
This is the **accepted version** of the journal article:

Salvia, Roser [et al.]. «Evaluation of blood cytotoxicity against tumor cells using a live-cell imaging platform». *Cytometry. Part A*, 2025. DOI 10.1002/cyto.a.24930

This version is available at <https://ddd.uab.cat/record/310426>

under the terms of the  ^{IN}
COPYRIGHT license.

Evaluation of blood cytotoxicity against tumor cells using a live-cell imaging platform

Roser Salvia¹, Laura G. Rico¹, Teresa Morán², Michael W. Olszowy³, Michael D. Ward⁴ and Jordi Petriz¹.

¹ Functional Cytomics Lab, Germans Trias i Pujol Research Institute (IGTP), Autonomous University of Barcelona (UAB), Badalona, Spain

² Medical Oncology Department, Catalan Institute of Oncology Badalona (ICO), Germans Trias i Pujol University Hospital (HUGTiP), Badalona, Spain; Applied Research Group in Oncology, Germans Trias i Pujol Research Institute (IGTP) Badalona, Barcelona, Spain; Department of Medicine, Autonomous University of Barcelona, Cerdanyola del Vallès, Spain

³ Sartorius Stedim North America, inc., Arvada, Colorado, USA

⁴ Thermo Fisher Scientific, Fort Collins, Colorado, USA

Correspondence

Jordi Petriz, PhD. jpetriz@igtp.cat

Abstract

Cellular cytotoxicity is an important mechanism of the immune system to clear infections and eliminate tumor cells. Its two main mediators are cytotoxic T lymphocytes and natural killer (NK) cells. In lung cancer, intratumoral NK cells show reduced cytolytic potential and one third of patients do not express HLA-I proteins, which activate NK cells in a process termed "absent self-recognition". In this work, we investigate NK cytotoxicity as a potential oncological biomarker that informs patient status and predicts response to treatment. We describe a simple and rapid test to analyze NK cytotoxicity without the need for large volumes of blood, requiring short processing time and reduced use of both reagents and blood samples, using the IncuCyte live imaging technique.

Keywords

Blood cytotoxicity; Live-cell imaging; Cancer

Data Availability

The data that support the findings of this study are available on request from the corresponding author [JP].

1. Introduction

Cellular cytotoxicity is an important mechanism of the immune system to resolve infections and cancer by killing compromised cells. The two major mediators of cellular cytotoxicity are cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. For their potent antitumor defense, these lymphocytes release perforins to open pores in the cell membrane and granzymes that enter the cell to activate caspase-dependent apoptosis [1], resulting in target cell cytolysis. Another mechanism of cytotoxicity described in NK cells and CTL is related to death receptors of the TNFR superfamily. TNF-related apoptosis inducing ligand (TRAIL) and CD95L (Fas ligand) signaling induce apoptosis in infected or tumor cells [2–4].

In lung cancer, the infiltration of T lymphocytes correlates with a good prognosis [5]. However, one third of these patients do not express HLA-I proteins due to the impaired expression of HLA-I maturation proteins or β 2-microglobulin, allowing the tumor to evade the immunosurveillance. This fact is also associated with decreased CTL infiltration and reduced levels of PD-L1 [6]. Fortunately, the absence of HLA-I proteins activates NK cells in a process called ‘missing-self recognition’ [7]. Furthermore, in the setting of lung cancer, intratumoral NK cells show reduced cytolytic potential with decreased degranulation and INF γ production [8]. Overall, the determination of NK cytotoxicity may be a good oncological biomarker to know the patient status and predict the response to treatment.

The gold standard technique for assessing cytotoxicity is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) assay [9]. The colorimetric-based result is obtained from intracellular MTT reduction into formazan in viable cells and allows the measurement of viability and, indirectly, cytotoxicity. The MTT test has several pitfalls, such as cell toxicity or death induced by MTT itself, media interference in reducing potential, specific cell rate of MTT uptake and formazan extrusion, incubation time, among others [10]. In addition to this gold standard technique, many other cytotoxicity assays have been described, such as the ^{51}Cr release assay or flow cytometry assays. A very interesting alternative is the fluorescence detection of a co-culture with an automated live cell analysis because of its ability to obtain cell culture images over time. The IncuCyte[®] Live-Cell Analysis System (Sartorius) is an automated cell microscope placed in a cell culture incubator that provides high quality fluorescence images and powerful analysis software. By co-culturing fluorescent target cells with effector cells, the IncuCyte system is able to accurately generate a growth curve of the target cells and calculate the cytotoxicity caused by the effector cells. The IncuCyte platform is a well-established method for cell cytotoxicity, proliferation and apoptosis [11–13], and in the work of Cichocki *et al.*, the authors analyze the cytotoxicity of induced NK cells against a K562 cell line with a NuLight Red transduction [12].

Considering the lack of HLA-I in some lung cancer tumors and the importance of NK cells in fighting cancer cells, in this work we have chosen the EGFP-K562 cell line as target cells because of its lack of HLA-I complex and its green fluorescent reporting. Thus, this method is independent of patient tumor biopsies, which in lung cancer are very small, invasive and paraffin-embedded.

