On the trail of innate immune responses: Plasmacytoid dendritic cells and beyond

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NAB2 and EGR-1 exert opposite roles in regulating TRAIL expression in human Natural Killer cells

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The transcriptional regulator NGFI-A binding protein 2 (NAB2) and the early growth response (EGR) genes are key regulators of effector molecules, such as cell death-inducing genes. We have previously shown that NAB2 modulates the levels of expression of the Tumor Necrosis Factor (TNF) family member TNF-related apoptosis inducing ligand (TRAIL) in T cells and plasmacytoid DCs. Provided that TRAIL plays a key role in NK cell cytotoxicity towards infected and tumor cells, we investigated whether NAB2 also mediates TRAIL expression in human NK cells, and if so through which mechanisms. We show that NAB2 is induced in NK cells upon IL-2 and IL-15 stimulation, and promotes the induction of TRAIL. In addition, we show that the transcription factor EGR-1, which is upregulated by the same stimuli as NAB2, rather acts as a brake on TRAIL expression in NK cells. Overall, these data provide new mechanistic insights in the regulation of TRAIL, and show that the gene regulation through the NAB2/EGR axis allows for a highly controlled expression pattern of this effector molecule in NK cells.
1. Introduction

The NGFI-A-binding protein NAB2 has been widely described as a critical regulator of gene transcription during immune cell development, function and cell fate (1-5). NAB2 is induced upon a variety of extracellular stimuli that trigger specific receptors located on the cell surface of immune cells. T cell receptor (TCR) or Toll-like receptor (TLR) triggering, for instance, leads to the induction of NAB2 in T cells and plasmacytoid DCs (2,4). Furthermore, extracellular factors such as increased levels of growth factors, or changes in the microenvironment mediated by hypoxia or irradiation lead to enhanced NAB2 expression in immune and non-immune cells (6,7). Because NAB2 does not contain any DNA binding domains itself, it regulates gene expression indirectly by interacting with the Early growth response (EGR) transcription factors, thus far the only transcription factors known to interact with NAB2 (7). EGR-1 (or NGFI-A), which has been the first EGR identified to be regulated by NAB2, is quickly induced upon extracellular triggering, and like NAB2, it controls gene expression that is required during development and immune cell functions (1,7,8).

Several studies have shown that gene regulation through NAB2 can both repress and activate EGR-mediated gene transcription. For instance, during macrophage development, NAB2 promotes macrophage-specific gene transcription, while it simultaneously blocks the expression of neutrophil-specific genes (1). In activated T cells, NAB2 enhances Interleukin 2 (IL-2) gene transcription through its interaction with EGR-1; this process is blocked by the transcriptional activity of EGR-2 and EGR-3 (2,3). Depending on the cellular context, NAB2 also has the capacity to activate or repress transcription of the same target gene, resulting in seemingly contradictory findings about its role in control of specific gene expression. This is highlighted by our own studies demonstrating that NAB2 co-activates TRAIL expression in activated human plasmacytoid dendritic cells (pDCs), while it behaves as a co-repressor of transcription of the same gene, TRAIL, in reactivated murine CD8+ T cells (4,5).

This observation brought us to question whether and how NAB2 expression influences the levels of TRAIL in other cell death-inducing immune cells, such as Natural Killer (NK) cells. TRAIL expression in activated NK cells plays a relevant role in cytotoxicity and immunosurveillance against virally-infected and tumor cells (9,10). With the exception of murine NK cells from the liver, which constitutively express TRAIL (11,12), resting NK cells do not express TRAIL, but markedly upregulate this cytotoxic molecule upon cytokine stimulation. Specifically, IL-2, IL-15, and other cytokines such as IL-12 and type I Interferons (IFNs) have been shown to be responsible for TRAIL induction in NK cells (12-14).

Our study reveals that NAB2 expression is increased upon IL-2 and IL-15 triggering in NK cells, but not upon stimulation with IL-12 or type I IFNs. Importantly, we also found

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that NAB2 can promote TRAIL expression upon IL-2 and IL-15-mediated stimulation. Furthermore, we found that EGR-1 expression was also induced by IL-2 and IL-15 stimulation. Surprisingly, however, EGR-1 expression limited the induction of TRAIL in activated NK cells. This seemingly divergent role of these two transcriptional regulators suggests a tightly regulated network between NAB2 and EGR-1 in the control of TRAIL expression in NK cell function.

