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American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
bloodadvances@hematology.org

Double-negative T cells utilize a TNFα-JAK1-ICAM-1 cytotoxic axis against acute myeloid leukemia

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Enoch Tin (University Health Network, Canada) JongBok Lee (University of Calgary, Canada) Ismat Khatri (University Health Network, Canada) Yoosu Na (University Health Network, Canada) Mark Minden (Princess Margaret Cancer Centre, Canada) Li Zhang (Toronto General Research Institute, Canada)

Abstract:
Allogeneic double-negative T cells (DNTs) are a rare T cell subset that effectively target acute myeloid leukemia (AML) without inducing graft-vs-host disease in an allogeneic setting. A phase I clinical trial demonstrated the feasibility, safety, and potential efficacy of allogeneic DNT therapy among patients with relapsed AML. However, the molecular mechanisms of DNT-mediated cytotoxicity against AML remain elusive. Thus, we utilized a flow cytometry-based high throughput screening to compare the surface molecule expression profile on DNTs during their interaction with DNT-susceptible or -resistant AML cells and identified a TNFα-dependent cytotoxic pathway in DNT-AML interaction. TNFα secreted by DNTs, upon encountering susceptible AML targets, sensitized AML cells to DNT-mediated killing, including those otherwise resistant to DNTs. Mechanistically, TNFα upregulated ICAM-1 on AML cells through a noncanonical JAK1-dependent pathway. DNTs then engaged with AML cells more effectively through an ICAM-1 receptor, LFA-1, leading to enhanced killing. These results reveal a TNFα-JAK1-ICAM-1 axis in DNT-mediated cytotoxicity against AML to improve therapeutic efficacy.

Conflict of interest: COI declared - see note

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Double-negative T cells utilize a TNFα-JAK1-ICAM-1 cytotoxic axis against acute myeloid leukemia

Running title: DNTs use a TNFα-based mechanism to kill AML

Authors and affiliations: Enoch Tin[1,2], Jong Bok Lee[3], Ismat Khatri[1], Yoosu Na[1], Mark D. Minden[4] and Li Zhang[1,2,5*]

[1]Toronto General Hospital Research Institute, University Health Network, Toronto, Ontario, Canada.

[2]Department of Immunology, University of Toronto, Toronto, Ontario, Canada.

[3]Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada.

[4]Princess Margaret Cancer Centre, University Health Network, Toronto, Ontario, Canada.

[5]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada.

Corresponding author:

Li Zhang
Toronto General Hospital Research Institute, University Health Network
101 College St., room 2-207, Toronto, Ontario, M5G 1L7
T. (416) 581-7521 / F. (416) 581-7515
ORCID: https://orcid.org/0000-0003-4649-0757
Email: Li.Zhang@uhnresearch.ca

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Abstract

Allogeneic double-negative T cells (DNTs) are a rare T cell subset that effectively target acute myeloid leukemia (AML) without inducing graft-vs-host disease in an allogeneic setting. A phase I clinical trial demonstrated the feasibility, safety, and potential efficacy of allogeneic DNT therapy among patients with relapsed AML. However, the molecular mechanisms of DNT-mediated cytotoxicity against AML remain elusive. Thus, we utilized a flow cytometry-based high throughput screening to compare the surface molecule expression profile on DNTs during their interaction with DNT-susceptible or -resistant AML cells and identified a TNFα-dependent cytotoxic pathway in DNT-AML interaction. TNFα secreted by DNTs, upon encountering susceptible AML targets, sensitized AML cells to DNT-mediated killing, including those otherwise resistant to DNTs. Mechanistically, TNFα upregulated ICAM-1 on AML cells through a noncanonical JAK1-dependent pathway. DNTs then engaged with AML cells more effectively through an ICAM-1 receptor, LFA-1, leading to enhanced killing. These results reveal a TNFα-JAK1-ICAM-1 axis in DNT-mediated cytotoxicity against AML to improve therapeutic efficacy.

Key points

- Upon engaging DNT-susceptible AML, DNTs secrete TNFα which sensitizes AML to DNT killing, including DNT-resistant AML
- TNFα noncanonically signals through JAK1 to upregulate ICAM-1 on AML for increased DNT anti-leukemic function

Introduction

Acute myeloid leukemia (AML) is the most common form of adult acute leukemia with poor long-term survival due to high disease relapse rates following induction chemotherapy.[1–4] Currently, allogeneic hematopoietic stem cell transplantation (allo-HSCT) provides a potential curative treatment option for high-risk AML patients by eliciting a graft-vs-leukemia (GvL) response, where donor-derived immune cells target residual AML cells.[3,5] However, treatment-associated toxicities such as graft-vs-host disease (GvHD), risks of infection, and significant relapse rates pose a challenge.[3] Nevertheless, the potency of the GvL effect has driven the development of adoptive cellular therapies (ACTs) for AML. With the remarkable success of chimeric antigen receptor (CAR)-T cell therapy in B cell malignancies,[6] a number of studies developed and assessed CAR-T cell therapy for AML patients.[7–9] Although early-phase clinical trials showed some promising results, on-target off-tumour toxicities, logistical obstacles, and AML heterogeneity limit a greater success of CAR-T cell therapy against AML.[10–13]

$CD3^+CD4^-CD8^-$ double-negative T cells (DNTs) are mature peripheral T cells that account for approximately 3-5% of T lymphocytes. *Ex vivo* expanded, non-genetically modified DNTs from healthy donors can target primary and chemotherapy-resistant AML cells without inducing GvHD in xenograft models.[14,15] Recently, a phase I clinical trial (ChiCTR1900022795) demonstrated the feasibility, safety, and potential efficacy of allogeneic DNT therapy to treat AML patients with relapsed disease after allo-HSCT.[16] However, some patients are unresponsive to DNT therapy, and some primary AML samples show high levels of resistance to DNTs in preclinical models.[14,16] Further dissecting DNT cytotoxic mechanisms will help identify why DNTs fail to target some AML cells and provide strategies to overcome the resistance to DNT-mediated killing.

In this study, we used a flow cytometry-based high throughput screening (HTS) assay to compare cell surface molecule expression profiles on DNTs after encountering DNT-susceptible or -resistant AML cells. By validating the functions of these molecules *in vitro* and *in vivo*, we identified the significance of the TNFα/TNFα-receptor (TNFR) pathway in DNT-AML interactions. Furthermore, our study highlighted the unappreciated contributions of JAK1 in TNFα signaling to induce ICAM-1 expression on AML cells and the subsequent role of LFA-1 on DNTs to recognize and target TNFα-sensitized AML cells. These data demonstrate the utility of the flow cytometry-based HTS method to discover important immune-cancer cell pathways and the role of the TNFα-JAK1-ICAM-1 axis in DNT-mediated cytotoxicity against AML.

Methods

Ex vivo DNT expansion

DNTs were enriched from peripheral blood mononuclear cells (PBMC) of healthy donors using $CD4^+$ and $CD8^+$ depletion cocktail (Stemcell Technologies). Enriched DNTs were then expanded *ex vivo* as previously described (Supplementary Figure S1).[14,15]

Flow cytometry-based in vitro cytotoxic assays

DNTs were co-cultured with primary AML patient blasts (Supplementary Table S1) for 3 hours or AML cell lines for 2-24 hours at optimal effector-to-target (E:T) ratios (0.125-4 : 1). AML viability was determined by Annexin V (2- to 24-hour assays) or DAPI (multi-day assays) in $CD3^-CD33^+$ or $CD3^-CD34^+$ gated populations for AML cell lines and $CD45^{low}CD3^-CD33^+$ or $CD34^+$ populations for primary AML samples. Percentage specific killing was calculated by $\frac{\%\ Dead_{\ with\ DNTs} - \%\ Dead_{\ without\ DNTs}}{100 - \%\ Dead_{\ without\ DNTs}}$ x 100%.

For TNFα pre-treatment assays, DNTs or AML cells were treated with recombinant human TNFα (rTNFα, R&D Systems, 100 ηg/mL) for 24 hours (or 16 hours with primary AML), then washed with phosphate-buffered saline (PBS) prior to the killing assay.

