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Transcription factor Dlx3 induces aryl hydrocarbon receptor promoter activity

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ABSTRACT

The Distal-less (Dlx) homeobox transcription factors (TFs) play a prominent role in regulating multiple facets of vertebrate biology. Though widely studied as mediators of tissue development, recent work has uncovered a role for this TF family in modulating the vertebrate hematopoietic compartment. Pertinent to our study, murine *Dlx1-3* are expressed in an innate lymphocyte population known as natural killer (NK) cells, and they are implicated to assume a functional role in the NK cell maturation pathway. However, Dlx target genes are poorly understood. In *Drosophila*, the invertebrate *Dlx* ortholog *Distal-less* (*Dll*) regulates another transcription factor called *Spineless* (*ss*), which is critical for specifying distal antennal segments. Importantly, the vertebrate ortholog of *ss* is the aryl hydrocarbon receptor (AhR), a transcription factor recently shown to be important in the regulation of a number of immune cell subsets, including NK cells. Given these findings, we investigated whether Dlx TF family members might analogously regulate *AhR* in an NK cell context. Our results demonstrate that *Dlx3* is constitutively co-expressed with *AhR* in murine and human CD127^{+} NK cells. Critically, we show that Dlx3 induces *AhR* promoter activity by binding to a regulatory region that resides ~5.5 kb upstream of the transcriptional start site. This mechanism is functionally relevant, as *Dlx3* expression in human NK cells significantly enhances TF activity at AhR DNA-binding elements (Xenobiotic Responsive Elements, XREs). Thus, our study defines Dlx3 as a positive regulator of the aryl hydrocarbon receptor.

1. Introduction

Natural killer (NK) cells are an innate lymphocyte population in vertebrates that provide immediate defense against viral pathogens and malignant cells via both cell contact-dependent and –independent mechanisms. In humans, NK cells are generally defined as CD3^{-}CD56^{+} cells, and in C57BL/6 mice, NK cells are defined as CD3^{-}NK1.1^{+} cells. Found in a wide range of organs (i.e. liver, bone marrow, spleen, lymph node, thymus, etc.), the development of this phenotypically and functionally heterogeneous lymphocyte population involves the sequential acquisition and loss of various cell surface markers and transcription factors (TFs) [reviewed in Ref. [1]]. In brief, conventional NK cells in both humans and mice are derived from a common lymphoid progenitor (CLP) cell present in the bone marrow [1]. Once commitment to the NK cell lineage has occurred in an interleukin (IL)-15-dependent manner, the early acquisition of CD56 expression on human NK cells (CD56^{bright}) and CD27 on mouse NK cells (CD27^{high}) marks an immature phenotype, which is functionally associated with robust cytokine secretion but not target cell killing [1]. However, downregulated CD56 expression and augmented CD16 expression correlates with human NK cell maturation, whereas a loss of CD27 and the acquisition of CD11b correlates with mouse NK cell maturation [1]. Furthermore, expression of T-bet, a T-box TF, is activated in immature NK cells to facilitate their developmental stability and terminal maturation [2–4]. In both humans and mice, these phenotypic changes are generally associated with NK cells exhibiting a heightened capacity for target cell killing coming at the expense of cytokine secretion. However, in addition to conventional NK cells, recent work has begun to uncover the vast tissue-specific developmental and functional heterogeneity characteristic of this lymphocyte population [reviewed in Ref. [5]]. Pertinent to this study, a subset of NK cells that uniquely expresses CD127 (IL-7 receptor α) has been previously suggested to predominately reside in the thymus and lymph nodes where their development uniquely depends on the TF GATA-3 [5]. Functionally, the “thymic” NK cells behave similar to immature conventional NK cells in that they are able to robustly secrete cytokines but fail to efficiently kill target cells [5]. Altogether, characterization of the developmental and

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functional regulators of these various NK cell subsets remains an intense area of investigation.

The Distal-less (Dlx) homeobox TF family in vertebrates consists of six members that are expressed during development according to tissue type and spatiotemporal dynamics [6]. These gene regulators are critical for the specification and function of various tissues and anatomical structures, such as the nervous system and craniofacial skeleton [7,8]. In addition, Dlx proteins have been attributed to regulating hematopoietic cell proliferation and survival, as well as lymphopoiesis [6,9]. Relevant to our study, *Dlx1-3* are expressed in NK cells, with *Dlx3* being most highly expressed in immature $CD11b^{lo}$ NK cells [9]. Furthermore, *Dlx1* and *Dlx3* play an important role in early NK cell development, as persistent expression of either gene arrests NK cells in an immature phenotype while modulating the downstream expression of TFs involved in NK cell maturation [9]. However, characterization of Dlx target genes in vertebrates remains incomplete.

The aryl hydrocarbon receptor (AhR) is a transcriptional regulator of the Per (period circadian protein)-Arnt (aryl hydrocarbon receptor nuclear translocator protein)-Sim (single-minded protein) (PAS) superfamily of proteins that undergo activation in response to numerous endogenous metabolites, dietary compounds, and environmental contaminants/toxins [10,11]. Upon ligand binding, AhR translocates from the cytoplasm to the nucleus where it binds the aryl hydrocarbon receptor nuclear translocator (ARNT) to modulate gene expression by engaging segments of DNA known as Xenobiotic Responsive Elements (XREs). Initially studied in the immune system as a regulator of T helper 17 (Th17) cell and regulatory T (Treg) cell differentiation and development [12,13], AhR has been further implicated to play a role in mediating the murine NK cell anti-tumor [14] and IL-10 responses [15], and its expression and function has been described in non-conventional, IL-22 producing human NK cells [16], among other immune cell types [17]. What determines *AhR* gene expression in these contexts is poorly understood.

