS expression in many cell types. It is clear that NO is an important proinflammatory cytotoxic mediator that defends the host against various pathogens by inactivating and destroying important proinflammatory mediators and in the upregulation of arginase 1 (Arg1) and IL-10 (refs 5,6). NO derived from iNOS is involved in the regulation of M1 innate immune responses or iNOS modulates the differentiation of M1 macrophages. It seems that IL-10 mRNA expression was comparable in WT and iNOS−/− M1 macrophages. Similarly, the frequency of IL-12 p40-producing cells by intracellular staining and IL-1R expressing cells using flow cytometry. Notably, the frequency of IL-12-producing cells or IL-1R-expressing cells generated from iNOS−/− macrophages was significantly greater than that of cells from WT cultures (Fig. 1a).

In the present study, we show that mice deficient in iNOS exhibited enhanced M1 macrophage polarization while exhibiting no significant effects on M2 macrophages. We demonstrated that L-NIL, an iNOS selective inhibitor, significantly enhanced M1 macrophage polarization in cell cultures from wild-type (WT) mice. Meanwhile, a NO donor, SNAP, suppressed M1 macrophage differentiation in WT and iNOS−/− cell cultures. Furthermore, NO nitrated the tyrosine residues of IRF5 protein, resulting in the suppression of M1 macrophage polarization. Systems analyses demonstrate a mutually inhibitory circuit that dynamically fine-tunes the competitive expression of iNOS and IL-12 in macrophages. Transfer of iNOS-deficient macrophages into C57BL/6 mice lead to higher susceptibility to endotoxin shock. These findings suggest that NO plays a critical suppressive role in the control of M1 macrophage activation and highlight the importance of myeloid cell-derived iNOS in modulating M1 macrophage-mediated innate immune responses.

Results

iNOS deficiency enhances M1 macrophage differentiation. To investigate the function of nitric oxide (NO) in macrophage cell differentiation, we first assessed the characteristics of macrophage development in iNOS-deficient mice. Bone marrow cells from iNOS−/− or WT control mice were incubated with GM-CSF (10 ng ml−1) or M-CSF (10 ng ml−1) in vitro for 7 days. The cells were activated with LPS (200 ng ml−1) plus IFN-γ (10 ng ml−1) for M1 macrophage differentiation and examined for the percentages of IL-12 p40-producing cells by intracellular staining and IL-1R expressing cells using flow cytometry. Notably, the frequency of IL-12-producing cells or IL-1R-expressing cells generated from iNOS−/− macrophages was significantly greater than that of cells from WT cultures (Fig. 1a).

Next, we performed micro-array experiment to determine M1 macrophage marker gene expression. Interestingly, the mRNA expression of the M1 marker genes including IL-12A, IL-6, TNFα, IL-1R, CXCL9 and CXCL10 was significantly enhanced in iNOS−/− M1 macrophages compared with that of WT cells (Fig. 1b). To confirm the micro-array results, we performed qPCR experiments and found that mRNA expression of IL-12A, IL-6, CXCL9 and CXCL10 was indeed increased (Fig. 1c, Supplementary Fig. 1). These observations correlated with enhanced IL-12A and IL-6 secretion by iNOS−/− M1 macrophages as determined by ELISA (Fig. 1d). To rule out the possibility that the enhanced M1 cell differentiation was due to abnormal myeloid cell development, we analysed myeloid cell population from spleens and bone marrows of WT and iNOS−/− mice (Supplementary Fig. 2). In contrast to the striking effect of iNOS deficiency on M1 macrophage differentiation, M2 macrophage signature gene expression was not significantly affected in iNOS−/− macrophage cultures (Supplementary Fig. 3). In addition, we analysed whether eNOS or nNOS deficiency affects M1 macrophage differentiation. Bone marrow cells from eNOS−/−, nNOS−/− or WT control mice were incubated with GM-CSF (10 ng ml−1) in vitro for 7 days. The cells were then either activated with LPS (200 ng ml−1) plus IFN-γ (10 ng ml−1) for M1 macrophage differentiation. The results showed that neither eNOS deficiency nor nNOS deficiency affects M1 macrophage differentiation (Supplementary Figs 4 and 5). Taken together, these results indicate that M1 macrophage cell differentiation is enhanced in macrophages deficient in iNOS, suggesting that NO plays a negative role in M1 macrophage differentiation.

To investigate whether the enhancement of M1 macrophage differentiation was the result of alteration of IL-10 production in iNOS-deficient mice, we examined IL-10 mRNA expression in WT and iNOS−/− M1 macrophages. It seems that IL-10 mRNA expression was comparable in WT and iNOS−/− M1 macrophages and NO donor SNAP did inhibit M1 macrophage signature gene expression in IL-10−/− mice (Supplementary Fig. 6). Thus, the enhanced M1 macrophage differentiation in iNOS−/− mice was not due to the alterations of IL-10 expression. To understand how iNOS−/− macrophages affect T-cell activation, we first analysed MHC II expression in iNOS-deficient...
indicated genes was determined by qPCR. The data shown were normalized to levels of ubiquitin expression. Each bar represents mean ± s.d. from three independent experiments, unpaired Student’s t-test, *P < 0.05 versus WT cells. (b) The cells prepared in (a) were stimulated with IFN-γ (10 ng ml⁻¹) plus LPS (200 ng ml⁻¹) for 6 h and microarray experiment was performed for the analysis of M1 macrophage gene mRNA expression. (c) The cells prepared in (b) in the presence of SNAP (500 μM) were stimulated with IFN-γ (10 ng ml⁻¹) plus LPS (200 ng ml⁻¹) for 6 h, and mRNA expression of indicated genes was determined by qPCR. The data shown were normalized to levels of ubiquitin expression. Each bar represents mean ± s.d. from three independent experiments, one-way ANOVA with a Bonferroni correction, *P < 0.05; **P < 0.01. (d) The cells prepared in (a) in presence of SNAP (500 μM) or L-NIL (40 μM) were stimulated with IFN-γ (10 ng ml⁻¹) plus LPS (200 ng ml⁻¹) for 24 h and the supernatants were analysed for IL-12 and IL-6 by ELISA. Each bar represents mean ± s.d. from three independent experiments, one-way ANOVA with a Bonferroni correction, *P < 0.05; **P < 0.01.

