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Targeting CD70 in combination with chemotherapy to enhance the anti-tumor immune effects in non-small cell lung cancer

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ABSTRACT

Despite the recent emergence of immune checkpoint inhibitors, clinical outcomes of metastatic NSCLC patients remain poor, pointing out the unmet need to develop novel therapies to enhance the anti-tumor immune response in NSCLC. In this regard, aberrant expression of the immune checkpoint molecule CD70 has been reported on many cancer types, including NSCLC. In this study, the cytotoxic and immune stimulatory potential of an antibody-based anti-CD70 (aCD70) therapy was explored as single agent and in combination with docetaxel and cisplatin in NSCLC *in vitro* and *in vivo*. Anti-CD70 therapy resulted in NK-mediated killing of NSCLC cells and increased production of pro-inflammatory cytokines by NK cells *in vitro*. The combination of chemotherapy and anti-CD70 therapy further enhanced NSCLC cell killing. Moreover, *in vivo* findings showed that the sequential treatment of chemo-immunotherapy resulted in a significant improved survival and delayed tumor growth compared to single agents in Lewis Lung carcinoma-bearing mice. The immunogenic potential of the chemotherapeutic regimen was further highlighted by increased numbers of dendritic cells in the tumor-draining lymph nodes in these tumor-bearing mice after treatment. The sequential combination therapy resulted in enhanced intratumoral infiltration of both T and NK cells, as well as an increase in the ratio of CD8+ T cells over Tregs. The superior effect of the sequential combination therapy on survival was further confirmed in a NCI-H1975-bearing humanized IL15-NSG-CD34+ mouse model. These novel preclinical data demonstrate the potential of combining chemotherapy and aCD70 therapy to enhance anti-tumor immune responses in NSCLC patients.

Background

Non-small cell lung cancer (NSCLC) remains one of the leading causes of cancer-related mortality and accounts for approximately 80–85% of all lung cancer cases worldwide[1]. One of the contributing factors to the high mortality rate in NSCLC is that patients are often diagnosed at a late stage of the disease[2]. In the last decade, a paradigm shift has occurred in the first-line treatment of metastatic NSCLC, which currently consists of chemotherapy, targeted therapy and the combination of immunotherapy and chemotherapy[3]. NSCLC patients that harbor driver mutations, such as molecular alterations in epidermal growth factor receptor (EGFR), BFAF or rearrangements in genes encoding for anaplastic lymphoma kinase (ALK), ROS, RET and TRK are eligible for targeted treatment, which has improved lung cancer mortality to some extent[4]. Nevertheless, this treatment option is only applicable to a minority of NSCLC patients. Most of the NSCLC patients (85–90%) in advanced stages of disease have no oncogenic alterations and are therefore in need of other effective treatment options. While a combination of immunotherapy and chemotherapy remains the standard of care for the majority of advanced NSCLC patients, this treatment modality usually shows limited efficacy[5] due to intrinsic or acquired drug resistance[6], pointing out the need for new therapeutic strategies.

Immunotherapy has been integrated as one of the pillars of cancer treatment in many cancer types[7]. Immune checkpoint inhibitors aim to treat cancer by either activating the immune system or blocking the immunosuppressive tumor microenvironment. Especially, immune checkpoint inhibitors targeting the PD-1/PD-L1 axis have shown efficacy in a broad range of tumor types, including NSCLC. It should be noted, though, that only a subset of patients responds to single agent treatment, leaving considerable room for improvement[8]. Inflamed tumors, which have a preexisting CD8+ T cell response to tumor cells, are limited by intra-tumoral PD-L1 expression on those cells and are therefore tackled by antibodies against PD-1/PD-L1. Alternatively, non-inflamed tumors lack the intratumoral cytotoxic T-cells[9]. A major challenge is how to change a non-inflamed ("cold") tumor into an inflamed ("hot") tumor.

Another approach to release the brakes on the immune system is by modulating the CD70-CD27 axis. The CD70-CD27 axis has gained much interest in the field of oncology over the years[10,11]. CD70 can be constitutively expressed on cancer cells of various solid tumors, while hematological malignancies can show co-expression. The CD70-CD27 axis also facilitates immune evasion through CD27 signaling in the tumor microenvironment[10,11]. CD70 has been previously described to be constitutively expressed in a subset of NSCLC specimens. Interestingly, expression of CD70 was found on both primary and metastatic lesions, suggesting a significant therapeutic window for anti-CD70 therapy in NSCLC[12]. Monotherapy and combination strategies using CD70-targeting antibody-mediated therapies, such as antibody-drug conjugates (ADCs), antibody-dependent cellular cytotoxicity (ADCC) and chimeric antigen receptor T cell therapy have shown promising efficacy in hematological malignancies[11]. In case of solid tumors, a combination of cusatuzumab (anti-CD70 antibody with enhanced effector functions, argenx BV) with radiotherapy and/or chemotherapy was only evaluated in a phase I study in patients with nasopharyngeal carcinoma (NCT02759250). Patients that received a higher number of chemotherapy/radiotherapy lines had longer progression-free survival compared to the cohort of patients that received lower numbers of chemotherapy/radiotherapy lines prior to treatment with cusatuzumab, suggesting a possible role for chemotherapy/radiotherapy in immunomodulation[13]. Currently, there are no ongoing clinical trials evaluating combinatorial approaches of anti-CD70 therapy with either chemotherapy or radiotherapy in solid cancers and more specifically in NSCLC.

Our previous work has demonstrated that low doses of docetaxel and cisplatin were able to induce important hallmarks of immunogenic cell death (i.e. ATP, ecto-calreticulin and HMGB1) in NSCLC cell lines in vitro. In addition, phagocytosis of treated NSCLC cells and maturation of DCs occurred after treatment with this chemotherapeutic regimen. These findings were validated in an in vivo vaccination assay where all mice vaccinated with NSCLC cells and treated with the above-mentioned chemotherapeutic regimen remained tumor-free after challenge[14]. In this regard, combining immunotherapy with a chemotherapy regimen that can prime and activate a tumor-specific adaptive immune response might be a promising treatment strategy in NSCLC.