For blocking assays, neutralizing antibodies (Biolegend) were added: anti-CD120b (3G7A02), anti-TNFα (MAb1), anti-CD18 (TS1/18), anti-CD54 (HCD54), anti-CD62ε (HAE-1f), or corresponding isotype controls at 5-10 μg/mL unless specified. Percentage inhibition of killing was calculated by $\frac{\%\,Specific\ Killing_{without\ Ab} - \%\,Specific\ Killing_{with\ Ab}}{\%\,Specific\ Killing_{with\ Ab}}$ x 100%.

For assays involving small molecule inhibitors, AML cells were incubated with Necrostatin 2 racemate (Nec-1S, MedChemExpress) or Itacitinib (Selleckchem) for 24 hours at specified concentrations in the figure legends, followed by rTNFα treatment prior to cytotoxicity assays.

Flow cytometry-based high throughput screening assay

DNTs from three different donors were co-cultured with or without OCI-AML3 or KG-1a for 2 hours and stained with anti-CD3, anti-CD33, and anti-CD34 antibodies. Then, the Princess Margaret Genomics Centre conducted highly multiplexed cell surface assessment using 385 commercially available antibodies and flow cytometry, as described by Gedye *et al.*[17] Cell surface protein expressions from three groups were analyzed: DNT-OCI-AML3 co-culture, DNT-KG-1a co-culture, and DNT alone.

Multi-day mixed co-culture assay

DNTs, DNT-susceptible AML cells (MV4-11 or OCI-AML3^{WT}), and DNT-resistant AML cells (KG-1a or OCI-AML3^{CD64KO}) were co-cultured in a 24-well plate at a 2:1:1 ratio

(DNT: DNT-susceptible AML: DNT-resistant AML) for 2 days. Anti-TNFα neutralizing antibodies were added on day 0 and 1. Aliquots were taken each day to track cell viability by DAPI and ICAM-1 expression. Cells were distinguished by flow cytometry: KG-1a ($CD3^-CD34^+CD33^-$), MV4-11 ($CD3^-CD34^-CD33^+$), OCI-$AML3^{CD64KO}$ ($CD3^-CD33^+GFP^+$), and OCI-$AML3^{WT}$ ($CD3^-CD33^+GFP^-$).

Transwell assay

DNTs and AML cells (KG-1a and MV4-11) were co-cultured in the Transwell ®-96 Permeable Support with 0.4μm PET membrane (Corning). The top compartment contained MV4-11 cells co-cultured with or without DNTs (E:T ratio of 1:1). The bottom compartment housed KG-1a cells with or without DNTs (E:T ratio of 2:1). After 2 days, KG-1a viability from the bottom compartment were determined by DAPI and flow cytometry.

Xenograft model

NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}*/SzJ* (NSG) mice (Jackson Laboratories) were used for xenograft experiments. 8-12 week-old female mice were sublethally irradiated (225 cGy) one day prior to an intravenous injection of $2x10^6$ OCI-AML2 cells. OCI-AML2 cells were treated with or without rTNFα (100 ηg/mL) for 24 hours prior to the injection. $2x10^7$ DNTs were intravenously injected on days 1 and 4 after AML cell injection. 10^4 IU rIL-2 (Proleukin) was given intravenously at the time of DNT infusion and intraperitoneally on day 11. Mice were euthanized on day 14. Cells from mice femurs were harvested to assess the bone marrow (BM) engraftments of AML cells by flow cytometry.

Statistical analysis

All graphs and statistical analyses were generated using GraphPad Prism 5. Student's *t*-test, ANOVA, and linear regression tests were used. ns = nonsignificant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate statistical significance between groups. Error bars represent ± SEM or ± SD.

Human blood collection and use was in accordance with University Health Network (UHN) Research Ethics Board (05-0221-T) and NHLBI-approved protocols. Animal use for experiments was approved by UHN Animal Care Committee (AUP: 741) and performed according to the Canadian Council on Animal Care Guidelines.

Results

Flow cytometry-based HTS assay identifies the involvement of CD120b/TNFR pathway in DNT-mediated cytotoxicity.

While the anti-cancer activity of *ex vivo* expanded DNTs has been demonstrated,[14,15,18,19] the underlying mechanisms by which DNTs mediate their activity is less well characterized. To identify cell surface proteins potentially involved in the DNT-mediated killing of AML cells, we used a flow cytometry-based HTS assay[17] to compare the changes in the expression of 385 cell surface proteins on DNTs during their interactions with DNT-susceptible (OCI-AML3) or -resistant (KG-1a) AML cell lines relative to DNT alone control (Figure 1A). KG-1a cells previously exhibited high intrinsic resistance to DNTs in *in vitro* cytotoxicity assays and in xenograft models compared to other AML cell lines, such as OCI-AML3, OCI-AML2, and MV4-11.[20] Using DNTs derived from three different donors, we identified molecules that were preferentially down- or up-regulated on DNTs, when co-cultured with OCI-AML3 cells relative

to KG-1a cells, with a minimum cut-off of >25% differential expression and relevant immune cytotoxic function (Figure 1B; Supplementary Table S2). The screen identified four molecules of interest: CD120b/TNFR2, CD65, CD66c, and CD172α.

Next, we validated the findings using two additional DNT-susceptible AML cell lines, OCI-AML2 and MV4-11.[20] Amongst the molecules examined, CD120b and CD65 were consistently down- or up-regulated after co-culture with all three DNT-susceptible AML cell lines, respectively (Figure 1C; Supplementary Figure S2). However, CD66c was only upregulated in the DNT-OCI-AML3 co-culture, and CD172α levels remained elevated regardless of the co-cultured AML cell line (Supplementary Figure S2). To assess the functional relevance of CD120b and CD65, DNTs were co-incubated with AML cells in the presence of neutralizing antibodies against CD120b or CD62ε, a receptor for CD65,[21] or their respective isotype antibodies as controls. Notably, anti-CD120b antibodies significantly reduced DNT-mediated cytotoxicity against AML cells, while blocking CD62ε had no significant effect (Figure 1D). Also, the magnitude of CD120b downregulation on DNTs correlated with the susceptibility of AML cells to DNT killing (Figure 1E). Collectively, these data demonstrate the involvement of the CD120b/TNFR pathway in DNT-mediated cytotoxicity towards AML cells.

TNFα sensitizes AML cells to the anti-leukemic activity of DNTs.

TNFα, the ligand of CD120b, is a well-known inflammatory cytokine involved in various diseases, but whether it has an anti- or pro-tumourigenic role in AML remains unclear.[22–24] Thus, we examined the effects of TNFα in the context of AML and DNT interactions. Unlike approximately 28% of cancers directly susceptible to TNFα *in vitro*, such as breast and colorectal cancers,[25–27] a minimal increase in AML apoptosis was observed after a 24-hour exposure to

rTNFα *in vitro* at the highest titrated dose (Supplementary Figure S3A). While direct cytotoxicity was not observed, 10 out of 11 rTNFα pre-treated AML cell lines (Figure 2A, top) and primary AML blasts (Figure 2A, bottom) with varying degrees of intrinsic DNT-resistance became significantly sensitized to DNT-mediated cytotoxicity (Figure 2A). Similar effects were seen in a highly DNT-resistant B-cell acute lymphoblastic leukemia cell line, NALM-6 (Supplementary Figure S3B), where rTNFα induced minimal direct cytotoxicity (Supplementary Figure S3C, left) but sensitized NALM-6 to greater DNT killing (Supplementary Figure S3C, right). In contrast to cancer targets, DNTs did not kill healthy allogeneic PBMC (allo-PBMC) with or without rTNFα pre-treatment (Figure 2A). We also observed that AML cells with higher resistance to DNT killing showed a greater relative degree of sensitization by rTNFα (Figure 2B). In line with this trend, DNT-resistant $CD34^+$ populations in primary AML samples (Figure 2C) became susceptible to DNTs after rTNFα treatment, while their $CD34^-$ counterparts, which were inherently more susceptible to DNTs, were not further sensitized by rTNFα treatment (Figure 2D). To further validate the functional relevance of TNFα in DNT-AML interactions, blocking studies were performed. The addition of anti-TNFα neutralizing antibodies to the DNT-AML co-cultures significantly reduced DNT-mediated cytotoxicity against AML cell lines (Figure 2E).