Bone marrow cells from iNOS⁻/⁻ or WT control mice were incubated with GM-CSF (10 ng ml⁻¹) in vitro for 7 days. The cells were then activated with LPS (200 ng ml⁻¹) plus IFN-γ (10 ng ml⁻¹) for 24 h, stained for intracellular IL-12 and surface IL-1R and analysed by flow cytometry. Representative FACS dot plots gated on CD11b⁺ cells and the percentages of IL-12-producing and IL-1R-positive CD11b⁺ cells are shown. Each bar represents mean ± s.d. from three independent experiments, unpaired Student’s t-test, *P < 0.05 versus WT cells. (b) The cells prepared in (a) were stimulated with IFN-γ (10 ng ml⁻¹) plus LPS (200 ng ml⁻¹) for 6 h and microarray experiment was performed for the analysis of M1 macrophage gene mRNA expression. (c) The cells prepared in (b) in the presence of SNAP (500 μM) were stimulated with IFN-γ (10 ng ml⁻¹) plus LPS (200 ng ml⁻¹) for 6 h, and mRNA expression of indicated genes was determined by qPCR. The data shown were normalized to levels of ubiquitin expression. Each bar represents mean ± s.d. from three independent experiments, one-way ANOVA with a Bonferroni correction, *P < 0.05; **P < 0.01. (d) The cells prepared in (a) in presence of SNAP (500 μM) or L-NIL (40 μM) were stimulated with IFN-γ (10 ng ml⁻¹) plus LPS (200 ng ml⁻¹) for 24 h and the supernatants were analysed for IL-12 and IL-6 by ELISA. Each bar represents mean ± s.d. from three independent experiments, one-way ANOVA with a Bonferroni correction, *P < 0.05; **P < 0.01.

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from WT and iNOS−/− mice were activated by LPS plus IFN-γ in the presence of SNAP (a NO donor) or L-NIL (an iNOS specific inhibitor) for 24 h. Western blotting showed that iNOS protein was indeed induced in WT M1 macrophages but not in iNOS−/− cells (Fig. 3a). Interestingly, SNAP significantly reduced iNOS protein expression while L-NIL clearly enhanced iNOS protein expression (Fig. 3a,b). To understand the effect of NO on the regulation of iNOS at the transcriptional level, we transfected iNOS promoter luciferase reporter plasmid into RAW264.7 cells for 18 h and the cells were then activated with IFN-γ in the presence of SNAP for additional 12 h. We found that SNAP dose dependently suppressed iNOS promoter activation (Fig. 3c). In addition, SNAP significantly suppressed iNOS mRNA expression (Fig. 3d). Furthermore, we found that SNAP impaired iNOS mRNA stability (Fig. 3e). These results suggest that NO regulates iNOS expression at the transcriptional level. To further explore whether NO affects iNOS protein posttranslational modification, we transfected iNOS overexpression plasmid into 293T cells for 36 h and the cells were then treated with SNAP (500 μM) in the presence of cycloheximide (10 μM) for different time intervals. The results showed that addition of SNAP had no significant effect on iNOS protein stability (Fig. 3f). Furthermore, co-immunoprecipitation experiment showed SNAP did not affect iNOS ubiquitination. To confirm these results, we stimulated macrophage RAW264.7 cells with IFN-γ (10 ng ml−1) for 24 h. Afterwards the cells were washed five times with fresh medium and incubated with fresh medium in the presence of SNAP for 12 h. Similarly, addition of SNAP had no clear effect on iNOS protein stability and iNOS ubiquitination (Fig. 3g,h). Thus, NO significantly suppresses iNOS expression at the transcriptional level and had no noticeable effects on iNOS protein stability.
NO suppresses iNOS DNA binding activity in macrophages. The above findings prompted us to probe the molecular basis for NO control of M1 macrophage differentiation. Since many studies have demonstrated that IRF5 plays a critical role in M1 macrophage differentiation both in vitro and in vivo, we asked whether NO might affect IRF5 expression, resulting in the control of M1 macrophage differentiation. As such, we first activated bone marrow-derived macrophages from WT and iNOS−/− mice with LPS plus IFN-γ for 6 h for real-time PCR or 20 h (protein) for western blotting. The results showed that IRF5 mRNA and protein expression was similar in iNOS−/− M1 macrophage compared with WT cells (Fig. 4a). In addition, the protein expression of other transcription factors including IRF4 protein expression was comparable between iNOS−/− and WT M1 macrophages (Fig. 4b). Furthermore, qPCR experiments showed that NO donor SNAP and iNOS-specific inhibitor L-NIL had any noticeable effect on IRF5 mRNA expression (Fig. 4b). To investigate whether IRF5 is responsible for enhancing M1 macrophage differentiation in iNOS−/− mice, bone marrow-derived macrophages from WT and iNOS−/− mice were transfected with IRF5 siRNA or control siRNA and the cells were then activated with LPS plus IFN-γ (Fig. 4c). Flow
cytometry analysis showed that the percentage of IL-12-positive cells was significantly reduced in IRF5-knockdown cells of WT and iNOS−/− mice (Fig. 4d). In addition, IRF5 knockdown clearly reduced IL-12/23 p40 cytokine release in WT and iNOS−/− culture (Fig. 4d). These results suggest that IRF5 is involved in the regulation M1 macrophage differentiation in iNOS−/− mice. Next we wanted to investigate how iNOS affects IRF5-targeted M1 macrophage signature genes. Bone marrow cells from iNOS−/− or WT control mice were incubated with GM-CSF (10 ng ml−1) in vitro for 7 days and then the cells were activated with LPS (200 ng ml−1) for 20 h. iNOS deficiency has no effects on the IRF5 activity (Fig. 4e). However, chromatin immunoprecipitation (ChIP) experiments showed the DNA binding activity of IRF5 to the promoter region of IL-12 p40 gene was significantly increased in iNOS−/− M1 macrophages while SNAP greatly suppressed IRF5 DNA binding activity (Fig. 4e). In addition, we also analysed the effects of NO on the expression of STAT1, a transcription factor in IFN-γ signalling cascade. Macrophage RAW264.7 cells were pretreated with SNAP (500 μM) for 30 min and then the cells were activated with IFN-γ for various time intervals (10, 20 and 60 min). Western blotting showed that SNAP had no effect on the phosphorylation of STAT1 in iNOS−/− mice (Fig. 5a). In addition, SNAP did not affect STAT1 translocation (Fig. 5b). The DNA binding activity of STAT1 to the GAS element of iNOS promoter was significantly induced by IFN-γ/LPS in WT M1 macrophages and SNAP clearly suppressed STAT1 DNA binding activity (Fig. 5c).

