Defining the AHR regulated transcriptome in NK cells reveal gene expression program relevant to development and function

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Abstract:
The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that regulates cellular processes in cancer and immunity, including innate immune cell development and effector function. However, the transcriptional repertoire through which AHR mediates these effects remains largely unexplored. To elucidate the transcriptional elements directly regulated by AHR in NK cells, we performed RNA- and ChIP-sequencing on NK cells exposed to AHR agonist or antagonist. We show that mature peripheral blood NK cells lack AHR, but its expression is induced by Stat3 during IL-21-driven activation and proliferation, coincident with increased NCAM1 (CD56) expression resulting in a CD56<sup>bright</sup> phenotype. Compared to control conditions, NK cells expanded in the presence of the AHR antagonist, StemRegenin-1, were unaffected in proliferation or cytotoxicity, had no increase in NCAM1 transcription and maintained the CD56<sup>dim</sup> phenotype. However, it showed altered expression of 1,004 genes including those strongly associated with signaling pathways. In contrast, NK cells expanded in the presence of the AHR agonist, kynurenine, showed decreased cytotoxicity and altered expression of 97 genes including those strongly associated with oxidative stress and cellular metabolism. By overlaying these differentially expressed genes with AHR chromatin binding we identified 160 genes directly regulated by AHR, including hallmark AHR targets <i>AHRR</i> and <i>CYP1B1</i>, and known regulators of phenotype, development, metabolism, and function such as NCAM1, KIT, <i>NQO1</i>, and TXN. In summary, we define the AHR transcriptome in NK cells, propose a model of AHR and Stat3 coregulation, and identify potential pathways that may be targeted to overcome AHR-mediated immune suppression.

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Defining the AHR-regulated transcriptome in NK cells reveal gene expression programs relevant to development and function

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Abstract

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that regulates cellular processes in cancer and immunity, including innate immune cell development and effector function. However, the transcriptional repertoire through which AHR mediates these effects remains largely unexplored. To elucidate the transcriptional elements directly regulated by AHR in NK cells, we performed RNA- and ChIP-sequencing on NK cells exposed to AHR agonist or antagonist. We show that mature peripheral blood NK cells lack AHR, but its expression is induced by Stat3 during IL-21-driven activation and proliferation, coincident with increased NCAM1 (CD56) expression resulting in a CD56bright phenotype. Compared to control conditions, NK cells expanded in the presence of the AHR antagonist, StemRegenin-1, were unaffected in proliferation or cytotoxicity, had no increase in NCAM1 transcription and maintained the CD56dim phenotype. However, it showed altered expression of 1,004 genes including those strongly associated with signaling pathways. In contrast, NK cells expanded in the presence of the AHR agonist, kynurenine, showed decreased cytotoxicity and altered expression of 97 genes including those strongly associated with oxidative stress and cellular metabolism. By overlaying these differentially expressed genes with AHR chromatin binding we identified 160 genes directly regulated by AHR, including hallmark AHR targets AHRR and CYP1B1, and known regulators of phenotype, development, metabolism, and function such as NCAM1, KIT, NQO1, and TXN. In summary, we define the AHR transcriptome in NK cells, propose a model of AHR and Stat3 coregulation, and identify potential pathways that may be targeted to overcome AHR-mediated immune suppression.

Key points:

AHR directly regulates gene expression in NK cells involved in a wide range of functions including cell signaling, oxidative stress, and metabolism.

Understanding the repertoire of genes regulated by AHR may help direct future research on NK cell dysfunction mediated by AHR ligands in cancer.
Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor which influences diverse biological functions including cellular differentiation, malignant transformation, metabolism, and immune regulation1-4. In the absence of agonist, AHR resides in the cytoplasm forming a complex with co-chaperone proteins. On binding a ligand or an agonist, the AHR translocates to the nucleus where it forms a heterodimer with ARNT5. The AHR/ARNT heterodimer binds to a xenobiotic response elements (XREs) or AHR binding sites, regulating the expression of a wide variety of genes, including cytochrome P450 (CYP)5.

Altered tryptophan (Trp) metabolism due to elevated levels of the Trp-metabolizing enzymes indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), leads to generation of suppressive molecules like kynurenine (Kyn) and kynurenic acid (KA) which is linked to immune dysfunction6-8. Elevated levels of KA have been reported in the serum of patients with hematological malignancies like AML, lymphomas and solid cancers such as colon, glioblastoma, and breast cancers9-15. Kyn/KA are endogenous ligands that mediate their suppressive effects via binding to AHR and activation of this pathway induces immune tolerance2,4,12.

