The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C− monocytes

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The transcription factors that regulate differentiation into the monocyte subset in bone marrow have not yet been identified. Here we found that the orphan nuclear receptor NR4A1 controlled the differentiation of Ly6C− monocytes. Ly6C− monocytes, which function in a surveillance role in circulation, were absent from Nr4a1−/− mice. Normal numbers of myeloid progenitor cells were present in Nr4a1−/− mice, which indicated that the defect occurred during later stages of monocyte development. The defect was cell intrinsic, as wild-type mice that received bone marrow from Nr4a1−/− mice developed fewer patrolling monocytes than did recipients of wild-type bone marrow. The Ly6C− monocytes remaining in the bone marrow of Nr4a1−/− mice were arrested in S phase of the cell cycle and underwent apoptosis. Thus, NR4A1 functions as a master regulator of the differentiation and survival of ‘patrolling’ Ly6C− monocytes.

NR4A1 (Nur77), NR4A2 (Nurr1) and NR4A3 (NOR-1) constitute the NR4A subfamily of orphan nuclear receptors in the steroid thyroid receptor family1. NR4A1 was originally identified as being encoded by a growth factor–inducible gene and is often overexpressed in a variety of cancer cells, including lung, prostate, breast and colon cancers2–5. Many growth factors and mitogens rapidly induce NR4A1 expression, which suggests a survival role for this molecule in mediating the growth of cancer cells. Conversely, NR4A1 has been linked to the programmed cell death of T lymphocytes and B lymphocytes.6–7. NR4A1 is rapidly induced by T cell antigen receptor signals, and dominant-negative NR4A1 inhibits the clonal deletion of developing T cells, which indicates a role for NR4A1 and its family members in thymocyte negative selection8–10. Nr4a1 mRNA is also rapidly (<1 h) and potently induced in macrophages in response to a variety of inflammatory stimuli, including lipopolysaccharide, cytokines and oxidized lipids11.

Structural and functional studies of NR4A1 and other members of the NR4A family suggest that these nuclear receptors do not need to bind small-molecule ligands to be activated12,13. Instead, they seem to be regulated mainly by post-translational modifications, such as phosphorylation14. Acting as a transcription factor, NR4A1 can directly bind specific DNA-response elements alone or can form heterodimers with the retinoid X receptor15,16. In response to apoptotic stimuli, NR4A1 may form a dimer with retinoid X receptor and translocate from the nucleus to the cytoplasm, where it can target mitochondria to induce the release of cytochrome c and apoptosis17. Consistent with its role as a mediator of apoptosis in lymphocytes, NR4A1 triggers mitochondrial apoptosis though its interaction with Bcl-2, converting Bcl-2 from an antiapoptotic molecule to a proapoptotic molecule18,19.

Little is known about the exact functions of NR4A1 in monocyte biology. Of clinical relevance, NR4A1 is located in macrophages in human atherosclerotic lesions and diminishes the loading of lipids into macrophages and their inflammatory responses20,21. NR4A1 also inhibits the accumulation of macrophages and vascular remodeling in mice22. Nr4a3−/−Nr4a1−/− mice develop acute myeloid leukemia with abnormal population expansion of myeloid progenitor cells23. Studies of NR4A3 and NR4A1 in hypoallelic (Nr4a3+/−Nr4a1−/− and Nr4a3−/−Nr4a1+/−) mice have shown that they recapitulate the pathological features of myelodysplastic–myeloproliferative neoplasms, with enhanced proliferation and excessive apoptosis of hematopoietic stem cells and myeloid progenitors24. However, mice deficient in either NR4A1 or NR4A3 have relatively subtle abnormalities and lack overt defects in general physiology, consistent with the idea that NR4A family members have some functional redundancy25. Notably, NR4A2, a closely related member of the NR4A family, has been shown to have a regulatory role in maintaining the quiescence of hematopoietic stem cells26. Such work has demonstrated an important role for members of the NR4A family of nuclear receptors in myeloid differentiation and inflammatory diseases.

In mice and humans, at least two distinct subsets of CD11b+CD11c+ blood monocyte exist. In mice, Ly6c+CCR2+CX3CR1+CD62L+ monocytes are recruited to inflamed or infected tissues and lymph node27–29 and can differentiate into antigen-presenting cells that...
produce tumor necrosis factor, nitric oxide and reactive oxygen species. Ly6C+ monocytes patrol the resting vasculature and can populate inflamed sites. Ly6C+ mouse monocytes are analogous to CD14+ human monocytes, which show a strong inflammatory response to lipopolysaccharide, and Ly6C+ mouse monocytes are most probably analogous to CD14+CD16+ human monocytes, which patrol blood vessels and develop a proinflammatory response consisting of tumor necrosis factor and interleukin 1β that is induced by a Toll-like receptor 7–adapter MyD88–MEK kinase pathway. Both populations of monocytes are recruited to sites of inflammation or injury. Both Ly6C+ and Ly6C− monocytes can also participate in the resolution of inflammation and tissue repair.

Monocyte subsets arise from a common macrophage dendritic precursor (MDP) in the bone marrow. Adoptive-transfer experiments have shown that Ly6C+ monocytes can downregulate Ly6C expression and move between blood and bone marrow, but whether the loss of Ly6C expression corresponds to the conversion of one monocyte subset into another has been questioned. Many transcription factors, including PU.1, JunB, C/EBP-α, C/EBP-β and IRF8, have important roles in myeloid-lineage differentiation, but the specific factors that drive the differentiation of Ly6C+ monocytes are unknown. Here we sought to identify previously unknown transcription factors that control the differentiation of monocyte subsets in vivo. We identified an absence of Ly6C+ monocytes arising from NR4A1-deficient MDP cells in the bone marrow. Ly6C− monocytes remaining in the bone marrow of NR4A1-deficient mice underwent apoptosis because of abnormal cell-cycle progression, which indicated a critical role for NR4A1 in the differentiation and survival of Ly6C− monocytes.