The data above showed that iNOS had no effect on IRF5 expression but significantly affected IRF5 DNA binding activity. We then proceeded to analyse whether NO modulates post-translational modification of IRF5 protein. The amino-acid residue sequence in IRF5 protein has tyrosine residues, which
Figure 5 | NO induces nitration of tyrosine residues of IRF5 protein in macrophages. (a) RAW264.7 macrophages were pretreated with SNAP (500 μM) for 30 min and the cells were then activated with IFN-γ (10 ng ml−1) for various time intervals (10, 20, 30 and 60 min). Western blotting was performed for the analysis of STAT1 phosphorylation. β-Actin was used as a control. (b) RAW264.7 macrophages were pretreated with SNAP (500 μM) for 30 min, and the cells were then activated with IFN-γ (10 ng ml−1) for various time intervals (10, 20, 30 and 60 min). The cytosolic fraction and nuclear fraction of protein was prepared, and western blotting was performed for the analysis of phosphorylated STAT1 and STAT1 protein. (c) WT and iNOS−/− mice were stimulated with IFN-γ (10 ng ml−1) and LPS (200 ng ml−1) in the presence of SNAP (500 μM) for 6 h, followed by ChIP assay. Three micrograms of an anti-phosphorylated STAT1 antibody or isotype-matched IgG as control antibody was used in the immunoprecipitation step. PCR was used to quantify the amount of precipitated DNA with primers flanking the STAT1-binding site of the iNOS promoter region. Each bar represents mean ± s.d. from three independent experiments, unpaired Student’s t-test, *P<0.05, versus normal IgG. (d) BMDMs from WT and iNOS−/− mice were stimulated with IFN-γ (10 ng ml−1) and LPS (200 ng ml−1) in the presence of SNAP (500 μM) or L-NIL (40 μM) for 24 h. The cell lysates were then immunoprecipitated with an anti-nitrotyrosine antibody and blotted with an anti-IRF5 antibody. Data are representative of three independent experiments. (e) The 293T cells were transfected with IRF5 plasmid for 40 h in the presence of SNAP (500 μM) or L-NIL (40 μM). Cell lysates were immunoprecipitated with an anti-nitrotyrosine antibody and immunoblotted with an anti-IRF5 antibody. (f) The 293T cells were cotransfected with an IL-12 promoter reporter construct and an IRF5 plasmid in the presence of SNAP (100, 200 and 500 μM) for 30 h. Luciferase assays were performed and luciferase activities were normalized to β-galactosidase activity. Each bar represents mean ± s.d. from three independent experiments, unpaired Student’s t-test, *P<0.05 and ***P<0.001 versus IRF5 plasmid only.

may be subject to nitration induced by NO. To investigate this possibility, we activated bone marrow-derived macrophages from WT and iNOS−/− mice with LPS plus IFN-γ for 20 h, and the cell lysates were immunoprecipitated with an anti-nitrotyrosine antibody and immunoblotted with an IRF5 antibody. The results showed that IRF5 protein was nitrated in macrophages activated with LPS plus IFN-γ in WT macrophages but not in iNOS−/− cells (Fig. 5d). To confirm these results, 293T cells were transfected with IRF5 overexpression plasmid in the presence of SNAP or L-NIL for 40 h. Cell lysates were immunoprecipitated with an anti-nitrotyrosine antibody and immunoblotted with an anti-IRF5 antibody. SNAP treatment clearly induced tyrosine nitration of IRF5 (Fig. 5e), suggesting that NO-induced alterations of tyrosine residues may affect IRF5 activation. To examine the effect of NO on IRF5 at the functional level, we co-transfected an IL-12 promoter reporter and IRF5 expression plasmids into 293T cells in the presence of various doses of SNAP for 36 h and analysed their IL-12 promoter activation. The data showed that SNAP suppressed IRF5-mediated IL-12 promoter activation in a dose-dependent manner (Fig. 5f). To investigate whether IRF5 is nitrated in vivo, we injected (i.p.) LPS (200 μg mouse−1) into WT and iNOS−/− mice. The results clearly showed that IRF5 was nitrated in WT mice but not in iNOS−/− mice (Fig. 6a). Bioinformatic analysis suggests that tyrosine residue Tyr74 in IRF5 protein is most likely to be subject to nitration. We found that mutating this tyrosine residue significantly impaired IRF5-mediated IL-12 p40 promoter activation, suggesting that Tyr74 is critical for IRF5 transcriptional function (Fig. 6b). In addition, mutating Tyr104 also impaired IRF5-mediated IL-12 p40 promoter activation (Supplementary Fig. 8). Taken together, these results suggest that NO suppresses M1 macrophage differentiation at the transcriptional level by nitration of tyrosine residues in IRF5.