AHR signaling plays a critical role in shaping innate and adaptive immune responses. AHR has an important role in the development of innate lymphoid cells (ILCs) that are considered as natural killer (NK) cell precursors16,17. NK cells are large granular lymphocytes that participate in the innate immune response to virally infected and neoplastic cells tumors via specialized receptors for major histocompatibility antigens and immunoglobulin G (FcR)18,19. In addition to their ability to mediate antibody-dependent cellular cytotoxicity (ADCC), NK cells secrete cytokines such as interferon IFNγ and TNFα that inhibit tumor cell proliferation, enhance antigen presentation, and aid in the chemotaxis of T cells20,21. Human NK cells have been broadly divided into two subsets: CD56bright and CD56dim22,23. The CD56dim subset comprises 80%–95% of peripheral blood NK cells and represent the mature NK cell population in humans (stage V). They express maturity-related inhibitory receptors, cytotoxic effector proteins (perforin and granzyme B), and high expression of CD16 (FcyRIIIa), permitting responses to antibody-opsonized targets24. In contrast, the less mature CD56bright cells represent ~10% of the
circulating NK cells and are considered as potent cytokine producers. Furthermore, there is evidence to suggest that CD56bright NK cells serve as precursors to CD56dim NK cells, since CD56bright NK cells appear first in the blood following bone marrow or stem cell transplantation, which is then followed by an accumulation of CD56dim NK cells.25

The molecular mechanism by which AHR effects the differentiation and regulates the function of human NK cells remains largely unknown. In the present study, we use an ex vivo NK-cell expansion system to demonstrate that inhibition of AHR using an antagonist, SR-1, does not impact proliferation or effector function but promotes the development of NK cells with CD56dim phenotype. In contrast, NK cells treated with AHR agonists Kyn and KA suppress the cytotoxic function of NK cells and effected the expression of genes involved in regulation of metabolism. Utilizing RNA sequencing (RNA seq) and chromatin immunoprecipitation sequencing (ChIP seq) approaches we show that AHR modulates these functions by directly binding on the proximal region of gene promoters, including NCAM1 (CD56). Based on our findings, we propose a model of AHR/STAT3 pathway interactions in the regulation of mature NK cell function.
Methods

NK Cell Isolation

NK cells were isolated from whole blood or buffy coats of healthy donors using Rosette-Sep (StemCell Technologies, Vancouver, Canada) and were expanded by stimulation with FC21 feeder cells for 14–21 days as previously described. Briefly, irradiated FC21 feeder cells were added to the NK cell culture conditions at a ratio of 1:2 (effector:target) on day 0 and 1:1 on days 7 and 14 with human IL-2 alone (50IU/ml) or with the following: 1uM SR-1 (StemCell Technologies #72342), 25uM kynurenine (Tcoris Bioscience) or 25uM kynurenic acid (Tcoris Bioscience). The media was changed every 2-3 days.

Complete details regarding methods for cytotoxicity, flow cytometry, cytokine assays, immunoblotting, RNA and CHIP seq and statistical analysis are provided in the supplementary methods.
Results

Increased expression of AHR in IL21-expanded NK cells

We utilized an irradiated K562 feeder cell line overexpressing membrane-bound IL21 and 4-1BBL (FC21) to examine the role of AHR in NK cell proliferation, differentiation, and function. NK cells isolated from healthy-donor leukopak were grown with FC21 and low dose IL-2 (50 IU/ml) for a period of two weeks. We observed that NK cells stimulated with FC21 had a modest (2.2-fold) but significant (p<0.05) increase in levels of AHR transcript (Figure 1A and Supplementary Figure S1A). Moreover, the expression of AHR target genes CYP1B1 and aryl hydrocarbon receptor repressor (AHRR) were also significantly increased (Figure. 1A and Supplementary Figure. S1A). Although, there was a modest increase in AHR transcript, a robust increase in AHR protein was observed, peaking at day 14 following FC21 stimulation (Figure. 1B and Supplementary Figure. S2). We previously shown that FC21-mediated expansion of NK cells leads to increased Stat3 phosphorylation, thus we determined whether Stat3 may be involved in the regulation of AHR expression during NK-cell expansion. ChIP seq analysis confirmed that Stat3 binds to the proximal region of AHR promoter in expanded NK cells (Supplementary Figure. S1E).

In addition, although we observed a small increase in AHR mRNA expression in NK cell expanded using cytokines IL2 alone by RT-PCR, a robust increase in AHR expression was observed post transcriptional at the protein level. Together, these results provide further evidence that STAT3 is involved in AHR regulation in NK cells (Fig S1B-S1D).

