

# CD56<sup>bright</sup> cytokine-induced memory-like NK cells and NK-cell engagers synergize against non-small cell lung cancer cancer-stem cells

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## ABSTRACT

**Background** Due to their enhanced responsiveness and persistence, cytokine-induced memory-like (CIML)-natural killer (NK) cells have emerged as new immunotherapeutic tools against malignancies. However, their effects on tumor-cell spread and metastases in solid tumors remain poorly investigated. Moreover, a clear identification of the most effective CIML-NK subsets, especially in controlling cancer stem cells (CSC), is still lacking.

**Methods** We performed combined phenotypical and functional analyses of CIML-NK cell subsets, either selected by flow-cytometry gating, or generated from sorted CD56<sup>bright</sup>/CD56<sup>dim</sup> NK cells.

By co-culture experiments, we analyzed the effect of CIML-NK cells on non-small cell lung cancer (NSCLC) cell spheroids, or patient-derived xenografts (PDX), assessing changes in their CSC content, tumorigenicity, and/or tumor disseminating capability in vivo. CIML-NK cells were also infused in PDX-bearing mice to validate their effect on the CSC dissemination from the PDX to the lungs.

Finally, we generated and functionally analyzed CIML-NK cells from patients with stages I/III NSCLC (n=6).

**Results** We show that CIML-NK cells exert antitumor activity mostly through their CD56<sup>bright</sup> cell subset, which greatly expands during CIML differentiation. Compared with NK cells conventionally activated with interleukin-2, CIML-NK cells express lower levels of check-point receptors, TIGIT and TIM3, and higher effector functions against NSCLC cells from PDX, and against in vitro-generated tumor spheroids. Remarkably, CIML-NK cells also significantly reduce the CSC-containing CD133<sup>+</sup> cell subpopulation within spheroids and PDX, and limit tumor cell tumorigenicity and ability to disseminate CSCs from primary tumors to distant sites. Sorting experiments on CIML or tumor cell subsets reveal that CD56<sup>bright</sup> cells drive most of this anti-CSC activity, and suggest that such functional advantage could be related to increased expression of LFA-1 and ICAM-1 on CD56<sup>bright</sup> cells and CSCs, respectively. We also show that the tri-specific killer cell engager (TriKE) 1615133 significantly enhances CIML-NK cell activity against CSCs. Finally, we demonstrate that CIML-NK cells, capable of killing autologous tumor cells

## WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Cytokine-induced memory-like natural killer cells (CIML-NK) recall the initial cytokine stimulus and display enhanced responses against tumor cells. These effector cells are being tested in clinics in the context of Acute myeloid leukemia (AML).
- ⇒ It is not clear if there are specific subsets driving their responsiveness, and if CIML-NK cells could target cancer stem cells and control tumor dissemination and metastasis.

## WHAT THIS STUDY ADDS

- ⇒ Here we show that the CD56<sup>bright</sup> NK cell subset expands and drives CIML NK cell effector functions against non-small cell lung cancer (NSCLC) cells and, in particular, against their CD133<sup>+</sup> cancer stem cells (CSC) component. In line with their CSC-targeting, CIML-NK cells limit tumorigenicity, tumor spreading, and metastasis formation. Moreover, such CIML-NK anti-CSC activity can be further increased through the use of a specific CD16-binding tri-specific killer engager.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ This study highlights the therapeutic potential of CIML-NK cells in controlling CSCs and metastatic spread in NSCLC, and shows that effective CIML-NK cells, capable of targeting autologous tumor cells, can be induced from patients with NSCLC, providing hints to develop new therapeutic strategies to tackle metastatic disease.

and responding to the 1615133 TriKE, could be induced from patients with NSCLC.

**Conclusions** Our study discloses for the first time the therapeutic potential of CIML-NK cells in controlling CSCs and metastatic spread, highlighting the role of the CD56<sup>bright</sup> subset expansion and 1615133 TriKE for optimizing CIML-NK-based therapies against metastatic tumors.

## BACKGROUND

Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung malignancies and is diagnosed at an advanced stage in nearly 70% of patients. The introduction of targeted therapies and immunotherapies, involving immune checkpoint inhibitors, has improved the prognosis in some cases, yet has proved poorly effective against metastatic spread.<sup>1</sup> These facts pose the control of metastases as the major therapeutic concern for this type of cancer.

Natural killer (NK) cells may represent a potential therapeutic tool in this context. Indeed, they are endowed with potent antitumor activity, and it has also been suggested that they can kill cancer stem cells (CSC), which are accountable for tumor initiation and dissemination.<sup>2–4</sup> Moreover, the mechanisms driving their capability to recognize and kill tumor cells have been in large part defined<sup>5–8</sup> providing useful hints for the development of new therapeutic strategies. Nevertheless, the issue of how to get effective and durable NK cell-based immunotherapies is still the object of intense investigation, particularly in solid tumor settings and metastases. Major challenges concern the identification of new strategies that can enhance and drive NK cell-cytotoxicity into the tumor nests, overcome tumor-induced immune suppression, and prolong NK cell activity and persistence in vivo.<sup>3,9,10</sup>

Cytokine-induced memory-like NK cells (CIML-NK) have emerged as a promising tool to address some of these concerns. CIML-NK cells can be easily induced from peripheral blood (PB)-NK cells after brief exposure to the cytokine cocktail, interleukin (IL)-12+IL-15+IL-18,<sup>11</sup> and can recall the initial cytokine stimulus by showing enhanced responses, particularly interferon (IFN)- $\gamma$  release, to either cytokines or tumor cells.<sup>12–14</sup> Thus, although IL-2 still represents a commonly used cytokine to activate ex vivo or in vivo adoptively transferred NK cells in clinical trials, the clinical relevance of CIML-NK cells is now under examination. A first in-human study conducted in Acute myeloid leukemia (AML) patients has provided evidence that allogeneic CIML-NK cells can differentiate, proliferate, and persist in vivo, showing antitumor effects in more than 50% of patients.<sup>15</sup> By a multidimensional analytic approach, the study also characterized CIML-NK cells from patients, highlighting that this cell population is heterogeneous, with only a fraction of cells responding to tumor cells. This observation poses the issue of whether and how CIML-NK effectors could be further optimized, especially considering their possible exploitation in the context of solid tumors and metastases.

Recent studies have reported that CIML-NK cells can effectively attack melanoma, head and neck, colon, and ovarian cancer cell lines both in vitro and after administration to human tumor-bearing NSG-mouse models,<sup>16–19</sup> suggesting that different solid tumors could be targeted by CIML-NK cells. However, no information is presently available on the possible role of these cells in controlling CSCs and metastasis development, which is crucial for a

concrete step forward in the cure of solid tumors. CSCs can be delivered into the pre-metastatic niches as single cells or through multicellular three-dimensional (3D) structures that leave the primary tumor and circulate as tumor-cell clusters.<sup>20–22</sup> Therefore, in vitro generated, or tumor-derived CSC-enriched spheroids could be a valuable platform to test enhanced NK cell functions.<sup>23,24</sup> In this regard, therapeutic antibodies or bi/tri-specific molecules, simultaneously engaging key NK receptors and tumor-expressed epitopes represent potential tools to address enhanced NK responses to specific targets.<sup>25–27</sup> Their potential in targeting CSC, however, remains poorly investigated. In NSCLC, CSCs are included within the CD133<sup>+</sup> tumor cell population, which displays elevated tumorigenicity and is considered responsible for therapeutic resistance.<sup>28,29</sup> Therefore, a recently generated tri-specific cell engager (1615133 TriKE)<sup>30</sup> targeting CD133 could be particularly appropriate to support CIML-NK cell activity against NSCLC CSCs.

In this study, we show that CIML-NK cells target NSCLC CSCs and highlight, for the first time, the unique role of their CD56<sup>bright</sup> cell subset and of a specific TriKE in maximizing the anti-CSC effect of both healthy-derived and patient-derived CIML-NK cells.

## METHODS

See online supplemental material and methods for details.

### Biological factors, chemicals, media, and antibodies

Details on these materials are reported in online supplemental table S1 (biologicals, chemicals, and media) and in online supplemental table S2 (antibodies).

### Cell lines and tumor spheroids

NSCLC cell lines A549, H3122, H661, and SW900 were cultured under standard cell culture conditions and were regularly tested for *Mycoplasma* contamination. Tumor spheroids were generated as previously described.<sup>23</sup> Briefly, tumor cells were cultured in DMEM/F-12 medium supplemented with B-27 without vitamin A, 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor, 5  $\mu$ g/mL heparin in ultra-low attachment 6-well or 96-well microplates.

### Preparation of CIML, IL-2-NK, or control-IL-15 NK cells

NK cell isolation and preparation are described in online supplemental figures S1 and S2.

### Surface phenotypic analysis

NK and tumor cells were phenotypically characterized by flow cytometry. Details of all antibodies used are found in online supplemental table S2.

### Sorting of tumor-cell or NK-cell subsets

Tumor cells or lymphocytes were incubated with viability dye for 20 min, washed, and stained with fluorescently-labeled antibodies. Viable CD133<sup>+</sup> cancer cells or

CD3<sup>+</sup>CD19<sup>+</sup>CD56<sup>bright/dim</sup> lymphocytes were sorted on an FACSymphony S6 system (BD Biosciences).

### CD107a and IFN- $\gamma$ intracellular staining

NK:tumor cell co-cultures (1:1 ratio) were performed for 6 hours in the presence of anti-CD107a antibody, with the addition of brefeldin A and monensin after the first hour. After 6 hours, cells were stained for viability and surface markers. Next, cells were fixed, permeabilized, and stained for intracellular markers. Where indicated, 1615133 TriKE was added to the co-cultures 10 min before the anti-CD107a antibody.<sup>30</sup>

### Killing assays

- Flow cytometry-based killing assays were performed by co-culturing for 24 hours NK cells with Carboxyfluorescein succinimidyl ester (CFSE)-labeled target cells at the indicated Effector (E):Target (T) ratios. After co-culture, cells were stained with viability dye (dead cell marker, DCM) and annexin V. Dead target cells were defined as CFSE<sup>+</sup> annexin V<sup>+</sup> and/or DCM<sup>+</sup>.
- For calcein-based killing assays, target cells were labeled for 30 min with 5  $\mu$ g/mL calcein, washed, and co-cultured with effector cells in 96-well U-bottom plates in assay medium (red-phenol-free RPMI, 1% FBS, 2.5 mM probenecid). After 4 hours, supernatants were collected, and fluorescence emission was measured using a plate reader.<sup>31</sup>

### IncuCyte measurement of spheroid killing

Real-time spheroid killing was evaluated using the IncuCyte S5 platform (Essen Bioscience, Sartorius). NK cells were added to wells containing a single-spheroid. Tumor cell apoptosis within spheroids was evaluated by including caspase 3/7 green detection dye in the media. Images were acquired using a 4 $\times$  objective lens and analyzed by IncuCyte Controller V.2020A. The means of the technical replicates (n=3) for each condition were compiled for N=3 donors.