RESULTS

NR4A4 expression in monocytes and progenitors

We first examined the expression of mRNA for NR4A4 and other members of the NR4A family in Ly6C+ and Ly6C− monocytes and myeloid progenitor populations in the bone marrow of wild-type C57BL/6J mice (Fig. 1a). Ly6C+ monocytes expressed on average sevenfold more Nr4a1 mRNA than did Ly6C− monocytes, MDPs or common myeloid precursors (Fig. 1a). Similarly, NR4A1 protein expression was highest in Ly6C+ monocyte populations (Fig. 1b). NR4A1 protein expression was much higher in monocyte populations than in nonmyeloid (CD11b−) cells (Fig. 1b). Nr4a2 mRNA was also expressed in monocytes and myeloid progenitor cells, whereas Nr4a3 mRNA expression was undetectable (Fig. 1a). Notably, Nr4a4 mRNA expression was highest in MDPs, which are the precursors to monocytes, but it was less abundantly expressed in differentiated monocyte populations (Fig. 1a). We confirmed that expression of NR4A1 distinguished Ly6C− ‘patrolling’ monocytes from Ly6C+ ‘inflammatory’ monocytes with a new transgenic reporter mouse model in which induction of the Nr4a1 promoter drives expression of green fluorescent protein (NR4A1-GFP). The majority of CD11b+Ly6Cneg−lo monocytes (over 85%) circulating in the blood (Fig. 1c) and spleen (data not shown) of these reporter mice had abundant expression of
NR4A1-GFP; the majority of Ly6C+ monocytes (over 95%) had low although not negligible expression of NR4A1-GFP (Fig. 1c). Notably, as Ly6C expression decreased, NR4A1-GFP expression increased among CD11b+F4/80+ monocytes (Supplementary Fig. 1). GFPhi monocytes in NR4A1-GFP mice had other defining phenotypic features of patrolling monocytes, including abundant expression of the integrin CD11a (LFA-1), low expression of CD62L (L-selectin) and intermediate expression of the common dendritic cell (DC) marker CD11c and, by quantitative PCR, higher expression of the chemokine receptor CX3CR1 and lower expression of the chemokine receptor CCR2 than their Ly6C+ counterparts (Fig. 1d and data not shown). All GFPhi cells circulating in the blood of NR4A1-GFP mice were F4/80+CD11b+ monocytes and had the horseshoe-shaped nuclear morphology characteristic of monocytes (data not shown). Thus, Ly6C+ monocytes had higher expression of NR4A1 than did Ly6C+ monocytes or monocyte progenitors, which suggested that NR4A1 may have a unique function in this particular monocyte subset.

**Nr4a1−/− mice lack Ly6C− monocytes**

Nr4a1−/− mice had significantly fewer monocytes in bone marrow, blood and spleen than did wild-type (Nr4a1+/+) mice (Fig. 2a; gating strategies for monocyte subsets in bone marrow, Supplementary Fig. 2, and in spleen and blood, Supplementary Fig. 3). Monocytes in bone marrow, blood and spleen were identified as cells with low side scatter that were lineage negative (CD19−CD45b−CD3e−Ly6G−) and CD115−CD11b+. This monocyte population was distinct from DCs identified as CD11c+CD11b−CD115+ (Supplementary Fig. 4a,b). Furthermore, we observed no difference in the number of CD11chi DCs in Nr4a1−/− mice versus wild-type mice (Supplementary Fig. 4a).

We refined our assessment of the CD115−CD11b+ monocyte population to distinguish between Ly6C+ and Ly6C− subsets (Fig. 2a,b) and found that the diminished monocyte population in Nr4a1−/− mice was due almost entirely to loss of the Ly6C− monocyte population (Fig. 2a,b). Ly6C− monocytes were missing from the bone

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**Figure 2** Absence of Ly6C− monocytes in Nr4a1−/− mice. (a) Expression of CD115 and CD11b by live Nr4a1−/− or wild-type (WT) cells with low side scatter (left), followed by further gating of CD115+CD11b+ monocyte populations for expression of Ly6C (right). Numbers adjacent to outlined areas indicate percent CD115+CD11b+ cells (left), Ly6C+CD11b+ cells (middle left) and frequency of Ly6C+ or Ly6C− monocytes among all live spleen cells (middle); frequency of Ly6C+ and Ly6C− monocyte populations in blood (middle right) and bone marrow (far right) of Nr4a1−/− mice and wild-type mice (n = 10 per group), analyzed by flow cytometry. *P < 0.001 (unpaired Student’s t-test). (c) Quantification of hematopoietic cell populations in the blood of Nr4a1−/− and wild-type mice (n = 10 per group), analyzed by flow cytometry. Mono, monocytes; Gran, granulocytes; NKT, natural killer T cells; B, B cells; T, T cells.