To examine TNFα sensitization effects *in vivo,* NSG mice were engrafted with OCI-AML2 cells, pre-treated with or without rTNFα, followed by DNT infusions (Figure 2F, Supplementary Figure S3D). Consistent with *in vitro* results, rTNFα did not significantly alter the AML BM engraftment as rTNFα-treated AML had similar leukemic engraftment levels as PBS-treated ones (Figure 2G, left). However, we observed significantly reduced engraftment in mice that were infused with rTNFα-treated AML followed by DNT treatment relative to mice

that received untreated AML cells and DNT infusions (Figure 2G, right). We also investigated if rTNFα potentiates DNTs to mediate superior anti-leukemic activity. Although DNTs pre-treated with increasing concentrations of rTNFα for 24 hours showed a dose-dependent downregulation of CD120b (Supplementary Figure S3E), the magnitude of DNT-mediated cytotoxicity against AML cells remained unchanged (Supplementary Figure S3F). Overall, these findings indicate that TNFα does not directly kill AML or potentiate DNT cytotoxic potency. Instead, TNFα sensitized AML cells to enhanced DNT killing for AML targets that have different levels of resistance to DNT-mediated cytotoxicity.

CD64 expression on susceptible AML cells trigger DNTs to produce TNFα.

With the observation that rTNFα can sensitize AML targets, we examined whether DNTs produced TNFα when engaging AML cells. We confirmed that DNTs express and secrete TNFα when co-incubated with DNT-susceptible AML cell lines but not with the DNT-resistant AML cell line, KG-1a (Figure 3A; Figure 3B, left). TNFα was not detected in the supernatant of AML cell alone or DNT alone cultures (Figure 3B, right).

We found that susceptible AML cells typically express high levels of CD64 (Figure 3C), and CD64 expression on primary AML cells correlates with their susceptibility to DNT anti-leukemic activity.[28] To determine whether the expression of CD64 was critical for inducing TNFα secretion in DNTs, we first knocked out (KO) CD64 on DNT-susceptible OCI-AML3 (OCI-AML3^{CD64KO}) cells (Figure 3C) and assessed their susceptibility to DNT-mediated cytotoxicity. We found that OCI-AML3^{CD64KO} cells were rendered resistant to DNT killing to a similar degree as KG-1a (Figure 3D). When examining a possible CD64-TNFα relationship, we detected a significant drop in TNFα in the supernatant of DNTs co-cultured with OCI-

AML3^{CD64KO} cells which was comparable to KG-1a, in contrast to OCI-AML3^{AAVS} control (Figure 3E). Furthermore, despite the increased DNT-resistance due to CD64 KO, pre-treatment of OCI-AML3^{CD64KO} cells with rTNFα significantly sensitized them to DNT-mediated killing (Figure 3F). These data suggest that DNTs may require CD64 expression on AML cells to release TNFα and initiate cytotoxic activities against susceptible AML cells, but this mechanism can be bypassed by providing exogenous TNFα.

TNFα produced by DNTs, upon encountering sensitive AML, renders DNT-resistant AML susceptible to DNT killing.

AML is a highly heterogenous disease comprised of subpopulations with varying degrees of susceptibility, contributing to resistance to different forms of therapies.[29–31] Now given that DNTs are potent producers of TNFα which is crucial for DNT-mediated cytotoxicity against AML, we tested whether DNTs can sensitize nearby DNT-resistant AML cells to DNT killing while interacting with susceptible AML targets. As a surrogate to heterogenous AML cells comprised of susceptible and resistant populations, we mixed DNT-susceptible AML cells (MV4-11 or OCI-AML3) and KG-1a cells, respectively, and assessed the cytotoxicity of DNTs against KG-1a. DNTs almost eliminated susceptible AML cells from the mixed co-culture regardless of the presence of KG-1a (Supplementary Figure S4A), suggesting that KG-1a does not actively suppress DNT anti-leukemic function. In contrast, DNTs effectively killed KG-1a only in the presence of MV4-11 or OCI-AML3 (Figure 4A). To avoid confounding effects between different AML cells in mixed co-culture assays, a transwell assay was conducted where MV4-11 cells were cultured in the top compartment with or without DNTs, while KG-1a cells with or without DNTs were seeded in the bottom wells (Figure 4B). While having DNTs with

MV4-11 in the top compartment had minimal effect on KG-1a cell growth and viability (Supplementary Figure S4B), it sensitized KG-1a to DNT-mediated cytotoxicity (Figure 4C).

Since DNTs may secrete other soluble factors in addition to TNFα, to investigate the role of DNT-derived TNFα in the above assays, the mixed co-culture experiment was conducted in the presence of anti-TNFα neutralizing antibodies. These neutralizing antibodies reversed the effect of having MV4-11 cells in the co-culture on the cytotoxicity of DNTs against KG-1a cells (Figure 4D). To validate these observations with another DNT-resistant AML cell line and further examine the contribution of CD64, a mixed co-culture assay with wild-type OCI-AML3 (OCI-AML3^{WT}) and OCI-AML3^{CD64KO} cells to simulate susceptible and resistant populations, respectively, was used. Similar to the MV4-11-KG-1a mixed assay, the presence of OCI-AML3^{WT} rendered OCI-AML3^{CD64KO} cells susceptible to DNT anti-leukemic activity, which was partially abrogated by anti-TNFα neutralizing antibodies (Figure 4E). Overall, these data demonstrate that DNTs produce TNFα, upon interacting with DNT-susceptible AML cells, which can then sensitize nearby DNT-resistant AML targets to DNT killing.

TNFα signals through JAK1 to upregulate ICAM-1.

To dissect the molecular mechanisms by which TNFα alters AML susceptibility to DNTs, AML cells were pre-treated with inhibitors against potential downstream pathways of TNFα receptor, such as receptor-interacting protein kinase-1 (RIPK1) and janus kinase-1 (JAK1).[32–34] Pre-treating AML cells with Nec-1S (RIPK1 inhibitor) to block canonical TNFα receptor signaling[32,33] did not abrogate the effect of rTNFα-mediated sensitization of KG-1a cells to DNTs (Figure 5A). In contrast, Itacitinib, a selective JAK1 inhibitor, significantly reduced the ability of rTNFα to increase AML susceptibility to DNTs for both KG-1a and OCI-AML2

(Figure 5A). To further confirm the contribution of JAK1 in TNFα-induced sensitization of AML cells, JAK1 was knocked down in AML cells (AML^{shJAK1}, Figure 5B), and the effects of rTNFα on AML^{shJAK1} cells were compared to control cells (AML^{shGFP}). Consistently, rTNFα was significantly less effective at increasing AML susceptibility to DNTs for AML^{shJAK1} cells compared to AML^{shGFP} cells (Figure 5C). Together, these data support that JAK1 is involved in TNFα-induced sensitization of AML cells.

To further investigate how the TNFα-JAK1 axis sensitizes AML cells to DNTs, the expression of inhibitory, cytotoxic, and adhesion molecules on AML cells with or without rTNFα treatment was compared. rTNFα did not affect the expression of inhibitory ligands (TGF-β and PD-L1; Supplementary Figure S5A) or DNAM-1 ligands (CD112 and CD155; Supplementary Figure S5B) on AML, which were previously reported to influence conventional T cell and DNT function.[14,35,36] Instead, rTNFα treatment drastically upregulated ICAM-1, intercellular adhesion molecule-1 or CD54, on AML cells after overnight stimulation, but not on normal PBMC (Figure 5D; Supplementary Figure S5C). Also, rTNFα induced a greater ICAM-1 upregulation on DNT-resistant $CD34^+$ primary AML populations relative to their DNT-susceptible $CD34^-$ counterparts (Figure 5E), suggesting that ICAM-1 may contribute to the rTNFα-mediated sensitization observed in the $CD34^+$ population. In addition, DNTs induced ICAM-1 expression on DNT-susceptible AML cells after co-culture, which was partially reversed by anti-TNFα neutralizing antibodies (Figure 5F), demonstrating ICAM-1 upregulation by DNT-derived TNFα. Also, soluble factors released from the co-culture of DNTs with a DNT-susceptible AML target significantly promoted ICAM-1 upregulation on KG-1a cells (Figure 5G), which was reduced in the presence of anti-TNFα neutralizing antibodies (Figure 5H).