Dynamic modulation of IL-12 expression by iNOS in macrophages. We next set up experiments to test the dynamic IL-12 expression in macrophages. To study this, we used LPS alone instead of LPS plus IFN-γ as the stimulant. The rationale is that LPS alone will not restrict macrophages to a fixed phenotype. Rather, we and other observed that varying dosages of LPS will induce dynamic modulation of both pro- and anti-inflammatory
mediators\textsuperscript{21–24}. Bone marrow macrophages from WT mice were treated with varying dosages of LPS (100 pg ml\(^{-1}\), 1, 10, 1,000 ng ml\(^{-1}\)) overnight. Intracellular IL-12 was stained with a specific fluorescence-conjugated antibody and stained macrophages were analysed by flow cytometry. We observed that the expression of IL-12 was dynamically modulated based on the concentration of LPS (Fig. 7a). Super low-dose LPS moderately elevated the IL-12-expressing population. The induction of IL-12 reached the maximum at moderate LPS concentration (1–10 ng ml\(^{-1}\)). As the concentration of LPS further rises, the trend reversed. The population of IL-12-expressing cells dropped in cells treated with 1\,\mu\text{g}\,\text{ml}^{-1}\,\text{LPS}. In contrast, the expression of iNOS was not induced by 100 pg ml\(^{-1}\) LPS, but was only markedly induced with higher-dose LPS (Fig. 7b). In addition, we observed that IL-12 production was increased with the increasing concentrations of LPS up to 1\,\mu\text{g}\,\text{ml}^{-1}\,\text{in iNOS-deficient macrophages (Supplementary Fig. 9), and NO donor suppressed IL-12 production (Supplementary Fig. 9). On the basis of our above-described mechanistic studies, we distilled the competitive nature of iNOS and IL-12 expression into a simple computational motif as shown in Fig. 7c. In this circuit, LPS is activating two competing signalling programmes that differentially induce either iNOS or IL-12. In the meantime, these two programmes are mutually inhibitory. The signal strength leading to iNOS or IL-12 expression varies based on LPS concentration. Computational simulation of this motif gave rise to similar dynamic expression profiles of IL-12 (Fig. 7d).

On the basis of this computational analysis, we further predict that the addition of NO donor SNAP would destroy the dynamic induction of IL-12 by varying dosages of LPS (Fig. 7e,f). Indeed, our experimental data confirmed this prediction and demonstrated that SNAP treatment significantly reduced the IL-12-expressing population induced by LPS (Fig. 7g,h).

\textbf{iNOS regulates M1 macrophage differentiation in vivo.} It is well-established that iNOS-derived NO plays an important role in host defense against bacterial infection by killing bacteria directly. Accumulating evidence indicates that iNOS-deficient mice are susceptible to bacterial infection. However, an important question remains unaddressed yet: what is the state of macrophage differentiation in iNOS-deficient mice during bacterial infection? To answer this question, we injected i.v. into WT and iNOS\textsuperscript{−/−} mice \textit{L. monocytogenes} (2 \times 10\(^4\) CFU per mouse) for 2 days and mice were then killed. Bacterial loads in the spleens and livers of iNOS-deficient mice were significantly increased compared with WT mice (Fig. 8a). Interestingly, the production of M1 macrophage signature molecule TNF\(\alpha\) and IL-12 in the sera was clearly enhanced in iNOS\textsuperscript{−/−} mice (Fig. 8b). In addition, mRNA expression of M1 macrophage signature genes was also increased in iNOS\textsuperscript{−/−} mice after \textit{L. monocytogenes} infection (Fig. 8c, Supplementary Fig. 10). These results indicate that M1 macrophage differentiation was actually enhanced in iNOS\textsuperscript{−/−} mice, although iNOS\textsuperscript{−/−} mice are susceptible to \textit{L. monocytogenes} infection. To confirm this result, we prepared thioglycollate-elicited macrophages or BMDMs from WT and iNOS\textsuperscript{−/−} mice and transferred the cells into WT mice. Twenty-fours later, mice were challenged with LPS. Consequently, susceptibility and the expression of M1 macrophage signature molecules were observed. The results showed that mice transferred with iNOS\textsuperscript{−/−} macrophages were more susceptible to endotoxin shock (Fig. 8d, Supplementary Fig. 10a), and the production and expression of M1 macrophage signature molecules were significantly enhanced.

