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Two-layered immune escape in AML is overcome by Fcg receptor activation and inhibition of PGE2 signalling in NK cells

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Abstract:
Loss of anticancer NK cell function in AML patients is associated with fatal disease progression and remains poorly understood. Here, we demonstrate that AML-blasts isolated from patients rapidly inhibit NK cell function and escape NK cell-mediated killing. Transcriptome analysis of NK cells exposed to AML-blasts revealed increased CREM expression and transcriptional activity, indicating enhanced cAMP signalling, confirmed by uniform production of the cAMP-inducing prostanoid PGE2 by all AML-blast isolates from patients. Phosphoproteome analysis disclosed that PGE2 induced a blockade of LCK-ERK signalling that is crucial for NK cell activation, indicating a two-layered escape of AML-blasts with low expression of NK cell-activating ligands and inhibition of NK cell signalling. To evaluate the therapeutic potential to target PGE2 inhibition, we combined Fcg-receptor-mediated activation with the prevention of inhibitory PGE2-signalling. This rescued NK cell function and restored the killing of AML-blasts. Thus, we identify the PGE2-LCK signalling axis as the key barrier for NK cell activation in two-layered immune escape of AML-blasts that can be targeted for immune therapy to reconstitute anti-cancer NK cell immunity in AML patients.

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Two-layered immune escape in AML is overcome by Fcγ receptor activation and inhibition of PGE_2 signalling in NK cells

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RNA-seq data are available at GSE191074 using clohwuccxxmvpcb as a password.

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Key Points

- AML-blasts suppress NK cells by secreting PGE_2, leading to a blockade of LCK and consequently to a functional blockade of NK cells
- Therapeutic approaches with antibodies targeting AML-blasts in combination with the blockade of PGE_2 signalling enable NK killing

Abstract

Loss of anticancer NK cell function in AML patients is associated with fatal disease progression and remains poorly understood. Here, we demonstrate that AML-blasts isolated from patients rapidly inhibit NK cell function and escape NK cell-mediated killing. Transcriptome analysis of NK cells exposed to AML-blasts revealed increased *CREM* expression and transcriptional activity, indicating enhanced cAMP signalling, confirmed by uniform production of the cAMP-inducing prostanoid PGE_2 by all AML-blast isolates from patients. Phosphoproteome analysis disclosed that PGE_2 induced a blockade of LCK-ERK signalling that is crucial for NK cell activation, indicating a two-layered escape of AML-blasts with low expression of NK cell-activating ligands and inhibition of NK cell signalling. To evaluate the therapeutic potential to target PGE_2 inhibition, we combined Fcγ-receptor-mediated activation with the prevention of inhibitory PGE_2-signalling. This rescued NK cell function and restored the killing of AML-blasts. Thus, we identify the PGE_2-LCK signalling axis as the key barrier for NK cell activation in two-layered immune escape of AML-blasts that can be targeted for immune therapy to reconstitute anti-cancer NK cell immunity in AML patients.

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Introduction

NK cells are important for anticancer immunity because they can efficiently kill cancer cells[1]. Cancer cell elimination requires ligand-dependent stimulation of NK cells through germ-line encoded activating receptors, such as NKG2D, NKp30 and DNAM-1, without signalling through inhibitory NK cell receptors[2]. NK cells are also activated through their Fcγ-receptors by antibodies bound to target cells, leading to antibody-mediated cellular cytotoxicity[1, 3]. After binding to stimulatory ligands, activating receptors on NK cells signal through ITAM or YINM motifs, which are coupled to Src-kinases and further downstream kinases such as Vav, Rac, Rho and Erk, to induce cytokine production and NK cell degranulation to achieve tumour cell killing[4, 5, 6]. However, in cancer patients, particularly in patients with acute myeloid leukaemia (AML), NK cells often lose their effector function[7]. Although this loss of NK cell effector function in AML is well known[8] and intact NK cell function is linked to the prevention of disease recurrence[9], it remains unclear which mechanisms are responsible for this loss of NK cell function.

AML is the most common cause of leukaemia-related death, and patients with AML are treated mainly with chemotherapy, but less than one-third of AML patients achieve durable remission[7], strengthening the need for effective immune therapies. AML is characterised by the emergence of immature myeloid leukaemia cells in the bone marrow and peripheral blood, indicating a loss of anticancer immune surveillance. In AML, impaired functional maturation of NK cells[10], slightly reduced expression levels of stimulatory receptors[11, 12, 13] and low expression of NKG2D ligands on AML-blasts have been reported[14], which may all contribute to cancer immune evasion but do not explain the loss of NK cell function in these patients.

Here, we characterised the mechanisms by which AML-blasts from patients escaped killing by NK cells and identified the inhibitory PGE_2-EP2/EP4 signalling axis as a molecular target for immune therapy to reconstitute the effector function of NK cells against AML-blasts.

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Materials and Methods

Flow cytometry and cell sorting

The cells were stained with the appropriate antibodies for 30 min in PBS supplemented with 0.2% BSA and 2 mM EDTA at room temperature (20°C, RT) in the dark, followed by analysis using an ID7000, SP6800 and SA3800 spectral analyzer (Sony Biotechnology, Japan). FACS-Sorting of cells was performed using a Sony SH800 Cell sorter in purity mode. The data were analyzed using FlowJo software 10.2.4 (TreeStar Inc., OR, USA).

AML patients

All experiments were performed by ethics approval 218-08. After providing written informed consent following the Declaration of Helsinki and approval from the Institutional Review Board of Ludwig-Maximilians-University (Munich, Germany), peripheral blood or bone marrow samples were collected from patients with AML at primary diagnosis or relapse of disease. At primary diagnosis or relapse, samples were analyzed at the Laboratory for Leukemia Diagnostics of the Clinic of Ludwig-Maximilians University, Munich[15, 16]. Patient characteristics are summarized in Supplementary Table I. Mononuclear cells from AML patients were isolated by density gradient centrifugation (Biocoll Separating Solution) and cryopreserved at < -80 °C in 80% fetal calf serum and 20% dimethyl sulfoxide.

Monocyte, NK cell and B-cell isolation

All experiments were performed in accordance with the ethics approval 564/18AS. Informed written consent was obtained from each blood donor. Peripheral blood mononuclear cells were isolated from freshly drawn blood by density gradient centrifugation (Pancoll gradient). Following the manufacturer's recommendations, NK cells were purified using a negative selection kit (eBioscience). The purity of $CD56^+$ $CD3^-$ NK cells was usually > 90%. Human monocytes and B cells from healthy donors were isolated via positive immunomagnetic isolation using anti-CD14 or anti-CD19 coupled microbeads (Miltenyi) according to the manufacturer’s recommendations.

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Coculture experiments

AML-derived blasts were cocultured with allogenic, healthy donor-derived, untreated NK cells at various E:T ratios (NK:AML-blasts 1:0.3/1/3/10) in RPMI medium supplemented with 25 IU rhu interleukin (IL)-2 (proleukin S), 10% FCS, penicillin (200 mg), streptomycin (200 U/ml) and L-glutamine (2 mM) in 96-well U-bottom plates in a humidified incubator at 37 °C. Unless otherwise stated, the cocultures were incubated overnight (18 h).