Immunotherapies are dependent on the intrinsic cytotoxicity of the immune compartment, and in this work we have hypothesized that greater cytotoxicity is a good prognostic factor for overall survival in non-small cell lung cancer (NSCLC) patients.

2. Methods

2.1 Blood

Peripheral blood was collected in EDTA-anticoagulated tubes from $n = 27$ NSCLC patients and $n = 5$ controls. All patients enrolled in this study provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board (IRB) of our institution (study code: RegTumTor2014). All procedures related to sample processing were in accordance with the internal protocols of our laboratory at IGTP, approved by HUGTiP-IRB in accordance with current Spanish legislation.

2.2 Cell culture

The EGFP-K562 cell line is an erythroleukemia derived from chronic myeloid leukemia in terminal blast crisis. It is transformed to constitutively express enhanced green fluorescent protein (EGFP). The EGFP-K562 cell line was kindly provided by Jordi Barquinero of the Vall d'Hebron Research Institute. EGFP-K562 cells were cultured in RPMI 1640 medium (Biowest) supplemented with 10% heat-inactivated fetal bovine serum (Capricorn), 4 mM L-glutamine (Gibco), 500 IU/mL penicillin/streptomycin (PAA Laboratories), and 1 mM sodium pyruvate (Biowest) at 37°C in 5% CO₂. Cells were subcultured three times per week.

2.3 IncuCyte Live-Cell Analysis System

The IncuCyte microscope (Sartorius, USA) contains an SX5 green, orange, and NIR optical module with the 2023A Rev2 controller and graphical user interface. It is placed in a cell culture incubator at 37°C/5% CO₂.

2.4 Cytotoxicity assay: Co-culture of blood with EGFP-K562 cells

EGFP-K562 cells are centrifuged at 400g for 5 minutes and resuspended in RPMI 1640 medium supplemented with 20% FBS, 4 mM L-glutamine, 500 IU/mL penicillin/streptomycin and 1 mM sodium pyruvate. After counting in a flow cytometer, 1.2×10^4 cells were diluted in 900 μ l of medium and seeded into all 24 wells of an anti-adhesive flat-bottomed plate (Nunc, Thermo Fisher) and maintained in a 37°C incubator with 5% CO₂.

Peripheral blood is first lysed with 1X sterile ammonium chloride in a 1:10 ratio to eliminate erythrocytes. Thus, 4 ml of blood is lysed in 44 ml NH₄Cl for 10 min with gently stirring. Centrifuge at 400g for 5 minutes and wash the pellet in sterile PBS. The pellet is then resuspended in RPMI 1640 20% FBS and analyzed in the flow cytometer to determine the lymphocyte concentration of the lysed blood.

Finally, blood (effector cells) is co-cultured with different ratios of EGFP-K562 cells (target cells) by adding 100 μ l of the given concentration to each well. The first row of 6

wells is the control condition, seeded with 1.2×10^4 target cells only. The second row is a 1:1 co-culture, seeded with 1.2×10^4 effector cells and 1.2×10^4 target cells. The third row is 5:1, seeding 6×10^4 effector cells, while the last row is 15:1, seeding 1.8×10^5 effector cells.

The plate is transferred to the IncuCyte microscope which is placed in a cell incubator at $37^\circ\text{C}/5\%\text{CO}_2$ until the end of the assay. The green and phase channels are selected and the green channel acquisition time is set to 100 ms. Using the 10x objective, 9 images per well are acquired every 1 hour for 4 days.

Flow cytometry

For cell counting, 50 μl of cell culture is diluted in 450 μl Hanks' balanced salt solution (HBSS, Capricorn) and stained with 1 μl DAPI (1 mg/ml stock solution). This was immediately collected in the Attune NxT flow cytometer (Thermo Fisher) to obtain the concentration of the cell culture. Doublets and necrotic cells (DAPI+ cells) were excluded, as well as cells with loss of EGFP fluorescence. In the case of lysed blood, an additional region is drawn in the FSC vs. SSC plot to select the lymphocyte population.

Analysis of data

Data obtained in the IncuCyte is analyzed to automatically select green cells using the IncuCyte software. The background noise of the green channel is subtracted using the top hat correction option with a radius of 100 μm and a threshold of 2. The edge split is configured with a sensitivity of 5 to better characterize fluorescent cells. A mean intensity cutoff of 3-4 is set to discriminate low or non-specific green fluorescence. The green area value is then calculated from the 9 images obtained for each well. Tumor growth curves are based on the green area of each row and are generated in GraphPad Prism. The percentage of cytotoxicity is calculated as the inverse of the normalization of cell growth to the control condition according to Equation 1.

$$\text{Percentage of cytotoxicity} = \left[1 - \left(\frac{\text{Co-culture cell growth}}{\text{Control cell growth}} \right) \right] \times 100 \quad \text{Equation 1}$$

where co-culture cell growth refers to 1:1, 5:1 or 15:1 conditions and control refers to EGFP-K562 cell culture.