In *Drosophila*, the invertebrate *AhR* ortholog, *Spineless* (*ss*), plays a key role in specifying distal antennal segments during development [18]. Critically, its expression is positively regulated by the *Dlx* ortholog, *Distal-less* (*Dll*), which serves as a primary activator of the *ss* antennal disc enhancer [19–21]. Thus, given these previous findings, along with what has been observed regarding the expression of Dlx TFs and AhR in lymphocytes, we hypothesized that Dlx proteins could similarly regulate *AhR* promoter activity in the vertebrate NK cell setting. In this study, we show that *Dlx3* and *AhR* are abundantly co-expressed in murine and human $CD127^+$ NK cells. Our data further reveal that a segment of the *AhR* promoter, ~5.5 kb upstream of the transcriptional start site, is uniquely bound by Dlx3 to induce *AhR* expression. In the human NK cell line NK-92MI, Dlx3 enhances AhR activity at XRE sequences, thus describing a previously unidentified mechanism whereby vertebrate Dlx3 positively regulates AhR.

2. Materials and methods

2.1. Mice and human subjects

C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and bred in facilities at Stanford University (Stanford, CA, USA). All mice were housed in pathogen-free conditions with free access to food and water. 6- to 8-week old mice were used for experiments. Procedures performed in this study were all in accordance with the Stanford University Institutional Animal Care and Use Committee guidelines and with the National Institutes of Health guide for the care and use of laboratory animals. Human blood samples, obtained with informed consent, were provided by the Stanford School of Medicine Blood Center (Palo Alto, CA, USA).

2.2. Cell culture

293T human embryonic fibroblast cells were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies) at 37 °C in 5% CO_2. The human NK-92MI cell line was cultured in RPMI Medium (Life Technologies) supplemented with 2 mM L-glutamine, 0.2 mM I-inositol, 20 mM folic acid, 100 μM β-mercaptoethanol, 12.5% FBS, and 12.5% horse serum (Stemcell Technologies, Vancouver, BC, CAN). Culture media were renewed every 2–3 days depending on cell density, and sub-culture was conducted when confluence was reached.

2.3. Cell sorting

Spleens and lymph nodes from C57BL/6 mice were isolated, mechanically homogenized, and stained with PerCP-Cy5.5-anti-mouse CD3, APC-anti-mouse NK1.1, and PE-anti-mouse CD127 antibodies (all from BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocols. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers after Ficoll gradient centrifugation (GE Healthcare, Piscataway, NJ, USA). Mononuclear cells were resuspended in RPMI media and stained with PerCP-Cy5.5-anti-human CD3, APC-anti-human CD56, and PE-anti-human CD127 antibodies (all from BD Biosciences) according to the manufacturer's protocols. Labeled cells were analyzed and sorted using a FACSAria III cell sorter (BD Biosciences).

2.4. Plasmid construction

To construct pMXs-Flag-Dlx3-IRES-EGFP, an *Eco*RI/*Xho*I fragment generated by PCR amplification of mouse and human *Dlx3* from the PCR cloning vector PCR-XL-Topo (Invitrogen, Carlsbad, CA, USA) was sub-cloned into the *Eco*RI/*Xho*I site of pMXs-Flag-IRES-EGFP (a kind gift from Wayne Yokoyama, Washington University, St. Louis, MO, USA). pMXs-Flag-mDlx1 and -mDlx2 were also constructed by sub-cloning PCR products. pEGFP-Oct4 was constructed by sub-cloning *Oct4* PCR products into pEGFP-C1 (Clontech Laboratories, Mountain View, CA, USA). Promoter regions AhR-8 (−5638/−5403), AhR-8/9 (−5638/−3981) and AhR-P (−1805/−375) of the *AhR* gene were incorporated into the *Xho*I/*Hin*dIII sites of the pGL3 basic luciferase reporter (Promega, Sunnyvale, CA, USA) using PCR amplification and sub-cloning to generate pGL3-basic-AhR vectors.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

For examining the effect of Dlx3 on *AhR* expression, pMXs-Flag-hDlx3 was transfected into human NK-92MI cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). RNA was isolated 48 h after transfection and subjected to qRT-PCR probing for *hAhR* expression. For all qRT-PCR assays, total RNA was prepared from cells with the RNeasy Mini Kit (Qiagen, Hilden, DEU) and treated with DNase I (STEMCELL Technologies, Seattle, WA, USA) to remove residual genomic DNA. cDNA was prepared with random primers using the First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was done in triplicate with TaqMan PCR mixture (Applied Biosystems, Foster City, CA, USA) for 2 min at 50 °C and 10 min at 95 °C for initial denaturing, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min using the 7900HT Fast Real-Time PCR System (Applied

Biosystems, Foster City, CA, USA). All human and mouse primers were purchased from Applied Biosystems, Foster City, CA, USA. The expression of genes was normalized to the housekeeping *HPRT* gene. Fold changes in expression for each gene were calculated as $2^{-\Delta(\Delta CT)}$ where $\Delta C_T = C_T$ (target) $- C_T$ (housekeeping), and $\Delta(\Delta C_T) = \Delta C_T$ (treated) $- \Delta C_T$(control). Reaction product purity was confirmed through examination of melting curves for a single peak.