**Figure 3** Cell-intrinsic defect in monocyte development and lack of patrolling ability of monocytes from Nr4a1−/− bone marrow. (a) Ly6C+ and Ly6C− monocyte populations in the blood of irradiated (two doses of 600 rads each) Nr4a1−/− recipients reconstituted by transplantation of Nr4a1−/− (Nr4a1−/−→Nr4a1−/−) or wild-type (WT→Nr4a1−/−) whole bone marrow (5 × 10^6 cells), followed by reconstitution for 6 weeks (assessed as in Fig. 2a). (b) Quantification of Ly6C+ and Ly6C− monocyte populations in blood from irradiated wild-type or Nr4a1−/− recipients (n = 7 per group) of wild-type or Nr4a1−/− bone marrow (as in a). *P < 0.001 (unpaired Student’s t-test). (c) Tracking of CD11b+ cells in irradiated (9.5 Gy) wild-type CD45.1+ recipients reconstituted with bone marrow from wild-type or Nr4a1−/− CD45.2+ donors; anesthetized recipients were injected intravenously with 10 μg phycoerythrin-conjugated antibody to mouse CD11b (M1/70), then cell tracks (left) and displacement vectors of individual cells (red arrows, right) were assessed after 6 hours. Scale bars, 60 μm. (d) Patrolling CD11b+ cells in mice reconstituted with wild-type bone marrow (n = 2; three fields per hour) or Nr4a1−/− bone marrow (n = 4; seven fields per hour) as described in c. *P < 0.01 (unpaired Student’s t-test). Data are representative of two independent experiments (mean and s.e.m. in b,d).
marrow, blood and spleens of Nr4a1−/− mice (Fig. 2b). Ly6C− monocyte numbers were also slightly lower in the blood of Nr4a1−/− mice, although this observation was somewhat variable and not statistically significant. Except for slightly more granulocytes (<10%) in Nr4a1−/− mice than in wild-type mice, we found little variation in other subsets of cells of the immune response in blood (Fig. 2c) or spleen (Supplementary Fig. 4a).

To determine if the defect in Ly6C− monocyte differentiation in Nr4a1−/− mice was bone marrow derived and cell intrinsic, we engrafted irradiated Nr4a1−/− or wild-type recipients with Nr4a1−/− or control wild-type bone marrow (Fig. 3a, b). Grafting of wild-type bone marrow into Nr4a1−/− recipients restored the population of Ly6C− monocytes to the normal frequency (Fig. 3b). However, at 6 weeks after reconstitution of wild-type mice with Nr4a1−/− bone marrow, we observed 90% fewer Ly6C− monocytes in blood and a more modest 40% fewer Ly6C− monocytes (Fig. 3b).

Ly6C− monocytes have been shown to patrol the endothelium of blood vessels28,32. Therefore, we investigated the patrolling activity of Nr4a1−/− monocytes. We transplanted bone marrow from either wild-type or Nr4a1−/− CD45.2+ mice into irradiated wild-type CD45.1+ recipients (Supplementary Fig. 5). Recipients of wild-type bone marrow had a normal frequency of the Ly6C− monocyte subset in the blood (33% ± 1.4%), whereas recipients of Nr4a1−/− bone marrow had a much lower frequency of the Ly6C− subset (4% ± 1.4%; Supplementary Fig. 5). The number of patrolling cells in the blood vessels of the ears of recipients of Nr4a1−/− bone marrow (1.7 ± 1.3 cells per field per hour) was also much lower than that of mice that received wild-type bone marrow (12 ± 1.5 cells per field per hour; Fig. 3c, d). Together these data indicate that NR4A1 selectively regulates the production of patrolling Ly6C− monocytes in vivo.

**Figure 4** Normal stem cell populations and abnormal Ly6C− monocytes in Nr4a1−/− mice. (a) Quantification of hematopoietic stem cells (HSC), common myeloid precursors and MDPs in wild-type and Nr4a1−/− bone marrow (n = 10 mice per group), analyzed by flow cytometry. (b) Hema 3 staining of CD115+CD11b+ Ly6C+ or Ly6C− monocytes isolated from wild-type or Nr4a1−/− bone marrow by cell sorting. Scale bars, 10 μm. Data are representative of three independent experiments. Mean ± s.e.m.

**Figure 5** Specific defect in the differentiation of Nr4a1−/− Ly6C− monocytes from MDPs in the bone marrow. (a) Frequency of Nr4a1−/− and wild-type donor cells among Ly6C− and Ly6C+ monocytes and DCs in the spleen and Ly6C− and Ly6C+ cells in the blood of lethally irradiated wild-type CD45.1+ recipients (n = 6 per group) of Nr4a1−/− CD45.2+ and wild-type CD45.1+ whole bone marrow (2.5 × 10^6 cells from each donor, mixed at a ratio of 1:1). (b) Frequency of Nr4a1−/− and wild-type donor cells among Ly6C− and Ly6C+ cells in the blood of lethally irradiated wild-type CD45.1+ mice (n = 4 per group) that received Nr4a1−/− CD45.2+ and wild-type CD45.1+ MDPs (isolated by cell sorting from bone marrow, with 1 × 10^6 cells from each donor mixed at a ratio of 1:1), followed by reconstitution for 7 d. Data are representative of two independent experiments.

**Nr4a1−/−** MDPs do not differentiate into Ly6C− monocytes

The number of myeloid progenitor cells, including MDPs, was unaltered in Nr4a1−/− bone marrow (Fig. 4a). The Ly6C− monocytes that did appear in the bone marrow of Nr4a1−/− mice were morphologically larger and seemed less differentiated, with enlarged nuclei that were not horseshoe shaped and with more granularity than that of their counterparts in wild-type mice (Fig. 4b). It has been hypothesized that both Ly6C+ and Ly6C− monocytes arise from MDPs in the bone marrow, but the cellular factors that mediate their specific differentiation are unknown13. Our findings suggested that NR4A1 is a factor that regulates the differentiation of Ly6C− monocytes from MDPs in bone marrow.