Evaluation of NK cell functions

NK cells were stimulated by the addition of equal numbers of K562 cells to the wells (1:1), followed by centrifugation at 200 × g for 1 min to achieve immediate cell contact. NK cell effector functions were determined by measuring cytokine/chemokine secretion, degranulation, and target cell lysis. NK cells were cocultured with K562 cells for 3 h at 37 °C.

RNA sequencing of NK cells

For sample preparation**,** healthy donor-derived NK cells were cocultured with AML-blasts or with 100 ng/ml PGE_2 for 6 h, followed by NK cells reisolation via FACS sorting in purity mode at 4 °C. K562 cells were added for the last 2 h of coculture if indicated. NK cell mRNA was isolated (Monarch RNA Miniprep Kit, NEB) according to the manufacturer's instructions. Library preparation for bulk sequencing of poly(A)-RNA was performed as described previously[17].

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Results

AML-blasts from leukaemia patients cause loss of NK cell effector function

$CD3^{neg}CD56^{+}$ NK cells isolated from AML patients during disease exacerbation, had lower expression levels of stimulatory receptors such as NKG2D and lost their responsiveness to stimulation compared to NK cells from healthy donors (**Figure 1A,B; Figure S1A**), consistent with previous reports on the loss of NK cell function in AML patients[18].

To investigate the mechanism underlying this loss of NK cell effector function, we cocultured $CD3^{neg}CD56^{+}$ NK cells from healthy donors (HDs) with AML-blasts, which were isolated from the blood of AML patients during disease exacerbation. While, NK cell viability was not reduced by AML-blasts (**Figure 1C**), we detected downregulation of the NK cell activating receptors NKG2D, NKp30, and DNAM-1 (**Figure 1D,E**), similar to the NK cell phenotype found in AML patients. NK cells cocultured with AML-blasts failed to respond to activation by K562 cells in a dose-dependent manner, exhibited decreased CD107 expression, did not produce effector cytokines such as IFNγ and TNF, and no longer killed K562 cells (**Figure 1F-H; Figure S1B**). Notably, the granzyme levels remained unchanged (**Figure S1C**). Allogeneic monocytes from healthy donors had no measurable effect on the functionality of NK cells, whereas AML-blasts suppressed the number of NK cells responding to stimulation in a dose-dependent fashion (**Figure 1I,J; Figure S1D**), demonstrating that AML-blasts but not monocytes had a suppressive effect. Moreover, the expression levels of TNF and IFNγ were reduced in NK cells that still produced these cytokines (**Figure 1K**), demonstrating that AML-blasts reduced the number of NK cells responsive to stimulation as well as the response of individual cells. Notably, specific mutations in cancer cells (FLT3-ITD, FLT3-TKD or NPM1) detected in AML patients at initial diagnosis and disease relapse or differences in the FAB disease classification (M1-M5) did not correlate with AML-blast-induced suppression of NK cell function (**Figure S1E,F**). Rather, all AML-blast populations isolated from a total of 38 patients exhibited a similar ability to disrupt NK cell function regardless of disease classification (**Suppl. Table I**). Taken together, coculture with AML-blasts isolated from leukaemia patients suppressed NK cell function without affecting their viability, indicating

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that this coculture can be used as a tool to identify the mechanism underlying the loss of NK cell function after contact with AML-blasts.

PGE_2-induced transcriptional signature in NK cells cocultured with AML-blasts and from AML patients

Several mechanisms may cause the loss of NK cell function, such as signalling through inhibitory receptors like PD-1[19, 20], depletion of extracellular L-arginine by arginase[21, 22], or depletion of intracellular L-arginine by methylglyoxal[23]. However, neither checkpoint inhibition through antibody-mediated blockade of PD-L1 nor pharmacological inhibition of iNOS/Arg-1 nor neutralisation of arginine-depleting methylglyoxal by dimethyl biguanides rescued NK cells from the suppressive effect of AML-blast (**Figure S2A-D**), suggesting a previously unappreciated mechanism. Therefore, after coculture with AML-blasts, we subjected NK cells to RNA sequencing to characterise the mechanisms responsible for the loss of NK cell function in an unbiased fashion. We found that only 32 genes were differentially expressed when NK cells were cocultured with AML-blasts, of which 28 were upregulated and four were downregulated (**Figure 2A**). Notably, genes encoding IFN-γ and TNF were among the downregulated genes. Transcription factor network analysis revealed eight active transcription factors, CREM, FOSL2, IRF1, JUND, IRF4, ZNF331, EPAS1, and STAT3, in AML-blast-exposed NK cells (**Figure 2B**). EPAS1 encodes HIF2α, which induces autophagic NK cell death[24] and, therefore, is unlikely to play a role as there were no differences in viability (see *Figure 1*). Since the transcription factors JUND, FOSL2, and ZNF331 are involved in differentiation processes and IRF1 and IRF4 are activated in response to interferon signalling[25, 26], we focused on the transcription factors STAT3 and CREM. STAT3 activity is induced by signalling in response to IL-6 and IL-10[27]. IL-6 protein levels were elevated in monocytes **(Figure S2E)** and, therefore, unlikely to cause AML-mediated NK cell dysfunction. Although AML-blasts are known to secrete IL-10[11]. Although IL-10 induced transient STAT3 phosphorylation in NK cells, it failed to cause NK cell dysfunction (**Figure S2F-H**).

The transcription factor cyclic AMP responsive element modulator (CREM) is induced upon increased cyclic AMP signalling in cells[28]. PGE_2 is among the most

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prominent inducers of cAMP signalling in immune cells[29]. PGE_2 treatment of NK cells led to upregulated *CREM* gene expression (**Figure S2I**). When we treated NK cells with PGE_2 and performed RNA sequencing, we detected a transcriptional signature similar to that of AML-blast-exposed NK cells (**Figure 2C**) and a similar transcription factor network (**Figure S2J**). By comparing the transcriptional profiles of PGE_2- and AML-blast-exposed NK cells via gene set enrichment analysis, we found very high similarity, with a normalised enrichment score (NES) of 2.5, and extremely high statistical significance, with a false discovery rate (FDR) of 0 (**Figure 2D, Suppl. Table II**).

We found that AML-blasts but not monocytes or NK cells expressed high levels of the *PTGES2/COX2* gene, which encodes the PGE_2-producing enzyme cyclooxygenase-2 (**Figure 2E**). Indeed, we detected high concentrations of PGE_2 in the supernatants of AML-blasts from patients (**Figure 2F**). Importantly, the extent of the AML-blast-induced suppression of NK cell function directly correlated with the amount of PGE_2 produced by AML-blasts (**Figure 2G**). To test whether NK cells are similarly influenced by PGE_2 in AML patients, we identified NK cells in an AML single-cell RNA-seq database[30] and analysed their transcriptional profile for a PGE_2 signature (**Figure S2J-L**). NK cells from AML patients showed a characteristic PGE_2 signature, indicating PGE_2-effect *in vivo* (**Figure 2H; Figure S2K-N**).

PGE_2 promotes rapid AML-blast-induced suppression of NK cell function

PGE_2 exposure decreased the cytokine production, degranulation, and killing activity of NK cells in a dose-dependent manner, and impaired IFNγ and TNF production was observed at low PGE_2 concentrations (**Figure 3A; Figure S3A-B**). Furthermore, the cAMP-inducing agent forskolin and a cell-permeable cAMP analogue similarly inhibited NK cell function, suggesting a cAMP-dependent mechanism underlying the suppression of NK cell function (**Figure S3C**).