2.6. Chromatin immunoprecipitation (ChIP) assay

To elucidate the *AhR* binding site for mouse Dlx3, ChIP assays were conducted with a ChIP-IT Enzymatic Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. In brief, 293T cells were transiently transfected with pMXs-Flag-mDlx3-IRES-EGFP and an *AhR*-containing bacterial artificial chromosome (BAC) clone (AhR genomic clone RP23-157I22; BACPAC Resources, Oakland, CA, USA). Cells were treated with 1% formaldehyde followed by glycine stop solution, collected, centrifuged at 4 °C, and resuspended in lysis buffer. After an additional 30 min of lysis and 10 strokes in a dounce homogenizer, nuclei were isolated by 10 min of centrifugation at $2400 \times g$ and resuspended in shearing buffer. Genomic DNA was sheared by sonication on ice, and after centrifugation, 10 µl of the supernatant (containing sheared chromatin) was used as assay input. Immunoprecipitation was carried out overnight at 4 °C using an incubation mixture containing 10 µl sheared chromatin, protein G magnetic beads, BSA, sheared sperm DNA, and 3 µg of anti-Flag M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation, washing, and elution, samples were reverse cross-linked and treated with ribonuclease A and proteinase K. DNA was then purified using DNA minicolumns provided with the kit, and PCR was performed with primers indicated in Supplementary Table 1.

2.7. Transient transfection and luciferase reporter assay

293T cells (1×10^5 per well) were seeded in a 6-well plate one day prior to plasmid transfection with Lipofectamine 2000. For the *AhR* promoter reporter assay, 0.1 µg of reporter plasmid pGL3-basic-AhR was co-transfected with 0.2, 0.4 or 0.6 µg of Dlx1, Dlx2, Dlx3 or Oct4 expression vectors, and with 0.1 µg pRL-TK (Renilla luciferase; Promega, Madison, WI, USA). Cells were harvested 24 h after DNA transfection and firefly luciferase and Renilla luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Fluorescence intensity was measured using FLUOstar OPTIMA (BMG LABTECH, Ortenberg, DEU). Promoter activity was determined from the firefly luciferase activity in each reaction, normalized against Renilla luciferase activity. To measure the effect of Dlx3 protein on downstream AhR activity, NK-92MI cells (1×10^5) containing a Cignal XRE reporter construct [14] were plated onto 96-well plates and transiently transfected with variable concentrations of the human Dlx3 expression vector. Luciferase activity was measured after 48 h. shRNA targeting human AhR was obtained from Genecopoeia (Rockville, MD, USA).

2.8. Statistical analysis

Standard deviation and standard error of the mean were calculating using Microsoft EXCEL software. Two-tailed Student's *t*-test (EXCEL) was used to determine statistical differences between samples analyzed by qRT-PCR and luciferase reporter assays. A value of $P \leq 0.05$ was considered to be significant.

3. Results

3.1. AhR and Dlx3 are co-expressed in murine and human CD127$^+$ NK cells

As *AhR* expression has been described in human NK cells isolated from lymphoid tissue [16], we examined the expression of this TF in murine NK cells isolated from the lymph node and spleen. Similar to what was seen with human NK cells, NK cells (defined as CD3$^-$NK1.1$^+$) sorted from murine lymph nodes also robustly expressed *AhR* (Fig. 1). A significant proportion of NK cells in murine lymph nodes express the IL-7 receptor α (CD127), and the expression of *AhR* was significantly higher in the CD127$^+$ NK cells compared to what was observed in the CD127$^-$ NK cell compartment (Fig. 1B). The CD127$^+$ NK cells have previously been described to be of thymic origin and distinct from conventional NK cells [22,23]. Examination of additional transcription factor expression differences between the CD127$^+$ and CD127$^-$ NK cell populations demonstrated a higher level of expression of *Dlx3* and lower level expression of *T-bet* in the CD127$^+$ NK cells (Fig. 1B), and no expression of *Dlx1* and *Dlx2* was observed in either population (data not shown). Although the proportion of CD127$^+$ NK cells in the spleen is lower than what is seen in lymph node tissue (Fig. 1A), the same patterns of *AhR*, *Dlx3*, and *T-bet* expression were observed in NK cells sorted by CD127 expression from both secondary lymphoid organs (Fig. 1B and C).

To assess if the expression patterns of *AhR*, *Dlx3*, *T-bet*, and *CD127* also applied to human NK cells, we sorted CD3$^-$CD56^{dim} and CD3$^-$CD56^{bright} NK cells from human peripheral blood mononuclear cells (Fig. 2A). The CD3$^-$CD56^{bright} NK cells expressed higher levels of *CD127* (Fig. 2B), which has been previously described [23]. These cells also expressed higher levels of *AhR* and *Dlx3* and lower levels of *T-bet* (Fig. 2B), consistent with what was observed in the murine CD127$^+$ NK cells (Fig. 1). We also sorted CD3$^-$CD56$^+$CD127$^+$ and CD3$^-$CD56$^+$CD127$^-$ NK cells from human peripheral blood mononuclear cells (Fig. 2A), and the expression patterns of *Dlx3*, *AhR* and *T-bet* were again consistent with what was seen in murine NK cells, as *Dlx3* and *AhR* were more robustly expressed in the human CD127$^+$ NK cell compartment (Fig. 2C).