Hence, in this study, the in vitro and in vivo cytotoxic and immune stimulatory effects of anti-CD70 antibody-mediated immunotherapy with enhanced ADCC properties (aCD70) were evaluated in NSCLC as monotherapy and in combination with immunogenic chemotherapy

Methods

Cell lines and cell culture

Cell lines A549, NCI-H1975 and NCI-H1650 were purchased from the American Type Culture Collection (ATCC). In addition, the Lewis Lung carcinoma (LLC) cell line (gift from Dr. Carsten Riether, Department of Clinical Research, University of Bern; derived from the lung of a C57BL/6J mouse) was transduced with the viral vector pCH-EF1a-

MmCD70-Ires-eGFP-T2A-Puro (Leuven Viral Vector Core, KU Leuven, Belgium) to stably express CD70 (Suppl. Figure S3) A549 and LLC were cultured in Dulbecco's Modified Eagle Medium (DMEM, 10938–025, Life Technologies) supplemented with 10% FBS (10270–106, Life Technologies), 1% penicillin (100 U/mL)/streptomycin (100 µg/mL; 15140–122, Life Technologies) and 2 mM L-glutamine (L-Glut, 25030–024, Life Technologies). NCI-H1975 and NCI-H1650 were cultured in Roswell Park Memorial Institute Medium (RPMI, 52400–025, Life Technologies) supplemented as described above. Cells were grown as monolayers and were maintained in exponential growth in 5% CO_2 + 95% air in a humidified incubator at 37°C. All cell cultures were confirmed as Mycoplasma free using the Mycoalert® Mycoplasma detection kit (LT07–218, Lonza).

Compounds

Docetaxel and cisplatin were used (S1148 and S1166, respectively, Selleckchem) for in vitro studies and stored at −80°C at the desired stock concentrations prepared in either DMSO or NaCl (5 mM), respectively. Further dilutions of the chemotherapeutics were prepared in PBS. Combination regimens consisted of the IC_{20}-72 hours value of docetaxel (0.002 µM, 0.04 µM and 0.06 µM for NCI-1975, A549 and NCI-H1650, respectively) and cisplatin (8 µM, 3.8 µM and 2.9 µM for NCI-1975, A549 and NCI-H1650 respectively) for in vitro studies. Combination regimens of docetaxel (2.5 mg/kg) and cisplatin (1 mg/kg) were intraperitoneally (IP) used for in vivo studies. Mouse anti-CD70 antibodies (mFR70E), human anti-CD70 antibodies (cusatuzumab, argenx BV), both with enhanced effector function and isotype controls (I5029, Sigma-Aldrich, CB6, Sanbio) were used for in vitro (0.5 µg/ml) and in vivo (10 mg/kg) assays. Throughout this study, both mouse and human anti-CD70 antibodies will be referred to as 'aCD70'.

CD70 expression analysis

NSCLC cell lines were treated with docetaxel and cisplatin for 48 hours and stained with phycoerythrin (PE) mouse anti-human CD70 Abs (Clone Ki-24) or PE mouse IgG3 isotype control (eBioscience) to assess CD70 expression via flow cytometry. Samples were measured on the CytoFLEX (Beckman Coulter). The signal for aspecific binding (isotype control) was subtracted from the measured geometric fluorescence intensities (ΔgMFI). Dead cells were excluded from analysis by staining with 7-AAD. Analysis of all flow cytometry experiments was performed by using the FlowJo v10 software (TreeStar inc.). Experiments were performed in triplicate.

Immunohistochemical staining of CD70 was evaluated on dissected tumors of NCI-H1975-bearing Hu-NSG-IL15-CD34 + mice ($N = 50$, 25 mice per donor) treated with vehicle (PBS +NaCl) or chemotherapy (DOC+CDDP) on their endpoint (tumor size of 150 mm²). Five µm-thick sections were exposed to heat-induced antigen retrieval by incubation in a high pH buffer for 20 min. at 97°C (PT-Link) (DAKO, Glostrup, Denmark). Subsequently, endogenous peroxidase activity was quenched by incubating the slides in peroxidase blocking buffer (DAK0) for 5 min. CD70 staining was performed as previously described[15]. After blocking with normal goat serum

(30 min), anti-CD70 antibody (Invitrogen, PA5–102557, 1:750) was incubated at room temperature (35 min), followed by rabbit enhanced polymer-based linker (15 min), secondary HRP antibody (25 min) and visualized via a DAKO autostainer Link 48 instrument using the Envision FLEX+ detection kit (DAKO) according to the instructions of the manufacturer.

Purification of human natural killer cells

Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque Plus (Amersham Biosciences) gradient separation from buffy coats of healthy donors, purchased from the Blood Transfusion Centre of Red Cross-Flanders (Mechelen, Belgium). Primary natural killer (NK) cells were negatively isolated from PBMC using the NK cell isolation kit (Miltenyi Biotec, Utrecht, The Netherlands; CD56+ CD3- cells, purity 88.1 ± 4.7%) as previously described[15].

NK cell-mediated cytotoxicity assay

NucLight Red transduced NCI-H1960, A549 and NCI-H1975 cells were seeded in a 96-well plate[16]. After overnight incubation, cells were treated with docetaxel (IC_{20}) and cisplatin (IC_{20}) or vehicle (PBS) for 24 hours and then treated with human aCD70 or isotype control (0.5 μg/ml) in the presence of the IncuCyte Cytotox Green reagent (50 nM) whereafter NK cells were added to the co-culture at an effector:target (E:T) ratio of 5:1[16]. The plate was transferred to the temperature- and CO_2- controlled IncuCyte ZOOM for a total of 72 hours. Cell death was monitored by pictures that were taken every 24 hours to limit phototoxicity. For analysis, green object count (1/mm^2), red object count (1/mm^2) and green-red overlapping object count (1/mm^2) were determined with the IncuCyte ZOOM analyzer. NK cells were filtered out based on size and color. The percentage of tumor cell death was calculated with the formula: (Green object count)/(Red object count + Green object count – Overlapping object count) * 100. Experiments were performed minimum in triplicate using 3–5 NK donors.

NK cell activation

NCI-H1960, A549 and NCI-H1975 cells were seeded in 6-well plates, incubated overnight and treated with docetaxel and cisplatin (concentrations as described above). After 24 hours of treatment, the appropriate conditions were treated with human aCD70 or isotype control and co-cultured with NK cells at a 5:1 ratio. After washing, cells were stained with the following antibodies: anti-CD45 APC-Cy7 (1:50, Clone 2D1, Biolegend), anti-CD3 PE-Cy7 (1:100, Clone SK7, Biolegend), anti-CD56 PE-CF594 (1:25, Clone NCAM16.2, BD Biosciences), anti-CD69 PerCP-Cy5.5 (1:100, Clone FN50, Biolegend) and Live/Dead Fixable Aqua (1:50, Invitrogen) for 30 minutes at 4°C. Acquisition was performed on the Novocyte Quanteon (Agilent technologies).

Cytokine secretion

Supernatant was collected from the co-cultures of NSCLC cells and NK cells after 48 or 120 hours. Tumor necrosis factor (TNF)-α, Granzyme B (48 hours) and interferon (IFN)-γ (120 hours) secretion upon NK cell activation were measured using electrochemiluminescence detection on a SECTOR3000 (MesoScale Discovery/MSD, Discovery Workbench 4.0 software), as previously described [1]. Standards and samples were measured in duplicate and the assay was performed according to the manufacturer's instructions.