To evaluate whether prostanoids such as PGE_2, released from AML-blasts with increased COX2 expression, are indeed responsible for the loss of NK cell function, we used the selective COX2 inhibitor celecoxib to prevent PGE_2 production. Celecoxib treatment of cocultures of AML-blasts and NK cells prevented the AML-blast-induced inhibition of NK cell function (**Figure 3B**), suggesting that PGE_2 is a key factor in the

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AML-blast-induced inhibition of NK cell function. To provide direct evidence for the effect of PGE_2 on the inhibition of NK cell function, we blocked PGE_2 signalling by inhibiting the PGE_2 receptors EP2 and EP4. Blocking EP2/4 signalling prevented the reduction in cytokine expression and degranulation of NK cells that were cocultured with AML-blasts (**Figure 3B**). Comparable effects were also observed with regard to NKG2D surface expression. The inhibition of COX-2 activity by celecoxib, as well as the blocking of EP2/4 signalling, prevented the down-regulation of the activating receptor NKG2D (**Figure 3C**). Thus, the production of PGE_2 by AML-blasts is the key determinant of reduced NK cell effector functions.

Next, we investigated the time dynamics of the suppressive effect of prostanoids on NK cell effector functions. Five minutes of exposure to PGE_2 was sufficient to induce a suppressive effect, and further increasing the duration of exposure did not achieve additional suppression of NK cell effector function (**Figure 3D; Figure S3D**). We detected similar rapid kinetics for the suppression of NK cell function by AML-blasts (**Figure 3E**). In contrast, the suppressive effect of PGE_2 on the expression levels of NKG2D, DNAM-1 and NKp30 on NK cells was detected only after more than four hours (**Figure S3E**), indicating that the reduced expression of stimulatory receptors might not determine the rapid loss of NK cell effector functions. However, AML-blast-induced reduction of expression of activating receptors like NKG2D may contribute to NK cell dysfunction.

Overall, the short time period in which AML-blast-released PGE_2 inhibited NK cell function suggests a modification in signal transduction in NK cells and may explain the few transcriptional changes observed after the contact of activated NK cells with AML-blasts (**Figure S3F**).

Exposure to PGE_2 leads to inhibition of NK cell signalling in response to activation

We next explored whether PGE_2 exposure leads to the inhibition of signalling processes involved in NK cell recognition of target cells. Indeed, calcium influx in stimulated NK cells, a downstream event of activation-inducing signalling in immune cells[31], was impaired after PGE_2 exposure (**Figure 4A**). To characterise the signalling in NK cells

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following exposure to PGE_2 in detail, we performed mass spectrometric analysis. NK cells were incubated with PGE_2 for ten minutes, followed by LC/MS analysis. As expected from the short time period required for suppressing NK cell function, we did not observe changes in the proteome of NK cells after short-term exposure to PGE_2 (**Figure S4A**). In the phosphoproteome analysis, however, we found enrichment of phosphopeptides involved in NK cell signalling among the peptides downregulated by PGE_2 (**Figure 4B**). Therefore, we modified our experimental approach and incubated stimulated NK cells with K562, which are target cells, to uncover changes in the signalling pathways relevant to the NK cell activation caused by PGE_2 exposure. Compared with those in mock-treated NK cells, the intensities of 159 phosphopeptides differed significantly after PGE_2 treatment in NK cells exposed to K562 target cells (**Figure 4C; Figure S4B**). Pathway analysis suggested that several signalling pathways in NK cells are impaired by PGE_2, including the MAPK, mTOR and NK cell signalling pathways (**Figure 4D, Suppl. Table III**), which are important for mediating NK cell activation[5]. This led us to study the phosphorylation events in key signalling kinases in NK cells regulated by AML-blasts in more detail.

PGE_2 blocks Lck and ERK signalling, relevant for NK cell activation

NK cell-activating receptors, such as the ITAM-associated receptors NKp30, NKp44, NKp46, CD16, DNAM-1, 2B4, and NKG2D, require Src family kinases and, in particular, Lck to mediate activation-induced signalling and trigger NK cell effector functions[32, 33, 34]. Lck kinase activity is regulated at three phosphorylation sites (Y192, Y394, and Y505) that determine protein conformation and activity[35, 36]. Lck activation requires phosphorylation at residue Y394, whereas phosphorylation at residue Y505 leads to a closed conformation of Lck, rendering residue Y394 inaccessible for phosphorylation[36], and phosphorylation at residue Y192 also inhibits Lck activation[35, 37]. In PGE_2- and forskolin-treated NK cells, we detected increased Lck phosphorylation at residues Y505 and Y192 (**Figure 5A,B**), indicating that PGE_2 and increased cAMP signalling in NK cells caused an inactive/closed conformation of Lck with consequent loss of signalling.

Indeed, exposure to PGE_2 prevented the phosphorylation of residue Y394 in NK cells stimulated with K562 cells (**Figure 5C,D; Figure S5A**), confirming that PGE_2

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caused a loss of activation-induced signalling through Lck in NK cells. We characterised signalling events downstream of Lck in PGE_2-treated NK cells. PGE_2 induced a decrease in the phosphorylation of mTOR (S2448) and its downstream target S6 (S235/236) in NK cells stimulated with K562 cells (**Figure 5E,F**). Moreover, PGE_2 caused a complete decrease in ERK1/2 (T202/204) phosphorylation in stimulated NK cells (**Figure 5G,H**). Pharmacologic inhibition of the mTOR kinase by rapamycin, however, failed to affect NK cell function (**Figure 5I**), indicating that mTOR signalling is not critical for activation-induced signalling in NK cells. In contrast, inhibition of ERK activity led to a similar loss of NK cell function as PGE_2 treatment (**Figure 5I; Figure S5B,C**), consistent with the essential function of ERK for NK cell activation[38, 39, 40]. These results suggested that PGE_2 inhibited NK cell function by preventing signalling through Lck and ERK.

To address the question of whether contact with AML-blasts influences the activity of signalling kinases in NK cells through prostanoid production, we characterized the phosphorylation of the signalling kinases Lck, mTOR, S6 and ERK in NK cells in coculture experiments. Similar to PGE_2, AML-blasts efficiently suppressed the phosphorylation of Lck at residue Y394 (**Figure 5J,K**). Importantly, the addition of celecoxib or the EP2/4 antagonist rescued the phosphorylation of Lck at residue Y394 in NK cells exposed to AML-blasts (**Figure 5J,K**), consistent with the reversal of the inhibitory effect of AML-blasts on Lck signalling when prostanoid production in AML-blasts or PGE_2 signalling in NK cells was blocked. Moreover, celecoxib and the EP2/4 antagonist reversed the inhibition of mTOR and ERK phosphorylation induced by AML-blasts (**Figure 5L-O**). Taken together, these data demonstrate the key role of PGE_2 released from AML-blasts in suppressing NK cell effector function by inhibiting activation-induced signalling through Lck and ERK.