3. Results and Discussion

3.1 Co-culture setting

We selected three different ratios of lymphocytes to tumor cells to represent different scenarios of tumor burden. In addition, a control condition is performed to normalize the intrinsic cell growth of the tumor cells. In this way, we have cultured 4 conditions: EGFP-K562 alone, 1:1, 5:1 and 15:1 (ratio of lymphocytes to EGFP-K562), where 1:1 represents an increased tumor burden and 15:1 represents a decreased tumor burden.

Next, to determine the appropriate duration of culture, a thirteen-day culture was performed to analyze tumor cell growth over time. Figure 1 shows the growth of the tumor cells for the 4 conditions mentioned above. In the case of the tumor cell culture alone, its growth represents the average of six wells and follows the characteristic logarithmic expansion. On the other hand, when the cells are co-cultured with lymphocytes, the growth of the target cells is reduced, especially as the lymphocyte ratio increases. The cytotoxicity is then calculated based on equation 1. Since the curve of the cytotoxicity plot tends to stabilize at 96h, we have considered that a four-day culture is sufficient to visualize the cytotoxicity effect in this setting.

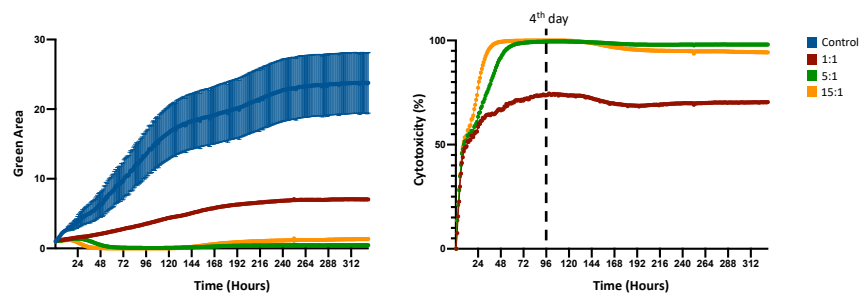


Figure 1. Lung cancer patient co-culture and derived cytotoxicity over 13 days. Control of tumor cell growth (blue), 1:1 (red), 5:1 (green), 15:1 (yellow) ratio of blood lymphocytes to EGFP-K562 tumor cells are shown, each condition is the average of $n=6$ wells. On the left, the green area is calculated from the 9 images taken in each individual well ($n=6$ wells/condition) and is representative of tumor cell growth. Values are normalized to the first time point and standard errors are shown. In the right graph, the percentage of cytotoxicity is calculated from the normalization to control growth and is therefore an indirect cytotoxicity. Day 4 is marked on the graph because it is the selected endpoint of the assay.

In total, we performed the cytotoxicity assay in 27 NSCLC patients and 5 controls. Images of the co-cultures on the IncuCyte microscope show that EGFP-K562 grow in clusters and that lymphocytes localize around these clusters (Figure 2A). More importantly, the blood effector cells induce EGFP-K562 cell death as indicated by the loss of green fluorescence (Figure 2B).