Mice

Female C57BL/6j mice ($n = 62$), age 6–8 weeks, were obtained from Charles River (Wilmington, Massachusetts, USA). Female Human (Hu)-NSG-IL15-CD34+ mice ($n = 50$, 2 different Hu CD34+ donors) were obtained from The Jackson Laboratory (Sacramento, USA). All mice were maintained at the Animal Core Facilities at University of Antwerp in randomized cages. All animal procedures (and determining of sample size) were conducted in accordance with, and approval of, the Animal Ethics Committee of the University of Antwerp under registration number 2020–49. All C57BL/6j mice were housed in filter-top cages, while HuCD34+ NSG-IL15 mice were housed under strict specific pathogen-free conditions. All cages were enriched with houses and nesting material. Mice were checked on a daily basis to inspect health and wellbeing. Mice were given a 7-day adaptation period upon arrival before being included in experiments to reduce stress levels.

Tumor kinetics and survival

Prior to injection, LLC or NCI-H1975 cells were harvested using TryplE (Life Technologies), washed thrice with sterile PBS and put through a 70 μm cell strainer under sterile conditions to assure single-cell suspension without any contaminants. Next, mice were injected subcutaneously with 0.25×10^6 cells (LLC) or 2.5×10^6 (NCI-H1975) cells suspended in 100 μL sterile PBS at the left abdominal flank. When tumors reached an average size of 25–35 mm^2, mice were randomized based on tumor size and divided over six different treatment groups (day 0): (1) vehicle (PBS+NaCl); (2) chemotherapy (DOC+CDDP) (3) isotype control; (4) aCD70; (5) sequential Chemo+aCD70; and (6) simultaneous Chemo+aCD70. Mice were given IP 1 mg/kg cisplatin and 2.5 mg/kg docetaxel at days 0, 2 and 4 or vehicle. Mouse aCD70 or corresponding isotype control was administered IP every other day (3×/week) for a total of 4 weeks at a dosage of 10 mg/kg per mouse. Tumor size was measured daily using a digital caliper (Chicago Brand, Medford, OR, USA). Tumor area was calculated using the formula length × width. Mice were sacrificed when a tumor size of 150 mm^2 was reached. Potential confounders were minimized by randomizing order of treatments, measurements and cage location. Blinding was used during allocation, tumor measurements and analysis of the different treatment groups.

Characterization of immune cells in LLC-bearing mice

LLC-bearing mice were randomized and treated as described above. At day 14, mice were sacrificed and both tumor and TDLN were removed. Tumors were weighed, minced and

enzymatically digested with digestion medium (DMEM+10% FBS+10 mm L-glutamine + Collagenase D + DNAse-I) for 60 minutes at 37°C and 5% CO_2 in a cell rocker. After digestion, all samples were washed with buffer (PBS+2% BSA+1 mm EDTA) and put through a 70-μm cell strainer to obtain single-cell suspension. Lymph nodes were dissociated mechanically, washed with FACS buffer and put through a 40-μm cell strainer to obtain a single-cell suspension. Single cell suspensions were stained with anti-mouse FoxP3 AF-647 (Clone MF-14), CD103 BV421 (Clone 2E7), CD4 BV570 (Clone RM4–5), NK1.1 BV605 (Clone PK136), CD45.2 BV650 (Clone 104), CD8a BV711 (Clone 53–6.7), CD11c BV785 (Clone N418), CD25 PE (Clone 3C7), CD3 PE/Fire 700 (Clone 17A2, all purchased from Biolegend) and Live/Dead Fixable Near IR APC-Cy7 (ThermoFisher). Prior to antibody staining, all cell suspensions were treated with Fc blocking antibody (1:100, Clone 2.4G2, BD Biosciences) to avoid aspecific binding of antibodies. The cell numbers in tumors were corrected for differences in tumor weight (Number of cells/Tumor weight). Fold changes were compared to the untreated control (vehicle) group. Intraperitoneal tumors were excluded from further analysis. All samples were analyzed using a Novocyte Quanteon flow cytometer.

Statistical analysis

Prism 8.02 software (GraphPad) was used for data comparison and graphical data representations. Both Prism software and SPSS Statistics 25 software (IBM) were used for statistical computations. The non-parametric Kruskal-Wallis test was used to compare means between more than two groups. If significant, the non-parametric Mann-Whitney U test was used to compare means between two groups. To assess differences between immune cells by flow cytometry after treatment, one-way ANOVA was performed (after testing for normality). All statistical tests were performed on a minimum of three independent experiments. The Log-rank test was used to compare survival probability between different groups. Differences were considered to be significantly different if $P < 0.05$.

Results

We previously showed that the combination of low doses of docetaxel and cisplatin induced important hallmarks of immunogenic cell death *in vitro*. In addition, this chemotherapeutic regimen was able to reject tumor growth after re-challenge in a vaccination assay *in vivo*[14]. Interestingly, it is also demonstrated that low doses of cisplatin increases CD70 expression on NSCLC cells, suggesting that chemotherapy could be an interesting partner to combine with anti-CD70 immunotherapy to broaden its therapeutic window in NSCLC patients and enhance the efficacy of anti-CD70 therapy[17]. Taking these findings into account, we first assessed the effects of the chemotherapeutic regimen of docetaxel and cisplatin on CD70 expression on NSCLC cell lines (Figure 1). Treatment of chemotherapy resulted in approximately 20% cell death among the
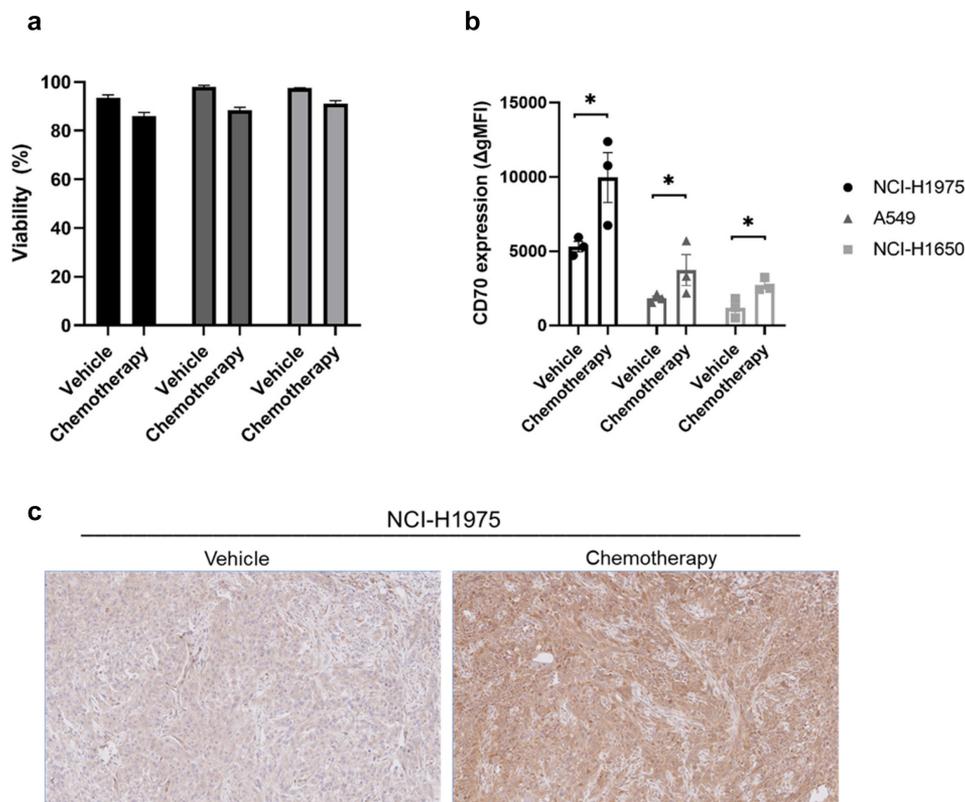