Since there was a strong activation of the AHR pathway in expanded NK cells, we therefore, examined the effect of the AHR antagonist StemRegenin-1 (SR-1) on FC21-driven proliferation/expansion. Treatment of NK cells with SR-1 resulted in >1,000-fold decrease in the expression of CYP1B1 and AHRR, but did not significantly impact the number of NK cells between the two groups at the end of two-week expansion period (Figure 1C, 1D and Supplementary Figure. S3). Together, these results demonstrate that even complete AHR inhibition does not impact the survival of NK cells.
AHR regulates genes involved in the NK cell development

Further, to investigate the role of AHR in NK cell phenotype, cells were expanded with SR-1 as described above. Consistent with previous studies, NK cells following the expansion with the FC21 were mostly CD56\textsuperscript{bright} at the end of two weeks\textsuperscript{27,29,30}. RT-PCR showed no change in CD56 (NCAM1) expression in NK cells after one week of treatment with SR-1, consistent with surface protein expression by flow cytometry. However, a 4-fold increase in CD56 expression was observed in FC21 expanded NK cells on day 14 (Figure 2A). Interestingly, a significant (p<0.001) increase in the percentage of CD56\textsuperscript{dim} NK cells was observed following their expansion with SR-1 (Figure 2B-2C). Moreover, we observed >50-fold reduction (p<0.001) in KIT (CD117) mRNA expression as well as a decrease (p<0.01) in KIT protein levels following two weeks of expansion with SR-1 (Figure 2D and 2E). However, AHR inhibition had no impact on the expression of other NK cell receptors at either D7 or D14 (Supplementary Figure. S4). In summary, these results suggest that AHR may regulate the expression of CD56 and KIT in NK cells.

Effect of AHR inhibition on NK cell functions

NK cells mediate their anti-tumor effects through the direct lysis of cancer cell by release of perforins and granzymes, and the secretion of cytokines such as IFN\textgamma and TNF-\alpha. To determine the effect of AHR inhibition on the lytic function of NK cells, FC21-expanded NK cells were treated with DMSO (control) or SR-1 overnight and then used in a standard cytotoxicity assay against MHC-I deficient K562 or MHC-I positive glioblastoma cells (SJGBM2) and triple negative breast cancer cells (HS578T) as target cells. AHR inhibition had no impact on the cytotoxic function of NK cells (Figure. 3A-3C and Supplementary Figure. S5). Next, to investigate whether long term AHR inhibition impacts the lytic function of NK cells, the cells were expanded for two weeks in the presence of SR-1 before testing their cytotoxicity against K562 or SJGBM2 target cells. Consistent with the above findings AHR inhibition had no effect on the lytic function of NK cells (Figure. 3D-3E). A similar trend was observed in primary NK cells treated overnight with the SR-1 (Supplementary Figure. S6).

Next, to determine whether AHR is involved in the regulating cytokine production, NK cells were treated overnight with SR-1 and then stimulated by K562 cells. The levels of cytokines in
the supernatant were measured using bead-based arrays. Inhibition of AHR had no impact on the levels IFN\(_\gamma\), TNF-\(\alpha\), IL-2 or other cytokines secreted in response to tumor targets (Supplementary Figure. S7 and data not shown). Together, these results demonstrate that AHR inhibition does not affect NK cell functions.

**AHR agonists suppress NK cell cytotoxic function**

Kynurenine and kynurenic acid are endogenous AHR ligands that mediate their suppressive effects via binding to AHR \(^12\). To examine the effect AHR agonists on proliferation and function of NK cells were expanded with FC21 for two weeks in the presence of either Kyn (25uM) or KA (25uM). In the presence of the AHR agonists, NK cells grew robustly and were similar to those without AHR agonists, demonstrating again that AHR does not affect the proliferation of NK cells (Supplementary Figure. S8). The NK cells expanded with AHR agonists were then assessed for cytotoxicity against K562 or SJGBM2 cells. When present during expansion, both AHR agonists were able to suppress the subsequent lytic function of NK cells (Figure. 4A-4B).

Next, to determine whether the expanded NK cells were sensitive to the inhibition of cytotoxic function during short-term exposure to AHR agonists, FC21-expanded NK cells were rested in low-dose IL2 (50IU/ml) overnight with or without KA, and were subsequently tested for cytotoxicity against HS578T or SJGBM2 target cells. A 50% reduction in the lytic activity of NK cells was observed following KA treatment (\(p<0.01\), Figure. 4C-4F). However, both agonists had no impact on the levels of IFN\(_\gamma\) and TNF\(\alpha\) (Supplementary Figure. S9). In summary, these results demonstrate that AHR activation by agonists does not impact activation-induced proliferation or cytokine production, but significantly suppresses the lytic function of NK cells.