3.2. Dlx3 binds the AhR promoter

Because *Distal-less*, the invertebrate ortholog of the Dlx TF family, induces expression of the *AhR* ortholog *Spineless* in *Drosophila* [19–21], we hypothesized that the co-expression of *Dlx3* and *AhR* observed in NK cells may reflect the regulation of *AhR* expression by Dlx3 protein. To investigate the ability of Dlx3 to regulate *AhR* expression, a BAC containing the *AhR* promoter region and a Flag-tagged Dlx3 expression construct were co-transfected into 293T cells, and chromatin immunoprecipitation (ChIP) of bound genomic DNA fragments using anti-Flag antibodies was then analyzed by PCR. Oligonucleotide primers spanning the 13 kb region upstream of the *AhR* transcription start site (Supplementary Table 1) were used to identify a sub-region to which Dlx3 bound. The DNA segment designated as AhR-8, −5638 to −5403 bp of the ATG start site, was uniquely enriched in precipitated DNA (Fig. 3A), thus indicating that Dlx3 is able to bind this region. Indeed, our analysis of AhR-8 identified 7 potential Dlx3 binding sites (data not shown). To confirm that Dlx3 binds this portion of the *AhR* promotor, we cloned the sequence spanning position −5638 to −5403 bp into a luciferase reporter construct. This reporter construct was then co-transfected with a Dlx3 expression vector into 293T cells. In agreement with our ChIP data, Dlx3 was able to induce AhR-8-controlled luciferase activity in a dose-dependent manner (Fig. 3B). This effect was specific to the Dlx3 family member since Dlx1 and Dlx2 were not able to induce expression of