Figure 1. Viability and CD70 expression on NSCLC cell lines after treatment with chemotherapy three NSCLC cell lines (NCI-H1975, A549 and NCI-H1650) were treated with either vehicle (PBS) or docetaxel (IC_{20}) and cisplatin (IC_{20}). (a) Percentage (%) viable cells and (b) CD70 expression (gMFI) were assessed after 48 h. (c) CD70 expression was assessed on tumors of NCI-H1975-bearing Hu-NSG-IL15-CD34+ mice after treatment with vehicle (Pbs+nacl) or chemotherapy (DOC+CDDP), dissected at study endpoint. Experiments (A&B) were performed in triplicate. All data represent mean ± SEM. *$P < 0.05$.

three NSCLC cell lines after 48 hours (Figure 1A). As hypothesized, the chemotherapeutic regimen resulted in an increase of CD70 expression on the NSCLC cells after 48 hours (Figure 1B). The cell lines used were CD70-high (NCI-H1975) intermediate (A549) and low (NCI-H1650) cell lines (approximately 70%, 50% and 20% CD70 positive cells in NCI-H1975, A549 and NCI-H1650 cell lines, respectively (data not shown). Similar findings were also observed in NCI-H1975-bearing Hu-NSG-IL15-CD34+ mice treated with either vehicle or chemotherapy (Figure 1C).

Next, we assessed the internalization rate of CD70 after incubation with anti-CD70 antibodies (aCD70). We found no internalization of CD70 in NSCLC cell lines compared to the positive control, the renal cancer cell line 786-O (Suppl. Figure S1). These data indicate that CD70 represents a potential target for ADCC-mediated antibody therapy rather than ADCs in NSCLC specifically. As such, we used monoclonal aCD70 antibodies (human or murine variant) with enhanced antibody-dependent cellular cytotoxicity (ADCC) properties (see material & methods) in this study. The effect of antibody-dependent NK cell-mediated killing of NSCLC cells was measured after treatment with aCD70, isotype control (IgG1), chemotherapy (Chemo+iso) or the sequential combination of aCD70 and chemotherapy (Chemo+aCD70) using real-time live cell imaging for 72 hours (Figure 2A). In two out of three NSCLC cell lines, aCD70 significantly improved NSCLC cell killing (20% killing of NCI-H1975 cells) as opposed to the isotype control (Figure 2B). The combination of Chemo +aCD70 significantly increased NSCLC cell killing in all cell lines compared to single treatment with aCD70 or chemotherapy (up to 62%, 21% and 15% killing of NCI-H1975, A549 and NCI-1650 cells, respectively). These findings demonstrate that the combination of Chemo+aCD70 results in enhanced NK cell-mediated killing of NSCLC cells compared to aCD70 or chemotherapy alone.

Next, the effect of mono- and combination treatment on NK cell activation was assessed through the following parameters: NK cell surface activation marker CD69, degranulation marker Granzyme B and secretion of pro-inflammatory cytokines TNF-α and IFN-γ (Figure 3). Overall, expression of CD69 was higher after treatment with aCD70 or Chemo +aCD70 in all cell lines, although these differences were not always significant.

Levels of degranulation marker Granzyme B were higher after treatment with aCD70 compared to isotype, and these differences were significant in cultures with NCI-1650 cells (Figure 3B). Overall, increased levels of Granzyme B were found in all cell lines. Secretion of pro-inflammatory cytokines associated with NK cell cytotoxicity were assessed as well. TNF-α levels were significantly increased after treatment with aCD70 alone and Chemo+aCD70 compared to isotype or chemotherapy in all NSCLC cell lines (Figure 3C). Levels of IFN-γ secretion were significantly higher after treatment with aCD70 compared to isotype in NCI-H1975 and NCI-H1650 cells. In addition, treatment with Chemo+aCD70 resulted in significantly higher IFN-γ levels compared to isotype in NCI-
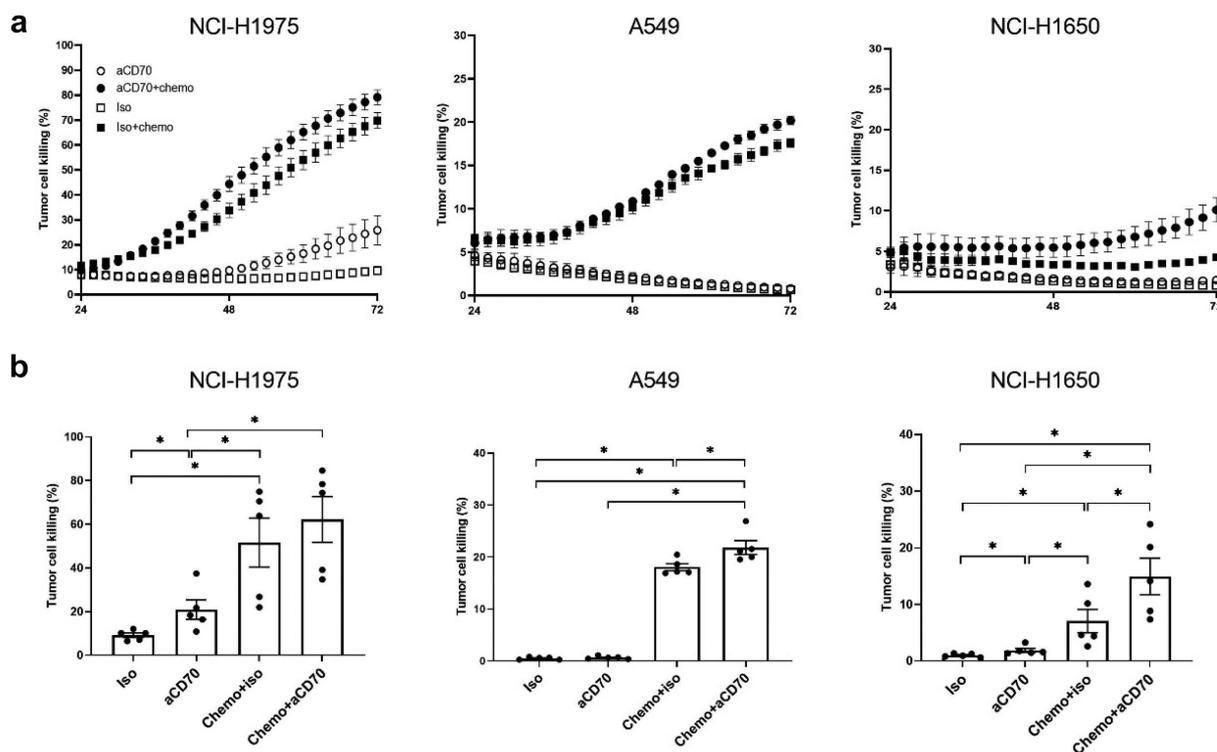

