Triad1 Granulopoiesis by Increasing Expression of HoxA10 Terminates Emergency

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HoxA10 Terminates Emergency Granulopoiesis by Increasing Expression of Triad1

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Expression of the E3 ubiquitin ligase Triad1 is greater in mature granulocytes than in myeloid progenitor cells. HoxA10 activates transcription of the gene encoding Triad1 (ARIH2) during myeloid differentiation, but the contribution of increased Triad1 expression to granulocyte production or function is unknown. Mice with bone marrow–specific disruption of the ARIH2 gene exhibit constitutive inflammation with tissue infiltration by granulocytes and B cells. In contrast, disruption of the HOXA10 gene in mice neither constitutively activates the innate immune response nor significantly alters steady-state granulopoiesis. This study explores the impact of HoxA10-induced Triad1 expression on emergency (stress) granulopoiesis. We found that mice with HOXA10 gene disruption exhibited an overwhelming and fatal emergency granulopoiesis response that was characterized by tissue infiltration with granulocytes, but reversed by re-expression of Triad1 in the bone marrow. We determined that HoxA9 repressed ARIH2 transcription in myeloid progenitor cells, antagonizing the effect of HoxA10 on Triad1 expression. Also, we found that differentiation-stage–specific ARIH2 transcription was regulated by the tyrosine phosphorylation states of HoxA9 and HoxA10. Our studies demonstrate a previously undescribed role for HoxA10 in terminating emergency granulopoiesis, suggesting an important contribution by Hox proteins to the innate immune response. The Journal of Immunology, 2015, 194: 5375–5387.

Granulocytes are produced by two distinct processes: steady-state granulopoiesis and emergency (or stress) granulopoiesis. Steady-state granulopoiesis is a homeostatic process that replaces cells lost to normal programmed cell death. In contrast, emergency granulopoiesis produces granulocytes in response to infectious or inflammatory stimuli, and contributes to innate immunity. In murine models, steady-state granulopoiesis is impaired by disruption of genes encoding G-CSF or GM-CSF, and requires the transcription factors PU.1 and C/EBPα (1–6). Emergency granulopoiesis is also impaired by loss of G-CSF in mice, but it is completely abolished by loss of the IL-1R (2, 7).

Emergency granulopoiesis has four stages: release of granulocytes from the bone marrow, expansion of hematopoietic stem cells and granulocyte/monocyte progenitor cells, acceleration of differentiation, and termination of the response. CXCR proteins regulate the first step; cells are protected from genotoxic stress during the second step by the Fanconi DNA–repair pathway; and the third step requires Stat3 and CEBPβ (8–11). Less is known about termination of emergency granulopoiesis, but dysregulation of this step is implicated in tissue damage during infectious challenge and in autoimmune diseases (12–16).

The hypothesis of this study is that increased expression of Triad1, an E3 ubiquitin ligase, is involved in termination of emergency granulopoiesis. Expression of Triad1 is known to increase during granulopoiesis and impair proliferation of myeloid progenitor cells, but substrates for Triad1 in hematopoietic cells are not defined (17–21). In epithelial cells, Triad1-dependent ubiquitination results in lysosomal degradation versus recycling (and sustained signaling) of the receptors for epidermal growth factor and growth hormone (22). In mice, homozygous knockout of the gene encoding Triad1 (ARIH2) is embryonic lethal at approximately embryonic day 16 due to hepatocyte apoptosis (19). ARIH2−/− fetal hematopoietic cells reconstitute hematopoiesis in irradiated wild type (Wt) mice, but recipients developed constitutive inflammation with tissue infiltration by B cells and granulocytes, suggesting involvement of Triad1 in immune regulation (19).

We previously identified tandem cis elements in the ARIH2 promoter that are activated by the homeodomain transcription factor, HoxA10 (21). Unlike ARIH2−/− mice, HOXA10−/− mice are viable; unlike mice transplanted with ARIH2−/− bone marrow, HOXA10−/− mice do not exhibit steady-state immune activation (23). We previously found that total protein ubiquitination increased during granulocyte differentiation in a HoxA10- and Triad1-dependent manner (21). Increased production of IL-1β during emergency granulopoiesis significantly increases expression of G-CSF relative to steady-state levels (1, 2). Therefore, it was of interest that we found HoxA10-dependent, increased Triad1 expression in murine myeloid progenitor cells stimulated with G-CSF in vitro (21). Also, we found that differentiation of myeloid cell line transfecants with retinoic acid/dimethyl formamide augmented activation of the ARIH2 promoter by HoxA10 (21). Retinoic acid/dimethyl formamide both differentiates and activates these cells, more closely modeling emergency granulopoiesis rather than steady-state (24). These results suggested conditional, rather than constitutive, Triad1 regulation by HoxA10.

Hox genes are found in four groups on four chromosomes in mouse and human (25). Although the HOX7-11 genes are maximally transcribed in committed progenitor cells, HoxA9 and HoxA10 proteins are present in granulocytes (26–29). Engineered overexpression of HoxA9 or HoxA10 in murine bone marrow induces granulocytosis in vivo that progresses to acute myeloid...
leukemia (30–32). However, preservation of steady-state granulopoiesis in mice with homozygous knockout of either HoxA9 or HoxA10 suggests that these proteins might be redundant with each other, or other Hox proteins, for this function (23, 33).

Most investigations of HoxA9 and HoxA10 have focused on identifying target genes that are relevant to the roles of these proteins in leukemogenesis. Work in our laboratory also identified target genes for HoxA9 and HoxA10 that are involved in phagocyte functions, including genes encoding gp91phox and p67phox, components of the phagocyte NADPH-oxidase (34–37). HoxA10 represses these genes in myeloid progenitors, but they are activated by HoxA9 during myelopoiesis (34, 37). Cytokine-induced phosphorylation of conserved, homeodomain tyrosine residues in HoxA9 and HoxA10 mediates these differentiation-specific effects (34–37). In contrast, we found cooperative, phosphorylation-independent activation of FGF2 transcription by HoxA9 and HoxA10 (41).