We did competitive reconstitution experiments to further examine the role of NR4A1 in monocyte development. We mixed bone marrow from CD45.2+ Nr4a1−/− mice and CD45.1+ wild-type control mice at a ratio of 1:1 and transplanted the mixture into CD45.1+ recipient mice. At 6 weeks after bone marrow transfer, only 25% of Ly6C− monocytes and less than 5% Ly6C− monocytes in spleen (Fig. 5a) or blood (Fig. 5a) were derived from Nr4a1−/− bone marrow. In contrast, about equal numbers of CD11c+ DCs and all other CD45− cells were derived from CD45.2+ and CD45.1+ donors (Fig. 5a). Therefore, Nr4a1−/− progenitor cells were inefficient specifically in generating monocytes in general and Ly6C− monocytes in particular.

To determine if the expression of NR4A1 in MDPs was required for monocyte differentiation, we did mixed chimeric experiments with MDPs purified by flow cytometry. We mixed isolated MDPs (lineage-negative CD115+CD117int cells) from CD45.2+ Nr4a1−/− mice and CD45.1+ wild-type control mice at a ratio of 1:1 and transplanted the mixture into irradiated CD45.2+ recipient mice. At 7 d after bone marrow transfer, recipients had less than 10% Ly6C− monocytes and 30% Ly6C+ monocytes derived from Nr4a1−/− donors, whereas most monocytes were derived from wild-type donors (Fig. 5b). These results were similar to those obtained in the bone marrow–transplantation studies (Figs. 3a, b and 5a). Together the results indicate that NR4A1 expression is required for optimal differentiation of Ly6C− monocytes. These data also suggest that NR4A1 deficiency influences the competitive advantages of Ly6C− monocytes or their precursors, although to a lesser extent than it influences Ly6C+ monocytes.

**Nr4a1−/−** Ly6C− monocytes accumulate in S phase

Given that other members of the NR4A family have been associated with cell-cycle regulation, we next examined the progression of monocytes through the cell cycle. Unexpectedly, we found that Nr4a1−/− bone marrow had approximately threefold more Ly6C− monocytes in S phase and twofold more Ly6C− monocytes in G2 phase than did wild-type control bone marrow (Fig. 6a, b). To assess potential DNA damage in the normally dividing Ly6C− monocytes, we examined phosphorylation of histone H2AX at Ser139, a marker used to identify double-stranded DNA breaks during progression through the cell cycle.
We found evidence of greater DNA damage in Nr4a1−/−Ly6C− monocytes that were in S or G2 phase than in their wild-type counterparts (Fig. 6c,d). Transcripts of the S-phase mediators cyclin A2 and Cdk1 were about twofold greater in abundance in Ly6C− monocytes from Nr4a1−/− than those from wild-type bone marrow (Fig. 6e), which indicated an acceleration of entry into the cell cycle. Consistent with that, expression of the transcription factor E2F2, which represses entry into S phase, was about 45% lower in Ly6C− monocytes from Nr4a1−/− mice than in those from wild-type mice (Fig. 6e). We observed no difference in the cell-cycle progression of Ly6C+ monocytes or other cells in Nr4a1−/− bone marrow (data not shown). Thus, in Ly6C− monocytes, NR4A1 is required for progression through the cell cycle during bone marrow differentiation.

**Nr4a1−/− Ly6C− monocytes die in the bone marrow**

To determine the nature of the defect in monocyte production and cell-cycle arrest, we measured apoptosis of Ly6C+ and Ly6C− populations in wild-type and Nr4a1−/− mice. Annexin V and propidium iodide staining of monocyte populations in bone marrow, blood and spleen showed that Nr4a1−/− bone marrow had significantly more apoptotic and dead Ly6C− monocytes than did wild-type bone marrow (Fig. 7a,b). Approximately twofold more apoptotic and dead Ly6C− monocytes were present in Nr4a1−/− bone marrow than in wild-type control bone marrow (Fig. 7a,b). Additionally, we observed higher expression of cleaved caspase-3, another hallmark of apoptosis, in Ly6C− monocytes obtained from Nr4a1−/− bone marrow than in monocytes from wild-type control bone marrow (Fig. 7c,d). We found no

**Figure 6** Abnormal cell cycle and DNA damage in Ly6C− monocytes from Nr4a1−/− mice. (a) Flow cytometry analysis of the cell-cycle progression of wild-type and Nr4a1−/− Ly6C− bone marrow monocytes, stained with propidium iodide. Numbers above bracketed lines indicate percent cells in phases G0–G1 (left), S (middle) and G2 (right). (b) Quantification of results in a (n = 6 mice per group). P < 0.009, Nr4a1−/− versus wild-type, for G0–G1 and S (unpaired Student’s t-test). (c) Flow cytometry analysis of DNA damage in wild-type and Nr4a1−/− Ly6C− bone marrow monocytes during cell-cycle progression, assessed as phosphorylation of histone H2AX at Ser139 (p-H2AX(S139)) and propidium iodide staining. Numbers adjacent to outlined areas indicate percent cells in phases G0–G1 (left) or S–G2 (right). (d) Phosphorylation of histone H2AX at Ser139 in wild-type and Nr4a1−/− Ly6C− bone marrow monocytes. (e) Quantitative real-time PCR analysis of the expression of transcripts encoding cyclin A2 (Ccna2), Cdk1 (Cdc2a) and E2F2 (E2F2) in Ly6C− monocytes isolated by flow cytometry from Nr4a1−/− or wild-type bone marrow (n = 6 mice per group), presented relative to expression by wild-type cells. *P < 0.05 (unpaired Student’s t-test). Data are representative of three (a,b), two (c,d) or four (e) experiments (mean and s.e.m. in e).