Figure 2. NK cell-mediated killing of NSCLC cell lines after treatment with monotherapy or the combination of chemo-immunotherapy three NSCLC cell lines (NCI-H1975, A549 and NCI-H1650) were treated with either vehicle (PBS) or docetaxel (IC$_{20}$) and cisplatin (IC$_{20}$) after 24 hours. The following day, cells were treated with aCD70 or isotype (0.5 μg/ml) and (healthy donor) NK cells were added to the co-culture (ratio 5:1). (a) Follow-up of killing for 72 hours and (b) percentage of tumor cell killing (%) at 72 hours was assessed using the IncuCyte ZOOM. Experiments were performed minimum in triplicate using 3–5 NK donors. All data represent mean ± SEM. *$P < 0.05$.
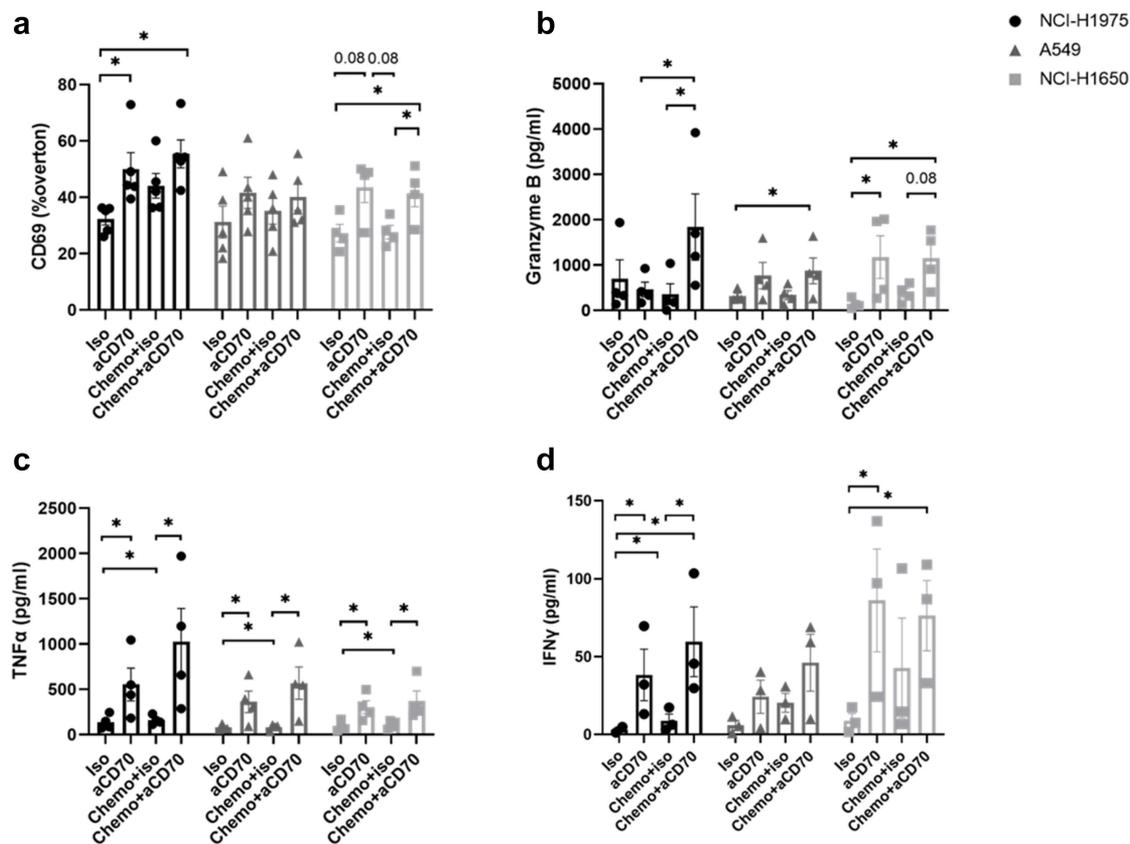
Figure 3. Activation status of NK cells in co-cultures with NSCLC cell lines after treatment with monotherapy or the combination of chemo-immunotherapy three NSCLC cell lines (NCI-H1975, A549 and NCI-H1650) were treated with either vehicle (PBS) or docetaxel (IC$_{20}$) and cisplatin (IC$_{20}$) after 24 hours. The following day, cells were treated with aCD70 or isotype (0.5 µg/ml) and (healthy donor) NK cells were added to the co-culture (ratio 5:1) using 3–4 donors. (a) Flow cytometric assessment of percentage CD69 positive cells (%overton) was measured after 24 hours. (b) Granzyme B and (c) TNFα were assessed after 48 hours and (D) IFNγ was measured after 120 hours in supernatant of the co-cultures. Experiments were performed minimum in triplicate using 3–4 NK donors. All data represent mean ± SEM. *$P < 0.05$.

H1975 and NCI-H1650 cells or chemotherapy in NCI-H1975 cells. Altogether, these findings suggest that NK cell-mediated NSCLC killing is accompanied by increased NK cell activity after treatment with either aCD70 or Chemo+aCD70.

Anti-tumor immunity involves not only NK cells but also other important immune cell types. To take this into account, we assessed the effects of aCD70 alone or Chemo+aCD70 in a syngeneic CD70+ LLC-bearing subcutaneous mouse model. The treatment schedule (Figure 4A) consisted of either aCD70, chemotherapy, sequential (SEQ) or simultaneous (SIM) administration of Chemo+aCD70 or their corresponding controls. Treatment with aCD70 or chemotherapy did not affect survival of LLC-bearing mice. Simultaneous Chemo+aCD70 did improve survival but this was only compared to isotype-treated mice. Interestingly, the sequential Chemo+aCD70 treatment further improved survival of the mice and was the only regimen that significantly increased survival of LLC-bearing mice compared to all the single agents (Figure 4B).

Furthermore, treatment of LLC-bearing mice with sequential Chemo+aCD70 significantly delayed tumor growth compared to all other treatment regimens, which was also the case compared to the simultaneous combination therapy at day 14 (Figure 4C-D). To gain more insights into the immune cells responsible for the observed responses, we sacrificed LLC-bearing mice at day 14 and performed multicolor flow cytometry on tumors and TDLNs (Figure 5 and Suppl. Figure S4).