To evaluate the contribution of JAK1 in TNFα-mediated ICAM-1 upregulation, AML cells were treated with Itacitinib to selectively inhibit JAK1 followed by rTNFα stimulation. The inhibition of JAK1 effectively disrupted TNFα-mediated ICAM-1 upregulation on KG-1a and OCI-AML2 cells compared to vehicle control (Figure 5I). Similarly, rTNFα was significantly less effective at increasing the expression of ICAM-1 on JAK1 knockdown AML cells relative to control AML cells (Figure 5J). Therefore, these results indicate that the TNFα-JAK1 signal upregulates ICAM-1 on AML cells.

ICAM-1-LFA-1 interaction is critical for TNFα to sensitize AML to DNT-mediated cytotoxicity.

To determine the functional relevance of TNFα-mediated ICAM-1 upregulation in DNT-mediated killing of AML cells, blocking assays were conducted with rTNFα-treated AML. ICAM-1 blocking antibody significantly reduced the intrinsic ability of DNTs to kill AML cell lines and lessened rTNFα-mediated sensitization effects (Figure 6A). A similar effect was also observed in 3 out of 4 primary AML patient samples (Figure 6B). This finding was further confirmed by genetic KO of ICAM-1 in AML cells (Supplementary Figure S6), as ICAM-1KO in OCI-AML2 significantly disrupted the ability of rTNFα to enhance DNT killing compared to control (Figure 6C). These data indicate the importance of ICAM-1 expression on AML cells for the sensitization effect of TNFα.

ICAM-1 is a high-affinity ligand for lymphocyte function-associated antigen-1 (LFA-1) receptor, an adhesion molecule comprised of two subunits, CD18 and CD11a,[37] highly expressed on DNTs (Figure 6D). To determine the importance of LFA-1 on DNTs to interact with ICAM-1 on AML cells, CD18KO DNTs or control DNTs (sgRND) (Figure 6E) were co-cultured with rTNFα pre-treated AML cells. We observed a significant decrease in the ability of rTNFα to

sensitize AML cell lines to CD18KO DNTs compared to control DNTs (Figure 6F). Consistent with this, the presence of anti-CD18 neutralizing antibodies to block LFA-1[38] significantly reduced rTNFα-mediated sensitization of KG-1a (Figure 6G) and 3 of 4 primary AML samples (Figure 6H) to DNTs, compared to isotype controls. Thus, ICAM-1-LFA-1 ligand-receptor interaction is crucial for TNFα-mediated sensitization of AML to DNTs.

Discussion

In this study, we investigated possible mechanisms for DNT-mediated cytotoxicity against AML cells by utilizing a flow cytometry-based HTS assay to compare cell surface protein expression after co-culture with DNT-susceptible versus -resistant AML cells. We identified a TNFα-JAK1-ICAM-1 cytotoxic axis, whereby DNTs secreted TNFα upon interacting with $CD64^+$ AML targets, which upregulated ICAM-1 on AML cells. This allowed DNTs to better target AML cells, including those otherwise resistant to DNTs, through LFA-1. These findings uncovered a novel mechanism involved in the DNT-mediated anti-leukemic response through a noncanonical TNFα-JAK1 pathway in AML. It also highlighted the potential combination of TNFα-based therapies with DNTs to improve the treatment of heterogenous malignancies such as AML.

Previously, we described the role of IFNγ, NKG2D, DNAM-1, and CD64 in DNT anti-leukemic activities. However, interference of these molecules did not completely abrogate DNT killing,[14,28] suggesting the presence of additional pathways. Hence, the flow cytometry-based HTS assay explored other potential mechanisms involved in DNT-mediated cytotoxicity against AML. The technique assessed immune-cancer cell interactions by incorporating target cells with differential susceptibilities and cell surface molecule expression data. Given the use of

monoclonal antibodies to augment contact-dependent mechanisms and CAR-T cells in cancer immunotherapy,[6,39] this approach can be potentially applied to other cancer models to identify targetable cell surface proteins by CARs or antibodies to improve ACTs.

TNFα is a prominent inflammatory cytokine able to elicit pro- and anti-tumourigenic effects.[22,24,40] In AML, the discrepancy remains as high serum TNFα is reported to be an adverse prognostic factor for newly diagnosed patients with AML,[23] while several clinical case reports describe the development of AML following the use of anti-TNFα inhibitors.[41,42] Consistent with the case reports, we demonstrated that anti-TNFα neutralizing antibodies reduced the anti-tumour activity of DNTs against AML cells *in vitro*. Also, rTNFα pre-treatment sensitized AML cells to DNT-mediated killing *in vitro* and *in vivo* in an ICAM-1-LFA-1-dependent manner. Notably, the TNFα effect was more pronounced in AML cells with a higher degree of resistance to DNTs and was not observed in healthy allo-PBMC. These findings support the potential combination therapy of DNT therapy with other approaches that augment the TNFα-ICAM-1 axis[43,44] to enhance treatment outcomes.

The release of TNFα appears to be induced by the expression of CD64, a high-affinity $F_c\gamma$ receptor, typically found on DNT-susceptible AML cells compared to DNT-resistant ones.[28] TNFα stimulation can bypass the initial CD64-dependent mechanism and increase DNT-mediated cytotoxicity against AML cells. However, the exact mechanisms between DNTs and $CD64^+$ AML remain unclear. CD64 expression is associated with more mature AML phenotypes, such as AML-M5,[45] and AML-M5 blasts are more susceptible to DNTs compared to other AML subtypes.[14] It is possible that DNTs directly bind to CD64 or another molecule regulated by CD64 expression on AML. Nevertheless, these observations uncover a novel function of CD64

on AML to initiate DNT cytotoxic activity and warrant additional investigations to clarify DNT activation mechanisms for improved therapeutic purposes.

Cancer heterogeneity refers to genotypic and phenotypic differences between a tumour across different patients, cancer cells within a single tumour, or the same tumour throughout disease progression.[31] The cellular non-uniformity is a major driver for therapeutic resistance and disease relapse, predisposing patients to poorer clinical outcomes, especially in patients with AML.[3,29,30] DNT therapy is not an exception where ~30% of primary AML blasts show resistance to DNT-mediated cytotoxicity, partially explained by CD64 expression profiles on AML.[14,28] Interestingly, TNFα increased the susceptibility of AML to DNTs, expanding the therapeutic window against various AML cells. Furthermore, TNFα produced by DNTs, after encountering susceptible targets, sensitized nearby DNT-resistant AML cell lines. Ultimately, these observations favourably support the use of DNT therapy for AML or other highly heterogeneous cancers,[46] possibly comprised of a mixture of DNT-susceptible and -resistant populations.

TNFα signals through TNFRs to elicit NF-κB activation and inflammatory responses.[32] TNFα produced from DNT-AML co-cultures contribute to the downregulation of CD120b on DNTs in an autocrine manner. Although TNFα stimulation of DNTs did not alter cytotoxic function, CD120b signaling has been investigated in regulatory T cells and may be involved in the ability of DNTs to resist host-versus-graft rejection.[15,47] In contrast, TNFα sensitized AML to greater DNT-mediated cytotoxicity, independent of a canonical mediator of TNFR/NF-κB, RIPK1. Instead, we identified a TNFR pathway involving JAK1 that consistently mediated ICAM-1 upregulation and AML sensitization. Although the TNFR pathway has been characterized, the molecular relationship and immunotherapeutic significance between TNFRs

and JAK1 remain unclear, except some studies demonstrating that TNFRs could signal through JAK1 in B cells, adipocytes, and epithelial cells.[34,48,49] JAK1 is one of four mammalian JAK proteins commonly found at the cytoplasmic domain of interferon and interleukin receptors. Upon ligand-receptor binding, receptor-associated JAKs are activated and lead to phosphorylation of Signal Transducer and Activator of Transcription (STAT) proteins. Phosphorylated STAT molecules translocate into the nucleus to upregulate inflammatory factors and cytotoxic ligands.[50] Given that DNTs secrete a wide range of inflammatory cytokines,[14,51] the significance of JAK-STAT signaling in DNT-AML interactions is currently being explored.