In these studies, we hypothesize that transcriptional activation of ARH2 by HoxA10 downregulates emergency granulopoiesis in a manner that is antagonized by HoxA9. This identifies modulation of protein ubiquitination/degradation as a novel mechanism for regulation of the innate immune response by Hox proteins. We hypothesize that modulation of innate immunity is an important, underexplored role for late Hox proteins in normal myelopoiesis.

Materials and Methods

Plasmid vectors

Human HoxA10 cDNA was obtained from C. Largman (University of California, San Francisco, CA) (42, 43). HoxA9 cDNA was obtained by PCR (12). Y326F/Y343F-HoxA10 (or HD-Y-mutant HoxA10) and Y212F/Y225F-HoxA9 (or HD-Y-mutant HoxA9) were generated by site-directed mutagenesis (37, 44). Triad1 cDNA was obtained from B.A. van der Reijden (Radboud University, Netherlands). Triad1-, HoxA10-, or HoxA9-specific shRNAs and scrambled controls were designed with the Promega Web site (Promega, Madison, WI) and subcloned into pLKO.1-promoter vector (21). Oligonucleotides with mutations in the Hox binding sites were determined in EMSA with a classical CCAAT box probe.

Additional constructs were generated with three copies of the ARIH2 promoter sequences in pGL3-promoter vector (Promega, as described previously (21)).

Oligonucleotides

Oligonucleotides were synthesized by MWG Biotech (Piedmont, NC). Double-stranded oligonucleotides used in EMSAs represented −22 to −48 bp (proximal: 5′-TTAAAAATATAAAATAATCCCTTTGTTCA-3′) or −174 to −198 bp (distal: 5′-CTTGCTCAATTTCAATATATCCATGGA-3′) from the ARH2 promoter (21). Oligonucleotides with mutations in the Hox binding sites were used in competition studies (mutant proximal, 5′-TTAAAAATATCCTTTAGAAATCTCTTTCA-3′; mutant distal, 5′-CTTGCTCAACGGAACGAATCATCCATGGA-3′) (21). Mutations are underlined.

Myeloid cell lines, culture, and analysis

The human myelomonocytic cell line U937 (24) was obtained from AS Kraft (Hollings Cancer Center, Medical University of South Carolina, Charleston, SC). Cells were treated for 48 h with IL-1β for granulocyte differentiation, as described previously (9, 45).

Stable transfecants. U937 cells were transfected by electroporation with a HoxA10 or HoxA9 expression vector, or both (or control vector), plus a vector with a neomycin resistance cassette (pSR207) (30 μg total) (37, 44). Stable transfecant pools were selected in G418 and aliquots tested for HoxA10 and HoxA9 by real-time PCR and Western blot. Other cells were transfected with a lentiviral vector for expression of Triad1-, HoxA10-, or HoxA9-specific shRNAs (or scrambled controls) (30 μg), selected in puromycin and tested for Triad1, HoxA10, or HoxA9 expression as described earlier (21).

Reporter assays. U937 cells (1.5 × 10⁵) were cotransfected with plasmids containing ARH2 promoter sequences linked to a Luciferase reporter (or control vector; 10 μg), and a vector to express shRNAs specific to HoxA9, HoxA10, HoxA9 + HoxA10 (or scrambled control vector; 30 μg) (0.24 V960 μg) (21). Other cells were cotransfected with a Luciferase reporter vector containing a minimal promoter and the −22 to −48 bp (proximal) or −174 to −198 bp (distal) ARH2 cis elements (or control vector; 10 μg) (21) and combinations of vectors to overexpress or knock down HoxA9 or HoxA10 (30 μg). Reporter assays were performed 24 h posttransfection ± IL-1β (to induce granulocyte differentiation) as described previously (9, 21). Cells were transfected with a β-galactosidase reporter plasmid to control for transfection efficiency (2 μg).

Real-time PCR

RNA was isolated with TRIzol reagent (Life Technologies-BRL, Gaithersburg, MD). Primers were designed with Applied Biosystems software and real-time PCR performed using SYBR green “standard curve” method. Results were normalized to 18S and γ-actin (for mRNA) or input chromatin (for chromatin immunoprecipitation). Primers were described previously (21).

Chromatin coimmunoprecipitation

Cells were incubated briefly in media supplemented with formaldehyde to generate DNA-protein cross-links (46). Lysates were sonicated to generate chromatin fragments with an average size of ∼100 bp, chromatin was immunoprecipitated with HoxA10, HoxA9, or control Ab, and amplified by real-time PCR, as described previously (47). HoxA10 antisera was obtained from Covance Research Products (Richmond, CA) and Ab to HoxA9 from Santa Cruz Biotechnology (Santa Cruz, CA). Each assay used 2 × 10⁶ murine bone marrow cells.

Protein assays

Western blot. Cells were lysed by boiling in 2× SDS sample buffer, lyase proteins were separated by SDS-PAGE, transferred to nitrocellulose, and Western blots probed with Abs to ubiquitin, HoxA9, HoxA10, Triad1, or Gapdh (loading control). Abs to Triad1 and Gapdh were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Each experiment was repeated at least three times with different lysates. For some studies, cells were pretreated with the lysosome inhibitor E64 to stabilize ubiquitinated proteins (48).

EMSAs. Nuclear proteins were isolated by the method of Dignam et al. (45, 49). Oligonucleotide probes were prepared and EMSA performed as described previously (21, 34). Three batches of nuclear proteins were tested in at least two independent experiments. Nuclear protein loading was determined in EMSA with a classical CCAAT box probe.

Statistical analysis

Statistical significance was determined by Student t test and ANOVA using SigmaPlot and SigmaStat software. Error bars represent SE. Statistical significance was a p value <0.02.

Murine studies

Ex vivo bone marrow studies. Mononuclear cells were obtained from the femurs of 5-week-old C57BL/6 mice. Lin−Sca1+ cells were separated using the Miltenyi magnetic bead system (according to manufacturer’s instructions; Miltenyi Biotech, Auburn, CA). Cells were cultured for 48 h in DMEM supplemented with 10% FCS, 1% pen-strep, and murine rGM-CSF (20 ng/ml), Sca1 (100 ng/ml), and IL-3 (10 ng/ml) (R&D Systems, Minneapolis, MN). 1 × 10⁶ cells/ml. Some CD44+ cells were separated for analysis (using the Miltenyi magnetic bead system), referred to as myeloid progenitor cells in this study. Other cells were differentiated after 24 h with G-CSF or IL-1β, as described previously (9, 30).