**Figure 7** Greater apoptosis exclusively of Ly6C− bone marrow monocytes from Nr4a1−/− mice. (a) Apoptosis of Ly6C+ and Ly6C− monocytes from the bone marrow, spleen and blood of wild-type and Nr4a1−/− mice (n = 6 per group), assessed by flow cytometry analysis of annexin V (AnnV) staining. *P < 0.01 (unpaired Student’s t-test). (b) Apoptosis and cell death of Ly6C− bone marrow monocytes from wild-type and Nr4a1−/− mice (n = 6 per group), assessed as annexin V and propidium iodide staining, respectively (left). Numbers in quadrants indicate percent cells in each. Right, quantification of apoptotic and dead cells. *P < 0.01 (unpaired Student’s t-test). (c) Frequency of wild-type and Nr4a1−/− bone marrow monocytes (n = 8 mice per group) expressing cleaved (active) caspase-3, assessed by flow cytometry. *P < 0.009 (unpaired Student’s t-test). (d) Immunofluorescence microscopy of cleaved (active) caspase-3 (green) in Ly6C− bone marrow monocytes isolated by flow cytometry from wild-type and Nr4a1−/− mice; nuclei are stained with the DNA-intercalating dye DAPI. Scale bar, 5 μm. (e) Apoptosis of myeloid stem cell populations in bone marrow from wild-type and Nr4a1−/− mice (n = 6 per group), assessed by flow cytometry analysis of annexin V staining. Data are representative of three (a,b,e) or two (c,d) experiments (mean and s.e.m. in a–c,e).
alteration in the annexin V staining of Ly6C− monocytes in the blood or spleen (Fig. 7a), which suggested that the Ly6C− monocytes or their precursors underwent apoptosis and death in the bone marrow before they were able to egress to blood. We did not observe alterations in the apoptosis of myeloid progenitor populations in Nr4a1−/− bone marrow (Fig. 7e), which suggested that apoptosis occurs during monocyte maturation after the MDP stage. Notably, we did not observe apoptosis of Ly6C+ monocytes in Nr4a1−/− bone marrow (Fig. 7a), which indicated that in the steady state, NR4A1 expression is not critical for the differentiation or survival of Ly6C+ monocytes.

**Phenotype of Ly6C− monocytes in Nr4a1−/− mice**

Next we measured the expression of mRNA for factors known to be important for monocyte and macrophage differentiation. We found significantly lower expression of CX3CR1 (70% lower), C/EBPβ (90% lower) and JunB (70% lower) in Nr4a1−/− Ly6C+ monocytes than in their wild-type control counterparts (Fig. 8), which suggested less differentiation potential of the Ly6C+ subset. Pu.1 expression was not significantly different in Nr4a1−/− Ly6C− monocytes versus wild-type control Ly6C− monocytes. Moreover, Nr4a1−/− Ly6C− monocytes had more activation of the transcription factor NF-κB than did Nr4a1+/+ Ly6C− monocytes (Supplementary Fig. 6), which suggested alterations in the inflammatory phenotype of this monocyte subset in the absence of NR4A1. At the protein level, the expression of CX3CR1 and CCR2 in Ly6C− monocytes from Nr4a1−/− bone marrow was also lower than that in Ly6C− monocytes from wild-type bone marrow (Fig. 8a). Notably, the surface expression of CX3CR1 and CCR2 in Nr4a1−/− Ly6C− monocytes was similar to that on wild-type monocytes. Expression of CD11a (LFA-1), an integrin that is important for the patrolling function of Ly6C− monocytes, was about 60% lower in Ly6C− monocytes and 30% lower in Ly6C+ monocytes from Nr4a1−/− mice than in their counterparts from wild-type mice (Fig. 8a). Thus, Ly6C− monocytes in Nr4a1−/− bone marrow did not express phenotypic markers consistent with normal, differentiated Ly6C− monocytes, in further support of the proposal of impaired differentiation of this monocyte subset in the absence of NR4A1.

**DISCUSSION**

In this study we have shown that mice deficient in NR4A1 had a defect in bone marrow production of Ly6C− monocytes and, as a consequence, these mice lacked mature Ly6C− monocytes circulating in the blood and spleen and patrolling the vascular endothelium. The loss of this subset in the absence of NR4A1 was probably due to an intrinsic defect in hematopoietic cell differentiation. Resident MDP populations in the bone marrow and DC populations in the spleen were unaffected by the absence of NR4A1, which suggested a specific role for NR4A1 in mediating monocyte differentiation. The few Ly6C− monocytes that remained in the bone marrow in the absence of NR4A1 seemed unable to proliferate and differentiate properly; they were larger than Ly6C− monocytes from control mice and had lower expression of CX3CR1, CCR2 and CD11a (LFA-1). Nr4a1−/− Ly6C− monocytes also accumulated in S phase of the cell cycle, although they were unable to complete cell division, and underwent apoptosis during phases S and G2 of the cell cycle. Thus, our data have shown that NR4A1 functions in the bone marrow as a critical transcription factor required for the production of monocytes, particularly those of the Ly6C− subset.

Lower expression of the transcription factor E2F2, which is considered a critical repressor for cell commitment to S phase,44 may have been partially responsible for the premature entry into and lack of completion of S-phase cycling observed for Nr4a1−/− Ly6C− monocytes. Published findings showing that E2F family members are required for the survival of myeloid cells and their ability to respond properly to colony-stimulating factor 1 signals45 are consistent with our observations. The finding that NR4A1 may regulate cell-cycle progression in monocytes is perhaps not unexpected given that studies have shown that the closely related member of the NR4A family, NR4A2, has a similar regulatory role in maintaining the quiescence of hematopoietic stem cells through associated upregulation of cell-cycle inhibitors.26. Our finding of higher expression of NR4A2 in MDPs, the precursor that has been identified as being closest to monocyte populations, indicates that NR4A2 may regulate the homeostasis of monocytic and DC precursor populations. Upregulation of the cell-cycle initiators cyclin A2 and Cdk1 in Nr4a1−/− monocytes indicated that NR4A1 may have a similar role in maintaining the production of Ly6C− and possibly Ly6C+ monocytes.