Blocking the inhibitory PGE_2-EP2/4 axis allows NK cells to kill anti-CD123-coated AML-blasts

Our results demonstrated that blocking the inhibitory PGE_2-EP2/EP4 signalling rescued NK cells from suppression by AML-blasts and enabled them to kill target cells such as K562 cells. Because AML-blasts do not express NKG2D ligands[14] (**Figure 6A**), we

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assumed that AML-blasts were protected from NK cell killing by two-layered immune escape, i.e., the absence of NK cell activation and the inhibition of NK cell signalling in response to activation. Therefore, we reasoned that enhancing NK cell activation in combination with blocking PGE_2 signalling would overcome this two-layered immune escape and enable NK cells to kill AML-blasts.

Activation-induced signalling through the Fcγ-receptor enhances the killing activity of NK cells, which is mediated by signalling through Lck[38, 41] and may thus serve as a possible route of NK cell activation. First, we evaluated whether the activation of NK cells through Fcγ-receptors by an anti-CD20 antibody coating of autologous B cells was inhibited by PGE_2. Indeed, PGE_2 attenuated the enhanced cytokine production and killing of anti-CD20 antibody-coated autologous B cells (**Figure 6SA**). The PGE_2-mediated attenuation of NK cell function was only partially overcome by increasing the dose of anti-CD20 antibodies (**Figure S6B**), as expected from the PGE_2-induced inhibition of Lck and ERK phosphorylation that led to a block in signalling downstream of Fcγ-receptors (**Figure S6C**). Thus, PGE_2 affects NK cell signalling through Fcγ-receptors, indicating that PGE2 completely controls activation-induced signalling in NK cells.

Next, we investigated whether NK cell killing of AML-blasts could be rescued by activating signalling through Fcγ-receptors in combination with blocking the inhibitory PGE_2-EP2/4 signalling axis. Current immune therapies for AML target the IL-3 receptor (CD123) on the surface of AML cells by employing anti-CD123 antibodies[42 43]. Given the high expression of CD123 on AML blasts (**Figure 6B**), we employed anti-CD123 antibodies to coat AML blasts, thereby providing activation signals through Fcγ receptors for NK cells. As expected from our results, NK cells encountering AML-blasts failed to become activated and produce cytokines in response to blocking the inhibitory PGE_2-EP2/4 signalling axis or activation signals from Fcγ-receptors when AML-blasts were coated with anti-CD123 antibodies (**Figure 6C**). However, when an anti-CD123 antibody coating of AML-blasts was combined with blocking of the inhibitory PGE_2-EP2/4 signalling axis, NK cells produced the effector cytokines TNF and IFNγ (**Figure 6C**). More importantly, the anti-CD123 coating of AML-blasts combined with the inhibitory PGE_2-EP2/4 signalling axis enabled NK cells to efficiently kill AML-blasts

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(**Figure 6D,E**). Thus, combining activation signals from Fcγ-receptors and preventing PGE_2-induced inhibition of activation-induced NK cell signalling is sufficient to overcome the two-layered immune escape of AML-blasts and boost NK cell-mediated killing of cancer cells.

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Discussion

There is an urgent need to develop novel therapeutic strategies for AML because of the frequent disease relapse after chemotherapy[7]. Immune therapies with antibodies or T cells targeting the IL-3 receptor α (CD123), often expressed by AML cells, have shown some effects but are limited by treatment-related toxicity. Recently, therapeutic strategies have increasingly focused on NK cells to achieve anti-cancer immune surveillance because of their unique ability to recognise and eliminate cancer cells[6]. NK cells are key for anti-leukaemia immune surveillance in the bone marrow and are, therefore, crucial for AML patients who received bone marrow transplants[44]. Accumulation of unconventional NK cells with reduced expression of activating receptors is associated with poor clinical outcome[45]. Adoptive NK cell transfer, and in particular transfer of cytokine-stimulated memory-like NK cells, is a promising strategy that can induce remission in patients with relapsed/refractory AML[46, 47, 48]. Moreover, the transfer of donor-derived memory-like NK cells results in improved tumour control and is associated with the long-term persistence of NK cells in bone marrow transplanted AML patients[47]. However, memory-like NK cells appear to be particularly sensitive to inhibitory signalling through NKG2A-ligation through HLA-E expression on AML blasts[49]. In cancer patients, however, NK cells decrease in numbers and lose their effector functions[50]. Current immune therapeutic strategies, therefore, aim to restore NK cell function against cancer[50] and block inhibitory pathways, such as the NKG2A/HLA-E axis[51]. Decreasing the expression of inhibitory receptors such as NKG2A by CRISPR/Cas9-editing improved CAR-NK cell effector function against AML-blasts[52]. Furthermore, in addition to preventing signals through checkpoint inhibitor molecules that render NK cells exhausted, NK cell engager molecules that deliver activation signals through NKp46 and CD16 to NK cells are currently being evaluated in clinical trials. In particular, for immune therapy of AML, a novel trifunctional NKp46-CD16-NK cell engager molecule targeting CD123 has recently been developed[43].

Here, we demonstrate that AML-blasts evade anticancer NK cell surveillance through a two-layered immune escape, i.e., the lack of NK cell stimulatory signals and the PGE_2-mediated inhibition of NK cell signalling. Our findings demonstrate that only

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the combination of NK cell engager molecules together with the prevention of the inhibitory PGE_2-EP2/4 signalling axis reconstituted the NK cell-mediated killing of AML-blasts, which has important consequences for the design of future NK cell therapies for AML patients.

The development of immune therapies against AML remains a challenge. Although the induction of a dysfunctional state of NK cells in AML patients and its association with the loss of immune control of cancer and disease relapse have been recognised for decades, their mechanism remains elusive[8, 9]. Different mechanisms have been implicated in causing immune cell dysfunction in AML, ranging from inhibitory mediators acting in a paracrine fashion and checkpoint molecules to cell-intrinsic mechanisms driving NK cell exhaustion[53]. Using unbiased transcriptome and proteome profiling to characterise the interaction between AML-blasts and NK cells, we identified PGE_2 as the most prominent AML-blast-derived inhibitor of NK cell function, which is corroborated by our finding that NK cells in AML patients have a characteristic PGE_2 signature. In a cohort of 38 AML patients, we demonstrated that through the production of the prostanoid PGE_2, AML-blasts inhibited NK cell signalling via EP2/4 blocking LcK and ERK functions as key signalling kinase that are relevant for the induction of anti-cancer NK cell effector functions, such as cytokine expression and the killing of cancer cells. Our data suggest that the rapid induction of this block in NK cell signalling through PGE_2 is likely sufficient for AML-blasts to escape killing even if they encounter fully functional NK cells. However, prevention of the inhibitory PGE_2-EP2/4 axis alone did not reconstitute NK cell function against AML-blasts, pointing towards a two-layered immune escape where AML-blasts fail to provide NK cell activating signals and, at the same time, prevent activation signalling in NK cells.