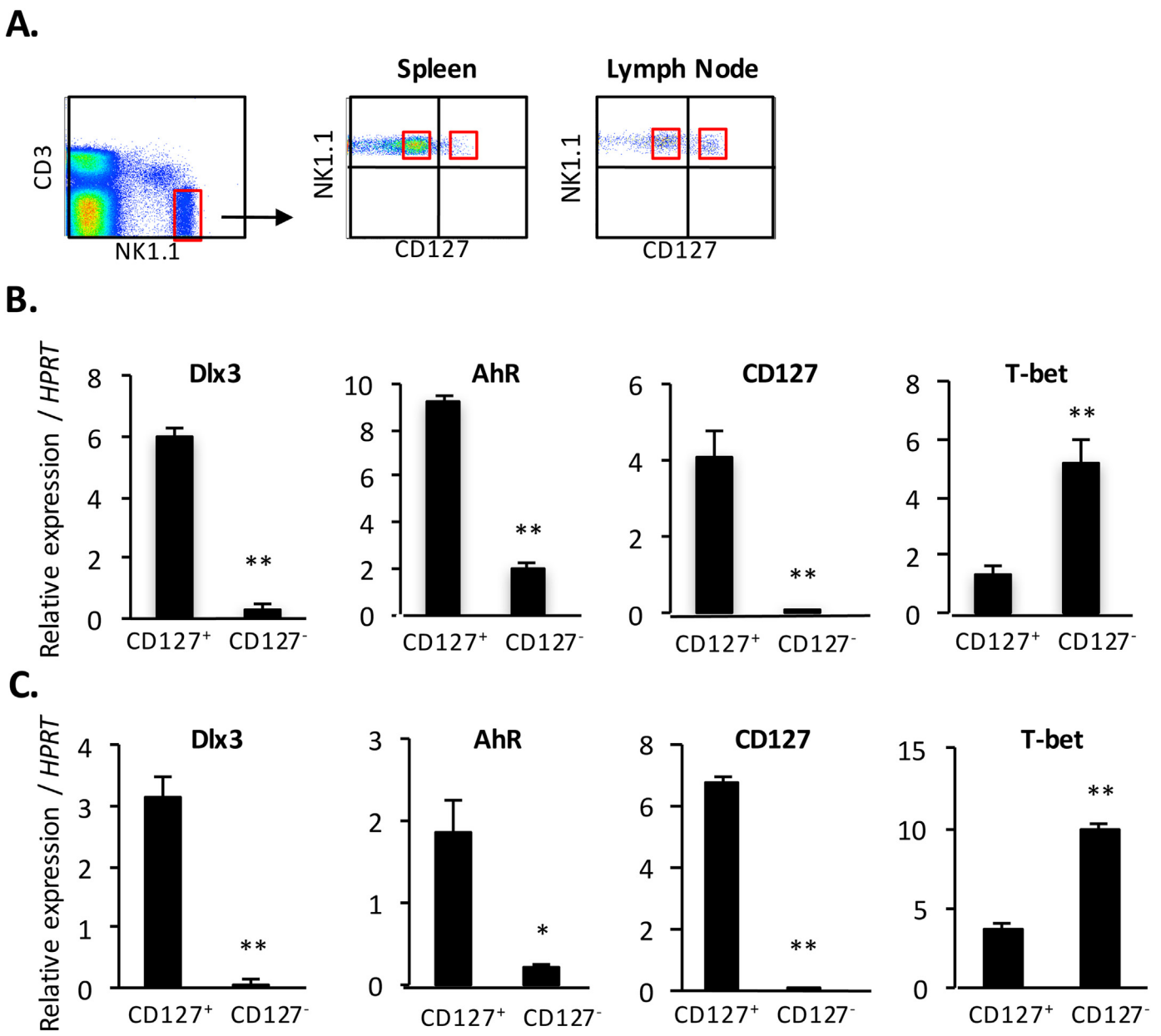

Fig. 1. *Dlx3* and *AhR* are co-expressed in mouse CD127$^+$ NK cells. (A) CD3$^-$NK1.1$^+$CD127$^+$ and CD3$^-$NK1.1$^+$CD127$^-$ NK cells were sorted by FACS (as indicated by red gates) from the spleen and lymph node of wild-type C57BL/6 mice. (B-C) The expression of *Dlx3*, *AhR*, *CD127*, and *T-bet* (normalized to *HPRT* expression) were assessed by qRT-PCR in CD127$^+$ and CD127$^-$ spleen (B) and lymph node (C) NK cells. ($^*P \leq 0.05$ and $^{**}P \leq 0.01$; n=3; experiments were performed three times).

luciferase in this reporter system, similar to Oct4 control samples (Fig. 3B). Dlx3 binding activity in this region was further substantiated by performing the reporter assay again, but with a larger segment of the promoter (AhR-8/9), which included the −5638 to −3981 bp region upstream of the transcription start site. Indeed, Dlx3 induced luciferase expression when controlled by the AhR-8/9 region (Fig. 3B), and Dlx3 activity was specific to this segment of DNA as the TF was not able to induce luciferase activity when controlled by a region of the promoter more proximal (−1805 to −375 bp) relative to the ATG start site (Fig. 3C).

3.3. *Dlx3 promotes AhR expression and function in human NK cells*

To provide functional evidence for Dlx3 as a regulator of AhR, we took advantage of the well-characterized human NK cell line NK-92MI. These cells were transfected with an expression vector encoding human *Dlx3*. As expected, after 48 h, NK-92MI cells receiving this construct highly expressed *hDlx3* whereas those receiving a control vector did not (Fig. 4A). Importantly, persistent *hDlx3* expression was accompanied by an increase in *hAhR* expression (Fig. 4A), thus supporting our luciferase reporter data (Fig. 3), which indicated that Dlx3 positively regulates *AhR* gene expression. To then show that this Dlx3-mediated induction of *AhR* was accompanied by increased AhR activity, we co-transfected NK-92MI cells with our hDlx3 expression vector and a Cignal XRE reporter construct, which has been previously described [14]. As AhR modulates downstream gene expression via engagement of XRE DNA sequences, we hypothesized that increases in Dlx3 would elicit greater XRE reporter activity due to augmented *AhR* expression. Indeed, persistent *hDlx3* expression led to a dose-dependent increase in XRE reporter activity (Fig. 4B)—an effect attributable to changes in AhR expression (Fig. 4C)—thus providing further evidence that Dlx3 functionally enhances AhR.