### Colony forming assay

Tumor spheroids were co-cultured with NK cells for 24 hours. Then, CD45-depleted viable tumor cells were seeded in 6-well plates (500 cells/well). Colonies were stained with 0.5% crystal violet. After image acquisition, crystal violet was dissolved (10% acetic acid) for absorbance quantification using a BioTek microplate reader.

### Patient-derived xenograft and patient-derived tumor cells

For NSCLC patient-derived xenograft (PDX) establishment, small tumor fragments from surgical specimens were implanted subcutaneously (s.c.) in the flanks of SCID mice, as previously described<sup>32</sup> and PDX were maintained by serial s.c. passages of fragments of growing tumors into mice. When indicated, tumor cells derived either from PDX or directly from patients' surgical specimens were analyzed in vitro in functional assays.

### In vivo tumorigenicity studies

- A549 spheroids 6–8-week male NSG mice were housed at the University of Minnesota Animal Facility.

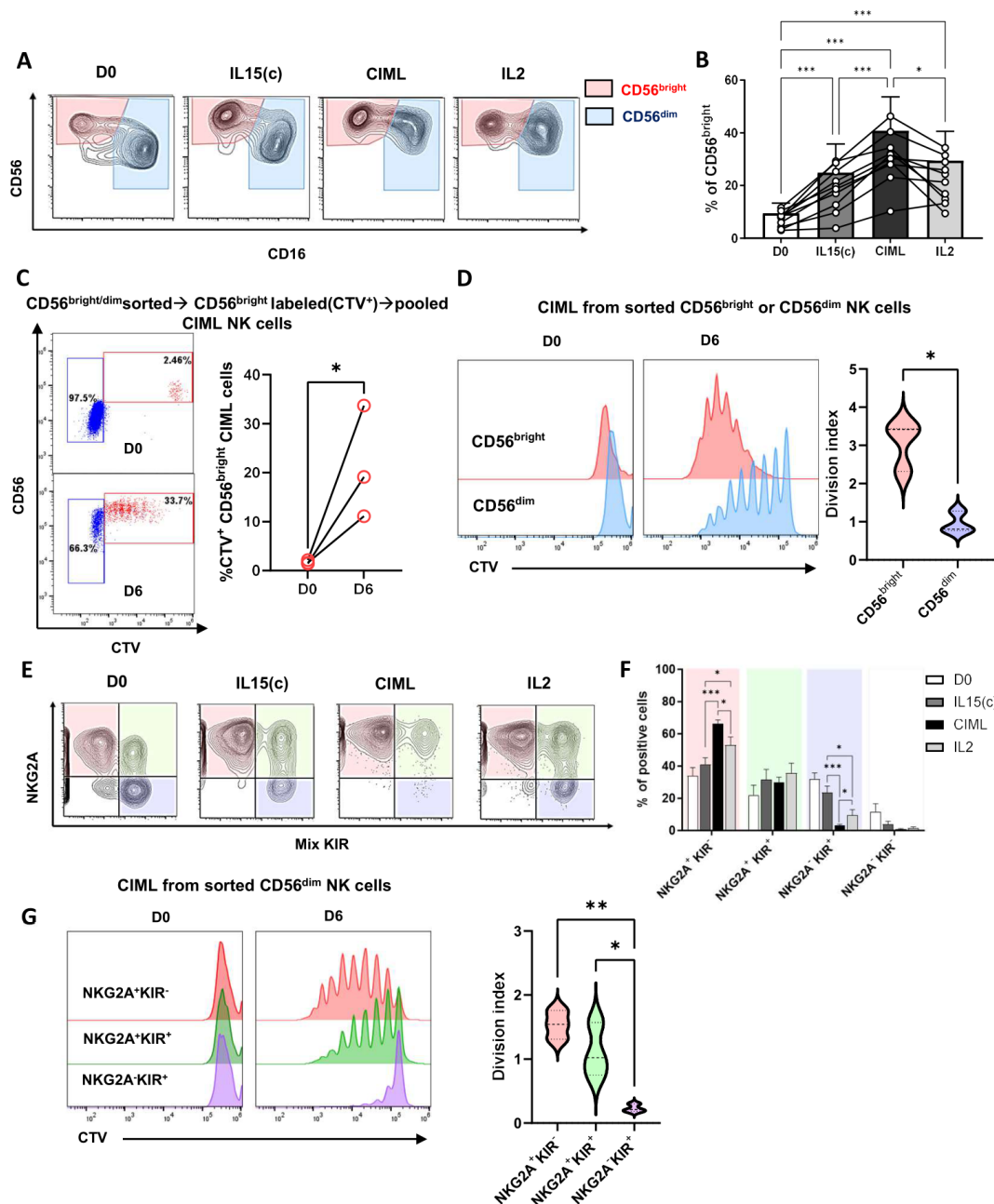
Spheroids from A549 cells were cultured alone or co-cultured with the indicated NK cell types for 24 hours (1:1 E:T ratio). After spheroid dissociation, and NK cell removal, A549 cells were assessed for purity and vitality by flow cytometry. 10<sup>5</sup> viable A549 cells, resuspended in 100  $\mu$ L phosphate-buffered saline (PBS)+Matrigel (1:1 v/v), were subcutaneously inoculated to the mouse flank (n=6 mice/group).

- PDX 7–10-week female SCID mice were housed at the INT (Milan) animal facility. PDX-derived single-cell suspensions were co-cultured with the indicated NK cell types for 4 hours (1:1 E:T ratio). After NK cell removal by the use of anti-CD45-microbeads and their immunomagnetic depletion with AutoMACS (all from Miltenyi), and purity and vitality assessment, 10<sup>5</sup> viable tumor cells, resuspended in PBS+Matrigel (1:1 v/v), were subcutaneously inoculated to the mouse flank. At endpoint, mice were euthanized and lungs were collected for immunohistochemistry (IHC) analysis or processed for flow cytometry analysis.<sup>28</sup> For in vivo evaluation of CIML-NK cell function, PDX were directly implanted into the mice. After 4 weeks 5 $\times$ 10<sup>6</sup> CIML-NK cells were injected intravenous and 2 days after mice were sacrificed to analyze lungs (n=5 mice/group).

## RESULTS

### Identification of the CD56<sup>bright</sup> cell subset as the major driver of CIML-NK cell functions

Following well-established protocols, we generated CIML-NK cells from the PB of healthy donors (HD) by stimulating freshly isolated NK cells for 16 hours with IL-15+IL-12+IL-18, followed by a 7-day resting period in the presence of low-dose IL-15. Afterward, we assessed the cells by cytofluorimetry for the expression of informative markers to identify possible characterizing cell subsets and evaluate their contribution to the functional CIML-NK cell features. As a comparison, NK cells were also analyzed before stimulation, at day 0, or after parallel 7-day culture in the presence of IL-2 (IL-2-NK), still used for clinical grade preparations, or low dose IL-15, as classical control of CIML-NK cells (IL-15 control cells: IL-15(c)-NK) (online supplemental figure S1). This analysis revealed that CIML-NK cells were characterized by an important expansion of the CD56<sup>bright</sup>CD16<sup>dim</sup> (hereafter CD56<sup>bright</sup>) cell subset, which was significantly larger than that observed in IL-2-NK and IL-15(c)-NK cells (figure 1A,B), reaching in one case 60% of total CIML-NK cells. To confirm that such expansion originated from “bona fide” CD56<sup>bright</sup> cells, we sorted naïve CD56<sup>bright</sup> and CD56<sup>dim</sup>CD16<sup>bright</sup> (hereafter CD56<sup>dim</sup>) cells, fluorescently labeled the CD56<sup>bright</sup> fraction, rejoined the two sorted subsets to reconstitute original PB-NK cell population, and induced CIML differentiation (online supplemental figure S2). This experiment showed that, indeed, real (labeled) CD56<sup>bright</sup> NK cells could significantly expand after CIML differentiation (up to 20-fold size increase) (figure 1C). In additional experiments, both CD56<sup>bright</sup> and CD56<sup>dim</sup>



**Figure 1** Characterization of freshly isolated NK (D0), IL-15(c)-NK, IL-2-NK and CIML-NK cells by flow cytometry. (A) Representative plots of CD56 versus CD16 expression showing the gating of CD56<sup>bright</sup> and CD56<sup>dim</sup> subsets. (B) Percentage of CD56<sup>bright</sup> cells in the different types of NK cells. Bars show mean±SD, and dots connected by lines represent percentages from each individual donor (n=11 donors). (C) Expansion of CD56<sup>bright</sup> cells in CIML-NK cells assessed at day 6 following cytokine priming. Prior to memory-like differentiation, naïve CD56<sup>bright</sup> and CD56<sup>dim</sup> cells were sorted, CD56<sup>bright</sup> were labeled with CellTrace Violet (CTV) and pooled with unlabeled CD56<sup>dim</sup> cells. The percentage of (CTV<sup>+</sup>) CD56<sup>bright</sup> cells was assessed by flow cytometric analysis before (day 0) and after (day 6) CIML differentiation (C, left). Representative flow cytometry plots and (C, right) graph displaying the percentage of CTV<sup>+</sup> cells from each donor (individual dots) at days 0 and 6 (n=3 donors). (D) Proliferation of CIML-NK cells assessed at day 6 following cytokine priming. The assessment was done by flow cytometric analysis of CTV dilution on CIML derived from sorted CD56<sup>bright</sup> (red) or CD56<sup>dim</sup> (blue) cells (D, left). Representative flow cytometry plots and (D, right) violin plot summarizing proliferation data of CD56<sup>bright</sup> and CD56<sup>dim</sup> CIML-NK cells at day 6 quantified as division index (n=3 donors). (E, F) Characterization of the NK cell subsets as identified by the combined expression NKG2A and KIRs (Mix KIR) in the different indicated cell types. (E) Representative flow cytometry plots of NKG2A versus KIR expression and (F) bar graphs displaying the mean percentage of the subsets±SD (n=6 donors). (G) Proliferation of NKG2A<sup>+</sup>KIR<sup>-</sup> (red), NKG2A<sup>+</sup>KIR<sup>+</sup> (green), and NKG2A<sup>-</sup>KIR<sup>+</sup> (violet) in CIML-NK generated from sorted CD56<sup>dim</sup> cells assessed by flow cytometric analysis of CTV dilution (G, left). Representative flow cytometry plots and (G, right) violin plot showing the division index in each subset at day 6 (n=3 donors). Groups were compared using a one-way repeated measures analysis of variance with post hoc Tukey test (B, F, G) and paired two-tailed t-test (C, D) (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001). CIML, cytokine-induced memory-like; IL, interleukin; NK, natural killer.

sorted cells were labeled and induced to CIML differentiation as separated populations to evaluate proliferation. Consistently, CD56<sup>bright</sup> cells showed a significantly higher proliferation rate compared with CD56<sup>dim</sup> cells (figure 1D). In agreement with these results, the majority of CIML-NK cells expressed an NKG2A<sup>+</sup>KIR<sup>-</sup> phenotype (figure 1E,F), which typically characterizes CD56<sup>bright</sup> and a fraction of less mature CD56<sup>dim</sup> cells (online supplemental figure S3). It is noteworthy that in CIML-NK cells, the NKG2A<sup>-</sup>KIR<sup>+</sup> cell population poorly proliferated and nearly disappeared during culture, indicating that these terminally differentiated cells minimally participate in the generation of CIML-NK cells (figure 1E–G and online supplemental figure S3). Differential proliferation of the CD56<sup>bright</sup>/CD56<sup>dim</sup> or KIR<sup>+</sup>/KIR<sup>-</sup> cell subsets could be also confirmed in CIML-NK cells induced from unsorted cells (online supplemental figure S4).