An alternative explanation for our findings is that NR4A1 may act as a survival and/or differentiation factor in Ly6C− monocytes by encouraging the expression of receptors important for monocyte...
function, such as CX3CR1. CX3CR1 has been identified as a factor important for the survival of Ly6C− monocytes46. However, the absence of CX3CR1 or its ligand CX3CL1 in mice results in only modestly fewer Ly6C− blood monocytes under steady-state and inflammatory conditions46,47. The relationship between NR4A1 and the potential monocyte-survival factor CX3CR1 is uncertain, as are the identities of additional factors that drive the survival and differentiation of Ly6C− monocytes. Our data suggest that the defect in monocyte production in Nr4a1−/− mice is more probably upstream of CX3CR1, as Nr4a1−/− mice had fewer Ly6C− monocytes in the bone marrow, whereas CX3CR1-deficient mice do not46.

Although it is accepted that both monocyte subsets arise from a common MDP in the bone marrow47, the molecular control of their differentiation and the putative intermediates between MDPs and mature monocyte subsets have remained unclear. Specifically, whether Ly6C− monocytes differentiate directly from a bone marrow progenitor or from the conversion of mature Ly6C+ monocytes has been a matter of debate. Although mature monocytes recirculate in the bone marrow40, our data do not support the proposal that mature Ly6C+ monocytes convert into Ly6C− monocytes in the peripheral blood. Instead, our findings suggest that the precursor of Ly6C− monocytes is an immature proliferating cell.

Under conditions of competitive bone marrow transplantation, both Ly6C+ and Ly6C− monocyte subsets were diminished in the absence of NR4A1. Given that irradiation and reconstitution inevitably enhance inflammation, it is possible that stress or inflammatory factors resulted in NR4A1-mediated regulation of the Ly6C+ and Ly6C− monocyte populations. Expression of NR4A1 was present but low in Ly6C− monocytes and probably had some role in Ly6C− monocyte function. Notably, activation of the NF-kB pathway, a pathway important for the regulation of inflammation, was greater in both populations of monocytes from Nr4a1−/− bone marrow. Active NR4A1-binding sites have been identified on the promoter of the gene encoding the NF-kB inhibitory regulator IkBα and have been suggested as a means of downregulating NF-kB inflammatory signaling48. Such findings suggest that in both Ly6C+ and Ly6C− monocytes, NR4A1 has an important role in regulating NF-kB-mediated inflammatory responses and that the absence of NR4A1 may lead to an exaggerated inflammatory response11.

Inflammation also leads to substantially more circulating monocytes32,49. NR4A1 is well known to rapidly respond to early inflammatory events and may partially drive monocyte proliferation and/or extravasation. Ly6C− monocytes have been observed to extravasate and respond rapidly32,38,47 in a time frame very similar to that of NR4A1 induction in response to early inflammation11. However, inflammation would probably not overcome the defect in production of the Ly6C− monocyte subset in Nr4a1−/− mice. One example of this is the finding that irradiation of Nr4a1−/− mice did not correct the impaired production of the Ly6C− subset in our studies. However, inflammation may indeed modulate the number of Ly6C− monocytes and may also influence the functions of both monocyte subsets. This may vary depending on the type or model of inflammation studied. Further research is needed to understand exactly how NR4A1-mediated signaling pathways are involved in regulating both monocyte-subset differentiation and the specific function of these monocyte subsets in disease.

In summary, we have identified NR4A1 as a critical regulator of monocyte homeostasis in the bone marrow. Deletion of NR4A1 from hematopoietic cells led to loss of monocytes in vivo, particularly of the Ly6C− subset. Thus, NR4A1-mediated signaling pathways may be key targets for the development of therapeutics to regulate monocyte differentiation and function to control early inflammatory events in disease.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

R.N.H. and J.M.C. designed and did experiments, analyzed data and contributed to the writing of the manuscript; H.G.H. did experiments with NR4A1-GFP mice; D.N. and A.M.G. did experiments; I.A.P. conceived of the studies of NR4A1-GFP mice, analyzed data and contributed to the writing of the manuscript; F.G. conceived of and directed the research related to intravital microscopy; analyzed data and contributed to the writing of the manuscript; and C.C.H. conceived of the research, directed the study, assisted with experimental design and contributed to the writing of the manuscript.

COMPETING FINANCIAL INTERESTS

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**ONLINE METHODS**

Mice. C57BL/6 wild-type mice (006646) and Nr4a1−/− mice25 on a congenic C57BL/6 background (006187) were from The Jackson Laboratory, and B6.SJL-Ptprca/BoyJTac mice (004007) were from Taconic Farms. Mice were fed a standard rodent chow diet and were housed in microisolator cages in a pathogen-free facility. The NR4A1-GFP reporter mice were generated as described26 and are available from The Jackson Laboratory (016617). All experiments followed guidelines of the La Jolla Institute for Allergy and Immunology Animal Care and Use Committee, and approval for use of rodents was obtained from the La Jolla Institute for Allergy and Immunology according to criteria outlined in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Mice were killed by CO₂ inhalation.

**Flow cytometry.** Spleens were excised and pushed through a 70-μm strainer, bone marrow cells from both femurs and tibias were collected by centrifugation, and blood was obtained by cardiac puncture with an EDTA-coated syringe. All samples were collected in Dulbecco's PBS (Gibco) with 2 mM EDTA and were stored on ice during staining and analysis. Red blood cells were lysed in RBC Lysis Buffer according to the manufacturer's protocol (BioLegend).