In summary, our findings highlight the utility of the flow cytometry-based HTS platform to identify underlying cytotoxic mechanisms between immune-cancer cell interactions and the significance of the novel TNFα-JAK1-ICAM-1 axis in DNT-mediated cytotoxicity against AML. These data support the potential combinatorial use of drugs that influence this axis to enhance the anti-leukemic activity of DNT therapy.

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Author Contributions

ET, JL, and LZ conceived and designed the study experiments. ET, JL, IK, and YN conducted experiments. MDM provided primary patient samples. ET and JL prepared the manuscript. JL, IK, MDM, and LZ provided feedback and edited the manuscript.

Disclosure of Conflicts of Interest

MDM is a consultant for Astellas, Abbvie, and Celgene. LZ has financial interests (e.g., holdings/shares) in *WYZE Biotech Co Ltd* and previously received research funding and consulting fee/honorarium from the Company. LZ and JL are co-inventors of several DNT cell technology related patents and intellectual properties for the treatment of AML. The remaining authors have declared no conflicts of interest.

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Figure Legends

Figure 1 - Flow cytometry-based HTS assay identifies the involvement of CD120b/TNFR pathway in DNT-mediated cytotoxicity.

A) Schematic of the flow cytometry-based HTS assay using DNT-resistant (KG-1a) and -susceptible (OCI-AML3) AML cell lines.

B) DNTs alone or co-cultured with OCI-AML3 or KG-1a in 96-well plates were stained with 385 fluorophore-conjugated antibodies. Molecule expression on DNTs ($CD3^+CD33^-CD34^-$) co-cultured with OCI-AML3 ($CD3^-CD33^+CD34^-$) or KG-1a ($CD3^-CD33^-CD34^+$) relative to DNT alone was determined. From left to right, the graph shows molecules that are upregulated or downregulated on DNTs during the interaction with OCI-AML3, relative to DNT-KG-1a interactions. The experiment was performed with three biological replicates, and the data were pooled together.

C) DNTs stained for CD120b after a 2-hour co-culture with DNT-resistant (KG-1a) or -susceptible (OCI-AML2, OCI-AML3, MV4-11) AML cell lines. A representative histogram (left) and corresponding median fluorescence intensity (MFI) values (right) of CD120b expression are shown. Experiments were done in triplicates. The data shown are representative of three independent experiments.

D) DNTs were co-cultured with DNT-susceptible AML cell lines, OCI-AML2 (top) or MV4-11 (bottom), for 24 hours in the presence of anti-CD62ε or anti-CD120b neutralizing antibody or corresponding isotype controls. Experiments were done in triplicates. The data shown are representative of two independent experiments.

E) Linear regression analysis performed between the MFI fold change in CD120b expression on DNTs co-cultured with AML cells relative to DNT alone and the

563 percentage specific killing of AML cell lines and primary AML samples by DNTs. AML
564 cells and DNTs were co-incubated for 2 hours. Each symbol represents an AML cell line
565 or primary AML sample. Numbers represent the AML patient ID. Experiments were
566 done in triplicates, and the data shown are representative of two independent
567 experiments.

568 Student's *t*-test, one-way ANOVA, and linear regression analysis were used. ns =
569 nonsignificant, *** $p < 0.001$.

570 **Figure 2 – TNFα sensitizes AML cells to the anti-leukemic activity of DNTs.**

571 (A and B) AML cell lines (top), primary AML blasts, and allogeneic peripheral blood
572 mononuclear cells (allo-PBMC) (bottom) were pre-treated with or without rTNFα,
573 washed with phosphate-buffered saline (PBS), then co-cultured with DNTs for 2-4 hours.
574 Percentage specific killing of AML cells are shown with a red line indicating the 10%
575 specific killing as resistance threshold (A). Linear regression analysis performed between
576 percentage specific killing and the relative percentage increase in specific killing of
577 rTNFα-sensitized AML (B). Each symbol represents an AML cell line or primary AML
578 sample. Experiments were done in triplicates and were performed with at least two DNT
579 donors. Numbers represent the AML patient ID.

580 (C and D) Primary AML samples (130794 and 140176) were treated with or without rTNFα,
581 washed with PBS, then co-cultured with DNTs. Gating strategy (C) and percentage
582 specific killing of rTNFα-treated and non-treated groups of the primary AML samples
583 gated on specified leukemic blast populations in triplicates are shown (D).

E) DNTs were co-cultured with AML cell lines for 24 hours, in the presence of increasing (for OCI-AML3) or fixed (for OCI-AML2 and MV4-11, 10 µg/mL) concentration of anti-TNFα blocking antibody or isotype control antibody. The graphs shown are representative of two independent experiments.

(F and G) Sublethally irradiated (225 cGy) NSG mice were intravenously injected with OCI-AML2 untreated or treated with rTNFα, followed by two infusions of DNTs or PBS. Schematic of the *in vivo* xenograft mouse model with treatment schedule (F). Bar graphs represent the mean AML bone marrow engraftment levels (left) and engraftment levels normalized to the PBS group (right) (G). Each symbol represents an individual mouse. Data represent the mean ± SD and pooled from three independent experiments (n=3-5/group).

Student's *t*-test, one-way/two-way ANOVA, and linear regression analysis were used. ns = nonsignificant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3 – CD64 expression on susceptible AML cells trigger DNTs to produce TNFα.

(A and B) DNTs and AML cells were cultured alone or together for 2 hours. The intracellular expression of TNFα was measured on DNTs ($CD3^+CD33^-$) by flow cytometry. The bar graph shows the percentage expression (left), and the flow plot shows a representative histogram with MFI values (right) (A). The level of TNFα in the supernatants of co-cultures (left) and AML or DNT cell alone groups (right) were determined by ELISA (B). The experiments were performed in triplicates. The data shown are representative of two independent experiments.

C) CD64 expression by OCI-AML3^{AAVS} control (red), OCI-AML3^{CD64KO} (blue), KG-1a (orange), and MV4-11 (green) cells. Representative histogram shows expression measured by flow cytometry with MFI values.

(D and E) OCI-AML3^{AAVS} control, OCI-AML3^{CD64KO}, and KG-1a cells were co-cultured with DNTs for 2 hours. Specific killing of AML cells was measured using flow cytometry (D). The level of TNFα from the co-culture supernatants was determined by ELISA (E). The experiment was performed in triplicates, and the data are representative of two independent experiments.

F) OCI-AML3^{CD64KO} cells were untreated or pre-treated with rTNFα (100 ηg/mL), washed with PBS, then co-cultured with DNTs for 24 hours. Specific killing of AML cells by DNT was determined by flow cytometry. Data shown are representative of two independent experiments done in triplicates.

Student's *t*-test and one-way/two-way ANOVA were used. n.d. = not detected, ns = nonsignificant, ** $p < 0.01$, *** $p < 0.001$.

Figure 4 – TNFα produced by DNTs, upon encountering sensitive AML, renders DNT-resistant AML susceptible to DNT killing.

(A) DNTs were co-cultured with DNT-resistant KG-1a in the presence or absence of DNT-susceptible AML (MV4-11 or OCI-AML3) at a 2:1:1 (DNT: KG-1a: DNT-susceptible AML) ratio for 24 hours. Specific killing of KG-1a was measured by flow cytometry. The experiments were done in triplicates, and the data shown are representative of two independent experiments.

(B and C) MV4-11 were cultured with or without DNTs in the top compartment of a transwell. KG-1a alone or co-cultured with DNTs were placed in the bottom

compartment of the transwell. Cells were then incubated for 2 days as shown in the schematic (B). Specific killing of KG-1a cells in the bottom compartment was measured and compared between MV4-11 and MV4-11 + DNT conditions (C). The experiments were done in triplicates. The data shown are representative of two independent experiments.

(D and E) DNT-resistant AML, KG-1a (D) or OCI-AML3^{CD64KO} (E), were incubated alone or with DNT-susceptible AML, MV4-11 (D) or OCI-AML3^{WT} (E), and DNTs in the presence of anti-TNFα blocking antibody (10 μg/mL) or isotype control for 2 days. Antibodies were added on day 0 and day 1 of the co-cultures. Specific killing of DNT-resistant AML cells was measured by flow cytometry. The experiments were done in triplicates. The data shown are representative of two independent experiments.