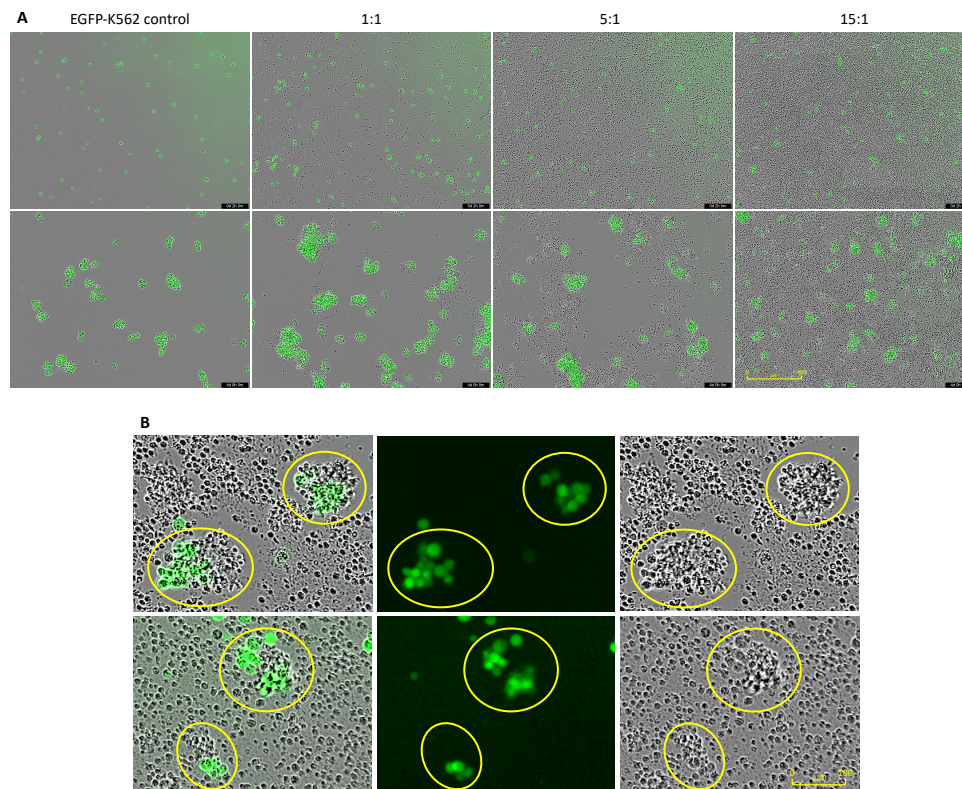


Figure 2. IncuCyte images of the different co-culture conditions at baseline and endpoint. The tumor cell line expresses green fluorescence while blood is visualized in the contrast phase. **A)** The top row corresponds to the second hour of culture and the bottom row corresponds to the fourth day endpoint of the same well. Columns represent control, 1:1, 5:1 and 15:1 ratio (lymphocytes to EGFP-K562). **B)** Enlarged images of 5:1 (top) and 15:1 (bottom) co-cultures displaying the green fluorescence and phase contrast in combination and separately, where tumor cell death is indicated by the loss of green fluorescence (yellow circles).

3.2 Cell cytotoxicity as a putative biomarker of disease progression

Current cytotoxicity assays usually isolate peripheral blood mononuclear cells by density gradient centrifugation. This step can cause cellular depletion in a biased manner depending on the specific cell liability and also cause cell dysfunction, which is critical as we want to preserve cell functionality [14]. These limitations may result in erroneous measurements, especially when very few cytotoxic cells are present or when sample size is very small (e.g., pediatric samples). Red cell lysing procedures can similarly cause depletion and dysfunction and should be avoided when possible to circumvent their potential effect on cell cytotoxicity. We have observed depletion of up to nearly 80% of leukocytes by comparing lysed and unlysed specimens [14].

Also, human eosinophils are more toxic than neutrophils in antibody-independent killing [15]. Thus, although neutrophilic and eosinophilic cytotoxicity cannot be ruled out, most of this cytotoxic activity should be originated in natural killer cells, as they are specialized in recognizing and killing tumor cells without prior sensitization. Likewise, cytotoxic T lymphocytes recognize and kill cells that present specific antigens (usually peptides

derived from pathogens or tumor-specific proteins) on their surface [16]. CTLs are crucial for controlling viral infections and tumor growth, but in this case cytotoxicity of CTLs can be ruled out, since they are HLA-I dependent and K562 cells do not present this complex. Importantly, cytotoxic activity is not limited to immune cells.

Obviously, there is an intrinsic variability of blood cytotoxic activity among control subjects due to a given unreported immune response. However, lung cancer patients show an increased variability of this blood cytotoxic potential, with patients showing very low activity (Figure 3).

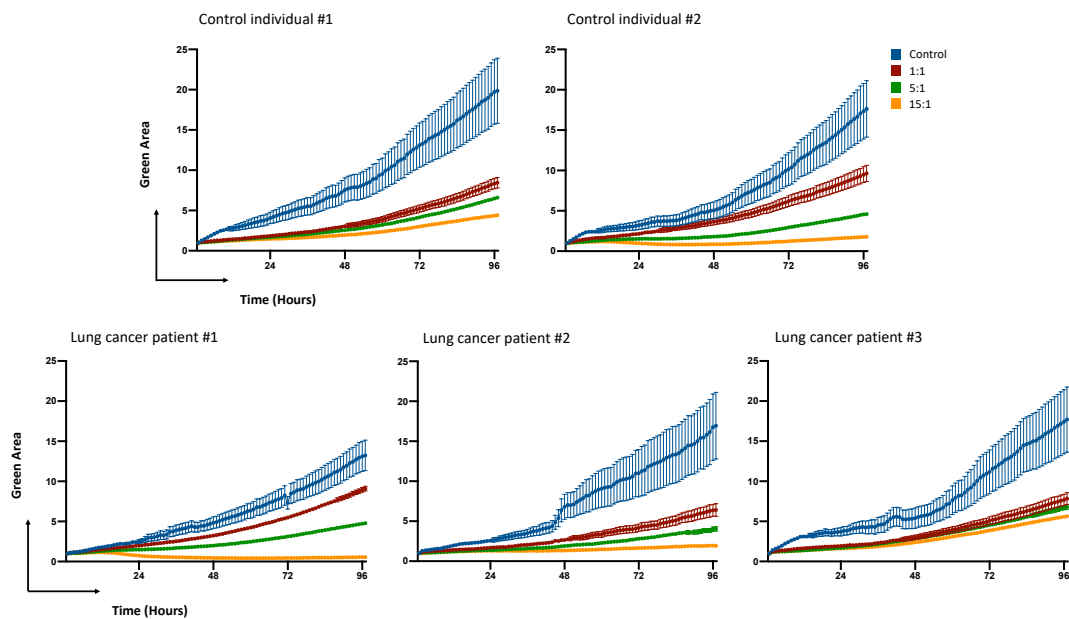


Figure 3. Control subjects and lung cancer patients show different cytotoxicity patterns. The control refers to EGFP-K562 green tumor cells cultured alone, and the ratios 1:1, 5:1, 15:1 refer to the ratio of blood lymphocytes to EGFP tumor cells. Values are normalized to the first time point and standard errors are presented.