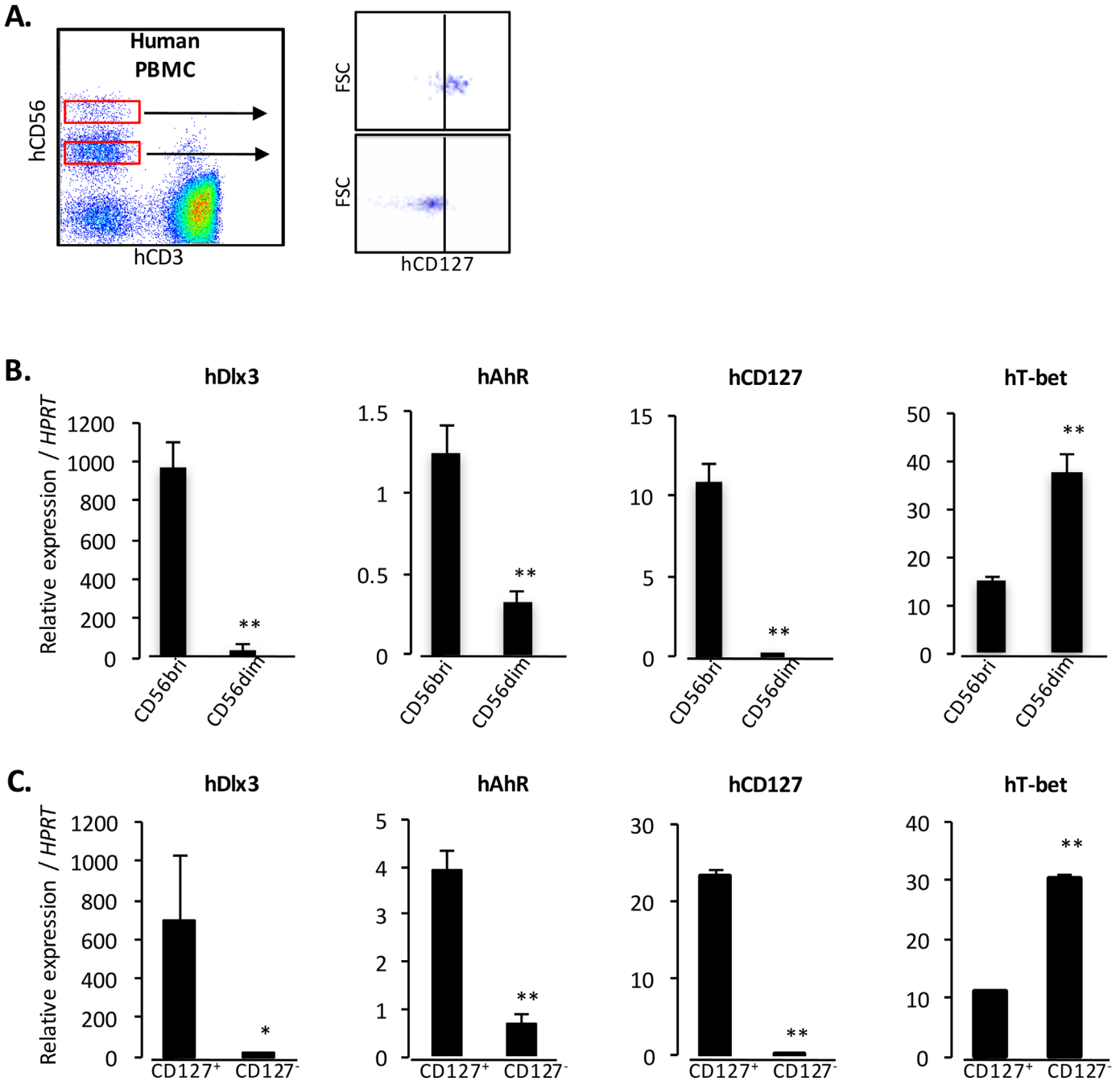

Fig. 2. *Dlx3* and *AhR* are co-expressed in CD56^{bright} and CD127^{+} human NK cells. (A) CD56^{bright} and CD56^{dim}CD3^{-} human NK cells were sorted by FACS from peripheral blood mononuclear cells isolated over a Ficoll gradient. Red boxes indicate the sorting gates. FACS profiling of CD127 is shown for each sorting gate (i.e. CD56^{bright} and CD56^{dim} cells). FSC=forward scatter. (B-C) The expression of *Dlx3*, *AhR*, *CD127*, and *T-bet* (normalized to *HPRT* expression) was assessed by qRT-PCR in human peripheral blood NK cells sorted by FACS into CD3^{-}CD56^{dim} and CD3^{-}CD56^{bright} subsets (B), and CD3^{-}CD56^{+}CD127^{+} and CD3^{-}CD56^{+}CD127^{-} subsets (C) ($^{*}P \leq 0.05$ and $^{**}P \leq 0.01$; n=3; experiments were performed three times). Note, CD56^{+} includes CD56^{bright} and CD56^{dim} cells.

4. Discussion

The transcriptional regulator *Distal-less* (*Dll*) was first identified in *Drosophila* as a single gene that is critical for proximodistal patterning in limb development. During specification of distal antennal segments, *Dll* serves as a primary inducer of *Spineless* (*ss*), the invertebrate ortholog of *AhR* [19–21]. The Distal-less (Dlx) TF family members in vertebrates similarly function to mediate development of the skin, limbs, nervous system, and craniofacial skeleton [24], though recent evidence has implicated an additional role for these proteins in regulating immune development and hematopoietic function. As high expression of murine *Dlx1-3* has been described in the immature conventional NK cell compartment [9], and because *AhR* expression has been observed in immature NK cells isolated from human lymphoid tissue [16,25], we wondered whether Dlx TFs might promote AhR activity in a vertebrate NK cell setting, akin to the regulation observed in *Drosophila*.

Indeed, the results of our study suggest a mechanism whereby Dlx3 acts as a positive regulator of *AhR* expression. Specifically, we observed robust co-expression of *Dlx3*—but not *Dlx1* or *Dlx2*—and *AhR* in murine and human CD127^{+} NK cells, which are known to exhibit the phenotypic characteristics of immature conventional NK cells as defined by low CD11b (Mac-1) expression [26]. To