We next characterized in more detail the expanded CIML CD56<sup>bright</sup> cells, to evaluate their potential contribution to the “CIML-functional traits”. Specifically, we analyzed the expression levels of major activating, inhibitory, and chemokine receptors in the different NK cell subsets (online supplemental figure S5). This analysis indicated that CD56<sup>bright</sup> NK cells undergoing CIML differentiation and expansion acquired a favorable antitumor functional profile, characterized by increased expression of certain activating receptors (including Nkp44), reduced TIGIT and TIM-3 inhibitory checkpoints, and a slight increase of the chemokine receptors CXCR3 and CXCR4. To check the potential functional advantage of CIML-NK cells, we evaluated their response to NSCLC cell lines derived from tumors of the three main subtypes: adenocarcinoma (A549 and H3122), squamous cell carcinoma (SW900), large cell carcinoma (H661). Overall, CIML-NK cells showed enhanced IFN- $\gamma$  production, compared with IL-15(c)-NK cells, and higher cytotoxic degranulation over both IL-15(c) and IL-2-NK cells (figure 2A,B). Moreover, the combined phenotypical and functional analysis suggested that the CD56<sup>bright</sup> subset could be the main driver of CIML-NK cell responses (online supplemental figure S6A). Consistent with this observation, in most of the effectors analyzed in functional experiments CIML-NK cells showed maximal CD56<sup>bright</sup> expansion when compared with IL-15(c)-NK or IL-2-NK cells (online supplemental figure S7). To confirm these latter findings, CIML-NK cells were generated from naïve CD56<sup>dim</sup> or CD56<sup>bright</sup> sorted cells, or from pooled populations after distinctive labeling of CD56<sup>bright</sup> cells, and assessed for their IFN- $\gamma$ , degranulation, and cytotoxic response to NSCLC targets. These experiments demonstrated that, indeed, CD56<sup>bright</sup> cells were more responsive than CD56<sup>dim</sup> cells (figure 2C–E and S6B,C) and supported most of the CIML-NK cytotoxic effect against NSCLC cells (figure 2F). CD56<sup>bright</sup> cells showed higher responses also in IL-2-NK and IL-15(c)-NK cells (online supplemental figure S6A), with the remarkable difference that in these latter cases the CD56<sup>bright</sup> cell subset was generally less expanded (figure 1A,B). Moreover, at variance with IL-2-CD56<sup>bright</sup> and IL-15(c)-CD56<sup>bright</sup> cells, CIML-CD56<sup>bright</sup>

NK cells showed a high granzyme B content (figure 2G), which could support their effective killing capabilities.

Overall, these experiments indicate that CIML-NK cells have a superior functional response over IL-2-NK cells against NSCLC cells of different tumor subtypes, with the CD56<sup>bright</sup> subset serving as the main driver of CIML effector functions.

### CIML-NK cells show superior ability to attack NSCLC spheroids

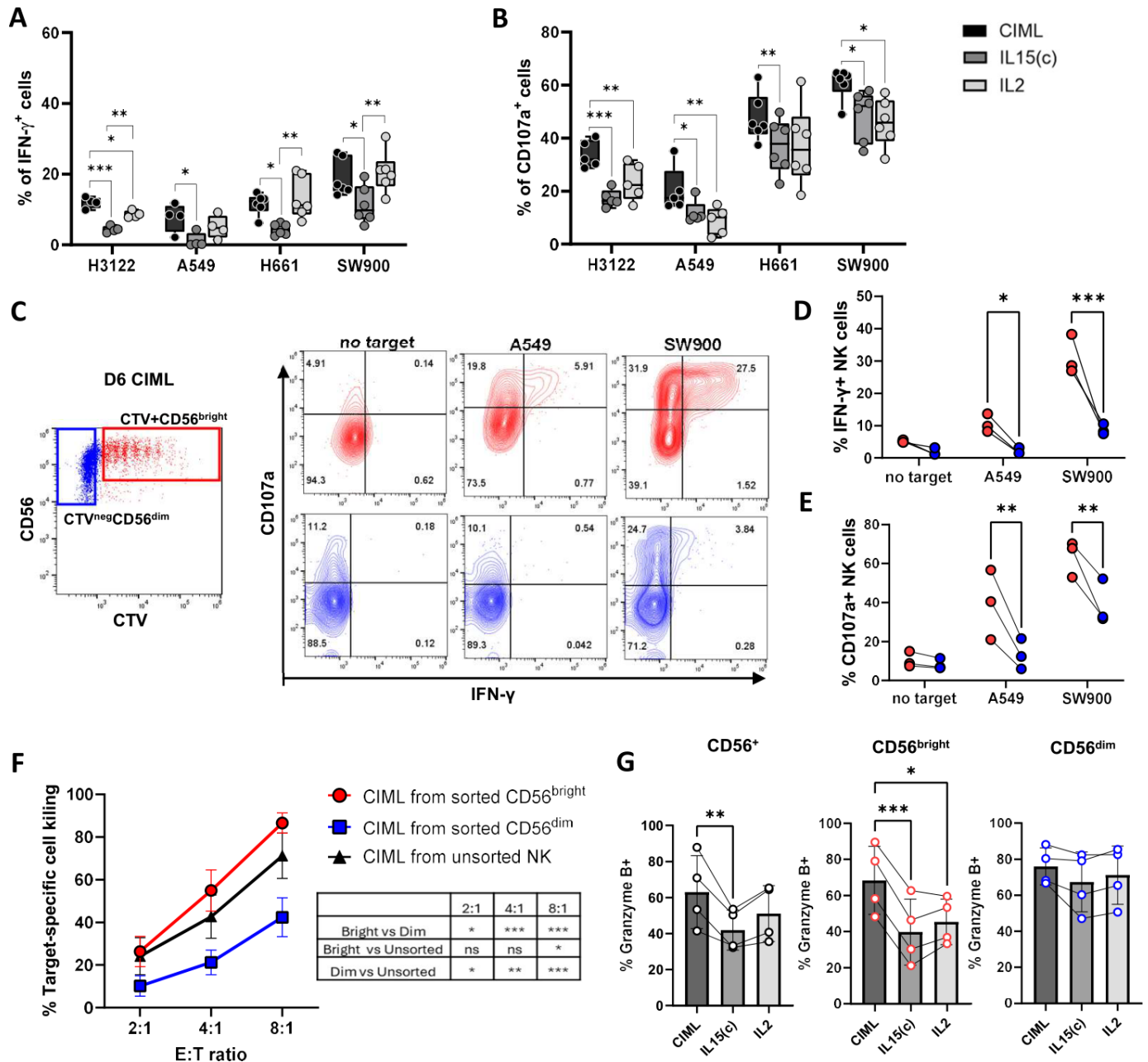
Tumor cells can disseminate through circulation as cellular clusters, therefore we generated spheroids from the above NSCLC cell lines following a previously described protocol<sup>23</sup> and assessed NK cell reactivity. We co-cultured NK cells in functional assays with either spheroid-derived cells or 3D spheroids (online supplemental figure S8A,B), to dissect the functional features of dissociated tumor cells and the general effects of the organized 3D structures. Since CIML-NK cells can upregulate the expression of the IL-2R $\alpha$  subunit,<sup>33</sup> we also investigated the possible additional effect of overnight exposure to low-dose IL-2 (50 IU/mL). The CD56<sup>bright</sup>/CD56<sup>dim</sup> cell subset composition of this latter effector was similar to that of CIML-NK cells (online supplemental figure S7).

The functional response of the different NK cell preparations, evaluated as cytotoxic degranulation and IFN- $\gamma$  production, significantly decreased when target cells were switched from adherent to spheroid-derived cells (online supplemental figure S9). Nevertheless, CIML-NK cells displayed superior functional responses to these targets over IL-2-NK and IL-15(c)-NK cells (figure 3A,B). Moreover, CIML-NK cells' responsiveness was further increased by IL-2 boost, particularly in terms of IFN- $\gamma$  production. NK cells were also tested in a cytotoxicity assay against A549 and SW900 cells (the lowest and highest NK cell stimulators, respectively), confirming that, indeed, CIML-NK cells could exert superior killing activity of spheroid-derived cells over IL-2-NK and IL-15(c)-NK cells (figure 3C,D).