Clinical outcome of lung cancer patients shown: LC#1 died because of disease progression, after receiving two cycles of pembrolizumab as first-line treatment. LC#2 was treated with 6 cycles of pembrolizumab as first-line combined with chemotherapy and died due to septic shock after a pneumonia. LC#3 was treated with 15 cycles of pembrolizumab as first-line combined with chemotherapy and did not progress nor die.

As expected, there is a strong relationship between cytotoxicity and the treatment received, as the patients who have not received any treatment, called naïve, have a better cytotoxicity ability compared to the patients who have received surgery or chemotherapy (Figure 4). Therefore, many naïve patients achieve 50% cytotoxicity (50%Cyt), whereas almost none of the treated patients achieve 50%Cyt. It is noteworthy that 67% of patients who progress do not reach 50%Cyt and only 8.3% do so within the first two days. On the other hand, the 53.3% of patients with better outcome who suffer disease progression cannot reach the 50%Cyt while the 33.3% have a cytotoxicity higher than 50%Cyt within the first two days (Figure 5 & 6 and Table 1). Additionally, the types of histology and staging at diagnosis do not have a relationship with the time to reach

50% of cytotoxicity at 1:1 ratio, according to Pearson’s chi-squared test (p-value = 0.7114 and 0.4274 respectively).

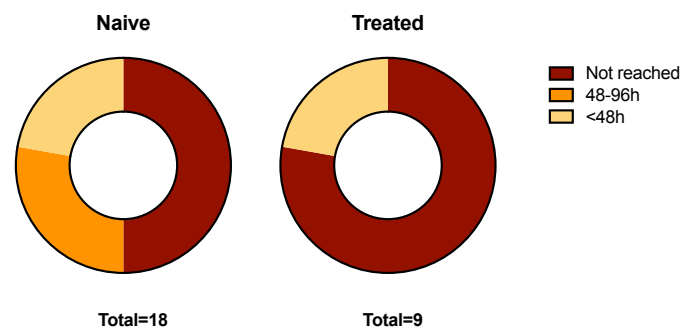


Figure 4. Time to 50% cytotoxicity according to treatment. Patients receiving first-line treatment, such as ICI alone or ICI in combination with chemotherapy, are considered treatment-naïve (n=18). Patients who received a previous treatment, such as second-line ICI, consolidation ICI, or adjuvant ICI, were considered treated (n=9). Cell cytotoxicity of 1:1 lymphocytes to tumor cells is considered.

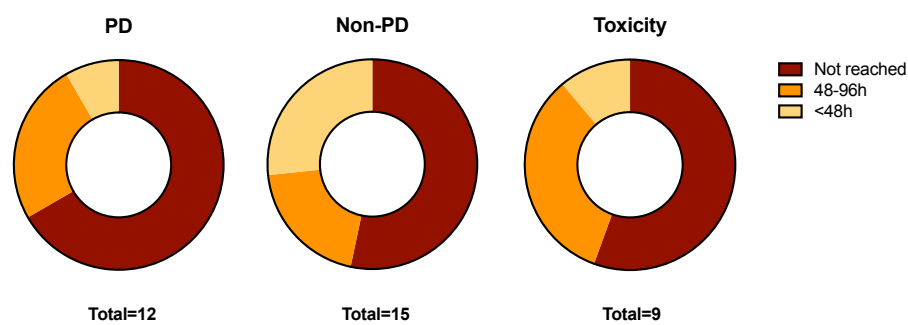


Figure 5. Time upon reaching 50% cytotoxicity, according to clinical outcome. Patients with progression of disease (PD, n=12) and without disease progression (Non-PD, n=15). Also, from those 27 patients, 9 demonstrated toxicity to the treatment. Cell cytotoxicity from 1:1 lymphocytes to tumor cells is considered.

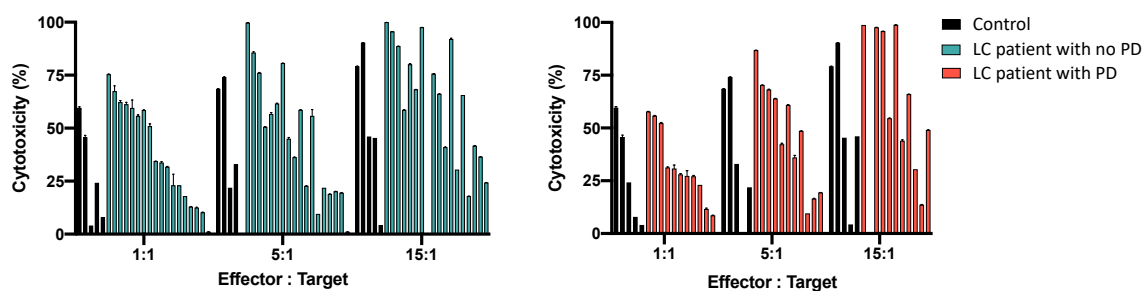


Figure 6. Cell cytotoxicity at baseline and disease progression. The percentage of cytotoxicity is shown for each NSCLC patient along the 1:1, 5:1, and 15:1 ratios (lymphocytes to tumor cells). Controls (n=5) are shown in black, patients without disease progression (n=15) are shown in green, and patients with disease progression (n=12) are shown in red.