DCs play critical roles in antigen processing and presentation and are key players in the activation of NK and T cells. In the context of chemotherapy-induced immunogenic cell death, we explored the presence of DCs in the LLC tumor model. Here, we observed an increase in the number of DCs in the TDLN of mice mainly after treatment with chemotherapy, sequential Chemo+aCD70 or simultaneous Chemo+aCD70 therapy (Figure 5A). We then assessed the amount of CD103+ DCs in the TDLN, which is the subtype responsible for cross-presentation and cross-priming of CD8+ T cells. Again, we observed a significantly higher percentage of CD103+ DCs in the TDLN of chemotherapy-treated mice compared to control or aCD70 (Figure 5B). These findings suggest that the regimen of DOC+CDDP is able to stimulate DCs to capture antigens at the tumor site, migrate to the TDLN and potentially activate NK and T cells.

To assess whether there are actual differences in the tumor microenvironment of treated LLC-bearing mice, we assessed NK and T cell numbers in tumors using multicolor flow cytometry (Figure 5C-F). Chemotherapy and sequential Chemo+aCD70 therapy resulted in a sixfold increase of CD4+ and CD8+ T cells T cells within the tumors of LLC-bearing mice (Figure 5C-D). Higher intratumoral ratios of CD8+/Tregs (a twofold increase), which are suggestive of a positive treatment response, were observed in the sequential Chemo+aCD70 therapy compared to all other groups within the tumors
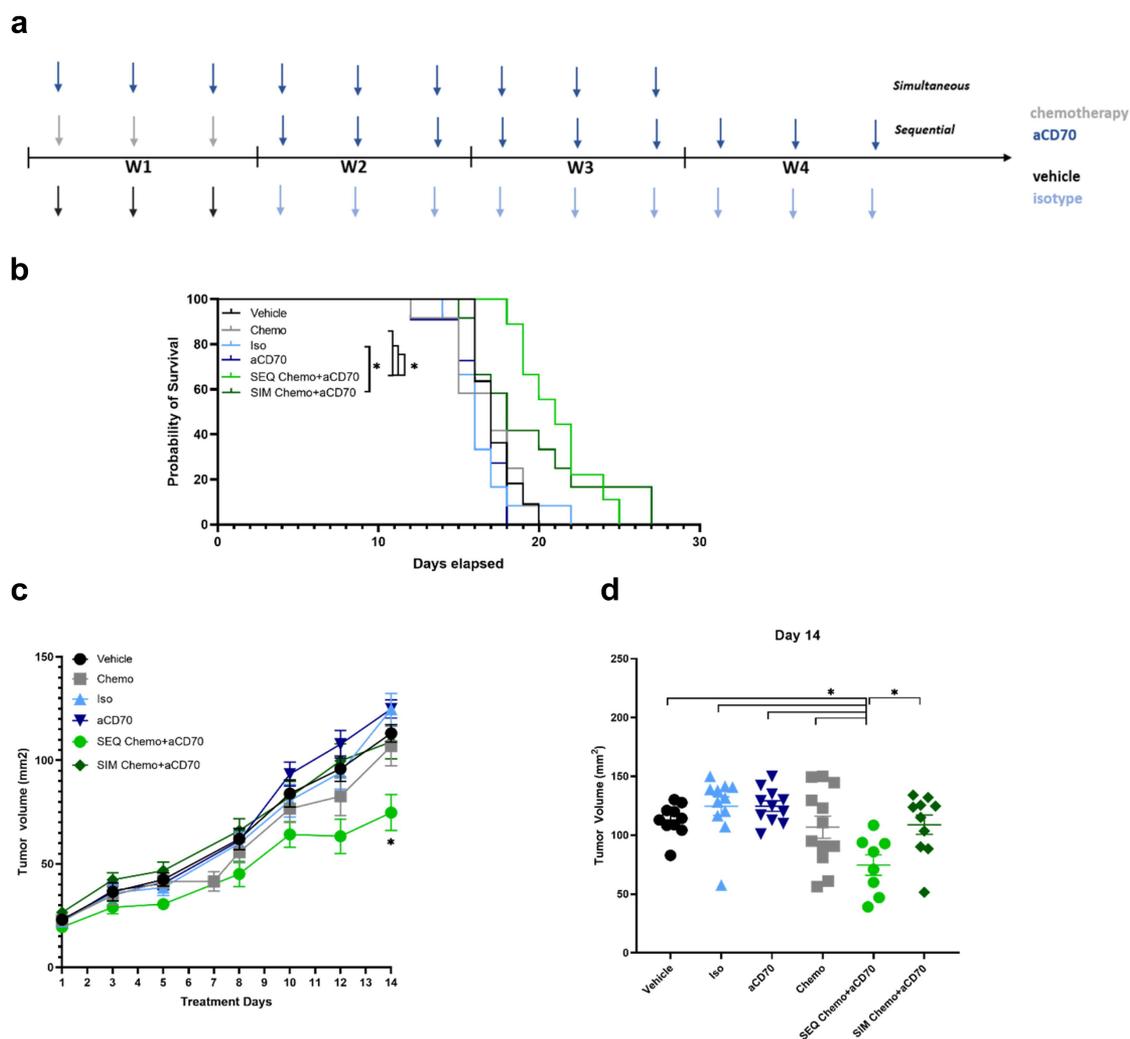
Figure 4. Survival data and tumor kinetics after treatment with monotherapy or the combination of chemo-immunotherapy in the syngeneic LLC-bearing mouse model CD70+ LLC cells were subcutaneously injected in the left flank of C57BL/6 mice ($n = 62$). After 10 days, when tumors reached an average size of 25–35 mm2, mice were randomized and divided over six different treatment groups; vehicle, chemotherapy (chemo), isotype (iso), mouse aCD70, sequential combination (SEQ) and simultaneous combination (SIM). (a) Treatment scheme showing timing of dosing for aCD70 or the corresponding isotype (10 mg/kg) with blue arrows and for chemotherapy (cisplatin, 1 mg/kg; docetaxel, 2.5 mg/kg) with gray arrows. (b) Survival and (c) tumor growth kinetics of LLC tumors for the different treatment regimens and (d) tumor volume at day 14 is shown. Data pooled from two independent experiments with $n = 4$–7 per condition per experiment. All data represent mean ± SEM. *$P < 0.05$.

(Figure 5E). Of note, chemotherapy alone did not appear to reduce the frequency of Tregs compared to the other treatment groups (Suppl Figure 5). Furthermore, a fourfold increase in the number of NK cells was observed in tumor-bearing mice treated with chemotherapy or sequential Chemo+aCD70 therapy (Figure 5F).

Overall, our findings suggest that the chemotherapeutic regimen of docetaxel and cisplatin can induce an immunogenic tumor microenvironment that has the potential to enhance the effect of CD70 targeting treatment.