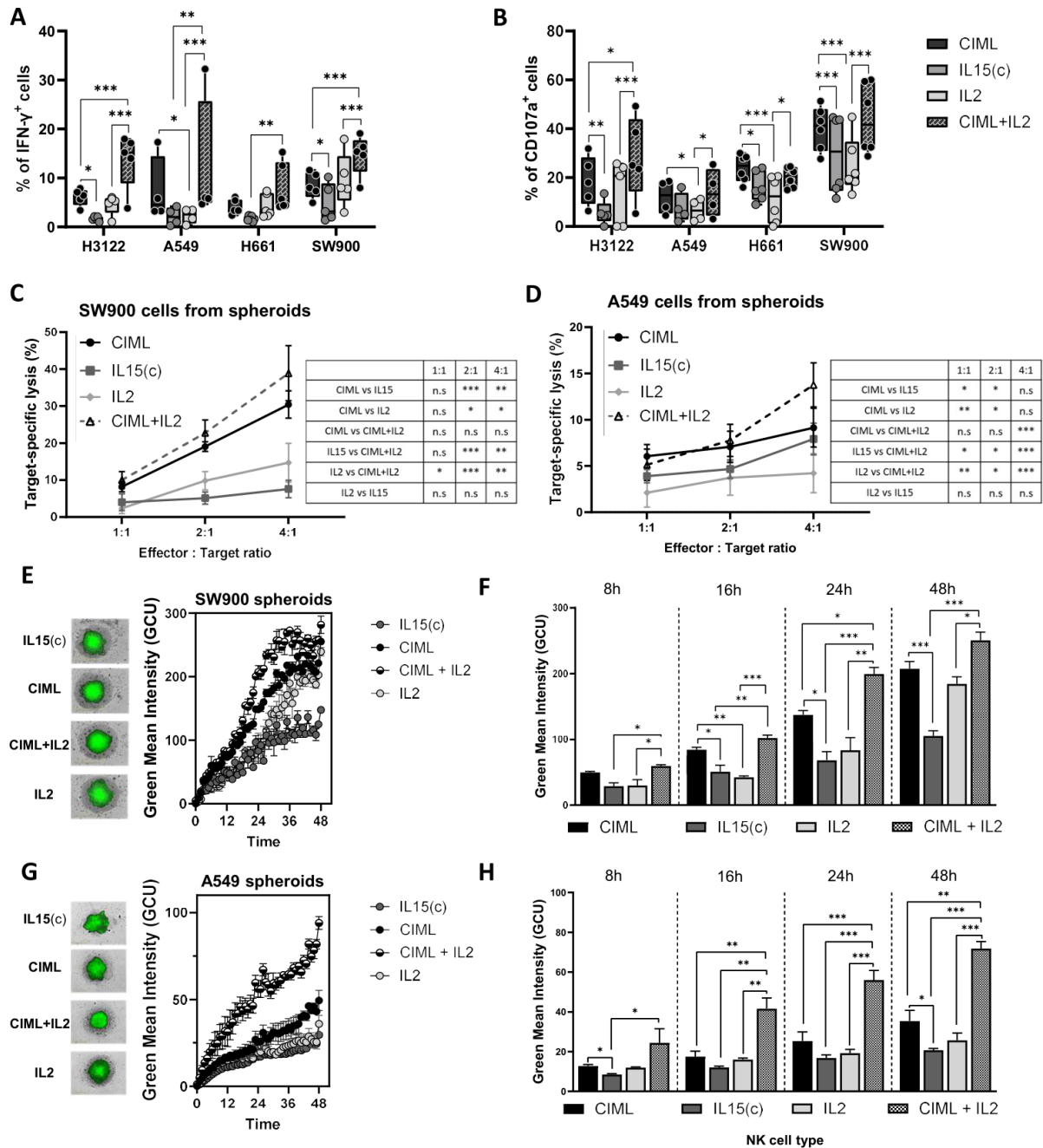
We next assessed the effects of NK cells directly on spheroids (one spheroid/well online supplemental figure S8B—). Spheroids from A549 and SW900 cells were loaded with caspase 3–7 substrate, co-cultured with NK cells, and evaluated by real-time live cell imaging (Incucyte). This analysis revealed that NK cells could kill tumor cells even when they are aggregated in 3D spheroids. More importantly, CIML-NK cells, especially when supplemented with IL-2, showed higher killing efficiency over IL-15(c)-NK and IL-2-NK cells. Remarkably, CIML-NK cells boosted with IL-2 showed persistent killing capabilities even after 36–48 hours of co-culture (figure 3E–H), suggesting that these effectors could minimize tumor-related exhaustion and suppression effects.

### CIML-NK cells decrease the CSC content and tumorigenicity of 3D tumor cell clusters

Given their superior response to spheroids, CIML-NK cells may be effective in attacking CD133<sup>+</sup> CSC, which



**Figure 2** Functional characterization of CIML, IL-15(c), and IL-2-NK cells against NSCLC cell lines, evaluation of the CD56<sup>bright</sup> and CD56<sup>dim</sup> CIML subsets. (A–B) The different NK cell types were co-cultured with the indicated NSCLC cell lines (E:T ratio of 1:1) for 6 hours, and the production of IFN- $\gamma$  or the expression of CD107a on NK cells (gated as CD56<sup>+</sup> cells) was measured by flow cytometry. Data are shown as box and whisker plots with median $\pm$ minimum to maximum of n=5–6 donors. (C–E) CIML-NK cells were generated from pooled labeled (CTV<sup>+</sup>) CD56<sup>bright</sup> and unlabeled (CTV<sup>neg</sup>) CD56<sup>dim</sup> naïve NK cells, and then cultured with or without the indicated cell lines for functional assessments. (C) Representative flow cytometry plots showing: (left) the gating of CTV<sup>+</sup> (red) and CTV<sup>neg</sup> (blue) cells in day 6 CIML-NK cells, and (right) the CD107a versus IFN- $\gamma$  expression on the gated subsets of CIML-NK cells in the different co-culture conditions. (D,E) Cumulative data on the frequencies of IFN- $\gamma$ <sup>+</sup> or CD107a<sup>+</sup> cells in CD56<sup>bright</sup> (CTV<sup>+</sup>, red) and CD56<sup>dim</sup> (CTV<sup>neg</sup>, blue) subsets of unstimulated/stimulated CIML NK cells (n=3 donors). (F) Calcein-based cytotoxicity of SW900 cells on co-culture with CIML-NK cells differentiated from sorted CD56<sup>bright</sup> (red), sorted CD56<sup>dim</sup> (blue), or unsorted (black) NK cells. The graph displays the mean cytotoxicity $\pm$ SD of n=4 donors at different E:T ratios performed in technical duplicates. (G) Flow cytometry analysis of granzyme B expression in the total (CD56<sup>+</sup>), CD56<sup>bright</sup>, and CD56<sup>dim</sup> NK cell populations of CIML-NK, IL15(c)-NK, and IL-2-NK cells. Bars display the mean percentage of granzyme B-positive cells of n=4 donors. Dots represent the percentage of positive cells in each individual donor. Comparisons between groups were performed using one-way ANOVA with Tukey post hoc test (A, B, G) or a two-way ANOVA with Sidak post test (D–F). Only significant values are shown (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001). ANOVA, analysis of variance; CIML, cytokine-induced memory-like; CTV, CellTrace Violet; Effector, (E): Target, (T); IFN, interferon; IL, interleukin; NK, natural killer; NSCLC, non-small cell lung cancer.



**Figure 3** Functional characterization of CIML, IL-15(c) and IL-2 NK cells against non-small cell lung cancer spheroids. (A–D) Evaluation of NK cell functional response to spheroid-derived cells. (A, B) CIML-NK, IL-15(c)-NK, IL-2-NK cells and CIML-NK cells boosted with IL-2 (CIML+IL-2) were co-cultured for 6 hours with H3122, A549, H661 or SW900 spheroid-derived cells. Afterward, the expression of IFN- $\gamma$  and CD107a was measured on NK cells by flow cytometric analysis. Data are shown as box a whisker plots with median $\pm$ minimum to maximum, dots represent the value of each single donor. Number of NK cell donors analyzed, n=6 (SW900, H661), n=5 (H3122), n=4 (A549). (C, D) Evaluation of NK cell cytotoxicity against spheroid-derived cells. The specific lysis of CFSE-labeled SW900 or A549 spheroid-derived cells was measured after 24 hours of co-culture with the indicated NK cell types at different E:T ratios. After co-culture, target cells were stained with dead cell marker and annexin V, and assessed by flow cytometry gating on CFSE-positive events. (E–H) Evaluation of NK cell cytotoxicity in the context of cell aggregates (spheroids). SW900 and A549 spheroids were co-cultured with the different NK cells (E:T ratio 8:1) in the presence of caspase 3–7 green dye in a 96-well plates. Wells were imaged every hour for 48 hours to quantify the caspase 3–7 activation (green signal) within the spheroids using the IncuCyte system. (E, G) Representative images of SW900 and A549 spheroids co-cultured with the different NK cell types displaying caspase activation (at time=48 hours), and quantification of caspase activation (green mean intensity) normalized to control in spheroid boundary. Graphs show mean $\pm$ SEM of n=3 donors ran in triplicates. (F, H) Visualization of target-specific caspase 3–7 activity at the indicated time points of the co-culture as bar graphs showing mean $\pm$ SEM. Groups were compared using a one-way (A–B) or two-way analysis of variance (C–H). (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001). Carboxyfluorescein succinimidyl ester, (CFSE); CIML, cytokine-induced memory-like; Effector, (E): Target, (T); IFN, interferon; IL, interleukin; NK, natural killer.

have been shown to be enriched in NSCLC spheroids.<sup>23</sup> To assess this possibility, we determined the percentage of viable CD133<sup>+</sup> cells within A549-derived spheroids before and after co-culture with CIML-NK IL-2-NL, IL15(c)-NK cells (Control (CTRL)-spheroids, CIML-spheroids, IL-2-spheroids, IL-15(c)-spheroids, respectively) (online supplemental figure S8C). We observed that CIML-NK cells were effective at reducing the CD133<sup>+</sup> CSC content in spheroids, and such capability of CIML-NK cells could not be maintained after the IL-2 boost (figure 4A). Remarkably, neither IL-2-NK nor IL-15(c)-NK cells could reduce the fraction of CD133<sup>+</sup> cells within spheroids.

We next analyzed whether CIML-NK cells, consistent with their effect on CD133<sup>+</sup> cells, could impact spheroid tumorigenicity. Thus, viable tumor cells were isolated from spheroid-NK cell co-cultures and assayed for clonogenicity in vitro, or evaluated for in vivo tumorigenic potential (online supplemental figure S8C). As shown in figure 4B, tumor cells from spheroids that were co-cultured with CIML-NK cells (either boosted or not with IL-2) formed significantly fewer colonies than those derived from CTRL-spheroids, IL-2-spheroids, or IL-15(c)-spheroids. For the in vivo studies, viable A549 cells from CTRL-spheroids or sorted from co-cultures were subcutaneously inoculated in NSG mice, and in situ tumor formation was monitored. Mice inoculated with tumor cells from CIML-spheroids displayed lower tumor incidence compared with mice receiving equal numbers of tumor cells from CTRL-spheroids, IL-2-spheroids, or IL-15(c)-spheroids (online supplemental figure S10). Moreover, tumor cells from CIML-spheroids, and not those from IL-15(c)-spheroids or IL-2-spheroids, generated significantly smaller tumors compared with those induced by CTRL-spheroids (figure 4C). On the whole, these data indicate that CIML-NK cells affect clonogenicity and tumorigenicity by targeting CD133<sup>+</sup> cells. However, IL-2-boosted CIML-NK cells, which do not affect the CD133<sup>+</sup> cell population, may target different clonogenic tumor cell subsets, with less pronounced in vivo effects (ie, no statistical significance in the tumor volume reduction).