Cells (2 × 10⁶ to 4 × 10⁶) were resuspended in 100 μl flow staining buffer (1% BSA (wt/vol) and 0.1% (wt/vol) sodium azide in PBS). Fc receptors were blocked for 15 min and surface antigens on cells were stained for 30 min at 4°C (flow cytometry antibodies, Supplementary Fig. 7). LIVE/DEAD Fixable Dead Cell Stain (Invitrogen) was used for analysis of viability, and forward- and side-scatter parameters were used for exclusion of doublets from analysis.

For additional intracellular staining, cells were fixed and made permeable with the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences). Cells were stained for 30 min at 4°C with directly conjugated fluorescent antibodies (Supplementary Fig. 7). Cells were stained for 1 h at 4°C with indirect antibodies, then were washed two times and stained for 30 min at 4°C with secondary antibody. CCR2 was stained as described27. Intracellular NR4A1 and CX3CR1 were stained with the appropriate secondary antibody: fluorescent isothiocyanate–conjugated antibody to mouse IgG (11-4011-85; eBiosciences) or to goat IgG (sc-2777; Santa Cruz Biotechnology).

The absolute number of cells was calculated by multiplication of the percentage of live cells in individual subsets by the total cell count before staining. Calculations of percentages were based on live cells as determined by forward and side scatter and viability analysis. Cell fluorescence was assessed with a FACSCalibur (BD Biosciences) and was analyzed with FlowJo software (version 9.2). Mean fluorescence intensity was quantified, and expression was calculated relative to that of the wild-type control.

Monocytes were identified as described (Supplementary Figs. 2 and 3). Hematopoietic stem cells were identified as lineage-negative CD117^Sca-1^-; common myeloid precursors were identified as lineage-negative CD117^Sca-1^-CD4^+^; MEPs were identified as lineage-negative CD115^CD117^-; DCs were identified as CD11c^hi^-; granulocytes were identified as Ly6G^-; natural killer T cells were identified as CD49b^-; B cells as CD19^-; and T cells were identified as CD3ε^-.

For staining of monocytes from NR4A1-GFP mice, blood was collected from the tail vein and heparin was added to inhibit clotting. Red blood cells were lysed in hypotonic ammonium chloride–potassium bicarbonate solution, and 5 × 10⁵ cells were incubated for 30 min at 4°C in 30 μl flow staining buffer (1% (wt/vol) BSA and 0.1% (wt/vol) sodium azide in PBS) with the appropriate antibodies in the presence of Fc Block (BD Biosciences). Cellular fluorescence was assessed with an LSR II, FACSaria II or FACScalibur (BD Biosciences) and data were analyzed with FlowJo software (TreeStar).

**Flow cytometry.** Before cell sorting, bone marrow was enriched for monocytes and stem cell populations by negative selection with isolation kits according to the manufacturer’s protocol (CD19, CD4, CD8, Ter119 custom ‘cocktail’ for monocytes, and lineage cell–depletion kit for stem cells (130090885); Miltenyi Biotec). Surface antigens on cells were then stained as described above, followed by cell sorting with a FACSaria (BD Biosciences). Approximately 5 × 10⁵ to 10 × 10⁵ events were collected for mRNA samples. The purity of monocyte and stem cell populations was verified by microscopy of cys otpin preparations of sorted cells stained with HEMA 3 (Fisher Scientific).

Quantitative real-time PCR. Monocyte and stem cell populations were isolated by flow cytometry and total cellular RNA was collected with an RNeasy Plus Micro Kit according to the manufacturer’s protocol (Qiagen). RNA purity and quantity was measured with a nanodrop spectrophotometer (Thermo Scientific). Approximately 500 ng RNA was used for synthesis of cDNA with an Iscript cDNA Synthesis Kit (Bio-Rad). Total cDNA was diluted 1:20 in H₂O, and a volume of 9 μl was used for each real-time condition with a MyQ Single-Color Real-Time PCR Detection System (Bio-Rad) and TaqMan Gene Expression Mastermix and TaqMan primers (Supplementary Fig. 8). Data were analyzed and presented on the basis of the relative expression method. The formula for this calculation was as follows: relative expression = 2^{(ΔΔCt)}

Bone marrow and MDP transplantation studies.Recipient mice (wild-type or Nr4a1−/−) were irradiated in two doses of 550 rads each (for a total of 1,100 rads) 4 h apart. Bone marrow cells from both femurs and tibias of donor mice (wild-type or Nr4a1−/−) were collected under sterile conditions. Approximately 5 × 10⁷ nucleated bone marrow cells were obtained from each donor mouse. Bones were centrifuged for the collection of marrow, then cells were washed and resuspended in Dulbecco's PBS for injection. Approximately 5 × 10⁶ unfractionated bone marrow cells in 200 μl media were delivered retro-orbitally into each recipient mouse. Recipient mice were housed in a barrier facility under pathogen-free conditions before and after bone marrow transplantation. After bone marrow transplantation, mice were provided autoclaved acidified water with antibiotics (trimethoprim–sulfamethoxazole) and were fed autoclaved food. Mice were used for experiments after 6 weeks of bone marrow reconstitution.