**Figure 6 | NO induces nitration of tyrosine residues of IRF5 protein in macrophages.** (a) WT or iNOS\textsuperscript{−/−} mice were injected (i.p.) with LPS (200\,\mu\text{g}/mouse) for 6 or 12 h. Mice were then killed and spleen cells were prepared. The cells were stained for IRF5 and nitrotyrosine and analysed by flow cytometry. Representative FACS dot plots gated on CD11b\(^+\) cells, and the percentages of IRF5 and nitrotyrosine-positive CD11b\(^+\) cells are shown. Each bar represents mean \pm s.d. from three independent experiments, unpaired Student’s t-test, **P<0.01, versus iNOS\textsuperscript{−/−} cells. (b) The 293T cells were transfected with an IL-12 p40 promoter reporter construct and wild-type IRF5 or mutant IRF5Y74H plasmids for 30 h. Luciferase assays were performed, and luciferase activities were normalized to β-galactosidase activity. In addition, IRF5 protein expression was analysed by western blotting. Each bar represents mean \pm s.d. from three independent experiments, unpaired Student’s t-test, ***P<0.001, versus cells transfected with WT IRF5 plasmid.
in mice transferred with iNOS−/− macrophages (Fig. 8e,f, Supplementary Fig. 11b,c). Thus, iNOS deficiency in macrophages promotes M1 macrophage differentiation in both infection and endotoxin shock models, suggesting that iNOS expressed in macrophages may play a negative role in the regulation of innate immune response.

To further investigate the role of iNOS in macrophage function in vivo, we extended our studies to include a tumour cell inoculation model. C57Bl/6 mice were inoculated with melanoma cell line B16 cells. Then mice were treated either with PBS or iNOS specific inhibitor L-NIL. Interestingly, L-NIL treatment significantly decreased the tumour size in tumour-bearing mice (Supplementary Fig. 12). In addition, tumour-infiltrating cell analysis showed that L-NIL treatment significantly increased the percentage of M1 macrophages in the tumour microenvironment (Supplementary Fig. 12). Thus, iNOS inhibition in tumour microenvironment facilitates M1 macrophage differentiation, resulting in the decrease of tumour size, further confirming that NO negatively regulates M1 macrophage differentiation in vivo.

Discussion
Classically activated macrophages (M1) play an important role in host defense against pathogen infection and are also involved in the pathogenesis of autoimmune and inflammatory diseases. Therefore, understanding the intrinsic modulating programmes for M1 macrophage differentiation will help us understand the
mechanisms for the control of innate immune responses and dissect the mechanism involved in the development of human inflammatory diseases. In the present study, we demonstrate that iNOS-deficient mice displayed enhanced M1 macrophage differentiation but without major effects on alternatively activated macrophages (M2). Addition of N6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL), the iNOS inhibitor, significantly enhanced M1 macrophage differentiation, and S-nitroso-N-acetylpencillamine (SNAP), the NO donor, suppressed M1 macrophage differentiation. NO derived from iNOS-mediated nitration of tyrosine residues in an IRF5 protein leading to the suppression of IFN5-targeted M1 macrophage signature gene activation, indicating that NO regulates macrophage differentiation by modulating IRF5. Finally, studies of an experimental model of endotoxin shock showed that iNOS deficiency results in more severe inflammation with an enhanced M1 macrophage activation phenotype. These results suggest that iNOS derived from macrophages selectively modulates M1 macrophage differentiation.

iNOS is expressed in different cell types including, macrophages, dendritic cells, NK cells and primary tumour cells.\textsuperscript{11,25} NO derived from iNOS in macrophages and other innate immune cells is pro-inflammatory and an essential component of host defenses against various pathogens including bacteria, parasites and viruses.\textsuperscript{15,25} Therefore, iNOS is an important signature molecule for M1 macrophage activation. Although increasing evidence indicates that iNOS is involved in the modulation of immune responses in addition to its killing effect of pathogens, the importance of iNOS involved in the control of M1 macrophage differentiation is incompletely understood. Previously we demonstrated that IL-12 mRNA and protein expression were significantly increased in iNOS KO mice, indicating that iNOS indeed contributes the regulation of proinflammatory cytokines by macrophages.\textsuperscript{18} In addition, Giordano et al.\textsuperscript{20} reported that expression of inflammatory cytokines including TNF-\textgreek{z}, IL-6, IL-12p70 and IL-23 was upregulated in iNOS\textsuperscript{−/−} bone marrow-derived dendritic cells. Taken together, these results indicate that iNOS expressed in
innate immune cells including macrophages can modulate inflammatory cytokine production, and these cytokines are generally produced by M1 macrophages.

In the present study, we clearly demonstrated that iNOS expressed by M1 macrophages plays a negative role in the regulation of M1 macrophage differentiation. This conclusion was supported by the following observations: (1) the expression of M1 macrophage signature genes is significantly increased in iNOS+/− M1 macrophages while eNOS and nNOS deficiency has no effect on M1 macrophage differentiation; (2) iNOS−/− macrophages induce strong T-cell activation; (3) The iNOS-selective inhibitor, L-NIL, significantly increased the expression of M1 macrophage signature genes. In addition, the NO donor, SNAP, significantly suppressed M1 macrophage differentiation; (4) C57BL/6 mice transferred with iNOS−/−/C0 macrophages are more susceptible to endotoxin shock with enhanced M1 macrophage cytokines. Thus, our observations support the concept that iNOS expressed in M1 macrophages modulates M1 macrophage differentiation by regulation M1 macrophage signature gene expression.

IL-10 is an anti-inflammatory cytokine and has been reported to inhibit macrophage function, resulting in the control of inflammation. IL-10 can be produced by different cells types including macrophages, T cells and B cells. It is well established that M2 macrophages produce IL-10 resulting in the modulation of immune response. To exclude the possibility that the enhanced M1 macrophage differentiation was due to effects of IL-10 in iNOS−/− mice, we examined IL-10 expression in WT and iNOS−/− macrophages under M1 conditions. IL-10 mRNA expression was comparable in WT and iNOS−/− macrophages. Furthermore, we found that NO donor SNAP significantly suppressed the expression of M1 macrophage signature gene expression. This indicated that enhanced M1 macrophage differentiation in iNOS−/− mice is independent of the effects of IL-10.