Student's *t*-test and one-way/two-way ANOVA were used. ns = nonsignificant, * $p < 0.05$, *** $p < 0.001$.

Figure 5 - TNFα signals through JAK1 to upregulate ICAM-1

A) KG-1a and OCI-AML2 cells were exposed to increasing or fixed (40 μM) concentrations of receptor-interacting protein kinase-1 (RIPK1) inhibitor, janus kinase-1 (JAK1) inhibitor, or DMSO for 24 hours, followed by rTNFα treatment. AML were then co-cultured with DNTs for 24 hours (for KG-1a) or 2 hours (for OCI-AML2). Percentage increase in specific killing after rTNFα pre-treatment was determined. The data shown are representative of two independent experiments.

(B and C) Wild-type (WT) OCI-AML2 and OCI-AML3 cells were untreated or transduced with shRNAs against JAK1 (shJAK1) or GFP control (shGFP). RNA expression of JAK1 was normalized to HPRT housekeeping gene (B). Transduced AML cells were pre-

treated with or without rTNFα, washed with PBS, then co-cultured with DNTs for 2 hours. Specific killing (left) and percentage increase in specific killing (right) after rTNFα pre-treatment are shown (C).

D) AML cell lines (top), primary AML samples with patient IDs, and healthy PBMCs (bottom) were untreated (blue) or treated (orange) with rTNFα. Representative histograms of ICAM-1 expression with MFI values including fluorescence-minus-one (FMO) control (red) are shown.

E) $CD33^{+}CD45^{low}CD3^{-}CD34^{+}$ ($CD34^{+}$) and $CD33^{+}CD45^{low}CD3^{-}CD34^{-}$ ($CD34^{-}$) primary AML blasts from two patients were treated with rTNFα. The ICAM-1 MFI fold change is shown.

F) ICAM-1 expression with MFI values of AML cell lines co-cultured with or without DNTs for 24 hours in the presence of anti-TNFα or isotype control. The data shown are representative of two independent experiments.

G) MV4-11 and KG-1a were co-cultured with or without DNTs in separate compartments of the transwell from Figure 4B. Representative histograms of ICAM-1 expression on KG-1a with MFI values after 2 days is shown.

H) KG-1a cells were alone or were incubated with MV4-11 or OCI-AML3 and DNTs in the presence of anti-TNFα blocking antibody or isotype control for 24 hours. ICAM-1 expression on live KG-1a cells was determined. The data are representative of two independent experiments.

I) KG-1a and OCI-AML2 were treated with JAK1 inhibitor or DMSO for 24 hours, followed by stimulation with or without rTNFα and stained for ICAM-1. The MFI fold change of ICAM-1 expression from rTNFα-treated AML cells relative to untreated AML

cells is shown and compared between vehicle and JAK1 inhibitor conditions. The data displayed are two pooled independent experiments.

J) JAK1 knockdown (shJAK1) or control (shGFP) AML cells were untreated or treated with rTNFα (100 ηg/mL), then stained with anti-ICAM-1 antibody. The MFI fold change of ICAM-1 expression from rTNFα-treated AML cells relative to untreated AML cells is shown and compared between control and JAK1 knockdown conditions. The data shown are representative of two independent experiments.

Student's *t*-test and one-way/two-way ANOVA were used. Ns = nonsignificant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6 - ICAM-1-LFA-1 interaction is critical for TNFα to sensitize AML to DNT-mediated cytotoxicity.

(A and B) AML cell lines (A) and primary AML samples (B) were pre-treated with or without rTNFα, washed with PBS, then co-cultured with DNTs in the presence of isotype control or anti-ICAM-1 blocking antibody. Percentage specific killing of AML cells (A, left) and percentage increase in specific killing between rTNFα-treated AML and non-treated AML (A, right) were determined. The experiments were performed in triplicates with at least two DNT donors. Numbers represent AML patient ID.

C) Cas9 and ICAM-1^{KO} OCI-AML2 cells were untreated or treated with rTNFα, washed with PBS, then co-cultured with DNTs for 2 hours. The data shown are the percentage increase in specific killing of rTNFα-treated AML cells by DNTs relative to untreated AML cells. The graph shown is representative of two independent experiments.

D) DNTs were stained for LFA-1 subunits (red), CD11a and CD18, and compared to FMO control (blue).

E) Representative histogram of random sgRNA control DNTs (sgRND, orange) and $CD18^{KO}$ DNTs (CD18KO, blue) to show CD18 expression relative to FMO control (red).

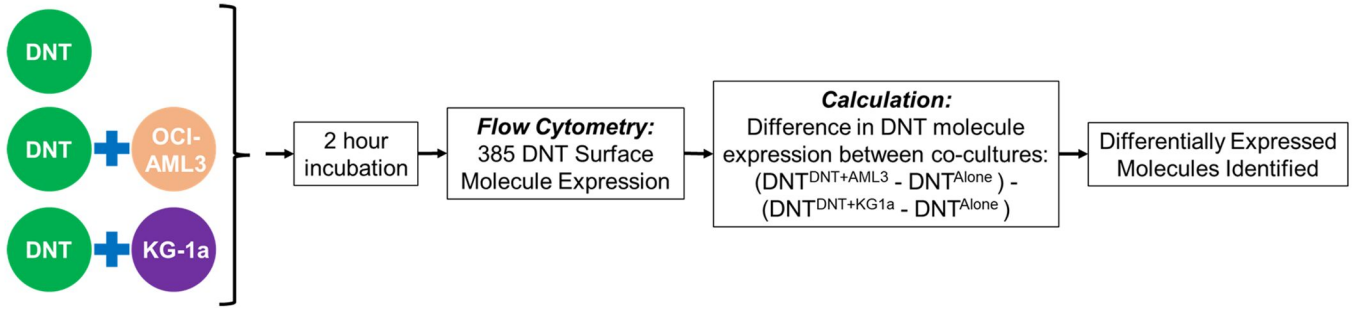
F) AML cells were pre-treated with or without rTNFα, washed with PBS, then co-cultured with $CD18^{KO}$ DNTs (CD18KO) or control DNTs (sgRND) for 24 hours (for KG-1a) and 2 hours (for OCI-AML2). The percentage increase in specific killing due to rTNFα pre-treatment was determined. The data shown are representative of two independent experiments.

(G and H) KG1a (G) and 4 primary AML samples (H) were pre-treated with or without rTNFα, washed with PBS, then co-cultured with DNTs, in the presence of isotype control or anti-CD18 blocking antibody for 24 hours (for KG-1a) and 3 hours (for primary AML). Percentage change in specific killing between rTNFα-treated AML and non-treated AML is shown. The experiments were performed in triplicates with at least two DNT donors. Numbers represent AML patient ID.

Student's *t*-test and one-way/two-way ANOVA were used. ns = nonsignificant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 1

A)



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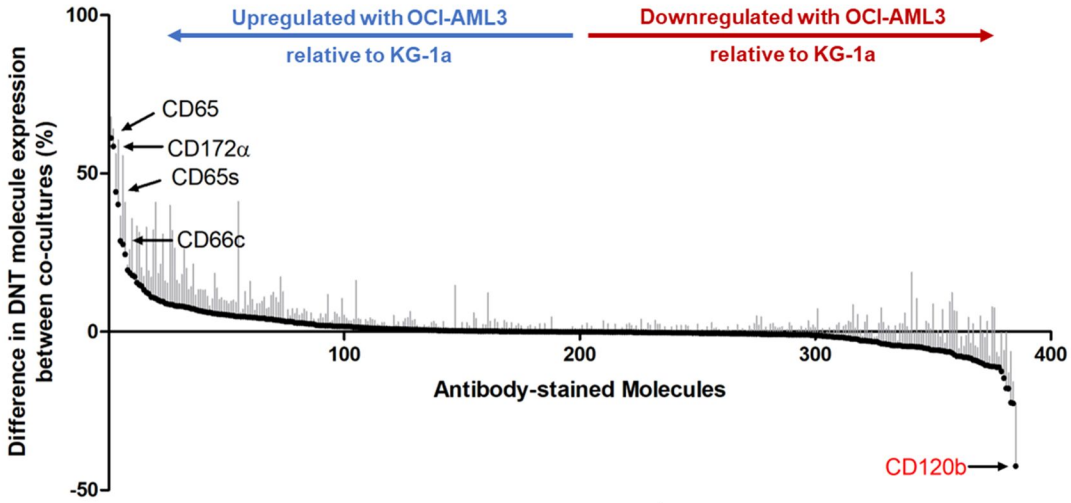