### CIML-NK cells target disseminated CSC in PDX models

To investigate in more detail the effects of CIML-NK cells boosted or not with IL-2 on CD133<sup>+</sup> NSCLC cells, and to extend the study to patient-derived tumor cells, we analyzed in co-culture experiments four different targets: A549 and SW900 spheroids, and cells derived from two NSCLC PDX models (PDX-LT710 and PDX-LT111) (see extended M&M section). These experiments indicated that CIML-NK cells consistently reduced the CD133<sup>+</sup> cell fraction in all the analyzed targets, whereas IL-2-boosted CIML showed variable behavior (online supplemental figure S11).

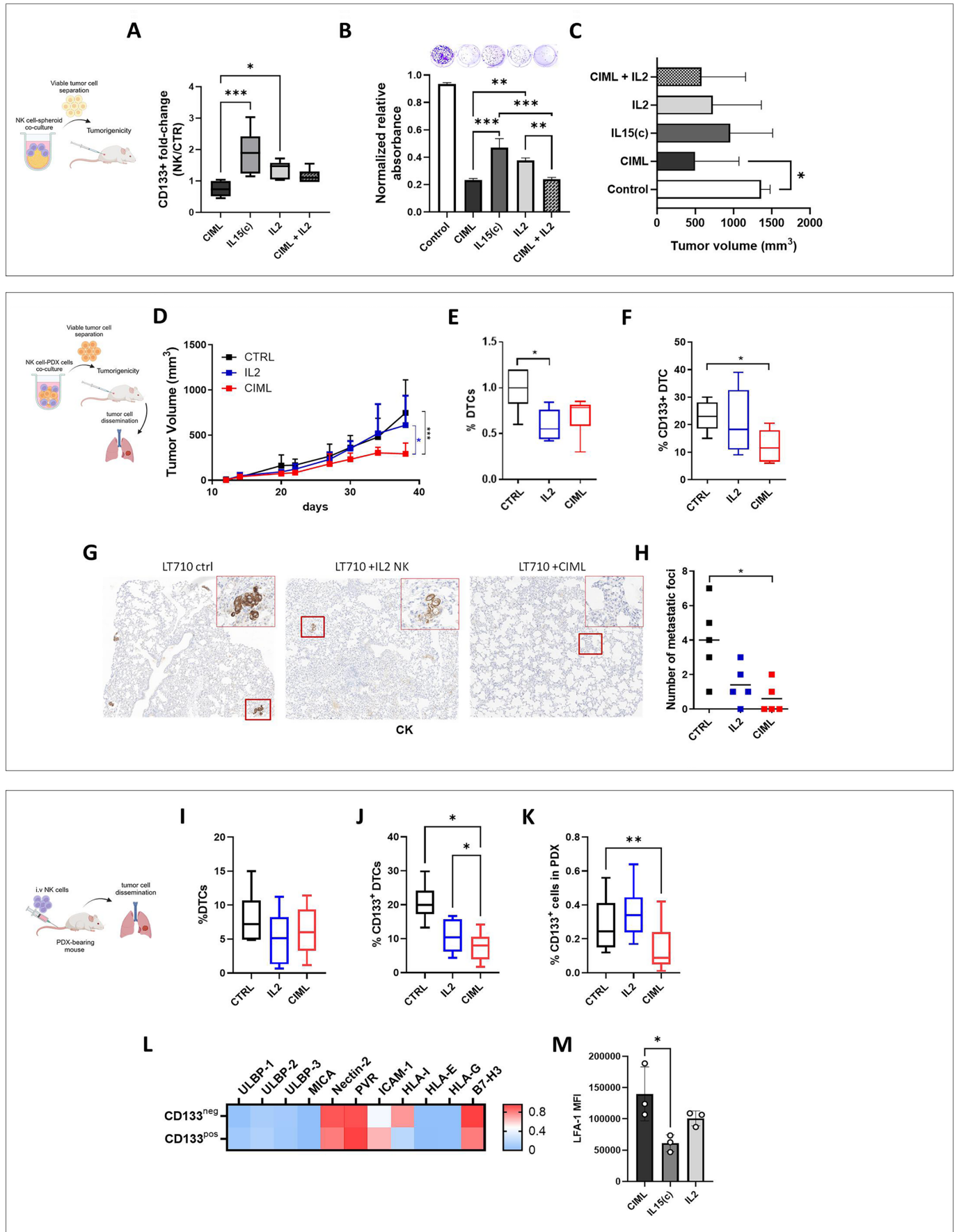
We then focused on CIML-NK cells and asked whether they could be effective in influencing PDX capability to support dissemination driven by CSCs. We first analyzed PDX-LT710. On co-culture, CIML-NK cells, besides reducing the PDX CD133<sup>+</sup> cell content, also showed

higher cytotoxic degranulation and IFN- $\gamma$  production over IL-15(c)-NK and IL-2-NK cells (online supplemental figure S12). Accordingly, after co-culture with CIML-NK cells, PDX cells displayed reduced tumorigenic capability, generating significantly smaller subcutaneous tumors (CIML-PDX) compared with those induced by PDX cells cultured alone (CTRL-PDX) or in the presence of IL-2-NK cells (IL-2-PDX) (figure 4D). Flow cytometry analysis of lung-derived cells in PDX-bearing mice revealed decreased numbers of disseminated tumor cells (DTC) in both CIML-PDX and IL-2-PDX mice (figure 4E) compared with CTRL. However, only CIML-PDX mice showed a significant reduction in CD133<sup>+</sup> cells within DTCs (figure 4F). Coherently with this reduction, immunohistochemistry analysis of the lungs showed significantly lower numbers of lung metastatic nodules in CIML-PDX compared with CTRL-PDX mice (figure 4G,H). Remarkably, a similar ability of CIML NK cells to reduce tumor growth and dissemination of CD133<sup>+</sup> cells in vivo was also confirmed in the PDX-LT111 model (online supplemental figure S13A–C). We next verified whether CIML-NK cells could effectively reduce the CSC dissemination to the lungs in vivo. Specifically, we compared CIML-NK and IL-2-NK cells for their engraftment/biodistribution, and for their effects on the tumors. We injected intravenously NK cells in mice bearing near end-point subcutaneous PDX and evaluated the frequency of NK cells in the tissues 2/5 days after injection, and the CD133<sup>+</sup>/CD133<sup>−</sup> lung DTCs at day 2. The flow cytometry analysis of NK cells from mouse tissues revealed a higher frequency of CIML-NK, compared with IL-2-NK cells in different tissues, reaching statistical significance in lungs and blood. The analysis at day 5 indicated that CIML-NK cells and, at a lower extent, IL-2-NK cells could persist in lung and tumor tissues (online supplemental figure S14). Regarding tumor cell dissemination, lung DTC numbers were only slightly modified in NK-injected mice (figure 4I). However, DTCs from mice receiving CIML-NK cells showed a significantly reduced fraction of CD133<sup>+</sup> cells, compared with DTCs from mice receiving no NK or IL-2-NK cells (figure 4J). Significant CD133<sup>+</sup> cell reduction was also observed within the subcutaneous tumors in CIML-NK and not in IL-2-NK injected mice (figure 4K).

A further experiment, focused on the effects of CIML-NK cells on lung DTCs, gave similar results. Indeed, 2 days after injection CIML-NK cells were present in the lungs of the PDX mice (online supplemental figure S15A) and, coherently, a significant reduction of lung CD133<sup>+</sup> cells could be observed within DTCs (online supplemental figure S15B).

On the whole, these data on the PDX models indicate that CIML-NK cells could effectively target disseminating CD133<sup>+</sup> cells in vivo.

To investigate why CD133<sup>+</sup> cells could be eliminated more efficiently than their CD133<sup>−</sup> counterpart by CIML-NK cells, we comparatively analyzed the surface expression of major NK-receptor ligands on CD133<sup>+</sup> and CD133<sup>−</sup> PDX cells. This analysis drew attention to some