IRF5, a member of interferon regulatory factor (IRF) family, has a variety of functions including activation of genes encoding inflammatory cytokines, type I interferon and tumour suppressors32–34. Recently because M1 macrophages expressed high amount of IRF5 and overexpression of IRF5 in M2 macrophages induced global expression of M1 macrophage signature genes, IRF5 has been defined as a key transcription factor for M1 macrophage differentiation35. In addition, IRF5-deficient mice are resistant to lethal endotoxin shock. The evidence suggests that activation of IRF5 expression defines commitment to the M1 macrophage lineage. Interestingly, microarray analysis showed that IRF5 expression in iNOS−/− M1 macrophages was comparable to WT cells. Furthermore, SNAP or L-NIL had no significant effect on IRF5 protein expression in WT and iNOS−/− M1 macrophages, indicating that the enhanced M1 macrophage differentiation is not the result of increased IRF5 protein levels. However, knockdown of IRF5 in iNOS−/− M1 macrophages reduced the expression of M1 signature cytokines, suggesting that iNOS may control IRF5 activation during M1 macrophage differentiation. How might iNOS regulate IRF5 activation? Recently we have shown that tyrosine residues in RORγt are nitrated. Such nitration of RORγt significantly impaired the binding of RORγt to the promoter region of the IL-17 gene, resulting in the inhibition of IL-17 transcription36. We assume that iNOS-derived NO may also nitrates tyrosine residues of IRF5 protein. The results indeed showed that the tyrosine residues in IRF5 protein were nitrated in WT M1 macrophages but not in iNOS−/− M1 macrophages. In addition, ChIP assay showed that IRF5 DNA binding activity was clearly enhanced in iNOS−/− macrophages and SNAP suppressed IRF5 DNA binding activity. Furthermore, we identified Tyr74 and Tyr104 residues of IRF5 are important for induction of IL-12p40 promoter activation. A previous study demonstrated that tyrosines of IkBβ are nitrated as a consequence of NO synthase activation, resulting in dissociation of IkBβ from NF-kB37. Some other studies have presented that nitration of a specific tyrosine in proteins can have structural and functional significance32–34. Taken together, our study reveals a novel mechanism for the modulation of M1 macrophage differentiation by nitration of IRF5 tyrosine residues.

M1 macrophages have been believed to play critical roles in host defense against bacterial infection, endotoxin shock and tumour growth38–40. It is well known that iNOS-derived NO contributes to the direct killing of various pathogens. However, the modulation of iNOS on M1 macrophage differentiation in vivo is still unknown. In the present study, we found that iNOS-deficient mice were susceptible to L.monocytogenes infection as reported previously since NO is directly involved in the killing of bacteria. However, the expression of M1 macrophage signature molecules was significantly increased in iNOS-deficient mice after infection, indicating that M1 macrophage activation was enhanced in iNOS-deficient mice during L.monocytogenes infection. Consistent with the results, mice transferred with iNOS−/− macrophages were susceptible to lethal endotoxin shock, with enhanced expression of M1 macrophage signature molecules. Thus, these results suggest that iNOS expressed in macrophages plays a negative role in M1 macrophage differentiation in vivo, although it is a key marker for M1 macrophages. In another in vivo model of tumour inoculation of melanomas, L-NIL treatment significantly decreased the tumour size in tumour-bearing mice. In addition, tumour-infiltrating cell analysis showed that L-NIL treatment significantly enhanced the percentage of M1 macrophages in the tumour microenvironment. Thus, iNOS inhibition in tumour microenvironment facilitates M1 macrophage differentiation, resulting in the decrease of tumour size, further confirming that NO negatively regulates M1 macrophage differentiation in vivo.

Our systems analyses reveal a scenario that may best reflect and explain dynamic and balanced innate immune modulation in real-life settings. In vivo, macrophages likely exist as a mixed population of M1/M2 or hybrid phenotypes instead of an exclusively polarized M1 or M2 state38. Balanced macrophage portfolio would enable proper adaptation to changing environments. Similar phenomena are increasingly recognized in other cellular systems such as T-helper cells39. Our recent computational studies reconciled the current debate regarding the ‘plasticity’ versus ‘stability’ of T-helper cells40. A dynamic and mutually inhibitory motif differentially senses the signal strength activating the couple induction of Th17 and Treg cells40. On the basis of external signal strength, CD4 T-helper cells may readily adopt Th17, Treg or a double-positive phenotypes40. This would allow timely immune activation and homeostasis. Similarly, our current study provides initial evidence that macrophages possess similar dynamic circuit capable of fine-tuning the expression of iNOS and IL-12.

Taken together, our studies clearly demonstrate that in addition to acting as a signature marker for M1 macrophages, iNOS expressed in M1 macrophages dedifferentiate M1 macrophages. According to our observations, we suggest a novel molecular mechanism for the effects of iNOS-derived NO on M1 macrophage dedifferentiation that involves the suppression of IRF5 activation. The results established a novel concept that iNOS expressed in macrophages not only selectively regulates M1 macrophage gene expression but also modulates the M1 macrophage dedifferentiation, as a whole resulting in control of innate immune responses.
Methods

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**Methods**

**Mice.** C57BL/6/J (B6, stock#000064), iNOS-deficient mice (B6.129P2-Nos2<sup>tm1(Lau)</sup>, stock#002609), cNOS-deficient mice (B6.129P2-Nos2<sup>tm1(Lau)</sup>, stock#002684), nNOS<sup>−/−</sup> mice (B6.129s4x1-Nos2<sup>tm2(Plj)</sup>, stock#008519) and CD4<sup>+</sup> OVA TCR transgenic (OT-II) mice (B6. C57BL/6J transgenic mouse, stock#004194) were obtained from Jackson Laboratory and maintained in the barrier facility at the Mount Sinai School of Medicine. For all the experiments, 6- to 8-week-old female mice were used. All animal study protocols were approved by the Institutional Animal Care and Use Committees of Mount Sinai School of Medicine and Virginia Tech.