**AHR regulates the NK cell transcriptome**

Next, we performed RNA seq analysis to examine the transcriptome of NK cells expanded with FC21 with or without AHR antagonist (SR-1) or agonist (Kyn). RNA was isolated from donor-matched NK cells prior to their expansion at day 0 (DO) and after expansion with FC21 alone or with SR-1 or Kyn on days 7 (D7) and 14 (D14). An unbiased gene expression analysis pipeline was used to identify the differentially expressed genes (DEGs) between IL-2 (control) and SR-1 expanded NK cells (Figure. 5A-5B). Principal component analysis (PCA) showed that NK cells treated with SR-1 clustered together and were distinct from the control NK cells (Figure. 5C and Supplementary Figure. S10B). Furthermore, analysis of NK cells treated with SR-1 at D7
showed change in expression of 132 genes (1.5fold; p<0.05) (Figure. 5D and S10A), which increased to 1,004 DEGs at D14 in SR-1 treated NK cells (Figure. 5A-B and 5D). Next, we used AHR-ChIP to differentiate direct and indirect effects of SR-1 and Kyn on the transcriptome and identify specific genes regulated by AHR. Using this analysis, we found 24/132 genes and 148/1,004 were direct AHR target genes following the treatment of NK cells with SR1 on days 7 and 14 respectively (Figure. 5D). Gene ontology analysis using ingenuity pathway analysis (IPA) revealed an enrichment of genes involved in the regulation of processes related to cell migration and adhesion in D7-SRI treated group (data not shown). The DEGs were highly associated with NF-kB, interleukin-9 (IL9), and G-protein coupled receptor (GPCR) signaling pathways in D14 NK cells treated with the AHR antagonist (Figure. 5E and Supplementary Figure. S11).

Although there was some overlap between the DEGs of SR-1 and Kyn treated NK cells, they were mostly distinct. NK cells expanded with the AHR agonist Kyn showed no change in gene expression at D-7. However, 97 genes were differentially expressed at D14, and further analysis revealed that 12/97 were AHR target genes (Figure. 6A-6B). Importantly, the expression of all 12- AHR target genes were increased when compared to the control group, consistent with the role of AHR agonists functioning as transcriptional activators (Supplementary Figure. S12). These genes were mostly involved in the regulation of metabolic processes, including antioxidant enzymes, pentose phosphate pathway (PPP), and iron transport (Figure. 6C and 6D). Together, our results identify AHR as a key transcriptional factor involved in the regulation of NK cell transcriptome, particularly of signaling and metabolic pathways.

**AHR regulates CD56 expression in NK cells**

Bioinformatic analysis revealed presence of XRE sites on promoters of several genes that are involved in the regulation of NK cell function. ChIP-seq analysis was performed on D14 FC21-expanded NK cells, as we had identified this time point as having the highest AHR expression (Figure. 1B). ChIP seq analysis showed that most peaks were within ± 5kb of the transcriptional start sites (TSS) around the gene promoters. Importantly, no binding was observed in the total input sample, suggesting specificity of the AHR ChIP-antibody (Figure. 7A-B). Analysis of ChIP-seq data using HOMER revealed an enrichment of the known AHR motif GCGTGC/A (Figure. 7C). Furthermore, to validate the specificity of ChIP seq we confirmed AHR
recruitment to the promoters of the known AHR target genes \textit{CYP1A1} and \textit{AHRR} (Figure 7D-7E). Consistent with \textit{CD56} gene expression alterations identified by flow cytometry and RNA seq, analysis of the \textit{NCAM1} promoter showed presence of XRE binding sites and ChIP seq results confirmed AHR binding to the proximal sites of the gene promoter (Figure 7F-7G). We had also observed that SR1 treatment decreases \textit{KIT} expression in NK cells. Analysis of ChIP seq also showed AHR binding to \textit{KIT} promoter in FC21 expanded NK cells (Figure 7H-7I). Together, we conclude that AHR is involved in the transcriptional regulation of \textit{CD56} and \textit{KIT} genes in NK cells.

**AHR activation attenuates NK cell function**

To better understand the underlying mechanism(s) of how AHR activation suppresses the lytic function of NK cells, IPA was applied to the DEGs from NK cells treated with Kyn to identify major pathways impacted. \textit{NAD(P)H quinone dehydrogenase-1 (NQO1)} and \textit{thioredoxin (TXN)} were among the most upregulated genes in Kyn treated NK cells. Furthermore, CHIP seq analysis of the \textit{TXN} and \textit{NQO1} showed loading of AHR to their promoters (Figure 7J-7K and Supplementary Figure. S13). Antioxidant enzymes such as NQO1 and TXN play a major role in mitigating the toxic effect of free radicals. Similarly, there was also significant binding of AHR to promoters of genes involved in PPP (e.g., 6-phosphogluconate dehydrogenase (\textit{PGD})), glutathione synthesis (e.g., glutamate-cysteine ligase (\textit{GCLC})), and iron metabolism (e.g., ferritin light chain (\textit{FTL})) (Supplementary Figure. S13).