**Figure 4** (Continued)

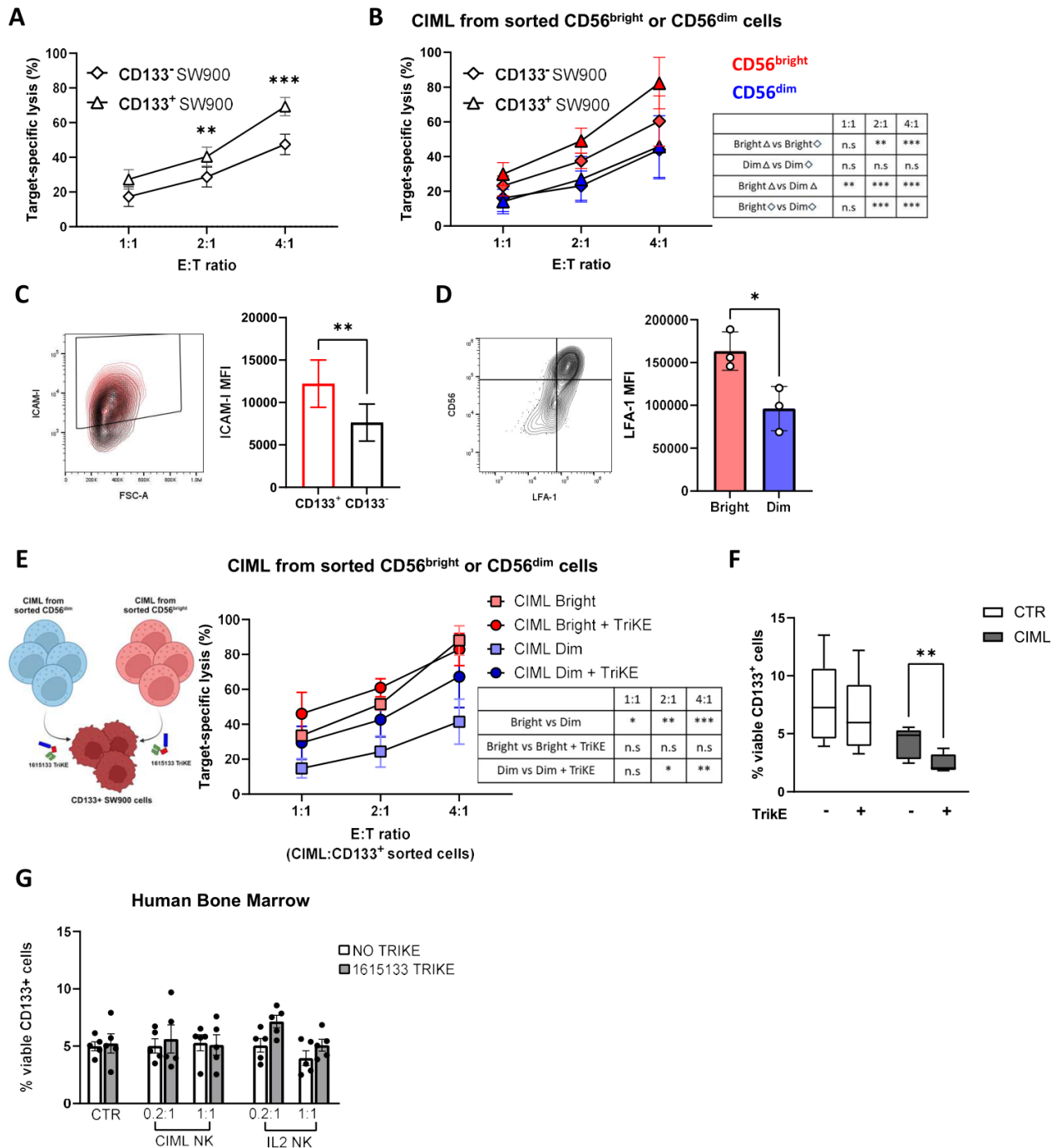
**Figure 4** CIML-NK cells target spheroid and PDX CD133<sup>+</sup> cells and limit tumorigenicity and tumor dissemination in vivo. (A–C) Analysis of the effect of NK cells on spheroids. A549 spheroids were co-cultured for 24 hours at a 1:1 E:T ratio with CIML-NK, IL15(c)-NK, IL-2-NK cells, or CIML-NK cells boosted with IL-2 (CIML+IL-2), or were cultured alone (CTRL). Next, spheroids were dissociated, NK cells were removed by depleting CD45<sup>+</sup> cells (using CD45-specific microbeads) and recovered tumor cells were analyzed for viability and CD133 expression, and assayed for clonogenicity, in vitro, and tumorigenicity, in vivo. (A) Flow cytometry assessment of CD133<sup>+</sup> percentage in the different co-cultures, reported as fold-change to control untreated spheroids. Data are shown as box and whisker plots with median±5–95 percentiles (n=3 donors). (B) In vitro clonogenicity assay of spheroid-derived cells. Viable tumor cells from dissociated spheroids were appropriately diluted and seeded in 6-well plates to follow colony formation (B, top). Image of a representative experiment. Wells were stained with crystal violet for clone evaluation (B, bottom). Bar graphs showing the mean normalized relative absorbances of the crystal violet dissolved with 10% acetic acid of n=3 donors ran in technical triplicates. (C) In vivo tumorigenicity of spheroid-derived tumor cells. Viable tumor cells from dissociated spheroids were s.c inoculated to NSG mice, and tumor formation and growth was followed over time. Bar graph shows the mean maximum tumor volume±SD reached per group (n=6 mice per group). (D–H) Analysis of the effect of NK cells on PDX (patient LT710). PDX cells were co-cultured for 4 hours with the different NK cell types or cultured alone (CTRL). Next, NK cells were removed from co-cultures by depleting CD45<sup>+</sup> cells and tumor cells were analyzed for tumorigenicity in vivo. (D) Assessment of in vivo tumorigenicity of PDX cells from the indicated cultures. Mean group tumor growth curve±SD are shown (n=4 mice/group). (E–H) Evaluation of tumor cell dissemination to the lungs from subcutaneous PDXs. Subcutaneous tumors were generated from PDX cells from the indicated cultures. (E) Percentage of lung disseminating tumor cells (DTC) assessed by flow cytometry. DTC were identified as viable-H2K<sup>+</sup> (non-murine) cells. (F) Percentage of CD133<sup>+</sup> cells within the lung DTCs assessed by flow cytometry. Data are shown as box and whisker plots with median±minimum to maximum values (n=4 mice per group). (G) Representative images of IHC staining to evaluate the presence of CK+metastatic nodules (at higher magnification in the insets) in lung tissue sections. (H) Graph showing the number of CK+lung metastatic foci from each mouse (dots) and the mean foci number (line) per group. (I–K) Effect of CIML-NK cells in vivo. 5×10<sup>6</sup> CIML-NK cells were inoculated intravenous in near end-point PDX-bearing mice. 48 hours after, mice were sacrificed, lungs and primary s.c tumors were dissociated, and analyzed. (I) Percentage of lung DTC assessed by flow cytometry. (J) Percentage of CD133<sup>+</sup> DTC and (K) percentage of CD133<sup>+</sup> tumor cells in s.c tumors evaluated by flow cytometry. Data are shown as box and whisker plots with median±minimum to maximum values (n=6 mice per group ran in technical duplicates). (L) Heatmap showing the expression levels of major activating and inhibitory NK-cell ligands measured by flow cytometry on CD133<sup>+</sup> and CD133<sup>neg</sup> PDX cells. (M) Flow cytometry analysis of the surface expression of LFA-1 in IL-15(c), IL-2-NK, and CIML-NK cells. Bars report mean MFI±SD and dots represent MFI values of individual donors (n=3 donors). Comparisons between groups were assessed using one-way ANOVA with Dunnett's multiple comparison test (A, C), Turkey post hoc test (B, E, F, H–K, L) or two-way ANOVA (D). Only significant values are shown (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001). ANOVA, analysis of variance; CIML, cytokine-induced memory-like; Effector, (E):Target, (T); IL, interleukin; Immunohistochemistry, (IHC); Mean Fluorescence Intensity, (MFI); NK, natural killer; PDX, patient-derived xenograft.

markers that could explain the differential killing of the two subsets. Indeed, compared with their CD133<sup>−</sup> counterpart, CD133<sup>+</sup> cells expressed higher levels of ICAM-1, lower HLA class I, and slightly lower B7-H3 (an additional inhibitory ligand for NK cells). Overall, PDX cells virtually did not express non-classical HLA-E and HLA-G (figure 4L). Our group showed in a recent study<sup>34</sup> that ICAM-1 was consistently upregulated in the CD133<sup>+</sup> subset in a large panel of NSCLC cell lines of different tumor subtypes, whereas differential expression of HLA-I in CD133<sup>+</sup>/CD133<sup>−</sup> cells was uncommon. Moreover, B7-H3 expression in CD133<sup>+</sup> cells could be even upregulated in certain cell lines. Therefore, only increased ICAM-1 expression appears as a distinctive feature of CD133<sup>+</sup> NSCLC cells. Based on this thought, we analyzed the expression of LFA-1, which binds ICAM-1, on CIML-NK, IL-2-NK, and IL-15(c)-NK cells. CIML-NK cells expressed the highest LFA-1 levels (figure 4M), suggesting a role for the enhanced LFA-1:ICAM-1 axis in CIML-mediated reduction of CD133<sup>+</sup> cells.

#### CD56<sup>bright</sup> CIML-NK cells are the major drivers of the CD133<sup>+</sup> NSCLC cell targeting

To confirm that CIML-NK cells could more effectively kill CD133<sup>+</sup> than CD133<sup>−</sup> cells, and to define whether there is a specific subset driving such activity,

we performed functional analyses using sorted cells (ie, CD133<sup>+</sup>/CD133<sup>−</sup> and CD56<sup>bright</sup>/CD56<sup>dim</sup> cells). These experiments were done on the SW900 cell line, particularly enriched in CD133<sup>+</sup> cells (1–2% of total) (online supplemental figure S16). We first analyzed CIML-NK cells in cytotoxicity assays against sorted CD133<sup>+</sup> and CD133<sup>−</sup> SW900 cells and found that, indeed, they could kill at higher efficiency CD133<sup>+</sup> cells (figure 5A). Next, we derived CIML-NK cells from sorted naïve CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells and analyzed their killing capabilities against CD133<sup>+</sup> or CD133<sup>−</sup> cells. As shown in figure 5B, CD56<sup>dim</sup> CIML-NK cells killed both targets to a similar extent, while CD56<sup>bright</sup> CIML-NK cells exhibited superior killing of CD133<sup>+</sup> over CD133<sup>−</sup> cells. Like CD133<sup>+</sup> PDX-derived cells, CD133<sup>+</sup> SW900 cells showed higher ICAM-1 expression over their CD133<sup>−</sup> counterpart (figure 5C), while, in line with our recent data,<sup>34</sup> they did not show evident differences on HLA-I or B7-H3 expression (online supplemental figure S17). We then analyzed CIML-NK cells for the surface expression of LFA-1, and, remarkably, we could find higher LFA-1 expression on CD56<sup>bright</sup> compared with CD56<sup>dim</sup> cells (figure 5D) providing further evidence of a possible role of enhanced LFA-1:ICAM-1 interaction in driving the capability of CD56<sup>bright</sup> CIML-NK to kill CD133<sup>+</sup> cells.



**Figure 5** CD56<sup>bright</sup> CIML-NK cells drive the killing of CD133<sup>+</sup> non-small cell lung cancer cells, and 161513 TriKE maximize this effect by triggering CD56<sup>dim</sup> CIML-NK cell activity. (A) Calcein-based cytotoxicity assay of CIML-NK cells against sorted CD133<sup>+</sup> and CD133<sup>-</sup> SW900 cells. (B) Specific lysis of sorted CD133<sup>-</sup> or CD133<sup>+</sup> SW900 cells by CIML-NK derived from sorted CD56<sup>bright</sup> or CD56<sup>dim</sup> cells. Graphs (A, B) show the mean percentage $\pm$ SD of  $n=4$  donors ran in technical duplicates. (C) ICAM-1 expression on CD133<sup>+</sup> (red) or CD133<sup>-</sup> (black) SW900 cells. (D) LFA-1 expression on CD56<sup>bright</sup> and CD56<sup>dim</sup> CIML-NK cells. Bars (C, D) show the mean MFI $\pm$ SD of 3 independent experiments. (E–F) Study of the effect of 161513 TriKE on the targeting of CD133<sup>+</sup> by CIML NK cells. (E) CD133<sup>+</sup> SW900-specific lysis by CIML-NK derived from sorted CD56<sup>bright</sup> or CD56<sup>dim</sup> cells in the presence or absence of 161513 TriKE. Graphs show the mean percentage $\pm$ SD of  $n=3$  donors ran in technical duplicates. (F) 161513 TriKE enhances the CD133<sup>+</sup> cell targeting capability of CIML-NK cells. Box and whisker plot showing the median percentage $\pm$ min to max of CD133<sup>+</sup> viable SW900 cells after co-culture with CIML-NK cells in the presence or absence of 161513 TriKE ( $n=4$  donors). (G) CIML-NK cells and 161513 TriKE do not modify the CD133<sup>+</sup> cell content in human bone marrow. Human bone marrow cells were co-cultured with the indicated NK cells (CIML-NK or IL-2-NK) at the indicated E:T ratios in the presence/absence of TriKE. The percentage of viable CD133<sup>+</sup> cells was assessed by flow cytometry. Comparisons between groups were performed through two-way analysis of variance (A, B, E–G) two-tailed t-tests (C, D) (\* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ ). CIML, cytokine-induced memory-like; Effector, (E):Target, (T); IL, interleukin; Mean Fluorescence Intensity, (MFI); NK, natural killer; TriKE, tri-specific cell engager.