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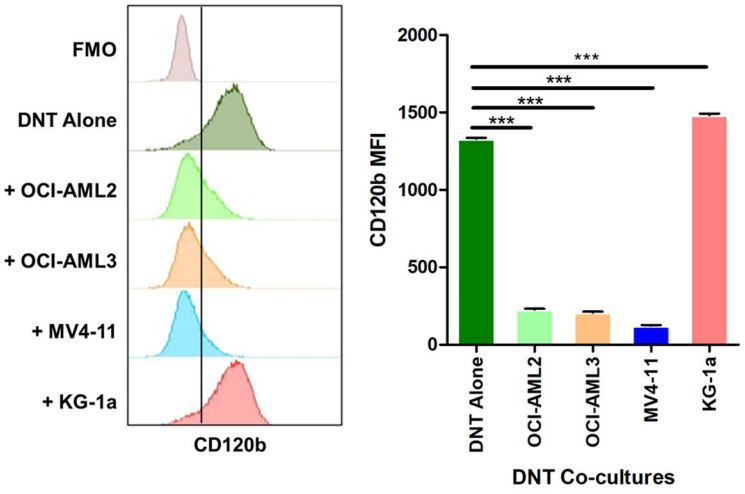

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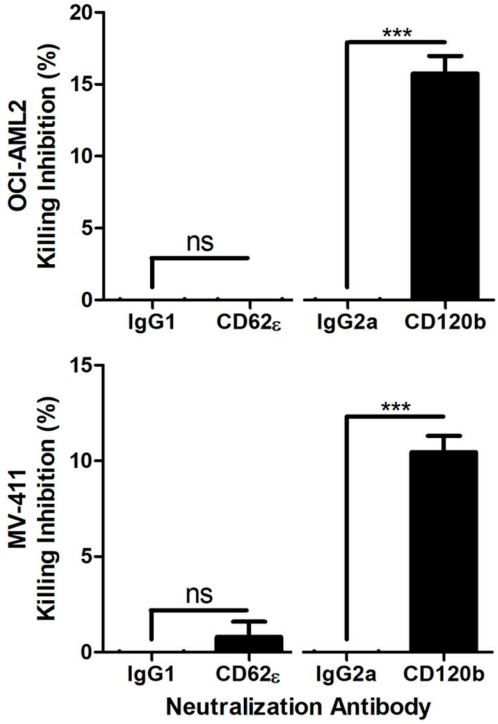

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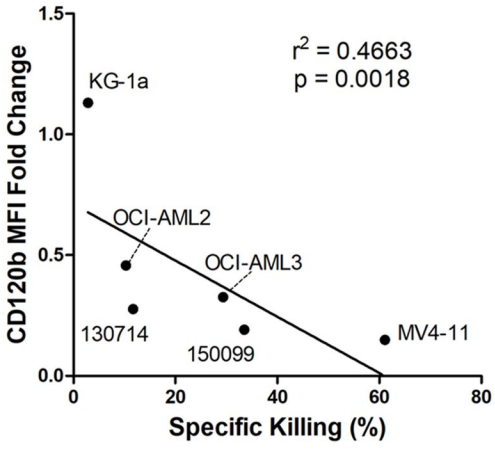