Next, to determine the clinical relevance of our results, we analyzed patient gene expression using the Oncomine database. We found a direct correlation between elevated expression of \textit{IDO-1/TDO-2} and AHR target gene \textit{CYP1B1, NQO1} and \textit{TXN} in three independent lymphoma datasets (Supplementary Figure 14). Together, these results suggest the activation of AHR signaling by its agonists induce oxidative stress, which perturbs cellular metabolism, either of which may result in NK cell dysfunction.
Discussion

The development of human NK cells is a complex process and AHR has been implicated in playing a dual role regulating NK cell differentiation and function, although the repertoire of genes directly regulated by AHR in NK cells is largely undefined. Here, we assessed gene expression and AHR chromatin binding assays in combination with AHR agonists and antagonists to define the genes directly regulated by AHR through binding to regulatory sites on gene promoters. We found AHR regulates gene sets that highly associated with multiple signaling pathways, metabolic pathways, and oxidative stress response. In addition, AHR directly and positively regulates CD56 expression such that inhibition/loss of AHR signaling promotes the generation of NK cells with a CD56\textsuperscript{dim} phenotype.

AHR inhibition promotes the development of CD56\textsuperscript{dim} NK cells.

Human peripheral blood NK cells are broadly divided into CD56\textsuperscript{bright} and CD56\textsuperscript{dim} subsets\textsuperscript{24,31}. NK cells have been postulated to undergo a linear pattern of development with CD56\textsuperscript{bright} NK cells becoming CD56\textsuperscript{dim} NK cells in the secondary lymphoid tissue\textsuperscript{22,32}. Studies show that CD56\textsuperscript{bright} NK cells appear first in the blood following bone marrow transplantation followed by an accumulation of CD56\textsuperscript{dim} NK cells\textsuperscript{25}. The present study confirms previous observations that NK cell expansion with feeder cells expressing IL-21 and 4-IBBL results in CD56\textsuperscript{bright} NK cells, but also demonstrate that blocking of AHR signaling abrogates the upregulation of CD56 in this system. The presence of AHR ligands in the media is evident from the upregulation of AHR and its target genes \textit{CYP1B1} and \textit{AHR}, which is blocked by the addition of the AHR antagonist SR-1. Our results show that inhibition of AHR reduces CD56 expression resulting in NK cells with a CD56\textsuperscript{dim} phenotype. Similarly, the presence of AHR ligands in cell culture media has been shown to promote the generation Th17 cell \textit{in vitro}, and AHR antagonist have been shown to promote the expansion of hematopoietic stem cells (HSC)\textsuperscript{33,34}.

Moreover, recent study from Kaufman lab show that SR-1 promotes the development of NK cell from early HSC progenitors, providing further evidence regarding the role of AHR in NK cell development\textsuperscript{35}. Here, we identified \textit{KIT} (CD117) as a gene that is highly regulated by AHR, observing $>50$-fold reduction in the expression of \textit{KIT} following treatment with SR1. KIT is a transmembrane protein with tyrosine kinase activity and is expressed on normal bone marrow
cells. Stem cell factor (SCF) is a ligand for the KIT receptor and plays a major role in the development of HSCs. CD56\textsuperscript{bright} cells have been shown to express KIT and proliferate in response to IL-2 and SCF stimulation. NK cells lose KIT expression as they mature to stage V CD56\textsuperscript{dim} cells.

Our analysis of the NCAM1 (CD56) promoter shows that Stat3 and AHR regulatory elements that are in close proximity and the ChIP data confirmed that AHR directly regulates CD56 expression at the transcriptional level by binding to the promoter. However, CD56 expression is not altered during the first week of expansion. IL21 stimulation leads to increased Stat3 phosphorylation during NK cell expansion, so binding of Stat3 to the NCAM1 promoter may be an early event, which opens the chromatin, facilitating the binding of AHR to XRE sites that then enhance CD56 expression during the second week of expansion. AHR is been shown to cooperate with Stat3 in other systems, such as in regulation of Aiolos (IKZF3) during early stages of Th17 cell differentiation. Oncostatin M has been shown to regulate AHR expression in a Stat3-dependent manner in HepG2 cells. Our data supports the possibility that decreased AHR signaling as immature NK cells emigrate from bone marrow to peripheral blood may be responsible for the phenotypic maturation from CD56\textsuperscript{bright} to CD56\textsuperscript{dim}, consistent with previous observations of higher expression of AHR in CD56\textsuperscript{bright} NK cells compared to CD56\textsuperscript{dim} cells.