**1615133 TriKE rescues the cytotoxicity of CD16<sup>+</sup> CIML-NK cells against CD133<sup>+</sup> NSCLC cells**

Our findings revealed that CIML-NK cell-mediated killing of CD133<sup>+</sup> cells is essentially driven by their CD56<sup>bright</sup> cell fraction. We then considered rescuing the cytotoxicity of CD56<sup>dim</sup> (CD16<sup>+</sup>) CIML-cells to maximize the overall anti-CSC effectiveness. To this end, we decided to evaluate the “ad-hoc” TriKE (1615133 TriKE)<sup>30</sup> capable of targeting CD16 and CD133, and harboring the IL-15 moiety, which could support CIML-NK cell survival (online supplemental figure S18A). We generated CIML-NK cells from sorted naïve CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells and comparatively analyzed their ability to kill sorted CD133<sup>+</sup> SW900 cells in the absence/presence of the TriKE (figure 5E). CD56<sup>bright</sup> CIML-NK cells efficiently killed CD133<sup>+</sup> cells, while their CD56<sup>dim</sup> counterpart showed lower cytotoxic effects. The 1615133 TriKE had no significant functional effects on CD56<sup>bright</sup> CIML-NK cells, while it increased the cytotoxic activity of CD56<sup>dim</sup> CIML-NK cells against CD133<sup>+</sup> cells. The functional effect of 1615133 TriKE, was specific, as CIML-NK cell-mediated killing of CD133<sup>+</sup> SW900 cells was increased by 1615133 TriKE and not by the 161,519 TriKE (targeting CD19—not expressed on CD133<sup>+</sup> SW900 cells) (online supplemental figure S18B). As CD16-engager, 1615133 TriKE could also trigger IL-2-NK cells and even the IL-15(c)-NK cells. Its efficacy in enhancing CD133<sup>+</sup> cell killing appeared highest in CIML-NK cells, which is in line with the memory-like features of these cells (online supplemental figure S19A,B). The advantage of TriKE-induced cytotoxicity of CIML over IL-2-NK cells is even more evident in the case of targets (CD133<sup>+</sup>) particularly resistant to NK cell activity (online supplemental figure S19C,D). To further confirm the effectiveness of the TriKE in boosting CIML-NK cell activity against CD133<sup>+</sup> cells, we co-cultured (unsorted) CIML-NK cells with SW900-single spheroids in the absence/presence of the 1615133 TriKE and evaluated the impact on the CD133<sup>+</sup> cell population within the spheroids, however, and even more importantly, the combination with the TriKE significantly amplified this effect (figure 5F). We therefore evaluated in vitro whether this synergistic effect could be safe for hematopoietic stem cells, considering possible therapeutic developments for such CIML-TriKE combination. Co-culture experiments showed that neither CIML-NK, nor TriKE, nor their combination had significant effects on the vitality of human bone marrow cells and on their content of CD133<sup>+</sup> cells (online supplemental figures S20 and 5G).

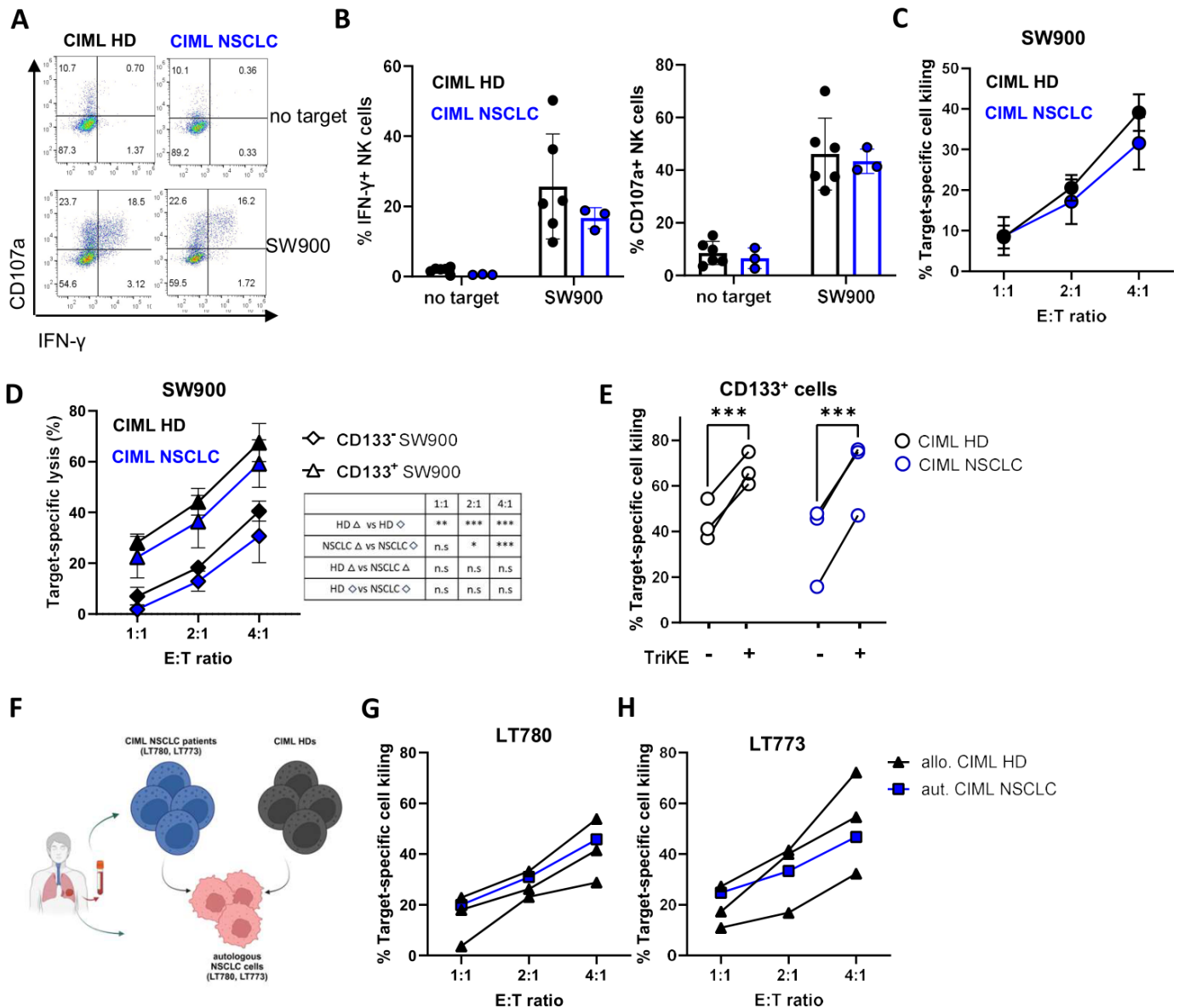
**CIML-NK cells derived from patients with NSCLC kill autologous tumor cells and respond to 1615133 TriKE**

Therapies based on optimized autologous NK cells present the advantage of avoiding lymphodepletion before infusion. However, the KIR:HLA-I interaction in the autologous setting could limit NK cell efficacy. CIML-NK effectors, showing an expansion of KIR<sup>neg</sup> cells, combined to TriKEs, may partly overcome these

| Table 1 Features of patients and healthy donors (HD)                            |     |       |        |     |
|---------------------------------------------------------------------------------|-----|-------|--------|-----|
| Histological subtypes                                                           |     | Stage | Gender | Age |
| Patients ID                                                                     |     |       |        |     |
| LT710                                                                           | ADC | IIB   | Female | 63  |
| LT780                                                                           | ADC | IB    | Male   | 76  |
| LT773                                                                           | ADC | IIIB  | Male   | 65  |
| NEO065                                                                          | ADC | IIIB  | Female | 65  |
| NEO067                                                                          | ADC | IIIA  | Female | 70  |
| NEO068                                                                          | NOS | IIIA  | Male   | 76  |
| Healthy donor ID                                                                |     |       |        |     |
| IMD233                                                                          | HD  | –     | Female | 65  |
| IMD213                                                                          | HD  | –     | Male   | 65  |
| IMD241                                                                          | HD  | –     | Male   | 69  |
| IMD239                                                                          | HD  | –     | Male   | 61  |
| IMD240                                                                          | HD  | –     | Female | 61  |
| IMD222                                                                          | HD  | –     | Male   | 61  |
| ADC, adenocarcinoma; HD, healthy donor; NOS, not otherwise specified carcinoma. |     |       |        |     |

problems. Therefore, we asked whether CIML-NK cells could be derived from patients with NSCLC, and whether they could display effector functions comparable to those from HDs. We derived CIML cells from six patients with NSCLC ranging from stage I to III (tables 1–2) and found that their phenotype, in terms of expansion of CD56<sup>bright</sup> and distribution of NKG2A/KIR subsets mirrored that of age-matched (over 60 years old) HDs (table 1) (online supplemental figure S21). Furthermore, NSCLC and HD CIML-NK cells displayed similar functional capabilities in terms of degranulation,

| Table 2 Patients’ cumulative data                        |            |
|----------------------------------------------------------|------------|
| Patients with NSCLC                                      |            |
| Number of patients                                       | 6          |
| Histological subtypes:                                   |            |
| ADC                                                      | 5          |
| Non-ADC                                                  | 1          |
| Stage                                                    |            |
| IB                                                       | 1          |
| IIB                                                      | 1          |
| IIIA                                                     | 2          |
| IIIB                                                     | 2          |
| Gender                                                   |            |
| Male                                                     | 3          |
| Female                                                   | 3          |
| Age at the time of surgery median (min/max)              | 69 (63–76) |
| ADC, adenocarcinoma; NSCLC, non-small cell lung cancer . |            |



**Figure 6** Effective CIML NK cells can be generated from patients with NSCLC at different disease stages. (A–C) Functional assessment of CIML NK cells derived from PBMCs from healthy donors (CIML HD, black) or patients with stage I–III NSCLC (CIML NSCLC, blue) against SW900 cells. (A) Representative flow cytometry plots of CD107a versus IFN- $\gamma$  expression by CIML-NK cells. (B) Bar graphs showing the mean $\pm$ SD expression of IFN- $\gamma$  (left) and CD107a (right) gated on CD56 $^{+}$  cells. Dots represent the values from each individual donor. (C) SW900-specific lysis by CIML HD (black) or CIML NSCLC (blue) at different E:T ratios. Graphs show the mean percentage $\pm$ SD of  $n=6$  donors per group ran in technical duplicates. (D) Specific lysis of sorted CD133 $^{-}$  or CD133 $^{+}$  SW900 cells by CIML HD (black) or CIML NSCLC (blue) at different E:T ratios. Graph shows mean percentage $\pm$ SD of each group of  $n=3$  donors per group ran in technical duplicates. (E) CD133 $^{+}$  SW900-specific lysis by CIML HD or CIML NSCLC at a 2:1 E:T ratio in the presence or absence of 1615133 TriKE. Graphs show the percentage of each donor ( $n=3$ ) ran in technical duplicates. (F–H) NSCLC CIML NK cells are effective against autologous tumor cells. (F) Cartoon summarizing the strategy. Primary tumor cells were isolated from LT780 and LT773 patients and assessed in a calcein-based cytotoxicity assay against autologous or HD (allogeneic) CIML-NK cells. LT780- (G) and LT773- (H) specific cytotoxicity by autologous (aut. CIML NSCLC, blue) or allogeneic (allo. CIML HD, black) CIML-NK cells at different E:T ratios. Graphs show the mean values of each donor ran in technical duplicates ( $n=3$  HD and 1 NSCLC donors). Groups were compared using a two-way analysis of variance. (\* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ ). CIML, cytokine-induced memory-like; Effector, (E):Target, (T); HD, healthy donors; IFN, interferon; NK, natural killer; NSCLC, non-small cell lung cancer; Peripheral Blood Mononuclear Cell, (PBMC); TriKE, tri-specific cell engager.