Signalling through the Src kinase Lck is important for downstream signalling of NK cell-activating receptors like NKG2D and Fcγ-receptors mediating antibody-dependent cellular cytotoxicity. Thus, the PGE_2-induced block in signalling may render NK cells nonresponsive to NK cell engager molecules. We used anti-CD123 antibodies that target the IL-3 receptor as an NK cell engager molecule to direct antibody-dependent NK cell cytotoxicity through the activation via the Fcγ-receptor towards AML-blasts. Yet, anti-CD123 antibodies did not induce anticancer NK cell effector function,

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consistent with the potent inhibition of NK cell signalling by AML-blast-derived PGE_2. Combining Fcγ-receptor activation signals with preventing the inhibitory PGE_2-EP2/4 axis, however, restored Lck and ERK signalling and, consequently, enabled NK cells to kill anti-CD123-coated AML-blasts. Thus, our results reveal a combinatorial strategy that can be used to overcome the two-layered immune escape of AML-blasts and to render NK cells competent to execute protective anticancer immune surveillance.

Acknowledgements

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Competing interests statement: The authors declare to have no competing interests.

Author contributions

T.B., C.R., A.M.P., C.K., B.B., A.N., G.H., A.M., A.H., C.K., C.M., J.H., and C.S. performed the experiments and analyzed the data. S.D., C.U., J.M. and R.Ö. performed the bioinformatics analyses. T.B., R.J., L.S., B.S., C.D., A.P., K.S.G., V.B., R.R., and J.A. contributed specific technologies and reagents. T.B., M.S., P.A.K., J.B. and B.H. designed the experiments and wrote the manuscript. All the authors have read and approved the manuscript.

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Figure 1: AML-blasts inhibit NK cell function and escape NK cell killing. **A**, representative NKG2D expression by $CD3^{neg}CD56^{+}$ NK cells from healthy donors (HDs) and AML patients. **B**, IFNγ- and TNF-producing NK cells after K562 cell (n=5) stimulation. **C**, NK cell viability after overnight coculture with AML-blasts from different patients (n=5). **D,E**, NK cell expression of NKG2D, DNAM-1 and NKp30 in the presence of AML-blasts from different patients (n=9). **F-H**, IFNγ, TNF, and CD107a expression and cytotoxic effector function (% of apoptotic K562 target cells) of NK cells in the presence of AML-blasts. **I**, suppression of NK cell functions after coculture with different ratios of AML-blasts to NK cells; monocytes served as controls. **J**, IFNγ, TNF, and CD107a expression and lysis (cytotoxic effector function) of NK cells after stimulation with K562 cells in the presence of AML-blasts or monocytes (10X) (n≥7). **K**, normalized geometric means of IFNγ, TNF, and CD107a in NK cells after coculture with AML-blasts (n≥7). Statistical analysis by Student's t-tests (B,D) and one-way ANOVA (I). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 2: The transcriptional profile of NK cells by AML-blasts is mediated via PGE_2. A-D, RNA sequencing of NK cells treated with 100 ng/ml PGE_2 or cocultured (6h) with AML-blast and exposed to K562 cells (for the last 2 h of the experiment). **A,B**, heatmap and TF interaction network analysis of DEGs in NK cells cocultured with AML-blasts (n=3). **C,D**, heatmap and gene set enrichment analysis comparing NK cells exposed to PGE_2 and cocultured with AML-blasts. **E**, relative expression levels of PTGES1/2 mRNA in NK cells (n=2), monocytes (n=8), and AML-blasts (n=6) quantified by qPCR. **F**, PGE_2 concentrations in the supernatants of AML-blasts (n=22), monocytes (n=3) and NK cells (n=8). **G**, correlation of PGE_2 concentrations in AML-blast supernatant with the suppression of NK cell function (n≥5). **H**, Violin plot distribution of UCell scores for the enriched PGE_2 signature according to single-cell RNA-seq in cells from AML patients. Statistical analysis: one-way ANOVA (F) or Student's t-test (G,I). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 3: Suppression of NK cell effector functions by PGE_2 is rapid and reversible. A, suppression of NK cell effector functions towards K562 cells after exposure to increasing concentrations of PGE_2 (n=5). **B,C**, Effector function and NKG2D surface expression of NK cells cocultured with AML-blasts (ratio 1:3) after exposure to K562 cells in the presence of celecoxib (1 µM) or an EP2/4 antagonist (5 µM) (n≥5). **D,E**, time-resolved changes in NK cell function in response to the addition of PGE_2 (100 ng/ml) to K562 cells or coculture with AML-blasts followed by K562 stimulation (n=5). Statistical analysis by Student's t-test (B,C,E). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 4: Regulation of NK cell signalling by PGE_2. A, Ca^{2+} influx into NK cells after stimulation with K562 cells in the presence of PGE_2 (100 ng/ml) (n=4). **B-D**, NK cells were treated with 100 ng/ml PGE_2, stimulated with K562 cells for 10 min, reisolated and subjected to phosphoproteome analysis (n=6). **B**, significantly enriched Gene Ontology pathways in PGE_2-treated NK cells vs NK cells alone ordered by Z score, colour code represents statistical significance. **C**, volcano plot analysis of differentially expressed phosphopeptides, red circles identify significant hits. **D**, enriched pathways in NK^{Stim}+PGE_2 vs NK^{Stim} cells ordered by Z score, colour code represents statistical significance. Statistical analysis: Student's t-test (c). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; two-sided Welch's t test with a p value threshold of < 0.05 was used (B,D).

Figure 5: PGE_2 inhibits NK cell signalling through Lck and ERK. A,B, Time kinetics of Lck phosphorylation at residues Y505 and Y192 in NK cells exposed to PGE_2 (100 ng/ml) or forskolin (50 µM) detected by western blot and quantification (n≥3). **C-H**, phosphorylation of signalling kinases in NK cells treated with PGE_2 (100 ng/ml) and stimulated with K562 cells for Y394 Lck (**C,D**), for S6 (S235/S236) and mTOR (S2448) (**E,F**) and for ERK1/2 (Y202/Y204) (**G,H**) (n≥3). **I,** NK cell function after treatment with PGE_2 (100 ng/ml), the MEK1/2 inhibitor PD 0325901 (1 µM) and rapamycin (20 nM) (n=3). **J-O.**

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phosphorylation of signalling kinases in K562-stimulated NK cells cocultured with AML-blasts and treated with celecoxib (1 μM) or an EP2/4 antagonist (5 μM) (n≥4). Statistical analysis by two-way ANOVA (**I**). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

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Figure 6: Inhibition of PGE_2 signalling restores NK cell killing by anti-CD123-coated AML-blasts.
A, B, AML-blasts were analysed for NK cell stimulatory ligands and CD123 expression by flow cytometry of three different patients. **C,** cytokine production by NK cells after contact with AML-blasts and treatment with anti-CD123, celecoxib or EP2/4 inhibitor or the combination of both (n=3). **D**,**E,** NK cell-mediated killing of AML-blasts (annexinV^{pos}) after adding an anti-CD123 antibody, celecoxib or an EP2/4 inhibitor (n=3). Statistical analysis: two-way ANOVA (**C-F**). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Graphical summary: AML-blasts escape anticancer NK cell surveillance by (1) lack of NKG2D-ligand expression and (2) PGE_2 secretion blocking the LcK-signalling pathway relevant for NK cell activation. Anti-CD123 antibody application, in combination with PGE_2 signalling blockade, enables NK cell activation and leads to the killing of AML-blasts. This demonstrates the importance of targeting the inhibitory PGE_2 signalling pathway to render NK cell responsive to NK cell engager molecules and increase anticancer NK cell activity.