2. Materials and Methods

2.1. Isolation of primary NK cells and NKL cell cultures

Primary NK cells were obtained from peripheral blood of healthy donors as previously described (15). After PBMC isolation by ficoll gradient (Ficoll-Paque PLUS, GE Healthcare), primary NK cells were isolated using the NK cell negative isolation kit (Dynal, Life Technologies). NK cells were between 95 and 99% CD3−, CD56+. Local ethical committee approval was received for the studies and informed consent of all participating subjects was obtained.

The human NK cell line NKL (16) was cultured in RPMI supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FCS, and with 50 U/ml recombinant human IL-2. Cells were maintained at 37°C in 5% CO2.

2.2. Generation of NKL cells expressing constitutive NAB2, NAB2E51K, or short hairpins targeting EGR-1.

Lentiviral vectors containing human NAB2 and NAB2E51K, or the control empty vectors (EV) were generated as previously described (4) and used to transduce NKL cells. GFP expressing cells were sorted on the FACSARia II (BD Biosciences). EGR-1 knock-down was achieved in NKL cells with lentiviral vectors containing shRNA targeting EGR-1 (CloneID: NM_001964.2-1135s1c1, shEGR-1) or Luciferase (SHC007), obtained from Sigma MISSION shRNA library (Sigma-Aldrich), in which the puromycin selection was replaced with the GFP reporter gene. Lentiviral particles were generated by calcium phosphate transfection in 293T cells. NKL cells were activated for 2 hours with 50 U/ml IL-2 and 100 ng/ml IL-12 prior to spin transduction with 8 μg/ml polybrene at 2000 rpm for 90 min. EGR-1 knock-down efficiency was assessed by RT-PCR on sorted GFP+ NKL cells.

2.3. Activation of primary NK cells and NKL cells

Primary human NK cells were stimulated for indicated time points with 300 U/ml IL-2 (National Institutes of Health cytokine repository), 15 ng/ml IL-15 (PAN Biotech), or left untreated. NKL cells were seeded overnight in culture medium deprived of IL-2, followed
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2.4. Flow cytometry and antibodies

TRAIL cell surface staining was performed with unlabelled anti-TRAIL antibody (clone 2E5; Enzo Life Sciences), or with control mouse IgG1 (BD Biosciences), followed by anti-mouse IgG1-Biotin (Enzo Life Sciences) and APC- or PE-conjugated Steptavidin (BD Pharmingen). Dead cell exclusion was performed with Propidium Iodide. Flow cytometry was performed with LSRII (BD Biosciences), and data were analysed with FlowJo software (Tristar).

2.5. RNA extraction and Real Time PCR

Total RNA was isolated with TRIZOL (Invitrogen). cDNA was generated with SuperScript RT II or SuperScript RT III (Invitrogen) using Random Primers (Promega). Real-time RT-PCR was performed with ABsolute QPCR SYBR Green mix (Abgene) or SyBR Green Master Mix (Applied Biosystems) using the CFX96 (Bio-Rad) or Step One Plus (Applied Biosystems) RT-PCR systems. The following primers were used for analysis: TRAIL (5’T-ATGGCTATGATGGAGGTCCAG-3’; 5’T-TTGTCCTGCATCTGCTTCAGC-3’), NAB2 (5’T-CCCGAGAGAGCACCTACTTG-3’; 5’T-GGGTGACTCTGTTCCTCCAACC-3’), EGR-1 (5’T-AGCCCTACGAGCACCTGAC-3’, 5’T-GGTTTGGGCTGGGTAACTG-3’). Each sample was analyzed in duplicates or triplicates and expression levels were normalized to the housekeeping gene 18s (5’T-AGACAACAAGCTCCGTGAAGA-3’; 5’T-CAGAAGTGACGCAGCCCTCTA-3’).