This idea is also supported by previous observations that inhibition of AHR promotes the differentiation of stage-3 human tonsillar ILC3 into NK cells. Based on our findings, we propose a model that AHR regulates NK cells development at two stages- first regulating the expression of KIT during early stem cell differentiation to toward NK cell precursors, and later cooperating with Stat3 to control CD56 expression as the NK cells acquire a more mature phenotype.

**Mechanism of NK cell suppression by AHR agonist**

Subversion of the anti-tumor function of NK cells by cancer cells is an important process in tumor progression and metastasis. Endogenous AHR ligands such as kynurenine (Kyn) and kynurenic acid (KA) products of altered tryptophan (Trp) metabolism that are implicated in immune suppression and evasion. However, the mechanism by which AHR agonist suppress the function of NK cells remains largely unknown. Here, we confirm that NK cells treated with...
AHR agonist leads to AHR signaling and suppressed lytic function of NK cells. Interestingly, treatment of NK cells with Kyn/KA had no impact on proliferation or induce apoptosis of NK cells. RNA-seq analysis of agonist-treated NK cells revealed that AHR activation alters the expression of genes that are associated with oxidative stress and cellular metabolism.

Tumor cells and suppressive myeloid cells (MDSC) are principle sources of the Trp-metabolizing enzymes IDO and TDO in the TME. IDO and TDO suppress immune cell function through depletion of Trp and generation of Trp metabolites, but the mechanism by which these metabolites suppress immune function is unclear. AHR ligands such as TCDD have been shown to promote the production of ROS by uncoupling electron transfer in microsomes isolated from liver extracts. In addition, there is evidence that elevated levels of Trp metabolites resulting from IDO expression causes cellular damage associated with increased levels of intracellular ROS in many pathological conditions.

The regulation of redox homeostasis is fundamental to maintaining normal cellular functions and ensuring cell survival. Moreover, there is an intimate connection between cellular metabolism and oxidative stress. Free radicals that are byproducts of mitochondrial respiration and cellular metabolism can severely compromise the function of cells by damaging DNA and protein. Oxidative stress occurs due to an imbalance between generation and elimination of reactive oxygen species (ROS). Activated T cells undergoing clonal expansion dramatically raise their bioenergetic needs, increasing their glucose and glutamine utilization. This, in turn, increase the generation of ROS which triggers an antioxidative GSH response to prevent cellular damage, suggesting that antioxidant enzymes like GSH are essential for effector T-cell functions. Similarly, there is now evidence that impaired cellular metabolism attenuates NK cell function in chronic diseases and cancer. Excessive ROS production is linked to NK cell dysfunction in patients with metastatic breast cancer. Moreover, Kyn-mediated oxidative stress can lead to defects in memory CD4 T cells during HIV-1 infection. Similarly, reactive nitrogen species (RNS) can affect the function of cells- we previously reported that the production of nitric oxide by MDSC leads to the nitration of key signaling proteins suppressing NK cell functions in tumors.
Thus, immune cells need to regulate antioxidant enzymes to protect them from oxidant-induced injury. Activated NK cells have been shown to resist to oxidant-induced cell death by increasing the expression of antioxidant enzymes like thioredoxin (TXN)\textsuperscript{53,54}. Here, our ChIP data suggests that AHR can potentially control the expression of genes involved in the regulation of antioxidant enzymes, pentose phosphate pathway (PPP), and iron transport by binding to XRE sites on gene promoters. Antioxidants enzymes such as NQO1 and TXN play a key role in mitigating the toxic effect of free radicals, including reactive oxygen species (ROS). The PPP runs in parallel to glycolysis, oxidizing glucose to generate large quantities of NADPH, a critical cofactor in reducing glutathione disulfide into the antioxidant glutathione\textsuperscript{55}. In addition, iron is an important component in the regulation of metabolic homeostasis and FTH downregulation has been shown to increase the susceptibility of cancer cells to NK cell recognition\textsuperscript{56}.

IDO and TDO overexpression is associated with poor survival outcome in number of malignancies including lymphomas\textsuperscript{14,15}. Altered Trp metabolism which leads to the production of suppressive molecules like Kyn has been linked to immune dysfunction\textsuperscript{6-8}. Based on our findings we propose that AHR ligands (Kyn/KA) may induce a stress response that perturbs cellular homeostasis attenuating the lytic function of NK cells. However, more studies are required to rigorously test this concept.