IFN- $\gamma$  production and cytotoxicity against SW900 cells (figure 6A–C). NSCLC CIML-NK cells, just like HD CIML, showed a preferential killing of CD133 $^{+}$  SW900 cells, and could respond to 1615133 TriKE by significantly

increasing the killing of CD133 $^{+}$  cells (figure 6D,E). Notably, from two patients with NSCLC we could also obtain primary tumor cells and test them in cytotoxicity assays to functionally assess autologous CIML-NK cells

or allogeneic CIML-NK cells derived from HDs. These experiments showed that NSCLC CIML-NK cells were able to kill autologous tumor targets at levels comparable to those of allogeneic HD CIML (figure 6F–H).

Overall, these data demonstrate that CIML-NK cells with effective therapeutic potential can be derived from patients in different disease stages.

## DISCUSSION

The generation of strengthened immune effector cells, endowed with enhanced specificity, cytotoxicity, and persistency, represents one of the most pursued strategies to define new crucial tools for adoptive cell therapy. A still open issue on such immune cell products regards the heterogeneity of their cellular composition, which may affect their real effectiveness. Chimeric antigen receptor (CAR)-T cells, for instance, can show variable content of CD4, CD8, T regulatory cells (Treg), or exhausted cells. Similarly, CAR-NK, or cytokine-stimulated NK cells can be also characterized by abundance/scarcity of various functionally relevant NK cell subpopulations, which generally reflect different maturation stages and/or capabilities in terms of response readiness and intensity, such as the CD56<sup>bright</sup>, CD56<sup>dim</sup>, NKG2A<sup>+</sup>, KIR<sup>+</sup> cell subsets, or cells that have been “licensed”, or have acquired “adaptive” features. Recent studies have further highlighted the complexity of the NK cell diversity,<sup>35</sup> suggesting how a comprehensive understating of phenotypical, functional, and metabolic features of NK cell subsets is required to develop effective and standardized NK cell products.

CIML-NK cells have been characterized in different studies, and several of their features have been defined in vitro, in animal models and even after their transfer to patients. However, it has not been investigated whether they could be generated by certain “driver” cells.

In the present study, we shed light on the CIML-NK cell subset composition and demonstrate that CIML-differentiation is associated with an up to 20-fold expansion of CD56<sup>bright</sup> cells. Focusing on NSCLC, we also demonstrate that CD56<sup>bright</sup> cells account for most of the CIML-NK antitumor activity, crucially killing CD133<sup>+</sup> CSCs. In line with this finding, CIML-NK cells are shown to limit NSCLC tumorigenicity and pro-metastatic cell dissemination in a PDX model. Remarkably, we also demonstrate that the anti-CSC activity of CIML-NK cells can be maximized by triggering their latent CD56<sup>dim</sup>CD16<sup>+</sup> component with a TriKE engaging CD16 and targeting CD133.

A recent study on inpatient differentiated CIML-NK cells, showed that KIR<sup>+</sup> cells, both licensed and unlicensed, could increase their function on CIML differentiation, specifically acquiring anti-leukemia activity.<sup>36</sup> Moreover, the expression of high NKG2A levels together with CD8 $\alpha$  correlated with minor CIML's therapeutic effects in AML.<sup>15</sup> Our observations indicated that in vitro generated CIML-NK cells were mostly characterized by an NKG2A<sup>+</sup>KIR<sup>-</sup> profile, with the most mature NKG2A<sup>-</sup>KIR<sup>+</sup> population nearly disappearing. Such NKG2A<sup>+</sup> cells,

however, showed an advantage over the KIR<sup>+</sup> cell subset, exhibiting higher proliferative potential (figure 1D and G) and reduced propensity to become exhausted, as witnessed by the poor expression of the TIGIT and TIM-3 exhaustion markers of the CD56<sup>bright</sup> CIML-NK cells.

CIML-NK cells have been recently evaluated in two phase 1 clinical trials of patients with relapsed AML.<sup>15 37</sup> Additionally, recent studies in animal models have also provided evidence that CIML-NK cells can be effective against different solid tumors.<sup>16–19</sup> Here, for the first time, we investigate the potential of CIML-NK cells in contrasting the metastatic spread, a crucial therapeutic concern for many solid tumors. We first provide evidence of the superior responsiveness of CIML-NK cells over NK cells activated by IL-2, which have been standardly used in clinical trials, showing that CIML-NK cells have an increased reactivity against NSCLC cell lines representative of the three main tumor subtypes. Then, we show that CIML-NK cells eliminate CD133<sup>+</sup> cells within the NSCLC aggregates, possibly implying that they could affect circulating tumor cell clusters. Finally, we demonstrate that they could contribute to controlling tumor cell dissemination and metastasis formation in animal models.

The main driver of these functions appears to be the CD56<sup>bright</sup> cell subset which, besides expanding significantly, also acquires enhanced IFN- $\gamma$  production and cytotoxic degranulation against NSCLC targets. The potential antitumor properties of CD56<sup>bright</sup> cells have been highlighted by Wagner *et al*,<sup>38</sup> showing that CD56<sup>bright</sup> cells from both HD and AML or Multiple myeloma (MM) patients could respond to IL-15 or ALT-803, an IL-15 superagonist, by significantly enhancing their reactivity to AML or MM target cells. At variance with such IL-15 stimulation, however, CIML differentiation results in maximal expansion of this rather small CD56<sup>bright</sup> cell population, which reaches levels that can positively impact the effectiveness of CIML-based therapies. In this regard, it should be considered that a certain degree of variability in the levels of the CD56<sup>bright</sup> cell expansion does exist in CIML-NK cells (figure 1B and online supplemental figure S7). Therefore, an investigation of the mechanisms regulating such an expansion during CIML differentiation could be crucial to optimize this effector in clinical settings.

The enhanced function of CD56<sup>bright</sup> CIML-NK cells could be partly explained by their increased expression of granzyme B. Nevertheless, the crucial advantage of these cells in killing preferentially highly tumorigenic cells would rather depend on the combined increased expression of LFA-1 and ICAM-1 on CD56<sup>bright</sup> CIML and CD133<sup>+</sup> cells, respectively. In this regard, the LFA-1/ICAM-1 binding is critical for the “lytic synapse” formation,<sup>39</sup> and increased expression of open-conformation (ie, active) LFA-1 has been shown on CD56<sup>bright</sup> cells on stimulation with IL-15 and interaction with target cells.<sup>38</sup> In addition, ICAM-1 expression and signaling could increase the pro-metastatic properties of circulating tumor cells<sup>40</sup> and support stemness,<sup>41</sup> therefore its expression could be favored in highly tumorigenic cells. Along

these lines, we found an increased ICAM-1 expression on the CD133<sup>+</sup> cell fraction of both PDX and SW900 cells. Moreover, our recent studies indicated ICAM-1 upregulation in the CD133<sup>+</sup> fraction as a common feature of NSCLC cell lines of different tumor subtypes and EMT status.<sup>34</sup>

PDX-derived CD133<sup>+</sup> cells also showed variable downregulation of classical HLA-I molecules, which are recognized by KIRs, and of B7-H3, recognized by a still undefined inhibitory NK receptor. These features, however, could not significantly contribute to the advantage of CD56<sup>bright</sup> CIML-NK cells in killing CD133<sup>+</sup> targets. Indeed, CD56<sup>bright</sup> CIML-NK cells marginally expressed KIRs. In addition, CIML and IL-2-NK cells were similarly inhibited by the expression B7-H3 on the target cells, as demonstrated in cytotoxicity assays against B7-H3<sup>neg</sup> or (transfected) B7-H3<sup>+</sup> target cells (online supplemental figure S22).

CD56<sup>bright</sup> and most CD56<sup>dim</sup> CIML-NK cells express the NKG2A<sup>+</sup>KIR<sup>-</sup> phenotype, which is advantageous to eliminate autologous tumor cells not expressing (or expressing at low levels) the NKG2A-ligand HLA-E. This is a condition that can occur in a variable fraction of different tumor types, including NSCLC.<sup>42–43</sup> Consistent with this observation, the NSCLC cell lines used in our study virtually do not express surface HLA-E. Nevertheless, also depending on the allelic HLA-E variants and on the possible different associated peptides, HLA-E can be expressed in several tumors, with consequent inhibitory effects on NKG2A<sup>+</sup> effector cells.<sup>44</sup> In this case, it should be considered combining CIML-NK cells with NKG2A-blocking molecules, such as monalizumab<sup>42</sup> or cell engagers overcoming inhibitory stimuli.<sup>27</sup> More broadly, possible synergies to optimize CIML activity are just being investigated, even considering CD16-triggering-based strategies.<sup>16–25</sup> In our study we go further, focusing on CSCs, and demonstrating how a specific TriKE could optimize CIML-NK cell activity in this context.

CSCs-targeting represents a longstanding and still missed goal for a real step forward in the cure of many tumors. In this study, by describing the synergy between CIML-NK cells and 1615133 TriKE we offer a new attractive element in the field. On this basis, we also demonstrate that effective CIML-NK cells, synergizing with the TriKE, could be obtained from patients with stage I to III NSCLC, posing the premises for the investigation of their clinical efficacy in preventing or controlling metastatic disease.

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**Competing interests** DAV and MF, and the University of Minnesota, are shared owners of the TriKE technology licensed by the University to GT Biopharma Inc. In addition, MF receives research support, consults for, and holds stock options in GT Biopharma Inc. No GT Biopharma funds were used in the creation of the TriKE molecule used in this study. These interests have been reviewed and managed by the University of Minnesota in accordance with its conflict of interest policy.

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