2.6. Statistical analysis

Data are represented as mean ± standard deviation (SD), and evaluated using a two-tailed, unpaired Student’s T test. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Activation of NK cells with IL-2 or IL-15 results in enhanced NAB2 expression

To assess whether the transcriptional regulator NAB2 mediates the expression of TRAIL in NK cells, comparable to what is found in T cells and pDCs (4,5), we first determined if NAB2 expression was induced by two key mediators of NK cell activation and cytotoxicity, IL-2 and IL-15 (9,17). We found that in primary human NK cells the

by stimulation with 100 U/ml IL-2, 10 ng/ml IL-15, 10 ng/ml IL-12 (R&D Systems) or 100 ng/ml IFN-β (PBL Medical Laboratories).
expression of NAB2 mRNA upon overnight stimulation with IL-2 and IL-15 was increased by a two-three fold compared to unstimulated NK cells (Fig. 1A, \( p = 0.0035, p = 0.001 \)). As expected, also TRAIL mRNA and protein levels were significantly enhanced in primary NK cells that were stimulated with IL-2 and IL-15 (Fig 1B, \( p = 0.0038, p = 0.0001 \)). We further investigated the kinetics of NAB2 expression in activated NK cells, and found that upon IL-2 and IL-15 stimulation, NAB2 mRNA was maximally induced between 4 and 8 h of stimulation and was back to basal levels after 20 h of stimulation (Fig. 1C). The kinetics of TRAIL mRNA induction and its protein expression were similar to that of NAB2, only that TRAIL protein induction peaked between 8 and 24 h post activation, and the TRAIL expression level remained high for longer periods of time (Fig. 1D).

We also assessed the expression pattern of NAB2 and TRAIL in the NK cell line NKL (16). Upon stimulation with IL-2 or IL-15 for 4 h, also NKL cells showed significantly increased expression of NAB2 (Fig. 1E, \( p < 0.0001 \)) and induction of TRAIL mRNA and protein (Fig. 1F). These results and the kinetics of NAB2 and TRAIL in NKL cells stimulated with IL-15 (Supplemental Fig. 1A and B) corroborated with our findings in primary NK cells, and allowed us to use this cell line to further dissect the role of NAB2 in the regulation of TRAIL expression.

### 3.2. High NAB2 levels increase TRAIL expression in activated NK cells

Having established the expression pattern of NAB2 and TRAIL in IL-2 and IL-15 stimulated NK cells, we next investigated whether modulating the expression levels of NAB2 influenced the induction of TRAIL upon NK cell stimulation. To this end, we transduced NKL cells with wild type NAB2 (NKL-NAB2), or with a dominant negative mutant of NAB2 that impairs the activity of endogenous NAB2 by sequestering it from its EGR binding partners (NKL-NAB2\textsubscript{E51K}) (4,18,19). As control, we used NKL cells transduced with the empty vector (NKL-EV).

In resting NKL cells, the modified NAB2 levels did not affect the protein levels of TRAIL, which were comparable between NKL-NAB2, NKL-NAB2\textsubscript{E51K} and NKL-EV (Fig. 2A). Also, mRNA levels were not affected by the altered levels of NAB2 (Fig. 2B, \( p = 0.42 \) and \( p = 0.63 \)). Interestingly, upon IL-2 and IL-15 stimulation, the exogenous expression of wild type NAB2 in NKL-NAB2 cells led to higher expression levels of TRAIL protein when compared to the expression levels in activated NKL-EV cells (Fig. 2A). TRAIL mRNA levels were also more potently induced in NKL-NAB2 cells compared to NKL-EV cells upon IL-2 and IL-15 stimulation (Fig. 2B, \( p = 0.005 \) and \( p = 0.009 \)). Provided that high NAB2 levels lead to increased TRAIL levels in activated NKL cells, these findings indicate that NAB2 acts as a positive transcriptional regulator of TRAIL expression in NK cells.
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Figure 1. IL-2 and IL-15 stimulation induce NAB2 and TRAIL in human primary NK cells and in the human NKL cell line. (A-D) Human primary NK cells were stimulated overnight (A, B) or for indicated time (C, D) with 300 U/ml IL-2, 15 ng/ml IL-15, or left untreated (Ctrl). (A, C) mRNA levels of NAB2 were measured by RT-PCR and (B, D) TRAIL mRNA (left) and protein (right) levels were measured by RT-PCR and flow cytometry. Isotype control IgG1 staining was performed on activated NK cells. (E, F) NKL cells were seeded overnight in the absence of IL-2 and stimulated for 4 h with 10 ng/ml IL-15, 100 U/ml IL-2, or left untreated (Ctrl). mRNA levels of NAB2 (E) and TRAIL (F, left), and TRAIL protein levels (F, right) were measured. Data are shown as mean ± SD of 2-3 replicates normalized to the 18s reference gene, and are representative of independent donors (A-D) or 3 independently performed experiments (E, F). Data are analyzed with a two-tailed, unpaired Student’s T test (**p < 0.005; ***p < 0.001).
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Figure 2. Overexpression of NAB2 leads to increased TRAIL levels in IL-2 and IL-15 activated NKL cells. (A) NKL cells transduced with NAB2 (NKL-NAB2), NAB2E51K (NKL-NAB2E51K), or the empty vector (NKL-EV) were stimulated for 4 h with IL-2, IL-15, or left untreated (Ctrl) and TRAIL protein expression was measured. (A) Data represent one of 4 (IL-2) or 5 (IL-15) independent experiments. (B) mRNA levels of TRAIL were measured in NKL cells stimulated for 6 h with IL-2, IL-15 or left untreated (Ctrl). Data are depicted as mean ± SD of 3 replicates normalized to the 18s reference gene, and are analyzed with a two-tailed, unpaired Student’s T test (**p < 0.005).