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Figure 1

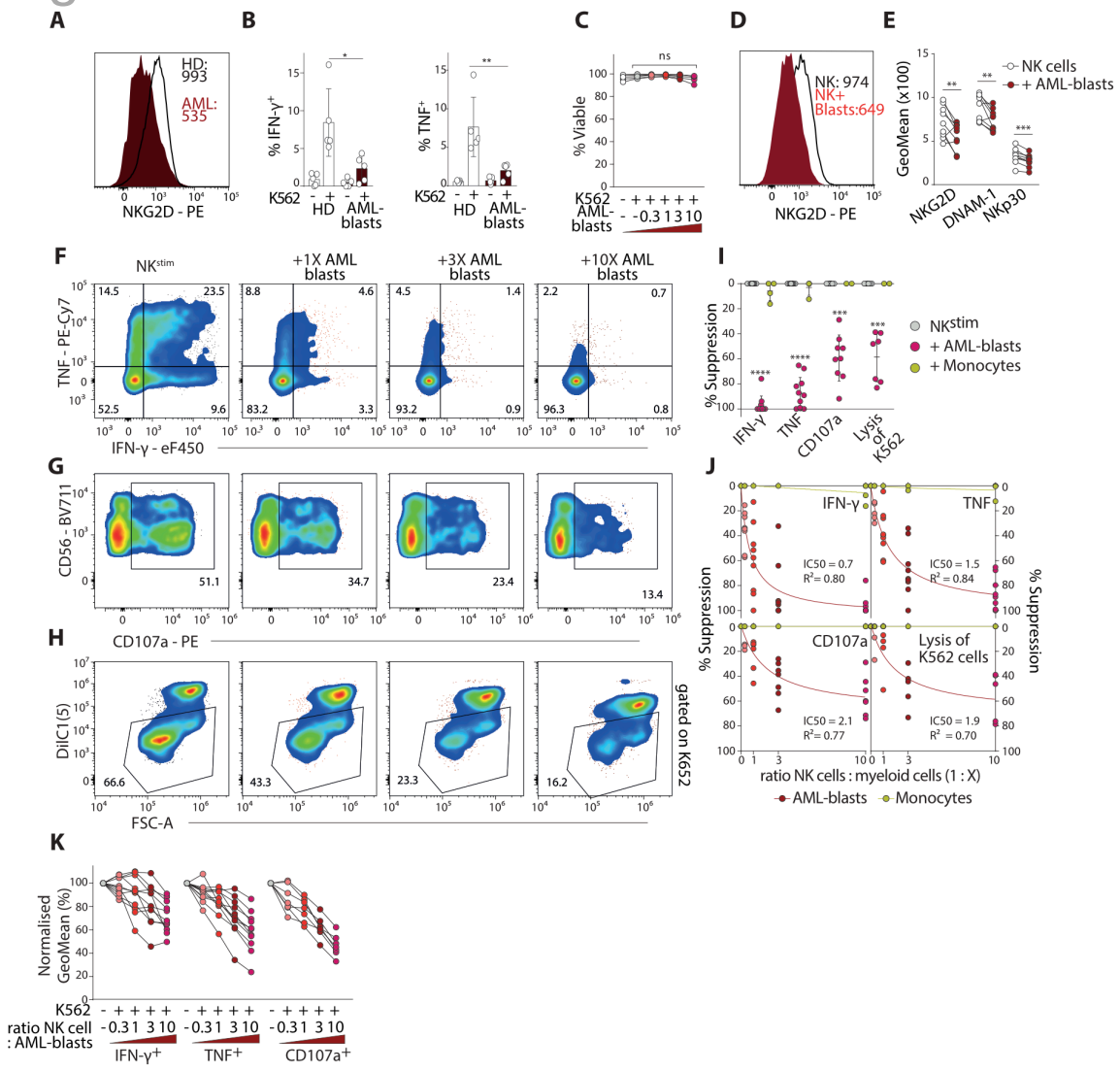

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Figure 2

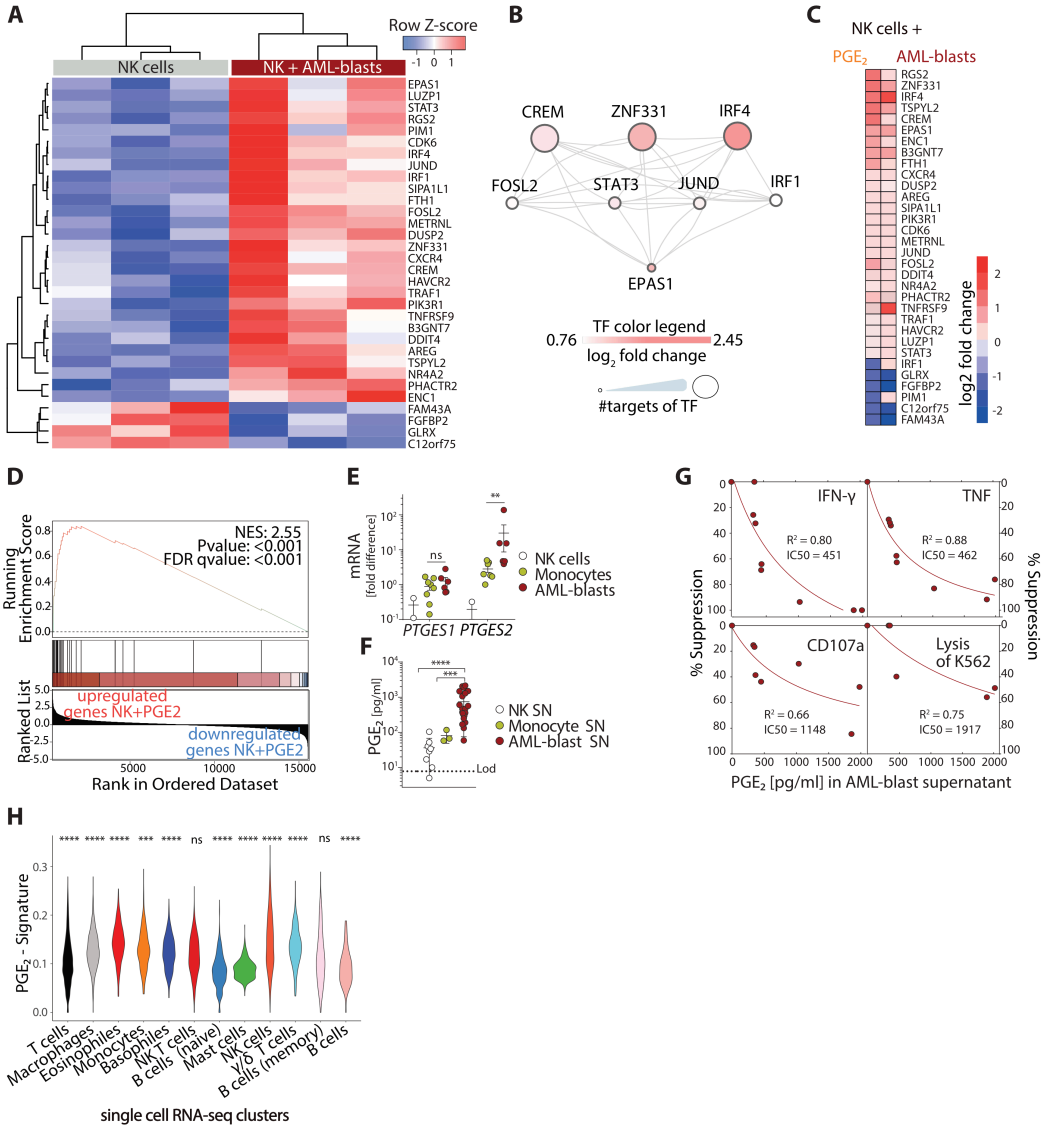

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Figure 3

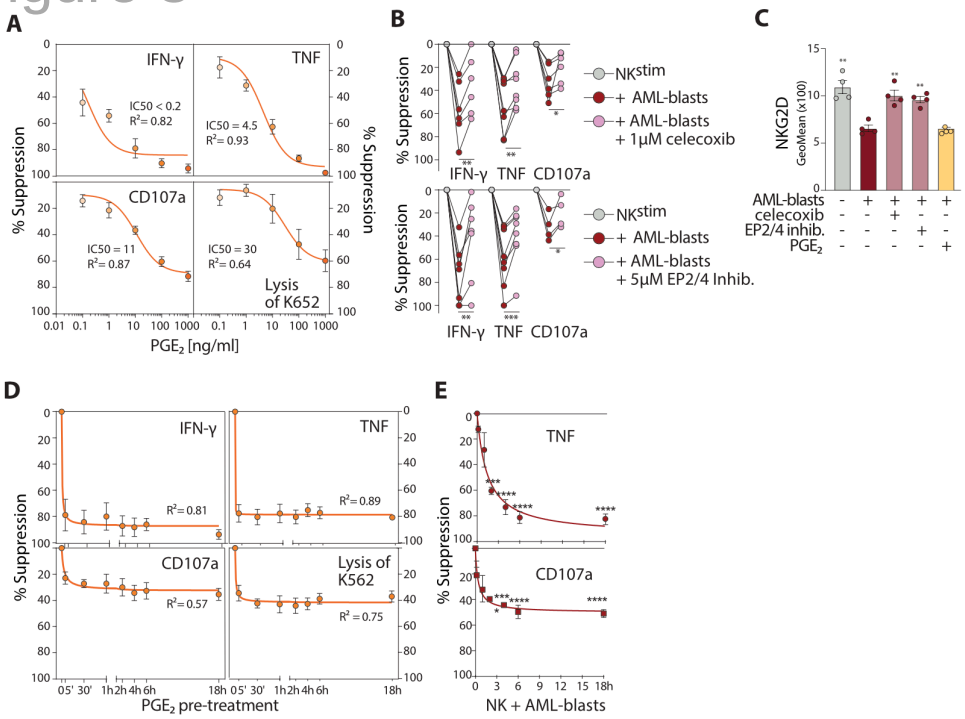

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Figure 4

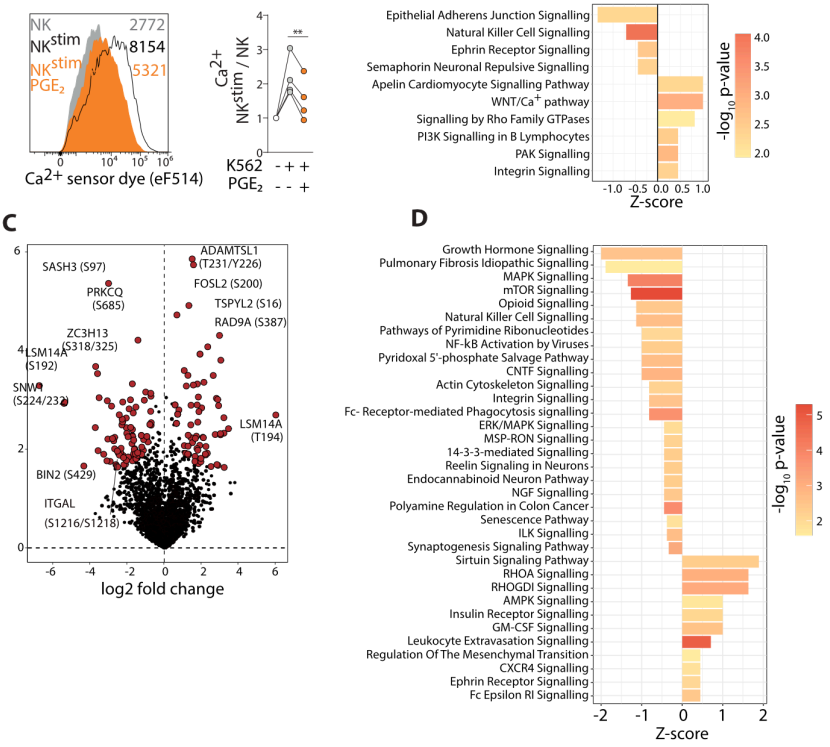

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Figure 5