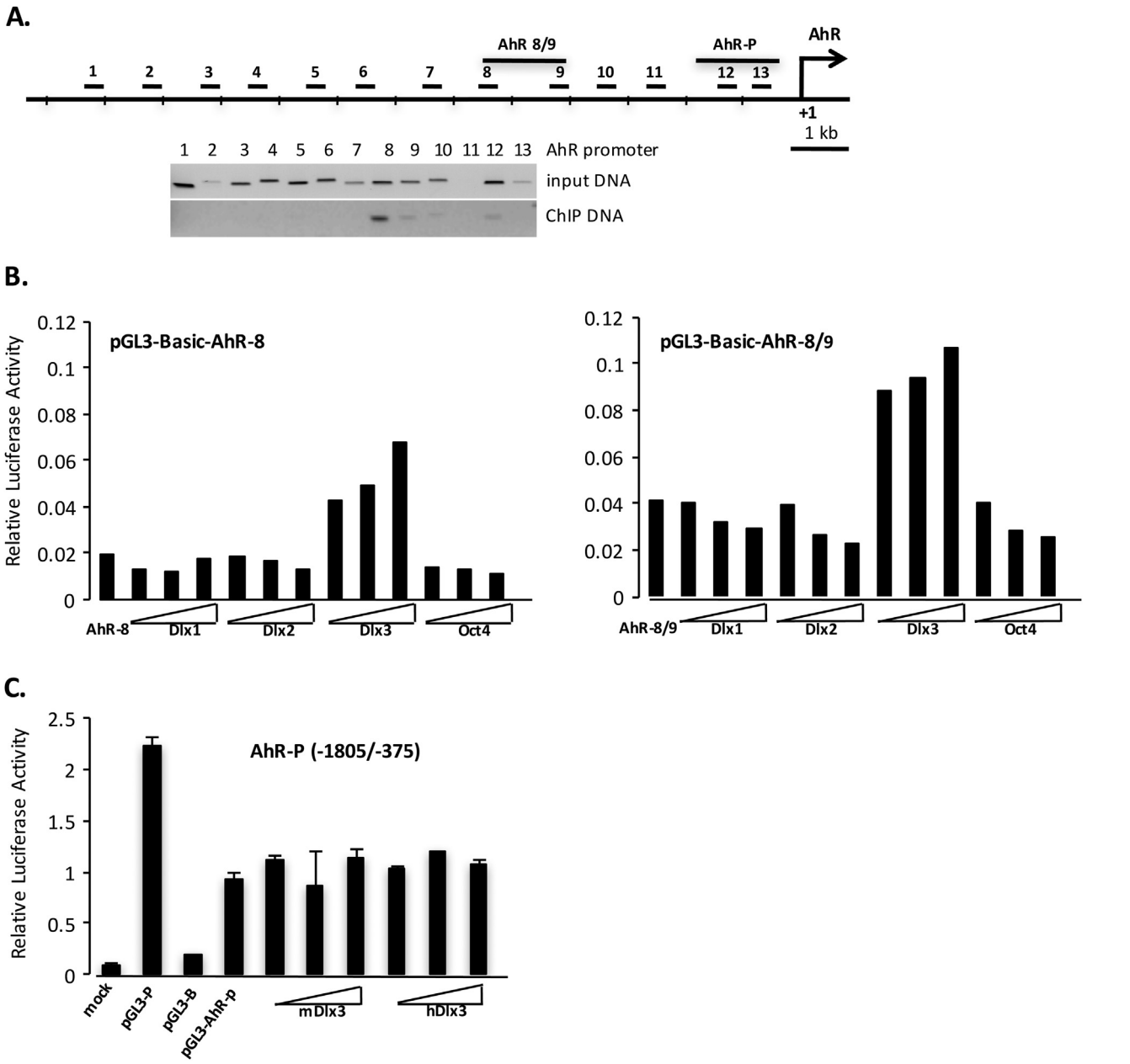

Fig. 3. Dlx3 binds and activates the *AhR* promoter. (A) A Flag-mDlx3 (mouse Dlx3) expression vector and *AhR*-containing BAC clone were co-transfected into 293 T cells and mDlx3 was immunoprecipitated with a specific anti-Flag antibody. After isolation of genomic DNA fragments bound to mDlx3, PCR using oligonucleotide primers specific to 13 *AhR* promoter regions, spanning 13 kb upstream of the transcription start site, was performed. (B-C) A transient-transfection analysis was performed with AhR-8 (−5638/−5403) and AhR-8/9 (−5638/−3981) luciferase reporters transfected into 29T cells in the presence of mouse Dlx1, mouse Dlx2 or mouse Dlx3 expression vectors, or a control mouse Oct4 expression vector (B). (C) Activity of an AhR-P (−1805/−375) luciferase reporter, or control luciferase reporters, was analyzed in 293T cells co-transfected with a human Dlx3 (hDlx3) or mDlx3 expression vector. "mock"=no reporter construct transfected. "pGL3-P"=the "pGL3-Promoter" construct, which contains the SV40 promoter. "pGL3-B"=the "pGL3-Basic" construct, which lacks any promoter or enhancer. "pGL3-AhR-P"=the AhR-P segment of the AhR promoter (−1805 to −375 upstream of the ATG start site) cloned into the pGL3-Basic vector.

explore whether *Dlx3* was able to directly modulate *AhR* promoter activity, we co-transfected 293T cells with a Dlx3 expression vector and an *AhR*-containing BAC. Using ChIP analysis, we found that Dlx3 protein uniquely bound to a DNA segment −5638 to −5403 bp of the *AhR* transcriptional start site. We used luciferase reporter assays to confirm this observation. Finally, to provide evidence for this mechanism in the context of NK cell biology, we transfected NK-92MI cells with a human Dlx3 expression vector and subsequently observed both augmented *AhR* expression and increased reporter activity (regulated by XRE DNA sequences) indicating an increase in AhR activity. Because *Dll* has been shown to interact cooperatively with Homothorax and Extradenticle (orthologs of vertebrate Meis1 and Pbx1, respectively) in binding to the *Spineless* promoter [21], we examined the AhR promoter region but were unable to find Meis1 and Pbx consensus binding sites [27,28] near the Dlx3 binding site. Nevertheless, we did check to see if Meis1 and Pbx are expressed in murine NK cells, and we found that they are expressed (Supplementary Fig. 1).