3.3. The transcription factor EGR-1 limits TRAIL expression in activated NK cells

The transcriptional regulator NAB2 does not bind directly to DNA to exert its function, because it lacks a DNA binding domain (7). Instead, NAB2 regulates transcription of target genes interacting with its binding partners, the EGR transcription factors (7). As EGR-1 is also implicated in the regulation of cytotoxic molecules (20), we asked whether it plays a role in the transcriptional regulation of TRAIL in NK cells. To this end, we first assessed whether cytokine stimulation induces EGR-1 levels. We found that overnight stimulation of primary human NK cells with IL-2 or IL-15 resulted in increased EGR-1 mRNA levels (Fig. 3A, p < 0.0001). Given the fact that EGR proteins belong to the immediate responders to stimulation (21), we also assessed EGR-1 expression at earlier time points after NK cell activation, and found that its levels were highest at 1 h post IL-2 or IL-15 stimulation (Fig. 3B). This expression pattern was also observed in NKL cells stimulated with IL-15 (Supplemental Fig. 2A).

To assess whether EGR-1 can modulate the expression of TRAIL in activated NK cells, we transduced NKL cells with lentiviruses that constitutively express a short hairpin (sh)RNA directed against EGR-1 (shEGR-1) together with the GFP reporter gene. shRNA
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against Luciferase was used as control (shLuc) (Supplemental Fig. 2B). The reduction of basal EGR-1 mRNA levels in the presence of the shRNA against EGR-1 was approximately 50% ($p = 0.01$; Supplemental Fig. 2C). Strikingly, the diminished EGR-1 levels in NKL cells resulted in an increased capacity to respond to IL-2 and IL-15 stimulation, leading to higher TRAIL levels when compared to the shLuc expressing control cells (Fig. 3C, upper panel). Furthermore, we found that the increased levels of TRAIL expression was intrinsic to the GFP$^+$ shEGR-1 expressing NKL cells and not mediated by soluble factors, because the untransduced, GFP$^-$ NKL cells present in the same culture (Supplemental Fig. 2B) did not show any differences in TRAIL expression upon stimulation (Fig. 3C, lower panel). Overall, these data reveal that, while high NAB2 levels increase TRAIL expression in activated NK cells, EGR-1 acts as a corepressor of TRAIL expression.