**Antibodies.** The following antibodies were used at the indicated dilutions for immunoblotting and immunoprecipitation. Antibodies for Ub (sc-8017, 1:300), OVA (sc-9048, 1:300), OVA257-264 (sc-156913, 1:50), from Cell Signaling Technology (USA); IRF5 (ab-21689, 1:300) and Nitrotyrosine (sc-65385, WB 1:300, IP 2), IRF1 (sc-640, 1:300), IRF4 (sc-6059, 1:300), IRF7 (sc-9083, 1:300), IRF8 (sc-6058, 1:300) were purchased from Cell Signaling Technology (USA); STAT1 (9172, 1:50), IRF1 (sc-640, 1:300), IRF4 (sc-6059, 1:300), IRF7 (sc-9083, 1:300), IRF8 (sc-6058, 1:300) were isolated from tibias and femurs of C57BL/6 mice, and the cells were cultured and lysed for 15 min on ice in 0.5% Nonidet P-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol and 10 mM HEPES (pH 7.5). Cell lysates were clarified by centrifugation (4 °C, 15 min, 14,000 r.p.m.) and protein was subjected to 10% sodium dodecyl sulfate–PAGE (SDS–PAGE) and immunoblotting was performed. Anti-iNOS (Santa Cruz), anti-IRF5 (MBL), anti-STAT1 and anti-β-actin (Sigma) antibodies were used according to the manufacturers’ instructions. Secondary antibodies were from Santa Cruz. The full scans of western blots are provided in the Supplementary Figs 13–18.

**Preparation of bone marrow-derived macrophages.** Bone marrow (BM) cells were isolated from the tibia and femurs of C57BL/6 mice, and the cells were cultured in the complete DMEM medium supplemented with GM-CSF (10 ng ml<sup>−1</sup>) and M-CSF (10 ng ml<sup>−1</sup>). On day 6 or 7, bone marrow-derived macrophages (BMDMs) were harvested and then seeded in fresh complete DMEM medium at a density of 2 × 10<sup>5</sup> cells ml<sup>−1</sup> for experiments.

**Intracellular staining and flow cytometry.** Bone marrow–derived macrophages were either activated with LPS (200 ng ml<sup>−1</sup>) or LPS alone at different concentrations overnight, and brefeldin A was added to the culture for 5 h before intracellular staining. Cells were fixed with IC Fixation Buffer (BD Bioscience), incubated with permeabilization buffer and stained with PE-anti–mouse IL-12 p40, APC-anti-iNOS and PE-Cy5.5 anti–mouse C11b antibodies. Flow Cytometry was performed on a FACScalibur (BD Biosciences).

**RNA isolation and quantitative real-time RT-PCR (qPCR).** Total RNA was extracted using an RNasy plus kit (QIAGEN, Valencia, CA), and cDNA was generated with an oligo (dT) primer and the SuperScript II cDNA synthesis kit (Invitrogen, USA) followed by analysis using iCycler PCR with SYBR Green PCR master Mix (Applied Biosystems). Program was chosen to compare the CT value of target gene to the CT. The following primer sets were used: IL-6 sense, 5′-CTCCAAGCCAAAGTCCTAGA-3′; IL-6 anti-sense, 5′-TGG-CTATTAATTA-3′; TNF<sub>α</sub> sense, 5′-CCAGAACACCTTGATGGTCTC-3′; TNF<sub>α</sub> anti-sense, 5′-TGG-CTATTAATTA-3′; Ubiquitin sense, 5′-GCA-AGTGGCTAGAGTG-3′; Ubiquitin anti-sense, 5′-CTTGTTAATATTCTGCTGC-3′; CAT-3 sense, 5′-CTTCTAAAGGCTTCTTCGAA-3′; CAT-3 anti-sense, 5′-GGT-GTCCTGGCAAGCTG-3′; β-actin sense, 5′-GTAACCTCATCCATCACTACA-3′; β-actin anti-sense, 5′-GGAAGCTACCTGGCAAGCTG-3′; C280 mM NaCl, 0.5% Nonidet P-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol and 10 mM HEPES (pH 7.5). Cell lysates were clarified by centrifugation (4 °C, 15 min, 14,000 r.p.m.) and protein was subjected to 10% sodium dodecyl sulfate–PAGE (SDS–PAGE) and immunoblotting was performed. Anti-iNOS (Santa Cruz), anti-IRF5 (MBL), anti-STAT1 and anti-β-actin (Sigma) antibodies were used according to the manufacturers’ instructions. Secondary antibodies were from Santa Cruz. The full scans of western blots are provided in the Supplementary Figs 13–18.