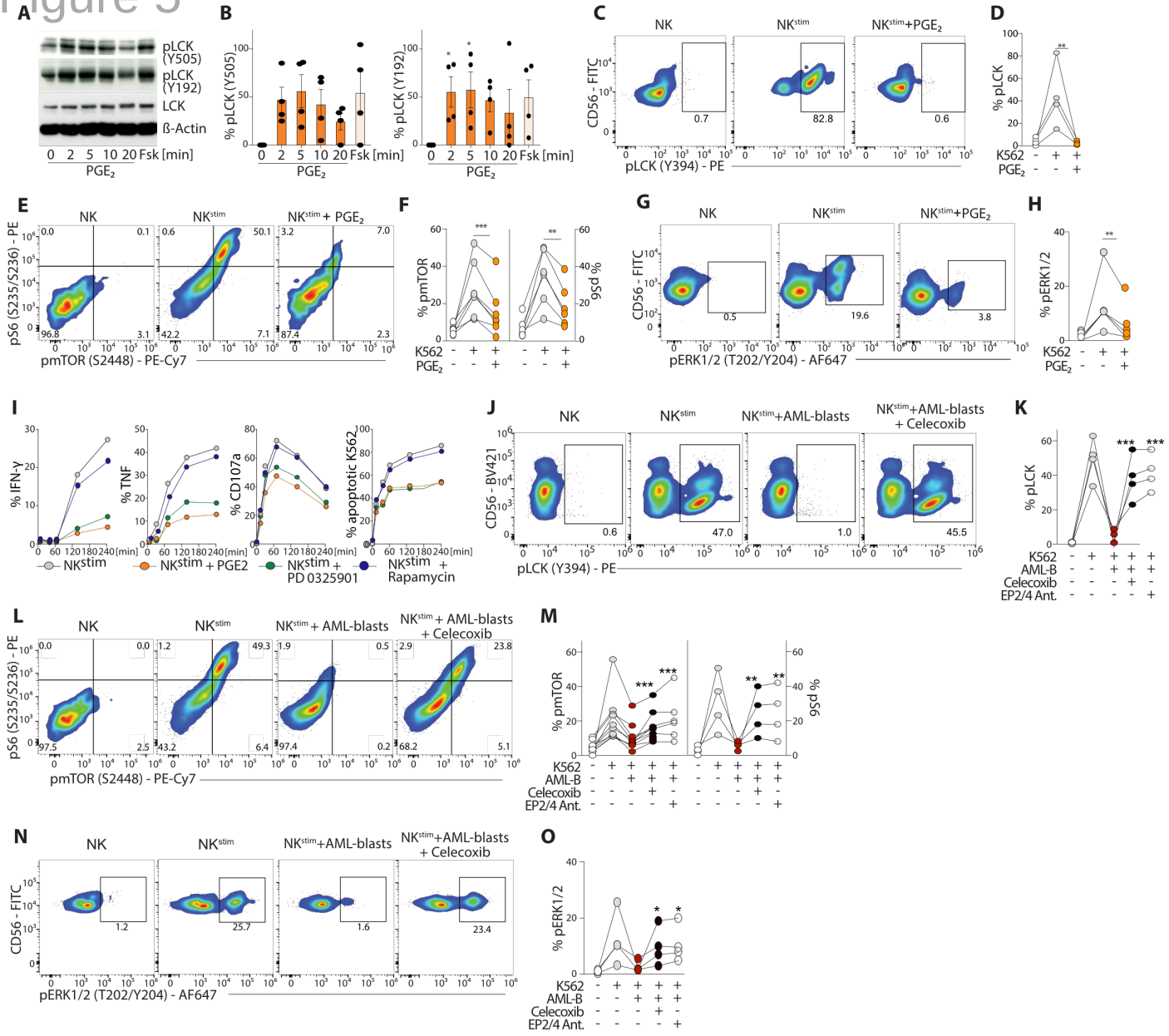

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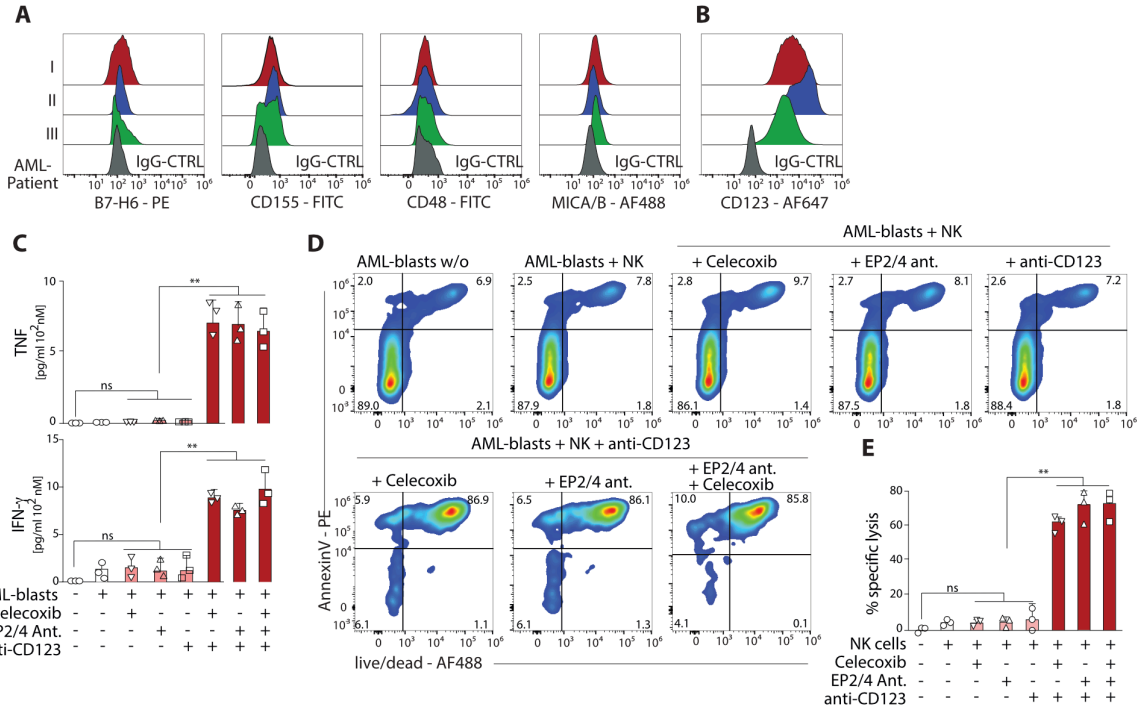
Figure 6



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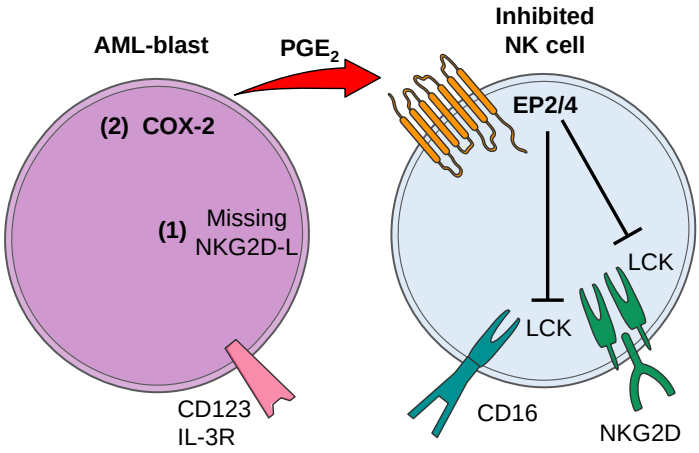
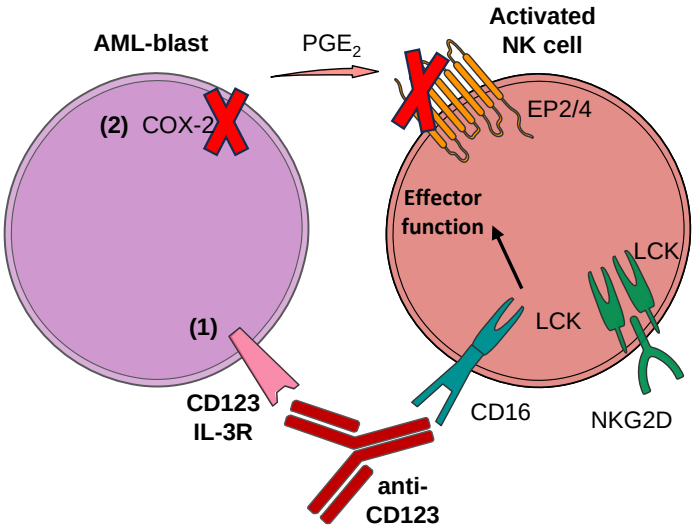
Functional Blockade of NK Cell Function by PGE_2-Secreting AML Blasts

Two-layered immune escape of AML-blasts from NK cell killing



(1) Lack of stimulatory signal for NK cell activation
(2) PGE_2-induced inhibition of NK cell signaling

Reconstituting NK cell killing of AML-blasts



(1) NK cell engaging through CD16 via anti-CD123
(2) Prevention of PGE_2-EP2/4 inhibitory signaling

Conclusions: **AML-blasts suppress NK cell effector function by secreting PGE_2, leading to a blockade of LCK and a functional blockade of NK cells. Antibodies targeting AML-blasts in combination with blockade of PGE_2 signaling enable NK killing.**

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