Bone marrow transplantation. Retrovirus was generated with Phoenix packaging cells according to manufacturer’s instructions (Strategene, La Jolla, CA). Lin−Sca1− bone marrow cells were incubated with retroviral supernatant (∼10⁶ PFU/ml) supplemented with polybrene (6 μg/ml) (30). Transgene expression was confirmed by real-time PCR. Transduction studies were repeated three times with at least two batches of retroviruses. Emergency granulopoiesis assay and analysis of peripheral blood and tissues. WT or HoxA10−/− mice were injected i.p. with OVA/Alum (referred to as Alum) or saline every 4 wk (seven mice per group). Alum was...
prepared as described previously and a 0.5 ml volume was injected i.p. (9). Some Wt mice transplanted with transduced HoxA10+/− bone marrow (six mice per group; see later) were similarly treated. Wt mice were littermates of HoxA10+/− mice (HoxA10+/− breeding pairs used because of HoxA10−/− infertility).

Peripheral blood was obtained from the tail vein every 2 wk, and complete blood counts were determined using an automated cell counter. Mice were sacrificed if Hgb was ≥6.0, platelets were ≥100,000, or granulocytes were ≥100,000. Sternal bone marrow and lung samples were stained using H&E by the Pathology Core Facility of the Robert H. Lurie Comprehensive Cancer Center. Light microscopy was performed and digital images were captured (40× magnification). Differential cell counts were obtained from 300 cells (in duplicate) on slides from three different animals.

Bone marrow transplantation. Lin−Sca1+ cells from the bone marrow of HoxA10+/− mice were transduced with a retroviral vector to express Triad1 (Triad1/murine stem cell vector [MSCV]) or empty control vector (MSCV), as described earlier. Viable cells were obtained by negative selection for Annexin V using Ab-conjugated magnetic beads (Miltenyi Biotech). Lethally irradiated Wt C57 Black 6 mice were injected (retro-orbital) with Lin−Sca1+ cells (2×105 cells).

Approvals. Animal studies were approved by the Animal Care and Use Committees of Northwestern University and Jesse Brown VA Medical Center.

Results

HoxA10−/− mice exhibit an overwhelming and fatal emergency granulopoiesis response

The survival of mice with disruption of the HOXA10 gene is comparable with Wt mice in a pathogen-free environment, consistent with a lack of constitutive activation of the innate immune response in HoxA10−/− mice (21, 23). In these studies, we investigated the contribution of HoxA10 to emergency granulopoiesis. Emergency granulopoiesis is studied in mice by i.p. injection method because it permits analysis of multiple episodes of emergency granulopoiesis without causing death in Wt mice (9). We compared Alum-injected HoxA10−/− or Wt mice with matched cohorts injected with saline, as controls for steady-state granulopoiesis. Mice were injected every 4 wk.

Although 100% of Wt mice survived three cycles of Alum injection, only 20% of HoxA10+/− mice were alive 2 wk after the first Alum injection, and no HoxA10−/− mice survived more than a few days after the second injection (Fig. 1A). At necropsy, Alum-injected HoxA10−/− mice exhibited enlarged livers and spleens in comparison with saline-injected HoxA10+/− mice, or Alum- or saline-injected Wt mice (~50% increase). Lungs appeared to have areas of consolidation, Wt and HoxA10−/− mice tolerated saline injection without morbidity of mortality.

In the first 24 h after Alum injection, we found an equivalent increase in circulating granulocytes in Wt or HoxA10+/− mice, representing release from the bone marrow (p > 0.2, n = 6). Initial granulocytosis resolved by 72 h in all Alum-injected mice (during the first cycle), consistent with transit from circulation to tissues. In Wt mice, circulating granulocytes began to increase again 10 d after Alum injection, were maximal at 14 d, and returned to baseline at 4 wk (Fig. 1B) (9). The increase in circulating granulocytes 14 d after Alum injection was significantly greater in HoxA10−/− mice versus Wt mice (p < 0.001, n = 6), with highest counts in the most severely affected animals (Fig. 1B). In HoxA10−/− mice, the number of circulating granulocytes did not return to baseline before the second injection.

Histological examination of sternal bone marrow from Wt mice revealed an increase in mature granulocytes 2 wk post Alum injection, as anticipated (Fig. 1C). At steady-state, HoxA10−/− murine bone marrow was not significantly enriched in granulocytes in comparison with Wt, but 2 wk post Alum-injection, the percent of granulocytes in HoxA10−/− sternal bone marrow was significantly greater than in Wt mice (44.6 ± 2.9% of nucleated cells for Wt mice versus 60.9 ± 2.6% for HoxA10−/− mice, n = 6, p = 0.005; Fig. 1C). Two weeks after Alum injection, granulocyte infiltration of the lungs was observed in HoxA10−/− mice, but not Wt (Fig. 1C). Pulmonary infiltration was not found in saline-injected Wt or HoxA10−/− mice. Granulocyte infiltration of the spleen and liver was also observed in Alum-treated HoxA10−/− mice, but not in Alum-injected Wt mice or saline-injected HoxA10−/− or Wt mice. At steady-state, expression of Triad1 mRNA in the bone marrow of HoxA10−/− mice was slightly less than Wt (p = 0.02, n = 4), and this difference increased significantly 2 wk post Alum injection (p = 0.001, n = 4; Fig. 1D). HoxA10 expression in Wt murine bone marrow was not significantly different 2 wk postinjection with Alum versus saline (p = 0.1, n = 4; Fig. 1D). Expression of HoxA10 decreases as steady-state granulopoiesis proceeds, but it has not been investigated in emergency granulopoiesis. Because HoxA9 shares some HoxA10 target genes, we also investigated HoxA9 expression. We found that HoxA9 mRNA was slightly decreased 2 wk post Alum injection in Wt and HoxA10−/− mice. HoxA9 expression was not altered by HoxA10 knockout (Fig. 1D).