CD45.1 in B6.SJL-Ptprca/BoyJTac mice (wild-type CD45.1) and CD45.2 in Nr4a1−/− and C57BL/6 mice (wild-type CD45.2) were used for tracking cells in chimeras that received mixed–bone marrow transplantation. For this transplantation, 2.5 × 10⁶ cells from Nr4a1−/− mice (CD45.2^-^) and 2.5 × 10⁶ cells from B6.SJL-Ptprca/BoyJTac mice (wild-type CD45.1^-^) were mixed at a ratio of 1:1 for reconstitution of recipients (wild-type CD45.1^-^ or wild-type CD45.2^-^) as described above. For MDP chimeras, approximately 1 × 10⁵ cells from each donor (Nr4a1−/− mice and wild-type CD45.1^-^ mice) mixed at a ratio of 1:1 were used for reconstitution of recipients (wild-type CD45.1^-^ or CD45.2^-^). MDP transplantation was done as described above, except that MDP recipients received only one dose of radiation of 600 rads and were analyzed after only 7 d of reconstitution.

Annexin V analysis of apoptotic cells. Annexin V and propidium iodide were used for the identification of apoptotic and dead cell populations by flow cytometry. Bone marrow, blood and spleen cells were stained with annexin V (Invitrogen) and propidium iodide (Invitrogen) according to the manufacturers' protocols, after surface markers were stained to determine whether populations were monocytes or stem cells.

**Cell-cycle analysis.** For analysis of the cell cycle, first, identifying surface antigens on monocyte populations in the bone marrow were stained, then cells were fixed and made permeable with a Cytofix/Cytoperm Kit (BD Biosciences). Permeable cells were then stained for 30 min at 25°C with propidium iodide (50 μg/ml; Invitrogen), followed by one wash and resuspension in the flow staining buffer described above. For analysis of DNA damage, cells were also stained with fluorescence-conjugated antibody to H2AX, phosphorylated at Ser139 (2F3; BioLegend) during the incubation with propidium iodide. Cells were then analyzed on a linear scale for DNA content and then gated and were analyzed for the frequency of cells in phases G0–L, S and G2 of the cell cycle with FlowJo software.

**Immunofluorescence.** Cytospin preparations of bone marrow monocytes sorted from wild-type and Nr4a1−/− mice by flow cytometry were stained

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with antibody to cleaved (active) caspase-3 (Asp175; 9661; Cell Signaling), antibody to the p65 subunit of NF-κB (sc-8008; Santa Cruz Biotechnology) and DAPI (4,6-diamidino-2-phenylindole) and were visualized by immuno-fluorescence microscopy. Cytospin preparations were dried overnight, fixed for 20 min with 4% (wt/vol) paraformaldehyde, washed in PBS, made permeable for 5 min in a solution of 0.1% (wt/vol) citrate and 0.1% (vol/vol) Triton X-100 and washed three times in PBS. Cells were then incubated for 60 min in a solution of 5% (vol/vol) donkey serum with 0.2% (vol/vol) Triton X-100 in PBS and then were incubated overnight at 4 °C with primary antibody diluted in a solution of 0.2% (vol/vol) Triton X-100 and 1% BSA (wt/vol) in PBS. The next day, slides were washed three times in PBS and incubated for 1 h at 25 °C with fluorescent secondary antibodies at a dilution of 1:1,000; slides were then washed three more times in PBS and were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Rabbit antibody to mouse cleaved caspase-3 was used at a dilution of 1:500, followed by staining with Alexa Fluor 488–conjugated donkey antibody to rabbit (A-21206; Invitrogen). Mouse antibody to mouse p65 was used at a dilution of 1:33, followed by staining with Alexa Fluor 594–conjugated donkey antibody to mouse (A21203; Invitrogen). Images were acquired with a FluoView FV10i confocal laser-scanning microscope (Olympus) and analyzed with Imaris image-processing and analysis software (Bitplane). The frequency with which p65 fluorescence overlapped DAPI staining was quantified for wild-type and Nr4a1−/− Ly6C+ monocytes from the bone marrow.

Analysis of patrolling cells. Wild-type CD45.1+ mice were irradiated (9.5 Gy) and 3 h later were injected intravenously through the tail vein with 2 × 10⁷ bone marrow cells from wild-type (CD45.2+) mice or Nr4a1−/− (CD45.2+) mice (in 150 ml PBS). Before and after the procedure, mice were maintained in a sterile environment and were provided water containing an antimicrobial agent (Baytril; Bayer). Then, 6 weeks later, blood samples were obtained from the tail vein and mice were assessed for full chimerism and monocyte subset phenotype by flow cytometry. After blood was obtained, mice were anaesthetized by intraperitoneal injection of ketamine-xylazine-ACP and were given intravenous injection of 10 μg phycoerythrin-conjugated antibody to mouse CD11b (M1/70; Becton Dickinson). Blood vessels in the ear were imaged through a glass cover slip with a TCS SP5 inverted confocal microscope (Leica) with a diode-pumped solid-state laser line of 561 nm and a 20× water-immersion objective with a numerical aperture of 0.7. Both blood vessels (circulating antibodies) and CD11b+ cells (bound antibodies) were visualized over 1 h in several fields per mouse. These data were deconvolved (least-square constant-modulus blind algorithm; Autoquant X2; Media Cybernetics) and blood vessels of the ear were visualized and analyzed with Imaris software (Bitplane). CD11b+ cells in the blood vessels were tracked with the autoregressive-motion algorithm and tracks were filtered for a minimum track length of 30 μm from their origin and a minimum track duration of 3 min, then were manually assessed and edited for track continuity. For each field, tracks were ‘translated’ to a common origin in space to allow comparison (number, direction and displacement). Tracks were then counted and patrolling cells per field were plotted.

Statistical analysis. Data for all experiments were analyzed with Prism software (GraphPad). Unpaired t-tests and two-way analysis of variance were used for comparison of experimental groups. P values of less than 0.05 were considered significant.