Table 1. Time to reach 50% cytotoxicity according to clinical outcome and treatment.

Sample	Hours to 50% cytotoxicity	Disease progression	Toxicity	Treatment	Histology	Diagnostic stage
LC74	83	Yes	Yes	Naïve	Adenocarcinoma	IV
LC76	NR	Yes	No	Treated	Adenocarcinoma	IIIB
LC79	NR	Yes	Yes	Naïve	Adenocarcinoma	IV
LC80	NR	Yes	Yes	Treated	Squamous cell c.	IIIB
LC82	NR	Yes	Yes	Treated	Adenocarcinoma	IVC
LC83	NR	No	No	Treated	Adenocarcinoma	IIIA
LC86	NR	Yes	No	Naïve	Adenocarcinoma	IVC
LC87	NR	No	No	Naïve	Adenocarcinoma	IIA
LC88	NR	Yes	No	Naïve	Squamous cell c.	IVC
LC89	94	No	Yes	Naïve	Squamous cell c.	IVC
LC90	NR	No	No	Naïve	Adenocarcinoma	IVC
LC92	58	Yes	No	Naïve	Squamous cell c.	IV
LC93	NR	No	No	Treated	Squamous cell carcinoma	IIB
LC95	53	No	Yes	Naïve	Squamous cell c.	IVC
LC96	60	No	No	Naïve	Adenocarcinoma	IV
LC98	56	Yes	No	Naïve	Adenocarcinoma	IIIA
LC99	31	No	No	Treated	Squamous cell c.	IIA
LC100	45	No	No	Naïve	Adenocarcinoma	IIB
LC101	27	No	No	Naïve	Squamous cell c.	IIIB
LC102	14	No	Yes	Naïve	Squamous cell c.	IIIB
LC103	11	Yes	No	Naïve	Adenocarcinoma	IV
LC106	NR	Yes	Yes	Naïve	Adenocarcinoma	IB
LC109	NR	No	Yes	Treated	Adenocarcinoma	IIIB
LC110	NR	Yes	No	Naïve	Squamous cell c.	IIIA
LC113	NR	No	No	Treated	Adenocarcinoma	IV
LC115	NR	No	No	Naïve	Adenocarcinoma	IV
LC117	NR	No	No	Treated	Adenocarcinoma	IIIA

Note: Those patients that have not reached the 50% of cytotoxicity are shown as NR (not reached). Patients receiving first-line treatment, such as ICI alone or ICI in combination with chemotherapy, are considered treatment-naïve. Patients who received a previous treatment, such as second-line ICI, consolidation ICI, or adjuvant ICI, were considered treated.

This rapid and HLA haplotype-independent assay allows the measurement of cytotoxic activity in the blood while correlating it with different tumor burdens. In this case, the samples correspond to NSCLC patients at baseline, before receiving anti-PD-(L)1 ICI, although any other malignancy can be assessed for cytotoxicity. By correlating the clinical outcome of the patients with the cytotoxic potential at baseline, we could observe that those patients who did not experience disease progression could reach 50% cytotoxicity earlier than those patients who eventually progressed. However, the 1:1 (effector:target) cytotoxicity has an accuracy of 48%, and we would need to increase the number of patients in order to draw significant conclusions.

It is important to note that the tumor burden of the co-culture does influence the number of patients reaching 50% cytotoxicity (Table 2). A high tumor burden significantly hinders the immune system's ability to eliminate the malignant cells. This effect is also influenced by the individual immunological status of each patient. The sheer volume of cancer cells can overwhelm and suppress cytotoxic T cells. Cancer cells can also develop evasion mechanisms, such as expressing inhibitory proteins or creating protective microenvironments, to avoid immune attack. Furthermore, they may release factors that directly impair the function of cytotoxic T cells, diminishing their cancer-killing capacity and promoting tumor growth. However, the effectiveness of these evasion tactics and the overall impact of tumor burden on cytotoxicity can vary considerably depending on the individual's immune competence. A patient with a robust immune system may be better equipped to combat the suppressive effects of a high tumor burden compared to a patient with a weakened or compromised immune system.

Table 2. Percentages of cell cytotoxicity on day 4 of co-culture. The percentage of cytotoxicity is shown for each control and NSCLC patient along the 1:1, 5:1, and 15:1 ratios (lymphocytes to tumor cells), representing a decreasing tumor burden.

Sample	Disease	1:1	1:5	1:15	Disease progression
CTRL07	Control	3.92	21.82	45.98	-
CTRL09	Control	59.50	68.44	79.16	-
CTRL10	Control	45.59	74.06	90.28	-
CTRL13	Control	24.13	32.85	45.28	-
CTRL14	Control	7.85	-28.13	4.17	-
LC74	NSCLC	55.70	70.26	-	Yes
LC76	NSCLC	11.58	16.45	13.55	Yes
LC79	NSCLC	27.07	48.51	65.94	Yes
LC80	NSCLC	30.70	42.22	54.52	Yes
LC82	NSCLC	27.18	35.95	43.89	Yes
LC83	NSCLC	31.72	22.67	40.99	No
LC86	NSCLC	8.53	19.34	48.92	Yes
LC87	NSCLC	22.98	55.74	91.94	No
LC88	NSCLC	31.23	63.88	95.81	Yes
LC89	NSCLC	50.86	44.99	-	No
LC90	NSCLC	34.34	36.22	75.60	No
LC92	NSCLC	52.18	68.15	97.65	Yes
LC93	NSCLC	33.72	58.58	66.09	No
LC95	NSCLC	59.44	56.62	80.04	No
LC96	NSCLC	61.22	50.54	58.57	No
LC98	NSCLC	57.73	86.75	98.68	Yes
LC99	NSCLC	67.36	85.63	95.50	No
LC100	NSCLC	62.21	76.05	88.59	No
LC101	NSCLC	55.72	61.55	68.17	No
LC102	NSCLC	58.45	80.60	97.51	No