Re-expression of Triad1 in HoxA10−/− bone marrow normalizes emergency granulopoiesis

To determine whether impaired Triad1 expression contributes to the abnormal emergency granulopoiesis phenotype of HoxA10−/− mice, we transduced HoxA10−/− Lin−Sca1− bone marrow cells with a retroviral vector to re-express Triad1 (Triad1/MSCV) or empty expression vector (MSCV). Transduced bone marrow was transplanted into lethally irradiated Wt mice. Cohorts of mice were injected with Alum or saline and analyzed as described earlier.

We found that mortality, numbers of circulating granulocyte, and granulocyte infiltration of the lungs after Alum injection were not significantly different in HoxA10−/− mice in comparison with Wt mice transplanted with control-vector–transduced HoxA10−/− bone marrow (Fig. 1A–C). In contrast, survival of Alum-injected mice transplanted with Triad1-transduced HoxA10−/− bone marrow was significantly better than HoxA10−/− mice (Fig. 1A). One hundred percent of mice transplanted with Triad1-transduced HoxA10−/− bone marrow survived the first cycle of Alum injection and 80% survived a second cycle. Circulating granulocytes returned to baseline between Alum injections in these mice (Fig. 1B).

Pulmonary infiltration with granulocytes was not found post Alum injection in mice transplanted with Triad1-transduced HoxA10−/− bone marrow (Fig. 1C). Two weeks after Alum injection, the percent of granulocytes in the bone marrow of control-vector–transduced HoxA10−/− recipients was significantly greater than in recipients of Triad1-transduced HoxA10−/− bone marrow (58.6 ± 3.2% of total nucleated cells for control vector versus 43.8 ± 2.7% with Triad1 vector; p = 0.006, n = 5; Fig. 1C). We found similar Triad1 expression in the bone marrow of Alum-injected Wt mice and mice transplanted with Triad1-transduced HoxA10−/− bone marrow (p = 0.6, n = 4; Fig. 1D).

HoxA10 and Triad1 influence protein ubiquitination and Fgf-R1 stability during emergency granulopoiesis

We hypothesize that HoxA10-induced expression of Triad1 during emergency granulopoiesis results in ubiquitination and degradation of proteins that facilitate proliferation of myeloid progenitor...
cells and/or enhance phagocyte function. Fgf-R1 is regulated by ubiquitin-mediated lysosomal degradation, and we investigated involvement of Triad1 in this process. Fgf-R1 was of interest for several reasons. First, Fgf2 binding to Fgf-R1 enhances proliferation of progenitor cells, but also primes mature granulocytes for activation (27, 38–40). Second, HoxA10 activates FGF2 transcription, but does not influence Fgf-R1 mRNA (27). Ubiquitination of Fgf-R1 by Triad1 would be a mechanism for HoxA10 to terminate effects of Fgf2.

To pursue this, we first determined whether loss of HoxA10 influenced total protein ubiquitination in murine bone marrow cells. For these studies, CD34+ cells were isolated from Wt or HoxA10<sup>2/2</sup> mice after 24 h of culture in GM-CSF, IL-3, and Sca1<sup>2</sup>Gr1<sup>2</sup> under myeloid progenitor conditions in these studies) and analyzed with or without IL-1β–induced differentiation (9, 30). We found that >70% of cells were Sca1<sup>-</sup>kit<sup>+</sup>CD34<sup>-</sup>CD38<sup>-</sup>Gr1<sup>-</sup> under myeloid progenitor conditions and >80% were CD34<sup>-</sup>CD38<sup>-</sup>Gr1<sup>-</sup> after treatment with IL-1β.

We analyzed cell lysates for protein expression and ubiquitination by Western blot, and for mRNA expression by quantitative real-time PCR. We found a significantly greater increase in total protein ubiquitination upon differentiation of Wt myeloid progenitor cells in comparison with HoxA10<sup>2/2</sup> cells (Fig. 2A). Differentiation with IL-1β significantly increased Triad1 protein (Fig. 2A) and mRNA (Fig. 2B) in Wt myeloid progenitor cells, but not HoxA10<sup>2/2</sup> cells, consistent with earlier in vivo results. Fgf-R1 protein was relatively less abundant in Wt versus HoxA10<sup>2/2</sup> cells, with and without IL-1β–induced differentiation (Fig. 2A). Expression of Fgf-R1 mRNA was not altered by HoxA10 knockout, consistent with our previous studies (data not shown) (21).
We next investigated the role of Triad1 in the decrease in total protein ubiquitination, and increase in Fgf-R1 protein, in HoxA10<sup>−/−</sup> bone marrow. For these studies, we transduced Lin<sup>−</sup>Sca1<sup>+</sup> bone marrow mononuclear cells with a retroviral vector to express Triad1 or with control MSCV vector. Cells were analyzed as described earlier. We found that re-expression of Triad1 in HoxA10<sup>−/−</sup> cells increased total protein ubiquitination and decreased Fgf-R1 protein (Fig. 2A). In control experiments, we demonstrated that expression of HoxA9 and HoxA10 mRNA were not altered by Triad1 re-expression (Fig. 2C).