Exploration into the physiologic relevance of Dlx3-regulated *AhR* expression is complicated by the fact that Dlx3 deficiency in mice causes early embryonic lethality. However, as mice homozygous for targeted *AhR* mutation exhibit moderate developmental

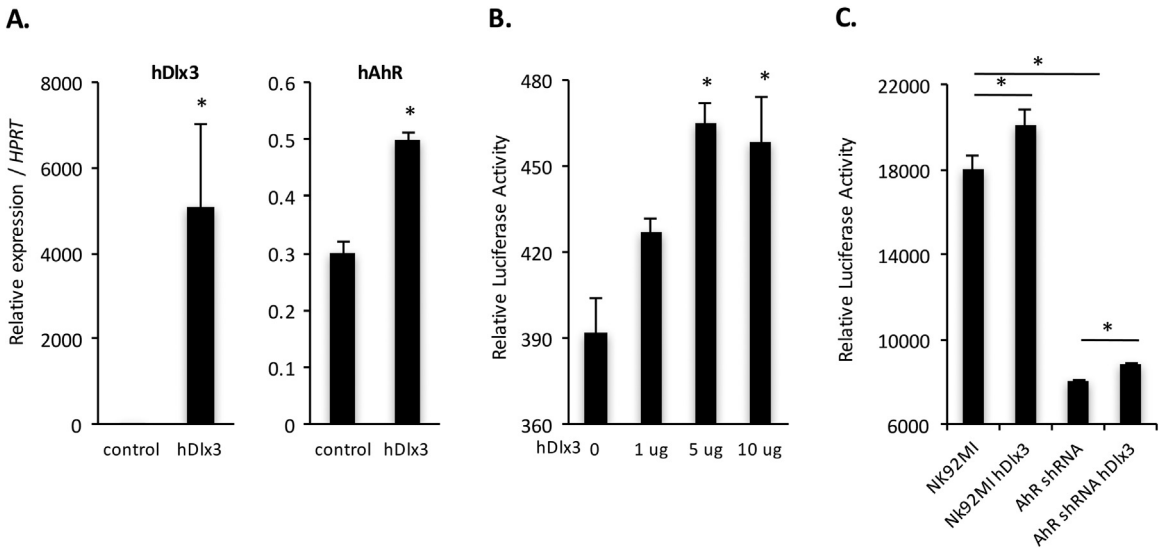

Fig. 4. Dlx3 induces AhR activity in NK cells. (A) Total RNA was isolated 48 h after transfection of NK-92MI cells with a hDlx3 expression vector and was subjected to qRT-PCR probing for *hDlx3* and *hAhR* expression ($^{*}P \leq 0.05$). (B) NK-92MI cells stably transfected with a luciferase reporter vector containing AhR binding sites (XRE sequences) in its promoter were transiently transfected with varying concentrations of an hDlx3 expression vector. Luciferase activity was measured after 48 h to assess for AhR activity ($^{**}P \leq 0.01$; n = 3; experiments performed at least three times). (C) NK-92MI cells, stably expressing the XRE-luciferase reporter construct, were transfected with human Dlx3, shRNA targeting AhR, or both, and luciferase activity was measured after 48 h to assess for AhR activity ($^{*}P \leq 0.05$; n = 3; experiment performed three times).

defects, but are nonetheless viable [29,30], it appears as though *AhR* is not a critical Dlx3 target during early vertebrate development. However, it is interesting that loss of AhR has been shown to significantly reduce the number of certain hematopoietic cell types in post-natal mice, most notably type 3 innate lymphoid cells (ILC3) [31,32], which—for human ILC3 cells—have been shown to undergo differentiation to NK cells upon AhR silencing [25]. Furthermore, because AhR is induced upon NK cell exposure to cytokines such as IL-2 and can modulate NK cell activity [14], AhR appears to serve a post-developmental homeostatic and/or functional role for regulating the NK cell response. This is intriguing as robust *Dlx3* and *AhR* co-expression has been noted in NK cell populations that are specialized for cytokine secretion and immunomodulation, for example, $CD127^{+}$ NK cells (Figs. 1 and 2), $CD27^{+}CD11b^{-}$ immature conventional NK cells in mice [unpublished data], and immature $CD56^{bright}$ NK cells in humans (Fig. 2). Furthermore, AhR has been implicated to regulate both the NK cell killing and cytokine responses in mice [14,15]. As a result, experiments that make use of NK cell-specific Dlx3 ablation will be useful for exploring the functional relevance of this mechanism in mediating tumor control and viral immunity, two parameters of immune function that are prominently affected by NK cell activity.

In addition, given the emerging role of AhR in regulating multiple immune pathways, it will be interesting to explore the relevance of Dlx3-AhR crosstalk in other hematopoietic cell types. B and T lymphopoiesis has been previously linked to Dlx TF function [9], though a specific role for Dlx3 in controlling the hematopoietic compartment has yet to be described. Experiments using mice derived from $Dlx3^{-/-}$ embryonic stem cells complemented with $Rag^{-/-}\gamma_c^{-/-}$ mouse blastocysts—which do not develop T, B, and NK cells—will allow for the examination of *AhR* expression differences throughout these lymphocyte populations, as compared to Dlx3-immunosufficient mice. Thus, our elucidation of a previously undefined mechanism, whereby Dlx3 induces *AhR* promoter activity, is likely to assume a role in regulating downstream gene expression throughout many facets of vertebrate biology and immunology.

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Transparency Document. Supplementary material

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.06.023.

Appendix A. Supplementary material

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