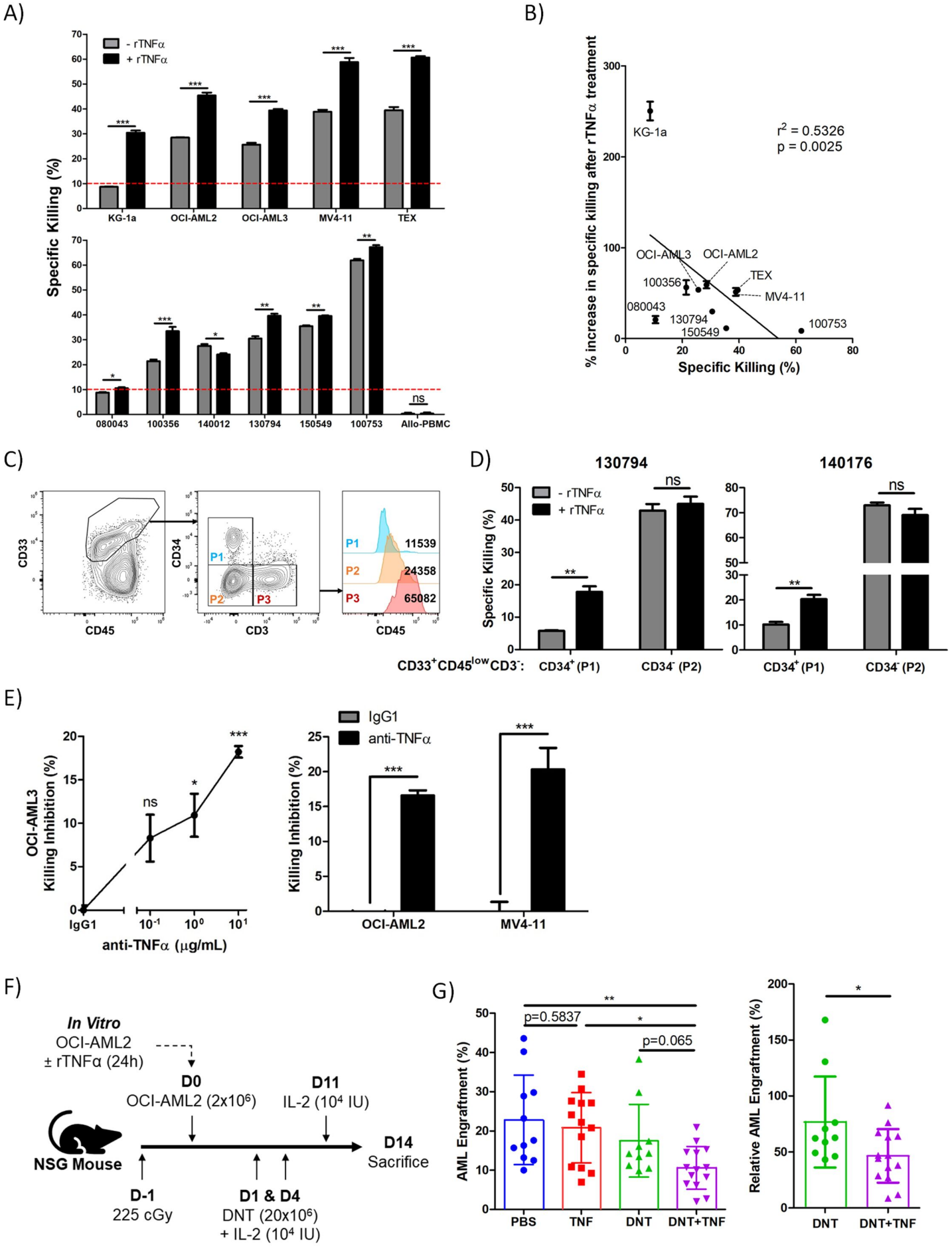


Figure 3

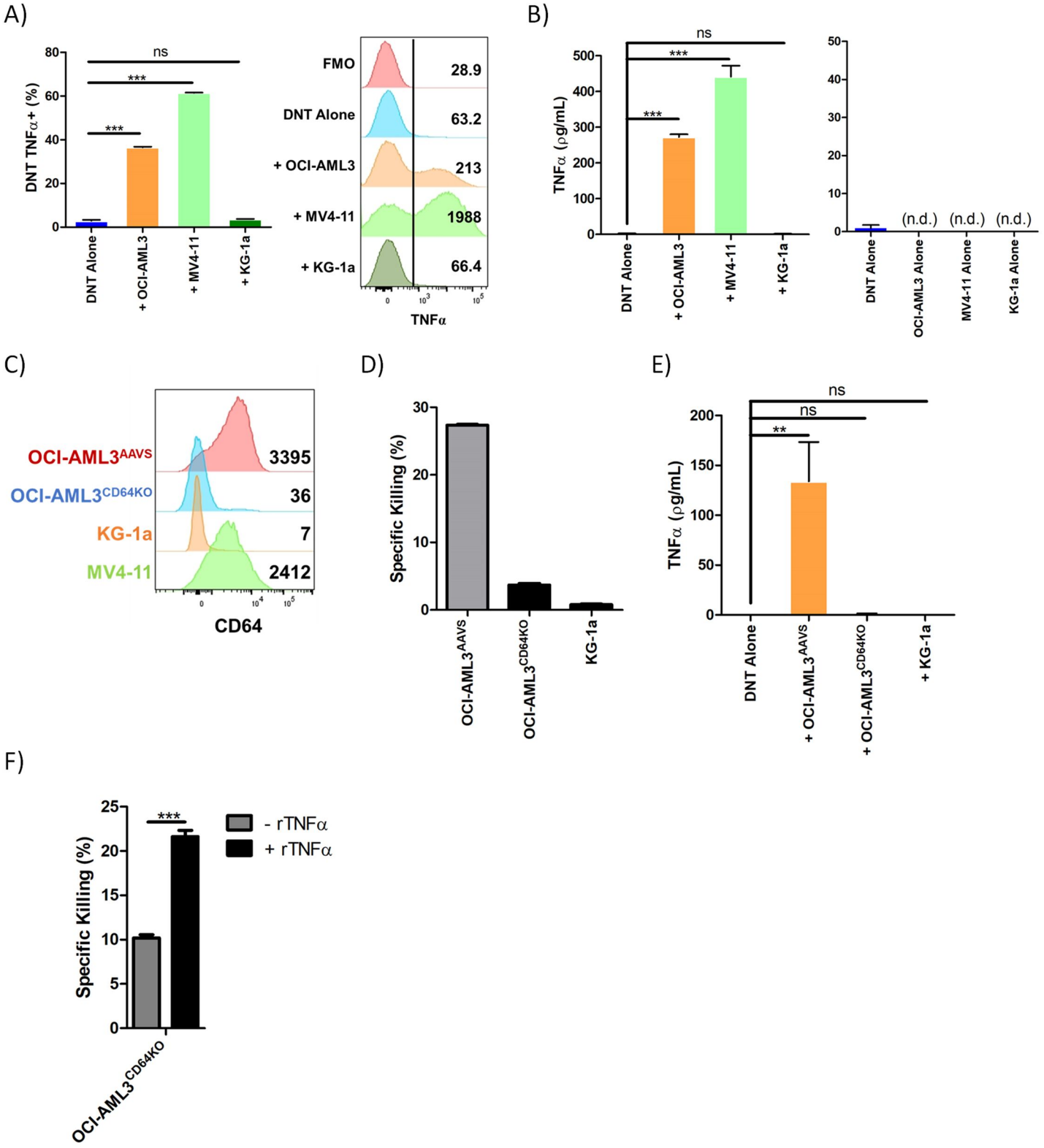
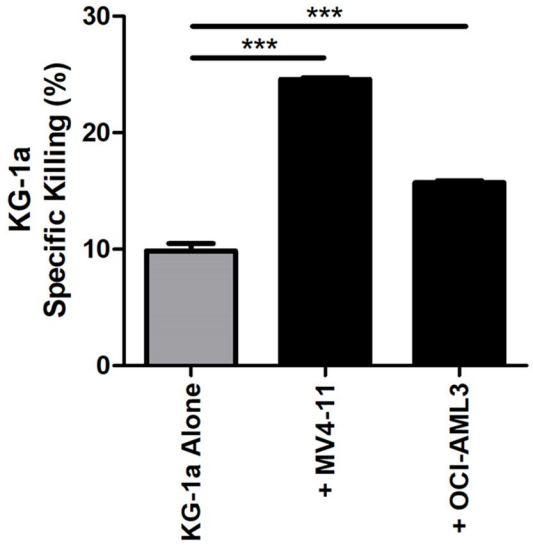
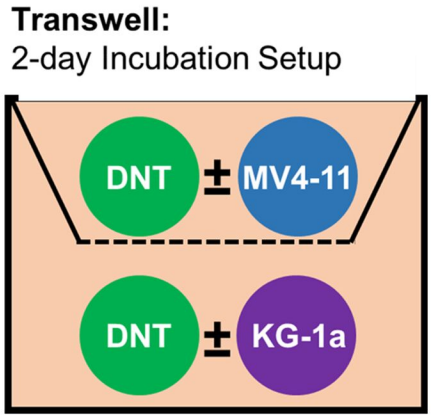


Figure 4

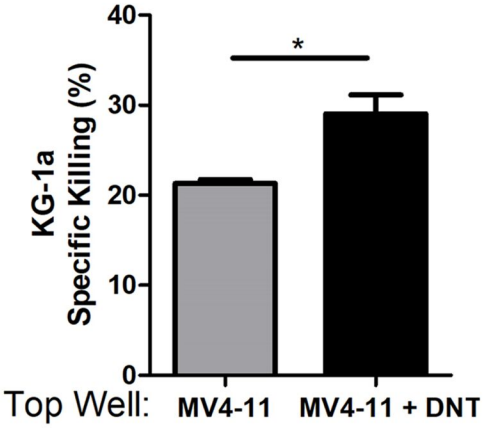
A)



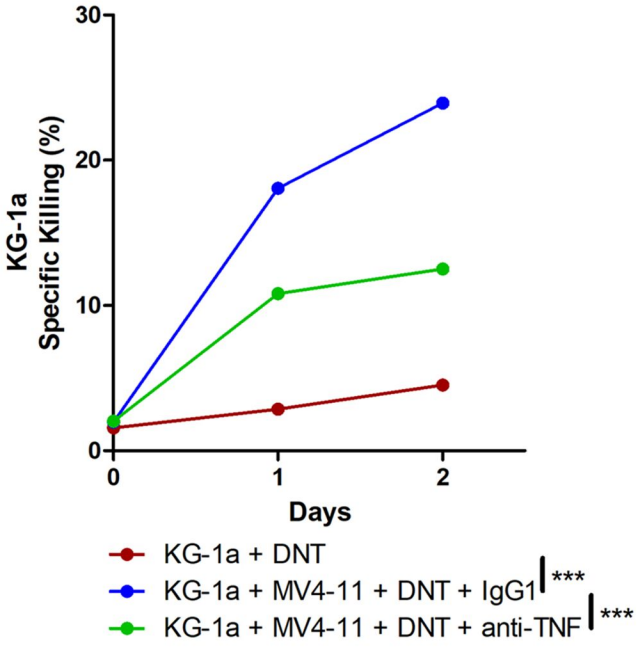
B)



C)



D)



E)

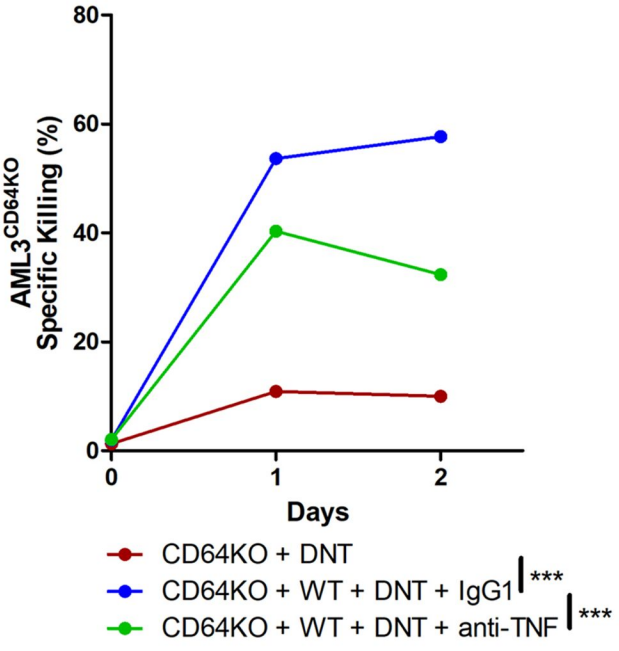


Figure 5