**FIGURE 2.** HoxA10 influences total protein ubiquitination and Fgf-R1 protein stability in a Triad1-dependent manner. Bone marrow from Wt or HoxA10<sup>−/−</sup> mice was assayed under myeloid progenitor conditions (GM-CSF, IL-3, and Scf followed by CD34<sup>+</sup> separation) or after IL-1β-induced differentiation. Some cells were transduced with a Triad1 expression vector or MSCV control vector. (A) Loss of HoxA10 decreases IL-1β-induced total protein ubiquitination and increases Fgf-R1 protein, but this is reversed by re-expression of Triad1. Cell lysates were analyzed by Western blots probed with Abs to total ubiquitinated (Ub) protein, Triad1, Fgf-R1, or Gapdh (as a loading control). (B) HoxA10 knockout prevents increased Triad1 mRNA expression during IL-1β-induced differentiation. These cells were also analyzed for Triad1 mRNA expression by real-time PCR. Statistically significant differences in Triad1 mRNA abundance with versus without Triad1 vector are indicated by an asterisk (*). Statistically significant difference after IL-1β differentiation is indicated by double asterisks (**). (C) Triad1 overexpression does not alter HoxA9 or HoxA10 expression, and HoxA10 knockout does not alter HoxA9 expression. Cells were analyzed for HoxA9 or HoxA10 mRNA by real-time PCR. Statistically significant differences in HoxA10 mRNA abundance with HoxA10 knockout versus Wt cells are indicated by single (*) or double asterisks (**). (D) HoxA10 knockout decreases Fgf-R1 ubiquitination in a Triad1-dependent manner. The cells described earlier were treated with a lysosomal stabilizer (E64) before harvesting. Cell lysates were immunoprecipitated with an anti-ubiquitin Ab followed by Western blotting with Ab to Fgf-R1. Western blots of total cell lysates were probed with Ab to Gapdh as a loading control. Lysates that were immunoprecipitated with an irrelevant, control Ab demonstrated no immunoreactive Fgf-R1 on Western blot (data not shown). The p values <0.02 were considered statistically significant. All symbols represent p < 0.02.
To determine whether the observed increase in Fgf-R1 protein in HoxA10−/− cells was due to decreased ubiquitination, some of the cells (described earlier) were treated with a lysosomal stabilizer (E64) before harvesting, and lysate proteins were immunoprecipitated with an anti-ubiquitin Ab followed by Western blot for Fgf-R1. We found increased Fgf-R1 ubiquitination during IL-1β differentiation of Wt cells, but not HoxA10−/− cells. Fgf-R1 ubiquitination was greater in Wt versus HoxA10−/− cells but was increased by re-expressing Triad1 in HoxA10−/− cells (Fig. 2D).

HoxA9 and HoxA10 regulate ARIH2 transcription during emergency granulopoiesis

Mechanisms that regulate ARIH2 transcription during myelopoiesis are undefined. We hypothesized that activation of ARIH2 by HoxA10 during emergency granulopoiesis might be antagonized by HoxA9. This would be the opposite of the mechanism for regulation of phagocyte effector genes, which are repressed by HoxA10 in myeloid progenitor cells, but activated by HoxA9 during myelopoiesis (34–37). We investigated this hypothesis using ARIH2 promoter/reporter constructs designed around Hox-DNA-binding consensus sequences (previously described) (21, 52). U937 myeloid cells were cotransfected with these constructs and vectors to express shRNAs specific to HoxA9, HoxA10, or both, or control scrambled shRNA. Transfectants were assayed for reporter activity with or without IL-1β–induced differentiation (21, 24). We found that knockdown of HoxA9 significantly increased activity of constructs with 198 bp of ARIH2 promoter (p < 0.001, n = 6; similar results with 333 and 629 bp constructs), but not constructs with ≤167 bp (p > 0.6, n = 6; Fig. 3A). Differentiation significantly decreased this effect of HoxA9 knockdown (p < 0.01, n = 6 for percent repression by HoxA9 with versus without differentiation). Knockdown of HoxA10 decreased activity of the ARIH2 promoter constructs in a manner that was consistent with known HoxA10-binding cis elements between −32 to −41 and −182 to −191 bp (Fig. 3B) (21). Knockdown of HoxA9 + HoxA10 increased activity of 198-bp construct in untreated transfectants (p < 0.01, n = 6), but decreased activity in differentiated transfectants (p < 0.001, n = 6). Control experiments confirmed equivalent knockdown of HoxA9 and HoxA10 (Fig. 3C).

**FIGURE 3.** HoxA9 and HoxA10 regulate ARIH2 transcription during emergency granulopoiesis. (A) HoxA9 represses an ARIH2 cis element between −198 and −167 bp, but this effect is decreased by differentiation with IL-1β. U937 cells were cotransfected with promoter-reporter gene constructs with truncations of the ARIH2 5′ flank and vectors to knockdown HoxA9, HoxA10, or both. Asterisks or number signs denote statistically significant differences in reporter activity with versus without HoxA10-specific shRNA (* or #), with versus without HoxA9 shRNA (** or ##), and with versus without both shRNAs (*** or ###). Statistically significant difference in reporter activity with versus without IL-1β is indicated by ampersand (&). (B) Schematic identifying the ARIH2 cis elements. Human sequences are in black, murine in blue, and conserved sequences in gray. Hox-consensus sequences are in red, and the HoxA10-binding cis elements are underlined. Truncations used in reporter assays are indicated by arrows. (C) Control studies demonstrate equivalent shRNA knockdown of HoxA9 or HoxA10. Hox protein expression was determined by real-time PCR or Western blot (inset). Statistically significant differences in HoxA9 or HoxA10 mRNA with versus without knockdown of HoxA9 or HoxA10 are indicated by asterisks (* or **, respectively). The p values <0.02 were considered statistically significant. All symbols represent p < 0.02.
FIGURE 4. HoxA9 and HoxA10 are antagonists for ARIH2 transcription during emergency granulopoiesis. (A) HoxA9 antagonizes activation of the distal ARIH2 cis element by HoxA10 in untreated, but not in differentiating, transfectants. U937 cells were cotransfected with a reporter construct with three copies of the distal ARIH2 cis element linked to a minimal promoter and vectors to overexpress and/or knock down HoxA9 and HoxA10. Asterisks or number signs indicate statistically significant differences in reporter activity with versus without overexpression of HoxA10 (*) and with versus without overexpression of HoxA9 (** or ##). Statistically significant difference reporter activity in HoxA9 + HoxA10 overexpressing cells with versus without IL-1β is indicated by triple asterisk (***). Symbols indicate statistically significant differences in reporter activity in HoxA9 + HoxA10 overexpressing cells with versus without HoxA9 shRNA (### or &). Statistically significant differences in reporter activity with versus without expression of HoxA10-specific shRNA (@@@ or ^). (B) Blocking tyrosine phosphorylation decreases ARIH2 activation by HoxA10 and increases ARIH2 repression by HoxA9. U937 cells were cotransfected with the distal ARIH2 cis element reporter construct and vectors to express HoxA9 or HoxA10, forms of these proteins with mutation of tyrosine residues in the homeodomain (HD-Y-mut), or Wt Hox proteins + constitutively active Shp2 (E76K). Symbols indicate statistically significant differences in reporter activity in cells overexpressing Wt versus HD-Y-Mut HoxA9 or Wt versus HD-Y-Mut HoxA10 (*) or ***) in undifferentiated transfectants and in IL-1β differentiated transfectants (# or &). Symbols indicate statistically significant differences in reporter activity in HoxA9 or HoxA10 overexpressing cells with versus without E76K-Shp2 expression (** or #, respectively) in undifferentiated transfectants and in IL-1β differentiated transfectants (### or &). The p values <0.02 were considered statistically significant. All symbols represent p < 0.02.
To further investigate repression of *ARIH2* by HoxA9, we co-transfected U937 cells with a reporter construct that included three copies of the proximal or distal cis element linked to a minimal promoter plus vectors to knock down or overexpress various combinations of HoxA9 and HoxA10 (or relevant control vectors). Transfectants were analyzed for reporter activity with or without IL-1β differentiation. HoxA9 knockdown did not alter activity of the proximal *ARIH2* cis element (data not shown). However, distal cis element activity was increased by expression of HoxA9-specific shRNA (*p* < 0.001, *n* = 6) and decreased by HoxA10 knockdown (*p* < 0.01, *n* = 6; Fig. 4A). Simultaneously knocking down HoxA9 and overexpressing HoxA10 activated the distal cis element in comparison with overexpressing HoxA10 alone (*p* < 0.0001, *n* = 6; Fig. 4A). Also, HoxA10 knockdown in HoxA9-overexpressing transfectants further decreased cis element activity in comparison with overexpressing HoxA9 alone (*p* < 0.01, *n* = 6; Fig. 4A).