To validate our findings in a more humanized setting, we repeated the *in vivo* study using a NCI-H1975-bearing HuIL15-NSG-CD34+ mouse model which stably expresses human CD4 +, CD8+ T cells and CD56+ NK cells and assessed tumor growth kinetics and survival (Figure 6). Of note, follow-up of treated mice did not show any significant changes in body weight in both mouse models (Suppl. Figure S2). Comparable to the LLC-bearing C57BL/6 model, aCD70 monotherapy did

not result in a significant delay in tumor growth or improved survival. In this model, chemotherapy treatment on its own considerably improved survival of NCI-H1975 bearing mice compared to the control group as opposed to the LLC-bearing C57BL/6 mouse model. These *in vivo* findings are in line with our *in vitro* results, which suggest a high sensitivity of the NCI-H1975 cell line to the chemotherapeutic regimen of docetaxel and cisplatin. In addition, both combination therapies resulted in a significant increase in survival compared to all monotherapies. Importantly, the sequential combination therapy again showed the highest efficacy, by significantly improving survival of tumor-bearing mice compared to all other therapies including the simultaneous combination therapy.

Discussion

While immunotherapy as single agent has shown only modest responses, combination strategies, such as chemo-
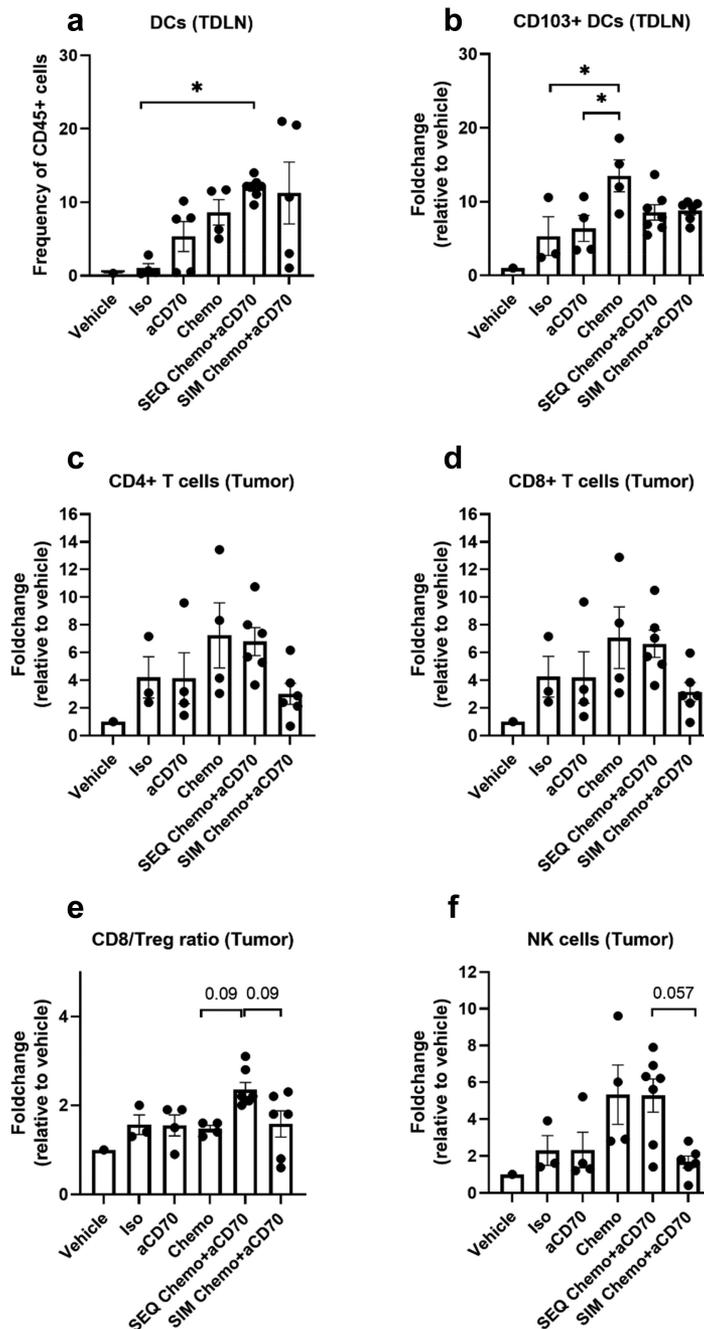
Figure 5. Characterization of immune cell populations in tumor and tumor-draining lymph nodes C57BL/6j mice bearing LLC tumors were treated with vehicle, isotype, aCD70, chemotherapy or the combination (sequential vs simultaneous). (a,b) DCs or CD103+ DCs in TDLN, (c) DCs in tumors, (d) T cells in tumors, (e) CD8+ T cells in tumors and (f) CD4+ T cells in tumors (g) CD8/Treg ratios in tumor and (H) NK cells in tumors. Gating strategy is shown in supplementary. Data from one experiment, $n = 3–7$ mice/group, except for the vehicle group. Fold change of (number of cells/tumor weight) were compared to the untreated control (vehicle) group. All data represent mean ± SEM. One-Way ANOVA test. *$P < 0.05$.

immunotherapy clearly enhance the anti-tumor response in the clinic[18]. We previously showed that the chemotherapeutic regimen of docetaxel and cisplatin was able to induce immunogenic cell death in NSCLC, which is a form of tumor cell death that can prime and activate tumor-specific cytotoxic T cell responses. In addition, it has been described that cisplatin can increase CD70 expression on NSCLC cells[14,17]. Based on these findings, we hypothesized that by including the chemotherapeutic regimen of docetaxel and cisplatin to aCD70 immunotherapy, improved anti-tumor immune effects could occur in NSCLC. In this study, we are the

first to demonstrate *in vitro* and *in vivo* enhanced innate and adaptive immune effects of anti-CD70 antibody-based immunotherapy in combination with the chemotherapeutic regimen of docetaxel and cisplatin in NSCLC.