**Chromatin immunoprecipitation assay.** The ChIP procedure was performed using an assay kit following the manufacturer’s instruction (Upstate Biotechnology, Lake Placid NY). In brief, activated macrophages were crosslinked by 1% formaldehyde for 10 min at 37 °C. Nuclei were prepared and subjected to sonication to obtain DNA fragments. Chromatin fractions were precluded with protein A-agrose beads followed by immunoprecipitation overnight at 4 °C with 3 μg of anti-IRF5 (Santa Cruz) or control antibody. Crosslinking was reversed at 65 °C for 4 h, followed by proteinase K digestion. DNA was purified and subjected to qPCR. The input DNA was diluted 200 times before PCR amplification. The input and PCR–amplified DNA were precipitated with DNA PCR using primers encompassing the IRF5-binding sites of the mouse IL-12 promoter regions.

**Infection of L.monocytogenes in mice.** Mice were infected i.v. with 2 × 10<sup>8</sup> CFU viable L. monocytogenes in a volume of 0.2 ml PBS and mice were killed 2 days later<sup>41</sup>. Spleens and livers were homogenized and viable bacteria were grown on BHI agar plates and enumerated by the pour plate method after serial dilution. Colonies were enumerated 24 h after incubation at 37 °C. In addition, spleens and livers were homogenized in Trizol (Life Technologies, USA) and total RNA was extracted using an RNeasy kit following the manufacturer’s instructions (QIAGEN, Valencia, CA) and the array was performed on an Illumina MouseWG-6 v2 expression beadchip (Illumina, USA) by the Genomics Core Facility at the Mount Sinai School of Medicine. Microarray data were normalized with background subtraction and rank invariant normalization. The value ‘1’ substituted any negative values for calculating fold change. All signals were Log2 transformed. Linear modelling of the transformed data was determined by using Limma in R with the Benjamini and Hochberg correction. Probes were selected based on fold change and P values. The expression values were plotted with heatmap. 2, Acension number is available from the NCBI under GEO accession number (GSE65436).
applying quasi-equilibrium approximation, we use a sigmoidal function to determine whether the net activation or inhibition on \( \text{IL12} \) is activated or inhibited when there are no regulatory influences from species in the motif. We purposely developed this generic mechanism and function to simulate potential cellular outcomes. We were able to derive dependencies in a simple manner. Instead, this approach aimed at performing bifurcation analyses of integrated skeletal motifs to simulate potential cellular outcomes. We were able to derive dependencies in a simple manner. Instead, this approach aimed at performing bifurcation analyses of integrated skeletal motifs to simulate potential cellular outcomes. We were able to derive dependencies in a simple manner.

Model implementation. The basic motif shown in Fig. 7c was based on existing reports that suggest the existence of a mutually inhibitory circuit responsible for the generation of iNOS and IL-12 by varying dosages of LPS. This motif was translated into ordinary differential equations (ODEs) to enable simulation. The framework we used to create the ODEs was not based on standard reaction rate equations, but rather on a formalism that allows us to capture complex dependencies in a simple manner. We purposely developed this generic modelling approach that does not depend on minute details of complex pathways. Instead, this approach aimed at performing bifurcation analyses of integrated skeletal motifs to simulate potential cellular outcomes. We were able to derive dependencies in a simple manner. Instead, this approach aimed at performing bifurcation analyses of integrated skeletal motifs to simulate potential cellular outcomes. We were able to derive dependencies in a simple manner.

\[
\frac{dx}{dt} = \gamma x (s(x) - X) + F(\xi(t))
\]

where \( s(x) \) defines the steady-state level of \( X \) and \( F(\xi(t)) \) is a Gaussian white noise process. The equilibrium second moment of the variable \( X <(\langle x(t) - x_0 \rangle^2)_{eq} = \theta_x \) is related to \( \gamma_x \) and the second moment of the noise by a fluctuation-dissipation theorem, follows:

\[
\langle F(\xi(t))\rangle = 2 \gamma_x \theta_x \delta(t - t')
\]

Thus, we can rewrite Equation 9 as:

\[
\frac{dx}{dt} = \gamma_x (s(x) - X) + D_X \cdot \xi(t)
\]

Here \( \xi(t) \) is a temporally uncorrelated, statistically independent, Gaussian white noise process, which is formally defined by \( \xi(t) = \lim_{\tau \to 0} N(0, 1/\tau) \) with \( <\xi(t)\xi(s)> = \delta(t-s) \). We set \( \gamma_x = 1 \) for all species for simplicity in our model. Thus, we can vary \( D_X \) as parameters to control the strength of noise to manually fit the model. Values of \( D_X \) are listed in Supplementary Table 2.

The Langevin equations are integrated and propagated by the explicit method:

\[
X(t + \Delta t) = X(t) + \gamma_x \cdot (s(X(t) - x_0) - X(t)) \cdot X(t) + \Delta t \cdot \Delta x(t) \cdot \eta(t)
\]

where the \( \eta(t) \) are independent random variables.

The stochastic simulations were performed in Matlab Version 7.9.0.

Statistical analysis. The results are shown as means ± s.e.m., and statistical analysis was performed using Student’s t-test. Where more than two groups were compared, one-way ANOVA with a Bonferroni’s correction was performed.

Kaplan–Meier method was used to estimate overall survival and the Log-rank test was applied to determine the difference of survival rate. P values <0.05 were considered statistically significant.

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Author contributions

G.L. and H.X. initiated the project. H.X. supervised the project. G.L., R.Z. and L.P. did most of the experiments. P.J., F.X., J.Y., Q.L., H.Z., K.S., J.W., X.L., Z.Z. and A.S. performed experiments. W.W., C.Q., C.S., J.H. and S.L. provided critical reagents and gave important advice throughout the study. S.G., C.C. and L.L. contributed to dynamic experimental analysis and computational modelling. G.L., L.L. and H.X. wrote the manuscript.

Additional information

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