To determine whether phosphorylation of conserved, tyrosine residues in the homeodomains HoxA9 and HoxA10 influenced *ARIH2* promoter activity, we cotransfected U937 cells with the distal cis element/reporter construct and vectors to overexpress tyrosine-mutant forms of HoxA9 (referred to as HD Y-mut-HoxA9) or HoxA10 (HD Y-mut-HoxA10) (36, 37). As another method to prevent phosphorylation, we cotransfected other cells with vectors to overexpress HoxA9 or HoxA10 plus a constitutively active form of Shp2 (E76K). HoxA9 and HoxA10 are substrates for Shp2 only in myeloid progenitor cells, but E76K-Shp2 dephosphorylates these proteins throughout IL-1β–induced myelopoiesis (30, 36, 37). For these studies, we used an amount of E76K-Shp2 that had a minimal effect on the *ARIH2* cis element alone (determined by dose titration studies; data not shown). We found that distal cis element activation was less efficient in transfectants overexpressing HD Y-mut-HoxA10 or HoxA10 + E76K-

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**FIGURE 5.** Emergency granulopoiesis alters in vivo HoxA9 and HoxA10 binding to the *ARIH2* promoter. (A) Differentiation of murine myeloid progenitor cells with IL-1β or G-CSF increases in vivo HoxA10 binding and decreases HoxA9 binding to the distal *ARIH2* cis element. Murine bone marrow cells were assayed under myeloid progenitor conditions or after G-CSF– or IL-1β–induced differentiation. Chromatin was coimmunoprecipitated with Ab to HoxA9, HoxA10, or irrelevant control Ab and analyzed by real-time PCR with primers flanking the distal *ARIH2* cis element. Statistically significant differences in coprecipitation of cis element DNA with HoxA9 or HoxA10 in cells with versus without G-CSF or IL-1β–induced differentiation are indicated by asterisks or number signs (*, **, ***, or #). (B) Constitutive activation of Shp2-PTP increases HoxA9 binding to the distal *ARIH2* cis element, but decreases binding of HoxA10. Bone marrow cells from Wt mice were transduced with a retroviral vector to express E76K-Shp2 or with MSCV control vector. Cells were analyzed by chromatin immunoprecipitation with Abs to HoxA9 or HoxA10, as described earlier. Statistically significant differences in coprecipitation of cis element DNA with HoxA9 or HoxA10 in cells with versus without E76K-Shp2 expression are indicated by asterisks or number signs (*, **, ***, or #). (C) Constitutive activation of Shp2 blocks IL-1β–induced tyrosine phosphorylation of HoxA9 or HoxA10. Lysates from cells described earlier were subjected to immunoprecipitation with an anti-phospho tyrosine Ab (or irrelevant control Ab) followed by Western blots with Abs to total HoxA9 or HoxA10 protein. (D) Control studies demonstrate Shp2 overexpression in E76K-Shp2 transfused cells, but no change in expression of HoxA9 or HoxA10. Western blots of nonimmunoprecipitated proteins from the experiment described earlier were probed with Ab to Shp2, HoxA9, HoxA10, or Gapdh (as a loading control). The *p* values < 0.02 were considered statistically significant. All symbols represent *p* < 0.02.
Shp2 compared with transfectants with Wt HoxA10 alone, with or without IL-1β–induced differentiation (p < 0.0001, n = 6; Fig. 4B). Conversely, overexpression of either HD Y-mut-HoxA9 or HoxA9 + E76K-Shp2 more efficiently repressed the distal cis element than overexpressed Wt HoxA9 alone (p < 0.01, n = 6; Fig. 4B). We previously determined that Wt and HD Y-mutant proteins have comparable expression and stability in U937 cells (30, 36, 37).

**HoxA9 and HoxA10 interact with the ARIH2 promoter during emergency granulopoiesis**

To investigate interaction between HoxA9 or HoxA10 and the ARIH2 promoter during emergency granulopoiesis, we performed chromatin immunoprecipitation assays. For these studies, Wt murine bone marrow mononuclear cells were cultured under myeloid progenitor conditions (see earlier) and analyzed with or without differentiation with G-CSF or IL-1β (9, 30). We found that either cytokine significantly decreased in vivo interaction of HoxA9 with the distal ARIH2 cis element, but increased binding of HoxA10 (p < 0.001, n = 3; Fig. 5A).