LC103	NSCLC	75.37	99.69	99.99	Yes
LC106	NSCLC	27.93	60.82	98.82	Yes
LC109	NSCLC	10.23	19.48	36.49	No
LC110	NSCLC	12.91	18.88	17.90	Yes
LC113	NSCLC	12.48	20.13	41.64	No
LC115	NSCLC	-4.35	-0.54	24.28	No
LC117	NSCLC	22.98	9.43	30.38	No

In some cancers, a low level of natural cytotoxicity may be the norm. This may be due to factors such as immune evasion by the tumor, inherent weakness of the immune system, or other disease-specific mechanisms. In such cases, disease progression may depend more on other factors (tumor aggressiveness, treatment response, etc.) than on the level of cytotoxicity itself. In some cases, a high level of cytotoxicity may be a sign that the immune system is actively fighting the cancer, but the cancer is also aggressive and progressing despite the immune response. This could be due to immune exhaustion and tumor escape. It's possible that there is a threshold of cytotoxicity needed to effectively control the cancer. Below that threshold, even if there's some immune activity, it's not enough to prevent progression. Above the threshold, the immune system may be more likely to keep the cancer in check, but if the cancer is aggressive, it may still progress despite the higher cytotoxicity. With fewer cancer cells, the immune system has a better chance of keeping the cancer in check. There are simply fewer cells for cytotoxic T cells to target and destroy. Lower tumor burden can lead to less immune suppression. Cancer cells often release factors that dampen the immune response. With fewer cells, this suppression is reduced, giving the immune system a better chance to function effectively.

This personalized methodology is a simple and rapid test to analyze blood cytotoxicity without the need for large volumes of blood, requiring short processing time and reduced use of both reagents and blood samples, which stands as a powerful and complementary technique with great potential in personalized medicine.

Author contributions

Roser Salvia: Investigation; writing – original draft; writing – review and editing; methodology; validation; visualization; software; formal analysis; data curation. **Laura G. Rico:** Investigation; writing – review and editing; visualization. **Teresa Morán:** Writing – review and editing; funding acquisition; supervision; resources. **Michael W. Olszowy:** Writing – review and editing; resources. **Michael D. Ward:** Writing – review and editing; resources. **Jordi Petríz:** Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; writing – review and editing; software; formal analysis; project administration; data curation; supervision; resources.

Acknowledgments

This study was supported by Sartorius Stedim North America, Inc. and Thermo Fisher Scientific. We thank the CERCA Program/Generalitat de Catalunya and the Germans Trias i Pujol Research Foundation for institutional support and acknowledge the financial support of the Obra Social la Caixa. This work was also supported by the positive discussion of the Agency for the Management of University and Research Grants (AGAUR, Generalitat de Catalunya), Catalan Research Group (SGR) 2021 SGR 00002.

Disclosure

M.D.W. works for Thermo Fisher Scientific, which is in the business of selling flow cytometers and flow cytometry reagents. M.W.O. works for Sartorius Stedim North America, Inc., which is in the business of selling live cell analyzers and reagents. The remaining authors declare no potential conflicts of interest.