**Figure 3.** EGR-1 is induced upon IL-2 and IL-15 stimulation and limits TRAIL expression in NK cells. (A-B) Human primary NK cells were stimulated overnight (A) or for indicated time (B) with IL-2, IL-15, or left untreated (Ctrl), and EGR-1 mRNA levels were assessed by RT-PCR. Data are shown as mean ± SD of 3 experimental replicates normalized to the 18s reference gene. (C) NKL cells were transduced with short hairpins directed against EGR-1 (shEGR-1) or control Luciferase (shLuc) and TRAIL expression was measured by flow cytometry of transduced (GFP$^+$) and untransduced (GFP$^-$) cells upon 4 h stimulation with IL-2, IL-15, or left untreated (Ctrl). Numbers in the upper right corner represent TRAIL GeoMFI of shLuc/shEGR-1 populations. Data are representative of 2 independent experiments (**p < 0.001).
3.4. NAB2 and EGR-1 expression in NK cells is induced by IL-2 and IL-15, but not by IFN-β or IL-12

Here, we have shown that NAB2 and EGR-1 are induced in NK cells upon IL-2 and IL-15 activation and that these two transcriptional regulators modulate the expression of TRAIL, albeit in the opposite way. We next set out to determine whether EGR-1 and NAB2 were acting only downstream of the IL-2R and IL-15R, or also of other stimulatory receptors. Stimulation with IL-12 or IFN-β has been shown to activate NK cells and promote TRAIL expression (13,14). In order to understand whether signalling through these two cytokine receptors also increased NAB2 and EGR-1 expression that could regulate TRAIL levels, we activated NKL cells also with these two cytokines. Interestingly, we could not detect increases in NAB2 or EGR-1 levels upon 4 h stimulation with IL-12 (Fig. 4A and B). In addition, while previous studies have shown that NKL cells displayed increased TRAIL levels upon a 48 h culture with IL-12 (13), 4 h stimulation with 10 ng/ml IL-12 failed to do so, as opposed to IL-2 and IL-15 stimulation (Fig. 4C and D). Of note, IFNγ mRNA levels were increased upon IL-12 stimulation (data

Figure 4. NAB2 and EGR-1 are induced in NK cells upon stimulation with IL-2 and IL-15, but not IL-12 or IFN-β. (A-C) NKL cells were seeded overnight in absence of IL-2 prior to 4 h stimulation with 100 U/ml IL-2, 10 ng/ml IL-15, 10 ng/ml IL-12, 100 ng/ml IFN-β, or were left untreated (Ctrl). mRNA levels of NAB2 (A), EGR-1 (B) and TRAIL (C) were measured by RT-PCR. Data are shown as mean ± SD of 2 replicates normalized to the 18s reference gene. (D) TRAIL protein expression was measured by flow cytometry in NKL cells stimulated for 4 h with indicated cytokine, or left untreated (Ctrl). Data are representative of at least 3 independent experiments.
not shown), indicating that IL-12 stimulation - at least at this time point - failed to specifically induce TRAIL, NAB2 and EGR-1 expression.

In line with our findings that type I IFN stimulation of human pDCs induced TRAIL expression independently of the PI3K/NAB2 axis (4), IFN-β triggering of NK cells also completely failed to induce NAB2 (Fig. 4A). Similarly, the expression of EGR-1 mRNA was unchanged upon IFN-β triggering of NK cells (Fig 4B), even though TRAIL expression was optimally induced (Fig. 4C and D). Together, these findings provide further evidence that type I IFN-R mediated TRAIL induction is uncoupled from the NAB2-EGR axis, at least in the two cell types we analysed, pDCs and NK cells.

4. Discussion

Here, we show that NAB2 and EGR-1 determine the levels of TRAIL expression in NK cells that were activated with IL-2 or IL-15. While the transcriptional regulator NAB2 acts as a positive regulator for TRAIL expression, the transcription factor EGR-1 keeps the levels of TRAIL expression in check. Therefore, we propose that these two transcriptional regulators allow for a balanced TRAIL expression in NK cells.

Which signalling pathways mediate the induction of NAB2 and EGR-1 in NK cells is yet to be determined. One potential candidate for the induction of these two transcriptional regulators is PI3K. We have recently demonstrated that TLR-mediated induction of NAB2 in human pDCs occurs in a PI3K-dependent manner (4). Interestingly, signalling through the IL-2 Receptor (IL-2R) and the IL-15R, which is mediated by their common signal-transducing receptor chains CD122 and the common gamma chain also results in the activation of the PI3K-AKT pathway (22).