Based on the reporter assays in the previous section, we also investigated the role of tyrosine phosphorylation on binding of HoxA9 or HoxA10 to the ARIH2 cis element. For these studies, Lin"Scal" murine bone marrow mononuclear cells were transduced with a retroviral to express constitutively active Shp2 (E76K) or with empty control vector. Cells were cultured under myeloid progenitor conditions, with or without IL-1β–induced differentiation, and chromatin immunoprecipitation assays were performed as described earlier. We found that constitutive activation of Shp2 significantly decreased HoxA10 binding to the ARIH2 promoter in IL-1β–differentiated cells, but increased HoxA9 binding under these conditions (for both experiments, p < 0.01, n = 4; Fig. 5B). In control experiments, lysate proteins from these cells were also analyzed for tyrosine phosphorylation of HoxA9 and HoxA10 by immunoprecipitation and Western blot. IL-1β increased tyrosine phosphorylation of both HoxA9 and HoxA10, but this was blocked in cells expressing E76K-Shp2 (Fig. 5C). Other control experiments verified that Shp2 was overexpressed in E76K-Shp2 transduced cells, but expression of total HoxA9 or HoxA10 protein was not altered (Fig. 5D).

We also performed in vitro assays as an additional approach to evaluate this interaction. For these studies, U937 nuclear proteins were preincubated with HoxA9, HoxA10, or irrelevant control Ab, and analyzed by EMSA. We previously demonstrated that the distal ARIH2 cis element probe generates a low-mobility, HoxA10-containing complex in such assays (21). In these studies, we found that HoxA9 Ab disrupted the complex most efficiently in assays with untreated cells, and HoxA10 Ab was most efficient with differentiated cells (Fig. 6A). In binding assays with unlabeled oligonucleotide competitors, we found cross-competitive binding specificities between the ARIH2 cis element and HoxA9/A10 binding cis elements from several previously described HoxA9/HoxA10 target genes, but not irrelevant oligonucleotide competitors (Fig. 6B).

**HoxA9 influences Triad1 expression and total protein ubiquitination**

We first investigated the influence of IL-1β on expression of HoxA9, HoxA10, and their common target genes in primary murine bone marrow cells. We found that expression of HoxA9 and HoxA10 increased in IL-1β–treated cells at early time points but decreased by 48 h (Fig. 7A). We also found an IL-1β–induced increase in expression of other Hox target genes involved in the innate immunity, including β3 integrin, Fgf2, and gp91phox (Fig. 7A) (27, 36, 47).
Based on our studies of the ARIH2 promoter, we hypothesized that Triad1 expression and total protein ubiquitination are inversely related to HoxA9 expression level. To investigate this, we transduced Wt Lin<sup>−</sup>Sca1<sup>+</sup> murine bone marrow cells with vectors to express HoxA9-specific shRNAs or scrambled shRNA control vectors. Transduced cells were analyzed by Western blot and real-time PCR under myeloid progenitor conditions (as defined earlier) or after differentiation with IL-1β. We found that HoxA9 knockdown increased total protein ubiquitination (Fig. 7B) and expression of Triad1 protein (Fig. 7B) and mRNA (Fig. 7C), consistent with our hypothesis. Control studies verified knockdown of HoxA9.

To investigate the influence of Triad1 on HoxA9-related changes in protein ubiquitination, we also transduced Lin<sup>−</sup>Sca1<sup>+</sup> bone marrow cells with retroviral vectors to express Triad1-specific shRNAs, with or without HoxA9 shRNAs. Cells were analyzed as described earlier. We found that blocking Triad1 expression decreased total protein ubiquitination in cells with HoxA9 knockdown (Fig. 7B). Control studies demonstrated decreased expression of Triad1 protein (Fig. 7B) and mRNA (Fig. 7C) with these shRNA vectors.

**HoxA9 and Triad1 influence cell proliferation**

In previous studies, we found that the HoxA10-induced Triad1 expression antagonized the net proproliferative effect of overexpressing HoxA10 in myeloid progenitor cells (21). Based on the results in this work, we hypothesize that repression of ARIH2 by HoxA9 facilitates cytokine-induced proliferation in HoxA9 overexpressing cells. To investigate this, we transduced Lin<sup>−</sup>Sca1<sup>+</sup> murine bone marrow cells with retroviral vectors to express HoxA9, Triad, or both (or control vectors). Cells were analyzed for proliferation in response to a dose titration of GM-CSF. We found that GM-CSF induced significantly more proliferation in HoxA9-overexpressing cells in comparison with control cells at most doses ($p$, $0.01$, $n = 6$; Fig. 8A). We also found that re-expression of Triad1 decreased GM-CSF hypersensitivity in HoxA9-overexpressing cells (Fig. 8A). In control studies, we found that HoxA9 overexpression significantly decreased Triad1

**FIGURE 7.** HoxA9 and HoxA10 regulate Triad1 expression during IL-1β–induced differentiation of murine myeloid progenitor cells. (A) Expression of HoxA9, HoxA10, and target genes that are relevant to innate immunity increases during differentiation with IL-1β. Murine bone marrow cells were cultured under myeloid progenitor conditions with or without differentiation with IL-1β. Expression of mRNA for HoxA9, HoxA10, β3 integrin, Fgf2, gp91<sup>phox</sup>, or Triad1 was determined by real-time PCR. Statistically significant differences in mRNA expression of these genes in IL-1β–differentiated cells versus control cells are indicated by asterisks and number signs (*, **, ***. #, ##, or ###, respectively). (B) Knockdown of HoxA9 increases total protein ubiquitination in a Triad1-dependent manner. Bone marrow–derived myeloid progenitor cells were transduced with retroviral vectors to express shRNAs specific to HoxA9, Triad1, both, or control (scrambled) shRNAs and analyzed with or without IL-1β differentiation. Western blots of lysate proteins were probed with Abs to total ubiquitinated proteins, Triad1, or Gapdh as a loading control. (C) Knockdown of HoxA9 increases Triad1 mRNA expression. These cells were also analyzed by real-time PCR for mRNA abundance of Triad1 and HoxA9. Asterisks and number signs denote statistically significant differences in Triad1 expression with versus without HoxA9 knockdown (* or #) and with versus without knockdown of Triad1 (** or ##). Asterisks and number signs denote statistically significant differences in Triad1 expression in cells with Triad1 knockdown with versus without HoxA9 shRNA (*** or ###). Ampersands denote statistically significant differences in HoxA9 mRNA with versus without HoxA9 knockdown (& or &&). The $p$ values <0.02 were considered statistically significant. All symbols represent $p < 0.02$. 