NK cells serve as the first line of defense against tumor cells and have shown to mediate efficient anti-tumor responses by inducing innate and adaptive immune responses[19]. Once activated, NK cells can rapidly secrete pro-inflammatory cytokines, chemokines and cytotoxic mediators. In addition, NK cell cytotoxicity against cancer cells is due to binding of the Fc receptors, such as CD16, to the Fc portion of the ADCC-
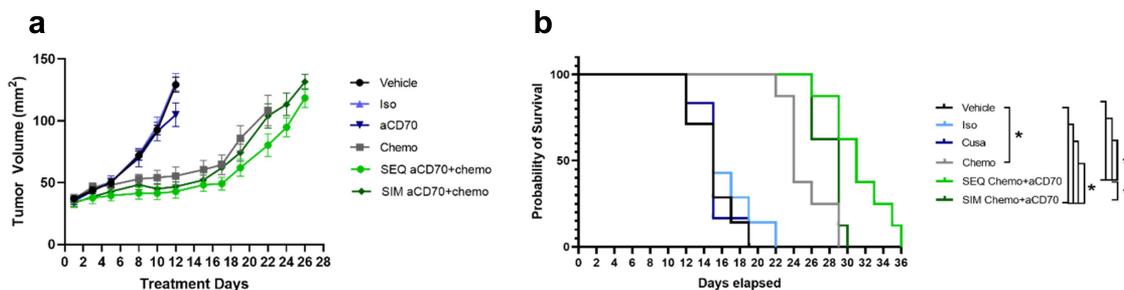
Figure 6. Survival data and tumor kinetics after treatment with monotherapy or the combination of chemo-immunotherapy in a humanized IL15-NSG-CD34+ mouse model NCI-H1975 cells were subcutaneously injected in the left flank of Hu IL15-NSG-CD34+ mice ($n = 50$, 2 Hu CD34+ donors). When tumors reached an average size of 25–35 mm^2, mice were randomized and divided over six different treatment groups; vehicle, chemotherapy (chemo), isotype (iso), human aCD70, sequential combination (SEQ) and simultaneous combination (SIM). (a) Tumor growth kinetics and (b) survival is shown. Data pooled from two human CD34+ immune cell donors with $n = 3–5$ per condition per experiment. All data represent mean ± SEM. *$P < 0.05$.

mediated antibody-coated target cells[20]. In this study, we showed that treatment with a CD70-targeting ADCC-mediated antibody increased NK cell-mediated killing of CD70 expressing NSCLC cell lines and resulted in increased NK activation (CD69), secretion of pro-inflammatory cytokines (TNFα, IFNγ) and degranulation (Granzyme B) at various levels, depending on the NSCLC cell line. Our findings show that the CD70-low NCI-H1650 cell line (20% CD70 positive) responded better to NK cell-mediated killing of NSCLC cells compared to the CD70-intermediate cell line A549 (50% CD70 positive). Studies have reported that tumor cells can evade immune recognition and thus efficient NK cell-mediated killing by downregulating the expression of NKG2D ligands, such as MHC class I chain-related A (MICA), MICB and several UL-16 binding proteins (ULBPs)[20], which might be a possible explanation for the NK cell insensitivity of this cell line. Interestingly, it has already been described that both docetaxel and cisplatin are able to increase expression of NKG2D-ligands (such as MICA and MICB), which resulted in increased antibody-mediated ADCC in HER2+ breast cancer cell lines[21] and NK cell-mediated killing of NSCLC cell lines[22,23]. Of note, we also observed significantly lower MICA/B expression on the A549 cells compared to the other cell lines. In addition, chemotherapy increased MICA/B expression on all tumor cell lines at varying levels (data not shown). Here, we demonstrated a significant increase in NK cell-mediated NSCLC cell killing after treatment with Chemo +aCD70 combination compared to chemotherapy or aCD70 as single agent *in vitro*.

As a next step, we explored aCD70 monotherapy and the combination of chemo-immunotherapy in an *in vivo* setting, taking both innate and adaptive immunity into account. Treatment of LLC-bearing mice with chemotherapy or aCD70 monotherapy did not affect survival or tumor growth *in vivo*. The use of chemotherapy in the aggressive syngeneic LLC model has been previously described, showing no significantly improved effects on tumor growth or survival of mice, which is in line with our findings[24,25]. In addition, the poor immunogenic profile of the LLC-bearing mouse model and thus absence of NK cells in the tumor might explain the lack of effect of aCD70 as single agent. Interestingly, treatment with the sequential combination of chemo-immunotherapy significantly improved survival and delayed tumor growth in LLC-

bearing mice. Moreover, our data show that pre-treatment with chemotherapy is able to an immunogenic tumor microenvironment, thereby improving the effector function of aCD70 therapy to a certain extent.

Chemotherapy treatment resulted in increased numbers of DCs and particularly CD103+ DCs with cross-presentation potential in TDLN of LLC-bearing mice. The importance of DCs in cancer has been extensively demonstrated as they initiate specific immune responses by capturing antigens and priming of tumor-specific T cells in addition to activating NK cells[26–28]. In support of this, our study showed that chemotherapy increased overall intratumoral T cell and NK cell numbers in tumors of mice, which demonstrates the ability of the chemotherapeutic regimen, consisting of docetaxel and cisplatin to prime the immune response in NSCLC.

Interestingly, a difference was observed between the sequential and simultaneous combination where only the sequential treatment resulted in overall higher numbers of T and NK cells, as well as increased ratios of CD8+ T cells over Tregs in the tumor microenvironment. These observations could explain the stronger anti-tumor effects and improved survival *in vivo*, since it has been shown that increased CD8/Treg ratios are associated with improved overall survival in cancer patients and response to therapy[29–31]. In addition, these findings show that the chemotherapeutic regimen of docetaxel and cisplatin has the potential to turn an immunologically 'cold' LLC tumor into a 'hot' tumor that has been infiltrated by immune cells. As such, the combination therapy can result in strong effects, even in cold tumors, of anti-CD70 antibody-based therapy with effector functions in NSCLC. Furthermore, in the context of the CD70-CD27 axis, interactions between CD70 and CD27 have shown to increase the frequency of Tregs in the tumor microenvironment, reduce tumor-specific T cell responses and promote tumor cell growth when CD27 is expressed on TILs[32]. In lung cancer specifically, expression of CD27 on Tregs has been reported in the tumor microenvironment of CD70 positive NSCLC specimens[15] and it has been shown that CD27 expression on human CD4+CD25+ Tregs positively correlates with their suppressive activity *in vitro* and the expression of FOXP3[33]. It is tempting to speculate that the sequential combination therapy increased CD8/Treg ratios due to blocking of CD70-CD27 interactions in the tumor microenvironment, although future studies evaluating immune patterns together

with CD70/CD27 expression on these cell subsets are necessary to elucidate possible underlying mechanisms observed in our study. In addition, it remains to be elucidated whether the effect observed *in vivo* could be (partially) dependent on NK cells and ADCC in the LLC-bearing mouse model.

Of note, there were also limitations in this study. For instance, the sample size of C57BL/6 mice was small, especially in the vehicle group, which was due to exclusion criteria of I. P. tumors.

Finally, the superior effect of the sequential combination therapy on improved survival was further validated in a more humanized *in vivo* setting, which enabled us to evaluate the human aCD70 antibody, cusatuzumab.

Overall, our findings demonstrate that the sequential combination therapy of chemotherapy and anti-CD70 therapy has the potential to elicit an immunogenic microenvironment that can enhance the effect of CD70-targeted immunotherapy.

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Data availability statement

All data relevant to the study are included in the article or uploaded as supplementary information. Additional data are available on reasonable request.

Ethics approval and consent to participate

All animal procedures were in approval of the Animal Ethics Committee of the University of Antwerp under registration number 2020–49.

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