NK cell-based therapies hold great promise, but high levels of suppressive molecules like kynurenine can dampen their response. The recent failure of IDO inhibitors suggest that new strategies are required to overcome immune suppression. This study provides a strong rationale for generating NK cells that lack AHR to overcome immune suppression imposed by AHR ligands in the TME. In summary, we provide evidence that AHR functions as a key transcriptional factor that plays a pivotal role in the development and function of human NK cells.
Data Sharing Statement:
RNA seq data have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE173220.

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Authors Contributions:
PT, JM, AT, AC, JF, EE, NC performed experiments, collected and analyzed the data. PT and DAL designed the experiments, wrote and edited the manuscript.

Conflict of interest:
DAL reports patents, ownership interest in Courier Therapeutics, and ownership interest, research funding, royalties, consulting fees, and advisory board membership with Kiadis Pharma, unrelated to this work. PT, JEM, AT, and NC received licensing fees for intellectual property from Kiadis Pharma, unrelated to this work. JAF has licensing agreements and received licensing fees from Kiadis and EMD Millipore, unrelated to this work. The remaining authors have no competing interests to declare.
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Figure Legends

Figure 1. AHR expression in IL-21 expanded NK cells.
A. RNA seq data showing the expression of *aryl hydrocarbon receptor* (AHR), *aryl hydrocarbon receptor repressor* (AHRR) and *cytochrome P450 isoform B1* (CYP1B1) from naïve (resting) and day 14 expanded NK cells. NK cells isolated from healthy donors were expanded for a period of two weeks with irradiated K562 feeder cells overexpressing membrane bound IL21 and 4-IBBL (FC21) in RPMI media containing IL-2 (50IU/ml).
B. Immunoblot showing the expression of AHR from FC21 expanded NK cells at the indicated time points. Protein lysates prepared from NK cells were probed with anti-AHR or anti-actin antibodies.
C-D. Quantitative real-time PCR showing the expression of AHR targets genes *CYP1B1* and *AHRR* from NK cells expanded with DMSO (control) or 1μm Stemregenin (SR-1) on day-7 (D7) and day-14 (D14). The value on the y-axis represents fold change in gene expression relative to the D7 control NK cells.

Figure 2. SR-1 promotes the development of CD56dim NK cells.
A. Real-time PCR showing CD56 expression from NK cells expanded with DMSO (control) or SR-1 on day-7 (D7) and day-14 (D14). The value on the y-axis shows fold change in gene expression relative to D7 control NK cells.
B. Representative FACS histograms showing CD56 expression in day 7 or 14 expanded NK cells with SR-1(1μm) or DMSO (control).
C. Quantification of CD56 expression by measuring mean fluorescence intensity (MFI) in NK cells expanded with SR-1 or control from multiple donors on days 7 or 14. Values show mean ± SD (n=6, p<0.001). ns: not significant
D. RNA seq data showing the expression of *KIT* in NK cells expanded with DMSO (control) or SR-1. Value show mean ± SD (n=3, p<0.001).
E. FACS analysis showing KIT expression in NK cells expanded with SR-1 or control (DMSO) on day 14. MFI was used to determine protein expression. The values show mean ± SD (n=3, p<0.01).
Figure 3. Effect of AHR inhibition on NK cell function.
A-C. NK cells cell expanded using FC21 feeder cells for two weeks. Following the expansion NK cells were treated overnight with SR-1(1um) or DMSO (control). NK cells were then co-cultured with calcein labeled- K562, SJGBM2 or HS578T cells at various effector to target (E:T) for 4hr. The calcein release in the supernatant was used to determine mean specific lysis.
D-E. NK cells were expanded using FC21 cells for two weeks with SR-1(1um) or DMSO (control). The expanded NK cells were co-cultured with calcein labeled tumors cells K562 cells or SJGBM2 cells at E:T for 4hrs.

Figure 4. AHR agonist suppress NK cell function.
A-B. NK cells expanded using FC21 for two weeks with AHR agonist 25μm- kynurenine (Kyn) or kynurenic acid (KA). The expanded NK cells were tested against calcein labeled tumors K562 or SJGBM2 cells. Mean specific lysis was determined using amount of calcein release.
NK cells cell expanded using FC21 for two weeks, following the expansion NK cell were treated overnight with KA (25uM) or DMSO (control) and then co-cultured with calcein labeled tumors at various effector to target (E:T) for 4hr. Representative plot showing standard cytotoxicity assay against C. HS578T cells E. SJGBM2 cells. Percent lysis calculated relative to the control group shown in the D and F. Data show mean ± SEM, (n=3; p<0.01).