References

- [1] J.A. Trapani, P. Jans, M.J. Smyth, C.J. Froelich, E.A. Williams, V.R. Sutton, D.A. Jans, Perforin-dependent nuclear entry of granzyme B precedes apoptosis, and is not a consequence of nuclear membrane dysfunction, *Cell Death Differ* 5 (1998) 488–496. <https://doi.org/10.1038/sj.cdd.4400373>.
- [2] I. Prager, C. Watzl, Mechanisms of natural killer cell-mediated cellular cytotoxicity, *J Leukoc Biol* 105 (2019) 1319–1329. <https://doi.org/10.1002/JLB.MR0718-269R>.
- [3] A. Zeytun, M. Hassuneh, M. Nagarkatti, P.S. Nagarkatti, Fas-Fas ligand-based interactions between tumor cells and tumor-specific cytotoxic T lymphocytes: A lethal two-way street, *Blood* 90 (1997) 1952–1959. <https://doi.org/10.1182/blood.v90.5.1952>.
- [4] W.D. Thomas, P. Hersey, TNF-Related Apoptosis-Inducing Ligand (TRAIL) Induces Apoptosis in Fas Ligand-Resistant Melanoma Cells and Mediates CD4 T Cell Killing of Target Cells, *The Journal of Immunology* 161 (1998) 2195–2200. <https://doi.org/10.4049/jimmunol.161.5.2195>.
- [5] F. Djenidi, J. Adam, A. Goubar, A. Durgeau, G. Meurice, V. de Montpréville, P. Validire, B. Besse, F. Mami-Chouaib, CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients, *J Immunol* 194 (2015) 3475–3486. <https://doi.org/10.4049/JIMMUNOL.1402711>.
- [6] C. Pereira, P. Gimenez-Xavier, E. Pros, M.J. Pajares, M. Moro, A. Gomez, A. Navarro, E. Condom, S. Moran, G. Gomez-Lopez, O. Graña, M. Rubio-Camarillo, A. Martinez-Martí, J. Yokota, J. Carretero, J.M. Galbis, E. Nadal, D. Pisano, G. Sozzi, E. Felip, L.M. Montuenga, L. Roz, A. Villanueva, M. Sanchez-Cespedes, Genomic profiling of patient-derived xenografts for lung cancer identifies B2M inactivation impairing immunorecognition, *Clinical Cancer Research* 23 (2017) 3203–3213. <https://doi.org/10.1158/1078-0432.CCR-16-1946/439396/AM/GENOMIC-PROFILING-OF-PATIENT-DERIVED-XENOGRAFTS>.
- [7] S. Kim, J. Poursine-Laurent, S.M. Truscott, L. Lybarger, Y.J. Song, L. Yang, A.R. French, J.B. Sunwoo, S. Lemieux, T.H. Hansen, W.M. Yokoyama, Licensing of natural killer cells by host major histocompatibility complex class I molecules, *Nature* 436 (2005) 709–713. <https://doi.org/10.1038/NATURE03847>.
- [8] S. Platonova, J. Cherfils-Vicini, D. Damotte, L. Crozet, V. Vieillard, P. Validire, P. André, M.C. Dieu-Nosjean, M. Alifano, J.F. Régnard, W.H. Fridman, C. Sautès-Fridman, I. Cremer, Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma, *Cancer Res* 71 (2011) 5412–5422. <https://doi.org/10.1158/0008-5472.CAN-10-4179/649842/AM/PROFOUND-COORDINATED-ALTERATIONS-OF-INTRATUMORAL>.
- [9] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J Immunol Methods* 65 (1983) 55–63. [https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4).
- [10] M. Ghasemi, T. Turnbull, S. Sebastian, I. Kempson, The MTT assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis, *Int J Mol Sci* 22 (2021) 12827. <https://doi.org/10.3390/IJMS222312827/S1>.
- [11] A. Single, H. Beetham, B.J. Telford, P. Guilford, A. Chen, A comparison of real-time and endpoint cell viability assays for improved synthetic lethal drug validation, *J*

- Biomol Screen 20 (2015) 1286–1293.
<https://doi.org/10.1177/1087057115605765>.
- [12] F. Cichocki, R. Bjordahl, S. Gaidarova, S. Mahmood, R. Abujarour, H. Wang, K. Tuininga, M. Felices, Z.B. Davis, L. Bendzick, R. Clarke, L. Stokely, P. Rogers, M. Ge, M. Robinson, B. Rezner, D.L. Robbins, T.T. Lee, D.S. Kaufman, B.R. Blazar, B. Valamehr, J.S. Miller, iPSC-derived NK cells maintain high cytotoxicity and enhance in vivo tumor control in concert with T cells and anti-PD-1 therapy, *Sci Transl Med* 12 (2020) eaaz5618.
<https://doi.org/10.1126/SCITRANSLMED.AAZ5618>.
- [13] B.H. Goldenson, H. Zhu, Y.Z.M. Wang, N. Heragu, D. Bernareggi, A. Ruiz-Cisneros, A. Bahena, E.H. Ask, H.J. Hoel, K.J. Malmberg, D.S. Kaufman, Umbilical Cord Blood and iPSC-Derived Natural Killer Cells Demonstrate Key Differences in Cytotoxic Activity and KIR Profiles, *Front Immunol* 11 (2020) 561553.
<https://doi.org/10.3389/FIMMU.2020.561553/BIBTEX>.
- [14] L.G. Rico, R. Salvia, J. Juncà, M.D. Ward, J.A. Bradford, M. Sorigue, J. Petriz, Erythrocyte lysing solutions have a detrimental effect in flow cytometric dendritic cell detection, *Cytometry Part A* 103 (2023) 383–391.
<https://doi.org/10.1002/cyto.a.24696>.
- [15] R.L. Roberts, B.J. Ank, E.R. Stiehm, Human eosinophils are more toxic than neutrophils in antibody-independent killing, *Journal of Allergy and Clinical Immunology* 87 (1991) 1105–1115. [https://doi.org/10.1016/0091-6749\(91\)92156-U](https://doi.org/10.1016/0091-6749(91)92156-U).
- [16] T.M. Holling, E. Schooten, P.J. Van Den Elsen, Function and regulation of MHC class II molecules in T-lymphocytes: of mice and men, *Hum Immunol* 65 (2004) 282–290. <https://doi.org/10.1016/J.HUMIMM.2004.01.005>.