While culturing with IL-12 can activate NK cells (9,13), we have observed that this stimulus does not suffice to induce TRAIL and/or NAB2 and EGR-1 within 4 h. Importantly, evidence has been provided that IL-12R does not signal through PI3K, and also fails to induce EGR-1 (22,23). This is also in line with our previous findings that IL-2 and IL-15 triggering of T cells affects TRAIL expression in murine T cells in a Nab2-dependent manner, while IL-12 triggering fails to do so (24).

Of note, also other early activation gene family members such as c-fos and junB, and the transcription factor AP-1 are induced upon IL-2R signalling, but not upon IL-12R-mediated signalling (23). Because AP-1 has been shown to induce NAB2 and EGR-1 expression in T cells upon T cell receptor triggering (3), it is tempting to speculate that this transcription factor is also involved in regulating NAB2 and EGR-1 induction in NK cells.

Interestingly, in line with our findings in human pDCs (4), type I IFN stimulation of NK cells induced TRAIL expression independently of NAB2 and EGR-1-mediated gene regulation (Fig. 4). This demonstrates that the STAT-dependent type I IFN receptor
signaling pathway (14,22,25) induces TRAIL expression in an entirely independent manner from NAB2-EGR-1 transcriptional regulation.

Intriguingly, we found that reduced levels of EGR-1 were instrumental for the induction of TRAIL in NK cells. The reduction of EGR-1 levels led to elevated TRAIL levels upon stimulation of NK cells with IL-2 and IL-15, suggesting that EGR-1 acts as a brake to prevent uncontrolled and fast expression of TRAIL (Fig. 3). This notion is supported by our observation that endogenous EGR-1 is very fast but transiently induced (Fig. 3). Conversely, NAB2 is induced several hours later upon NK cell activation (Fig. 1). The differential expression pattern of these two transcriptional regulators may result in a time-dependent control of TRAIL expression. This could therefore explain why NAB2 and EGR-1, although being induced in NK cells through the same stimuli, exert an opposite role in TRAIL regulation, EGR-1 acting as a repressor of TRAIL induction at early stages, and NAB2 being a co-activator that for TRAIL mRNA expression at later time points. Furthermore, the selective induction of the other NAB2 binding partners EGR-2 and EGR-3 could also dictate whether NAB2 and EGR-1 collaborate or modulate each other’s function, and whether they induce or repress their target genes.

A control of TRAIL by NAB2 and EGR-1 analogous to the one we described here has been shown in human vein endothelial cells, where overexpression of EGR-1 resulted in repression of TRAIL, while overexpression of NAB2 in this cell type led to upregulated TRAIL expression (20). In contrast, NAB2 and EGR-1 collaborate in inducing gene expression in activated T cells (3) as well as in neuroectodermal or epithelial cells (18), suggesting that the function of these two transcriptional regulators - a collaborative versus an antagonistic role in gene regulation - is strictly cell type-dependent.

Overall, our findings indicate that NAB2 and EGR-1 are important regulators of TRAIL expression in NK cells. TRAIL induction appears to be a highly regulated process that should allow NK cells to fine-tune their cytotoxic activity during the control of infection and tumors.

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References


Supplemental Information

Supplemental Figure 1. NKL cells rapidly induce NAB2 and TRAIL expression upon IL-15 stimulation. (A-C) NKL cells were stimulated for indicated time points with 10 ng/ml IL-15 or left untreated (Ctrl). NAB2 (A) and TRAIL (B) mRNA levels were assessed by RT-PCR. Data are shown as mean ± SD of 3 replicates normalized to the 18s reference gene. (C) TRAIL protein levels were measured by flow cytometry. Isotype control IgG1 staining was performed on activated NKL cells. Data are representative of 2 independent experiments.
Supplemental Figure 2. (A) NKL cells were stimulated for indicated time points with 10 ng/ml IL-15 or left untreated (Ctrl), and EGR-1 mRNA levels were measured by RT-PCR. (B, C) NKL were transduced with lentiviruses expressing the shRNA targeting EGR-1 (shEGR-1), or Luciferase (shLuc) as control, together with the GFP reporter gene. (B) The % of GFP+ cells was measured and (C) EGR-1 knock-down was measured by RT-PCR on GFP+ -sorted shLUC and shEGR-1 expressing NKL cells. Data are analyzed with a two-tailed, unpaired Student’s T test (*p < 0.05).