Figure 5. AHR inhibition leads transcriptional reprograming of NK cells
A. Heatmap showing hierarchical clustering of differentially expressed genes (DEGs) between SR-1 and DMSO (control) expanded NK cells on day 14. RNA isolated from control or SR-1 expanded NK cells was used for RNA seq. The transcripts were aligned to the human GRCh38 assembly. The results show the expression of genes that change 1.5-fold and p-value <0.05. Hierarchical clustering was performed using Pearson correlation.
B. Multivariate plot showing the expression of highest expressed gene upregulated or downregulated between the control (DMSO) and SR-1 treatment groups. Each dot represents a gene, with the x-axis representing expression and the y-axis representing fold-change. Dots colored red show genes whose differential expression is considered statistically significant (<10% FDR).
C. Principle component analysis (PCA) showing the genes from day 14 SR-1 or DMSO expanded NK cells. X- and Y-axis show principal component-1 (PC1) and principal component-2 (PC2) that explain 92% and 5% of the total variance, respectively.

D. Venn diagram showing the number AHR targets genes in NK cell expanded with SR-1 as identified by AHR-ChIP seq on day 14.

E. Ingenuity pathway analysis (IPA) showing the top 10 signaling pathways. Gene ontology analysis was performed using the list of differentially expressed AHR target genes (148) from day 14, SR-1 expanded NK cells.

**Figure 6. AHR agonists suppress NK cell function.**

A. Heatmap showing hierarchical clustering of DEGs between kynurenine (Kyn) and DMSO (control) in day 14 expanded NK cells. Donor matched control or Kyn expanded NK cells were used for RNA seq. The results show expression of genes that change 1.5-fold and p-value <0.05.

B. Venn diagram showing the number of the DEGs genes and AHR targets genes in NK cells expanded with Kyn.

C. IPA analysis showing the top 10 pathways. Gene ontology was performed using the list of differentially expressed AHR target genes from day 14 NK cells expanded with Kyn.

D. GSEA analysis showing an enrichment of glucose metabolic (top) and NRF2 (bottom) pathways in NK cells expanded with Kyn.

**Figure 7. AHR regulates NCAM1 and NQO1 expression**

A. Distributions of peaks across the transcriptional start site (TSS) of gene promoters. ChIP seq was performed using anti-AHR antibody on FC21 expanded NK cells from two donor on day 14. The reads were aligned to the human genome (hg38).

B. Heatmap showing the distribution of peaks across the gene promoter within 5kb of TSS. The peaks are clustered into 5-regions indicated as C1-C5.

C. De-novo motifs were identified by HOMER package using default parameters and input sequences comprising ±100 bp from the center of the top 1000 peaks. The highest-ranking motif from each sample is shown.
D-E. ChIP seq data showing recruitment of AHR to the proximal regions of gene promoters in day 14 FC21 expanded NK cells. Peaks were visualized using the UCSC genome browser. D. Cytochrome P450 A1 (left panel) and E. AHR repressor (right panel).

F. Schematic showing the position of Stat3 and AHR binding sites on the human NCAM1 (CD56) promoter relative to the transcriptional start site and 1st exon.

G. ChIP seq data showing the recruitment of AHR to NCAM1 promoter in day 14 FC21 expanded NK cells.

H. Schematic showing the position of AHR binding sites on the human KIT (CD117) promoter relative to the transcriptional start site and 1st exon.

I. ChIP seq data showing the recruitment of AHR to the promoter of KIT in day 14 FC21 expanded NK cells.

J. Schematic showing the position of Stat3 and AHR sites on the gene NADPH quinone oxidoreductase (NQO1) relative to the TSS.

K. Recruitment of AHR to the NQO1 gene promoter in D14 expanded NK cells.
Figure 2

A

Fold change CD56 expression

Day-7 Day-14

Control SR1

B

D-7

D-14

CD56

Control SR1

C

MFI CD56

D7 IL2 D7 SR1 D14 IL2 D14 SR1

n.s. p=0.00171

D

Normalized RNA counts

Control SR1

p<0.001

E

MFI KIT Expression

Control SR1

p<0.01
Figure 5

A

B

C

D

E

UVA-induced MAPK Signaling
Phospholipase C Signaling
Aryl Hydrocarbon Receptor Signaling
TNFR2 Signaling
NF-κB Activation by Viruses
G-Protein Coupled Receptor Signaling
Cholecystokinin/Gastrin-mediated Signaling
Systemic Lupus Erythematosus In B Cell...
Non-Small Cell Lung Cancer Signaling
IL-9 Signaling