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expression, with or without IL-1β–induced differentiation (p < 0.001, n = 4; Fig. 8B). These studies also found overexpression of Triad1 and that overexpressed Triad1 did not influence HoxA9 mRNA abundance.

Discussion
In this work, we found that mice with knockout of HoxA10 in the bone marrow have a fatal emergency granulopoiesis response, a phenotype this is rescued by re-expression of Triad1. Inflammatory infiltrates and granulocytosis are not observed at steady-state in HoxA10−/− mice, but constitutive inflammation is a characteristic of mice with Triad1−/− bone marrow (19, 23). Therefore, our studies suggest that conditional regulation of ARIH2 by HoxA10 contributes to terminating the innate immune response. Hox proteins were not previously known to modulate emergency granulopoiesis (or any other process) by facilitating ubiquitin-mediated protein degradation. We found that expression of Triad1 and transcription of ARIH2 during emergency granulopoiesis were dependent on tyrosine phosphorylation of HoxA10. Also, non-tyrosine phosphorylated HoxA9 antagonized HoxA10 by repressing ARIH2 in myeloid progenitor cells. Therefore, these Hox proteins cooperate to regulate the innate immune response in addition to performing their well-described proto-oncogene functions during leukemogenesis.

Mice with Triad1−/− bone marrow exhibit constitutive infiltration of tissues with granulocyte and B cells, suggesting that Triad1 controls inflammatory processes (19). We find only slightly decreased Triad1 expression in the bone marrow of HoxA10−/− mice at steady-state, but an absence of normal induction of Triad1 expression during emergency granulopoiesis. Although HoxA10-induced Triad1 expression is important for termination of emergency granulopoiesis, our studies do not exclude a role for Triad1 in the steady-state process. Conversely, steady-state granulopoiesis is relatively normal in HoxA10−/− mice, suggesting that regulation of emergency granulopoiesis may be a major, but previously unknown, function of HoxA10.

Total protein ubiquitination increases in a HoxA10- and Triad1-dependent manner in differentiating myeloid cells. Many proteins involved in innate immunity are metabolized by ubiquitination-mediated degradation, including Fgf-R1, gp91phox, and intermediates downstream from the IL-1R (29, 53, 54). HoxA9 protein may also be regulated by ubiquitination (55). Such proteins

FIGURE 8. Regulation of Triad1 by HoxA9 influences cell proliferation. (A) Triad1 reverses HoxA9-induced cytokine hypersensitivity. Murine myeloid progenitor cells were transduced with retroviral vectors to express HoxA9, Triad1, both, or control vector and analyzed for proliferation in response to a dose titration of GM-CSF. Statistically significant differences in cell proliferation at a given cytokine dose (as determined by [3H]thymidine uptake) are indicated by asterisks (* or **). (B) Overexpression of HoxA9 decreases Triad1 expression. Murine bone marrow cells described earlier were analyzed for Triad1 and HoxA9 expression by real-time PCR. Statistically significant differences in Triad1 mRNA abundance with versus without HoxA9 over-expression are indicated by asterisks or number signs (*, **, *** or #). Statistically significant difference in HoxA9 mRNA abundance with versus without HoxA9 overexpression is indicated by number signs (##). The p values <0.02 were considered statistically significant. All symbols represent p < 0.02.

FIGURE 9. Schematic representation of regulatory network involving HoxA10, HoxA9, Triad1, and Fgf2. Regulation of Triad1 influences progenitor proliferation and phagocyte function via Fgf2 and Fgf-R1.
may be direct substrates for Triad1 or for another ligase that is regulated by Triad1 (56). Previous studies indicated that HoxA10 inhibits granulocyte function by repressing phagocyte effector genes in myeloid progenitor cells, including genes encoding the rate-limiting NADPH-oxidase proteins and secondary granule proteins (34–36, 57, 58). Activation of ARIH2 is a novel immune-modulatory mechanism for HoxA10, involving induction of protein degradation. Conversely, HoxA9 has been shown to activate genes encoding NADPH-oxidase proteins and E-selectin in differentiating phagocytes (37, 41). ARIH2 repression may enhance proinflammatory effects of HoxA9 by stabilizing these or other inflammatory mediator proteins.

Although HoxA9 and HoxA10 mRNA are maximally expressed in myeloid progenitors, HoxA9 and HoxA10 proteins are relatively stable and found in granulocytes (25, 26). Consistent with this, we found that phosphorylation of conserved tyrosine residues in the homeodomains HoxA9 and HoxA10 during IL-1β-, G-CSF-, or M-CSF–induced differentiation of myeloid progenitor cells influences expression of some target genes (34–37). HoxA10 phosphorylation decreased affinity for cis elements in the NADPH-oxidase genes, but increases the affinity for ARIH2 (34–37). Conversely, HoxA9 phosphorylation increased affinity for NADPH-oxidase genes, but decreases affinity for ARIH2 (34–37). Differences between HoxA9 and HoxA10 in the impact of homeodomain phosphorylation on target gene regulation may reflect the influence of nonconserved domains outside of the homeodomain. For example, we previously defined activation and repression domains in HoxA10 that are not present in HoxA9 (47, 59).

In contrast with these genes, HoxA9 and HoxA10 cooperate to activate the FGFR2 gene in a manner that is not influenced by tyrosine phosphorylation (27, 38). Instead, our present studies suggest that HoxA10 regulates the effects of Fgfr2 via Triad1-induced ubiquitination and degradation of Fgfr-R1. This may terminate the effect of Fgfr2 on progenitor expansion and phagocyte function, and downregulate emergency granulopoiesis (Fig. 9).

The physiology of emergency granulopoiesis is relevant to human autoinflammatory joint diseases (12–16). IL-1 promotes myeloid progenitors (Blood 106: 418–428, 2005) and proinflammatory effects of HoxA9 by stabilizing these or other proinflammatory mediator proteins. HoxA9 has been shown to activate inflammatory mediator proteins (34–36, 57, 58). Activation of FGFR2 in the sustained hematopoietic response of Listeria monocytogenes–infected mice (J. Exp. Med. 201: 1487–1502).

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